Stanozolol treatment decreases the mitochondrial ROS generation and oxidative stress induced by acute exercise in rat skeletal muscle

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Stanozolol treatment decreases the mitochondrial ROS generation and oxidative stress induced by acute exercise in rat skeletal muscle. J Appl Physiol 110: 661–669, 2011. First published December 16, 2010; doi:10.1152/japplphysiol.00790.2010.—Anabolic androgenic steroids are used in the sport context to enhance muscle mass and strength and to increase muscle fatigue resistance. Since muscle fatigue has been related to oxidative stress caused by an exercise-linked reactive oxygen species (ROS) production, we investigated the potential effects of a treatment with the anabolic androgenic steroid stanozolol against oxidative damage induced on rat skeletal muscle mitochondria by an acute bout of exhaustive exercise. Mitochondrial ROS generation with complex I- and complex II-linked substrates was increased in exercised control rats, whereas it remained unchanged in the steroid-treated animals. Stanozolol treatment markedly reduced the extent of exercise-induced oxidative damage to mitochondrial proteins, as indicated by the lower levels of the specific markers of protein oxidation, glycoxidation, and lipoxidation, and the preservation of the activity of the superoxide-sensitive enzyme aconitase. This effect was not due to an enhancement of antioxidant enzyme activities. Acute exercise provoked changes in mitochondrial membrane fatty acid composition characterized by an increased content in docosahexaenoic acid. In contrast, the postexercise mitochondrial fatty acid composition was not altered in stanozolol-treated rats. Our results suggest that stanozolol protects against acute exercise-induced oxidative stress by reducing mitochondrial ROS production, in association with a preservation of mitochondrial membrane properties.

antioxidant enzymes; anabolic androgenic steroids; free radicals; protein oxidative damage; membrane unsaturation

DESPITE THE WIDESPREAD USE of anabolic androgenic steroids to enhance physical performance, the mechanisms of androgen action in skeletal muscle remain poorly understood. Androgens are required to maintain normal muscle mass and strength in men, since suppression of testosterone levels reduces these parameters, and, conversely, exogenously administered androgens have anabolic effects on muscle. Testosterone and anabolic androgenic steroids, administered at supraphysiological doses, can induce hypertrophy of type I and II muscle fibers (4, 23) and are effective in increasing skeletal muscle mass and strength in eugonadal males (24). There is also experimental evidence that treatment of rodents with testosterone and anabolic androgenic steroids may improve work capacity and fatigue resistance of skeletal muscles (2, 8, 17, 44, 46), but the molecular basis underlying these effects remains unclear. It has been suggested (2) that androgens could regulate muscle function and fatigue properties through a modification of oxidative metabolism, since an increased oxidative capacity is closely related to an augmented ability to resist fatigue. In favor of this hypothesis, androgens have been reported to modify metabolic enzyme activities (16, 26), mitochondrial size (41), and muscle cell composition (7). In other studies, however, no changes in muscle oxidative capacity were detected after treatment with testosterone and anabolic androgenic steroids (6, 17, 46).

The effect of androgens on the redox status of skeletal muscle has been scarcely investigated, despite its influence on muscle function and fatigue properties. Skeletal muscle cells continuously generate reactive oxygen species (ROS), which play a critical role in the modulation of muscle contractility: low and physiological levels of ROS are required for normal force production, but high levels of ROS promote contractile dysfunction, resulting in muscle weakness and fatigue, likely due to oxidative damage of several molecular targets (19). Exhaustive exercise induces an augmented generation of ROS within skeletal muscle (35), which alters intracellular antioxidant-antioxidant balance in favor of the former and can result in oxidative damage of exercising muscles when the production of ROS overwhelms the antioxidant defense systems. Multiple potential sites for ROS generation in skeletal muscle have been identified, including mitochondria. NAD(P)H oxidase enzymes, phospholipase A2-dependent processes, and xanthine oxidase (19). Mitochondria have been considered as the main ROS generator during exhaustive exercise and, at the same time, the primary target for oxidative modification, but, surprisingly, there is little evidence that mitochondria generate ROS in vivo and are under oxidative stress during exercise (20, 25, 27, 48).

Testosterone and anabolic androgenic steroids have been shown to increase antioxidant defenses in certain tissues and cell types (1, 6, 34, 43). These results support the idea that sex hormones may be involved in the redox homeostasis and suggest a mechanism by which androgens could influence muscle function and fatigue properties. In the present work, we tested the hypothesis that anabolic androgenic steroids could protect skeletal muscle mitochondria against exercise-induced oxidative modification by modulating mitochondrial ROS generation and/or scavenging. With this aim, we have isolated mitochondria from gastrocnemius muscles of sedentary and acutely exercised rats to study the effect of a treatment for 8 wk with the anabolic androgenic steroid stanozolol on: 1) the rate of mitochondrial ROS production; 2) the levels of specific markers of protein oxidation [the specific protein carbonyls glutamic (GSA) and aminoacidic semialdehydes (AASA)], glycoxidation [carboxyethyl-lysine (CEL) and carboxymethyl-lysine (CML)], and lipoxidation [malondialdehyde-lysine (MDAL)], and on the activity of the superoxide-sensitive...
enzyme aconitate; and 3) the antioxidant enzymatic activities. Since the lipid environment can affect membrane function, including mitochondrial electron transport chain, and, conversely, mitochondrial membranes can vary in sensitivity to oxidative damage, depending on their unsaturated fatty acid content (30), the full fatty acid composition of mitochondrial membranes was also determined.

