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Phosphorylation of p70^{s6} kinase is implicated in androgen-induced levator ani muscle anabolism in castrated rats

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Abstract

Androgens are known to increase muscle mass, strength and muscle protein synthesis. However, the molecular mechanisms by which androgens regulate skeletal muscle development remain poorly understood. The ribosomal protein kinase p70^{s6k} is a regulator of ribosome biogenesis and plays an important role in the regulation of growth-related protein synthesis. The phosphorylation of p70^{s6k} has been implicated in load-induced skeletal muscle hypertrophy. In the current study, we determined the effect of DHT on the phosphorylation of p70^{s6k} in the androgen-sensitive levator ani muscle of castrated rats. DHT induced a rapid increase in the phosphorylation of p70^{s6k}, which was detectable within 6 h after a single injection. Interestingly, DHT-induced phosphorylation of p70^{s6k} occurred only in androgen-sensitive muscles, but not prostate and seminal vesicle. Co-administration of flutamide, an AR antagonist, inhibited DHT-induced p70^{s6k} phosphorylation. While serum IGF-I levels were not changed by DHT treatment, IGF-I gene expression levels increased and the mRNA levels of IGFBP3 and IGFBP5 were suppressed in the LA muscle after DHT replacement in castrated rats. These results suggest that the phosphorylation of p70^{s6k}, likely via the IGF-I pathway, may play an important role in androgen-induced skeletal muscle hypertrophy.

Keywords: Androgen; p70^{s6k}; Levator ani; IGF-I

1. Introduction

Androgens are essential for the development and maintenance of male sex characteristics and other tissue and organs such as bone, muscle, brain, liver and kidney [1]. Androgen deficiency is associated with loss of fat-free mass and a decrease in fractional muscle protein synthesis [2]. Conversely, administration of replacement doses of testosterone to hypogonadal men [3–5] and of supraphysiological doses to eugonadal men [6,7] increased fat-free mass, muscle size and strength. These anabolic effects are associated with increases in muscle protein synthesis rates [4,7–10] and accretion [11–13] as well as increased satellite cell proliferation and DNA accumulation [14–16]. The effects of androgens are believed to be mediated through the androgen receptor (AR), a 110 kDa nuclear receptor that regulates the expression of target genes through binding to an androgen response element [17]. However, the molecular mechanisms underling androgen-mediated muscle hypertrophy remain poorly understood.

Recent studies have demonstrated that testosterone administration increased muscle protein synthesis with a concomitant increase in IGF-I mRNA levels in elderly men [8]. Conversely, androgen deficiency caused a decrease in muscle IGF-I expression [2]. These findings suggest the possibility that the IGF-I pathway may play a role in androgen-mediated anabolic effect in muscle. The 70 kDa S6 ribosomal protein kinase is a down-stream effector of the insulin/IGF-I pathway [18], and is known to phosphorylate the S6 ribosomal protein on a number of residues. Phosphorylation of these residues results in a selective increase in the translation of mRNAs

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containing a terminus of polypyrimidine tract (TOP) [19]. Most of these TOP-containing transcripts are known to encode components of the translational apparatus such as elongation factors and ribosomal proteins [20] and therefore may play an important role in translational regulation and protein synthesis.

Indeed, increasing evidence indicates that $p70^{s6k}$ is an important regulator of cellular growth. $p70^{s6k}$ knockout mice exhibited a reduced body and organ size [21]. Several recent reports have shown that agents that induce muscle hypertrophy can cause acute activation of $p70^{s6k}$ [22–24]. The activation of $p70^{s6k}$ has been shown to participate in the regulation of protein synthesis in skeletal muscle by insulin and IGF-I [25]. In L6 myoblast culture, rapamycin, a drug that blocks the phosphorylation of $p70^{s6k}$ through mammalian target of rapamycin (mTOR) kinase, eliminated insulin-stimulated increase in protein synthesis [26]. Furthermore, a recent study has demonstrated that a single bout of growth-inducing electrical stimulus caused a prolonged increase in $p70^{s6k}$ phosphorylation, which correlates closely with skeletal muscle hypertrophy [27]. Taken together, these findings lead to an attractive speculation that $p70^{s6k}$ may play a key role in muscle growth.

In the present study, we addressed the question whether phosphorylation of $p70^{s6k}$ is involved in mediating the effect of androgens on muscle hypertrophy. We investigated the effect of dihydrotestosterone (DHT) on $p70^{s6k}$ phosphorylation in the levator ani muscle of castrated rats. The results from this study have demonstrated that androgens induced rapid phosphorylation of $p70^{s6k}$ in levator ani in a dose-dependent manner. In addition, we have shown that DHT increased IGF-I mRNA levels and inhibited the expression of IGF binding proteins in the LA muscle. These results suggest that androgens may induce muscle hypertrophy in part through the IGF-I/$p70^{s6k}$ signaling pathway.

2. Materials and methods

2.1. Materials

DHT, flutamide, *N*,*N*-dimethylacetamide (DMAC), 0.2% Tween 80 and 0.25% carboxymethyl cellulose were purchased from Sigma. The following reagents were from ICN: sodium fluoride, EDTA, EGTA, benzamidine, phenylmethylsulfonyl fluoride (PMSF) and leupeptin hemisulfate. BCA reagent was purchased from Pierce. Polyvinylidene difluoride (PVDF) membranes and 7.5% polyacrylamide gels were from Bio-Rad. The $p70^{s6k}$ antibodies were from Santa Cruz and peroxidase-conjugated donkey anti-rabbit IgG antibody was from Jackson ImmunoResearch. ECL plus kit was purchased from Amersham. DHT was prepared in a solution containing 10% DMAC, 0.2% Tween 80, 0.25% carboxymethyl cellulose. Flutamide was prepared in a solution containing 8% ethanol, 1% gelatin and 0.9% NaCl.

