Testosterone Signals through mTOR and Androgen Receptor to Induce Muscle Hypertrophy

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ABSTRACT

BASUALTO-ALARCÓN, C., G. JORQUERA, F. ALTAMIRANO, E. JAIMOVICH, and M. ESTRADA. Testosterone Signals through mTOR and Androgen Receptor to Induce Muscle Hypertrophy. *Med. Sci. Sports Exerc.*, Vol. 45, No. 9, pp. 1712–1720, 2013. **Purpose**: The anabolic hormone testosterone induces muscle hypertrophy, but the intracellular mechanisms involved are poorly known. We addressed the question whether signal transduction pathways other than the androgen receptor (AR) are necessary to elicit hypertrophy in skeletal muscle myotubes. **Methods**: Cultured rat skeletal muscle myotubes were preincubated with inhibitors for ERK1/2 (PD98059), PI3K/Akt (LY294002 and Akt inhibitor VIII) or mTOR/S6K1 (rapamycin), and then stimulated with 100 nM testosterone. The expression of α-actin and the phosphorylation levels of ERK1/2, Akt and S6K1 (a downstream target for mTOR) were measured by Western blot. mRNA levels were evaluated by real time RT-PCR. Myotube size and sarcomerization were determined by confocal microscopy. Inhibition of AR was assessed by bicalutamide. **Results**: Testosterone-induced myotube hypertrophy was assessed as increased myotube cross-sectional area (CSA) and increased α-actin mRNA and α-actin protein levels, with no changes in mRNA expression of atrogenes (MAFbx and MuRF-1). Morphological development of myotube sarcomeres was evident in testosterone-stimulated myotubes. Known hypertrophy signaling pathways were studied at short times: ERK1/2 and Akt showed an increase in phosphorylation status after testosterone stimulus at 5 and 15 min, respectively. S6K1 was phosphorylated at 60 min. This response was abolished by PI3K/Akt and mTOR inhibition but not by ERK1/2 inhibition. Similarly, the CSA increase at 12 h was abolished by inhibitors of the PI3K/Akt pathway as well as by AR inhibition. **Conclusions**: These results suggest a crosstalk between pathways involving fast intracellular signaling and the AR to explain testosterone-induced skeletal muscle hypertrophy. **Key Words:** TESTOSTERONE, MUSCLE HYPERTROPHY, MYOTUBES, AKT, S6K1

BASIC SCIENCES

Hypertrophy is a physiological process that takes place in skeletal muscle as an adaptive response to some stimuli like exercise and hormones (13,34). These stimuli converge in common signaling pathways to induce muscle hypertrophy, a process characterized by increased cell size and protein synthesis (6,13,34). Different cellular events take place upon hypertrophy stimulus such as activation of specific signaling pathways and increased myogenic factor activities (13). A central link for the development of skeletal muscle hypertrophy is the activation of the mammalian target of rapamycin (mTOR) pathway (15,22), which also has been reported in testosterone-induced cardiomyocyte hypertrophy (1). This hormone plays important roles in the regulation of cell proliferation and differentiation of skeletal muscle (5). Both Type I and Type II skeletal muscle fibers have been shown to respond to testosterone treatment increasing muscle mass, cross-sectional areas (CSA), and satellite cell number after hormone administration (30).

Testosterone and its synthetic cognates have been used both clinically and illicitly to increase muscle mass (13); however, the cellular mechanisms explaining these effects are not completely understood. Different cellular and molecular mechanisms have been involved in skeletal muscle hypertrophy induced by testosterone including promotion of myonuclear accretion (29), entry of satellite cells into cell cycle (30), recruitment of mesenchymal pluripotent cells into the myogenic lineage (28), and activation of the intracellular androgen receptor (AR) (19). Besides regulating gene expression via the AR, testosterone also produces fast, nontranscriptional responses involving membrane-linked signal transduction pathways (12,14). A rapid nongenomic action exerted via G-protein–coupled receptor, intracellular calcium increases, and extracellular signal-regulated kinases 1/2 (ERK1/2) activation has been described for testosterone in skeletal myotubes (11). Recently, in a cellular lineage of myoblasts that lack the classical AR (L6 myoblasts) testosterone has been shown to promote proliferation and differentiation of

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L6 cells via a G-protein–coupled receptor (16). In the same cell line stably expressing human AR (L6.AR cells), both ERK1/2 and the AR have been involved in hypertrophy induced by testosterone (36), suggesting a role for ERK-induced mTOR activation.

Muscle hypertrophy induced by different stimuli has been preferentially studied in C2C12 and L6 myoblasts cell lines. In this work, we used a primary culture of rat myotubes to study the relationship between the hypertrophy induced by testosterone and its driving signaling pathways, looking for a model that resembles more the physiology of skeletal muscle fibers. The involvement of the intracellular AR and fast, nongenomic pathways were assessed to describe their roles in the hypertrophy process triggered by testosterone.

MATERIALS AND METHODS

Reagents. Testosterone enanthate, LY294002, and bicalutamide were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Rapamycin, PD-98059, and Akt inhibitor VIII were obtained from Calbiochem (La Jolla, CA). LY294002 is a PI3K inhibitor that acts on the ATP binding site of the protein (32). Akt inhibitor VIII was used as an inhibitor of Akt (inhibiting Akt1 and 2). The simultaneous binding of Akt inhibitor VIII to two different functional regions, PH and kinase domains, prevents Akt activation loop to be phosphorylated by its upstream activator, PDK1, locking it in an inactive conformation (3). Rapamycin was used as an inhibitor of mTOR, and it acts by binding to FKBP12. Both elements act by binding mTORC1 complex inhibiting its activity and so the activation of downstream targets as S6K1 (8). PD-98059 is a MEK (MAP kinase kinase) inhibitor that blocks ERK1/2 proteins (9). Bicalutamide is a competitive antagonist of the intracellular AR that competes with testosterone for the binding site of the testosterone on the receptor (17). Testosterone was diluted in absolute ethanol in a final concentration of 0.01% (v/v) in the stimulation solution; at this concentration, ethanol has no effects on biochemical determinations (11).

