Regulation of androgen receptor expression at the onset of functional overload in rat plantaris muscle

Won Jun Lee, Raymond W. Thompson, Joseph M. McClung, and James A. Carson

Integrative Muscle Biology Laboratory, Exercise Science Department, University of South Carolina, Columbia South Carolina 29208

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Regulation of androgen receptor expression at the onset of functional overload in rat plantaris muscle. Am J Physiol Regul Integr Comp Physiol 285: R1076–R1085, 2003; 10.1152/ajpregu.00202.2003.—Skeletal muscle androgen receptor (AR) expression at the onset of functional overload (OV) has not been well described. It is also not known if overload and/or anabolic steroid differentially regulate AR expression. The purpose of this study was to examine AR gene expression at the onset of functional OV in rat plantaris muscle with and without nandrolone decanoate (ND) administration. The functional significance of AR protein induction was examined using skeletal α-actin promoter activity in transiently transfected CV-1 fibroblast cells. Male Sprague-Dawley rats (~125 g) were functionally overloaded for 1, 3, 7, or 21 days. A subset of animals was given an ND (6 mg/kg) injection at day 0 and then overloaded for 3 days. Control animals underwent sham surgeries. AR protein concentration increased 106 and 279% after 7 and 21 days of OV, respectively. AR mRNA increased 430% after 7 days of OV. AR protein expression in C2C12 murine myotubes subjected to 1% chronic radial stretch for 18 h was elevated 101% compared with control. ND treatment increased AR protein concentration 1,300% compared with controls, and there was no additional effect when ND and OV were combined. ND with 3 days of OV treatment increased AR mRNA expression 50% compared with control. AR overexpression in transiently transfected CV-1 fibroblast cells increased ~424 bp skeletal α-actin promoter activity 80 to 1,800% in a dose-dependent fashion. Co-overexpression of either serum response factor (SRF) or active RhoA with AR overexpression induced a synergistic 36- and 28-fold induction of skeletal α-actin promoter activity. Cotransfection of AR, SRF, and active RhoA induced 180-fold increase in skeletal α-actin promoter activity. In conclusion, AR protein expression is increased after 7 days of functional OV, and this induction is regulated pretranslationally. AR induction in conjunction with SRF and RhoA signaling may be an important regulator of gene expression during overload-induced muscle growth.

SEX STEROID HORMONES have a multitude of biological targets. Androgens, in addition to having effects on the male reproductive system, have anabolic effects on skeletal muscle (3). Androgen actions are mediated by binding to its androgen receptor, which is localized to the cytoplasmic compartment of target cells (52). Unbound androgen receptors are maintained in a heterocomplex with chaperone proteins (17). Heat shock protein (Hsp) 90 regulates the ligand binding affinity of the androgen receptor by maintaining the receptor in a high-affinity ligand-binding conformation, which is important for efficient hormonal response (17). Ligand binding induces a conformational change that facilitates unmasking of the nuclear localization signal (NLS). Translocation of the ligand-activated androgen receptor to the nucleus is induced by NLS binding to importin proteins (42). In the nucleus this complex can bind androgen response elements (AREs) and interact with the transcription initiation complex (58). Androgen receptor regulation is extremely complex and dependent on both tissue-specific and developmental regulation (29). The androgen receptor can also be activated in the absence of ligand, and cellular signaling pathways can also modulate androgen receptor actions (37, 53). Insulin-like growth factor 1 (IGF-1), keratinocyte growth factor (KGF), and epidermal growth factor (EGF) can induce androgen-independent activation of the androgen receptor (15).

Androgen receptor protein expression and ligand binding capacity are increased in functionally overloaded rat skeletal muscle (22, 28). The skeletal muscle androgen receptor may exert both ligand-dependent and -independent actions in functionally overloaded skeletal muscle, which may be related to growth factor signaling (15). IGF-I and muscle DNA content are also increased in overloaded skeletal muscle (2). Rat soleus and plantaris muscles respond quickly to functional overload by increasing muscle mass after 1 wk, and this growth continues for several additional weeks (51). Functional overload also stimulates a rapid remodeling response in rat hindlimb muscle, which includes structural damage, myofiber growth, satellite cell activation, and immune cell infiltration (16, 25, 46). Each of these responses has been hypothesized to be important for the large increases in muscle mass and protein induced by functional overload. Androgens can also influence muscle DNA content, inducing myonuclei accumulation in the rat levator ani muscle (23). This induction is thought to be mediated by the proliferation of the androgen receptor (15).

