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Regulation of neuronal and endothelial nitric oxide synthase by anabolic-androgenic steroid in skeletal muscles

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Summary. Anabolic-androgenic steroids (AAS) and exercise share comparable effects on myogenic differentiation, force development, fiber growth and skeletal muscle plasticity. The participation of nitric oxide synthase (NOS) on these effects was only demonstrated in response to exercise. Using immunohistochemistry and western blotting we examined the effect of AAS on the expression of NOS I and III isoforms in three muscles, distinct metabolically and physiologically: *soleus* (SOL), *tibialis anterioris* (TA) and *gastrocnemius* (GAS). Mice with a lipid profile akin to humans were used. Sedentary mice (Sed-C) or exercised, submitted to six-weeks of aerobic treadmill running (one hour/day, 5 days/week) were administered mesterolone (Sed-M and Ex-M, respectively) or gum arabic (vehicle, Ex-C) during the last three weeks, three alternate days per week. Consistently, The TA showed the strongest labeling and the SOL the weakest with NOS III predominating over NOS I. Mesterolone administered to sedentary mice (Sed-C x Sed-M) significantly upregulated NOS I in TA and SOL and NOS III in all three muscles. Mesterolone administered to exercised mice (Ex-C x Ex-M) upregulated NOS I in all three muscles and NOS III in TA and SOL. The exercise to mesterolone-treated mice (Sed-M x Ex-M) produced a strong increase in NOS I expression in GAS; in contrast it antagonized the mesterolone-induced upregulation of NOS I in TA muscle and NOS III in SOL and GAS. The data show nitric oxide (NO) as a potential signaling mediator of AAS effects in skeletal muscle and that NOS I and NOS III upregulations were muscle phenotype-specific. These may be regarded as an indication of the complex NOS/NO signaling mechanism related with AAS effects vs. metabolic/physiological muscle characteristics.

Key words: Mesterolone, NOS I, NOS III, Chronic Aerobic Exercise, Transgenic Mice

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Introduction

Anabolic-androgenic steroids (AAS) are synthetic derivatives that can increase myogenesis, induce muscle mass gain, strength and fiber transition, decrease body fat, and improve physical performance (Brodsky et al., 1996; Bhasin et al., 2001; see reviews by Hartgens and Kuipers, 2004; Chen et al., 2005; Eriksson et al. 2005; Jasuja et al., 2005). In humans, such effects are also produced by power (resistance) exercise alone (Wackerhage and Ratkevicius, 2008; Wahl et al., 2008; Andersen et al., 2009), or combined with AAS (Kadi et al., 1999; Sinha-Hikim et al., 2003).

Similarly, in humans, studies documented that high intensity aerobic (endurance) exercise produced fiber type redistribution (Gollnick et al., 1972; Simoneau et

Abbreviations: AAS, anabolic-androgenic steroids or mesterolone; CETP, cholesteryl ester transfer protein; Sed, sedentary group; Ex, exercised group; Sed-C, sedentary animals treated with gum Arabic; Sed-M, sedentary animals treated with mesterolone; Ex-C, exercised animals treated with gum Arabic; Ex-M, exercised animals treated with mesterolone; NOS, nitric oxide synthase; NOS I, neuronal type; NOS III, endothelial type; GAS, *gastrocnemius*; SOL, *soleus*; TA, *tibialis anterioris*.

al., 1985), increases in the fiber size (Simoneau et al., 1985) and capillary growth (Jensen et al., 2004). In rats, endurance exercise induced fiber type transition (Dimauro et al., 1992), increases in capillary supply in extensor muscles (Gute et al., 1996) and increased glucose transport (Balon and Nadler, 1997). Using the very same current murine model, which is a transgenic mouse ($CETP^{+/-}LDLr^{-/+}$, with a lipid profile akin to humans) we recently showed the interaction of chronic treadmill training and mesterolone to produce additive adaptive changes in the *soleus* muscle (Fontana et al., 2010). Furthermore, it caused the largest muscle mass gain and hypertrophy in muscle fibers in the *soleus, tibialis anterioris* and *gastrocnemius* muscles, compared to those of sedentary mice treated with mesterolone or exercised treated with gum arabic (unpublished results).

It has been well documented that much of the reported exercise action in skeletal muscle such as strength development (Kobzik et al., 1994; Förstermann et al., 1998), glucose transport (Balon and Nadler, 1997), myogenic differentiation (Pisconti et al., 2006; Tatsumi, 2010) and fiber hypertrophy (Tatsumi, 2010; see also Green et al., 2004 for review) is mediated by NOS/NO system. Overall, it becomes obvious that AAS, exercise training and the NOS/NO system share common targets. However, to the authors knowledge the participation of NOS/NO has only been demonstrated in response to exercise, whereas it has not been addressed in relation to the AAS muscular effects. Here, we evaluate the hypothesis that mesterolone administration combined or not with high intensity aerobic exercise can affect NOS expression in mice. NOS I and III isozymes were assessed through western blotting and immunohistochemistry in three metabolically and physiologically different mouse muscles, *soleus, tibialis anterioris* and *gastrocnemius*. The findings should allow us to infer whether mesterolone ± chronic aerobic exercise training regulates the expression of the two isozymes and if the level of regulation varies accordingly with the muscle phenotype. The results will allow inferring a putative NO signaling mediating the effects produced by anabolic steroids in skeletal muscle.

