Nandrolone Normalizes Determinants of Muscle Mass and Fiber Type after Spinal Cord Injury

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Abstract

Spinal cord injury (SCI) results in atrophy of skeletal muscle and changes from slow oxidative to fast glycolytic fibers, which may reflect reduced levels of peroxisome proliferator-activated receptor gamma coactivator-1 α (PGC-1 α), increased myostatin signaling, or both. In animals, testosterone reduces loss of muscle fiber cross-sectional area and activity of enzymes of energy metabolism. To identify the molecular mechanisms behind the benefits of androgens on paralyzed muscle, male rats were spinal cord transected and treated for 8 weeks with vehicle, testosterone at a physiological replacement dose, or testosterone plus nandrolone, an anabolic steroid. Treatments were initiated immediately after SCI and continued until the day animals were euthanized. In the SCI animals, gastrocnemius muscle mass was significantly increased by testosterone plus nandrolone, but not by testosterone alone. Both treatments significantly reduced nuclear content of Smad2/3 and mRNA levels of activin receptor IIB and follistatin-like 3. Testosterone alone or with nandrolone reversed SCI-induced declines in cellular and nuclear levels of PGC-1 α protein and PGC-1 α mRNA levels. For PGC-1 α target genes, testosterone plus nandrolone partially reversed SCI-induced decreases in levels of proteins without corresponding increases in their mRNA levels. Thus, the findings demonstrate that following SCI, signaling through activin receptors and Smad2/3 is increased, and that androgens suppress activation of this signaling pathway. The findings also indicate that androgens upregulate PGC-1 α in paralyzed muscle and promote its nuclear localization, but that these effects are insufficient to fully activate transcription of PGC-1 α target genes. Furthermore, the transcription of these genes is not tightly coupled with their translation.

Key words: muscle atrophy; nandrolone; oxidative metabolism; PGC-1 α ; slow twitch muscle; spinal cord injury

Introduction

SPIRAL CORD INJURY (SCI) results in marked atrophy of skeletal muscle with a switch from oxidative slow-twitch toward glycolytic fast-twitch fibers and reduced endurance (Dudley-Javoroski and Shields, 2008; Qin et al., 2010a). Our understanding of the molecular mechanisms for atrophy following paralysis predominantly comes from studies of nerve transection. Following paralysis, a period of accelerated protein catabolism ensues, resulting from accelerated breakdown of muscle proteins by the ubiquitin-proteasome system (Furuno et al., 1990; Medina et al., 1991). Studies in transgenic mice have shown that muscle atrophy F-box (MAFbx) and muscle ring finger 1 (MuRF1), two ubiquitin E3 ligases, increase rates of denervation atrophy (Glass, 2010). Expression of these ligases is regulated, in part, by two transcription factors, FOXO1 and FOXO3A (Glass, 2010).

Studies in rats suggest that following SCI, these genes are upregulated for only a brief period of less than 2 weeks (Zeman et al., 2009). Such findings suggest that other mechanisms may also contribute to the atrophy of muscle after injury.

One potential alternative mechanism involves myostatin, which is a member of the TGF- β family of growth factors. Myostatin has been shown to reduce the size of skeletal muscle (Glass, 2010). The activity of myostatin is reduced by endogenous inhibitors, the follistatins (Glass, 2010). Myostatin signals by binding the activin receptor IIB, ultimately resulting in phosphorylation and nuclear translocation of the transcription factors Smad2 and Smad3 (Glass, 2010). Myostatin mRNA levels have been reported to be increased in muscle paralyzed by stroke (Ryan et al., 2011). How myostatin levels and downstream signaling are altered after SCI is not well understood. In one study of muscle biopsies from

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persons with chronic SCI, myostatin mRNA levels were reduced, although myostatin protein levels were not altered (Leger et al., 2009). In a separate study, the effects of long-term electrical stimulation of the soleus in individuals with SCI were assessed with mRNA levels. This form of training decreased mRNA levels for myostatin (Adams et al., 2011).

The mechanisms behind the fiber type alterations observed after paralysis may include reduced activity of peroxisome proliferator-activated receptor gamma coactivator-1 α (PGC-1 α) (Lecker et al., 2004), or increased activity of myostatin (Hennebry et al., 2009). The expression of genes encoding proteins necessary for oxidative metabolism in skeletal muscle is normally upregulated by PGC-1 α , a transcriptional co-regulator that interacts with several transcription factors, including NRF1/2 and MEF2 (Finck et al., 2006). Of interest, myostatin reduces MEF2 expression (Hennebry et al., 2009). PGC-1 α and the closely-related protein PGC-1 β are thought to be key determinants of muscle fiber type and energy metabolism (Finck and Kelly, 2006). PGC-1 α promotes mitochondrial biogenesis and oxidative metabolism. An increase in its level has been shown to be sufficient to increase the proportion of fibers with slow-twitch muscle phenotypes and endurance (Lin et al., 2002). Expression, nuclear localization, and transcriptional activity of PGC-1 α are increased by exercise (Akimoto et al., 2005). Expression of PGC-1 α and PGC-1 β mRNA is reduced after nerve transection or spinal isolation, a variant of SCI in which reflex arcs are disrupted by dorsal rhizotomy (Lecker et al., 2004). How the expression of PGC-1 α or PGC-1 β changes after SCI has not been reported. However, a study of the effects of electrical stimulation of the soleus muscle of two individuals with SCI showed that this form of exercise led to an increase in PGC-1 α mRNA levels and the expression of genes for energy metabolism (Adams et al., 2011).