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of muscle satellite cells. Although muscle androgen receptor expression is sensitive to both circulating androgen and mechanical stimuli, the regulation of muscle-specific gene expression by this receptor-ligand complex is not well understood.

The multitude of signaling pathways activated in functionally overloaded muscle requires the integration of a variety of stimuli, including hormonal, growth factor, and mechanical signals (14). RhoA associated signaling is an excellent candidate for linking androgen signaling and mechanical stimuli. RhoA is a member of a Rho family of small GTPases, and RhoA signaling is involved in muscle transcription factor activation (8, 49). The androgen receptor’s transcriptional activation capability can be modulated by RhoA signaling in the Xenopus vitellogenin A2 gene in mammalian cells (48). Functional overload and anabolic steroid administration increase RhoA gene expression (32). RhoA signaling regulates the activity of serum response factor (SRF), an MADS-box transcription factor that is essential for muscle-specific gene expression (36, 55). SRF protein expression is increased and its DNA binding activity altered in hypertrophying skeletal muscle (13, 20). RhoA and SRF signaling may be a potential integration site for functional overload and anabolic steroid induced signaling in skeletal muscle. However, signaling pathways integrating RhoA, SRF, and the androgen receptor are not well understood.

Anabolic steroids are structural derivatives of testosterone that can increase skeletal muscle mass (7), protein synthesis (44), myonuclei accumulation, and IGF expression (18). Nandrolone decanoate administration with functional overload for 7 days synergistically induces androgen receptor protein expression in 5-mo-old rat plantaris muscle, but not in the plantaris muscle of 25-mo-old animals (28). However, androgen receptor expression during the onset of overload-induced muscle growth has not been well described. It is also not known if overload or anabolic steroids regulate androgen receptor expression pretranslationally. The purpose of this study was to examine androgen receptor protein and mRNA expression at the onset of functional overload in rat plantaris muscle with and without nandrolone decanoate administration. The mechanical sensitivity of androgen receptor expression was also examined in cultured C2C12 myotubes subjected to chronic radial stretch. The functional significance of androgen receptor overexpression and modulation by proteins associated with mechanical signaling pathways that are upregulated during overload-induced hypertrophy was examined in transiently transfected CV-1 fibroblasts. It was hypothesized that androgen receptor expression would be sensitive to mechanical stimulation, and this induction would be regulated pretranslationally. It was also hypothesized that androgen receptor overexpression in conjunction with SRF and RhoA overexpression would synergistically induce skeletal α-actin gene expression in CV-1 fibroblasts.

METHODS

Animals and housing. Thirty-eight male Sprague-Dawley rats (~125 g) were acquired from Harlan rodent colony (Indianapolis, IN). 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 seven treatment groups as follows: 1) control (n = 15), 2) 1-day functional overload (n = 4), 3) 3-day functional overload (n = 5), 4) 7-day functional overload (n = 5), 5) 21-day functional overload (n = 4), 6) 3-day anabolic steroid (n = 5), and 7) 3-day anabolic steroid with functional overload (n = 5). All procedures were approved by the University of South Carolina Animal Care and Use Committee.

Anabolic steroid administration. The anabolic steroid nandrolone decanoate (Deca-Durabolin, Oranon) was used in the study because of its long biological half-life and previous studies demonstrating an anabolic effect in rat skeletal muscle (12, 28). The selected dose of nandrolone decanoate administration has been previously demonstrated to prevent hindlimb suspension-induced rat skeletal muscle atrophy (57). Nandrolone decanoate was injected (6 mg/kg body wt) intramuscularly into the hip region immediately after the ablation surgery (12), at the beginning of the 3-day experimental period. The anabolic steroid group received one injection of nandrolone decanoate in sesame seed oil, and sham controls received sesame seed oil alone. Animals were killed at the end of the 3-day experimental period.

Surgical ablation of synergists. The hindlimb soleus and plantaris muscle were functionally overloaded for 1, 3, 7, and 21 days by surgical ablation of the distal third of the lateral and medial gastrocnemius muscle as previously described (5, 28). Briefly, rats were anesthetized with an intramuscular injection of a cocktail containing ketamine hydrochloride (75 mg/kg), xylazine (3 mg/kg), and acepromazine (5 mg/kg). In a sterile aseptic environment, the dorsal surface of the hindlimb was shaved and cleaned. The gastrocnemius muscles were then exposed by a posterior longitudinal incision through the skin and biceps femoris muscle of each lower hindlimb, and the distal two-thirds of the heads of each gastrocnemius muscle were excised. Control animals underwent a sham procedure that consisted of the same procedure, except for gastrocnemius excision. When the rats were recovered from the anesthetic, they were returned to their cages. Rats were weighed weekly and were carefully observed for signs of failure to thrive, such as precipitous weight loss, disinterest in the environment, or unexpected gait alterations. Soleus and plantaris muscles were removed 1, 3, 7, and 21 days after the initial surgery, frozen in liquid nitrogen, and stored at −80°C until further analysis.