2.2. Animals

Male Sprague–Dawley orchidectomized (ORX) or sham-operated immature rats weighing around 150 g were purchased from Charles River. Animals were kept under controlled environmental conditions with 12 h light–dark cycle and free access of food and water and were treated according to IACUC regulations. The ORX animals were randomly divided into three groups. They began to receive daily subcutaneous injections (2 ml/kg bodyweight) at 10 days after the surgery with the following treatments: (1) vehicle; (2) 0.3 mg/kg DHT; (3) 3.0 mg/kg DHT. The sham-operated animals received vehicle only. Animals were euthanized by CO_2 at 6, 24 or 96 h after the first injection. Blood samples were collected at necropsy. Serum IGF-I levels were determined using a rat IGF-I ELISA kit (DRG International). After necropsy, perineal muscles (levator ani and bulbocavernosus), ventral prostate, and seminal vesicle were removed. The weights of the excised tissues were measured immediately after necropsy. In some experiments, castrated rats were injected subcutaneously with DHT (3 mg/kg), flutamide (120 mg/kg) alone or flutamide plus 3.0 mg/kg DHT and levator ani was collected at 24 h after the treatment. Wet tissues were immediately frozen in liquid nitrogen after weight measurement.

2.3. $p70^{s6k}$ Western blot analysis

The excised tissues were homogenized with Polytron in a lysis buffer containing 50 mM Tris–HCl (pH 7.4), 100 mM sodium fluoride, 10 mM EDTA, 2 mM EGTA, 1 mM benzamidine, 0.2 mM PMSF, and 10 μg/ml leupeptin hemisulfate. The homogenate was centrifuged at 500 × *g* for 30 min. The supernatant containing $p70^{s6k}$ was centrifuged for additional 30 min at 5000 × *g*. BCA reagent kit (Pierce) was used for protein assay and 15 μg protein was separated on a 7.5% polyacrylamide/Tris–HCl gel (Bio-Rad) at 70 V for 2–3 h. After electrophoretic separation, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) at 200 mA for 2 h at 4 °C. The membrane was incubated in a 10% Blotto (10% powdered milk in 1× Tris-buffered saline) solution for 1 h at room temperature (RT). The $p70^{s6k}$ antibodies (Santa Cruz) were diluted in 1% milk at 1:200 (p70, p-p70 T424/S421) and incubated for 1 h at RT. After wash, peroxidase-conjugated donkey anti-rabbit IgG secondary antibody (1:10,000) (Jackson ImmunoResearch) was added and incubated for 45 min at RT. Antibody binding was detected using an ECL plus kit (Amersham). The level of $p70^{s6k}$ phosphorylation was quantified using a Bio-Rad Fluor-s MultiImager and Quantity One software.

2.4. Quantitative RT-PCR

Total RNA was extracted from levator ani and bulbocavernosus muscles using RNeasy mini kit (QIAGEN) following the instructions provided by the manufacturer, and

then treated with DNase I (Ambion). cDNA was synthesized from 2 μg of total RNA using a high capacity cDNA archive kit (ABI). An aliquot of cDNA preparation containing 50 ng cDNA was mixed with Sybr green PCR master mix (ABI) and gene-specific primers (300 nM) in a 50 μl volume. Gene-specific forward (F) and reverse (R) primers (Biosource) were: IGF-I (F, GCTGTGCAGTTCGCCCATT; R, GGTATGCTAGGAGCCTGGGTAAA); IGFBP3 (F, CGTGGAGCTCAAATACGCCTTA; R, AGCCAGCTGCTGATCACGTT); IGFBP5 (F, TTCACAGGCTCTGACCTCCTC; R, TGACTAGGGACCAGAGAAGTGGG); 18S (F, CATTCTTGGCAAATGCTTTCG; R, CGCCGCTAGAGGTGAAATTCT). Real-time PRC was performed and analyzed using an ABI Prism 7000 sequence detection system. The mRNA levels were calculated as: $10^{-(Ct-40)/3.35}$, and normalized with 18S rRNA from the same cDNA preparation. Gene expression levels were expressed as ratio of DHT-treated or sham vehicle versus castrated vehicle control samples.

2.5. Statistical analysis

Results were expressed as the means ± S.E. One-way ANOVA was performed followed by Dunnetts's tests using JMP software. The level of statistical significance was set at $P < 0.05$.

3. Results

3.1. Anabolic and androgenic activities of DHT

An assay for anabolic activity of androgens was developed by Eisenberg and Gordan [28] and later modified by Hershberger et al [29]. The levator ani muscle was selected as the anabolic target tissue and prostate or seminal vesicle as androgenic target tissues because they were almost completely atrophied after castration and restored on administration of androgen. This assay, with minor modifications, has been used extensively. In the present study, the androgen administration was begun 10 days after castration and continued for 4 days. As expected, castration induced significant atrophies of the perineal muscles, ventral prostate and seminal vesicle (Fig. 1). Administration of DHT increased the wet weight of the levator ani and bulbocavernosus as well as ventral prostate and seminal vesicle in a dose-dependant manner. When the high dose of DHT was given to castrated rats, the atrophied levator ani underwent rapid growth and the wet weight was restored to levels similar to the sham control after 4 days of treatment (Fig. 1). Similarly, ventral prostate wet weight was restored to the sham level in the high DHT-dose group.