The administration of testosterone to spinal cord-transected rats or mice reduced muscle atrophy as assessed by myofiber cross-sectional area (Gregory et al., 2003; Ung et al., 2010). The specific mechanisms behind this benefit are uncertain. Several mechanisms have been suggested to be involved in other models of muscle atrophy. In skeletal muscle of individuals without neurological injuries, activation of Notch, Akt, p38 MAPK, and Wnt signaling and inhibition of myostatin/TGF- β /SMADS signaling have been implicated (Kovacheva et al., 2010; Singh et al., 2009). Other potential mechanisms are suggested by findings from studies in hypogonadal men that have shown that testosterone upregulates the muscle growth factor insulin-like growth factor -1 (IGF-1; Ferrando et al., 2002; Urban et al., 1995). Additionally, in hypogonadal rodents, testosterone has been found to activate a downstream target of IGF-1, mTOR (Xu et al., 2004).

Testosterone and anabolic steroids such as nandrolone have been shown to reduce atrophy caused by paralysis or glucocorticoid administration. Several studies have shown that testosterone or nandrolone reduced muscle atrophy caused by a lack of weight-bearing or immobilization (Taylor et al., 1999; Wimalawansa et al., 1999). In muscle paralyzed by nerve transection, nandrolone slowed atrophy and reduced expression of MAFbx, MuRF1, and FOXO1 (Qin et al., 2010b). In glucocorticoid-treated rats, testosterone reduced the expression of MAFbx, MuRF1, and FOXO1 (Qin et al., 2010c; Wu et al., 2010; Zhao et al., 2008b), increased expression of IGF-1, and increased protein levels and nuclear entry of PGC-1 α (Jones et al., 2010; Qin et al., 2010c).

Hypogonadism is observed in the majority of men acutely after SCI (Schopp et al., 2006); low testosterone levels are present in approximately 40% of men with chronic SCI (Tsitouras et al., 1995). Testosterone levels are also reduced acutely after SCI in rats (Huang et al., 1995). The goal of the present study was to determine the effects of physiological concentrations of testosterone (testosterone replacement therapy; TRT) alone or in combination with nandrolone on skeletal muscle mass in spinal cord-transected rats. An additional goal was to evaluate the effects of these agents on the signaling pathways that promote atrophy and fiber-type switching. We hypothesized that during the first 2 months after SCI, these androgenic agents would reduce the adverse effects of paralysis on PGC-1 α levels and myostatin signaling.

Methods

Animal studies

We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during the course of this research, and that the animal studies reported here were reviewed and approved by the Institutional Animal Care and Use Committee at the James J. Peters VA Medical Center. Male Wistar rats obtained from Taconic Farms (Hudson, NY) and weighing approximately 250 g were used. The animals were given food and water *ad libitum* and were housed in a facility providing controlled temperature and humidity and a 12-h:12-h day:night cycle.

For our studies, a rat model of complete transection of the spinal cord was used. A transection was chosen rather than incomplete models of SCI caused by contusion or compression to minimize the potential heterogeneity in muscle attributable to having some motor units remain active as a consequence of an incomplete injury. We reasoned that such heterogeneity would obscure alterations in paralyzed fibers. After being weighed, the animals were anesthetized using ketamine and xylazine. Hair was removed from the skin over the thoracic spine with a clipper, then cleaned with povidone-iodine and 70% alcohol. A complete transection of the spinal cord at the interspace between the 10th and 11th vertebrae was performed after exposure of the spinal cord by laminectomy. Some animals underwent a sham SCI in which the spinal cord was exposed but not transected. The wounds were closed with sutures. Alzet mini-osmotic pumps (model 2004; Alzet, Cupertino, CA) were implanted through stab wounds at a separate site on the back; the stab wounds were closed with sutures. The pumps infused vehicle (propylene glycol; Sigma-Aldrich Chemical Co., St. Louis, MO), a high replacement dose of testosterone (2.8 mg/kg/day), or nandrolone (0.75 mg/kg/week) combined with testosterone (2.8 mg/kg/day). This dose of nandrolone was selected because it was previously shown to reduce muscle atrophy after nerve transection (Zhao et al., 2008a). Group sizes were: sham-SCI, 11; SCI-vehicle, 9; SCI-testosterone, 12; SCI-nandrolone-testosterone, 8.

Postoperatively, the animals were treated with amoxicillin in drinking water for the first 3–5 days, then with enrofloxacin as indicated for urinary tract infections. Urine was manually expressed at least twice daily until automatic emptying of the bladder resumed, then as needed. Because the mini-osmotic pumps used infuse drug for only 28 days, at day 29 the animals were anesthetized with ketamine and diazepam, and the

pumps were replaced. At day 56, the animals were weighed then euthanized by inhalation of carbon dioxide, and the gastrocnemius muscles were carefully removed, placed in pre-weighed tubes, and flash-frozen on dry ice. The tubes were re-weighed to obtain the final muscle weights. Weights of the left and right gastrocnemius muscles were each divided by the body weight on the day of initial surgery, and these normalized values were averaged to obtain a mean gastrocnemius weight for each animal; this value was used in subsequent calculations. Gastrocnemius was chosen for these studies because it is mixed in fiber type, large enough to permit many assays, and had undergone substantial atrophy after SCI.