Crude protein extracts. Crude protein extracts were made as previously described (12). Frozen plantaris muscles were homogenized in Mueller buffer (50 mM HEPES, pH 7.4, 0.1% Triton X-100, 4 mM EGTA, 10 mM EDTA, 15 mM Na3P04, 100 mM β-glycerophosphate, 25 mM NaF, 1 mM Na3VO4, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin, and 0.3 μg/ml aprotonin), 2 ml per 1 g of tissue. Tissue was homogenized on ice with a Polytron homogenizer (Kinematica) using three 15-s pulses at a low setting. Homogenates were fractionated into soluble and insoluble fractions by centrifugation, and the protein concentration was determined by Bradford assay (Bio-Rad) and stored at −80°C until use for Western blotting.

Transfection assays in CV-1 fibroblast cell cultures. Cells were transfected 24-h postplating using LipofectAMINE (Life Technologies,) as described previously (11). Monkey
CV-1 fibroblasts were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS on 6 cm plate. Briefly, each transfection reaction contained 1 mg of luciferase reporter plasmid (γ-actin) and various amounts of transactivator plasmids (pCGN-AR, pCGN-V14RhoA, and/or pCGN-SRF) as previously reported (11). All transfections were performed with empty DNA vector to keep the level of DNA and CMV promoter constant in all transfection reactions. Cells were transfected, 16–18 h after which the transfection media were removed and replaced with Dulbecco’s modified Eagle’s medium supplemented with 2% horse serum and 10 mg/ml insulin for an additional 48 h. Cells were then harvested with PBS and then scraped in 250 μl of 1× Reporter Lysis Buffer (Promega). Cellular debris were removed by centrifugation, and 50 μl of supernatant was analyzed for luciferase activity by mixing with 100 μl of luciferase substrate (20 mM Tris-Tricine, pH 8.0, 4 mM MgSO4, 0.1 mM EDTA, 30 mM dithiothreitol, 0.5 mM ATP, 0.5 mM β-luciferin, 0.25 mM coenzyme A). Emitted luminescence was measured for 10 s. Protein concentrations were measured by Bradford assay (Bio-Rad) and used to normalize luciferase activity.

C2C12 cell culture and radial stretch. A static radial device was employed that applied a multidirectional radial stretch on a Silastic membrane in a single plane. The membrane was coated with rat-tail collagen, and C2C12 cells were cultured in sterile conditions as previously reported (32). Silastic membranes were removed and replaced with Dulbecco’s modified Eagle’s medium supplemented with 2% horse serum and 10 mg/ml insulin for an additional 48 h. Cells were then harvested with PBS and then scraped in 250 μl of 1× Reporter Lysis Buffer (Promega). Cellular debris were removed by centrifugation, and 30 μl of supernatant was analyzed for luciferase activity by mixing with 100 μl of luciferase substrate (20 mM Tris-Tricine, pH 8.0, 4 mM MgSO4, 0.1 mM EDTA, 30 mM dithiothreitol, 0.5 mM ATP, 0.5 mM β-luciferin, 0.25 mM coenzyme A). Emitted luminescence was measured for 10 s. Protein concentrations were measured by Bradford assay (Bio-Rad) and used to normalize luciferase activity.

Western blot analysis. Western blot analysis was performed as previously reported (12). Forty micrograms of crude protein homogenate was incubated (15 min, 65°C) with an equal volume of protein sample buffer and fractionated on a 8% SDS-polyacrylamide gel (150 V, 25°C, 1 h), and electrophoretically transferred to a nitrocellulose membrane (300 mA, 4°C, 14 h). Transfer was verified by Ponceau S staining. Dose-response analysis of the AR receptor demonstrated that 40 μg of crude protein extract gave signal in a linear range for quantitation (data not shown). The membranes were then probed with androgen receptor (N-20) polyclonal rabbit antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), as previously described (12, 28). The donkey anti-rabbit IgG horse-radish peroxidase-linked secondary antibody was visualized by ECL-plus (Amersham, Life Sciences) as per manufacturer instructions and quantified by densitometry scanning.