3.2. DHT-induced $p70^{s6k}$ phosphorylation in perineal muscles

Since $p70^{s6k}$ is an important regulator of cellular growth, we hypothesized that androgens may stimulate muscle growth by inducing $p70^{s6k}$ phosphorylation. Phosphorylation of $p70^{s6k}$ occurs in a hierarchical manner commencing with phosphorylation of carboxyl-terminal sites localized within the presumptive autoinhibitory pseudosubstrate domain [30]. These phosphorylation events have been suggested to transform the conformation of the protein so as to disrupt a putative interaction between the autoinhibitory region and the substrate recognition pocket. As a result, additional internal phosphorylation sites are exposed to activating kinases. In this study, the phosphorylation sites threonine-421 (T421) and serine-424 (S424) located within the autoinhibitory domain were examined using antibodies specific for these sites. The phosphorylation of $p70^{s6k}$ was significantly decreased in the levator ani after castration (Fig. 2). Administration of DHT to the castrated rats induced a rapid increase in $p70^{s6k}$ phosphorylation within 6 h. The $p70^{s6k}$ phosphorylation continued to increase at 24 h after a single injection of DHT with levels reaching above the sham control. These increases in $p70^{s6k}$ phosphorylation were maintained over a 4-day period when daily administration of DHT to the castrated rats continued. Interestingly, the total $p70^{s6k}$ protein levels were not changed over the entire 4-day experiment period, indicating that the increases in $p70^{s6k}$ phosphorylation occurred independently of changes in the total $p70^{s6k}$ levels. Similar to the changes in wet weight of levator ani in response to different doses of DHT, the increases in $p70^{s6k}$ phosphorylation were also dose-dependent (Fig. 3).

To determine if this androgen-induced $p70^{s6k}$ phosphorylation also occurs in other androgen-sensitive tissues, the phosphorylated $p70^{s6k}$ was measured in prostate and seminal vesicle as well as perineal muscles from castrated rats treated with 3 mg/kg DHT for 4 days. The phosphorylation of $p70^{s6k}$ was induced in the androgen-sensitive muscles, but not in prostate and seminal vesicle (Fig. 4), indicating that this effect is tissue-specific.

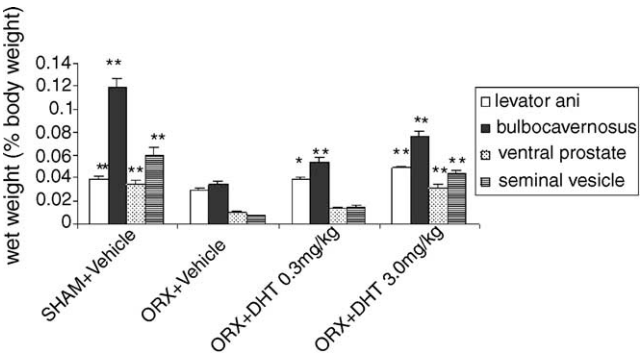

Fig. 1. Effect of castration and administration of DHT on the wet weights of levator ani, bulbocavernosus, ventral prostate and seminal vesicle. Rats were orchidectomized (ORX) or sham-operated and daily subcutaneous injections of vehicle or DHT started 10 days later. Animals were sacrificed after 4 days of treatment and wet tissue weights were recorded. Data are expressed as means ± S.E., $n = 8$. Significantly different from ORX animals treated with vehicle: $^{*}P < 0.05$, $^{**}P < 0.01$.

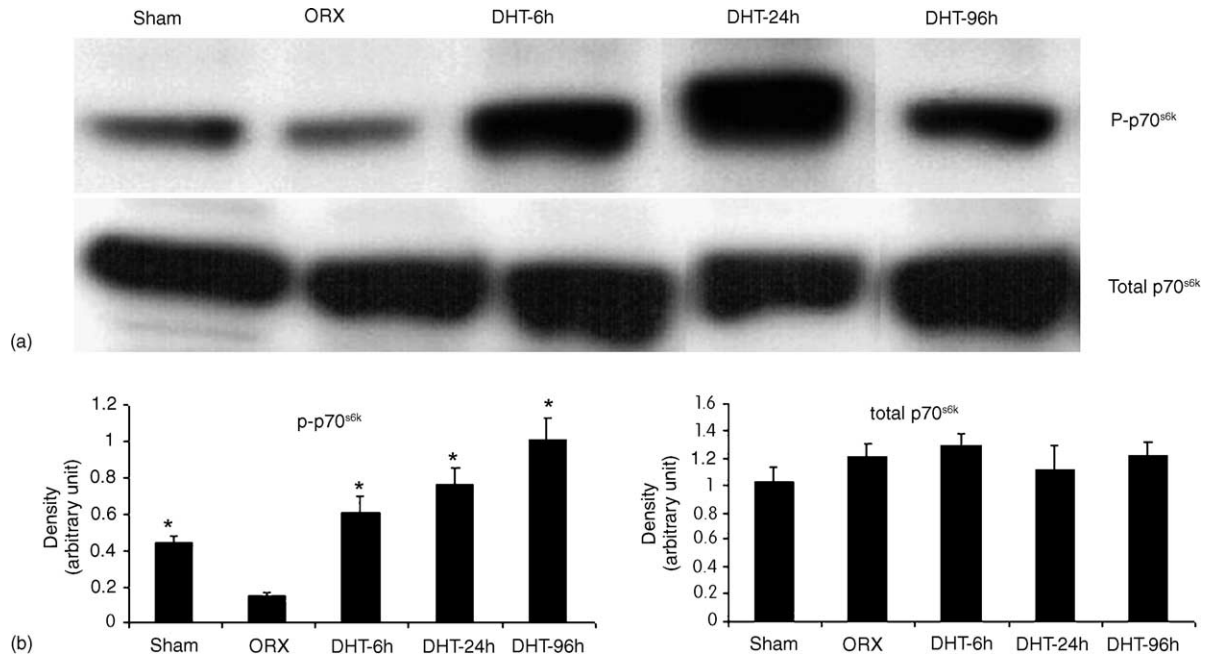

Fig. 2. Phosphorylation of p70^{s6k} in the levator ani muscle. Animals were treated with DHT (3 mg/kg) and sacrificed at the time point as indicated in the figure. Total and phosphorylated p70^{s6k} was detected by Western blot and the levels were quantified using Bio-Rad Fluor-s MultiImager as described in Section 2. (a) Representative autoradiogram of a Western blot. (b) Graphs of the densities of p-p70^{s6k} and total p70^{s6k} (means ± S.E., $n = 4$). Significant difference between castrated (ORX) controls and treatment group or sham animals: $^{*}P < 0.01$.