MATERIALS AND METHODS

Steroid treatment and single bout of exhaustive exercise. Thirty-two male Wistar rats (initial body weight, 283 ± 7 g; 8 wk old) were obtained from Charles River (Barcelona, Spain). They were housed in an animal room at 22 ± 2°C and 50 ± 10% relative humidity 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, 0800–2000) before the beginning of the steroid treatment period. All of the experimental procedures employed, as well as rat care and handling, were approved by the Ethics Committee of the Complutense University and complied with the "European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes." Initially, animals were randomly divided between a control (C) group (n = 16) and a stanozolol-treated (ST) group (n = 16). The animals selected for stanozolol (17β-hydroxy-17α-methyl-5α-androst-3-ene-17α-carbonitrile, 2 mg/kg body wt) (Zambon, Barcelona, Spain) treatment received, by gastric gavage, 2 mg steroid/kg body wt as a suspension in 1 ml of water 5 days/wk for 8 wk. Untreated C animals received the same amount of vehicle by the same procedure and with the same periodicity. Our laboratory has previously shown that oral administration of these doses of stanozolol reduced serum testosterone levels, indicating that the steroid was absorbed and distributed through the tissues of the rats (6). After completion of the 8-wk treatment period, eight rats from the C group (C-Ex) and eight rats from the ST group (ST-Ex) were arbitrarily selected to perform a single session of exhaustive exercise on a rodent motor-driven treadmill (Columbus Instruments, Columbus, OH) with a 15% slope. The exercise bout was performed at 20 m/min until exhaustion. Animals started with a 5-min warm-up at 15 m/min, after which the rats ran for 10 min at 20 m/min, and finally at 25 m/min until exhaustion. Animals were judged to be exhausted when they could no longer continue at 10 min at 20 m/min, and finally at 25 m/min until exhaustion. Animals were judged to be exhausted when they could no longer continue

Mitochondrial oxygen consumption. The rate of oxygen consumption by muscle mitochondria was measured polarographically at 37°C with a computer-controlled Clark-type O2 electrode (Oxygraph, Hansatech, UK) in 0.5 ml of incubation buffer (145 mM KCl, 30 mM HEPES, 5 mM KH2PO4, 3 mM MgCl2, 0.1 mM EDTA, 1 mg/ml fatty acid-free bovine serum albumin, pH 7.4) with 0.25 mg mitochondrial protein/ml. The substrates used were complex I (2.5 mM pyruvate/2.5 mM malate) or complex II linked (5 mM succinate + 2 μM rotenone). The assays were performed in the absence (state 4-resting) and in the presence (state 3-phosphorylating) of 500 μM ADP. The respiratory control index was calculated as state 3-to-state 4 oxygen consumption ratio. Respiratory control index values of 5.7 ± 0.7 and 2.5 ± 0.1 were obtained with pyruvate/malate and succinate plus rotenone, respectively, indicating a suitable degree of integrity of the mitochondrial preparations.

Mitochondrial H2O2 generation. The rate of mitochondrial H2O2 production was assayed by measuring the increase in fluorescence (excitation at 312 nm, emission at 420 nm) due to oxidation of homovanillic acid by H2O2 in the presence of horseradish peroxidase, essentially as described (3, 39). Reaction conditions were 0.1 mg of mitochondrial protein per ml, 6 U/ml horseradish peroxidase, 0.1 mM homovanillic acid, 50 U/ml of superoxide dismutase (SOD), and 2.5 mM pyruvate/2.5 mM malate, or 5 mM succinate plus 2 μM rotenone as substrates, added at the end (to start the reaction) to the same incubation buffer used for the oxygen consumption measurements, in a total volume of 1.5 ml. Unless otherwise stated, the assays with succinate as substrate were performed in the presence of rotenone to avoid the backwards flow of electrons to complex I. The assays were run in the absence and in the presence of 500 μM ADP. In some experiments, rotenone (2 μM) or antimycin A (10 μM) were additionally included in the reaction mixture to assay maximum rates of complex I or complex III H2O2 generation. Duplicated samples were incubated for 15 min at 37°C. The reaction was stopped by transferring the samples to an ice-cold bath and addition of 0.5 ml of stop solution (2.0 M glycine, 2.2 M NaOH, 50 mM EDTA, pH 12), and the fluorescence was measured at 25°C in a computer-controlled Jasco FP6200 fluorometer (Jasco, Essex, UK), equipped with a thermostatted and magnetic-stirred cell sample holder. Appropriate blanks, with all of the reaction components, but without substrates, were run in parallel to correct the fluorescence of the samples. The rate of peroxide production was calculated using a standard curve of H2O2 (ε230 = 71 M−1·cm−1). The sensitivity of the method (a minimum rate of 10 pmol·min−1·ml−1 can be determined reliably) allows the measurement of H2O2 release by isolated mitochondria that respired in the absence of respiratory chain inhibitors. Since the SOD added in excess converts all superoxide anion radical (O2−•) released by mitochondria (if any) to H2O2, the measurements represent the total (O2−• + H2O2) rate of mitochondrial ROS production.