Cell Cultures

Newborn rats were bred in the Animal Breeding Facility, Faculty of Medicine, University of Chile. Studies were approved by the Institutional Bioethical Committee, Faculty of Medicine, University of Chile (CBA #0334 FMUCH), in accordance with the *Guide for the Care and Use of Laboratory Animals* (from the National Institutes of Health (NIH), Bethesda, MD), which were in *adherence* with the animal care standards set by the American College of Sports Medicine. Rat myotubes were cultured as reported previously (11). Briefly, muscle tissue from 1 to 3 d postnatal rat pups was obtained from the hindlimbs of neonatal rats, utilizing the whole muscle mass from both hindlimbs to obtain the cell culture. The tissue was dispersed mechanically and then treated with 0.2% (w/v) collagenase for 15 min in phosphate-buffered saline (PBS). The suspension was filtered through a Nytex membrane and spun down at 1000 rpm. Then 1 h of preplating was performed for the enrichment of myoblasts; cells were plated at 30%–50% confluence. The growth medium was Dulbecco's modified Eagle's medium (DMEM)-Ham's F-12, 5% bovine serum, 10% fetal bovine serum plus antibiotics (penicillin and streptomycin), and antifungal agents (amphotericin B). After 3 d, myoblasts were differentiated in DMEM-Ham's F-12 without serum for 3–4 d. With these procedures, we obtained myotubes that have been used as a physiological model to study muscle signaling (11,12). To eliminate remaining fibroblasts, 10 μM cytosine arabinoside was added on the third day of culture for 24 h. The medium was then replaced with serum-free medium. Myotubes with an estimated purity of more than 90% were visible after the fifth day of culture. Unless indicated, we used 5- to 7-d-old cultures exhibiting a fairly homogeneous population of myotubes with central nuclei, corresponding to young, not fully differentiated, cells.

Western Blot

Total protein lysates were prepared from control and testosterone-stimulated myotubes by homogenizing them in a lysis buffer containing 20 mM Tris–HCl, pH 7.5, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 1 mM $Na_2P_2O_7$, 10% glycerol, 150 mM NaCl, 10 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitors (Complete™, Roche Applied Science). Cell lysates were sonicated for 1 min, incubated on ice for 30 min, and centrifuged to remove debris. Protein concentration of the supernatants was determined with Coomassie Plus™ Protein Assay (Thermo Scientific) using bovine serum albumin as standard. Cells lysates containing 40 μg of protein were separated using SDS-PAGE and transferred to Polyvinylidene difluoride (PVDF) membranes. PVDF membranes were incubated overnight at 4°C with primary antibody. Next, the membranes were washed three times in Tris-buffered saline with Tween (TBST), incubated with peroxidase-conjugated secondary antibodies either antirabbit or antimouse (1:2000, Pierce Thermo Scientific) for 1 h at room temperature, and washed three additional times in TBST. The following primary antibodies and their dilutions were used: sarcomeric α-actin (1:15,000) and β-tubulin (1:2000) from Sigma Chemical Company; phospho-S6K1/2 (Thr412/Thr389) (1:2000) and AR (1:1000) from Millipore corporation; total S6K1 (1:2000), phospho-Akt (Ser473) (1:1000), total Akt (1:1000) from Cell Signaling Technology; phospho-ERK1/2 (Thr202/Tyr204) (1:2000) and total ERK1/2 (1:2000) from Santa Cruz Biotechnology. Secondary Horseradish peroxidase-conjugated antirabbit and antimouse antibodies were used as described previously. Protein bands in the blots were visualized using an ECL Detection Kit (Thermo Scientific), and the intensity of the bands was determined by densitometry using the ImageJ free software (NIH).

Real-time Polymerase Chain Reaction

According to the manufacturer's protocol, total RNA was obtained from myotubes using TRIzol reagent (Invitrogen). cDNA was prepared from 1 μg of RNA using SuperScript II enzyme (Invitrogen). Real-time polymerase chain reaction (PCR) was performed using Stratagene Mx3000P (Stratagene) using the Brilliant III Ultra-Fast QPCR & QRT-PCR Master Mix amplification kit (Agilent Technologies). The primers used were as follows: α-actin 5′-TGTGTGTGACAACGC CTCTG-3′ (sense), 5′-CTGTGGTCACGAAGGAATAG-3′ (antisense); β-actin 5′-TCTACAATGAGCTGCGTGTG-3′ (sense), 5′-TAC ATG GCT GGG GTG TTG AA-3′ (antisense); MuRF1 5′-CTGGCTTGATTCCGGACGGAAA-3′ (sense), 5′-GGCAGCGGAAACGACCTCCA-3′ (antisense); Atrogin-1/MAFbx 5′-CAGACCTGCATGTGCTCAGT-3′ (sense), 5′-CCAGGAGAGAATGTGGCAGT-3′ (antisense). All primers used presented optimal amplification efficiency (between 90% and 110%). PCR amplification of the housekeeping gene β-actin was performed as a control. Thermocycling conditions were as follows: 95°C for 3 min and 40 cycles of 95°C for 10 s, 60°C for 20 s. Expression values were normalized to β-actin and are reported in units of $2^{-\Delta\Delta CT} \pm$ SEM (19). CT was determined by MXPro software when fluorescence was 25% higher than the background. PCR products were verified by melting curve analysis.

