Nandrolone decanoate modulates cell cycle regulation in functionally overloaded rat soleus muscle

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McClung, Joseph M., Kristen A. Mehl, Raymond W. Thompson, Larry L. Lowe, and James A. Carson. Nandrolone decanoate modulates cell cycle regulation in functionally overloaded rat soleus muscle. Am J Physiol Regul Integr Comp Physiol 288: R1543–R1552, 2005; doi:10.1152/ajpregu.00285.2004.—Functionally overloading rat soleus muscle by synergist ablation induces a rapid increase in mass. Muscle remodeling during the first week of overload is critical for the overload-induced growth. Anabolic steroid modulation of this overload-induced remodeling response is not well understood. The purpose of this study was to determine whether pretreatment with nandrolone decanoate, a clinically administered anabolic steroid, alters muscle morphology and gene expression related to muscle growth during the initiation of functional overload in the rat soleus muscle. Adult (5 mo) male Fisher 344 × Brown Norway rats were randomly assigned to control (Sham), 3-day functional overload (OV), nandrolone decanoate administration (ND), or 3-day functional overload with nandrolone decanoate administration (OV + ND) treatment groups. Morphologically, OV increased the percentage of small (36%) and large (150%) fibers and expanded the ECM 50%. ND administration decreased the 3-day OV induction of small fibers 51% (361%) and large (150%) fibers and expanded the ECM 50%. ND administration also attenuated the induction of cell cycle regulator p21 (64%) and myogenin (37%) mRNAs after 3 days of overload. These data demonstrate that nandrolone decanoate pretreatment can alter morphological and cell cycle regulator expression related to muscle growth at the onset of functional overload.

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anabolic steroids; hypertrophy; myogenic regulatory factors

OVERLOADING RAT HINDLIMB MUSCLE by synergist ablation stimulates rapid muscle remodeling (34, 49). Initially, chronically overloading muscle induces edema, macrophage infiltration, extracellular matrix expansion, and satellite cell proliferation (49). The large growth response of these overloaded muscles appears to require the integration of many signaling stimuli including mechanical, growth factor, steroid hormone, and cytokine pathways. Androgens have anabolic effects in skeletal muscle (5), and testosterone administration can interact with increased muscle loading to induce muscle mass accretion in healthy and diseased humans (6). Androgens can also interact with components of mechanical stimuli-induced signaling pathways to increase muscle mass in functionally overloaded rat skeletal muscle (38) and synergistically alter the response of overloaded rat skeletal muscle to disuse atrophy (53). Although anabolic steroid administration can alter muscle mass, the intramuscular processes regulated by anabolic steroid interaction with functional overload are not well defined.

Satellite cells are quiescent myoblasts located between the basal lamina and the muscle fiber sarcolemma and are critical for muscle growth and regeneration (42). Satellite cell entry and exit from the cell cycle can be regulated by a variety of alterations to the surrounding environment in the muscle including mechanical, growth factor, and hormonal signaling (8). Satellite cell activation is tightly regulated by the helix-loop-helix myogenic regulatory factor (MRF) family of DNA binding proteins (46). This family includes MyoD, myf-5, myogenin, and MRF4 (46). Cyclin-dependent kinases (Cdk2, Cdk4) complex with cyclin proteins to promote the G0-G1 transition and S-phase DNA synthesis. MyoD and cyclin D1 are associated with satellite cell proliferation, whereas myogenin and cyclin kinase inhibitors, such as p21, are associated with differentiation and cell cycle withdrawal (48). MyoD possesses two specific regions involved in the remodeling of repressed chromatin, mediating genetic transcriptional activation that myogenin is incapable of regulating (24). Targeted deletion or inhibition of MyoD expression also results in the downregulation of M-cadherin, a critical protein involved in cell adhesion and myoblast fusion in skeletal muscle (46). Myogenin knock-out results in severe skeletal muscle deficiency and early death in mice, primarily due to the inability of skeletal muscle myoblasts to fuse and form mature myofibers (40). MyoD overexpression increases myocyte number in culture but is unable to attenuate the myogenin−/− response in mice, suggesting distinct roles for MyoD and myogenin in the regulation of myoblast commitment and differentiation (40).