The H2O2 production and O2 consumption of muscle mitochondria were measured in parallel in the same samples under similar experimental conditions. This allowed the calculation of the fraction of electrons out of sequence, which reduce O2 to ROS at the respiratory chain [the percentage of free radical leak (FRL)], instead of reaching cytochrome oxidase to reduce O2 to water. Since two electrons are needed to reduce 1 mol of O2 to H2O2, whereas four electrons are transferred in the reduction of 1 mol of O2 to water, the percent FRL was calculated as the rate of H2O2 production divided by two times the rate of O2 consumption, and the result was multiplied by 100 (3). The lower the FRL, the higher is the efficiency of the mitochondria in avoiding oxygen radical generation at the respiratory chain.
Enzymatic activities. Aconitase activity was determined in muscle mitochondria according to Gardner (13). Previously, the mitochondrial samples were diluted with isolation buffer (220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4) to a final concentration of 3 mg/ml, sonicated for 30 s, and centrifuged at 14,000 g for 30 s, after which the supernatants were quickly used to determine the enzymatic activity. Aconitase activity is expressed as nanomoles of NADPH per minute per milligram mitochondrial protein.

Mn-SOD activity was measured in muscle mitochondria following the inhibition of the rate of pyrogallol autooxidation, according to Marklund and Marklund (29). One unit of SOD activity was defined as the amount required to inhibit the rate of pyrogallol oxidation by 50%. Glutathione peroxidase (GPX) was measured following NA-DPH oxidation at 340 nm in the presence of excess glutathione reductase, glutathione, and H2O2 as substrate, as described (12). GPX activity is expressed as micromoles of NADPH per minute per milligram mitochondrial protein.

Oxidation-derived protein damage markers. GSA, AASA, CML, and MDAL were determined as trifluoroacetic acid methyl esters derivatives in acid hydrolyzed delipidated and reduced mitochondrial protein samples by gas chromatography-mass spectrometry (GC-MS) using an isotope dilution method, as previously described (31). GC-MS analyses were carried out on a Hewlett-Packard 6890 Series II gas chromatograph (Agilent, Barcelona, Spain) equipped with a HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm), coupled to a Hewlett-Packard model 5973A mass selective detector using the previously described temperature program (31). Quantification was performed by external standardization using standard curves constructed from mixtures of deuterated and nondeuterated standards. Analyses were carried out by selected ion-monitoring GC-MS. The ions used were as follows: lysine and [2H5]lysine, mass-to-charge ratio (m/z) 180 and 187, respectively; 5-hydroxy-2-aminovaleric acid and [14C]5-hydroxy-2-aminovaleric acid (stable derivatives of GSA), m/z 280 and 285, respectively; 6-hydroxy-2-aminocaproic acid and [14C]6-hydroxy-2-aminocaproic acid (stable derivatives of AASA), m/z 294 and 298, respectively; CML and [14C]CML, m/z 392 and 396, respectively; CEL and [14C]CEL, m/z 379 and 383, respectively; and MDAL and [14C]MDAL, m/z 474 and 482, respectively. The amounts of product were expressed as the ratio micromoles of GSA, AASA, CML, CEL, or MDAL per mole of lysine.

Fatty acid analysis. Fatty acyl groups of muscle mitochondrial lipids were analyzed as methyl ester derivatives by GC-MS, as previously described (31). Separation was performed in a SP2330 capillary column (30 m × 0.25 mm × 0.20 μm) in a GC Hewlett Packard 6890 Series II gas chromatograph (Agilent, Barcelona, Spain). A Hewlett Packard 5973A mass spectrometer was used as detector in the electron-impact mode. Identification of fatty acyl derivatives of GSA, AASA, CML, and MDAL was performed by external standardization using standard curves constructed from mixtures of deuterated and nondeuterated standards. Analyses were carried out by selected ion-monitoring GC-MS. The ions used were as follows: lysine and [2H5]lysine, mass-to-charge ratio (m/z) 180 and 187, respectively; 5-hydroxy-2-aminovaleric acid and [14C]5-hydroxy-2-aminovaleric acid (stable derivatives of GSA), m/z 280 and 285, respectively; 6-hydroxy-2-aminocaproic acid and [14C]6-hydroxy-2-aminocaproic acid (stable derivatives of AASA), m/z 294 and 298, respectively; CML and [14C]CML, m/z 392 and 396, respectively; CEL and [14C]CEL, m/z 379 and 383, respectively; and MDAL and [14C]MDAL, m/z 474 and 482, respectively. The amounts of product were expressed as the ratio micromoles of GSA, AASA, CML, CEL, or MDAL per mole of lysine.