Immunofluorescence and Confocal Microscopy

Cells were plated on 35-mm coverslips. Differentiated myotubes were stimulated with testosterone for different times in free serum culture media; afterward, they were fixed in 4% paraformaldehyde for 10 min at RT and permeabilized with 0.1% Triton X-100. Cells were rinsed with PBS, then blocked with PBS–1% BSA for 1 h at room temperature, and incubated at 4°C overnight with the following antibodies: sarcomeric α-actinin (1:100) and myogenin (1:100). Cells were washed and then incubated for 1 h with Alexa Fluor-488 antimouse antibody (Molecular Probes Invitrogen). Immunofluorescence was observed in a confocal microscope (Carl Zeiss, Axiovert 200, LSM 5-Pascal), and images were deconvolved using Iterative Deconvolution software of ImageJ. Z-stack images were reconstructed using ImageJ. Intracellular AR was localized using indirect immunofluorescence. Myotubes (control and testosterone stimulated) were washed and then fixed in 2% paraformaldehyde/5% acetic acid for 10 min at RT and permeabilized with Triton X-100 0.1% for 6 min and treated with a blocking solution of 8% BSA in PBS for 60 min. Cells were incubated with the primary polyclonal antibody against the intracellular AR (1:50, PG-21; Millipore) overnight at 4°C. Later, myotubes were washed in PBS and incubated with Alexa-633–conjugated goat antirabbit secondary antibody (1:500, Invitrogen Molecular Probes) for 60 min at room temperature. Cells were washed, and Vectashield (Vector Laboratories, Inc., Burlingame, CA) was added to prevent bleaching. Myotubes were examined with a confocal microscope (Carl Zeiss, Axiovert 200, LSM 5-Pascal), and images were acquired in z-stack (four different experiments, at least three images per experiment). Later, images were deconvolved using iterative deconvolution, and z-stacks were reconstructed using ImageJ software (NIH). Controls performed both without the primary antibody and by displacement with the antigenic peptide as a test for specificity were made. At 4 h, a significative shift from cytoplasm to the nucleus was observed. Fluorescence intensity quantification was analyzed with ImageJ. Three regions of interest per image were analyzed for cytoplasm and nuclei, and the fluorescence intensities were finally tabulated in GraphPad Prism 5.

Hypertrophy Parameters

Differentiated myotubes grown on 35-mm coverslip were stimulated with testosterone for 12 h in free serum culture media. Indirect immunofluorescence against the sarcomeric protein α-actinin was made. Confocal images in the *z*-axis were taken. Arbitrary parameters have been set to measure transversal section area, only not branched myotubes, and the widest regions of each myotube were chosen to measure CSA. Data of myotube area were subjected to statistical analysis.

Statistics

All values are expressed as mean ± SEM from at least three different determinations. For statistical analysis, Mann–Whitney and Wilcoxon tests were used to determine significance ($P < 0.05$). GraphPad Prism 5.03 was used to make statistical analysis.

RESULTS

Testosterone induces hypertrophy in primary cultured skeletal muscle myotubes. Among all effects of testosterone in skeletal muscle, hypertrophy is the hallmark of the physiological effects for the exogenously administrated hormone. Figure 1A shows that testosterone (100 nM) induced hypertrophy after 12 h, measured as increase in myotube CSA. Cell size increased by 35.24% (134.5 ± 10.21 μm basal vs 181.9 ± 14.41 μm testosterone). The number of nuclei per myotube after 12 h of testosterone stimulation showed no significant change (Fig. 1E). In agreement with the former result, a significant increase in sarcomeric α-actin mRNA (219.58% compared with control myotubes, $P < 0.05$) and α-actin protein levels (291.15% compared with control myotubes, $P < 0.05$) was obtained at 6 and 12 h, respectively (Fig. 1B and C). It has been observed that some hypertrophic stimuli act via inhibition of atrophy pathways, such as FoxO family of transcription factors and subsequent inhibition of "atrogenes" (atrogin-1/MAFbx and MuRF-1) (13). mRNA levels of atrogin-1/MAFbx and MuRF-1 did not reveal changes after 6 h of testosterone stimulation (Fig. 1D). To assess effects of testosterone on myotube differentiation process, 5-d-old myotubes