Alterations in the expression of both MRFs and other cell cycle-regulating proteins can alter skeletal muscle’s ability to regenerate from injury. Antisense inhibition of MyoD prevents muscle regeneration in snake venom (notexin)-injured skeletal muscle, resulting in the increased occurrence of small fibers as late as 4 wk after initial injury (56). MyoD mRNA is induced early during skeletal muscle regeneration from cardiotoxin injection and is followed by the induction of differentiation markers such as MRF4 and myogenin (55). Irradiation suppresses skeletal muscle MyoD expression after injury, and there is also a delayed induction of myogenin and a deficient regeneration response. These outcomes have been associated with an inability of skeletal muscle satellite cells to fuse with regenerating myofibers (55). Adams et al. (2) demonstrated that myogenin and p21 mRNAs are induced as early as 12 h after functional overload in plantaris muscle, which is before increases are seen for MyoD and DNA content. Discerning the regulation of myogenic factors during skeletal muscle regener
eration is critical for understanding the control of satellite cell proliferation and differentiation that is essential for muscle growth and repair.

Testosterone administration can increase satellite cell number in both humans (47) and rodents (29). Additionally, testosterone administration modulates both skeletal muscle total DNA content (11, 34) and the corresponding myonuclear number (47). Androgen action in skeletal muscle is mediated through binding to its cytosolic androgen receptor and translocation to the nucleus, where this complex can regulate gene transcription. Androgen receptor ligand binding capacity and gene expression increase in functionally overloaded rat skeletal muscle (35). The functional role of androgen receptor induction in overloaded skeletal muscle is not certain. However, there is the possibility that increased androgen receptor expression is important for fiber adaptation to overload, possibly through the regulation of cell cycle proteins critical for satellite cell activity. Androgens can regulate notch signaling in the prostate (41), and in injured skeletal muscle, notch signaling is related to satellite cell proliferation (18). MRFs and other cell cycle regulatory factor genes are transcriptional target genes of androgens (36, 37). Androgens induce Cdk2, Cdk4, and p21 expression and can repress the Cdk inhibitor p16, resulting in increased Cdk kinase activity (37). Androgen receptor interaction with transcription factor Sp1 forms a preinitiation transcription complex that enhances p21 gene expression (36). Exogenous androgen administration increases androgen receptor expression in fast- and slow-type hindlimb skeletal muscle in adult rats (11) and can synergistically increase muscle androgen receptor expression during the initial growth response of rat soleus muscle to functional overload (34). The possibility of interactive effects between exogenous androgen administration and functional overload on myogenic and cell cycle regulatory gene expression has not been investigated in skeletal muscle.

Functional overload induced by synergist ablation is a potent growth stimulus (34, 49). Besides myofiber hypertrophy, the first several days after the application of the stimulus can be characterized by increased DNA content, satellite cell activation, inflammatory cell invasion, and fibroblast proliferation (30). Testosterone can potentially regulate all of these events (11, 47). Although testosterone can influence myonuclear number and satellite cell activity, the effect of anabolic steroid administration on initial overload-induced changes in muscle morphology and myogenic cell regulatory factor gene expression is not known. The overall purpose of this study was to determine whether anabolic steroid pretreatment would enhance overload-induced morphological and gene expression changes related to muscle growth in the rat soleus muscle. The study’s primary hypothesis was that anabolic steroid pretreatment would enhance myofiber enlargement, while attenuating the incidences of damaged or regenerating myofibers. The final hypothesis was that nandrolone decanoate pretreatment would accentuate overload-induced changes in cell regulatory factor gene expression related to proliferation, while suppressing gene expression related to differentiation. Further delineation of mechanisms related to anabolic steroid action in skeletal muscle is critical for understanding the potential and limitations of anabolic steroid’s therapeutic use in muscle mass maintenance and enlargement. Soleus muscles from 5-mo adult rats were analyzed for muscle morphology and gene expression related to cell cycle regulation after 3 days of functional overload with nandrolone decanoate pretreatment.

METHODS

Animals and housing. Twenty-two male Fisher 344 × F34B, Brown Norway rats (~4 mo at the start of the study) were acquired from the National Institute on Aging aged rodent colony. Animals were housed individually, kept on a 12:12-h light-dark cycle, and given ad libitum access to normal rodent chow and water for the duration of the study at the fully accredited animal care facilities at the University of South Carolina, Columbia. Rats were randomly assigned to four treatment groups as follows: 1) control-oil (Sham), 2) overload-oil (OV), 3) overload-steroid (OV + ND), and 4) control-steroid (ND). All procedures were approved by the University of South Carolina Animal Care and Use Committee.