Materials and methods

Animals

The experimental protocol was approved by the University's Committee for Ethics in Animal Use (CEUA/Unicamp, Protocol 700-1) and followed the "Brazilian College of Animal Experimentation" (COBEA, www.cobea.org.br). The transgenic mice ($CETP^{+/-}LDLr^{-/+}$) used in this study were supplied by Dr. Alan Tall of the Molecular Medicine Division, Columbia University, N.Y., USA and have been cross-bred and kept in the Institute of Biology (ABCPB Dept.) at Unicamp. The mice (C57/BL6 strain) were heterozygous for the human cholesteryl ester transfer protein (CETP) transgene and for the low density lipid (LDL)-receptor null allele (Casquero et al., 2006). The mice were housed in a temperature-controlled room (22±1°C), humidity of 55-65%, 12 h light/dark cycle and had free access to water and standard food (Nuvilab, Colombo, PR, Brazil).

Twenty four young adult 2 month-old male mice ($CETP^{+/-}LDLr^{-/+}$, ~20-22 grams body weight) at the beginning of the experiment and 3.5 months old at sacrifice were used in the experiments. The animals were divided into two groups (n=12/group): the sedentary group (Sed) and the exercised group (Ex). One half of the animals in each group received gum arabic (Sed-C and Ex-C, n=6 each) and the other half received mesterolone (Sed-M and Ex-M, n=6 each). Exercise consisted of treadmill running on a motorized device with 12 separate lanes and controlled speed, each mouse submitted to involuntary running in separate lanes. After a 5-day period of low to moderate level running adaptation (15 m/min during 20 min/day) on a 0 degree grade, the velocity and the duration of daily training was progressively increased from the 1st to the 3rd week (12.42 m/min during 20 min/day to 16.68 m/min for 45 min/day, respectively). Finally, the speed was kept constant at 17.04 m/min for 60 min/day during the last three weeks of training (from 4th to 6th wk). All exercises were carried out for 5 days/week as outlined in detail in Fontana et al. (2008). The animals received either mesterolone (AAS) (Proviron trademark of Schering, Schering do Brasil, São Paulo, SP, Brazil) or gum arabic (vehicle) (Sigma Chemical, St. Louis, MO, USA) (2 μg/g body weight) by orogastric via during the last 3 weeks (three days a week: Monday, Wednesday and Friday) of training or sedentary period. Gum arabic (extracted from Acacia genus), commonly used in the formulation of suspensions of hydropathic pharmaceuticals, was used as a vehicle given its non-toxic and pro-absorptive effect in the small intestine (Codipilly et al., 2006). Mesterolone (1-alpha-methyl-5-alpha-androstan-17-beta-ol-3-one) is a non-17-alpha-alkylated derivative of testosterone. $CETP^{+/-}/LDLr^{+/-}$ mice were used as they have a plasma lipid profile akin to that of humans and thus the data obtained with this study is relevant to those studying human response to AAS use and abuse since variations in the plasmatic lipids could influence the response to AAS and vice-versa.

Muscle samples

After 72 hours from the last period of training, overnight fasted mice were deeply anesthetized with a 1:1 mixture of ketamine chloride (Dopalen, 100 mg/kg of animal) and xylazine chloride (Anasedan, 10 mg/kg,) (2 μl/mg body mass, i.p.). Both anesthetics were obtained from Vetbrands (Jacareí, SP, Brazil). *Soleus* (SOL), *tibialis anterioris* (TA) and *gastrocnemius* (GAS) muscles were excised, and the middle portion of each muscle was separated, oriented in a mixture of gum tragacanth (Sigma, St. Louis, MO, USA) and Tissue-Tek

embedding medium (Sakura Finetechnical Co., Tokyo, Japan) to obtain cross sections of the fibers, and immediately frozen in isopentane cooled to -156-9°C in liquid nitrogen. The tissues were then stored at -70°C until processing. The whole *gastrocnemius* was considered without selection for any particular portion of the muscle.