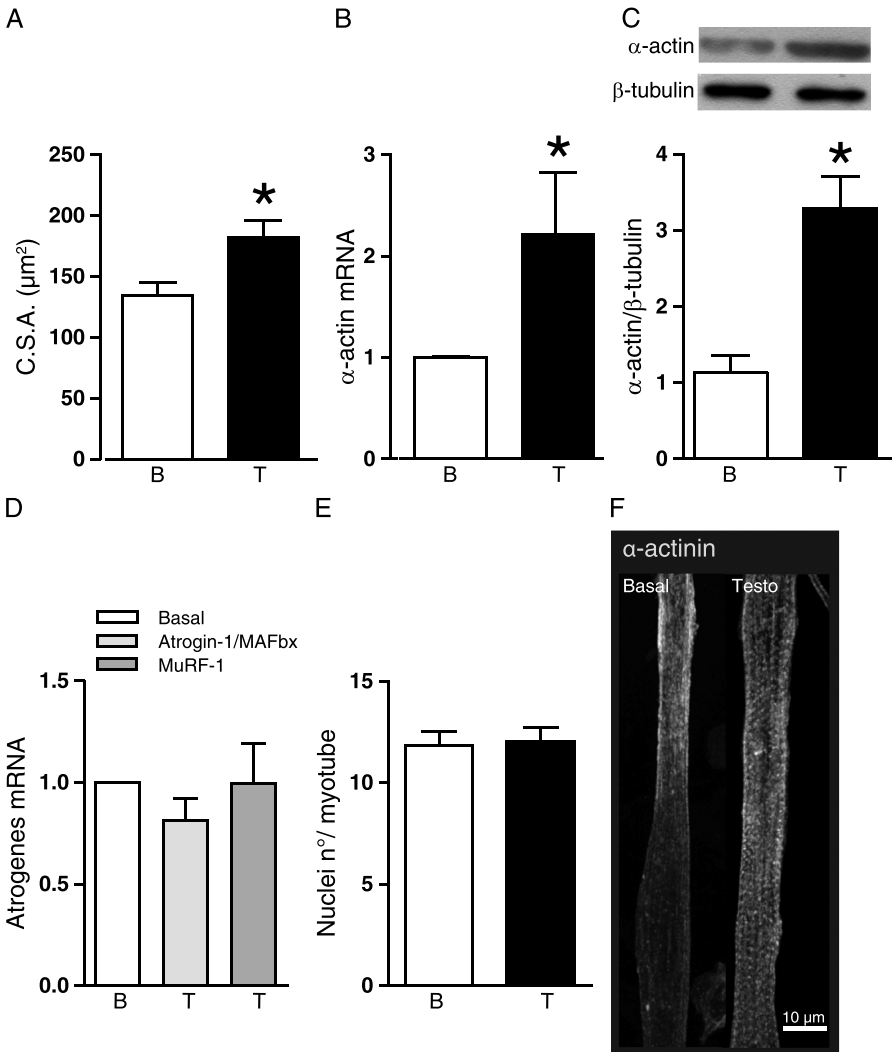

FIGURE 1—Testosterone induces hypertrophy in skeletal muscle myotubes. (A) CSA from myotubes were determined in basal conditions (B) or after 12 h of testosterone stimulation (100 nM, T) (n = 27 myotubes from three independent experiments). (B) α-Actin mRNA levels were evaluated after 6 h of testosterone by real-time PCR (n = 3 independent experiments). (C) α-Actin protein was determined by Western blot; β-tubulin is shown as a loading control (n = 4 independent experiments). (D) Atrogenes (atrogin-1/MAFbx and MuRF-1) mRNA levels were assessed in basal and after 6 h of testosterone stimulation (n = 4 independent experiments). (E) Myotubes grown on coverslips were stimulated or not with testosterone for 12 h. Myotube nuclei were identified by immunofluorescence against myogenin and different microscopic fields (40×) were counted. Data are expressed as number of nuclei per myotube. (F) α-Actinin was detected using anti–α-actinin with a conjugated secondary antibody (*green*). Cell sarcomerization was visualized in control and testosterone-stimulated myotubes. Values are mean ± SEM. $^{*}P < 0.05$ compared with basal value.

were stimulated with testosterone for 12 h, and sarcomerization was evaluated using immunofluorescence against α-actinin. A clear pattern of cross-striation was obtained in large areas of myotubes incubated with testosterone, whereas control myotubes show scarce regions that show a striation pattern along their structure (Fig. 1F).

Testosterone activates mTOR pathway. Ribosomal protein S6 kinase 1 (S6K1) is a main target for mTOR, and its activation has been directly related to hypertrophy (15,34). Testosterone induced gradual increases in S6K1 phosphorylation in myotubes, raising a peak of 3.5-fold over the basal value after 60 min of stimulation (Fig. 2A). ERK1/2 and phosphatidylinositol 3-kinase (PI3K)/Akt are potential upstream activators for mTOR (16). Testosterone (100 nM) induced an increase in phosphorylation of both ERK1/2 and Akt (Fig. 2B and C). Time course analysis for ERK1/2 phosphorylation shows a fast and transient peak at 5 min (Fig. 2B, $P < 0.05$ vs basal), whereas Akt phosphorylation displays an increase that peaked 15 min after testosterone stimulation, returning gradually to basal values (Fig. 2C, $P < 0.05$ vs basal). To test whether ERK1/2 or PI3K/Akt were responsible for S6K1 phosphorylation, we inhibited both pathways at different levels (Fig. 2D). Inhibition of ERK1/2 by use of PD-98059 still induced S6K1 phosphorylation at 60 min ($P < 0.01$ vs basal), whereas inhibition of Akt (Akt inhibitor VIII) abolished the S6K1 phosphorylation increases. PI3K inhibition (LY294002) diminished both basal and increased S6K1 phosphorylation induced by testosterone. As expected, rapamycin blocked testosterone-induced S6K1 phosphorylation (Fig. 2D).

