Exogenous testosterone treatment decreases diaphragm neuromuscular transmission failure in male rats

CESAR E. BLANCO,1 WEN-ZHI ZHAN,2 YUN-HUA FANG,2 AND GARY C. SIECK2,3
1Department of Biokinesiology and Physical Therapy, University of Southern California, Los Angeles, California 90033; and Departments of 2Anesthesiology and 3Physiology and Biophysics, Mayo Clinic and Foundation, Rochester, Minnesota 55905

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Blanco, Cesar E., Wen-Zhi Zhan, Yun-Hua Fang, and Gary C. Sieck. Exogenous testosterone treatment decreases diaphragm neuromuscular transmission failure in male rats. J Appl Physiol 90: 850–856, 2001.—The effect of chronic exogenous testosterone (T) treatment on neuromuscular transmission in the diaphragm (Dia) muscle of adult male rats was determined. The contribution of neuromuscular transmission failure (NTF) to Dia fatigue was evaluated by superimposing intermittent direct muscle stimulation on repetitive nerve stimulation of isometric contraction in vitro. T treatment significantly reduced the contribution of NTF to Dia fatigue by ~29% (P < 0.001). Fiber type-specific effects on NTF were determined by measuring Dia fiber glycogen levels subsequent to repetitive nerve or muscle stimulation. T treatment had no effect on glycogen depletion in Dia type I and IIa fibers regardless of stimulation route. In the control group, type IIX fibers demonstrated significantly less glycogen depletion after nerve stimulation compared with direct muscle stimulation (P < 0.05), suggesting the presence of NTF. In contrast, T treatment increased glycogen depletion of type IIX fibers during nerve stimulation to levels similar to those after direct muscle stimulation. These data indicate that testosterone treatment substantially improves neuromuscular transmission in the Dia.

PREVIOUS STUDIES ON THE EFFECT of pharmacological anabolic-androgenic steroids (AAS) levels on neuromuscular systems in animal models have principally focused on their modulation of skeletal muscle properties (7, 11, 12, 13, 15, 27, 28, 33, 34). Because both final components of neuromuscular systems (i.e., motoneurons and skeletal muscle fibers) express the androgen receptor (6, 18, 22, 35), AAS-induced alterations in muscle function may be mediated by either component. Our laboratory have shown that pharmacological testosterone treatment increases choline acetyltransferase (ChAT) mRNA levels in motoneurons throughout the spinal cord in adult male rats (4). The increase in motoneuronal ChAT mRNA levels could potentially lead to greater ChAT activity levels at the axon termi

nal, thereby increasing the presynaptic capacity to synthesize acetylcholine. Indeed, Tucek et al. (44) have shown that serum testosterone levels modulate the ChAT activity of the levator ani muscle in male rats and parallel the changes observed in ChAT mRNA levels among the innervating motoneurons (4). The levator ani muscle is almost exclusively composed of type IIB fibers (3). In contrast, ChAT activity of the soleus muscle (predominantly composed of type I fibers) is decreased by castration but is unaffected by pharmacological testosterone treatment (45). These observations suggest that AAS could potentially exert selective effects on synaptic transmission in specific skeletal muscle fiber subpopulations.

A hypothesized AAS-induced increase in presynaptic ChAT activity may allow sustained acetylcholine release during repetitive activation of neuromuscular transmission leading to a reduction in the contribution of neuromuscular transmission failure (NTF) to peripheral muscle fatigue. NTF during repetitive activation of muscle contraction may be due to many factors, including diminished neurotransmitter release, failure in axon action potential propagation, and reduced end-plate excitability (40). Such an effect would be most apparent among type IIX and IIB fibers because they are the most susceptible to NTF (17). NTF occurs in all muscle fibers, with type IIX or IIB fibers being most susceptible to failure (17). NTF has previously been shown to significantly contribute to peripheral muscle fatigue of the mixed fiber type diaphragm (Dia) muscle in gonadally intact male rats (17). The purpose of this study was to determine whether chronic pharmacological treatment of gonadally intact adult male rats with testosterone propionate (TP) decreases the contribution of NTF to peripheral Dia fatigue and differentially reduces the susceptibility of type-identified Dia muscle fibers to NTF.

MATERIALS AND METHODS

General surgical methods. All protocols were in accordance with the American Physiological Society animal care guidelines and were approved by the Institutional Animal Care Committee.

