

Testosterone represses ubiquitin ligases atrogin-1 and Murf-1 expression in an androgen-sensitive rat skeletal muscle in vivo

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Pires-Oliveira M, Maragno AL, Parreiras-E-Silva LT, Chiavegatti T, Gomes MD, Godinho RO. Testosterone represses ubiquitin ligases atrogin-1 and Murf-1 expression in an androgen-sensitive rat skeletal muscle in vivo. *J Appl Physiol* 108: 266–273, 2010. First published November 19, 2009; doi:10.1152/jappphysiol.00490.2009.—Skeletal muscle atrophy induced by denervation and metabolic diseases has been associated with increased ubiquitin ligase expression. In the present study, we evaluate the influence of androgens on muscle ubiquitin ligases atrogin-1/MAFbx/FBXO32 and Murf-1/Trim63 expression and its correlation with maintenance of muscle mass by using the testosterone-dependent fast-twitch levator ani muscle (LA) from normal or castrated adult male Wistar rats. Gene expression was determined by qRT-PCR and/or immunoblotting. Castration induced progressive loss of LA mass (30% of control, 90 days) and an exponential decrease of LA cytoplasm-to-nucleus ratio (nuclear domain; 22% of control after 60 days). Testosterone deprivation induced a 31-fold increase in LA atrogin-1 mRNA and an 18-fold increase in Murf-1 mRNA detected after 2 and 7 days of castration, respectively. Acute (24 h) testosterone administration fully repressed atrogin-1 and Murf-1 mRNA expression to control levels. Atrogin-1 protein was also increased by castration up to 170% after 30 days. Testosterone administration for 7 days restored atrogin-1 protein to control levels. In addition to the well known stimulus of protein synthesis, our results show that testosterone maintains muscle mass by repressing ubiquitin ligases, indicating that inhibition of ubiquitin-proteasome catabolic system is critical for trophic action of androgens in skeletal muscle. Besides, since neither castration nor androgen treatment had any effect on weight or ubiquitin ligases mRNA levels of extensor digitorum longus muscle, a fast-twitch muscle with low androgen sensitivity, our study shows that perineal muscle LA is a suitable in vivo model to evaluate regulation of muscle proteolysis, closely resembling human muscle responsiveness to androgens.

muscle atrophy; proteasome; androgen; nuclear domains; levator ani muscle

SKELETAL MUSCLE FIBERS ARE highly adaptable to changes in neuromuscular transmission, atrophying in response to either disuse or denervation (27, 52) and hypertrophying after electromechanical stimulation through exercise (42, 52). Several endocrine factors also regulate muscle trophic status; particularly, androgenic hormones have a well established anabolic action (8). Low testosterone levels have been associated to decreased human muscle mass (3, 20, 43, 58), whereas testosterone administration has been used to improve general muscle function in elderly men (17). These results show that general human musculature clearly responds to androgen deprivation or administration. In fact, anabolic androgens increase muscle

mass and strength in several atrophic states, such as cachexia in cancer, AIDS, and burn patients (25, 29, 33). Treatment with nutritional supplementation alone is ineffective, since proteolysis must be inhibited for substantial mass recovery (36).

There are several proposed mechanisms for the primarily anabolic actions of androgens (33). Classically, androgens exert their effects by increasing general muscle protein synthesis directly (13, 21, 34) or through increased activation (32) and proliferation (53) of myogenic satellite cells (64), progenitor cells that express high levels of androgen receptors (AR) (54).

Still, there is a lack of effective models to evaluate testosterone action in vivo, because at adulthood rodent skeletal muscles in general display a very modest or undetectable response to sex hormones (1, 2, 12, 14, 22, 23, 37). This low sensitivity to androgens clearly contrasts with the marked responses of the sexually dimorphic perineal muscles of rat, bulbocavernosus (BC), and levator ani (LA) muscles, which play an important role in copulation. Castration induces atrophy of these muscles (55, 62), with a significant reduction of synaptic proteins (9, 22, 24). In fact, hypertrophic action in the LA is a good predictor of effective anabolic androgens (30, 33). Also, although 50% of human myonuclei are AR⁺ (54), only 7% of myonuclei express AR in the extensor digitorum longus (EDL) of the rat (46). Therefore, it is to be expected that LA, with 74% AR⁺ myonuclei (46), mimic more closely human muscles than the general nonresponsive musculature of the rat.

Interestingly, LA has been recently used to evaluate the action of androgens over protein synthesis signaling (63), satellite cell function (47), and synaptic adaptation (49), but not androgenic regulation of proteolysis. Proteolytic activity is a hallmark in several models of skeletal muscle atrophy, and the ubiquitin-proteasome system (UPS) is responsible for up to 80–90% of cellular protein degradation (36). Several ubiquitin ligases catalyze the transfer of ubiquitin from a carrier protein to UPS substrates, thereby targeting these proteins for degradation by the 26S proteasome with a chain of ubiquitin molecules (36). Regulation of muscle proteolysis involves ubiquitin ligases, such as atrogin-1/MAFbx/FBXO32 (26) and Murf-1/Trim63 (10), specifically upregulated in muscle atrophy. Also, overexpression of atrogin-1 leads to atrophy of cultured myotubes, whereas atrogin-1 or Murf-1 knockout animals are resistant to muscle atrophy (10).