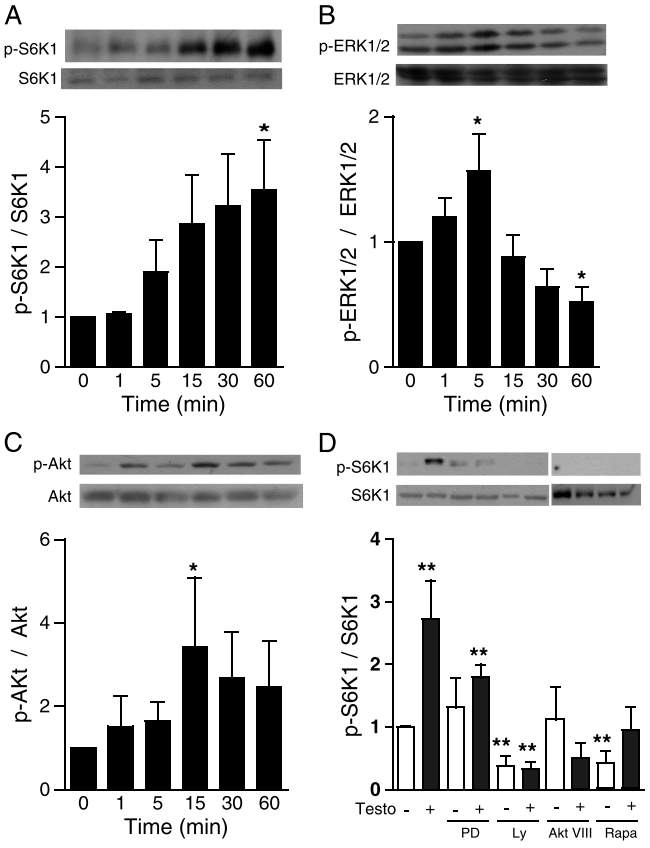

FIGURE 2—Testosterone increases S6K1 phosphorylation through PI3K–Akt. Skeletal muscle myotubes were stimulated with 100 nM testosterone for the indicated times. Phosphorylated protein levels were determined by Western blot from whole-cell lysates using specific antibodies against the active phosphorylated proteins. Next membranes were reprobed and normalized with their respective total protein. Densitometric analyses show the ratio of phosphorylated and total proteins. Testosterone increased the phosphorylation status of S6K1 (A), ERK1/2 (B) and Akt (C). D, S6K1 phosphorylation was analyzed at 60 min. Myotubes were preincubated with PD-98059 (PD), a MEK inhibitor; LY294002 (Ly), an inhibitor of PI3K; Akt inhibitor VIII (Akt VIII), an inhibitor of Akt; or rapamycin (Rapa), an inhibitor of mTORC1 complex. Inhibition of either PI3K or Akt blocked the increase in the S6K1 phosphorylation (n = 4). Values are mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with basal value.

PI3K/Akt and mTOR/S6K1 pathways, but not the canonical AR, are required to induce α-actin protein increases. Actin and myosin heavy chain are two sarcomeric proteins that account for the majority of the total myofibrillar protein mass, and it has been demonstrated that skeletal muscle hypertrophy selectively occurs through accumulation of myofibrillar rather than sarcoplasmic proteins (39). Considering this background, changes in α-actin protein levels were assessed to determine the role of PI3K/Akt or ERK1/2 in myotube hypertrophy induced by testosterone. Figure 3A shows that after 12 h, the inhibition of either PI3K/Akt or mTOR/S6K1 pathways blocked the increases in α-actin protein levels induced by testosterone, whereas ERK1/2 inhibition have no effect. The role of the intracellular AR was also checked on α-actin protein levels. We used bicalutamide, a nonsteroidal AR antagonist that blocks the classical effects of androgens (17). As shown in Figure 3B, the increase in α-actin protein levels was still observed after AR inhibition (Bic + T, $P < 0.05$ vs basal).

AR activation is required for myotube hypertrophy. The presence of the canonical AR was verified in skeletal muscle myotubes (Fig. 4). After testosterone stimulus during 12 h, a significant increase in the AR protein levels was observed (Fig. 4A, $P < 0.05$ vs basal). Nuclear

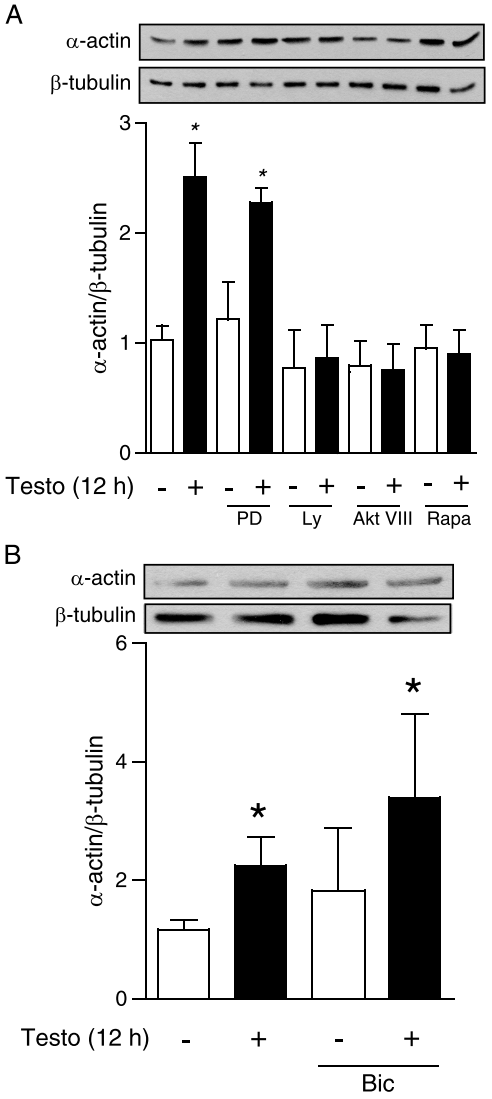

FIGURE 3—Testosterone increases α-actin protein via S6K1 signaling, independently of AR activation. Myotubes were stimulated with 100 nM testosterone for 12 h. (A) Myotubes were preincubated with PD-98059 (PD), a MEK inhibitor; LY294002 (Ly), an inhibitor of PI3K; Akt inhibitor VIII (Akt VIII), an inhibitor of Akt; or rapamycin (Rapa), an inhibitor of mTORC1 complex. Inhibition of PI3K, Akt, or mTOR, but not ERK1/2 inhibition, abolished the increase in α-actin protein levels induced by testosterone. (B) Bicalutamide, a competitive inhibitor of the intracellular AR, did not prevent the increase in α-actin levels induced by testosterone stimulation. α-Actin protein was determined by Western blot; β-tubulin is shown as a loading control. Values are mean ± SEM. $^{*}P < 0.05$ compared with basal value.