Mitochondrial rates of oxygen consumption and H2O2 production. The rate of oxygen consumption by muscle mitochondria was measured without (state 4) and with (state 3) ADP in the presence of either complex I- (pyruvate/malate) or complex II-linked (succinate plus rotenone) substrates (Table 1). The addition of ADP markedly increased the rate of oxygen consumption in all cases, indicating the good quality and tight coupling of the mitochondrial preparations. No significant differences in oxygen consumption were found among the experimental groups with any substrate in either state 4 or state 3.

Table 1. Oxygen consumption by skeletal muscle mitochondria from control and stanozolol-treated rats

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Control</th>
<th>C-Ex</th>
<th>ST</th>
<th>ST-Ex</th>
</tr>
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<tbody>
<tr>
<td>Pyr/Mal</td>
<td>22 ± 3</td>
<td>23 ± 3</td>
<td>25 ± 2</td>
<td>22 ± 3</td>
</tr>
<tr>
<td>Succ (Rot)</td>
<td>97 ± 12</td>
<td>104 ± 15</td>
<td>101 ± 12</td>
<td>104 ± 15</td>
</tr>
<tr>
<td>Pyr/Mal</td>
<td>127 ± 18</td>
<td>135 ± 23</td>
<td>138 ± 24</td>
<td>129 ± 30</td>
</tr>
<tr>
<td>Succ (Rot)</td>
<td>239 ± 34</td>
<td>271 ± 36</td>
<td>251 ± 33</td>
<td>254 ± 37</td>
</tr>
</tbody>
</table>

Values are means ± SE in nmol O2·min⁻¹·mg mitochondrial protein⁻¹ from 8 different animals. C, sedentary controls; C-Ex, exercised controls; ST, stanozolol-treated sedentary rats; ST-Ex, stanozolol-treated exercised rats; Pyr/Mal, 2.5 mM pyruvate/2.5 mM malate; Succ (Rot), 5 mM succinate plus 2 μM rotenone; State 4, substrate alone; State 3, substrate + 500 μM ADP. No significant differences between groups were found with any substrate.

RESULTS

Mitochondrial rates of oxygen consumption and H2O2 production. The rate of oxygen consumption by muscle mitochondria was measured without (state 4) and with (state 3) ADP in the presence of either complex I- (pyruvate/malate) or complex II-linked (succinate plus rotenone) substrates (Table 1). The addition of ADP markedly increased the rate of oxygen consumption in all cases, indicating the good quality and tight coupling of the mitochondrial preparations. No significant differences in oxygen consumption were found among the experimental groups with any substrate in either state 4 or state 3.

The results concerning the mitochondrial rate of H2O2 production are shown in Fig. 1. The exercise session increased the rate of mitochondrial H2O2 generation with both the complex I- and complex II-linked substrates in the C group, but not in the ST animals. Similar results were obtained when mitochondria respired in the presence of ADP (data not shown). Maximal rates of H2O2 production were measured using appropriate combinations of substrates and inhibitors of the respiratory chain. No significant differences in H2O2 generation among the experimental groups were observed with pyruvate/malate plus rotenone (full reduction of complex I) or succinate (+ rotenone) plus antimycin A (full reduction of complex III).

As shown in Fig. 2, the percentage of electrons directed to ROS generation in the respiratory chain (the FRL) with both pyruvate/malate and succinate as substrates was augmented by exhaustive exercise to 187 and 138%, respectively, with respect to the values of the sedentary C group. Stanozolol treatment prevented the exercise-induced increase in FRL.

Mitochondrial enzymatic activities. After the exercise session, mitochondrial aconitase activity was significantly reduced (P < 0.05) in C rats, whereas a nonsignificant small change was detected in the ST group (Fig. 3). On the other hand, stanozolol treatment had no effect on aconitase activity in sedentary animals.

Graphic). 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.
The activities of the mitochondrial antioxidant enzymes SOD and GPX are shown in Fig. 4. SOD activity was not affected in sedentary rats by stanozolol treatment. After the single session of exercise, the enzymatic activity increased in the C group, but not in the ST animals. On the other hand, neither steroid administration nor exhaustive exercise modified GPX activity. It is well documented that exercise induces an increase in SOD activity in active muscles, whereas the information regarding the effects of a single session of exercise on GPX activity is limited and controversial (21). In general, increases in this enzymatic activity are detected in highly oxidative muscles after intense sessions of exercise. Our data are consistent with these results, since rat gastrocnemius is a mixed muscle containing low percentages of type I and type IIa fibers, and the bout of exercise performed by our rats can be regarded as being of moderate intensity and medium duration.