Surgical ablation-induced overload. The hindlimb soleus and plantaris muscles were functionally overloaded for 3 days by bilateral surgical ablation of the distal third of the lateral and medial gastrocnemius muscle as previously described (34, 38). Rats were weighed and carefully observed for signs of failure to thrive, such as precipitous weight loss, disinterest in the environment, or unexpected gait alterations. The plantaris muscles were removed at 3 days after the initial surgery, frozen in liquid nitrogen, and stored at −80°C for future analyses. The soleus muscle was removed 3 days after the initial surgery, frozen in liquid nitrogen, and stored at −80°C for analysis in the present study.

Anabolic steroid administration. The anabolic steroid nandrolone decanoate (Deca-Durabolin; Orionon) was used in these studies because of its long biological half-life and previous studies demonstrating an anabolic effect in rat skeletal muscle (11). Nandrolone decanoate was injected (6 mg/kg body wt) intramuscularly into the hip region every 7 days as previously described (34). Animals were weighed before each injection, and the administration of anabolic steroid was adjusted to reflect a consistent 6 mg/kg body wt dose. Control animals received intramuscular injection of a similar volume of sesame seed oil. Controls and overloaded animals received three injections of either nandrolone decanoate in sesame seed oil or sesame seed oil alone for the 14-day period before surgical treatment. The last injection for animals was given at the time of the surgical treatment.

Morphological analysis. Transverse sections were cut from the soleus muscle midbelly on a cryostat at −20°C and mounted. Hema
toxyl and eosin staining was performed on sections taken from soleus in all treatment animals (n = 22). For the determination of nuclear localization, eight digital images were taken from each hematoxylin and eosin-stained muscle section at a ×40 magnification, and a minimum of 1,400 individual nuclei per sample were counted and analyzed for myofiber and extracellular matrix localization per square millimeter of muscle area by a blinded investigator as previously described (3). To reduce experimental bias, all nuclei present on digital images were quantified.

Fiber cross-sectional area and extracellular matrix percentage. For cross-sectional area (CSA) analysis, three distinct digital images from hematoxylin and eosin-stained muscle sections at a ×20 magnification were taken and analyzed for soleus fiber CSA (μm²) with National Institutes of Health imaging software (Scion Image). Each fiber was traced with a handheld mouse, and the number of pixels traced was calibrated to a defined area in square micrometers. Approximately 200 fibers were traced per sample, which, by examination of no additional change in standard deviation, was determined to be an appropriate fiber number. All fibers in the cross section images were quantified unless the sarcolemma was not intact. For extracellular matrix percentages, six digital images of hematoxylin and eosin-stained muscle sections at a ×40 magnification were analyzed (14). Middle segment sections of soleus muscle images were visualized and an 18 × 14 grid overlaid on each image. Each dot was counted by a blinded investigator as a part of the extracellular matrix if it was not
on a muscle fiber. Dots at least 75% in the extracellular matrix were counted. Dots that were not clearly distinguishable were omitted from the count. All analyses were performed by a researcher blinded to the treatment groups.

Sarcosome loss and central nuclei occurrence. Five digital images of hematoxylin and eosin-stained muscle sections at a ×20 magnification were analyzed by a blinded investigator as previously described (25), with the following modifications. Criteria for central nuclei were defined as normal fibers that contained a well-defined nucleus in the center of the fiber approximately equidistant from the sarcosome on all sides. Sarcosome loss was defined as the loss of >25% of the total surrounding myofiber sarcosome.

Crude protein extracts. Crude protein extracts were made as previously described (34, 35, 38). Frozen soleus muscles were homogenized in Mueller buffer on ice with a Polytron homogenizer (Kinematica Switzerland), using 3 × 15 pulses at a low setting. Homogenates were fractionated into soluble and insoluble fractions by centrifugation, with the protein concentration determined by Bradford assay (Bio-Rad), aliquoted, and stored at −80°C until use for Western blotting.

Western blot analysis. p70s6k protein and phosphorylation levels were determined in 3-day soleus skeletal muscle as previously described (38). After gel transfer, membranes were probed with p70s6k primary antibodies (C-18; Santa Cruz) diluted in 1% milk-Tris-buffered saline-Tween 20 (TBST; 1:6,500). The horseradish peroxidase-conjugated anti-rabbit secondary antibody was diluted in 1% milk-TBST (1:6,500), visualized by chemiluminescence (ECL, Amersham Life Sciences) as per manufacturer instructions, and quantified by densitometry scanning (Scion Technologies, Frederick, MD). Relative phosphorylation amounts were determined as previously described (38) by summing the total amount of phosphorylated p70s6k and dividing by the total p70s6k.