Western blotting

SOL, TA and GAS muscles (n=12, from the two hindlimbs of each experimental group of 6 animals) were homogenized in an extraction cocktail (10 mM EDTA, 2 mM PMSF, 100 mM NaF, 10 mM sodium pyrophosphate, 10 mM $NaVO_4$, 10 μL of aprotinin/mL and 100 mM Tris, pH 7.4). The homogenates were centrifuged at 3,000 g for 10 min and the supernatants stored at -70°C until use. Equal protein concentrations were obtained from SOL, TA and GAS homogenates as determined with a dye-binding assay using a Bio-Rad reagent (Bio-Rad Laboratories; Richmond, USA) and bovine serum albumin (BSA) as the standard. Aliquots from homogenates were loaded (equal amount of protein per lane for all samples) and run on 6% polyacrylamide gels. After SDS-PAGE, the proteins were transferred by electroblotting to a nitrocellulose membrane and then blocked overnight at 4°C in Tris-buffered saline (TBS, pH 7.4) containing 0.05% Tween 20 with 5% non-fat milk. The membranes were incubated for 2 h at room temperature (RT) with goat polyclonal primary antibody anti-rabbit NOS I and rabbit monoclonal primary antibody anti-mouse NOS III (1:50 in TBS; Transduction Labs, Lexington, KY, USA). Subsequently, the membranes were rinsed (6x10 min) in TBS and then incubated with goat HRP-conjugated secondary antibody anti-rabbit (Sigma, St. Louis, MO, USA) (1:1000 in TBS) for 1.5 h at RT. After rinsing in buffer, the immunoreactive bands were detected by chemiluminescence (Super Signal, Pierce West Pico Chemiluminescent Substrate, Rockford IL, USA) using X-ray film (BioMax XAR Film Kodak, Rochester, NY, USA). Proteins bound to the antibodies were detected by autoradiography using preflashed Kodak BioMax XAR film (Eastman Kodak; Rochester, NY). Band intensities were quantified by optical densitometry of the developed autoradiographs using UN- SCANN- IT version 6.1 (Silk Scientific Corporation, Utah, USA).

Immunohistochemistry

Cryostat cross-sections (12 μm thick) from SOL, TA and GAS muscles (n=12 muscles/per experimental group) were collected on silane-coated glass slides, air dried and fixed in cold acetone (4°C) for 4 min at RT. The sections were washed in 0.05 M Tris-buffered saline, pH 7.4 (TBS) for 5 min at RT. To quench endogenous peroxidase activity, sections were treated with methanol containing 0.3% H_20_2 for 30 min at RT, and then rinsed with TBS (4x5 min). Non-specific sites were blocked with TBS containing 5% non-fat milk at RT for 1 h, and then incubated at 4°C overnight with goat polyclonal primary antibody anti-rabbit NOS I and rabbit monoclonal primary antibody anti-mouse NOS III (1:50 in TBS containing 1% non-fat milk; Santa Cruz Biotechnol., Santa Cruz, CA, USA). After washing in TBS, the bound primary antibodies were detected by incubating sections with a goat HRP-conjugated secondary antibody against rabbit (Santa Cruz) (diluted 1:4 with serum blocking solution) for 30 min at RT, followed by washing in TBS and incubation with HRP-streptavidin (Santa Cruz) for 30 min. After washing in TBS (4x5 min), the reaction was developed using 3,3-diaminobenzidine (DAB, Sigma). The slides were counterstained with Harris's hematoxylin. For negative controls, the primary antibodies were omitted.

Statistical analysis

All numerical results were analyzed using the Origin software package (Microcal™ Software Inc., Northampton, MA, USA) and expressed as the mean ± standard deviation (SD). The statistical significance among the control and treated groups was set at $P<0.05$ and determined by One-way ANOVA followed by the Tukey test.

Results

The behavior of the animals was monitored during the exercise training. Ex-M and Ex-C animals exhibited a similar normal behavior. Interestingly, animals treated with mesterolone trained with the tail upwards whereas those treated with gum arabic did not. As regards exhaustion, mice from both exercised groups (either treated with gum arabic or mesterolone) showed signs of exhaustion in the first three weeks of training.

At the end of the 7th week of exercise (one of adaptation + six of training), the animals clearly demonstrated higher tolerance to exercise as they were able to run for 60 min with no apparent indication of exhaustion. However, invariably, after completion of exercise training the animals remained drowsy for about two hours. It is worth noting that sedentary animals treated with mesterolone (Sed-M group) occasionally showed aggressiveness towards their cage companions and needed to be separated. Such behavior was not observed in Ex-M mice.

Western blotting

The TA muscles of all groups of mice (Sed-C, Ex-C, Sed-M, Ex-M) showed the highest expression of NOS I and NOS III, always with prevalence of NOS III over NOS I in the present transgenic mouse model. In contrast, SOL muscles from all groups showed the least expression of both isozymes. The higher the proportion of fast glycolytic fibers in the muscle the higher the intensity of the NOSs reactivity, such that

SOL<GAS<TA. NOS I expression in TA muscle of Sed-C animals (taken as control) was higher by 1.8-fold than in GAS and by 2.3-fold than in SOL (P<0.05). NOS III expression in TA muscles was higher by 2-fold than in GAS and by 4-fold than in SOL (P<0.05). The administration of mesterolone to sedentary animals (Sed-M), or the exercise to animals treated with gum arabic (Ex-C), or both combined (Ex-M) affected NOS III expression less than NOS I expression.

Sed-C x Sed-M

The mesterolone treatment of sedentary mice significantly increased by 11% the expression of NOS I in SOL muscle and by 30.4% in TA. NOS III expression was increased by 9% in SOL and GAS muscles and by 3.7% in TA (P<0.05).