3.3. AR antagonist blocks DHT-induced p70^{s6k} phosphorylation

To determine if the effect of DHT on p70^{s6k} phosphorylation was mediated through AR, flutamide, an AR antagonist, which has been shown to effectively ablate testosterone-induced growth of levator ani and prostate in immature rats [31], was used in the subsequent study. Flutamide (120 mg/kg) was co-administered with DHT (3 mg/kg) to rats through subcutaneous injections. Phosphorylated p70^{s6k} levels were determined 24 h post-injection. As shown in Fig. 5, castrated rats had very low levels of phosphorylated

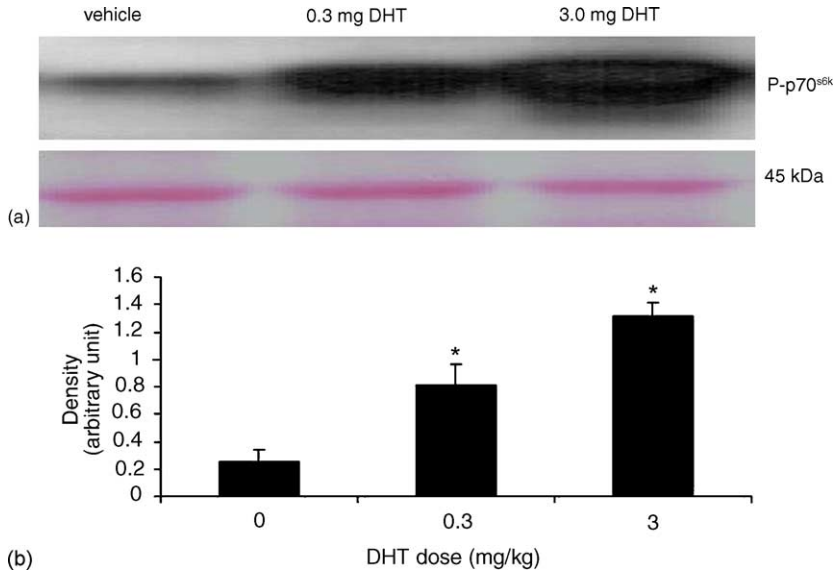

Fig. 3. Dose-dependent response of p70^{s6k} phosphorylation in castrated rats treated with DHT. Animals received subcutaneous injections with vehicle or DHT (0.3 and 3.0 mg/kg) and sacrificed at 24 h post-injection. The phosphorylated p70^{s6k} was detected by Western blot and quantified as described in Section 2. The 45 kDa protein bands after staining the membrane with Ponceu S showed the equal loading of proteins on the gel. (a) Representative autoradiogram of a Western blot. (b) Graph of p-p70^{s6k} density (means ± S.E., $n = 3$). Significant increases in p70^{s6k} phosphorylation between vehicle control and treated groups: $^{*}P < 0.05$.

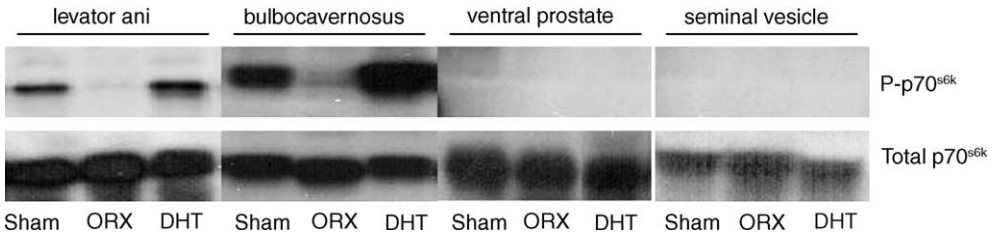

Fig. 4. Effect of DHT on p70^{s6k} phosphorylation in perineal muscles, ventral prostate and seminal vesicle. Castrated animals were treated with DHT (3 mg/kg) for 4 days and androgen-sensitive muscle (levator ani and bulbocavernosus) and non-muscle tissues (ventral prostate and seminal vesicle) were collected for Western analysis for total and phosphorylated p70^{s6k} as described in Section 2. Representative autoradiograms of Western blots obtained from different tissues are shown.

p70^{s6k} as compared to the sham group. Administration of flutamide alone to castrated rats did not have any effect on p70^{s6k} phosphorylation. When co-administrated with DHT, flutamide completely blocked the increase in p70^{s6k} phosphorylation induced by the AR agonist.

3.4. Coordinated regulation of IGF-I and IGFBPs by DHT in perineal muscles

The p70^{s6k} has been identified as a down-stream effector of the IGF-I/PI3K signaling pathway [18]. To investigate if IGF-I systems may be involved in the androgen-induced p70^{s6k} phosphorylation in the perineal muscles, serum IGF-I levels and the mRNA levels of IGF-I, IGFBP3 and IGFBP5 in the perineal muscles were determined in rats treated with DHT for 4 days. There were no significant differences in serum IGF-I concentrations among different groups (sham, 1219 ± 88; ORX, 987 ± 80; 0.3 mg DHT, 1023 ± 57; 3.0 mg DHT, 1038 ± 70 ng/ml; data are means $\pm$ S.E., $n = 8$). However, the expression levels of IGF-I in both muscles were decreased after castration (Fig. 6). Administration of DHT to the castrated rats increased IGF-I mRNA levels by two-fold in both muscles. Interestingly, there were concomitant changes in the expression levels of IGFBP3 and IGFBP5. In comparison with the sham, castration caused a 30% and 170% increase in IGFBP5 mRNA levels in the levator ani and bulbocavernosus, respectively. Administration of DHT to the castrated rats led to dramatic suppression of IGFBP5 expression in both muscles. Similarly, androgen deficiency induced a 170% and 470% increase in IGFBP3 expression in levator ani and bulbocavernosus, respectively. DHT treatment reduced their expression to 3% of the control levels in both muscles.