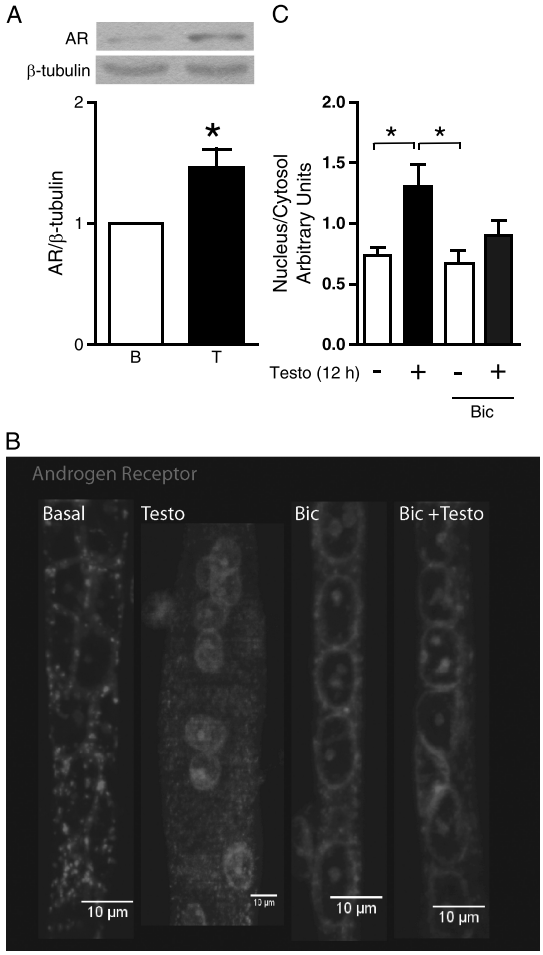

FIGURE 4—The AR is present and functional in skeletal muscle myotubes, and the AR inhibitor bicalutamide prevents its migration to the nucleus. Skeletal muscle myotubes were stimulated with 100 nM testosterone for the indicated times. (A) Western blot showing the increase in AR protein levels after 12 h of testosterone stimulation (*n* is at least five independent experiments). (B and C) Myotubes grown on coverslips were stimulated with testosterone 100 nM for 4 h. Immunofluorescences against the AR were made. B, Representative confocal stacks of every condition are shown. (C) Quantification of immunofluorescences against the AR (*n* = 4 independent experiments). The relation between AR stain in nuclei/cytoplasm was observed in basal and stimulated conditions, and with the AR inhibitor bicalutamide. Bicalutamide inhibits AR migration to the nucleus after 4 h. Values are mean ± SEM. $^{*}P < 0.05$ compared with basal value.

translocation of the AR after 4 h of testosterone stimulation was confirmed by immunofluorescence. Bicalutamide inhibited the translocation of the AR to the nucleus, a critical step for the activated AR (Fig. 4B and C).

To assess the role of the AR in testosterone-induced CSA increase, we inhibited the AR for 12 h. As shown in Figure 5B, the inhibition of the AR blocked the increase in CSA observed in testosterone-stimulated myotubes (Bic + T, $P < 0.01$ vs testosterone). Moreover, inhibition of PI3K and mTOR also blocked the CSA increase induced by testosterone (Fig. 5A, $P < 0.05$ vs testosterone). Collectively, these results indicate that AR and PI3K/Akt/mTOR activation mediated by testosterone are required to induce skeletal muscle myotube hypertrophy.

DISCUSSION

In this work, we have demonstrated that testosterone induces rapid activation of the mTOR/S6K1 pathway through PI3K/Akt, and this axis, together with the AR, induces hypertrophy in cultured skeletal muscle myotubes.

The mechanism of testosterone action to induce cell hypertrophy in skeletal muscle has long been hypothesized but not yet completely studied. To date, it has been established that testosterone has anabolic effects in human skeletal muscles, but the signaling pathways involved in this

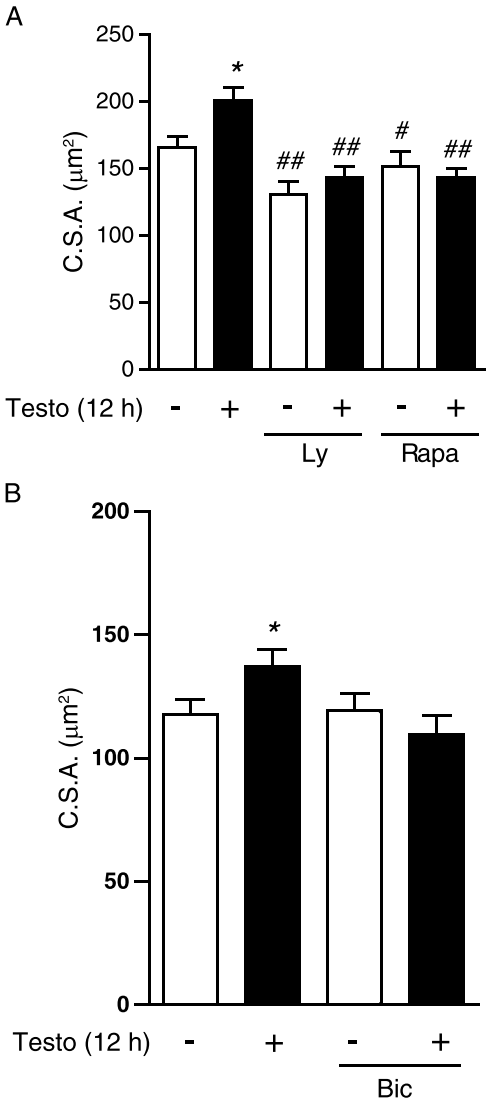

FIGURE 5—Interlink between AR and Akt–S6K1 activation in myotubes. Skeletal muscle myotubes were stimulated with 100 nM testosterone for 12 h in the presence of PI3K inhibitor, LY294002 (Ly); mTORC1 complex inhibitor, rapamycin (Rapa), or AR inhibitor bicalutamide (Bic) and changes in CSA of the myotubes were determined (*n* is at least three independent experiments). At a global cellular level, the inhibition of the nongenomic identified pathway as well as inhibition of intracellular AR abolished the increase in CSA induced by testosterone. Values are mean ± SEM. $^{*}P < 0.05$ compared with basal value, $^{**}P < 0.01$ compared with testosterone condition, $^{***}P < 0.001$ compared with testosterone condition.