Northern blot analysis. Northern blot analyses for p21, cyclin D1, and myogenin were performed as previously described (13). Briefly, 15–20 μg of total RNA were fractionated on a denaturing 1% agarose gel (1× MOPS, 6.7% formaldehyde) and then transferred to a nylon membrane by capillary action. All probes for Northern blot analysis were made by random priming as previously reported (13). Membranes were then visualized by autoradiography (−80°C, 3–40 h) and quantified by densitometry scanning (Scion Image, Frederick, MD). Relative phosphorylation amounts were determined as previously described (38) by summing the total amount of phosphorylated p70s6k and dividing by the total p70s6k.

Total RNA isolation and cDNA synthesis. Total RNA was isolated with TRIzol reagent (Life Technologies, Grand Island, NY) per the manufacturer’s instructions. cDNA was reverse transcribed from 4 μg of total RNA with 1 μl of random hexamers and 50 U of Superscript II RT (Invitrogen) in a final volume of 20 μl at 25°C for 10 min, followed by 42°C for 50 min and 70°C for 15 min.

Semiquantitative PCR. PCR amplification was performed with primers for MyoD and IGF-I as previously described (35). For the MyoD probe, the following oligonucleotides were used: sense 5′-CCCGACGGGCTCTGCTGCTT-3′, antisense 5′-CGCGTGGGCTTGCTGCA-3′. Amplification was carried out by repeating the following cycles: MyoD (34 cycles); denaturation at 94°C for 45 s, annealing at 61°C for 60 s, and extension at 72°C for 60 s, with a final extension cycle at 72°C for 10 min. IGF-I (200 bp, accession no. X06043) primer sequences are similar to those published by Adams et al. (1): sense 5′-GCAATGTGGAGATGTGTC-3′, antisense 5′-GGAGGCCGCTCCTCCTCATTC-3′. Primer sequences were verified by Primer3 Input and modified to optimize melting temperature. The number of cycles and the PCR conditions for mRNAs were optimized so that the amplified signal was on the linear portion of the amplification curve. Amplified products were subjected to electrophoresis through 1.0% agarose gels, stained with ethidium bromide, visualized by ultraviolet transillumination, photographed, and then quantified by densitometry scanning (Scion Image). Target fragment levels were normalized against GAPDH, a kind gift provided by Dr. James Reecy (Dept. of Animal Science, Iowa State University, Ames, IA). The sequence for GAPDH (240 bp, accession no. BC059110) primer is sense 5′-TGATGACATCAAGAAGGTGTTGAA-3′, antisense 5′-TCCCTGGAAGCCTGTAGGCCCT-3′. Data are presented as each mRNA IOD-to-GAPDH IOD ratio.

Data analysis. Frequency histograms and the frequency of fibers <500 μm² and >5,000 μm² were compared by a one-group χ² analysis. Statistical significance was set at P ≤ 0.05, and a Bonferroni post hoc analysis was used to determine differences between treatment groups. All other variables were analyzed by a two-way ANOVA to test for main effects (steroid treatment or functional overload) and interactions (steroid treatment × functional overload) with P ≤ 0.05 accepted as the level of statistical significance. Where significant interactions existed, independent t-tests were used as post hoc analyses between treatments. All results are reported as means ± SE.

RESULTS

Muscle weights. Nandrolone decanoate administration (ND; 173 ± 5 mg) had no effect on soleus muscle mass. Three days of overload (OV; 187 ± 7 mg) increased soleus muscle wet weight (mg) from Sham control values (Sham; 161 ± 3 mg). There was no interaction of overload and nandrolone decanoate administration (OV+ND; 181 ± 8 mg) on wet weight. OV (0.52 ± 0.01 mg/g) also increased soleus muscle-to-body weight ratio from Sham control values (0.44 ± 0.01 mg/g). There was no effect of nandrolone decanoate alone (0.44 ± 0.01 mg/g) or in combination with overload (0.51 ± 0.02 mg/g) on the muscle-to-body weight ratio.

Fiber CSA. Fiber CSAs were digitally traced from rat soleus sections taken at the midbelly after 3 days of overload. Mean CSA (μm²) was not different between Sham (3,029 ± 167 μm²), ND (3,079 ± 183 μm²), OV (2,913 ± 132 μm²), and OV+ND (3,108 ± 116 μm²) treatments. However, there were significant changes within the distribution of muscle fiber area that mean data cannot reflect (Fig. 1, B and C). The frequencies of fibers <500 μm² and >5,000 μm² were compared by a one-group χ² analysis as previously described (4). Overload increased the percentage of small fibers 361% from Sham treatment and 315% from ND administration alone. The combination of OV+ND significantly attenuated the overload-induced increase in small fiber incidence. The combination of OV+ND increased the percentage of small fibers 117% from Sham and 99% from ND alone. ND alone increased the percentage of large-diameter fibers (>5,000 μm²) 306% from Sham controls (0.76% vs. 3.1%). Overload also increased the percentage of large-diameter fibers 223% from Sham controls (0.76% vs. 2.5%). The combination of functional overload and ND administration (4.4%) also increased the percentage of large-diameter fibers 366% from Sham controls.