4. Discussion

The androgen-dependent levator ani muscle of the rat provides a suitable model to explore the molecular mechanism of steroid hormone action in the target tissue. As expected, androgen replacement caused rapid growth of LA muscle. A chief component of the cell growth response is the genera-

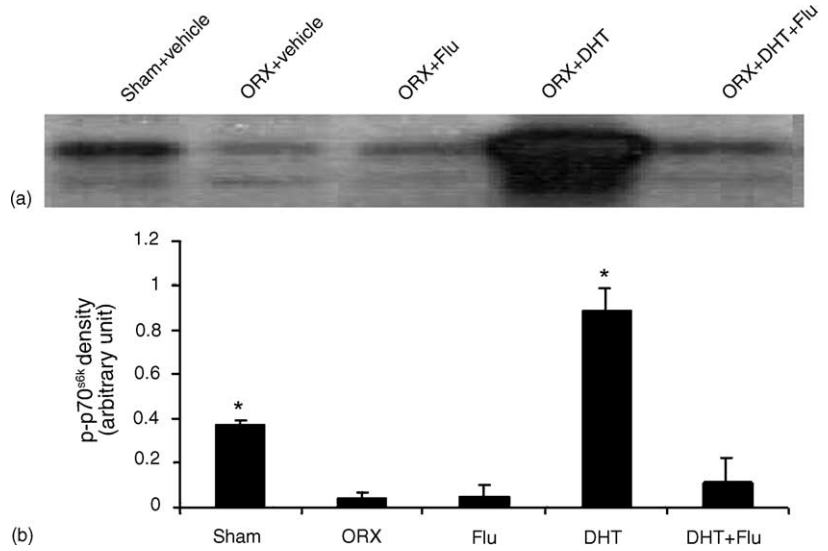

Fig. 5. Inhibition of DHT-induced p70^{s6k} phosphorylation by flutamide. Castrated rats received a single subcutaneous injection with vehicle, flutamide (120 mg/kg), DHT (3.0 mg/kg), or DTH plus flutamide. Levator ani muscle was collected 24 h later and phosphorylated p70^{s6k} levels were determined by Western blot and quantified as described in Section 2. (a) Representative autoradiogram of a Western blot. (b) Graph of p-p70^{s6k} density (means $\pm$ S.E., $n = 3$). Significant increases in p70^{s6k} phosphorylation between vehicle control and treated groups: $^{*}P < 0.05$.

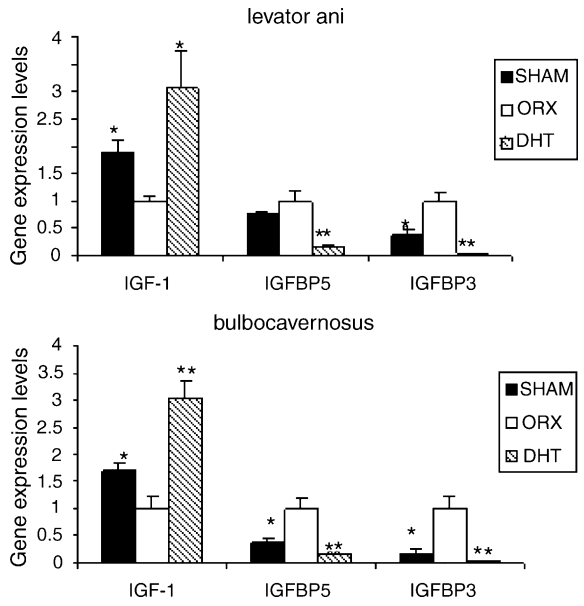

Fig. 6. Coordinated regulation of IGF-I and IGFBPs by androgens in the perineal muscles. Castrated animals received once daily injection of either vehicle or 3 mg/kg DHT for 4 days. Total RNA was isolated from the perineal muscles and gene expression levels were determined as described in the material and methods. Data are means ± S.E., $n = 4$. Significant difference between ORX and sham or DHT: $^{*}P < 0.05$, $^{**}P < 0.01$.

tion of new translational machinery, which is essential for the increased demand of the cell to perform an immense array of distinct anabolic processes. In light of the findings from recent studies that the p70^{s6k} signaling pathway plays a critical role in cell growth by modulating the translation of TOP mRNA, we hypothesized that androgens may regulate muscle growth through the p70^{s6k} pathway. We have demonstrated here that DHT induced p70^{s6k} phosphorylation, a process essential for its activation. Thus, androgens might cause increases in p70^{s6k} activity with subsequent changes in protein synthesis in skeletal muscle. Interestingly, this effect seems to be perineal muscle-specific as p70^{s6k} phosphorylation was not induced by DHT in prostate and seminal vesicle. Thus, it appears that the androgenic activity of DHT may be independent of p70^{s6k} activation. The increases in p70^{s6k} phosphorylation have been noted in skeletal muscle in response to a variety of other myotrophic stimuli [23,27]. Under those conditions, the phenotypic changes are associated with increased protein synthesis or rate of translation initiation. Boissonneault et al. [32] have shown that protein synthesis was decreased in androgen-deficient rats due to repressed translation capacity. This condition was associated with a 20% decrease in the LA polyribosome yield following castration [33], indicating that ribosome biogenesis might be altered in response to androgen deficiency. Our findings that p70^{s6k} phosphorylation is decreased in castrated animals and androgen replacement induced rapid increase in p70^{s6k} phosphorylation may provide a mechanistic explanation for the reduction in LA polyribosome in castrated rats. It is conceivable that androgen may stimulate ribosome biogenesis through the p70^{s6k} pathway and consequently increase protein synthesis in levator ani muscle.

