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Androgen Receptor Regulates Expression of Skeletal Muscle–Specific Proteins and Muscle Cell Types

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C_2C_{12} myoblasts expressing the androgen receptor (AR) were used to analyze the role of androgen–AR signaling pathway in skeletal muscle development. Marked up-regulation of AR expression was observed in differentiated myotubes. A nuclear run-on transcription assay demonstrated that transcription of the AR gene is increased during skeletal muscle cell differentiation. Regulation of skeletal muscle–specific protein expression by the androgen–AR signaling pathway was further analyzed using quadriceps skeletal muscle from wild-type (WT) and AR knock-out (ARKO) male mice. A histological analysis of quadriceps skeletal muscle indicates no morphological differences between ARKO and WT mice. However, the androgen–AR signaling pathway increases expression of slow-twitch–specific skeletal muscle proteins and downregulates fast-twitch–specific skeletal muscle proteins, resulting in an increase of slow-twitch muscle fiber type cells in quadriceps muscle.

Key Words: Androgen receptor; skeletal muscle; slow-twitch fiber type; testosterone.

Introduction

The androgen receptor (AR) is a member of the steroid receptor superfamily and is composed of a variable amino-terminal domain, a highly conserved DNA-binding domain, and a ligand-binding domain *(1–4)*. Numerous coregulators of nuclear receptors have been cloned and characterized based on their interactions with nuclear receptors *(5–7)*. In addition, several cytoskeletal proteins, such as actin monomer, supervillin, gelsolin, and filamin, have been reported to interact with and enhance transactivation of AR *(7,8)*, indicating a unique feature in mechanisms of AR transactivation.

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The androgen–AR signaling pathway has been reported to play key roles in determination of sexual differentiation as well as development of sex accessory organs (reviewed in refs. *1* and *2*). In addition, AR has been reported to be expressed in non-reproductive tissues, such as brain, kidney, liver, and muscle. Animal studies using frogs indicate that androgen is required for appropriate development of sexually dimorphic skeletal muscles (reviewed in ref. *2*). Animal studies showed that administration of testosterone (T) resulted in skeletal muscle hypertrophy and increase of AR expression *(9,10)*. Consistent with the animal studies, clinical studies showed that T might induce skeletal muscle cell hypertrophy by enhancing AR expression followed by increasing muscle protein synthesis (reviewed in refs. *11* and *12*).

Individual skeletal muscle fiber cells are distinguished by the presence of multiple elongated nuclei located just beneath the cell membrane (sarcolemma), and the presence of regular cross-striations resulting from the particular arrangement of the contractile proteins, such as actin and myosin (reviewed in ref. *13*). Individual muscle fibers are polygonal in shape, and bundles of muscle fibers are surrounded by a dense layer of collagen. On the basis of the adenosine triphosphatase histochemical reactions, muscle cell types of adult muscle are divided into type I, type IIA, and type IIB (reviewed in ref. *14*). In general, individual skeletal muscles contain both slow-twitch and fast-twitch fibers in different ratios to allow muscles to perform a range of tasks from being stationary for a long time to explosive power production.

The process of myogenesis is regulated by the key myogenic regulatory factors, MyoD and MEF-2 family proteins of transcription factors. The MEF-2 factors heterodimerize with the MyoD family proteins or MyoE proteins, and regulate skeletal muscle cell differentiation. A recent study suggested that the androgen–AR signaling pathway suppressed myoblast cell growth and accelerated cell differentiation to myotubes via enhanced myogenin expression *(17)*. Nevertheless, detailed molecular mechanisms by which the androgen–AR signaling pathway plays a role in skeletal muscle development need to be further studied.

In an attempt to analyze the detailed molecular mechanisms by which the androgen–AR signaling pathway regulates skeletal muscle development, we established C_2C_{12} myoblast cells stably transfected with full length AR cDNA under the control of upstream regulatory region of AR gene (C_2C_{12}/pIRESNP-fAR. The results using the C_2C_{12} stable cell variants showed that AR expression was markedly upregulated in differentiated myotubes. Nuclear run-on transcription assay demonstrated that the marked up-regulation of AR expression during skeletal muscle cell differentiation is at the transcriptional level.

In this study, we showed the regulation of skeletal muscle–specific protein expression by the androgen–AR signaling pathway was analyzed using quadriceps skeletal muscle from the wild-type (WT) and AR knockout (ARKO) mice. The histological analyses indicate little morphological differences in quadriceps skeletal muscle tissues between the WT and ARKO mice. We conclude the androgen–AR signaling pathway upregulates slow-twitch–specific skeletal muscle proteins and downregulates fast-twitch specific skeletal muscle proteins.