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and Use Committees at the University of Southern California and the Mayo Clinic and Foundation. Surgical procedures were done under aseptic conditions with postsurgical recovery carefully monitored. Adult male Sprague-Dawley rats (body weight 250–275 g) were used throughout this study. Animals were randomly assigned to two groups, Sham controls or testosterone treated. Sham controls were subcutaneously implanted with a single, empty 45-mm-long Silastic capsule, and the animals receiving testosterone treatment were subcutaneously implanted with two 45-mm capsules filled with TP (4-androsten-17β-ol-3-one 17 propionate; Sigma-Aldrich Chemical) under ketamine-rompun anesthesia (ketamine: 50 mg/kg; rompun: 10 mg/kg). The capsules were made from tubing with an inner diameter of 1.57 mm and an outer diameter of 3.17 mm. All capsules were checked for leakage and preequilibrated in sterile saline for 24 h before implantation. The encapsulated TP diffuses through the walls of the Silastic tubing (9).

In vitro determinations of the effect of testosterone treatment on the contribution of NTF to peripheral muscle fatigue. To determine the contribution of NTF to peripheral Dia fatigue, direct muscle stimulation was superimposed on nerve stimulation at 40 or 75 Hz (17, 19). Animals were deeply anesthetized with pentobarbital sodium (60 mg/kg); blood was drawn and kept on ice until processed; and the Dia muscle with motor nerve, muscle fiber origins, and insertions intact was rapidly excised, immediately immersed in Rees-Simpson solution (in mM: 135 Na+, 5 K+, 2 Ca2+, 1 Mg2+, 120 Cl−, 25 HCO3−; pH 7.4) aerated with 95% O2-5% CO2 and maintained at 26°C. The fatigue properties of two 10-mm-wide strips from the midcostal region of the left hemidiaphragm were used to physiologically estimate the contribution of NTF to Dia muscle fatigue. The central tendon was attached to a clamp in series with a force transducer (model 6350, Cambridge Technology). Muscle fiber length was adjusted until maximal isometric twitch force responses were obtained (i.e., optimal fiber length) using direct muscle stimulation (anodal monophasic rectangular pulses of 0.5-ms duration). Supramaximal stimulation of the phrenic nerve was achieved by using monophasic rectangular pulses (0.2-ms duration) delivered via a suction electrode. Twitch contraction time (CT), one-half relaxation time (RT1/2), twitch tension (Pt), and the tetanic tension (Pp) were measured for each Dia muscle strip. Immediately upon cessation of the fourth stimulus train each muscle strip was rapidly frozen, at optimal length) and rapidly frozen in liquid nitrogen-cooled isopentane and used as an unstimulated control for initial fiber glycogen content. A total of four 2-min stimuli (direct or nerve; 75 Hz, 330-ms train duration, 1 train/s) with 1-min recovery period between each stimulus were presented to each muscle strip. Immediately upon cessation of the fourth stimulus train each muscle strip was rapidly frozen, at optimal resting length, in liquid isopentane cooled to its melting point by liquid nitrogen. All glycogen levels are reported as the percentage of initial glycogen levels (i.e., unstimulated) after either repetitive nerve or direct muscle stimulation.

Immunohistochemical determination of fiber type and glycogen levels of Dia muscle fibers. Serial cross sections from each of the three frozen Dia segments (unstimulated, direct muscle stimulated, and nerve stimulated) were cut on a cryostat (Frigerut 2800E, Reichert-Jung) at −20°C from each animal. Duplicate 20-μm-thick sections were stained for glycogen content using the periodic acid-Schiff (PAS) reaction. The staining intensity of the PAS reaction and cross-sectional area of individual fibers were determined using a one-way analysis of variance.

Statistical analysis. To normalize the evoked tension responses across testosterone treatment group and across animals the results were expressed as the ratio of maximum tension produced after 2 min of stimulation to the initial maximum tension produced. Both the ratio of maximum tension produced after 2 min of total stimulation to initial maximum tension during nerve stimulation (P = Pm/Min; force loss during nerve stimulation) and that of the maximum tension produced after 2 min of total stimulation to initial maximum tension during the superimposed direct muscle stimulation (MF = Mm/Minit; force loss during the superimposed muscle stimulation) were calculated. In this stimulation paradigm, the level of force decline with direct muscle stimulation is primarily due to the activation history imposed by nerve stimulation. An estimate of the contribution of NTF to total force decline was calculated using the following equation

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NTF = \left(\frac{MF - F}{MF}\right) \times 100 \%
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Statistical significance of the results was determined using a two-way ANOVA with Bonferroni corrections for planned post hoc comparisons. Planned post hoc comparisons were evaluated using the Tukey test. Significance of the results were accepted if P < 0.05. All statistical analyses were done using SPSS software (SPSS Science, Chicago, IL).