Total RNA isolation and cDNA synthesis. Total RNA was isolated using TRIzol reagent (Life Technologies, Grand Island, NY). After phenol-chloroform extraction, RNA was ethanol precipitated and diluted in 75% ethanol and 8 μM LiCl. Total RNA was dissolved in water, absorbance was measured at 260 nm, and stored at −80°C. Extracted RNA was treated with RNase-free DNase I to remove contaminating genomic DNA. cDNA was reverse transcribed from 4 μg of total RNA using 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 75°C for 15 min.

Expression of AR mRNA was evaluated using known dilutions of AR, HPRT, and SKA plasmids (100 fg–100 pg; see Fig. 3A). PCR for androgen receptor started with an initial denaturation at 95°C for 5 min, followed by 28, 30, 32, and 34 cycles of denaturation at 94°C for 60 s, annealing at 61°C for 55 s, and extension at 72°C for 60 s, with a final extension cycle at 72°C for 10 min. Amplified products were subjected to electrophoresis through 1.5% agarose gels, stained with ethidium bromide, visualized by ultraviolet transillumination, and photographed. The density of each band was plotted to calculate slope and intercept. The density of each sample at 32 cycles then was computed by linear regression. Target fragment levels were normalized to β-actin mRNA, integrated optical density (IOD):HPRT IOD and skeletal muscle actin mRNA IOD:HPRT IOD ratio. The sequence of the upstream primer is 5’-CGAAGCCGAC-GACGCCTGAGA-3’, and the sequence of the downstream primer is 5’-GCGAGCGGAAAGTTGTAGTAGT-3’. PCR was performed with 2.5 U of Taq DNA polymerase in an automatic DNA thermal cycler by adding 10 μl (1 to 10 dilution) of the cDNA of each sample and a PCR mix consisting of 1% PCR buffer, 1.5 mM MgCl2, 50 μM dNTPs, and 0.15 μM of 5’- and 3’-primers in a final volume of 50 μl. The number of cycles and the PCR conditions for each target mRNA were optimized so that the amplified signal was constant on the linear portion of the amplification curve. Products were run on the 1.5% agarose gel. A 501-bp fragment was excised and purified using QIAEX II gel extraction kit (QIAGEN). Purified PCR products were subcloned in TA cloning PCR 2.1 vector (Invitrogen), amplified, and purified. The sensitivity of RT-PCR was evaluated using known dilutions of AR, HPRT, and SKA plasmids (100 fg–100 pg; see Fig. 3A). PCR for androgen receptor started with an initial denaturation at 95°C for 5 min, followed by 28, 30, 32, and 34 cycles of denaturation at 94°C for 60 s, annealing at 61°C for 55 s, and extension at 72°C for 60 s, with a final extension cycle at 72°C for 10 min. Amplified products were subjected to electrophoresis through 1.5% agarose gels, stained with ethidium bromide, visualized by ultraviolet transillumination, and photographed. The density of each band was plotted to calculate slope and intercept. The density of each sample at 32 cycles then was computed by linear regression. Target fragment levels were normalized against hypoxanthine phosphoribosyl transferase (HPRT), and the data were presented as androgen receptor mRNA, integrated optical density (IOD):HPRT IOD and skeletal muscle actin mRNA IOD:HPRT IOD ratio. The sequence of the upstream primer for HPRT is 5’-CTC TGT GTG CTC CAG CCT-3’, and the sequence of the downstream primer is 5’-GGG ACG CAG CAA CAG ACA TT-3’ (54).

Data analysis. Results are reported as means ± SE. All variables were analyzed by Kruskal-Wallis one-way ANOVA on ranks. Post hoc analysis between treatments was performed using independent t-tests or Dunn’s method. P < 0.05 was accepted as the level of statistical significance.

RESULTS

Muscle wet weight. Plantaris muscle wet weight increased at 24 h of functional overload (147 ± 8 mg) compared with sham controls (124 ± 4 mg). The plantaris muscle wet weight increased 31% (143 ± 3 mg) and 32% (144 ± 4 mg) after 3 days of functional overload and overload plus anabolic steroid administration, respectively. Plantaris muscle wet weight 3 days after nandrolone decanoate administration was increased at 24 h of functional overload (147 ± 8 mg) compared with sham controls (124 ± 4 mg). The plantaris muscle wet weight increased 31% (143 ± 3 mg) and 32% (144 ± 4 mg) after 3 days of functional overload and overload plus anabolic steroid administration, respectively. Plantaris muscle wet weight 3 days after nandrolone decanoate administration was
not different from controls. Twenty-one days of functional overload increased plantaris muscle wet weight 20% compared with sham control (309 ± 3 vs. 257 ± 7 mg). As previously demonstrated, 3 days of functional overload with nandrolone decanoate administration had no effect on muscle wet weight, protein concentration, or total protein above functional overload alone in young rats (32).