It remains unclear how androgens regulate p70^{s6k} activity. One possibility is that AR exerts transcriptional regulation of relevant genes that modulate p70^{s6k} activity. Since p70^{s6k} is a down-stream effector of IGF-I signaling pathways, we further investigated whether the IGF-I system is activated in response to androgen treatment. In this study, we did not find significant changes in the circulating IGF-I levels between sham and castrated rats. Administration of a high dose of DHT for 4 days also did not change serum IGF-I concentrations though levator ani mass was restored to sham levels, suggesting that circulating IGF-I does not play any significant role in the anabolic effect of androgens on the androgen-sensitive muscles. However, the muscle IGF-I mRNA levels were significantly elevated in response to androgen treatment. Interestingly, the muscle mRNA levels of both IGFBP3 and IGFBP5 were dramatically suppressed by DHT in the castrated rats. Alterations in the expressions of IGF-I and its binding proteins have also been reported previously in the skeletal muscle of hypogonadal men or healthy elderly men treated with testosterone [2,8,13]. The IGFBPs function not only as carrier proteins for the IGFs, but also modulators of IGF action. Recent studies have demonstrated that IGFBP5 secreted by C2 myoblasts inhibited IGF-I induced muscle differentiation [34,35]. IGFBP5 also inhibited proliferation and differentiation of L6A1 myoblasts when added in the presence of IGF-II [36]. IGFBP3, the most abundant IGFBP in the circulation, is also active in the cellular environment as a potent antiproliferative agent [37]. This coordinated up-regulation of IGF-I and suppression of IGFBPs by androgens is likely to lead to the activation of the IGF-I signaling pathway and induction of p70^{s6k} phosphorylation in the perineal muscles. Interestingly, the muscle hypertrophy induced by clenbuterol was also associated with local increases in muscle IGF content and p70^{s6k} phosphorylation [23,38]. Thus, IGF-I/p70^{s6k} may be a common signaling pathway activated by myotrophic agents.

Another hypothesis to explain the DHT-induced p70^{s6k} phosphorylation is that androgens may interfere with the inhibitory effect of glucocoticoids on p70^{s6k} activity. Glucocorticoids have been shown to inhibit insulin-induced p70^{s6k} phosphorylation in rat skeletal muscle [39]. The T421/S424 residues located in the autoinhibitory pseudosubstrate domain of p70^{s6k} are the primary targets of glucocorticoid action. This autoregulatory region has been speculated to confer glucocorticoid sensitivity [40]. The finding from the current study that androgens induce phosphorylation at the same site as glucocorticoids, raised the possibility that androgens may increase p70^{s6k} phosphorylation by acting as glucocorticoid antagonists. There is some indirect evidence that the anabolic effects of testosterone and other androgenic–anabolic agents on skeletal muscle may be mediated through antiglucocorticoid action [41,42]. In vitro study has demonstrated that testosterone can bind, albeit at low affinity, to glucocorticiod receptors [43]. Such low-affinity binding would not be

effective unless extremely high doses of androgens were used. In the current study, however, a low dose of DHT (0.3 mg/kg) was able to induce $p70^{s6k}$ phosphorylation. Thus, it is unlikely that DHT-induced $p70^{s6k}$ phosphorylation is mediated through direct binding of androgen to GR. This notion is further strengthened by the observation that AR antagonist blocked the DHT-induced $p70^{s6k}$ phosphorylation, indicating this effect is mediated through binding to AR. However, it is still possible that androgens inhibit glucocorticoid activity at the gene levels by interfering with the binding of GR to GREs in the glucocorticoid-responsive target genes [44], because DNA binding and transcriptional activation are essential for GR-mediated $p70^{s6k}$ inactivation [40].

Androgens may also regulate $p70^{s6k}$ activity through AR-mediated protein-to-protein interactions. AR has been found to interact with a number of transcription factors including AP-1, Smad3, NFκB, and the Ets family of transcription factors. AR also interacts with cdk7, a catalytic subunit of TFIIH, and PITALRE, a kinase subunit of positive elongation factor b [45]. These interactions may be important for the transcriptional activity of AR. Members of the SRC family of coactivators (SRC-1, SRC-3, and TIF-2) typically interact with the ligand binding domain of nuclear receptors through LXXLL motifs. The LXXLL domains of the coactivators interact with the hydrophobic surface of the receptor AF-2 domain [46]. Interestingly, a LXXLL motif (residue 324–328) is present in the $p70^{s6k}$. Whether AR directly interacts with $p70^{s6k}$ via LXXLL motif is an intriguing question to be addressed in the future.

In summary, we have shown for the first time that DHT induces $p70^{s6k}$ phosphorylation in rat perineal muscles. The phosphorylation of $p70^{s6k}$ induced by AR agonists may play a role in androgen-stimulated muscle hypertrophy and may be used as a potential biomarker to predict the anabolic effect of AR ligands on muscle mass.