Quantitative real-time PCR (qPCR)

Measurements of the levels of mRNA transcripts in total RNA from gastrocnemius muscles were performed as previously described (Wu et al., 2010; Zhao et al., 2008b). Real time PCR was performed using Assay on Demand probes and primers and Taqman 2×PCR mix (Applied Biosystems, Foster City, CA). Each measurement was performed in triplicate, and the mean of crossing points for the three technical replicates was used in subsequent calculations. Expression levels of target mRNAs were expressed relative to values for the sham-SCI group using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001); 18S RNA was used as the internal control. Levels of 18S RNA were unaffected by SCI, testosterone, or testosterone plus nandrolone. When performing the calculation for relative change the control used was the appropriate mean value for the crossing point for the sham-SCI group. Using a hypothetical sample1 as an example, fold-change would be calculated for MAFbx as $2^{[(CtMAFbx_{sample1} - CtMAFbx_{Avg-Sham-SCI}) - (Ct18S_{sample1} - Ct18S_{Avg-Sham-SCI})]}$.

Subcellular fractionation and Western blotting

Extracts of whole muscle containing soluble and myofibrillar proteins were prepared as described by Wheeler and Koohmaraie (1999). Briefly, 50 mg of tissue was homogenized in extraction buffer for 20 sec on ice using a Polytron

(Paterson, NJ). A 200- to 300- μ L aliquot was immediately transferred to a clean tube containing the same volume of 2× treatment buffer (0.25 M Tris, 4% sodium dodecylsulfate, and 20% glycerol), and heated at 50°C for 20 min, mixed to shear DNA, and heated at this temperature for an additional 5 min. Supernatants obtained after samples were centrifuged for 20 min at 16,000g were removed and their protein concentrations were determined using the micro-BCA kit (Pierce Protein Research Products, Rockford, IL). Samples were diluted to 3 mg/mL and mixed 1:1 with 2× treatment buffer containing mercaptoethanol and 20% bromophenol blue, and then heated for 10 min at 50°C.

Subcellular fractionation was performed using a kit (catalog no. 78833; Pierce). Cytosolic and nuclear fractions were isolated from samples of 20 mg of muscle tissue following the manufacturer's recommended procedures. Protein concentrations were determined using the BioRad protein assay with bovine serum albumin as the standard. Proteins were denatured by heating in SDS-PAGE sample buffer (BioRad, Hercules, CA). As a quality control, cytoplasmic fractions were probed with anti-histone H1, which was routinely undetectable, or present only in trace amounts; nuclear fractions were probed with anti- β -tubulin, which was undetectable.

Proteins were resolved by SDS-PAGE using acrylamide concentrations from 7.5–12% as appropriate for the size of the protein of interest. Proteins were electrophoretically transferred to PVDF membranes by wet (myosin heavy chain) or semi-dry (all other proteins) transfer. The membranes were blocked with 5% non-fat dried milk. The antibodies used were: rabbit anti-Smad2/3 diluted 1:1000 (cat. no. 5678; Cell Signaling, Danvers, MA), mouse anti-MyHC slow 1:500 (cat. no. Ab11083; Abcam, Cambridge, MA), anti-PGC-1 α (cat. no. NBP1-04676, 1:2000; Novus Biologicals, Littleton, CO), anti-ACADM (cat. no. NBP1-40810, 1:2000; Novus Biologicals), anti-troponin I slow (cat. no. sc20488, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA), anti-LDHA (cat. no. 3582, 1:1000; Cell Signaling), HRP-coupled goat anti-rabbit 1:4000 (Santa Cruz Biotechnology), and goat anti-mouse IgG (cat. no. sc-2005; Santa Cruz Biotechnology). Immunostaining was

FIG. 1. Nandrolone increases body weight and gastrocnemius mass at 56 days after spinal cord injury (SCI). The panels show body weights (A) and wet weights of gastrocnemius muscles (B). Data are mean values $\pm$ standard error of the mean with numbers for each group: sham-SCI, 11; SCI-vehicle, 9; SCI-Ts, 12; SCI-nandrolone-testosterone, 8. The p value under each panel refers to that obtained for the one-way analysis of variance. Those indicated for comparisons between pairs of means were obtained using the Newman-Keuls *post-hoc* test (NS, the indicated comparisons were not significant).

FIG. 2. Levels in the gastrocnemius for mRNA for myostatin signaling at 56 days after spinal cord injury (SCI). (A–G) mRNA levels for the indicated genes were determined by quantitative real-time PCR (qPCR) and are shown as mean fold-change versus sham-SCI animals $\pm$ standard error of the mean ($n=6-10$ per group). The p value under each figure refers to that obtained for the one-way analysis of variance, and those for comparisons between pairs of means were obtained using the Newman-Keuls *post-hoc* test (NS, the indicated comparisons were not significant; Ts, SCI + testosterone; Ts/Nan, SCI + testosterone + nandrolone; Activin R IIB, activin receptor IIB).

visualized by enhanced chemiluminescence and recorded with photographic film or a Kodak IS4000 imaging system. Digitized images were used for scanning densitometry using Imagequant TL (GE Life Sciences, Piscataway, NJ). Intensities of bands were normalized relative to β -tubulin or histone H1 as indicated, and expressed as fold-change relative to intensities for sham-SCI.