Androgen receptor protein expression. Androgen receptor protein concentration varied over 3 wk of functional overload in the rat plantaris muscle. Western blot analysis revealed a 69% decrease in androgen receptor protein expression after 1 day of functional overload (Fig. 1B). However, by day 7 of functional overload, androgen receptor protein concentration increased 106% above control level and further increased 279% after 21 days of functional overload (Fig. 1B). Three days of nandrolone decanoate treatment increased plantaris androgen receptor protein concentration 1,300% (Fig. 1C) compared with controls. The combination of functional overload and nandrolone decanoate treatment had no additive effect on androgen receptor protein concentration above nandrolone decanoate alone (Fig. 1C). The androgen receptor is expressed in a variety of cell types (42), and many cell types are located within skeletal muscle, including muscle fibers, satellite cells, fibroblasts, macrophages, and other immune cells. Androgen receptor protein expression was examined in C2C12 derived skeletal myotubes subjected to chronic radial stretch. Androgen receptor protein expression in cultured C2C12 murine myotubes was significantly elevated 101 and 45% by 1 and 2% chronic radial stretch for 18 h compared with control values (Fig. 2B). This increase is representative of a specific induction of androgen receptor protein in differentiated skeletal myotubes subjected to mechanical stress.

Overexpression of androgen receptor in transiently transected CV1 fibroblasts. Transient transfection experiments into CV-1 fibroblasts have proven a valuable tool in examining actin promoter regulation (11). Androgen receptor was overexpressed in CV-1 cells (Fig. 3A) by transient transfection along with the transcriptional target −424 bp skeletal α-actin promoter. The −424 bp skeletal α-actin promoter has been shown to be sufficient for activation during stretch-induced hypertrophy (13). Androgen receptor overexpression in CV-1 cells elevated −424 bp skeletal α-actin promoter activity by 1,800% at the highest AR expression vector concentration compared with the vector control, respectively (Fig. 3B). SRF is induced during overload-induced hypertrophy (20) and an important regulator of the skeletal α-actin promoter during stretch-overload induced hypertrophy (13). SRF overexpression in CV-1 cells significantly induced skeletal α-actin promoter activity sevenfold, as previously demonstrated (9, 55). Overexpression of SRF with a low dose of androgen receptor induced a synergistic 36-fold induction of skeletal α-actin promoter activity above control (Fig. 3C). Rhoa protein and activity have been shown to increase during overload-induced hypertrophy (32).
and are thought to signal nuclear gene expression through SRF (55). Cotransfection of constitutively active RhoA and SRF has been shown previously to activate skeletal \( \beta \)-actin (55), as in the current study. Cotransfection of constitutively active RhoA (V14 RhoA) with the androgen receptor induced a 28-fold increase in skeletal \( \beta \)-actin promoter activity. When androgen receptor, SRF, and RhoA were cotransfected together, there was a 180-fold synergistic induction of skeletal \( \beta \)-actin promoter activity above control (Fig. 3C). All three of these proteins are induced in functionally overloaded rat hindlimb muscle.

Androgen receptor mRNA abundance. Pretranslational regulation of the androgen receptor was examined in functionally overloaded rat plantaris muscle by semiquantitative RT-PCR. cDNA for each sample was amplified 28, 30, 32, and 34 cycles for AR and 24, 28, 32, and 36 cycles for HPRT and skeletal \( \alpha \)-actin to determine the linear range of amplification. A standard curve was calculated using the four data points, and linear regression was used to determine the IOD value at 32 cycles. HPRT mRNA abundance (\( P > 0.05 \)) was not different between groups. Cyclophilin was examined as a correction factor. However, cyclophilin expression was significantly lower (\( P = 0.009 \)) after 3 days functional overload muscles compared with controls (data not shown). Subsequently, androgen receptor mRNA was normalized to the HPRT. Representative ethidium bromide-stained gels for androgen receptor after RT-PCR are shown in Fig. 5A. Semiquantitative RT-PCR revealed that 7 days of functional overload significantly increased AR mRNA 430\% (\( P < 0.05 \); Fig.