Muscle nuclei localization and content. Myofibers with central nuclei and myonuclei content were quantified in sections taken at the midbelly of 3-day-overloaded soleus muscle. The identification of centrally located nuclei in intact mature myofibers is associated with regeneration and/or growth. Nandrolone decanoate increased the percentage of fibers with central nuclei across both groups. Nandrolone decanoate administration increased the percentage of fibers demonstrating central nuclei 1,300% (P < 0.0001) from Sham. Overload alone had no effect on the percentage of fibers with central nuclei, and there was also no interaction of nandrolone and functional overload (Fig. 2A).

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Nuclei were classified as myofiber-associated nuclei, extracellular matrix-associated nuclei, or the total number of nuclei and expressed as nuclei per square millimeter of muscle. Overload (647 ± 47 nuclei/mm²) or nandrolone decanoate administration alone (680 ± 61 nuclei/mm²) or in combination (683 ± 41 nuclei/mm²) had no effect on the number of nuclei classified as myofiber-associated nuclei compared with Sham controls (673 ± 41 nuclei/mm²). There was a significant main effect of nandrolone decanoate administration on total nuclear number, decreasing it 12% (nuclei/mm²). Overload also had a significant main effect on total muscle nuclei content, increasing it 22% (Fig. 2B). There was no interaction of overload and nandrolone decanoate on total muscle nuclei content. Nandrolone decanoate also had a significant main effect on extracellular matrix-associated nuclei content, decreasing it 20%. Overload also had a significant main effect on extracellular matrix-associated nuclei, increasing it 40% (Fig. 2C). Overload and nandrolone did not have any interactive effects on extracellular matrix-associated nuclei content.

Other muscle morphology variables. The volume percent of noncontractile tissue (%NCT) and fibers with sarcolemma disruption were quantified from histological sections at the midbelly of the soleus muscle from all treatment groups after 3 days of overload. %NCT is associated with the amount of extracellular matrix in the muscle. Nandrolone decanoate administration alone had no effect on %NCT in the soleus muscle. However, there was a significant main effect of overload, increasing the %NCT 50% (Fig. 3A). Nandrolone decanoate did not alter the overload induction of %NCT. Muscle fiber sarcolemma disruption is an indicator of myofiber damage. There was no effect of nandrolone decanoate treatment on myofiber sarcolemma disruption. Overload had a significant main effect of increasing the number of fibers exhibiting sarcolemma disruption (Fig. 3B). Nandrolone decanoate administration did not modulate the overload induction of sarcolemma disruption.

**p70s6k protein and relative phosphorylation.** The phosphorylation of p70s6 kinase is associated with increases in cellular translational efficiency. Western blot analysis revealed that there was no effect of nandrolone decanoate (0.95 ± 0.07 IOD) on total p70s6k protein expression or on the p70s6k phosphorylation state (0.8 ± 0.08 IOD) compared with Sham controls (1.0 ± 0.09). OV alone (0.94 ± 0.19 IOD) had no effect on total p70s6k expression, and there was no interaction of OV + ND (1.15 ± 0.07 IOD) on total p70s6k. OV (1.33 ± 0.16 IOD) had a significant main effect on the relative phosphorylation of soleus p70s6k (expressed as % of total p70s6k in the phosphorylated form, normalized to control values), increasing...
Nandrolone decanoate did not modulate the overload induction of p70s6k phosphorylation (1.31 ± 0.06 IOD). Cell cycle regulators p21 and cyclin D1. Cyclin D1 and p21 mRNA expression was quantified in 3-day-overloaded soleus muscle. There was a significant main effect of nandrolone decanoate on cyclin D1 mRNA, increasing it 110% (Fig. 4B). Overload also had a significant main effect on cyclin D1 mRNA expression, increasing it 152%. However, there was no significant interaction of overload and nandrolone decanoate on cyclin D1 mRNA abundance. Overload and nandrolone decanoate did have a significant interaction on p21 mRNA abundance (Fig. 4C). Post hoc analysis revealed that overload increased p21 mRNA 910% from Sham treatment or nandrolone administration. Additionally, the combination of overload with nandrolone decanoate significantly attenuated the overload induction of p21 mRNA, but p21 mRNA abundance remained increased above Sham or nandrolone decanoate-only treatments (Fig. 4C).