Elucidation of components involved in repression of catabolic signaling might lead to a better understanding of the trophic action of androgens in skeletal muscle and the deleterious muscle wasting effects of aging and hypogonadism. Therefore, a thorough knowledge of signaling in a model of skeletal muscle atrophy specifically caused by androgen deprivation

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vation seems justified. Because rat LA displays rapid and progressive responses to androgens, the goal of this study was to determine the *in vivo* effect of androgen on UPS, analyzing the possible change of skeletal muscle ubiquitin ligases atrogin-1, and Murf-1 expression induced by hormone deprivation and/or administration.

MATERIAL AND METHODS

Animals and treatments. Adult male Wistar rats from the institutional animal care facility were maintained on a 12:12-h light-dark cycle, with standard chow and water *ad libitum*. Rats were randomly assigned to control (N) or castrated groups for 2 (C2), 3 (C3), 6 (C6), 7 (C7), 15 (C15), 30 (C30), 60 (C60), or 90 (C90) days. As illustrated in Fig. 1, experimental groups treated with testosterone propionate (TP; 4 mg/kg sc; Sigma Aldrich, St. Louis, MO) consisted of C15+TP7 (rats castrated 15 days before and treated for 7 days with TP at days 8, 11, and 14), C7+TP5 (rats castrated 7 days before and treated for 5 days with TP at days 2, 4, and 6), C7+TP1 (rats castrated 7 days before and then treated with a single dose of TP on day 6), and C3+TP1 (rats castrated 3 days before and then treated with a single dose on day 2).

Castration reduces plasma testosterone by 82% after 1 day and further to constant, nondetectable levels (<0.04 ng/ml) after 5 days (44). Therefore, testosterone $t_{1/2}$ was at most 8 h, and all castrated groups were intensely androgen deprived. Testosterone propionate administrations were modified from Maróstica et al. (41) to achieve plasma testosterone concentrations similar or just slightly higher than those of control animals. Testosterone propionate is a more lipophilic testosterone ester that is slowly absorbed after injection in oil vehicles. Peak plasma concentration occurs after 24 h, and half-life is also ~24 h; therefore, a thrice-weekly scheme of testosterone propionate administration was chosen for reduced animal manipulation stress (18).

Castration was performed as previously described (22) under 75 mg/kg ketamine and 12 mg/kg xylazine anesthesia. All animals were killed by decapitation at 120 days of age (C2–30), 150 days (C60), or 180 days (C90), and seminal glands (SG), LA, and extensor digitorum

longus (EDL) muscles were dissected, weighted, and processed as described below. Preliminary experiments with tissues from sham-operated rats or animals injected with vehicle were also tested. Since no significant change was observed when these two experimental groups were compared with intact rats, all subsequent experiments were performed with tissues from intact, age-matched rats as controls.

In another set of experiments, adult male Swiss mice, obtained and maintained as described for rats, were randomly assigned to control (N); castrated 3 days (C3), or castrated 3 days before and then treated with a single dose of 4 mg/kg TP sc on day 2 (C3+TP1). All experimental procedures were approved by the institutional ethics committee (protocol no. 1604/2007).

Quantitative analysis of nuclear domains. Skeletal muscles were fixed overnight with 4% formaldehyde at 4°C, washed with PBS, pH 7.4, perfused, and then incubated for 1 h with a 5 µg/ml Hoechst dye 33258 (Invitrogen, Carlsbad, CA) solution on DMEM containing 10% horse serum for nuclear staining (19). Then muscles were washed with DMEM and PBS, pH 7.4, and dissected under a stereoscope by separation of progressively thinner bundles of fibers.

Images of stained myofibers were acquired on a confocal microscope (LSM 510 META, Carl Zeiss Optical, Chester, VA), equipped with a multiphoton laser (Coherent, Santa Clara, CA), with excitation at 360 nm and emission at 460 nm, a ×40 objective (oil immersion, NA 1.3; Carl Zeiss Optical, Chester, VA), and a CDC camera (AxioCam, Carl Zeiss Optical). Images were obtained as z-series of 1-µm sections and digitalized for tridimensional reconstruction of myofibers.

Myofiber volumes were approximated as cylindrical and calculated as:

$$\text{Volume} = \pi \times \left(\frac{\text{Diameter}}{2} \right)^2 \times \text{Length}$$

Confocal images were processed with Zeiss LSM Image Browser 4.2.0.121 (<http://www.zeiss.com/micro>) and measured with ImageJ 1.32 (<http://rsb.info.nih.gov/ij/>).