Ex-C x Ex-M

When mesterolone was administered to trained mice the expression of NOS I showed a significant 9.5% increase in SOL; however it dramatically increased by 56.6% in TA. Interestingly, NOS I which had remained unaffected in GAS muscles of mesterolone-treated sedentary animals (Sed-M) increased by 95.2% in Ex-M mice. In contrast, NOS III expression increased by 5.5% in SOL and by 6.8% in TA muscle whereas it was

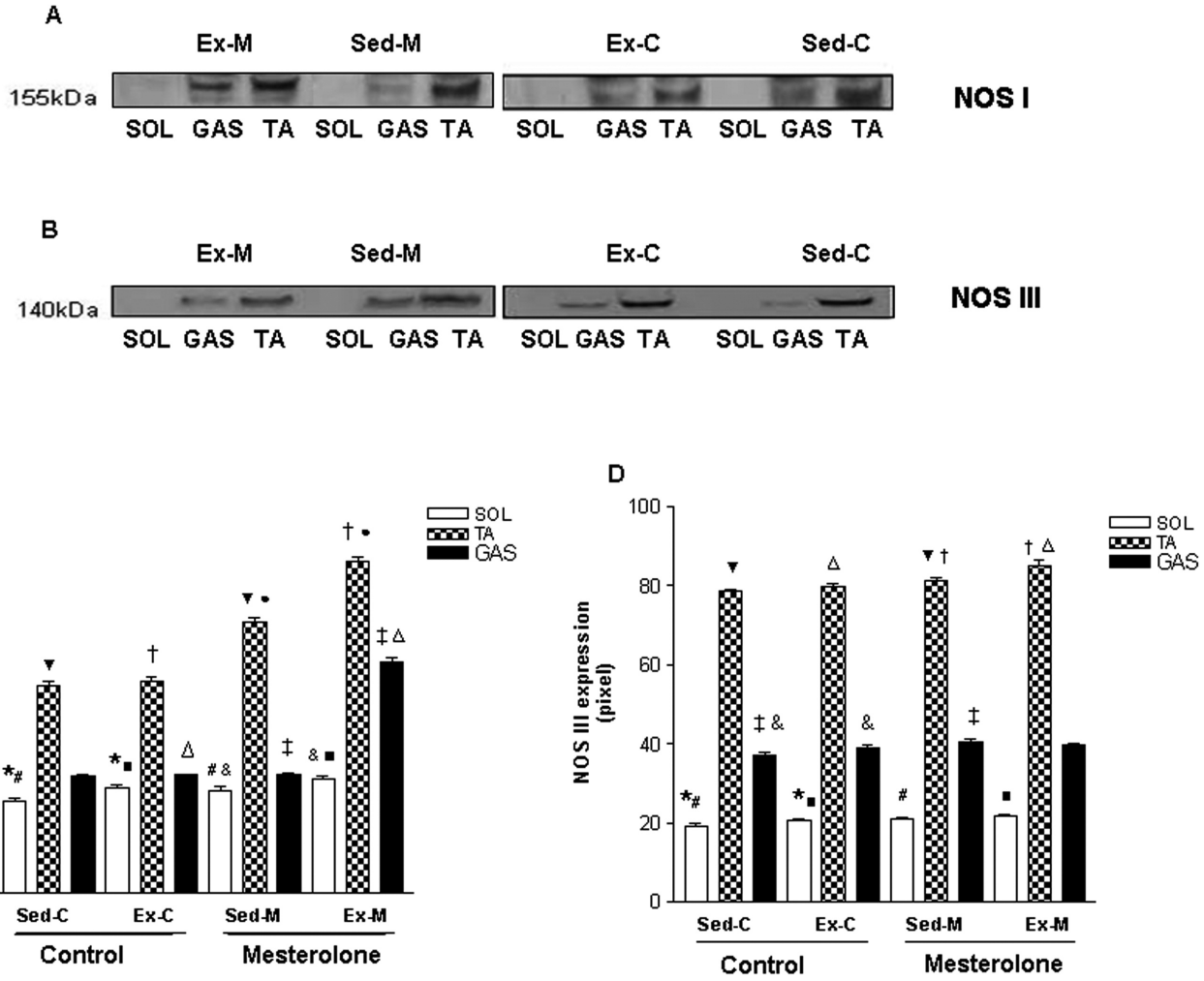

Fig. 1. NOS I **(A)** and NOS III **(B)** immunoblots in *soleus* (SOL), *tibialis anterioris* (TA) and *gastrocnemius* (GAS) of sedentary mice administered either gum arabic (Sed-C group) or mesterolone (Sed-M group) and of exercised mice administered gum arabic (Ex-C group) or mesterolone (Ex-M group). Note the weak expression level of NOS I and NOS III in the SOL muscle and the strong expression level in TA and GAS muscles; **C** and **D** panels are graphs representative of the density of pixels of each blot. Each column represents the mean ± S.D of the protein investigated. The results were confirmed in three sets of experiments (n=12 muscles/group) NOS I: SOL: #*& P< 0.001 and ■ P< 0.01; TA: • †▼ P< 0.001; GAS: Δ ‡ P< 0.001; NOS III: SOL: # P< 0.001 and *■ P< 0.01; TA: Δ†▼ P< 0.001; GAS: &‡ P< 0.001. One-way analysis of variance (ANOVA) followed by the Tukey test was used to compare groups.