Determination of the effect of testosterone treatment on NTF in type-identified Dia fibers. The stimulation paradigms and the microdensitometric determination of muscle fiber glycogen content described by Johnson and Sieck (17) were used to determine the susceptibility of immunohistochemically type-identified Dia fibers to NTF. Three days before the experiment, all animals received daily injections of 25 ml of a 5% glucose solution containing insulin to maximize initial muscle fiber glycogen content (39). Each animal was deeply anesthetized with pentobarbital sodium (60 mg/kg). The Dia muscle with intact motor nerve, muscle fiber origins, and insertions intact was rapidly excised and immediately immersed in aerated Rees-Simpson solution maintained at 26°C. The fatigue properties of two 10-mm-wide strips from the midcostal region of the left innervated hemidiaphragm muscle strips were determined at 75 Hz using nerve stimulation alone or direct muscle stimulation. Dia muscle strips that were repetitively activated through direct muscle stimulation were equilibrated for a minimum of 20 min in Rees-Simpson solution containing d-tubocurarine (12 μM). A third left midcostal diaphragm strip was immediately pinned at 1.4 times tonemontized length (to approximate optimal resting length) and rapidly frozen in liquid nitrogen-cooled isopentane and used as an unstimulated control for initial fiber glycogen content. A total of four 2-min stimuli (direct or nerve; 75 Hz, 330-ms train duration, 1 train/s) with 1-min recovery period between each stimulus were presented to each muscle strip. Immediately upon cessation of the fourth stimulus train each muscle strip was rapidly frozen, at optimal resting length, in liquid isopentane cooled to its melting point by liquid nitrogen. All glycogen levels are reported as the percentage of initial glycogen levels (i.e., unstimulated) after either repetitive nerve or direct muscle stimulation.

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MHCslow; Novocastra), MHC2A (anti-MHC2A; Blau n1.551; Ref. 16), MHC2B (anti-MHC2B; Schiaffino BF-F3; Ref. 37), and all but MHC2X (anti-MHC2X; Schiaffino BF-35; Ref. 37) as described by Prakash et al. (25, 26). The specificity of these antibodies against rat MHC isoforms has previously been established (16, 37). Dia fibers were classified (typed) subsequent to immunohistochemical determination of MHC isoform expression. The mean fiber optical density of the PAS-stained sections (i.e., glycogen content) was determined for each fiber type in each animal.

**Statistical analysis.** Statistical significance of the results for Dia fatigue was determined using a Bonferroni corrected repeated-measures one-way ANOVA with treatment group as the grouping variable and stimulus route as the repeated measure. Planned post hoc comparisons were evaluated using the Tukey test. Statistical significance of the results for glycogen levels was determined using a Bonferroni corrected repeated-measures one-way ANOVA with treatment group as the grouping variable and stimulus route as the repeated measure. Significance of the results was accepted if $P < 0.05$.

**RESULTS**

As illustrated in Fig. 1, serum testosterone levels were, on average, sixfold greater among the TP treatment group ($26.8 \pm 3.0$ ng/ml; $n = 7$) than among the animals implanted with an empty capsule (Sham, $3.5 \pm 1.3$ ng/ml; $n = 7$; $P < 0.05$). The serum levels of DHT and estradiol, two biologically active metabolites of testosterone, were also significantly increased by TP treatment (Fig. 1; $P < 0.01$ and $P < 0.05$, respectively). DHT levels in the TP-treated animals were ~20 times greater than among the Sham-operated animals (Sham: $18.9 \pm 1.3$ (n = 5) vs. $0.8 \pm 0.2$ ng/ml (n = 6)). Estradiol levels in the TP-treated animals (n = 5) were increased by 68% above Sham-operated controls (n = 6). TP treatment did not lead to significant changes in body weight (Fig. 1).

The effect of TP treatment on Dia contractile properties is shown in Fig. 2. Testosterone treatment significantly increased the specific $P_t$ (TP: $8.46 \pm 0.14$ N/cm$^2$; Sham: $7.26 \pm 0.35$ N/cm$^2$; $P < 0.01$). The specific $P_o$ of Dia muscle strips was also significantly increased in the animals receiving TP treatment (TP: $21.99 \pm 0.59$ N/cm$^2$; Sham: $19.13 \pm 0.21$ N/cm$^2$; $P < 0.001$). The ratio of twitch to tetanic tension was unchanged by TP treatment. TP treatment had no effect on Dia CT and RT$1/2$.