effect have not been fully unraveled (10,19). Sculthorpe et al. (27) described in their work that in human skeletal muscle myotubes, testosterone induces an increase in the fusion index of the cells. This effect was paralleled by an increase in IGF-1 mRNA expression levels in testosterone-treated myotubes. This response was shown to be a concentration-dependent response. We have previously reported that testosterone (100 nM) induces cardiomyocyte hypertrophy in newborn rats (1). Moreover, our previous studies in skeletal muscle myotubes have demonstrated that testosterone, at two different concentrations (10 and 100 nM), induces fast and transient intracellular calcium signals. The proportion of cells responding to the hormonal stimulus was approximately 40% for 10 nM testosterone and 70% for 100 nM testosterone. In the same study, testosterone increased ERK1/2 phosphorylation in a concentration-dependent manner, which reached statistical significance at 100 nM and 1 μM (11). In spite of this previous evidence, testosterone with 100 nM concentration was selected for the present study.

A common event in hypertrophy is the activation of mTOR, a central regulator of protein synthesis that integrates several upstream signaling inputs, which converge in coordinated cellular response (38). ERK1/2 and Akt have been shown to activate mTOR during hypertrophy (1,21). In myotubes, it has been reported that testosterone rapidly activated these two different kinases (21,26). S6K1 and 4E-BP1 represent mTOR downstream targets and effectors (18), and S6K1 phosphorylation has been extensively used to evaluate mTOR activation (39). Studies in animals and humans have demonstrated a direct relationship between resistance exercise hypertrophy and S6K1 phosphorylation (2,31). In the current study, S6K1 was found to be activated upon testosterone stimulation, and this event was dependent on PI3K/Akt. The involvement of Akt and its downstream targets, GSK-3β, mTOR, S6K1, and 4E-BP1 in hypertrophy, has been studied in humans under resistance training. In human quadriceps, increase in phosphorylation of both Akt and mTOR were observed after 8 wk of training and correlated to resistance training-induced hypertrophy (21). Two recent publications addressing testosterone signaling in skeletal muscle cell lines also pointed to mTOR as a target of androgen signaling and its role in hypertrophy. Wu et al. (37) observed in an AR-transfected cell line (L6.AR cells) increased mTOR phosphorylation after 2 h of testosterone stimulation, a time lapse that was consistent with ERK1/2 and S6K1 phosphorylation but appear unrelated to Akt activation. For Akt activation, phosphorylation at both Thr308 and Ser473 is necessary. Phosphatidylinositol 3 kinase (PI3K) activation increases PIP3 and allows the activation of PDK1 (phosphoinositide-dependent protein kinase-1). Then, PDK1 is able to phosphorylate the Thr308 of a PIP3-bound Akt. For full activation of Akt kinase, a second phosphorylation event in a hydrophobic loop containing Ser473 is necessary (4). mTOR could phosphorylate Akt at Ser473, but this site is phosphorylated by rapamycin-insensitive mTOR complex 2 (Rictor associated). This phosphorylation improves Thr308 phosphorylation by PDK1 and represents a positive loop for pathway activation (25). In cardiac tissue, pro-hypertrophic stimuli can regulate mTORC1 signaling, mainly activating either the PI3K/Akt (20) or the MEK/extracellular signal-regulated kinase 1/2 (ERK1/2) pathways (24). Here, we determined that testosterone activates both ERK1/2 and Akt in cultured myotubes. However, PI3K/Akt, but not ERK, is necessary for the testosterone-induced mTORC1/S6K1 axis activation. Both ERK and Akt pathways are activated by testosterone with similar kinetics, but only PI3K and Akt inhibition (LY294002 and Akt inhibitor VIII, respectively) reduces testosterone-induced S6K1 phosphorylation. These data suggest that testosterone activates mTOR axis through PI3K/Akt axis. More recently, White et al. (35) demonstrated in C2C12 cells that 24 h of testosterone stimulation increased mTOR and S6K1 phosphorylation. At low testosterone concentrations, this event was independent of Akt activation, but at a higher concentration (500 nM testosterone), Akt phosphorylation was observed. The authors suggest that Akt activation may have a dose-dependent concentration threshold. Besides the different testosterone concentrations used in those studies, the discrepancies between our current data and these two cited works could be due to differences in the time evaluated; in our work, rapid non-genomic effects of testosterone were studied, so we looked for fast changes in the phosphorylation status of the putative hypertrophic pathways. The inhibition of PI3K–Akt–mTOR pathway resulted in the inhibition of S6K1 phosphorylation and myotube hypertrophy.

In this study, both α-actin protein and CSA increases induced by testosterone were abolished by mTOR inhibition, suggesting that this pathway is required to achieve hypertrophy. The "genomic" AR-dependent effect seems to be required for the global response because bicalutamide preserved the α-actin increase but inhibited the hypertrophy induced by testosterone. It has been described that the increase in protein synthesis can be mediated at different levels (before, after, and translational level) and, even more, that net increase in protein accumulation can be due to an increase in protein synthesis or a decrease in protein degradation (4). It has also been suggested that hypertrophy is generated when atrophy pathways are inhibited (11,22). Others have reported a down-regulation of the main genes associated to atrophy (atrogin-1/MAFbx and MuRF-1 mRNAs, known as atrogenes) when muscle cells have been treated with testosterone (23,40). In our study, atrogin-1/MAFbx and MuRF-1 mRNAs were not modified by testosterone stimulation. Pires-Oliveira et al. (23) showed that a treatment with 4 $mg \cdot kg^{-1}$ of testosterone during 7 d is able to reduce strongly atrogenes expression in *levator ani* muscle from castrated rats. Like ours, other reports did not observe any change in these genes expression when they treated rats with androgen for short periods (6 h). Zhao et al. (40) also reported a down-regulation in atrogin-1/MAFbx expression and promoter activity when they treated C2C12.AR (cells transfected with AR) with 50 nM testosterone overnight.