Statistical analysis

Data are expressed as mean $\pm$ standard error of the mean (SEM). The significance of differences among groups was determined by one-way analysis of variance (ANOVA) with a Newman-Keuls *post-hoc* test to assess the significance of differences between pairs of means. A *p* value <0.05 was considered significant. Calculations were performed with Prism 4.0c (Graphpad Software, La Jolla, CA).

Results

Body weights of SCI animals administered vehicle (SCI-vehicle group) were reduced by 21% at 56 days after SCI ($p < 0.001$) compared to sham-operated animals administered vehicle (sham-SCI group; Fig. 1A). Body weights were increased for SCI animals administered testosterone plus nandrolone (SCI-nandrolone group) compared to the SCI-vehicle group ($p < 0.05$), but remained lower ($p < 0.01$) by 14% than the sham-SCI group (Fig. 1A). The weight of the gastrocnemius muscle was reduced ($p < 0.001$) by 54% at 56 days after SCI in the SCI-vehicle group (Fig. 1B), compared to the sham-SCI group. In the SCI-nandrolone group, muscle weight was 44% higher than in the SCI-vehicle group ($p < 0.01$). Muscle weights for the SCI-nandrolone group remained lower ($p < 0.001$) by 30% compared to the sham-SCI group (Fig. 1B). Administration of testosterone alone to SCI animals (SCI-testosterone group) did not alter body weight or gastrocnemius weight (Fig. 1A and B).

The mRNA levels for myostatin were not altered in muscle after SCI and were unaffected by testosterone or nandrolone administration (Fig. 2A). Expression of a receptor for myostatin (activin receptor IIB) was increased by SCI by more than 250% ($p < 0.001$), and was reduced by testosterone alone ($p < 0.001$), or in combination with nandrolone ($p < 0.001$); the latter agent reduced expression of activin receptor IIB to levels 65% lower than those present in the sham-SCI group (Fig. 2B). Increased expression of mRNA for follistatin ($p < 0.05$) and follistatin-like 3 ($p < 0.001$) was observed for the SCI-vehicle group compared to the sham-SCI group (Fig. 2C and E). Follistatin mRNA levels were not significantly reduced by testosterone, but were diminished by testosterone and nandrolone combined ($p < 0.05$; Fig. 2C). Follistatin-like 1 mRNA levels were not significantly altered by SCI or by administration of testosterone alone or together with nandrolone to animals with SCI (Fig. 2D). Levels of follistatin-like 3 mRNA were reduced by testosterone alone ($p < 0.001$), or combined with nandrolone ($p < 0.001$), to levels that were slightly, though not significantly, below levels for the sham-SCI group (Fig. 2E).

The levels of mRNA and total protein for Smad2 and Smad3 were unaffected by SCI, testosterone alone, or testosterone combined with nandrolone (Fig. 2F and G and Fig. 3A). However, nuclear levels of Smad2 and Smad3 were increased ($p < 0.001$) by 82% in the SCI-vehicle group compared to the

sham-SCI group. Nuclear levels of Smad2/3 were decreased by testosterone combined with nandrolone ($p < 0.01$), to levels only 28% greater than those present in the sham-SCI group (Fig. 3B). A similar reduction in nuclear Smad2/3 was seen for the SCI-testosterone group compared to the SCI-vehicle group ($p < 0.01$, Fig. 3B).

Expression of IGF-1 and its receptor were not significantly changed in the SCI-vehicle group compared to the sham-SCI group at 56 days; these mRNA levels were not significantly altered after SCI by testosterone alone or testosterone combined with nandrolone (Fig. 4A and B). For mRNA levels of FOXO1, FOXO3A, MAFbx, and MuRF1, there was no significant difference when comparing the SCI-vehicle with sham-SCI groups (Fig. 4C–F); neither testosterone alone nor testosterone combined with nandrolone significantly altered levels of these mRNAs.

At 56 days, PGC-1 α mRNA levels were reduced by 75% for the SCI-vehicle group compared to the sham-SCI group ($p < 0.01$, Fig. 5A), accompanied by smaller decreases in PGC-1 β mRNA levels, which did not reach significance (Fig. 5B). Total and nuclear PGC-1 α protein levels were reduced by SCI by 60% ($p < 0.001$), and 50% ($p < 0.05$), respectively (Fig. 5C and D). Testosterone alone or combined with nandrolone increased PGC-1 α mRNA expression to levels similar to those seen in the sham-SCI group ($p < 0.05$; Fig. 5A). Testosterone combined with nandrolone increased both total and nuclear PGC-1 α levels ($p < 0.05$, Fig. 5C and D). Nuclear PGC-1 α levels were not significantly different between the sham-SCI and SCI-nandrolone groups. Compared to the SCI-vehicle group, the SCI-testosterone group also demonstrated an increase in nuclear PGC-1 α protein levels ($p < 0.05$), which was similar to the increase observed for the SCI-nandrolone group (Fig. 5D).