MRF and IGF-I mRNA abundances. Overload and nandrolone decanoate treatments had a significant interaction on myogenin mRNA abundance. Overload induced myogenin mRNA 440% above Sham controls and 300% above nandrolone decanoate-only treatments. Nandrolone decanoate treatment alone did not alter myogenin mRNA abundance, but the combination of OV + ND attenuated the overload induction of myogenin mRNA (Fig. 4D).

Overload and nandrolone decanoate treatments had a significant interaction on myogenin mRNA abundance. Overload significantly increased MyoD mRNA abundance 275% across both overload treatments. Nandrolone decanoate administration did not modulate the overload induction of MyoD mRNA, and nandrolone decanoate alone also had no effect on MyoD mRNA abundance (Fig. 5B). IGF-I is a critical hormone in both growth and satellite cell activity. Overload had a significant main effect on IGF-I mRNA, increasing it 90%. There was no significant interaction of OV + ND on IGF-I mRNA abundance (Fig. 5C). IGF-I mRNA abundance was also not altered by nandrolone decanoate alone (Fig. 5C).
Skeletal muscle is a biological target of anabolic steroid action (54), and androgen status has been shown to interact with muscle loading to affect muscle mass and biological markers of muscle growth (7, 34). When studying anabolic steroids, many variables are important to consider, including species, age, gender, and muscle type (5, 11). Overload-induced muscle growth involves complex cellular signaling pathways that induce myofiber protein synthesis and several critical processes related to muscle remodeling. Initially, overload induces a remodeling response in rat hindlimb muscle that includes structural damage, myofiber growth, satellite cell activation, and immune cell infiltration (19, 30, 49). Morphological changes occurring during the first week of functional overload include increased mass, altered myofiber size, extracellular matrix expansion, and an increased cellularity associated with the extracellular matrix (49). Each of these responses has been hypothesized to be important for the large increases in muscle mass and protein induced by functional overload. To our knowledge, this is the first study to examine the effect of anabolic steroid administration on overload-induced remodeling processes at the onset of functional overload. Together, the results of this study clearly demonstrate that nandrolone decanoate administration is sufficient to alter skeletal muscle’s early response to overload. Alterations in muscle morphology and the temporal expression of cell cycle regulatory factors may have a critical impact on the muscle’s overall growth response.

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**DISCUSSION**

Skeletal muscle is a biological target of anabolic steroid action (54), and androgen status has been shown to interact with muscle loading to affect muscle mass and biological markers of muscle growth (7, 34). When studying anabolic steroids, many variables are important to consider, including species, age, gender, and muscle type (5, 11). Overload-induced muscle growth involves complex cellular signaling pathways that induce myofiber protein synthesis and several critical processes related to muscle remodeling. Initially, overload induces a remodeling response in rat hindlimb muscle that includes structural damage, myofiber growth, satellite cell activation, and immune cell infiltration (19, 30, 49). Morphological changes occurring during the first week of functional overload include increased mass, altered myofiber size, extracellular matrix expansion, and an increased cellularity associated with the extracellular matrix (49). Each of these responses has been hypothesized to be important for the large increases in muscle mass and protein induced by functional overload. To our knowledge, this is the first study to examine the effect of anabolic steroid administration on overload-induced remodeling processes at the onset of functional overload. Together, the results of this study clearly demonstrate that nandrolone decanoate administration is sufficient to alter skeletal muscle’s early response to overload. Alterations in muscle morphology and the temporal expression of cell cycle regulatory factors may have a critical impact on the muscle’s overall growth response.