RNA isolation and quantitative RT-PCR. RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA) from individual skeletal muscles. Reverse transcription into cDNA was performed using 1 µg of total cellular RNA, 20 pmol oligo(dT) primer (Invitrogen) and Advantage ImProm-II reverse transcriptase (Promega, Madison, WI). Real-time PCR was carried out on an ABI7000 sequence detection system (Applied Biosystems, Foster City, CA), using SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit with ROX (Invitrogen), and primers rat atrogin-1 (NM_133521) forward 5'-TGAAGACCGGCTACTGTGGAA-GAGAC-3' and reverse 5'-TTGGGGTGAAGTGAGACGGAGCAG-3' (amplimer size, 486 bp), rat Murf-1 (AY059627.1) forward 5'-TCGACATC-TACAAGCAGGAA-3' and reverse 5'-CTGTCCTTGGGAAGATGC-TTT-3' (amplimer size, 194 bp), rat cyclophilin B (NM_022536) forward 5'-AGACGAACCTGTAGGACGAG-3' and reverse 5'-AGATGCTCT-TTCCTCCTGTG-3' (amplimer size, 197 bp), mouse atrogin-1 (NM_026346) forward 5'-GCAGAGATCGGCAAGTC-3' and reverse 5'-CAGGTGGTGATCGTGAG-3' (amplimer size, 142 bp), mouse MuRF-1 (NM_001039048.2) forward 5'-TGTGCAAGGAACACGAAG-3' and reverse 5'-TGAGAGATGATCGTCTGC-3' (amplimer size, 208 bp), and mouse cyclophilin B (NM_011149.2) forward 5'-AAGGACTTCAT-GATCCAGGG-3' and reverse 5'-TGACATCCTCAGTGGCTTG-3' (amplimer size, 301 bp). Primers were previously tested in conventional reverse transcription PCR, where a single band of the expected amplimer size was obtained. Relative quantitation of mRNA levels was plotted as fold increase compared with noncastrated control group values. Cyclophilin B (CB) was used for normalization (4). C_t values were averaged, and ΔC_t values (target gene C_t minus CB C_t) were calculated for each group. $\Delta\Delta C_t$ values (ΔC_t from sample group minus ΔC_t from control group) were calculated, and mRNA amplification was determined as $2^{-\Delta\Delta C_t}$ (39).

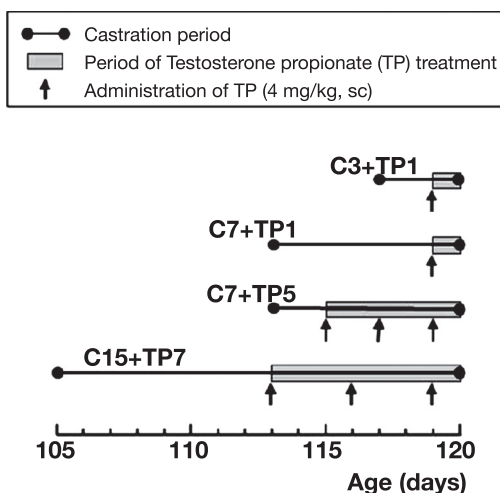


Fig. 1. Experimental treatments with testosterone propionate (TP). Group C15+TP7 was castrated for 15 days and treated with three doses of 4 mg/kg TP sc from day 8 to day 15 of castration; group C7+TP5 was castrated for 7 days and treated with three doses of 4 mg/kg TP sc from day 3 to day 7 of castration; group C7+TP1 was castrated for 7 days and then treated with a single dose of 4 mg/kg TP sc on the last day of castration; group C3+TP1 was castrated for 3 days and then treated with a single dose of 4 mg/kg TP sc on the last day of castration. Lines between filled circles show castration periods; gray rectangles show duration of treatments with TP; each arrow marks a 4 mg/kg TP sc administration.

Immunoblotting. Samples (40 μ g) of skeletal muscle protein were dissolved in 0.05 M Tris \cdot HCl, pH 7.4 with 0.5 mol/l NaCl and 0.1% Tween-20 (TBS-T) and loaded onto a 10% polyacrylamide gel with SDS. After electrophoresis, proteins were transferred (TransBlot Semi Dryer, Bio-Rad, Hercules, CA) to a nitrocellulose membrane (Transblot 0.45 μ M, Bio-Rad) at 10 mA/20 min, with 39 mM glycine, 48 mM Tris, 10% SDS on water/methanol 1:5 (vol/vol). Membranes were stained with Ponceau Red, blocked with 10% nonfat milk on TBS-T for 1 h and incubated with rabbit anti-rat atrogenin-1 primary IgG (a gift from Dr. Stewart H. Lecker, Renal Unit, Beth Israel Deaconess Medical Center, Boston, MA) (5) on blocking buffer for 2 h at room temperature. Secondary peroxidase-conjugated goat anti-rabbit IgG antibodies (a gift from Dr. Eduardo B. Oliveira, FMRP-USP, Ribeirão Pret, Brazil), diluted 1:10,000 on blocking buffer, were applied to previously washed membranes for 2 h at room temperature. Development was performed by the enhanced chemiluminescence method with luminol (Santa Cruz Biotechnology, Santa Cruz, CA). Atrogenin-1 pixel density levels were evaluated with NIH ImageJ 1.32 software, normalized to Ponceau Red density, and expressed as percentage of control values.

Statistical analysis. Results were expressed as means \pm SE, except where specified. For determination of statistical differences between averages, one-way ANOVA followed by Dunnett's post hoc test was used. All analyses were performed using GraphPad Prism for Windows 5.01. Statistical significance level was fixed at 5% ($P < 0.05$).

RESULTS

Androgenic regulation of levator ani muscle mass and morphology. Neither castration for 2–90 days nor androgen treatment for 1, 5, or 7 days significantly changed the body weight of control adult male rats (4-mo-old $N = 327.2 \pm 14.5$ g; 5-mo-old $N = 360.0 \pm 7.2$ g; 6-mo-old $N = 399.3 \pm 24.7$ g). However, androgenic deprivation was successfully induced by castration, as shown by progressive atrophy of the seminal gland, an androgen-dependent organ of the male reproductive tract that reached 20% of control weight ($N = 210.5 \pm 11.2$ mg; Fig. 2A) 30 days after castration.