Fig. 1. Rates of H$_2$O$_2$ production of skeletal muscle mitochondria from control and stanozolol-treated rats in the presence of substrates and complex I- and III-specific inhibitors. Pyr/Mal, 2.5 mM pyruvate/2.5 mM malate; Succ, 5 mM succinate in the absence or in the presence of 2 μM rotenone (Rot); AA, 10 μM antimycin A; C, sedentary controls; C-Ex, exercised controls; ST, stanozolol-treated sedentary rats; ST-Ex, stanozolol-treated exercised rats. Results are means ± SE from 8 different animals. *$P < 0.05$, significant differences between C and C-Ex groups. #$P < 0.05$, significant differences between ST-Ex and C-Ex groups.

Fig. 2. Free radical leak (FRL; %) of skeletal muscle mitochondria from control and stanozolol-treated rats. The FRL is the percentage of the total electron flow in the respiratory chain directed to oxygen radical generation (see MATERIALS AND METHODS). Results are means ± SE from 8 different animals. *$P < 0.05$, significant differences between C and C-Ex groups. #$P < 0.05$, significant differences between ST-Ex and C-Ex groups.
Levels of protein oxidative modification. The levels of all of the protein markers of oxidative, glycoxidative, and lipoxidative damage are shown in Fig. 5. All of the markers assayed were significantly increased in the C-Ex group compared with the sedentary group, suggesting an overall increased status of protein oxidative damage induced by the session of acute exercise. GSA, AASA, CEL, and CML augmented to 154, 186, 160, and 143% of control values, respectively, whereas, in the case of MDAL, the increase was particularly marked (415%). Stanozolol treatment reduced the extent of oxidative damage caused to mitochondria by the exercise session, since the levels of all of the markers surveyed were significantly lower in the ST rats than in the nontreated animals. It is interesting to note that steroid administration modified the levels of protein damage markers in mitochondria of sedentary animals as the values found in the ST group were lower than those of the C group, although the differences only reached statistical significance for GSA, CEL, and MDAL content.

Membrane unsaturation and fatty acid profile of muscle mitochondria. The full fatty acid composition of muscle mitochondrial lipids is shown in Table 2. Stanozolol treatment of sedentary animals did not change the fatty acid composition, except for an increase in the content of myristic acid (14:0) and a slight but significant decrease in the unsaturated 18:3 (n-3). Concerning the global indexes of fatty acid unsaturation, no significant differences of DBI and PI were detected between mitochondrial membrane lipids from C and ST rats. On the other hand, the exercise session altered the fatty acid composition of mitochondrial membranes in untreated rats, so that the total number of DBI and PI were significantly increased in the exercised group compared with the sedentary group. The fatty acid mainly responsible for these changes was the highly unsaturated docosahexaenoic acid [22:6 (n-3)] that augmented to 164% (P < 0.001) of control value, resulting in a significant increase in the PUFA and PUFA n-3 content. In contrast, the single bout of exercise did not modify the overall fatty acid composition of mitochondria from ST animals, except for a significant increase in the content of the saturated 14:0. Thus the fatty acid profile of mitochondria from the ST-Ex rats was markedly different from that of C-Ex animals: the more relevant changes were the higher content of saturated and mono-unsaturated fatty acids as 14:0 and 18:1 (n-9), and the lower content of the highly unsaturated arachidonic [20:4 (n-6)] and docosahexaenoic [22:6 (n-3)] acids. The global effect from the point of view of fatty acid unsaturation was that both the DBI and the PI were increased after the exercise session in the C group, whereas they remained unchanged in the ST group.

DISCUSSION

The main finding of the present work is that a treatment with the anabolic androgenic steroid stanozolol protected rat skeletal muscle mitochondria against oxidative damage of proteins and changes in membrane fatty acid composition induced by acute exercise. The protective effect of stanozolol was not due to an increase in mitochondrial antioxidant enzyme activities, but rather to a decreased mitochondrial ROS generation. Acute exercise has been consistently shown to result in oxidative damage of lipids, proteins, and DNA in exercising skeletal muscles (35), but the source(s) of oxidants during exercise is controversial at present. Mitochondria have been
considered as a main source of ROS during exercise, but a number of recent findings have argued against this idea (18, 47), and xanthine oxidase or NAD(P)H oxidase enzymes have emerged as physiological ROS generators (10, 14). Our results show that an acute bout of exercise augmented in C rats the rate of mitochondrial ROS generation with complex I- and complex II-linked substrates. These results are in agreement with previous reports showing that ROS production by isolated rat muscle mitochondria increased after a single session of swimming (48) or treadmill running exercise (22) and suggest that mitochondria is indeed a source of ROS during exercise.