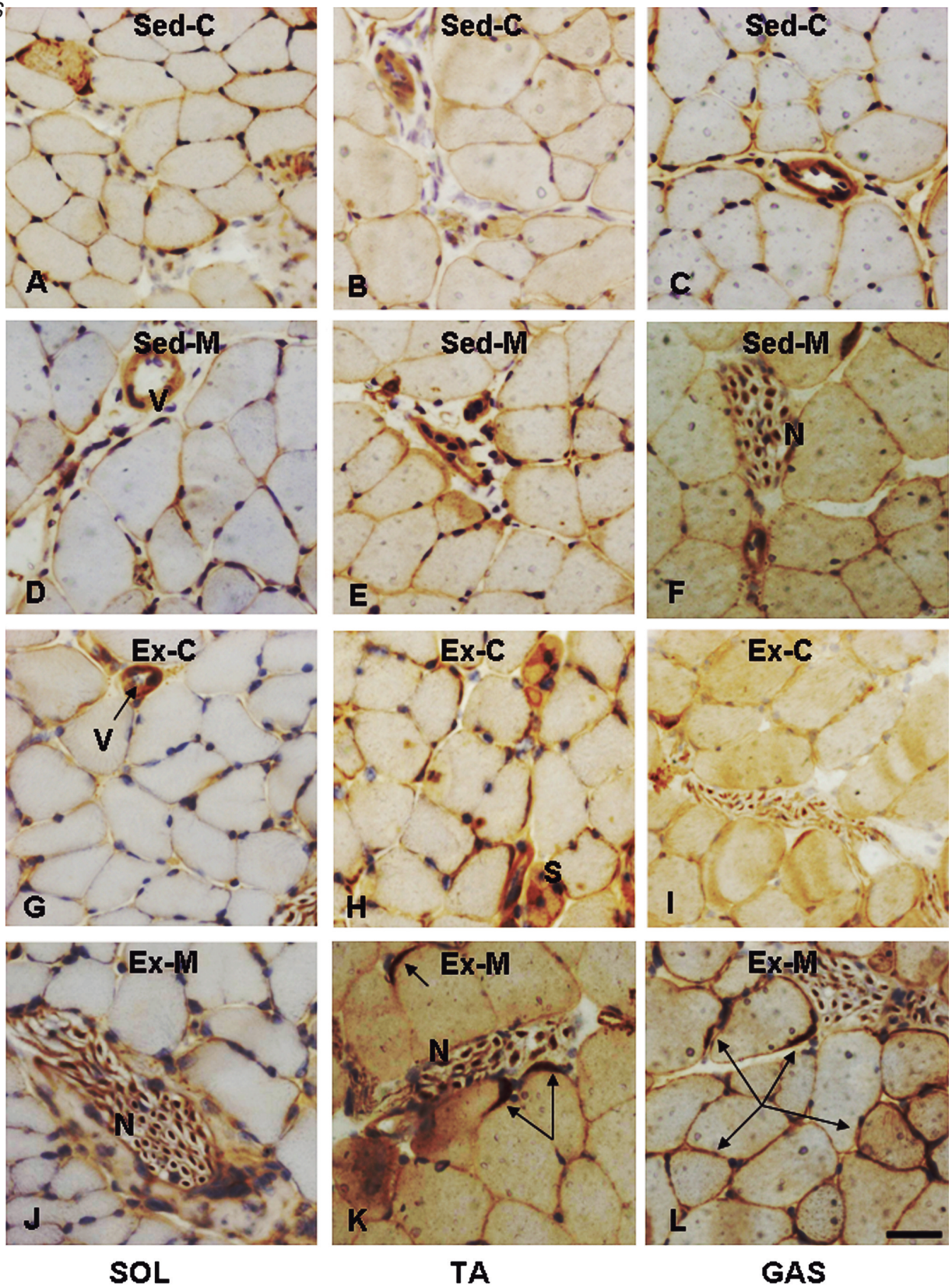

Fig. 2. Immunohistochemistry of NOS I in transversal sections of s*oleus, (SOL) tibialis anterioris (TA), and gastrocnemius (GAS)* muscles from sedentary animals treated with gum arabic (Sed-C) or mesterolone (Sed-M) or mice submitted to treadmill running treated with gum arabic (Ex-C) or with mesterolone (Ex-M). Morphology of fibers looks normal in all experimental groups. Note that difference in the immunoreactivity of NOS I among muscles was subtle. NOS I immunolabeling in SOL **(A-D-G-J)** was less intense than in TA **(B-E-H-K)** and GAS **(C-F-I-L)** muscle; however TA and GAS muscles from the Ex-M group exhibited stronger reactivity than the other groups; the changes in NOS I reactivity for SOL muscle were visually very subtle. The enzyme labeling appeared strong in the subsarcolema (arrows), in the blood vessel wall (V), in muscle spindles (S) and in motor nerve trunks (N). Bar: 40 μm