Comparatively, castration induced a progressive atrophy of the androgen-sensitive LA muscle, which reached 30% of control weight ($N = 164.3 \pm 6.3$ mg; Fig. 2A) after 90 days. Androgen administration for 1 or 5 days did not significantly increase LA muscle weight compared with values from nontreated animals subjected to the same castration periods (C3 = 149.8 ± 0.9 mg; C7 = 124.9 ± 6.6 mg, respectively). However, when rats castrated for 15 days were treated with testosterone propionate from days 8 to 15 (C15+TP7), LA atrophy was attenuated by 46% compared with nontreated castrated rats (C15 = 77.7 ± 2.3 mg; Fig. 2B). This androgen treatment also completely reversed SG atrophy induced by castration after 15 days (C15 = 109.4 ± 3.7 mg).

It is well known that atrophy of the androgen-deprived LA muscle occurs with no loss of skeletal muscle fibers (56, 60), whereas androgen treatment increases LA myofiber diameter (31). To follow the progressive adaptation of the atrophic fiber, we evaluated the effect of androgen deprivation on muscle fiber morphology of the LA muscle. We found an exponential decrease on myofiber diameter after castration, up to 39% of control values ($N = 35.3 \pm 0.4$ μ m; Fig. 3) after 60 days. Castration had no effect on EDL myofiber diameter compared with control ($N = 36.7 \pm 0.7$ μ m; Fig. 3).

Although androgen deprivation increased the number of apoptotic nuclei (Fig. 4B), which may lead to loss of some myonuclei, it was not sufficient to maintain a constant myo-

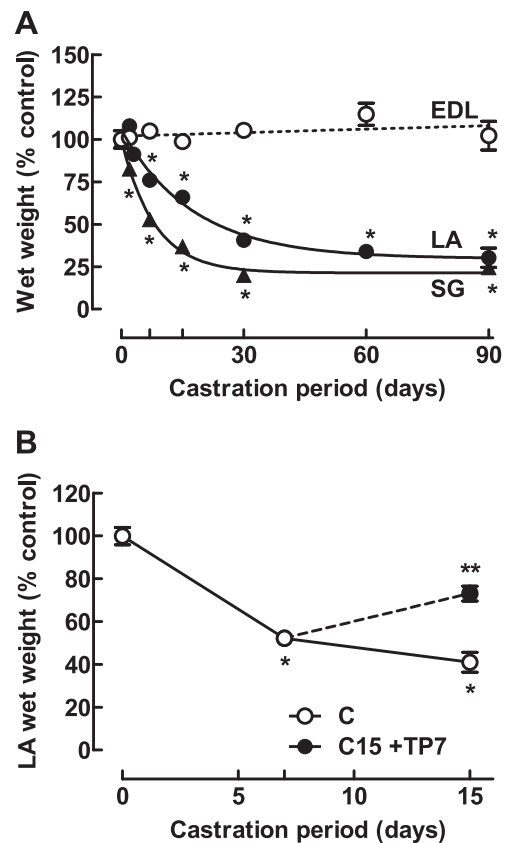


Fig. 2. Castration successfully induces atrophy of the androgen-sensitive seminal gland (SG) and levator ani muscle (LA) but does not change extensor digitorum longus (EDL) muscle weight. *A*: adult male rats were castrated for 0, 2, 3, 7, 15, 30, 60, and 90 days, and decreased SG weight was used as a control of successful castration. C60 and C90 values were normalized to age-matched controls (5-mo-old and 6-mo-old, respectively). *B*: although castration (C) significantly reduces LA wet weight, when rats were castrated for 15 days and treated with testosterone propionate (3 doses of 4 mg/kg sc) from day 8 to day 15 of castration (C15+TP7), LA muscle atrophy was significantly attenuated. Each point represents mean \pm SE of 3–4 individuals. *Significantly different from controls (0; $P < 0.05$). **Significantly different from castrated (C15; $P < 0.05$).

nuclear domain. In fact, cytoplasm-to-nucleus ratio decreased progressively and in parallel with LA myofiber diameter (Figs. 3 and 4A), reaching 22% of control value 60 days after castration (Fig. 4C).

Effects of androgen deprivation and replacement on ubiquitin ligase expression. To investigate the molecular basis of LA atrophy, we analyzed the expression of atrogenin-1, an F-box ubiquitin-ligase member whose expression is increased severalfold in a variety of muscle wasting conditions (26, 50) and Murf-1, a RING finger ubiquitin ligase also upregulated during muscle atrophy (10, 50). Atrogenin-1 mRNA expression peaked at a 31-fold increase after just 2 days of hormonal deprivation, returning to control levels after 30 days (Fig. 5A). Interestingly, even though androgen treatment for 24 h was too short to affect LA weight, it completely abolished the 20-fold increase of atrogenin-1 mRNA induced 3 days after castration (C3+TP1; Fig. 5B). Longer treatments with testosterone propionate (C15+TP7), which inhibit the progression of LA atrophy (Fig. 2B), also recovered atrogenin-1 mRNA level to control values (data not shown). These results show that LA atrophy induced

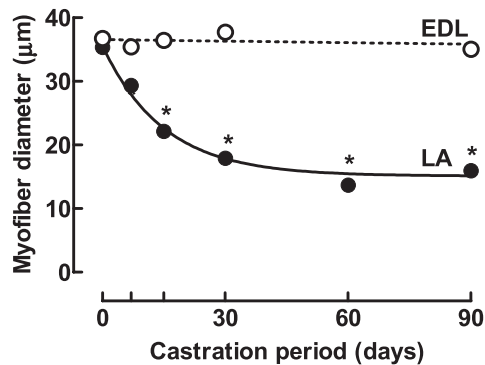


Fig. 3. Castration decreases myofiber diameter of the male rat LA. Adult male rats were castrated for 0, 7, 15, 30, 60, and 90 days, and myofiber diameter was determined on isolated myofibers from both muscles. LA myofiber diameter decreased exponentially with castration. There were no changes on EDL in all castration periods considered. Each point represents mean \pm SE of random myofibers (11–53) from 3–4 individuals. Symbols are larger than SE. *Significantly different from controls (0; $P < 0.05$).