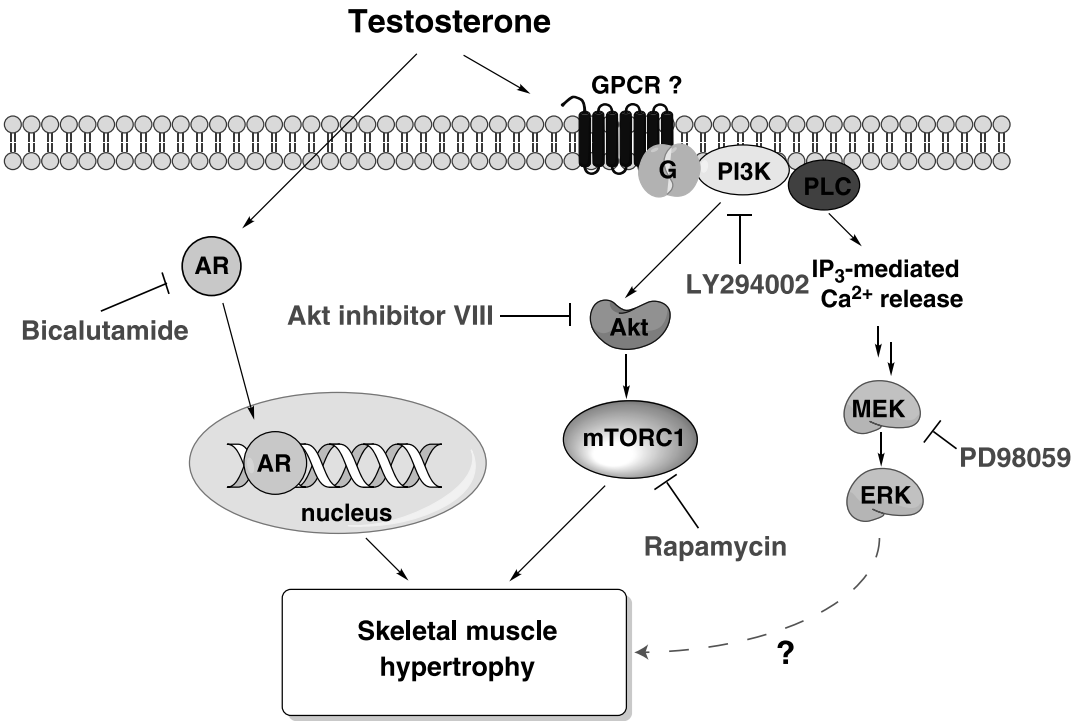

FIGURE 6—Scheme for hypertrophy effects of testosterone in skeletal muscle myotubes. Testosterone activates the genomic AR pathway and a putative membrane AR; Akt phosphorylation is triggered via PI3K. Activated Akt stimulates mTOR/S6K1 axis. These results indicate that activation of the intracellular AR as well as Akt/mTOR/S6K1 pathway represents an integrated step in the skeletal muscle hypertrophy response induced by testosterone.

Longer treatments with testosterone and improved sensitivity to testosterone effects due to overexpression of AR could explain the difference with our results. It would be important to study if longer treatment with testosterone is able to modify atrogenes expression in our model.

A recent work by White et al. (35) shows that when testosterone-stimulated myotubes were placed in conditions of testosterone withdrawal, mTOR and Akt signaling were amplified. Authors suggest this could represent a potential feedback mechanism regulated by testosterone. Whether the AR has a role in the generation of this feedback mechanism is still an open question. In our work, we identified a cooperative mechanism between nongenomic and genomic pathways where a rapid activation of the nongenomic Akt/mTOR/S6K1 pathway resulted to be necessary to induce increases in a specific sarcomeric protein and in cell size, and AR pathway complements the cell size regulation. We observed that the inhibition of AR did not affect a particular process such as an increase in the synthesis of a specific sarcomeric protein (α-actin), but it impacts when evaluating a whole-cell response such as changes in myotubes size. Thus, AR seems to be essential to coordinate the global hypertrophy response (11). There is evidence demonstrating that ERK1/2 can activate mTOR (24,33). In our work, the transient activation of ERK1/2 induced by testosterone was not directly related to the hypertrophy signaling cascade; however, activated ERK can influence the genomic response through a synergistic mechanism by phosphorylation of coactivators of the intracellular receptor at nuclear level as it has been reported for other intracellular steroid receptors (7). Finally, a scheme of the proposed Akt/mTOR-dependent action for testosterone in skeletal muscle cells is depicted (Fig. 6). It includes the genomic intracellular AR pathway and a putative membrane AR; Akt phosphorylation appears to be triggered via PI3K. Activated Akt stimulates mTOR/S6K1 axis. Taken together, our results indicate that activation of the intracellular AR as well as Akt/mTOR/S6K1 pathway represent an integrated step in the skeletal muscle hypertrophy response induced by testosterone

We can conclude that both signaling pathways (genomic and nongenomic) are necessary to elicit the myotube hypertrophy. It is feasible that key crosstalk can operate between the two identified pathways to ensure an orchestrated cellular response.

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