Compared to the sham-SCI group, in the SCI-vehicle group there were reductions in mRNA levels for the PGC-1 α target genes troponin I slow ($p < 0.001$), slow myosin heavy chain (MyHC7, $p < 0.01$), succinyl-CoA:3-ketoacid-coenzyme A transferase (Oxct1, $p < 0.01$), lactate dehydrogenase A (LDHA, $p < 0.05$), and medium chain acyl-CoA dehydrogenase (ACADM, $p < 0.05$); reductions in these target genes were between 60% to 95% (Fig. 6B–F). Troponin I fast mRNA levels were increased ($p < 0.01$) for the SCI-vehicle group compared to the sham-SCI group (Fig. 6A). Testosterone plus nandrolone increased expression of troponin I slow mRNA ($p < 0.01$) to levels that were not significantly different from the sham-SCI group (Fig. 6B), and reduced expression of troponin I fast mRNA ($p < 0.05$; Fig. 6A). For the SCI-testosterone group, troponin I fast mRNA levels were lower ($p < 0.01$) than the SCI-vehicle group, and similar to the sham-SCI group (Fig. 6A). Troponin I slow mRNA levels were not significantly altered by testosterone (Fig. 6B). When compared to the SCI-vehicle group, the mRNA levels for MyHC7, Oxct1, LDHA, and ACADM were not altered by testosterone alone or combined with nandrolone (Fig. 6C–F).

When comparing the SCI-vehicle group with the sham-SCI group, protein levels were reduced by SCI by 40–60% for ACADM ($p < 0.001$), slow myosin (MyHC7; $p < 0.001$), and slow troponin I ($p < 0.001$; Fig. 7B–D). Levels of LDHA tended to be reduced after SCI, but this trend was not significant (Fig. 7A). Testosterone alone increased protein levels for LDHA ($p < 0.01$), ACADM ($p < 0.001$), and slow troponin I ($p < 0.05$; Fig. 7A, C, and D). Compared to the SCI-vehicle group, the SCI-nandrolone group also demonstrated increased protein

FIG. 3. Effects of spinal cord injury (SCI), testosterone, and nandrolone on myostatin and nuclear importation of Smad2 and Smad3 at 56 days after SCI. Randomly selected samples of gastrocnemius muscle were assessed for: **A**, levels of Smad2 and Smad3 present in homogenates as assessed by Western blotting using β -tubulin as a loading control; and **B**, levels of Smad2 and Smad3 present in nuclear extracts as assessed by Western blotting using histone H1 as a loading control. The graphs depict the results of scanning densitometry as mean value $\pm$ standard error of the mean for fold-change relative to sham-SCI animals, while the insets show non-contiguous lanes from representative Western blots. The p value under each figure refers to that obtained for the one-way analysis of variance, and those for comparisons between pairs of means were obtained using the Newman-Keuls *post-hoc* test ($n=4-5$ per group; NS, the indicated comparisons were not significant; Ts, SCI + testosterone; Ts/Nan, SCI + testosterone + nandrolone; Ve, vehicle).

levels for LDHA ($p < 0.05$), ACADM ($p < 0.01$), and slow tropomyosin I ($p < 0.01$), with the magnitude of the increases being similar to testosterone alone (Fig. 7A, C, and D). Compared to the SCI-vehicle group, slow MyHC7 levels were not significantly increased for the SCI-testosterone or SCI-nandrolone groups (Fig. 7C).

Discussion

Muscle mass and protein

At 56 days after SCI, a modest, though significant, preservation of body weight and gastrocnemius muscle mass was observed when testosterone was administered in combination with nandrolone, whereas administration of testosterone alone was not protective. These steroid agents have the same mechanism of action, which involves binding to and activation of the androgen receptor; a major difference between the SCI-testosterone and SCI-nandrolone groups then is the total dose of androgen administered. In studies of healthy men, clear dose-response relationships have been described for the effects of testosterone on muscle mass and strength (Bhasin et al., 2001). Thus, in part, the difference in effectiveness of androgens in the SCI-testosterone and SCI-nandrolone groups may reflect dose-response relationships.

The magnitude of protection afforded by testosterone plus nandrolone is similar to that observed in a report which found that administration of testosterone to male Sprague-Dawley rats partially protected against decreases in muscle fiber cross-sectional area (CSA) at 11 weeks (Gregory et al., 2003). In that study, fiber size was reduced by 49% by SCI for gastrocnemius, soleus, and tibialis anterior, whereas only a 30%

reduction in CSA was observed for muscle fibers in SCI animals administered testosterone. Thus, the findings indicated that testosterone protected against 40% of the loss that would have otherwise occurred. Testosterone was also found to increase body weight and muscle fiber CSA of the soleus and extensor digitorum longus muscles in male mice at 4 and 8 weeks after SCI (Ung et al., 2010). Of note, the mass of these muscles was not significantly altered by this agent. Why testosterone would increase muscle fiber CSA but not alter mass is unclear. Possible explanations include changes in fiber number, or replacement of inter-fiber fat with muscle.

SCI and anabolic and catabolic genes and signals

Shortly after paralysis, the catabolism of muscle proteins is increased in part through the activities of MAFbx and MuRF1 (Bodine et al., 2001). Expression of these ubiquitin ligases is upregulated during muscle atrophy by FOXO1 and FOXO3A (Sandri et al., 2004; Stitt et al., 2004; Waddell et al., 2008). Conversely, muscle anabolism is stimulated by IGF-1, which inactivates FOXO1 and FOXO3A and blocks atrophy-related increases in the expression of MAFbx and MuRF1 (Stitt et al., 2004). How expression of MAFbx and MuRF1 changes over time after SCI is not well understood. In a prior study using the same rat SCI model, the expression levels of MAFbx and MuRF1 were initially elevated after SCI (i.e., at 3 and 7 days), but returned to baseline values by 14 days after SCI (Zeman et al., 2009). The rapid return of the expression of these genes to baseline values was postulated to reflect the development of spasticity and increased neuromuscular tone (Zeman et al., 2009). The findings from the present study also argued against