by castration is preceded by increased atrogen-1 mRNA expression. Conversely, androgen administration promptly reversed the effect of castration on atrogen-1 mRNA expression, which might explain effective prevention of LA atrophy later. Ultrashort treatment (6 h) with androgen, however, was not enough to significantly reduce atrogen-1 mRNA expression (data not shown).

Neither castration nor androgen treatment had any effect on EDL weight ($N = 186.9 \pm 16.2$ mg; Fig. 2A) or atrogen-1 mRNA levels (Fig. 5E) compared with controls. Interestingly, atrogen-1 mRNA relative expression in control EDL muscle was 28-fold higher than the expression observed in LA muscle, and castration increased LA atrogen-1 transcript, abolishing the differences between both muscles.

Taking into account that biological impact of changes in atrogen-1 transcript levels depends on whether they are followed by changes in the encoded proteins, atrogen-1 protein expression was evaluated through an immunoblotting assay. Castration induced a progressive increase in LA atrogen-1 protein content up to 2.7-fold at 30 days (Fig. 6). When rats were castrated for 15 days and treated with testosterone propionate from days 8 to 15 (C15+TP7), atrogen-1 protein expression returned to control levels (Fig. 6). As observed in transcript analysis, castration had no effect on EDL atrogen-1 protein, which was higher in EDL than in LA.

In LA, testosterone appears to have broad control over muscle ubiquitin proteasome system and also modulates Murf-1 mRNA expression. After castration, there was an 18-fold increase in LA Murf-1 mRNA, which was maximal after 7 days and quickly returned to control levels after 15 days (Fig. 5C). Androgen treatment for 24 h also abolished the increment of Murf-1 mRNA induced after 7 days of castration (Fig. 5D). Neither castration nor androgen treatment had any effect on EDL Murf-1 mRNA levels compared with controls (Fig. 5E). In parallel to atrogen-1 mRNA results, Murf-1 mRNA is five times more abundant in control EDL than in LA. As seen with atrogen-1, castration increased LA Murf-1 transcript and inverted the pattern between muscles.

We also compared the effects of testosterone on atrogen-1 and Murf-1 mRNA from rat LA with those on mouse LA/BC transcripts. We found that 3 days after castration atrogen-1 and

Murf-1 mRNA levels increased by five- and threefold, respectively (Fig. 7). When mice were castrated for 3 days and then treated with testosterone propionate through the last day, atrogen-1 and Murf-1 mRNA levels were similar to control (Fig. 7).