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References

[1] M. Sheffield-Moore, Androgens and the control of skeletal muscle protein synthesis, Ann. Med. 32 (2000) 181–186.
[2] N. Mauras, V. Hayes, S. Welch, A. Rini, K. Helgeson, M. Dokler, J.D. Veldhuis, R.J. Urban, Testosterone deficiency in young men: marked alterations in whole body protein kinetics, strength, and adiposity, J. Clin. Endocrinol. Metab. 83 (1998) 1886–1892.
[3] S. Bhasin, T.W. Storer, N. Berman, K.E. Yarasheski, B. Clevenger, J. Phillips, W.P. Lee, T.J. Bunnell, R. Casaburi, Testosterone replacement increases fat-free mass and muscle size in hypogonadal men, J. Clin. Endocrinol. Metab. 82 (1997) 407–413.
[4] I.G. Brodsky, P. Balagopal, K.S. Nair, Effects of testosterone replacement on muscle mass and muscle protein synthesis in hypogonadal men—a clinical research center study, J. Clin. Endocrinol. Metab. 81 (1996) 3469–3475.
[5] S. Bhasin, L. Woodhouse, R. Casaburi, A. Singh, D. Bhasin, N. Berman, X. Chen, K.E. Yarasheski, L. Magliano, C. Dzekov, J. Dzekov, R. Bross, J. Phillips, I. Sinha-Hikim, R. Shen, T.W. Storer, Testosterone dose–response relationships in healthy young men, Am. J. Physiol. Endocrinol. Metab. 281 (2001) E1172–E1181.
[6] S. Bhasin, T.W. Storer, N. Berman, C. Callegari, B. Clevenger, J. Phillips, T.J. Bunnell, R. Tricker, A. Shirazi, R. Casaburi, The effect of supraphysiologic doses of testosterone on muscle size and strength in normal men, N. Engl. J. Med. 335 (1996) 1–7.
[7] R.C. Griggs, W. Kingston, R.F. Jozefowicz, B.E. Herr, G. Forbes, D. Halliday, Effects of testosterone on muscle mass and muscle protein synthesis, J. Appl. Physiol. 66 (1989) 498–503.
[8] R.J. Urban, Y.H. Bodenburg, C. Gilkison, J. Foxworth, A.R. Coggan, R.R. Wolfe, A.A. Ferrando, Testosterone administration to elderly men increases skeletal muscle strength and protein synthesis, Am. J. Physiol. 269 (1995) 820–826.
[9] A.A. Ferrando, K.D. Tipton, D. Doyle, S.M. Phillips, J. Cortiella, R.R. Wolfe, Testosterone injection stimulates net muscle protein synthesis but not amino acid transport, Am. J. Physiol. 275 (1998) E846–E871.
[10] M. Sheffield-Moore, R.J. Urban, S.E. Wolf, J. Jiang, D.H. Catlin, D.N. Herndon, R.R. Wolfe, A.A. Ferrando, Short-term oxandrolone administration stimulates net muscle protein synthesis in young men, J. Clin. Endocinol. Metab. 84 (1999) 2705–2711.
[11] C.D. Kochakian, Comparison of protein anabolic properties of various androgens in the castrated rat, Am. J. Physiol. 60 (1950) 553–558.
[12] C.D. Kochakian, Effect of male hormone on protein metabolism of castrated dogs, Proc. Soc. Exp. Biol. Med. 32 (1935) 1064–1065.
[13] A.A. Ferrando, M. Sheffield-Morre, C.W. Yeckel, C. Gilkison, J. Jiang, A. Achacosa, S.A. Lieberman, K. Tipton, R.R. Wolfe, R.J. Urban, Testosterone administration to older men improves muscle function: molecular and physiological mechanisms, Am. J. Physiol. Endocrinol. Metab. 282 (2002) E601–E607.
[14] I. Sinha-Hikim, S.M. Roth, M.I. Lee, S. Bhasin, Testosterone-induced muscle hypertrophy is associated with an increase in satellite cell number in healthy, young men, Am. J. Physiol. Endocrinol. Metab. 285 (2003) E197–E205.
[15] Y. Joubert, C. Tobin, Testosterone treatment results in quiescent satellite cells being activated and recruited into cell cycle in rat levator ani muscle, Dev. Biol. 169 (1995) 286–294.
[16] Y. Joubert, C. Tobin, Satellite cell proliferation and increase in the number of myonuclei induced by testosterone in the levator ani muscle of the adult female rats, Dev. Biol. 131 (1989) 550–557.
[17] F. Claessens, G. Verrijdt, E. Schoenmakers, A. Haelens, B. Peeters, G. Verhoeven, W. Rombauts, Selective DNA binding by the androgen receptor as a mechanism for hormone-specific gene regulation, J. Steroid Biochem. Mol. Biol. 76 (2001) 23–30.
[18] J. Avruch, Insulin signal transduction through protein kinase cascades, Mol. Cell. Biochem. 182 (1998) 31–48.
[19] H.J.B. Jeffries, C. Reinhard, S.C. Kozma, G. Thomas, Rapamycin selectively represses translation of the 'polypyrimidine tract' mRNA family, Proc. Natl. Acad. Sci. U.S.A. 91 (1994) 4441–4445.
[20] O. Meyuhas, D. Avni, S. Shama, Translational control of ribosomal protein mRNAs in eukaryotes, in: J.W.B. Hershey, M.B. Mathews, N. Sonenberg (Eds.), Translational Control, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1996, pp. 363–388.
[21] H. Shima, M. Pende, Y. Chen, S. Fumagalli, G. Thomas, S.C. Kozma, Disruption of the $p70^{s6k}/p85^{s6k}$ gene reveals a small mouse phenotype and a new functional S6 kinase, EMBO J. 17 (1998) 6649–6659.
[22] M.O. Boluyt, J.S. Zheng, A. Younes, X. Long, L. O, H. Neil, E. Silverman, G. Lakatta, M.T. Crow, Rapamycin inhibits α1-adrenergic receptor-stimulated cardiac myocyte hypertrophy but not activation of hypertrophy-associated genes, Circ. Res. 81 (1997) 176–186.