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**Fig. 4. Cyclin D1, p21, and myogenin mRNA abundance with anabolic steroid administration in functionally overloaded rat soleus skeletal muscle. RNA (10–15 μg) was fractionated on denaturing 1% agarose gels (1 × MOPS, 6.7% formaldehyde) and then transferred to a nylon membrane by capillary action. Membranes were visualized by autoradiography (~80°C, 3–40 h) and quantified by densitometry scanning.**

A: representative Northern blot of 3-day cyclin D1, p21, and myogenin abundance, as well as 28s and 18s mRNA abundance in Sham, ND, OV, and OV+ND soleus muscle. B: fold induction of cyclin D1 mRNA abundance in Sham, ND, OV, and OV+ND treatments. ND administration significantly increased cyclin D1 mRNA across both groups. OV also significantly increased cyclin D1 mRNA across both groups. C: fold induction of p21 mRNA abundance in rat soleus muscle. There was an interaction of functional overload and nandrolone decanoate on p21 mRNA. D: fold induction of myogenin mRNA abundance in rat soleus muscle. There was an interaction of functional overload and nandrolone decanoate on myogenin mRNA. Values are expressed as means ± SE and normalized to Sham values. *Significantly different from Sham (P < 0.05); †significantly different from ND (P < 0.05); ‡significantly different from OV+ND (P < 0.05).
myofiber or extracellular matrix, the overload induction was only significant in regard to extracellular matrix-associated nuclei. The lack of change in myofiber-associated nuclei may be related to similar mean CSAs at this early time point of overload. Extracellular matrix-associated nuclei in overloaded muscle may reflect proliferating fibroblasts and infiltrating immune cells (15). Androgens can also influence muscle DNA content, inducing DNA content in the rat levator ani muscle (29) and aged rat soleus muscle (11, 34). In the current study, overload-induced increases in extracellular matrix nuclei were suppressed by the coadministration of nandrolone decanoate. Nandrolone decanoate administration also decreased the total nuclear content of overloaded skeletal muscle, which may be related to the suppression of overload-induced immune cell infiltration and/or fibroblast proliferation. Androgen status can alter immune system function, and there is a large inflammatory response and infiltration of immune cells during skeletal muscle regeneration (30). Neutrophil and macrophage infiltration occurs in coordination with muscle injury and fiber degeneration during periods of increased loading (23, 43, 50, 51). Leukocytes accentuate muscle damage by releasing enzymes associated with proteolysis and/or free radicals capable of inducing muscle damage (10, 31). It is also possible that nandrolone decanoate could alter fibroblast cell activity in the regenerating soleus muscle. Androgens can suppress signaling related to fibroblast cell-dependent fibrosis development after injury (17). Decreased fibrosis is thought to aid tissue regeneration after injury (44). Another anabolic steroid, stanozolol, has been shown to increase dermal fibroblast collagen synthesis without altering fibroblast replication (21). In the current study, nandrolone decanoate alone or in conjunction with overload did not affect muscle extracellular matrix volume. Extracellular matrix expansion after 3 days of overload is likely related to multiple factors, including edema and fibrosis. Transforming growth factor-β (TGF-β) signaling is important for fibroblast cell activation and regulates gene transcription through signaling cascades utilizing intracellular Smad proteins (22, 44). Androgen receptor activation can repress TGF-β signaling through protein-protein interaction with Smad3 in prostate cells (17). However, this relationship appears complex because anabolic steroids can induce TGF-β signaling in cultured dermal fibroblasts (21). TGF-β signaling related to fibroblast cell activation and fibrosis in overloaded skeletal muscle has not been well described and warrants further investigation.

Satellite cell activity is necessary for the process of overload-induced skeletal muscle growth (45). Satellite cell activity is also regulated by testosterone and its synthetic derivatives. Although satellite cell activity was not directly quantified in the current study, MRF expression is an accepted index of satellite cell activity (28, 55). However, MRF gene expression is also found in mature myofibers (28). The expression patterns of several cell cycle regulatory proteins in the current study suggest that nandrolone decanoate pretreatment impairs satellite cell exit from the cell cycle in overloaded skeletal muscle, by both the coordinated delay of differentiation and stimulation of proliferation. The regulation of satellite cell proliferation and differentiation in overloaded muscle involves MRF regulation of the cell cycle (2). MRFs, including MyoD, myogenin, and cell cycle regulatory factors (Cdk2, Cdk4, and p21) are induced in skeletal muscle at the onset of functional overload (2). MyoD mRNA expression is increased during satellite cell proliferation in culture, whereas myogenin mRNA induction occurs at the onset of differentiation (48). MRFs and other cell cycle regulatory factor genes are transcriptional target genes of androgen regulation (33, 37). Nandrolone decanoate pretreatment significantly attenuated the increase in myogenin mRNA levels after 3 days of overload. The suppression of overload-induced myogenin expression may be directly associated with the nandrolone decanoate attenuation of small-diameter fibers. Additionally, inflammatory cytokine production is induced in functionally overloaded muscle (12). The inflammatory cytokine TNF-α is a transcriptional regulator of the MyoD promoter (32, 52). Because testosterone is a potent anabolic hormone with multiple biological targets, it is extremely likely that it is regulating several pathways that ultimately alter skeletal muscle’s regenerative response to 3 days of functional overload.