A complementary approach to determine whether mitochondrial oxidant production is increased in vivo is to quantify stable end-products of oxidative reactions in the organelle itself. There is some experimental evidence for the accumulation of oxidative damage within muscle mitochondria after a single bout of intense exercise. Acute swimming increased ortho-tyrosine, meta-tyrosine, and o,o'-dityrosine levels in mitochondrial proteins of rat heart muscle (27), and hydroperoxide and protein-bound carbonyl content in mitochondria of rat skeletal muscle (48). The effect of acute treadmill running is unclear, since increases or no changes in thiobarbituric acid reactive substances levels in skeletal muscle mitochondria have been reported (20, 25). These discrepant results are likely related to the use of the nonspecific thiobarbituric acid assay to estimate lipid peroxidation. In this work, we used GC-MS to quantify levels of highly specific markers of irreversible protein oxidation. GSA and AASA are the main carbonyl products of metal-catalyzed protein oxidation and arise from the direct reaction of proline and arginine, and lysine, respectively (37). CEL is derived from the reaction of methylglyoxal, a by-product of glycolysis, with lysine, thus being used as surrogate for protein glycoxidation. MDAL arises from the addition of the carbonyl compound malondialdehyde, derived from lipid peroxidation, and lysine. For this reason, its levels express protein lipoxidative modification. Finally, CML is a mixed glycoxidative-lipoxidative product, as its precursor glyoxal is originated by both reactions. Our results show that detectable levels of these markers were present in mitochondrial proteins of sedentary rats. This observation suggests that a baseline level of protein oxidative damage exists in skeletal muscle mitochondria. It is noteworthy that chronic stanozolol administration reduced GSA, CEL, and MDAL levels in sedentary rats. The steady-state level of oxidatively modified proteins in mitochondria of a given tissue is dependent on ROS production, ROS removal, protein susceptibility, and oxidized protein repair and degradation (5). We show that neither mitochondrial ROS production nor antioxidant enzymatic activities were modified by steroid treatment. Thus it is tempting to speculate that chronic stanozolol administration might favor the turnover of irreversibly oxidized proteins, increasing the expres-

![Fig. 5. Markers of protein oxidation [glutamic (GSA) and aminoadipic semialdehydes (AASA)], glycoxidation [carboxethyl-lysine (CEL) and carboxymethyl-lysine (CML)], and lipoxidation [malondialdehyde-lysine (MDAL)] in skeletal muscle mitochondria from control and stanozolol-treated rats. Values are means ± SE from 7 different animals. Significant differences between *exercised and nonexercised groups, and #stanozolol-treated and nontreated groups: P < 0.05.](http://jap.physiology.org/)
sion of the mitochondrial proteases responsible for their degrada-
tion (45).

The exercise session markedly increased the levels of all of the
protein oxidation markers and reduced the activity of the super-
oxide-sensitive enzyme aconitase in C rats. These results provide
direct evidence for protein oxidative damage within mitochondria,
from which we can infer that mitochondrial ROS production was
increased in vivo during acute exercise. The fact that mitochon-
drial SOD activity augmented suggests an activation of the enzy-
me to cope with the increased levels of superoxide. Therefore,
our data strongly support the idea that muscle mitochondria are
physiologically relevant sources of ROS during intense running
exercise.

Taking into account the aforementioned results, we consider
that the increased rate of ROS production by isolated mitochon-
dria from exercised C rats is not an artifact resulting from the
intrinsic limitations of the in vitro measurement, but rather a
consequence of the exercise-induced changes in mitochondrial
function. Mitochondrial ROS release increased by pyruvate/ 
malate and succinate plus rotenone as substrates, indicating that
both the complex I and the complex III are responsible for the
augmented exercise-linked ROS generation. This effect was not
due to an increase in the concentration of respiratory complexes,
as maximal rates of complex I, or complex III H2O2 generation,
measured in the presence of rotenone and antimycin A, were not
different in the sedentary and the C-Ex groups. However, the
exercise session increased FRL with both respiratory substrates,
suggesting a reduced efficiency of the respiratory chain for elec-
don transport, especially at the level of complex I.

Interestingly, stanozolol treatment was able to protect muscle
mitochondria from exercise-induced oxidative stress, since levels of
all of the protein oxidation markers were lower in the ST than
in the nontreated group, and aconitase activity was not signifi-
cantly diminished. This effect could be due to a more efficient
ROS scavenging or to a reduced ROS generation during the
exercise session. The first possibility appears unlikely, since
stanozolol administration did not modify antioxidant activities.
However, the rate of ROS production by isolated mitochondria
from steroid-treated rats and the FRL parameter were not in-
creased after acute exercise, suggesting that stanozolol treatment
preserves, in some way, mitochondrial function.

Our study does not provide direct evidence to clarify the
mechanism responsible for this effect, but a possible explanation
is presented. The fatty acid composition of mitochondrial lipids
was modified in C rats after the exercise session, the most
significant change being a marked increase of the highly unsatu-
rated docosahexaenoic acid [22:6 (n-3)]. Some correlate of physi-
ological activity rather than the activity itself could be responsible
for this rapid response. Gudbjarnason (15) showed that catechol-
amine stress can alter the fatty acid composition of cardiac
phospholipids, causing an increase in the content of arachidonic
and docosahexaenoic acids. This is similar to the effect observed
in muscle mitochondria from C-Ex rats in the present study, and
thus it is conceivable that these changes may have been the result
of catecholamine stress during the exercise session. An increased
activity of Δ5 and Δ6 desaturases could explain the modification
of fatty acid composition. However, it seems unlikely that a 64%
increase in docosahexaenoic acid content could be accomplished
during the time of exercise session. A more likely alternative
explanation would be a rapid recycling of phospholipid fatty
acids. Lipid remodeling requires deacylation and reacylation of
phospholipids by phospholipase A2 and acyltransferase and transacylase enzymes; it is a very rapid process (30, 42) that may
have occurred during the ~56 min of exhaustive exercise per-
formed by the C-Ex rats.