Fig. 3. Effect of overexpression of AR on transiently transfected CV-1 fibroblasts. A: lanes 1 and 2 demonstrate native AR expression in CV-1 fibroblasts by Western blot analysis. Lanes 3 and 4 are crude CV-1 protein extracts transfected with pCGN-AR plasmid and demonstrate AR overexpression. B: CV-1 cells were transfected with 424 bp skeletal \( \beta \)-actin (SKA) promoter-luciferase reporter gene (1 \( \mu \)g of DNA) and CMV promoter-directed expression vectors as described in METHODS. Expression vectors for AR were assayed (0.2, 0.4, and 0.8 \( \mu \)g of DNA). C: expression vectors for serum response factor (SRF) and V14 RhoA were assayed singularly and in combination with AR. All transactivation procedures were balanced to 1 \( \mu \)g of DNA content by addition of empty CMV promoter vector. These data were generated from a minimum of 4 experiments, performed in duplicate. The fold induction represents the luciferase activity measured from lysates from various transactivation plasmids compared with the lysate activity from cells that received 424 bp skeletal \( \alpha \)-actin promoter-luciferase reporter gene and empty vector. *Significantly different from control (\( P < 0.05 \)).
expression did not increase until receptor gene expression. Androgen receptor protein expression of skeletal muscle androgen receptor substrate an interaction of hormonal and mechanical stimuli on the regulation of skeletal muscle androgen receptor. The differential regulation of androgen receptor expression by androgen receptor mRNA expression and suggests combined with 3 days of functional overload did induce androgen receptor protein expression. How-

Androgen receptor expression and functional overload. Androgen receptor expression increases after resistance training in human muscle (6) or in functionally overloaded rat muscle (28). The current study extends these findings by reporting that plantaris androgen receptor protein and mRNA are not induced during the first 3 days of functional overload. In the current study androgen receptor protein concentration decreased after 1 day of functional overload, which may be the result of increased protein turnover at the onset of functional overload. There is the possibility that a reduction of androgen receptor protein at the early onset of functional overload could be compensated for by a significant increase in androgen ligand binding affinity. However, androgen receptor protein concentration increased after 7 and 21 days of functional overload. Mechanical stimuli could induce an-

Fig. 4. The linear and equal efficiency of the amplification of AR, hypoxanthine phosphoribosyl transferase (HPRT), and skeletal α-actin (SKA) plasmid. A: representative gel electrophoretic pattern for AR, HPRT, and SKA plasmid. AR, HPRT, and SKA plasmids were diluted to $1 \times 10^{-11}$, $1 \times 10^{-12}$, $1 \times 10^{-13}$, and $1 \times 10^{-14}$ μg/ml and amplified 32 cycles. B: AR cDNA was amplified for 28, 30, 32, 34 cycles while HPRT and SKA cDNA were amplified for 24, 28, 32, 36 cycles. The IOD of each band of AR was plotted to calculate slope and intercept.

Fig. 5. AR mRNA abundance after functional overload. A: representative ethidium bromide staining of 1.5% agarose gel of AR PCR products of control and 7 days of functionally overloaded plantaris muscle. AR mRNA abundance was quantified by semiquantitative PCR. AR mRNA was amplified for 28, 30, 32, 34 cycles. The density of each sample at 32 cycles then was computed by linear regression. The density of each band was plotted to calculate slope and intercept. The density of each sample at 32 cycles then was computed by linear regression. B: AR mRNA abundance increases after 7 days of functional overload rat plantaris muscle. The graph represents the change in plantaris AR mRNA abundance corrected by HPRT. Data are representative of 4–6 animals/treatment group. CON, rat receiving sham operation. *Significantly different from control group ($P < 0.05$).

5B). Nandrolone decanoate treatment did not change androgen receptor mRNA concentration (Fig. 6B). However, androgen receptor mRNA increased 50% in nandrolone decanoate-treated muscle with 3 days of functional overload group compared with controls ($P = 0.04$; Fig. 6B). Skeletal α-actin mRNA was not changed by 3 (1.0 ± 0.33 vs. 0.58 ± 0.06) and 7 days (1.0 ± 0.33 vs. 1.46 ± 0.40) of functional overload. Skeletal α-actin mRNA concentration was not affected by either nandrolone decanoate (1.0 ± 0.19 vs. 1.49 ± 0.31) or nandrolone decanoate with 3 days of functional overload (1.0 ± 0.19 vs. 1.01 ± 0.07).