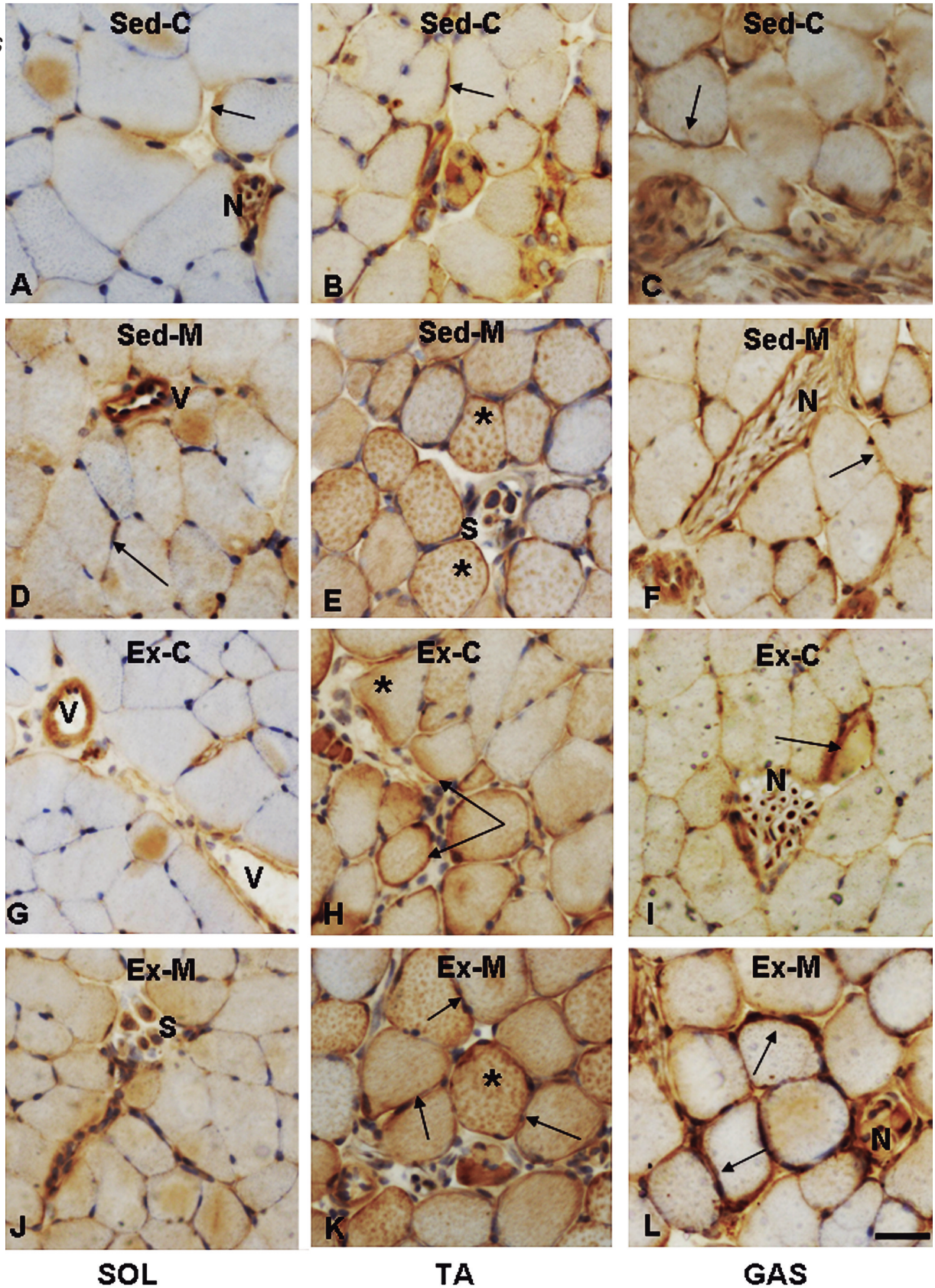

Fig. 3. Immunohistochemistry of NOS III in transversal sections of *soleus* (SOL), *tibialis anterioris* (TA) and *gastrocnemius* (GAS) muscles from sedentary mice treated with gum arabic (Sed-C) or mesterolone (Sed-M) or mice submitted to treadmill running treated with gum arabic (Ex-C) or with mesterolone (Ex-M). Muscle fibers of all the three muscles regardless the experimental group exhibit normal morphology. Note that SOL showed the weakest anti-NOS III positivity (**A-D-G-J**), whereas TA showed the strongest (**B-E-H-K**) followed by GAS (**C-F-I-L**). Muscles from Sed-M and Ex-M groups exhibit stronger immunoreactivity than Sed-C and Ex-C groups. Anti-NOS III immunolabeling was found in the endothelial wall of blood vessels (V), axons of motor nerve trunks (N), intrafusal fibers (spindle fibers) (S), and both in the sub-sarcolemmal region (arrows) and intermyofibrillar sarcoplasmic region (asterisks) of extrafusal fibers. Bar: 40 μm

unaltered in GAS (P<0.05).

Sed-C x Ex-C

The exercise alone significantly increased NOS I expression only in SOL (by 13.7%) whereas it increased NOS III by 7.4% in SOL and by 5% in GAS muscle. Exercise did not affect the expression of either isozymes in TA and NOS I in GAS (P<0.05).

Sed-M x Ex-M

Exercise training to mesterolone-treated mice also differentially modulated NOS I and NOS III expression in each muscle. In Ex-M mice, NOS I expression increased significantly by 95.3%, 22.3% and 11.5% in GAS, TA and SOL, respectively. In contrast, the six-week aerobic exercise for mice that received the steroid in the last three-week period increased NOS III expression by merely 4.5% in TA whereas it was unaltered in SOL and GAS muscles (P<0.05).