FIG. 4. Effects of spinal cord injury (SCI), testosterone, and nandrolone on mRNA levels of insulin-like growth factor -1 (IGF-1) and its receptor and genes for muscle protein catabolism at 56 days after SCI. (A–F) mRNA levels for the indicated genes were determined by quantitative real-time PCR (qPCR) and are shown as mean fold-change versus sham-SCI animals $\pm$ standard error of the mean. The *p* value under each figure refers to that obtained for the one-way analysis of variance, and those for comparisons between pairs of means were obtained using the Newman-Keuls *post-hoc* test (numbers for each group: sham-SCI, 11; SCI-vehicle, 9; SCI-Ts, 12; SCI-nandrolone-testosterone; NS, the indicated comparisons were not significant; Ts, testosterone; Nan, nandrolone).

a role for classical atrophy-related genes at 56 days, because at this time point expression levels for MAFbx and MuRF1 were comparable to those for controls. Thus, one might infer that any decline in muscle mass beyond the first 14 days after SCI must reflect negative influences beyond those of MAFbx and MuRF1.

After activation of activin receptor IIB by myostatin, Smad2/Smad3 are phosphorylated, then translocated to the nucleus (Ruegg and Glass, 2011). Activation and nuclear translocation of Smad2/Smad3 mediate muscle atrophy in-

duced by myostatin and other TGF- β family members (Sartori et al., 2009). The expression of the activin receptor IIB was increased at 56 days after SCI associated with increased nuclear levels of Smad2/Smad3 without alterations in myostatin mRNA levels. The findings suggested that at 56 days after SCI, signaling through Smad2 and/or Smad3 was increased in gastrocnemius muscle. These findings are consistent with increased myostatin signaling, possibly through increased availability of the myostatin receptor activin receptor IIB. Increased expression of follistatin and follistatin-like 3 was also

FIG. 5. Levels of PGC-1 mRNA and protein after SCI, and the effects of testosterone alone or with nandrolone on them at 56 days after SCI. Real-time PCR was used to determine mRNA levels for PGC-1 α (A), and PGC-1 β (B; $n=9-11$ per group). (C) Protein levels of PGC-1 α present in homogenates of gastrocnemius muscle were determined by Western blotting. The graph shows results of scanning densitometry as mean value $\pm$ standard error of the mean for fold-change relative to sham-SCI animals ($n=4$ per group; samples of gastrocnemius muscle were randomly selected for each group). The insets depict non-contiguous lanes of a representative Western blot. (D) Levels of PGC-1 α protein present in nuclear extracts are shown ($n=4$ per group; samples of gastrocnemius muscle were randomly selected for each group). The insets depict non-contiguous lanes of a representative Western blot. The *p* value under each figure refers to that obtained for the one-way analysis of variance, and those for comparisons between pairs of means were obtained using the Newman-Keuls *post-hoc* test (NS, the indicated comparisons were not significant; SCI, spinal cord injury; PGC-1, peroxisome proliferator-activated receptor gamma coactivator-1; PCR, polymerase chain reaction; Ts, testosterone; Nan, nandrolone; Veh, vehicle).

observed after SCI. These changes are most likely feedback inhibitory responses to increased myostatin signaling. This possibility is supported by a report that follistatin expression is increased by Smad3 when bound to a cognate regulatory element in the follistatin gene (Blount et al., 2008).

Taken together, the findings suggest that by 56 days after SCI, increased myostatin signaling through Smad2/Smad3 is one of the major negative influences on muscle size. The findings also suggest that by 56 days after SCI the initial phase of rapid catabolism of muscle proteins characteristic of the acute phase of muscle atrophy has resolved. The findings do not exclude the possibility that other pathways contribute to muscle atrophy at earlier times after SCI. Additional studies at earlier time points after SCI are needed to determine when after SCI Smad2/Smad3 become activated. Further studies are also needed to more specifically characterize the role of

Smad2/3 activation and myostatin in stimulating muscle atrophy after this injury.

One must also consider the possibility that signaling through myostatin declines with time after SCI, just as expression of MAFbx and MuRF1 does. How myostatin signaling changes in humans after SCI is unclear. A study of skeletal muscle from humans with chronic SCI revealed depressed myostatin mRNA levels, but myostatin protein levels were not changed (Leger et al., 2009). In a separate study, analysis of gene expression changes in the soleus muscles of two individuals with SCI who had undergone long-term training using selective electrical stimulation of the soleus were reported. These studies revealed significant down-regulation of myostatin mRNA (Adams et al., 2011).