### Table 2. Fatty acyl composition of total lipids in skeletal muscle mitochondrial from control and stanozolol-treated rats

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Control</th>
<th>C-Ex</th>
<th>ST</th>
<th>ST-Ex</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>0.82 ± 0.14</td>
<td>0.54 ± 0.07</td>
<td>2.44 ± 0.55&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.08 ± 0.71&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>16:0</td>
<td>20.39 ± 0.45</td>
<td>19.46 ± 0.49</td>
<td>20.04 ± 0.29</td>
<td>19.67 ± 0.37</td>
</tr>
<tr>
<td>16:1 (n-7)</td>
<td>0.67 ± 0.05</td>
<td>0.58 ± 0.08</td>
<td>0.71 ± 0.06</td>
<td>0.67 ± 0.08</td>
</tr>
<tr>
<td>18:0</td>
<td>18.34 ± 0.73</td>
<td>17.74 ± 0.70</td>
<td>18.14 ± 0.31</td>
<td>16.78 ± 0.48</td>
</tr>
<tr>
<td>18:1 (n-9)</td>
<td>8.44 ± 0.98</td>
<td>6.21 ± 0.42</td>
<td>8.99 ± 0.82</td>
<td>9.71 ± 0.90&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:2 (n-6)</td>
<td>17.73 ± 1.23</td>
<td>17.60 ± 1.02</td>
<td>17.19 ± 0.61</td>
<td>15.39 ± 0.45</td>
</tr>
<tr>
<td>18:3 (n-3)</td>
<td>0.80 ± 0.12</td>
<td>0.27 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.53 ± 0.05&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.59 ± 0.07&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>20:4 (n-6)</td>
<td>13.96 ± 0.79</td>
<td>16.12 ± 0.40</td>
<td>13.72 ± 0.95</td>
<td>12.44 ± 0.76&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>20:5 (n-3)</td>
<td>1.66 ± 0.44</td>
<td>0.28 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.51 ± 0.46</td>
<td>2.17 ± 0.52&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>22:4 (n-6)</td>
<td>1.54 ± 0.35</td>
<td>0.41 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.17 ± 0.21</td>
<td>1.32 ± 0.32&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>22:5 (n-6)</td>
<td>1.99 ± 0.23</td>
<td>1.11 ± 0.13</td>
<td>1.79 ± 0.25</td>
<td>2.13 ± 0.36&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>22:6 (n-3)</td>
<td>2.95 ± 0.23</td>
<td>2.13 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.55 ± 0.19</td>
<td>2.83 ± 0.22&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>ACL</td>
<td>10.65 ± 0.96</td>
<td>17.49 ± 0.62&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.16 ± 0.33</td>
<td>12.16 ± 0.96&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>SFA</td>
<td>18.54 ± 0.02</td>
<td>18.75 ± 0.02</td>
<td>18.45 ± 0.04</td>
<td>18.46 ± 0.02</td>
</tr>
<tr>
<td>UFA</td>
<td>39.56 ± 1.04</td>
<td>37.75 ± 1.08</td>
<td>40.64 ± 0.85</td>
<td>40.54 ± 0.81</td>
</tr>
<tr>
<td>MUFA</td>
<td>60.43 ± 1.04</td>
<td>62.24 ± 1.08</td>
<td>59.35 ± 0.85</td>
<td>59.45 ± 0.81</td>
</tr>
<tr>
<td>PUFA n-6</td>
<td>9.11 ± 1.01</td>
<td>6.80 ± 0.46</td>
<td>9.71 ± 0.87</td>
<td>10.38 ± 0.98&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>PUFA n-3</td>
<td>51.32 ± 1.41</td>
<td>55.44 ± 1.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>49.64 ± 1.05</td>
<td>49.07 ± 0.44&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>DBI</td>
<td>36.91 ± 1.18</td>
<td>35.54 ± 1.22</td>
<td>35.39 ± 1.13</td>
<td>33.47 ± 0.65</td>
</tr>
<tr>
<td>PI</td>
<td>14.41 ± 0.88</td>
<td>19.89 ± 0.66&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14.25 ± 0.38</td>
<td>15.59 ± 0.69&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>206.05 ± 4.69</td>
<td>231.60 ± 3.38&lt;sup&gt;b&lt;/sup&gt;</td>
<td>201.56 ± 3.55</td>
<td>206.74 ± 1.67&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>206.54 ± 6.37</td>
<td>245.61 ± 4.51&lt;sup&gt;b&lt;/sup&gt;</td>
<td>202.55 ± 4.12</td>
<td>212.10 ± 3.40&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are means ± SE in mol% from 7 different animals. ACL, acyl chain length; SFA, saturated fatty acids; UFA, unsaturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; DBI, double bond index; PI, peroxidizability index. Fatty acid indexes and their calculation are
detailed in MATERIALS AND METHODS. Significant differences between groups: C-Ex vs. C: *P < 0.05, *P < 0.001; ST vs. C: *P < 0.05; ST-Ex vs. C-Ex: 4P < 0.05, 5P < 0.01, 6P < 0.001; ST-Ex vs. ST: 6P < 0.05.
Whatever the mechanism involved, the changes in lipid fatty acid composition could alter mitochondrial function, leading to an enhanced ROS production. The increased PUFA n-3 content can modify mitochondrial membrane properties, particularly membrane fluidity. These changes could affect the electron transport through the respiratory chain complexes. In this regard, mitochondrial membrane fluidity has been shown to increase after acute exercise in heart and oxidative muscles (32). Furthermore, docosahexaenoic acid has been reported to increase oxidant production in HT-29 cells by accumulating in cardioplin in the inner mitochondrial membrane, where its presence alters electron transport efficiency (50). On the other hand, the higher content of PUFA would increase the susceptibility of mitochondrial membranes to lipid peroxidation. Our finding that postexercise levels of MDAL increased in a greater extent than those of GSA, AASA, CEL, and CML is consistent with this idea.