Figure 1 summarizes the effects of mesterolone alone (Sed-C x Sed-M), exercise alone (Ex-C x Ex-M), mesterolone to exercised mice (Ex-C x Ex-M), and exercise to mesterolone-treated mice (Sed-M x Ex-M).

Immunohistochemistry analysis

Imunohistochemistry was undertaken to detect the distribution of NOS I and NOS III isoforms in SOL, TA and GAS muscles in Sed-C, Se-M, Ex-C and Ex-M mice (Figs. 2, 3). Both isoforms were immunostained variably in the sub-sarcolemmal region and sarcoplasm among myofibrils of extrafusal muscle fibers. Labeling of NOS I and NOS III was also found in intrafusal muscle fibers (muscle spindles), blood vessel walls and intramuscular motor nerve trunks. In SOL muscle, NOS I (Figs. 2A,D,G,J) and NOS III (Figs. 3A,D,G,J) immunostaining was weaker, but stronger however in the two fast muscles. Figs. 2B,E,H,K and 2C,F,I,L show immunostaining of NOS I and Figs. 3B,E,H,K and 3C,F,I,L show immunostaining of NOS III for TA and GAS muscles, respectively. The differences in the intensity of immunolabeling in response to treatment were more obvious for NOS I than for NOS III (compare Figs. 2 and 3), confirming the immunoblotting findings. Apparently there was a descending level in the immunostaining intensity of NOS I and III: TA>GAS>SOL, consistent with immunoblotting data. Figures 2 and 3 shows that there were no changes in the morphology of fibers in any of the three muscles regardless the experimental group: sedentary or exercised either treated with gum arabic or mesterolone.

Discussion

The present results seems to indicate that there is a significant contribution from both NOS I and NOS III in the three skeletal muscles studied, principally in the two fast-twitch skeletal muscles (TA and GAS) in response to mesterolone ± exercise. Despite NOS I (or neuronal type) and NOS III (or endothelial type) are known to be NO-synthetizing constitutive enzymes, their expression can be regulated (induced) by a number of endogenous or exogenous stimuli. Here, the expression of NOS I and III assessed in three skeletal muscles with different physiological and metabolic phenotype was differentially regulated in response to the AAS mesterolone, meaning that the chronic administration of the steroid is able to induce changes in the level of these protein isoforms. The interaction of the chronic AAS treatment (3 wk) with chronic endurance exercise (7 wk considering the week of adaptation) regulated differentially the expression of the two NOS isoforms depending on muscle phenotype.

Mesterolone induced upregulation of NOS I in the SOL and TA of sedentary mice (Sed-C vs. Sed-M) which was potentiated in the TA of exercised mice (Ex-C vs. Ex-M) suggesting a synergism between the exercise and the drug. Interestingly, NOS I expression in GAS, which was unaltered by the steroid alone (Sed-C vs. Sed-M) or exercised mice treated with gum arabic (Sed-C vs. Ex-C), showed an almost 100% increase in Ex-M animals suggesting a positive stimuli interaction. Conversely, the exercise training to mesterolone-treated mice (Sed-M vs. Ex-M) antagonized the steroid upregulation of NOS I in the TA muscle. In contrast, NOS III expression which showed a small increase in Ex-C (5%) or in Sed-M (9%) remained unchanged in the GAS of Ex-M animals. NOS III regulation in SOL and TA muscles in response to mesterolone plus exercise was minor than for NOS-I. From our data, we cannot know whether the inducible changes in the expression of NOS I and NOS III caused by the steroid mesterolone ± endurance exercise reflected correlative changes in the activities of the two isozymes.

Here, the higher levels of NOS I expression in the TA and GAS muscles over SOL muscle is consistent with the predominance of the enzyme in fast-twitch glycolytic (Kobzic et al., 1994; Stamler and Meissner, 2001) and fast-oxidative-glycolytic myofibers (Planitzer et al., 2001), both of them Type II fibers preponderantly part of TA and GAS muscles, respectively. Type II fibers have been associated with force generation, increased strength and strong contractile properties, events to which NOS I has been shown to be involved (Folland et al., 2000). In addition, the association of NOS I with the dystrophin-glycoprotein complex in the fibers sarcolemma and with the neuromuscular junction and mitochondria (Kobzic et al., 1995; Reid, 2001; for review see Grozdanovic and Baumgarten, 1999) is in accordance with the described role of NOS I in excitation-contraction coupling and mechanical stability (Grozdanovic, 2001; Stamler and Meissner, 2001), a requisite for force generation in response to endurance exercise or to steroid intake (Kadi, 2000). In fact, the prevalence of NOS I, found here, in the two fast muscles (TA and GAS), compared with the slow-twitch muscle