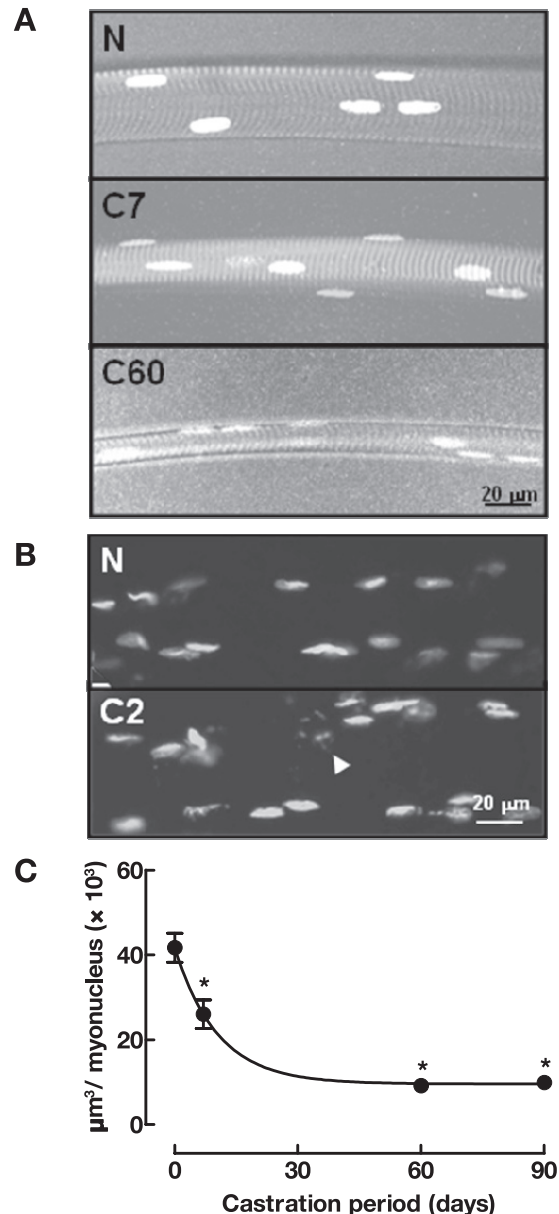
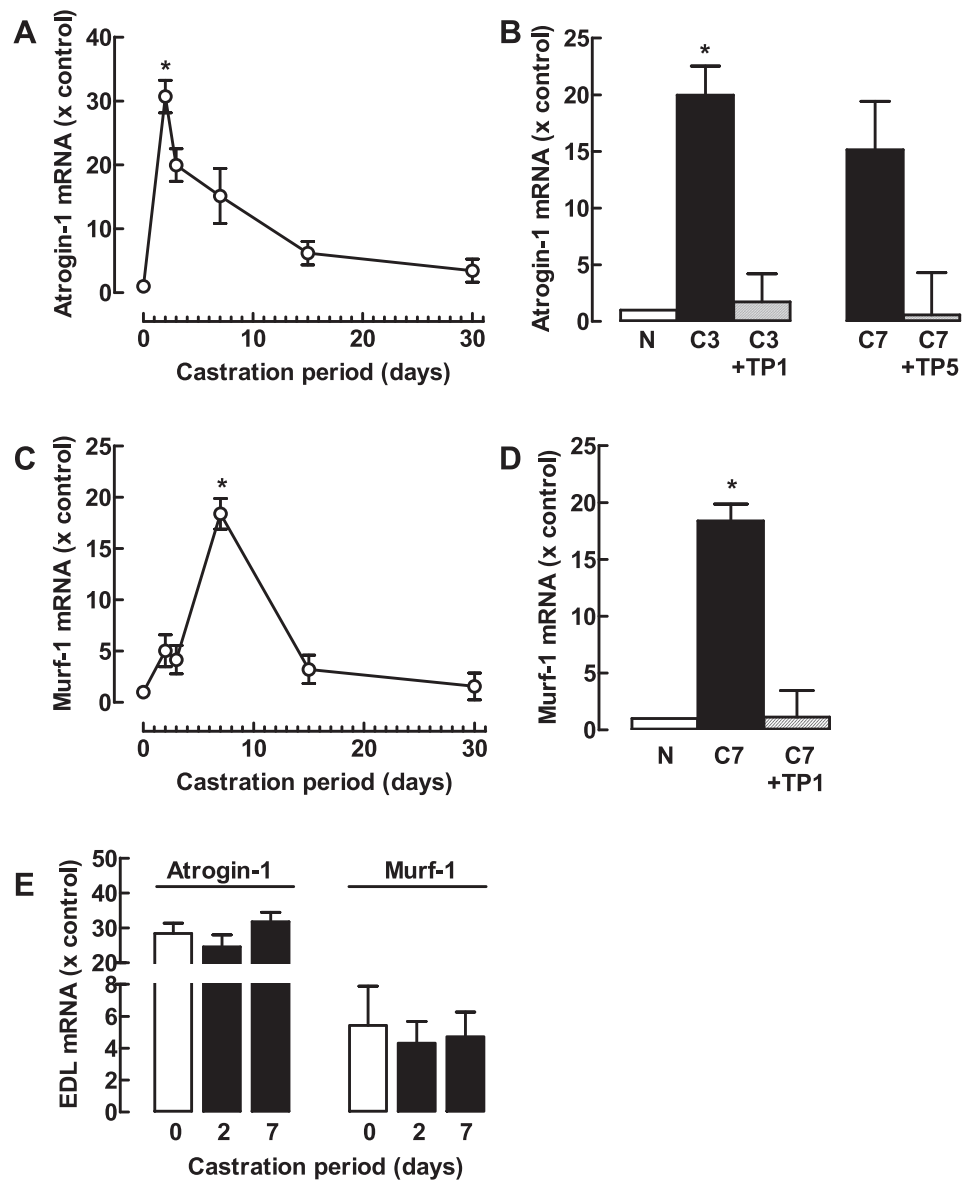


Fig. 4. Castration leads to decreased myofiber diameter and nuclear domains in the LA. A: castration for 7 (C7) or 60 days (C60) significantly reduced LA myofiber diameter, volume, and cytoplasm-to-nucleus ratio compared with fibers from control rats (N). Z-axis projections were obtained through confocal microscopy of Hoechst 33258-stained isolated myofibers and superposed on differential interference contrast images (representative images). B: fluorescence images of Hoechst 33258-stained cryosections showed a representative fragmented myonuclei (arrowhead) in a LA myofiber after castration for 2 days (C2). No fragmentation was seen in control muscles (N). C: cytoplasm-to-nucleus ratio results (nuclear domain) of myofibers from 3 individuals were quantitated. Each point represents mean \pm SE of random myofibers (14–29) from 3 individuals. Some symbols are larger than SE. *Significantly different from controls (0; $P < 0.05$).

Fig. 5. Androgen inhibits ubiquitin ligase mRNA expression in the LA of male rats. *A*: adult male rats were castrated for 0, 2, 3, 7, 15, or 30 days; atrogen-1 mRNA expression increased after only 2 days of castration and returned to control levels after 30 days. *B*: when rats were castrated for 3 days and then treated with a single dose of 4 mg/kg TP sc on the last day (C3+TP1), atrogen-1 mRNA returned to control levels (N), whereas nontreated rats castrated for 3 days (C3) showed a 20-fold increase in atrogen-1 mRNA; castration for 7 days and then treatment with three doses of 4 mg/kg TP from day 3 to day 7 (C7+TP5) also reduced atrogen-1 mRNA compared with nontreated rats castrated for 7 days (C7). *C*: Murf-1 mRNA expression also increased after 7 days of castration and returned to control levels after 15 days. *D*: when rats were castrated for 7 days and then treated with a single dose of 4 mg/kg TP sc on the last day (C7+TP1), Murf-1 mRNA returned to N, whereas nontreated rats castrated for 7 days (C7) showed an 18-fold increase in Murf-1 mRNA. *E*: castration had no effect on EDL atrogen-1 or Murf-1 mRNA. Both transcript levels were higher in EDL than in LA from control rats. Each point or bar represents the mean \pm SE of 3–6 individuals, normalized to expression of each gene in control rat LA (0). Standard errors were calculated as $2^{SE \text{ of } \Delta\Delta Ct}$ values. *Significantly different from controls ($P < 0.05$).