In comparison to the findings of the present study of muscle after SCI, somewhat different results were obtained in

FIG. 6. Levels of mRNA for PGC-1 α target genes at 56 days after SCI. (A–F) mRNA levels for the indicated genes were determined by PCR and are shown as mean fold-change versus sham-SCI $\pm$ standard error of the mean. The *p* value under each figure refers to that obtained for the one-way analysis of variance, and those for comparisons between pairs of means were obtained using the Newman-Keuls *post-hoc* test ($n = 7$ –11 per group; NS, the indicated comparisons were not significant; SCI, spinal cord injury; PGC-1, peroxisome proliferator-activated receptor gamma coactivator-1; PCR, polymerase chain reaction; Ts, testosterone; Nan, nandrolone; Veh, vehicle; MyHC7, slow myosin heavy chain; Oxct1, succinyl-CoA:3-ketoacid-coenzyme A transferase; LDHA, lactate dehydrogenase A; ACADM, medium chain acyl-CoA dehydrogenase).

studies of paralyzed muscle after stroke or nerve injury. In stroke patients, myostatin mRNA levels were increased in the vastus lateralis from the paretic versus non-paretic side and were reduced by resistance training (Ryan et al., 2011). In studies of rodents that have examined skeletal muscle after nerve transection or nerve crush, there have been persistent elevations of myostatin protein in some studies, but only a transient increase reported in others (Baumann et al., 2003; Lima et al., 2009).

Nandrolone and anabolic and catabolic genes and signals

Although IGF-1 is an androgen-responsive gene that is upregulated in skeletal muscle in response to these steroid hormones, nandrolone did not alter mRNA levels for IGF-1 or its receptor after SCI. These findings argue against a role for upregulation of IGF-1 signaling in preserving muscle mass after SCI. Nandrolone reduced nuclear localization of Smad2/Smad3 after SCI, associated with reduced expression of the activin receptor IIB. These findings suggest that nandrolone reduced signaling in response to myostatin or another TGF- β superfamily member, which would be expected to be

anabolic to muscle. This conclusion is further supported by findings that these changes in nuclear localization of Smad2/Smad3 occurred without changes in expression of FOXO1, MAFbx, or MuRF1.

Myostatin signaling has been a target for the action of androgens in one other report, although the mechanisms by which this occurred appear to be distinct from those observed after SCI. In that report, testosterone upregulated the myostatin inhibitor follistatin in the CH3 10T1/2 cell line and neurologically intact mouse muscle (Singh et al., 2009). Conversely, testosterone-induced upregulation of MyoD and myosin heavy chain were blocked by anti-follistatin antibodies (Singh et al., 2009).

Our studies do not address the interesting question of whether gains in muscle mass persist after discontinuation of testosterone combined with nandrolone. Clinical studies have indicated that whether gains in muscle mass continue after discontinuation of androgen therapies depends on context. In burn victims, individuals administered oxandrolone leave the hospital with more muscle, and this benefit persists after discontinuing the therapy (Demling and DeSanti, 2003). Conversely, in able-bodied older men, gains in muscle from exogenous androgens are lost once treatment

FIG. 7. Levels of protein encoded by PGC-1 α target genes at 56 days after SCI. Gastrocnemius muscle homogenates were subjected to Western blotting for LDHA (**A**), ACADM (**B**), slow myosin heavy chain (MyHC7, **C**), and troponin slow I (**D**). The graphs depict results of scanning densitometry as mean $\pm$ standard error of the mean using β -tubulin as the loading control. Insets above each graph show non-contiguous lanes from representative Western blots. The p values below each graph are for the one-way analysis of variance, and those above the bars reflect results of the Newman-Keuls test for the indicated comparisons ($n=7-11$ per group; NS, the indicated comparisons were not significant; PGC-1, peroxisome proliferator-activated receptor gamma coactivator-1; Ts, testosterone; Nan, nandrolone; Veh, vehicle; Oxct1, succinyl-CoA:3-ketoacid-coenzyme A transferase; LDHA, lactate dehydrogenase A; ACADM, medium chain acyl-CoA dehydrogenase).

is discontinued (Schroeder et al., 2004). Some effects of androgens appear to be more long lasting, however. In a study of the effects of oxandrolone on elderly men, a continued decrease in adipose tissue mass was observed 12 weeks after discontinuing oxandrolone administration (Schroeder et al., 2004).

SCI and determinants of oxidative metabolism

Expression of PGC-1 α is sufficient to promote slow-twitch oxidative fiber transformation (Lin et al., 2002); this and other evidence indicates that PGC-1 α is a key determinant of muscle

fiber type and muscle fiber capacity for oxidative metabolism (Finck and Kelly, 2006). PGC-1 α is a transcriptional co-regulator, and consequently must enter the nucleus and coordinate with transcription factors, such as PPAR- γ and MEF2, to regulate muscle gene expression (Finck and Kelly, 2006). Thus in this study we used nuclear levels of PGC-1 α as an indicator of whether this protein was activated. To gain insight into whether any nuclear PGC-1 α was transcriptionally active at key PGC-1 α -responsive genes, the expression of selected PGC-1 α target genes encoding proteins necessary for energy metabolism and slow-twitch myofiber phenotypes was examined.

At 56 days after SCI, expression of PGC-1 α mRNA and protein was markedly reduced, and PGC-1 α was not present in detectable amounts in the nucleus. These changes in PGC-1 α levels were accompanied by a marked decline in the expression of PGC-1 α -regulated genes of energy metabolism. Genes downregulated after SCI included LDHA, ACADM, and Oxct1, and slow muscle proteins such as slow myosin heavy chain and slow troponin I. Thus, paralysis after SCI excluded PGC-1 α from the nucleus, reduced its expression, and downregulated several of its known target genes.