(SOL), plus its stronger expressional modulation in response to mesterolone alone (Sed-M) or mesterolone + exercise (Ex-M) support the notion of NOS I implicated in the fiber Type II attributes related to force/strength development and fast contractility (Kobzik et al., 1994, Reiser et al., 1997); and which was positively affected by the AAS mesterolone and exercise, as shown here. Acute exercise has been shown to increase NOS activity associated with increased glycogen depletion after the exercise session (Roberts et al., 1999; Harris et al., 2008); but the authors do not know whether inducible activity was due to more protein or an increase in the activity of the existing protein expressed in the skeletal muscle. Likewise, from our data we cannot infer whether the changes in the amount of NOS I and NOS III expressed in all three muscles studied here results from the conversion of L-arginine to L-citrulline (NOS activity) or reflect more protein present in the muscle, or both. However, the two latter possibilities can be considered since strength and contractile force can be increased by both anabolic-androgenic steroid and exercise. In this sense, Reiser et al. (1997) reported that chronic electrical stimulation increased total muscle NOS activity in fast-twitch muscles and that this correlated with an increase in nNOS production; the authors suggested that increase in the contractile activity caused by electrical stimulation regulates nNOS expression and activity.

On the other hand, increases in the NOS III expression have been correlated with increase of blood flow through the lumen vessels (shear stress). As a result, NOS III-derived NO would lead to vessel relaxation and consequent structural normalization of shear stress (see Green et al., 2004, for review). Whether the lower modulation of NOS III regulation compared to that of NOS I would represent adaptive compensation of muscular microvasculature/hemodynamics and a reflex of mice conditioning in response to intensive exercise is not known. Nevertheless, it represents a possibility since autoregulation of blood flow is one among the skeletal muscle functions controlled by NOS III-derived NO (Stamler and Meissner, 2001). Moreover, the fact that NOS III density of pixels in immunoblot bands of all groups (Sed-C, Sed-M, Ex-C and Ex-M) was higher even than the highest density of pixels or higher level of protein) of NOS I (see Ex-M animals) may suggest that such a high level of NOS III expression seen in this murine transgenic model is deemed to undergo lower modulation. In the current study, mesterolone, exercise, or both combined caused small increases (not exceeding 9%) or had no effect on NOS III expression in all three muscles. This is in agreement with the increase in number of mitochondria and capillaries per muscle fiber seen in the soleus of sedentary or exercised mice administered mesterolone (Fontana et al., 2010). In this recent study, we also found that both mesterolone and exercise (per se or in combination) induced hypertrophy of Type I fibers which was greater than that of Type II of soleus muscle; and that there was increase in the proportion of Type I fibers and decrease in the proportion of Type II fibers in this slow-twitch muscle; in addition, a small number of satellite cells showed signs of activation which was greater in the mesterolone than in the exercised mice but any hyperplasia was noticed (Fontana et al., 2010), what agrees with reports on the regulation of satellite cells function by androgens (Chen et al., 2005) and exercise (Wahl et al., 2008). On the other hand, the fast-to-slow fiber transition seen in the *soleus* using the same protocol and murine transgenic model (Fontana et al., 2010) and the increase of NOS I, an isoform typical of fast twitch fibers, presently found in the soleus of Ex-C, Sed-M and Ex-M mice, seem to indicate that NOS I regulation could be not associated with transition of muscle phenotype (fast-to-slow here seen) as already suggested by Reiser et al. (1997).

Altogether, the findings have a connection with roles attributed to NO in myofibers, such as modulation of oxidative metabolism (Cleeter et al., 1994), regulation of glucose uptake by muscle fibers (Ballon and Nader, 1997; Kapur et al., 1997), transference of signals related to fiber and muscle growth (Stamler and Meissner, 2001), and muscle mass gain (Pisconti et al., 2006). All these cellular events are influenced by anabolic steroids. The reason why different muscles responded differently to AAS in their expression of NOS I and NOS III isoform is unclear. However, androgen receptors in the SOL, GAS and TA muscles, if different in number or differently challenged in response to mesterolone has probably effect on muscle responsiveness to hormone (Kadi, 2000; Monks et al., 2004). Taken together, such muscle peculiarities would have a role in the level of modulation of NOS I and III expression regulation, and consequently the NO produced would mediate differentially the force production, muscle blood flow, respiration, glucose homeostasis, protein synthesis in each muscle type. We suggest that the differential modulation of NOS I and NOS III isoforms would imply endogenous NO as a relevant signaling player in the different muscle responses to mesterolone.

In conclusion, several studies have demonstrated that NOS I and NOS III expression varies under different myo-physiological and myo-pathological conditions with NO mediating the response of skeletal muscles to such conditions (Maiorana et al., 2003; Green et al., 2004; McAllister and Laughlin, 2006). Despite the bulk of evidence that indicates the skeletal muscle as a main target for AAS action (Hartgens and Kuipers, 2004; Chen et al., 2005) and that skeletal muscles are major sources of NO, studies on either the possible modulation of NOS expression by AAS or the mediation of NO on AAS effects had not yet been carried out. Given the severe health risk caused by AAS abuse among recreational and professional athletes (Bahrke and Yesalis, 2004), or either the benefits of its clinical use for treatment of hypogonadism and sarcopenia (Vermeulen, 2001), studies dealing with exogenous NOS/NO system regulation are very relevant. The data obtained with the transgenic mice with a “humanized lipid profile” used in

this study are relevant to those studying human response to AAS use and abuse since variations in the plasmatic lipids could influence the response of the muscle with a different metabolic and physiological profile to anabolic steroid.

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