[23] A.A. Sneddon, M.I. Delday, J. Steven, C.A. Maltin, Elevated IGF-II mRNA and phosphorylation of 4E-BP1 and $p70^{s6k}$ in muscle showing clenbuterol-induced anabolism, Am. J. Physiol. Endocrinol. Metab. 281 (2001) E676–E682.
[24] H. Takano, I. Komuro, Y. Zou, S. Kudoh, T. Yamazaki, Y. Yazaki, Activation of p70 S6 protein kinase is necessary for angiotensin II-induced hypertrophy in neonatal rat cardiac myocytes, FEBS Lett. 379 (1996) 255–259.
[25] D. Dardevet, C. Sornet, T. Vary, J. Grizard, Phosphatidylinositol 3-kinase and p70 s6 kinase participate in the regulation of protein turnover in skeletal muscle by insulin and insulin-like growth factor I, Endocrinology 137 (1996) 4087–4094.
[26] M.G. Thompson, M. Pascal, S.C. Mackie, A. Thom, K.S. Morrison, F.R. Backwell, R.M. Palmer, Evidence that protein kinase C and mitogen activated protein kinase are not involved in the mechanism by which insulin stimulates translation in L6 myoblasts, Biosci. Rep. 15 (1995) 37–46.
[27] K. Baar, K. Eser, Phosphorylation of $p70^{s6k}$ correlates with increased skeletal muscle mass following resistant exercise, Am. J. Physiol. Cell Physiol. 276 (1999) C120–C127.
[28] E. Eisenberg, G.S. Gordan, The levator ani muscle as an index of myotrophic activity of steroidal hormones, J. Pharmacol. Exp. Ther. 99 (1950) 38–44.
[29] L.G. Hershberger, E.G. Shipley, R.K. Meyer, Myotrophic activity of 19-nortestosterone and other steroids determined by modified levator ani muscle method, Proc. Soc. Exp. Biol. Med. 83 (1953) 175–180.
[30] S. Ferrari, W. Bannwarth, S.J. Morley, N.F. Totty, G. Thomas, Activation of $p70^{s6k}$ is associated with phosphorylation of four clustered sites displaying Ser/Thr–Pro motifs, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 7282–7286.
[31] H.S. Kim, S.Y. Han, T.S. Kim, S.J. Kwack, R.D. Lee, I.Y. Kim, J.H. Seok, B.M. Lee, S.D. Yoo, K.L. Park, No androgenic/anti-androgenic effects of bisphenol-A in Hershberger assay using immature castrated rats, Toxicol. Lett. 135 (2002) 111–123.
[32] G. Boissonneault, J. Gagnon, M.A. Kim-Ho, P.A. Rogers, R.R. Tremblay, Depressed translational activity in the androgen sensitive levator ani muscle of the rat, J. Steroid Biochem. 32 (1989) 507–513.
[33] G. Boissonneault, J. Gagnon, C. Simard, R.R. Tremblay, Effect of the androgenic status on the phenotype of the plantaris muscle of the rat, Comp. Biochem. Physiol. B 93 (1989) 157–162.
[34] P. Rotwein, P. James, K. Kou, Rapid activation of insulin-like growth factor binding protein-5 gene transcription during myoblast differentiation, Mol. Endocrinol. 9 (1995) 913–923.
[35] P.L. James, C.E. Stewart, P. Rotwein, Insulin-like growth factor binding protein-5 modulates muscle differentiation through an insulin-like growth factor-dependent mechanism, J. Cell Biol. 133 (1996) 683–693.
[36] J.R. Florini, D.Z. Ewton, S.A. Coolican, Growth hormone and the insulin-like growth factor system in myogenesis, Endocr. Rev. 17 (1996) 481–517.
[37] R.C. Baxter, Signalling pathways involved in antiproliferative effects of IGFBP-3: a review, J. Clin. Pathol. Mol. Pathol. 54 (2001) 145–148.
[38] B.L. Awede, J.P. Thissen, J. Lebacq, Role of IGF-I and IGFBPs in the changes of mass and phenotype induced in rat soleus muscle by clenbuterol, Am. J. Physiol. Endocrinol. Metab. 282 (2002) 31–37.
[39] W. Long, L. Wei, E.J. Barrett, Dexamethasone inhibits the stimulation of muscle protein synthesis and PHAS-I and p70 S6-kinase phosphorylation, Am. J. Physiol. Endocrinol. Metab. 280 (2001) E570–E575.
[40] O.J. Shah, J.A. Iniguez-Lluhi, A. Romanelli, S.R. Kimball, L.S. Jefferson, The activated glucocorticoid receptor modulates presumptive autoregulation of ribosomal protein S6 protein kinase, p70 S6K, J. Biol. Chem. 277 (2002) 2525–2533.
[41] R.C. Hickson, S.M. Czerwinski, M.T. Falduto, A.P. Young, Glucocorticoid antaganism by exercise and androgenic–anabolic steroids, Med. Sci. Sports Exerc. 22 (1990) 331–340.
[42] M. Konagaya, S.R. Max, A possible role for endogenous glucocorticoids in orchiectomy-induced atrophy of the rat levator ani muscle: studies with RU 38486 a potent and selective antiglucocorticoid, J. Steroid Biochem. 25 (1986) 305–308.
[43] P.A. Danhaive, G.G. Rousseau, Binding of glucocorticoid antagonists to androgen and glucocorticoid hormone receptors in rat skeletal muscle, J. Steroid Biochem. 24 (1986) 481–487.
[44] S. Chen, J. Wang, G. Yu, W. Liu, D. Pearce, Androgen and glucocorticoid receptor heterodimer formation, J. Biol. Chem. 272 (1997) 14087–14092.
[45] C.A. Heinlein, C. Chang, Androgen receptor (AR) coregulators: an overview, Endocr. Rev. 23 (2002) 175–200.
[46] B.D. Darimont, R.L. Wagner, J.W. Apriletti, M.R. Stallcup, P.J. Kushner, J.D. Baxter, R.J. Fletterick, K.R. Yamamoto, Structure and specificity of nuclear receptor-coactivator interactions, Genes Dev. 12 (1998) 3343–3356.