The contribution of NTF to peripheral Dia fatigue, estimated by intermittently superimposing direct muscle stimulation on repetitive nerve stimulation, was significantly decreased (2-way ANOVA; $P < 0.001$) by testosterone treatment regardless of stimulation frequency (Fig. 3). At a stimulus frequency of 40 Hz, TP treatment significantly reduced the contribution of NTF to Dia fatigue by 22% (TP: $57.8 \pm 4.1\%$, $n = 7$; $P < 0.05$).
Sham: 74.1 ± 1.5%, n = 7; post hoc analysis, P < 0.05). At 75 Hz, testosterone treatment decreased the contribution of NTF to Dia fatigue by only 14% (TP: 66.9 ± 5.9%, n = 7; Sham: 77.7 ± 3.8%, n = 7; post hoc analysis, P < 0.05).

The fatigue indexes (FIs) of Dia muscle strips from sham and TP-treated rats determined during nerve or muscle stimulation are shown in Fig. 4. Dia fatigue was significantly greater (i.e., lower fatigue indexes) during nerve stimulation than during direct muscle stimulation in both treatment groups (P < 0.004). Testosterone treatment did not significantly alter Dia fatigue regardless of the stimulus delivery (P < 0.072). The results suggest that testosterone treatment reduced the difference between nerve stimulation-induced fatigue and direct muscle stimulation-induced fatigue [TP-treated animals (nerve stimulation vs. muscle stimulation): FI = 21.20 ± 1.6 vs. 24.1 ± 2.8%; untreated controls (nerve stimulation vs. muscle stimulation): fatigue index = 17.0 ± 0.5 vs. 24.1 ± 2.0%].

Glycogen levels decreased to a significantly greater extent during repetitive direct muscle stimulation among type I and IIa fibers (a further 10% decrease from baseline glycogen levels) regardless of treatment group (Fig. 5; P < 0.05). These observations are in agreement with the results of Johnson and Sieck (17) and suggest that NTF does occur among type I and IIa fibers. Statistical analysis indicated that among type IIx fibers there were significant interactions between treatment group and mode of stimulation (P < 0.018). As shown in Fig. 5, glycogen levels in type IIx fibers subsequent to repetitive nerve stimulation were significantly greater than compared with direct muscle stimulation among the Sham animals (61.1 ± 9.6% of baseline vs. 22.9 ± 6.1% of baseline levels, respectively; P < 0.01). In contrast, among TP-treated males, repetitive nerve and muscle stimulation decreased glycogen content of type IIx fiber glycogen content to the same extent (28.8 ± 1.6 and 29.1 ± 5.5% of baseline levels, respectively). Glycogen utilization in type IIx fibers during nerve stimulation was significantly increased by testosterone treatment (P < 0.05). These data suggest that TP treatment significantly increased glycogen utilization during repetitive nerve stimulation and selectively decreases NTF among type IIx Dia fibers. Due to the absence of type IIb fibers in every Dia muscle strip from each animal a similar analysis was not performed on this population. Interestingly, fiber cross-sectional area was unaffected by TP treatment (Fig. 6).

**DISCUSSION**

The results of this study indicate that treatment of sedentary adult male rats with pharmacological levels of testosterone via continuous infusion of TP improves Dia function. TP treatment significantly increased isometric force generation (P_t and P_o) and significantly decreased the contribution of NTF to peripheral Dia fatigue. Testosterone treatment selectively improved neuromuscular transmission among the type IIa muscle fibers in the Dia muscle. Based on the TP-induced increase in P_o, the force output after 2 min of repetitive nerve stimulation of Dia contraction (75 Hz) was calculated to be 43% greater among the TP treatment group. This suggests that AAS treatment may benefit patient populations at risk for respiratory failure due to impaired Dia function by improving force generation and decreasing the susceptibility of the muscle to NTF.

In contrast to the results of the present study, previous studies have reported that AAS treatment has minimal effects on rat Dia contractile characteristics, including P_t, P_o, and fatigue (27, 28). However, these studies altered circulating levels of androgens in male rats episodically via intramuscular injections (TP and nandrolone decanoate, respectively) and activated Dia

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**Fig. 3.** Effect of TP treatment on contribution of neuromuscular transmission failure (NTF) to diaphragm fatigue at 40- and 75-Hz stimulus frequencies. *Post hoc comparisons indicated that values were significantly different between the Sham and TP-treated groups (P < 0.001) at both 40 and 75 Hz.

**Fig. 4.** Effect of TP treatment on contribution of neuromuscular transmission failure (NTF) to diaphragm fatigue at 40- and 75-Hz stimulus frequencies. *Post hoc comparisons indicated that values were significantly different between the Sham and TP-treated groups (P < 0.001) at both 40 and 75 Hz.