Surprisingly, stanozolol treatment prevented the exercise-induced increase in PUFA content. The maintenance of the fatty acid composition in mitochondrial membranes may have preserved their properties and the efficiency of the respiratory chain for electron transport, reducing ROS production during the exercise session and, hence, oxidative damage to mitochondria. How stanozolol treatment avoids the exercise-induced changes in fatty acid composition is unknown. Recently, it has been shown that steroid hormones can modulate PUFA synthesis; in particular, dihydrotestosterone decreased the long-chain n-3 PUFA and the Δ5 desaturase mRNA contents in neuroblastoma cells supplemented with the precursor α-linolenic acid (11). However, the possibility that stanozolol inhibited the synthesis of the 22:6 (n-3) acid can be discarded, because this effect should have been detected in the sedentary rats treated with the steroid. Another possibility is that stanozolol acts through an indirect mechanism modulating the hormonal response to the stress imposed by the acute exercise, i.e., reducing adrenomedullary activation. In support of this hypothesis, it has been shown that, in situations of metabolic stress, there is an inverse relationship between plasma catecholamine and testosterone levels (9).

Despite stanozolol treatment, which reduced the mitochondrial oxidative damage induced by the exercise session, the resistance to fatigue was not improved, since the running time to exhaustion was similar for both the ST and nontreated exercised groups. This is not surprising, because endurance capacity during whole body exercise depends on a combination of multiple factors at different levels of organization, and, hence, a change in a particular system of skeletal muscle would not be sufficient to improve endurance capacity in a physiological model of increased contractile activity. In contrast, in vitro studies have previously shown that treatment of sedentary rats and mice with testosterone and anabolic androgenic steroids was effective in increasing work capacity and fat-free mass in sedentary rats and mice with testosterone and anabolic androgenic steroids was effective in increasing work capacity and fat-free mass. The exercise-induced increase in ATP production by isolated mitochondria. It is interesting to point out that the lack of increase in mitochondrial ROS generation observed in the ST rats after the running session might entail an interference with the physiological patterns of skeletal muscle response to exercise. Growing evidence indicates that the increased ROS production induced by acute exercise and the resultant alteration of intracellular redox status play an important role in the regulation of signaling pathways that are required to promote muscle adaptation in response to exercise (36). Thus contraction-induced ROS are reported to be involved in the activation of glucose transport in fast-twitch skeletal muscle (40) and in the regulation of glycolysis and expression of glucose metabolism-related genes in skeletal muscle cells (33). Moreover, supplementation with high dose of antioxidants blocks endurance exercise-induced upregulation of transcriptional factors and genes involved in mitochondrial biogenesis, and it precludes the beneficial effects of endurance exercise on insulin sensitivity (28, 38). Hence, stanozolol administration could decrease endurance training efficiency by preventing some ROS-induced cellular adaptations to exercise. On the other hand, our finding that stanozolol treatment reduced the baseline level of oxidized proteins in muscle mitochondria of sedentary rats suggests that the steroid could be of therapeutic value in situations such as aging, sarcopenia, and muscle disuse, in which ROS production is increased and turnover of damaged proteins is reduced. Further work is required to study this possibility, as well as to determine the long-term safety of anabolic androgenic steroid administration, since the potential hepatotoxicity of these compounds, in particular, the 17α-alkylated steroids, cannot be dismissed.

In conclusion, stanozolol treatment protects skeletal muscle mitochondria against protein oxidative modification, avoids the changes in membrane fatty acid composition caused by acute exercise, and prevents the exercise-induced increase of ROS production by isolated mitochondria.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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