DISCUSSION

To our knowledge this is the first study to demonstrate an interaction of hormonal and mechanical stimuli on the regulation of skeletal muscle androgen receptor gene expression. Androgen receptor protein expression did not increase until day 7 of functional overload, and this induction was regulated pretranslational. Anabolic steroid administration alone also induced androgen receptor protein expression. However, there was no corresponding increase in androgen receptor mRNA. Anabolic steroid administration combined with 3 days of functional overload did induce androgen receptor mRNA expression and suggests differential regulation of androgen receptor expression by functional overload and androgen treatments. The functional significance of androgen receptor induction in overloaded skeletal muscle is not solely related to available ligand because its biological action can be altered by other signaling pathways (53). The current study demonstrates that androgen receptor overexpression in conjunction with SRF and RhoA overexpression induces skeletal α-actin promoter activity in CV-1 fibroblast cells. SRF, RhoA, and androgen receptor proteins are all increased during the first week of functional overload (10, 28, 32).

The current study demonstrates that androgen receptor overexpression induces skeletal α-actin promoter activity in CV-1 fibroblast cells. SRF, RhoA, and androgen receptor proteins are all increased during the first week of functional overload (10, 28, 32).
Fig. 6. AR mRNA abundance after 3 days of ND treatment and ND with functional overload. A: representative ethidium bromide staining of 1.5% agarose gel of AR PCR products of control and ND with 3 days of functionally overloaded plantaris muscle. B: AR mRNA abundance increases after 3 days of functional OV with ND treatment rat plantaris muscle. The graph represents the change in plantaris AR mRNA abundance. AR mRNA abundance was corrected by HPRT. Data are representative of 4–6 animals/treatment group. Data are presented as means ± SE and normalized to control group. ND, rat receiving ND (6 mg/kg) injections; ND + OV, rat functionally overloaded for 3 days and treated with ND (6 mg/kg).

*Significantly different from control group (P < 0.05).

The functional significance of increased androgen receptor abundance is not certain. Androgen receptor overexpression in CV-1 fibroblasts induces skeletal α-actin promoter activity in a dose-dependent manner. This identifies a muscle structural gene as a target of androgen action, due to increased receptor concentration. Skeletal α-actin mRNA, however, was not elevated by 3 and 7 days of functional overload in the rat plantaris muscle. This result may be due to the increase in both mRNA synthesis and turnover rate. At the onset of functional overload in the chicken anterior latissimus dorsi (ALD) muscle, skeletal α-actin protein synthesis rates increase (21), while actin mRNA concentration decreases (13). Proteins associated with mechanical signaling through focal adhesion complex formation at the sarcolemma are upregulated as early as 12 h, 1, or 3 days after functional overload (32). Phosphatidylinositol 3-kinase (PI3K), p70S6K, and the eukaryotic initiation factors have been shown to be correlated with protein synthesis immediately after mechanical stimulation (4, 26). RhoA and PI3-kinase signaling interactions appear to have an important role for focal adhesion complex formation and PI3K translocation to the sarcolemma (56).

RhoA has been shown to be involved in mechanical signaling and skeletal muscle hypertrophy (19, 32). RhoA expression can also be altered by hormonal manipulation (32). RhoA-mediated signaling through interaction with focal adhesion complex formation at the sarcolemma can regulate skeletal α-actin promoter activity (56). This regulation is dependent on SRF binding at serum response elements (SREs); −424 bp skeletal α-actin promoter activity is induced during stretch overload hypertrophy of the ALD muscle, and SRF binding to SRE1 is critical for this response (13). Our functional data demonstrate that androgen receptor activity can be influenced by proteins associated with mechanical signaling pathways to induce muscle-specific gene expression. Although skeletal α-actin gene regulation is responsive to androgen receptor overexpression alone, co-overexpression of androgen receptor with proteins involved in mechanical signaling pathways induces a synergistic response. The current study demonstrates that skeletal α-actin promoter is induced by RhoA and SRF expression, as previously shown, but co-overexpression of the androgen receptor with SRF and RhoA can create a large synergistic induction. Additionally, our current data demonstrate that the androgen receptor and RhoA co-overexpression without SRF overexpression is also a potent stimulator of skeletal α-actin promoter activity. Overload-induced skeletal muscle hypertrophy increases androgen receptor, SRF, and RhoA protein abundance (12, 28, 32). These results suggest the possibility of a functional interaction between mechanical signaling and the androgen receptor for the regulation of muscle gene expression during functional overload. Further work is needed to examine this functional interaction in vivo.

Transcriptional activation capability of the androgen receptor can be modified by ligand-independent mechanisms or an alteration in ligand-receptor complex. Steroid receptor coactivator-1 (SRC-1) interacts with the androgen receptor to modulate both ligand-dependent and ligand-independent receptor transactivation (53). Elevated skeletal muscle inflammatory cytokine expression (IL-6, TNF-α) is a documented response to exercise or overload (24, 39). IL-6 can activate androgen receptor transcriptional activity (53), and SRC-1 phosphorylation by mitogen-activated protein kinase (MAPK) signaling is required. MAPK pathways can also directly modify ligand-dependent activation of androgen receptor (53) and is associated with RhoA-mediated signaling (30, 53). Three days of functional overload induce IL-6, RhoA, and SRF expression. Inflammatory, hormonal, and mechanical signaling are all
critically important stimuli needing integration in overloaded skeletal muscle.