**Fig. 5.** Effect of TP treatment on contribution of neuromuscular transmission failure (NTF) to diaphragm fatigue at 40- and 75-Hz stimulus frequencies. *Post hoc comparisons indicated that values were significantly different between the Sham and TP-treated groups (P < 0.001) at both 40 and 75 Hz.

**Fig. 6.** Effect of TP treatment on contribution of neuromuscular transmission failure (NTF) to diaphragm fatigue at 40- and 75-Hz stimulus frequencies. *Post hoc comparisons indicated that values were significantly different between the Sham and TP-treated groups (P < 0.001) at both 40 and 75 Hz.
contraction solely through direct muscle stimulation. Continuous elevation of serum AAS levels, rather than episodic increases could potentially account for the differences between the present study and previous studies. Kujawa et al. (20) have shown that the effect of testosterone treatment on recovery from facial paralysis is enhanced by continuous elevation (subcutaneous capsules) compared with episodic increases (subcutaneous injection) in circulating testosterone levels. Lewis et al. (21) have also shown that continuous elevation of circulating AAS levels with subcutaneous capsules containing nandrolone propionate increases Dia force generation in adult male hamsters (21). These observations suggest that the route of AAS delivery, episodic vs. continuous, may impact the clinical efficacy of AAS action on muscle function.

TP treatment reduced Dia fatigue during repetitive nerve stimulation. In contrast to the sham-treated animals, no significant differences were observed in Dia fatigue induced by repetitive nerve or muscle stimulation. Peripheral determinants of muscle fatigue include neuromuscular transmission failure, sarcoplemal action potential propagation failure, excitation-contraction failure, and contraction failure. The data indicate that the decrease in the contribution of Dia fatigue is principally due to the TP-induced reduction of NTF among type IIx fibers. The reduction in glyco- gen levels subsequent to repetitive nerve or muscle stimulation was similar among the type IIx fibers. Interestingly, TP treatment did not appear to have an effect on NTF in type I or IIa fibers. Type IIX and IIb fibers belong to fast-twitch motor units that have been shown to be more susceptible to NTF than motor units composed of type s or IIa fibers (39). Indeed, Reid et al. (31) have shown that the amplitude of endplate potentials and the quantal content of neuromuscular junctions on extensor digitorum longus muscle fibers belonging to fast-twitch fatigable motor units decreases at a greater rate during phasic or tonic stimulation at frequencies varying from 20 to 80 Hz than among soleus slow-twitch fibers in the rat.

The TP-induced reduction in NTF among type IIX fibers may be due to reductions in axonal action potential propagation failure, synaptic transmission failure...
and failure to generate a sarcolemmal action potential. Our laboratory has previously shown that TP-treatment of gonadally intact male Long-Evans rats increases ChAT mRNA levels in motor neurons in the lateral motor columns of the cervical and lumbar spinal cord (4). We hypothesized that increased ChAT mRNA levels could result in an increase in ChAT activity levels at the axon terminal. In addition, other investigators have shown that factors that increase ChAT mRNA levels also increase the vesicular acetylcholine transporter mRNA levels (1, 2, 23, 32). Potentially, a TP-induced increase in ChAT and vesicular acetylcholine transporter activities may be responsible for the observed reduction in NTF in the Dia by improving the availability of acetylcholine for synaptic release during repetitive activation of muscle contraction. Theoretically, a TP-induced increase in presynaptic acetylcholine stores could reduce the decremental fall in quantal content during repetitive activation of type IIX fibers and maintain the amplitude of the endplate potentials at a level sufficient to attain threshold, thereby preventing failure in the repetitive generation of sarcolemmal action potentials. Thus the TP-induced reduction in NTF is potentially due to modulation of presynaptic events that affect synaptic transmission at the neuromuscular junction.

Understanding how AAS, such as testosterone, affect the neuromuscular system has become increasingly important. Clinicians prescribe AAS, with and without resistive exercise training, to ameliorate I) hypogonadal function in adult men (24); 2) age-related decrements in muscle strength in men (10, 43, 46); 3) muscle wasting and hypogonadal function in human immunodeficiency-positive men (8, 29, 30, 36, 42); and 4) muscle atrophy, specifically diaphragm muscle atrophy, in chronic obstructive pulmonary disease (38). The results of a recent clinical study, indicate that AAS treatment of paraplegics improves Dia function and may reduce the possibility of respiratory failure during respiratory illnesses (41). The preponderance of these studies have shown significant AAS-induced increases in muscle mass and strength. The results of the present study strongly suggest that the AAS also improve muscle function by reducing the susceptibility of skeletal muscles to NTF.

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