These findings agree well with results from studies of human muscle biopsy samples after SCI that showed a decline in the levels of enzymes of oxidative metabolism, and a shift away from slow-twitch oxidative fibers toward fast-twitch glycolytic fibers (Dudley-Javoroski and Shields, 2008). The marked decline in PGC-1 α expression after SCI observed in the current study, and the associated exclusion of remaining protein from the nucleus, provide one likely molecular mechanism for these changes in biochemical and physiological properties. The reduced expression and nuclear entry of PGC-1 α at 56 days after SCI in rats in the present study agrees with and extends reports of changes in PGC-1 α levels in skeletal muscle in rodent models of other states of paralysis. Expression of PGC-1 α has been reported to be reduced in rat muscle by paralysis from nerve transection or spinal isolation, a variant of SCI in which reflex arcs are disrupted by dorsal rhizotomy (Sacheck et al., 2007).

Nandrolone and determinants of oxidative metabolism

Expression of PGC-1 α mRNA and protein were increased by testosterone alone or in combination with nandrolone, as was nuclear content of PGC-1 α . Testosterone plus nandrolone increased mRNA levels for slow troponin I and reduced levels of fast troponin I, but did not significantly alter mRNA levels of slow myosin heavy chain or enzymes of oxidative metabolism. Thus, the nuclear PGC-1 α that accumulates after administering this combination of androgens is either not fully active, or is repressed by other transcriptional regulators upregulated or activated by SCI. Of interest, it is thought that PGC-1 α coordinates with NRF1 and NRF2 to upregulate expression of mitochondrial genes, and MEF2 to promote expression of slow-twitch fiber genes (Lin et al., 2005). Thus, the limitations on PGC-1 α action observed in our study may relate in part to the gene expression programs activated by specific transcription factors with which this transcriptional coregulator associates. In addition, the activation of PGC-1 α by androgens may preferentially stimulate MEF2-dependent gene expression programs over programs regulated by NRF1/2.

Testosterone plus nandrolone increased levels of the proteins slow troponin I, LDHA, and ACADM, without significant increases in corresponding mRNA levels. One interpretation for this finding is that there is uncoupling between mRNA and protein levels for these genes, which presumably reflects additional, post-transcriptional layers of regulation, such as changes in protein stability or synthesis. Our findings agree with those reported in a rat model in which testosterone significantly increased enzymatic activities of succinate dehydrogenase and α -glyceryl phosphate dehydrogenase at 11 weeks after SCI caused by a complete mid-thoracic transection (Gregory et al., 2003).

Levels of PGC-1 α have also been found to be regulated by androgens in normally innervated muscle (Ibebunjo et al., 2011). In these studies, orchiectomy reduced PGC-1 α levels in normally innervated muscle. Conversely, such levels were upregulated in orchiectomized mice by testosterone replacement therapy (Ibebunjo et al., 2011). Of interest, and in agreement with our studies, the effects of administration of testosterone on PGC-1 α mRNA levels increased without corresponding elevations in the expression of PGC-1 α target genes. In a rat model of glucocorticoid-induced muscle atrophy, PGC-1 α protein levels and nuclear content were reduced by 7 days of dexamethasone, and co-administration of testosterone increased nuclear import and absolute levels of PGC-1 α protein. However, mRNA levels were not altered by either agent (Qin et al., 2010c), indicating that PGC-1 α levels are subject to regulation at the level of transcription and translation and/or post-translational pathways.

Because PGC-1 α expression and nuclear localization are highly regulated by neuromuscular activity (Akimoto et al., 2005), one must consider the possibility that one action of testosterone alone or combined with nandrolone was to increase the activity of animals. We did not formally examine activity levels or behavioral measures. However, during observations of the animals, there was no appreciable change in hindlimb function. The animals were unable to place their foot or bear weight. We saw no effect of androgens on foot placement or the ability to bear weight in this study or three other similar studies. If subtle changes in activity did occur, the magnitude of such changes is thus small, and would not appear to correlate with the complete normalization of Smad2/Smad3 levels, activin receptor IIB mRNA levels, or PGC-1 α levels we observed in animals treated with androgens. This is particularly true for the SCI-testosterone group, for which no change in muscle mass was observed despite large changes in signaling. These discrepancies together with the independent evidence discussed above that androgens modulate myostatin signaling and PGC-1 α mRNA and protein levels in animals without paralysis (Ibebunjo et al., 2011), lend credibility to the interpretation that at least some of the changes observed in our studies of muscle after SCI are directly attributable to actions of androgens on skeletal muscle.

Conclusions

SCI results in a marked reduction of PGC-1 α levels and exclusion of remaining PGC-1 α from the nucleus, associated with downregulation of genes for oxidative metabolism and slow-twitch muscle phenotype. These changes are associated with increased signaling through Smad2 and Smad3, likely due to increased expression of the activin receptor IIB, and/or increased levels of myostatin protein or that of related TGF- β family members. Testosterone alone or combined with nandrolone blocked SCI-related signaling through Smad2 and Smad3, and increased PGC-1 α levels and nuclear entry, but only selectively upregulated PGC-1 α -regulated genes. The effects of testosterone plus nandrolone on LDHA protein exceeded the corresponding changes in mRNA levels, suggesting that these agents exert additional post-transcriptional layers of regulation of proteins regulated by PGC-1 α . Further study will be needed to establish the effects of these agents on muscle oxidative metabolism, fiber type, and function.

Acknowledgments

This work was supported by the Veterans Health Administration, Rehabilitation Research and Development Service (B4162C, B4616R, and B3347K).

Author Disclosure Statement

No competing financial interests exist.

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