Although MRFs are key cell regulators in skeletal muscle, there are also other more ubiquitously expressed cell cycle...
regulatory proteins that are induced by muscle overload and/or regeneration (55). Regulation of cell cycle exit involves the p21 protein (26). In the current study, 3 days of functional overload increased p21 mRNA expression 10-fold. However, nandrolone decanoate pretreatment significantly attenuated this overload induction by 70%. p21 protein is expressed in many cell types, and this attenuated expression could be related to fibroblast cell differentiation and macrophage activation. Cyclin D1 is a protein involved in the progression from G1 to S-phase of the cell cycle. Although cyclin D1 is expressed in many cell types, recent studies examining regenerating skeletal muscle have associated changes in cyclin D1 expression with satellite cell activity (55). Nandrolone decanoate treatment alone induced cyclin D1 expression and suppressed both p21 and myogenin mRNA expressions. Although previous studies have demonstrated androgen stimulation of skeletal muscle satellite cell activity (47), during overload-induced growth nandrolone decanoate may function to suppress satellite cell differentiation. This may indirectly increase cell proliferation, because nandrolone decanoate treatment did not modulate the overload induction of cyclin D1 mRNA. Additional study is needed to further examine androgen-mediated signaling pathways important for the suppression of myogenic differentiation in overloaded skeletal muscle. However, the androgen receptor is likely a key mediator of this biological action.

Nandrolone decanoate pretreatment alone or in conjunction with overload induces alterations in the soleus muscle fiber size distribution profile. The mean soleus fiber CSA was not changed by 3 days of overload or nandrolone decanoate pretreatment. However, mean CSA is not an accurate description of the remodeling events occurring in overloaded skeletal muscle (20). An increased incidence of very small-diameter myofibers in skeletal muscle is an established indicator of regeneration from injury (15, 30, 55, 56). Small fiber incidence has been associated with degeneration/regeneration of preexisting fibers due to initial overload-induced damage and at 3 days of overload is not thought to be related to de novo synthesis of new fibers. Total fiber number has not been shown to change with functional overload in rat hindlimb muscle (27). The overload induction of small-diameter muscle fibers in the current study is consistent with previous research. Miller and Stauber (39) demonstrated that functional overload of the soleus muscle results in the occurrence of small fibers at a relative frequency of 24% 14 days after initial surgery in rats. Nandrolone decanoate administration appears not to alter processes related to small fiber formation, which may be more related to mechanical stimulation that is independent of sex hormone status. Increased muscle mass at the onset of functional overload is associated with both inflammation and edema (30). The current data suggest that nandrolone decanoate pretreatment does not regulate processes related to overload-induced edema at the onset of functional overload. Overload increased extracellular matrix volume and number of fibers exhibiting sarcolemma disruption by 3 days of overload, and nandrolone decanoate treatment had no effect on these variables. However, additional work is warranted to examine whether anabolic steroid administration alters the overall inflammatory response at the onset of functional overload. Nandrolone decanoate pretreatment alone increased the number of fibers demonstrating centrally located nuclei and may be related to the nandrolone decanoate-induced increase in large-diameter muscle fibers. The well-accepted nuclear domain theory suggests that myogenic precursor cell-derived nuclei are required for myofiber growth (16), and these nuclei may be located centrally in the myofiber for a period after fusion (9, 15). During functional overload, large fiber incidence is attributed to myofiber hypertrophy. Both nandrolone decanoate pretreatment and functional overload induced three- to fourfold increases in large myofiber incidence. However, there was no interaction between nandrolone decanoate and overload on large fiber incidence.

In summary, signaling pathways in functionally overloaded muscle require the integration of stimuli that include hormonal, growth factor, and mechanical signals. The present study demonstrates that nandrolone decanoate administration has significant effects on both muscle morphology and gene expression related to overload-induced muscle growth (Table 1). Nandrolone decanoate pretreatment suppresses the overload-induced gene expression of myogenin and p21, which are associated with satellite cell differentiation. Nandrolone decanoate had no effect on the expression of the cell cycle regulator cyclin D1 mRNA after 3 days of functional overload. In the present study, rats treated with nandrolone decanoate exhibited an altered temporal pattern of cell cycle regulatory expression and muscle morphology after 3 days of functional overload. These findings will lead to insight into important mechanisms of action of androgen therapy for increasing muscle mass in clinically relevant populations.

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