DISCUSSION

In the present study, we used the LA muscle of the adult male rat as a particularly sensitive model to study the effects of androgen on skeletal muscle mass and muscle ubiquitin ligases atrogen-1 and Murf-1 expression. Here, muscle atrophy was induced specifically by removal of the androgen, without invasive neuromuscular lesion, as in denervation, or systemic metabolic disturbance caused by cachexia or fasting.

LA atrophy induced by hormonal withdrawal was paralleled by a progressive decrease in nuclear domains due to myofiber volume reduction (Fig. 3), indicating that apoptotic loss of myonuclei is not widespread enough to maintain cytoplasm-to-nucleus ratio in the atrophied fiber. These data are in agreement with those of Nnodim (48), who showed a decreased distance between myofiber nuclei 8 wk after castration.

Trophic effects of androgens on skeletal muscle involves multiple mechanisms including increment of protein synthesis (34), recruitment of myogenic satellite cells (32), and/or re-

pression of catabolic pathways and factors, such as myostatin (45) and glucocorticoid receptor signaling (65, 67). Here, our results give new insight to this field, showing that androgens act *in vivo* to quickly and largely repress expression of atrogen-1 and Murf-1, which affect UPS activity, a system responsible for most proteolysis in the cell (36). Our results showed that androgen withdrawal induces a 31-fold increase in atrogen-1 and 18-fold increase in Murf-1 mRNA content (Fig. 5). These effects were larger than those observed in other models of severe skeletal muscle atrophy induced by cachexia or fasting (26), while similar to those seen in surgically denervated muscles (50).

There are some candidate pathways for androgenic regulation of atrogen-1 and Murf-1 expression. For instance, atrogen-1 regulation is known to involve FOXO3a, a member of the forkhead box (FOXO) transcription factor family. FOXO3a is inactivated through phosphorylation by several kinases (51), such as the phosphatidylinositol-3 kinase (PI3K)/Akt pathway

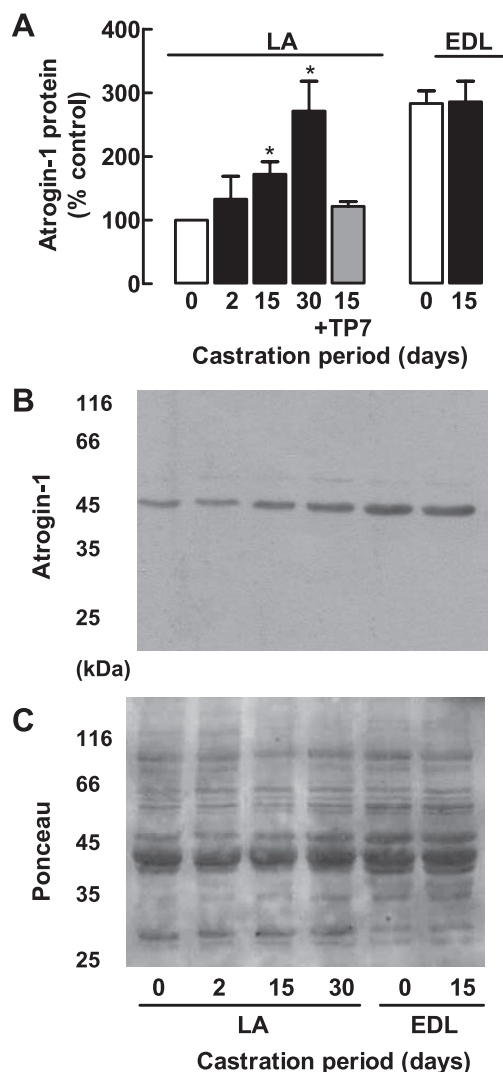


Fig. 6. Castration induces a linear increase of atrogin-1 protein in the rat LA. *A*: castration for 15 days and treatment with testosterone propionate (3 doses of 4 mg/kg sc) from day 8 to day 15 of castration (C15+TP7) significantly reduced atrogin-1 protein expression to control levels (0). *B*: adult male rats were castrated for 0 to 30 days, and protein samples from LA and EDL were used for immunoblotting analysis with anti-atrogin-1 antibody. *C*: expression was determined relative to Ponceau red staining through densitometric analysis. Each bar represents mean \pm SD of 2–5 individuals.

recruited by insulin-like growth factor 1 (IGF-1). Akt phosphorylates FOXO3a, blocking its nuclear translocation and thus decreasing atrogin-1 expression and protein degradation. Interestingly, androgen deprivation reduces LA IGF-1 content, whereas dihydrotestosterone treatment increases its amount (63), suggesting that androgens might act indirectly through the trophic IGF-1 pathway. Anabolic androgens might repress atrogin-1 expression through other mechanisms, since Zhao et al. (66) did not see changes in phosphorylated FOXO3a after treatment of AR-transfected C2C12 cells with testosterone. Since disruption of the AR DNA binding domain does not block androgen-mediated atrogin-1 promoter repression, the authors suggest an indirect inhibition mediated by Oct-1 (66). In vivo atrogin-1 expression may also be affected by localized changes in phosphorylated FOXO or even other pathways, such as an indirect inhibition of atrogin-1 promoter mediated

by Oct-1 (66), p38 mitogen-activated protein kinase (p38 MAPK) (38), or NF- κ B, whose activity is also implicated in skeletal muscle atrophy (36). These signaling pathways are still under investigation in our laboratory.