Androgen receptor expression and anabolic steroid administration. Skeletal muscle androgen receptor expression is sensitive to circulating androgen levels (3). Sprague-Dawley rats achieve sexual maturity and maximal testosterone production at ~90 days of age (45). Therefore, circulating testosterone levels and androgen-regulated targets in the present study's animals could still be categorized into an immature development phase. However, androgen receptor expression was induced by anabolic steroid administration in these young animals. Examining overload-stereo interaction on androgen receptor expression in castrated rats subjected to functional overload may provide further insights into the effect of circulating testosterone levels on the overload-induced expression. Nandrolone decanoate administration was sufficient to increase androgen receptor protein expression without increasing androgen receptor mRNA. Our observed disparity between androgen receptor protein and mRNA expression levels point to translational efficiency being important for the anabolic steroid induction of androgen receptor expression. Castrated rats receiving testosterone treatment increase androgen receptor protein in epithelial cells of the ventral prostate, without a corresponding induction of androgen receptor mRNA level (35). Testosterone also increases androgen receptor mRNA association with polyribosomes, which may increase the translation efficiency and mRNA stability (34). Besides having different effects on the regulation of androgen receptor mRNA expression, hormonal and mechanical stimuli also have differential effects on plantaris muscle mass, total RNA, and total DNA. Androgen receptor induction 3 days after a nandrolone decanoate injection does not correspond with an increase in plantaris muscle mass, total RNA, or total DNA content (32).

Androgen receptor and myogenic cell cycle regulation. Androgens can stimulate skeletal muscle satellite cell activation (23). Satellite cell proliferation/differentiation is critical for muscle regeneration after injury, and a necessary component of postnatal and functional overload-induced muscle growth (40, 41). Growth factor gene expression, including IGF-I, is upregulated after functional overload-induced muscle hypertrophy in rat skeletal muscle. Mechanical load-sensitive IGF-I induces muscle satellite cell proliferation and differentiation, which is followed by fusion with hypertrophying muscle fibers (2). It is not known if androgens have an additive effect on satellite cell activation in functionally overloaded muscle. However, in the current study androgen receptor protein was not induced until 3 days of functional overload. This time course suggests that androgen receptor induction may not be associated with satellite cell proliferation, which is initiated rapidly after the onset of functional overload (33). However, the androgen receptor's role in the regulation of myoblast differentiation is just beginning to be understood (27).

Myogenic regulatory factors (MRFs) and other cell cycle regulatory factor genes are transcriptional target genes of androgen regulation (27, 31). MRFs, including MyoD, myogenin, and cell cycle regulatory factors (CDK2, CDK4, and p21) regulate satellite cell activity (43) and are upregulated in skeletal muscle at the onset of functional overload (1). MRF mRNA induction in overloaded muscle coincides with the satellite cell activation. Androgens can induce CDK2, CDK4, and p21 expression, while repressing CDK inhibitor p16. The result is an increased CDK kinase activity (31). Myogenin expression is also induced by androgen receptor expression, while MyoD expression is not affected, and suggests that androgens may accelerate myoblast differentiation (27). The androgen receptor normally resides in an unbound state with the bound fraction being ~25% of total androgen receptor pool (38). Androgen ligand binding capacity has been reported to increase in functionally overloaded rat skeletal muscle (22). It remains a possibility that functional overload increases the percentage of androgen receptor in bound state without a corresponding increase in the total androgen receptor pool at the onset of functional overload. However, an overall increase in the total androgen receptor pool may be important to sustain longer lasting hypertrophic growth.

In summary, the present study demonstrates that androgen receptor protein expression is not induced until after 3 days of functional overload, and this induction is regulated posttranslationally. Nandrolone decanoate administration is sufficient to induce androgen receptor protein but not mRNA abundance. However, the combination of anabolic steroid administration and functional overload alters the time course of androgen receptor mRNA induction. Cotransfection of androgen receptor, SRF, and RhoA synergistically induces skeletal α-actin gene expression in CV1 cells and suggests that androgen receptor, in conjunction with SRF and RhoA signaling, can regulate muscle gene expression. This functional interaction may be an important signaling paradigm for functional overload-induced muscle growth.

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DISCLOSURES

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