As for Murf-1, our study is the first to demonstrate upregulation of its mRNA after androgen deprivation in vivo. The mechanisms involved are unknown, but Murf-1 promoter has a highly conserved glucocorticoid response element (61) that could potentially mediate direct inhibitory AR binding (16). Another recent report demonstrated the relevance of extracellular signal-regulated kinases (ERK) as yet another androgen-sensitive pathway that might regulate either Murf-1 or atrogin-1 expression (52a).

The possible regulation of ubiquitin ligases by androgenic hormones was recently questioned by MacLean et al. (40), who did not detect increased expression of atrophy-related ligase genes at gastrocnemius muscle of AR knockout mice. In fact, the low responsiveness of mouse muscle to androgenic action is consistent with the inability of castration to increase atrogin-1 and Murf-1 mRNA at EDL of adult rat. The levels of atrogin-1 and Murf-1 in EDL, even in control adult male rats with normal serum testosterone, were high (Figs. 5E and 6), indicating that physiological levels of androgens are not enough to repress ubiquitin ligases in EDL, probably due to low AR expression (2, 35). Therefore, our results strongly support the idea of a normal pattern of low sensitivity of general rodent musculature to androgen, whereas adult rat LA provides a model in which androgen-mediated signaling is amplified.

Since expression of ubiquitin ligases is higher in EDL, it is likely that its protein turnover is faster than that of LA. A corroborating example is acetylcholinesterase (AChE), whose activity is higher in LA than in EDL, whereas castration reduces total LA AChE activity to EDL levels (22). AChE is intensely degraded by endoplasmic reticulum-associated UPS (7), and testosterone could repress this degradation and increase AChE content in LA through reduced ubiquitin ligase expression.

Conversely, Beehler et al. (6) proposed that previously described apoptotic phenomena (11) thoroughly explain mus-

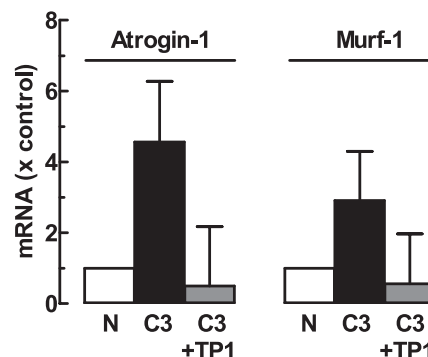


Fig. 7. Androgen also inhibits ubiquitin ligase mRNA expression in the androgen-dependent perineal muscles of male mice. Castration for 3 days (C3) increased both atrogin-1 and Murf-1 mRNA expression compared with controls (N). When mice were castrated for 3 days and then treated with a single dose of 4 mg/kg TP sc on the last day (C3+TP1), atrogin-1 and Murf-1 mRNA returned to control levels. Perineal muscles levator ani and bulbocavernosus were pooled from each individual. Each bar represents the mean \pm SE of 3 individuals, normalized to expression of each gene in control mice. Standard errors were calculated as $2^{SE \text{ of } \Delta\Delta Ct \text{ values}}$.

cle mass loss in the androgen-deprived LA instead of proteasome activation, since treatment with proteasome inhibitor bortezomibe (3 mg/kg) has no effect on atrophy induced by 10-day castration. However, as observed following denervation (50), we showed here that LA atrogen-1 and Murf-1 mRNA increased drastically and rapidly after castration (Fig. 5, A and C), preceding any loss of muscle mass (Fig. 2A). Probably, inefficiency of the bortezomibe treatment described by Beehler et al. (6) only shows that UPS activation is still in its early phases after 10 days of castration or that effective dose for proteasome inhibition changes with the target and/or atrophy model.

In this study, we also address whether mice ubiquitin ligases are repressed by androgen. In fact, similar to rat LA, castration increases atrogen-1 and Murf-1 mRNA, whereas androgen treatment decreases it (Fig. 7). The detection of a conserved response in mice gives new perspectives for further studies of androgenic repression of ubiquitin ligases and proteolysis using knockout mice, including atrogen-1^{-/-} and Murf-1^{-/-} mice themselves (10).

Meanwhile, we have shown that testosterone quickly and widely inhibits the expression of ubiquitin ligases atrogen-1 and Murf-1 in vivo, which may be critical for the anabolic action of sex male hormones. Deprivation of androgens, especially in conditions such as aging and hypogonadism, is therefore likely to induce loss of muscle mass through degradation of substrates of atrogen-1 and Murf-1, such as myogenic factor MyoD (59) and contractile machinery proteins titin or troponin I (15). Moreover, the high sensitivity to androgen makes the rat perineal LA muscle an outstanding model to study muscle atrophy instead of general rat musculature, which responds much more poorly to androgens than human muscle. Identification of androgenic regulation of ubiquitin ligases atrogen-1 and Murf-1 opens leeway to characterization of common mechanisms involved in skeletal muscle atrophy and to anabolic therapy targets with far fewer side effects than those currently used in practice.

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