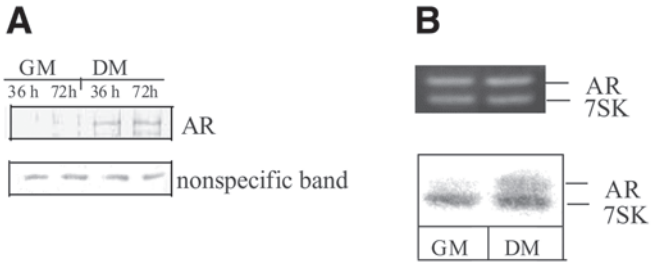

Fig 1. AR expression is upregulated in cell differentiation into myotubes. (**A**) Total cell lysates from C_2C_{12}/pIRESNP-fAR cells grown in growth medium (GM) or differentiation medium (DM) were analyzed on SDS polyacrylamide (7%) gel and transferred to a membrane. AR bands were visualized by Western blot with anti-AR antibody (NH27). Nonspecific bands appearing are used as internal control to show that equal amounts of proteins were analyzed. (**B**) Nuclear run-on transcription assay demonstrated that upregulation of AR expression in differentiated myotubes was at the transcription level. DNA fragments containing the AR gene and 7SK gene on agarose gel (upper panel) were denatured, neutralized, and transferred to a membrane. Denatured DNA fragments on a membrane were hybridized with radioactively labeled RNA transcripts (lower panel) as described in Materials and Methods.

Marked Upregulation of AR Protein Level in C_2C_{12}/pIRESNP-fAR Myotube

The skeletal myoblast C_2C_{12} cell line has successfully been used to study skeletal muscle differentiation *(20)*. C_2C_{12} myoblast cells in a high mitogen environment, such as media containing fetal bovine serum (FBS), undergo cell division. However, a low mitogenic environment, such as with media containing horse serum, induces C_2C_{12} cells to differentiate into myotubes. Appearance of multi-nucleated syncytia by cell fusion is an obvious indication of skeletal muscle cell differentiation.

Consistent with a previous report, the androgen–AR signaling pathway enhanced myogenin expression in C_2C_{12}/pIRESNP-fAR cells (see Materials and Methods) grown in a low mitogenic differentiation medium *(17)*. To our surprise, Western blotting analyses using anti-AR or anti-flag antibodies showed that the fAR protein level in C_2C_{12}/pIRESNP-fAR cells was markedly upregulated during differentiation into myotubes (Fig. 1A).

The mechanism implicated in the marked upregulation of AR protein level during myoblast cell differentiation into myotubes was analyzed using nuclear run-on transcription and pulse/chase labeling assays. In nuclear run-on transcription assays, the synthesis of radioactively labeled RNAs from isolated nuclei can be monitored in the presence of [α-^{32}P]UTP. New initiation of transcription is not allowed, while transcription from existing complexes is allowed to synthesize RNA in isolated nuclei *(22)*. Thus, nuclear run-on transcription assays can tell us whether a gene is regulated at the transcriptional level. As shown in Fig. 1B, the amount of AR mRNA was upregulated in C_2C_{12} myotubes grown in a low mitogenic differentiation medium for 3 d, while the amount of 7SK RNA was not. This result clearly indicates that the marked upregulation of AR expression in myotubes results from upregulation of AR mRNA synthesis. We also carried out pulse/chase labeling assays to determine whether AR protein stability in myotubes has been changed. The half-life for AR in myotubes was approx 2.5 h in the absence of androgens, judged by results of pulse/chase labeling assays (data not shown). Considering the half-life for AR protein in prostate cancer LNCaP cells in the absence of androgens is 3 h *(23)*, changing in the AR proteins stability may not contribute to the marked upregulation of AR protein level in myotubes.

Histological Analysis of Hind Limb Quadriceps Skeletal Muscle Tissues from the WT and ARKO Mice

The functional significance of the androgen–AR signaling pathway in skeletal muscle development was further analyzed using hind limb skeletal muscle tissues from WT and ARKO mice. Since mice reach puberty starting at around 6 wk after birth, mice at ages from 8 to 20 wk were selected for various analyses below. Quadriceps tissues from the mice hind limb skeletal muscles were fixed, dehydrated, paraffin-embedded, and analyzed by hematoxylin & eosin (H&E) staining as shown in Fig. 2 upper panel. H&E staining of cross-section of the quadriceps tissues showed typical skeleton muscle polygonal cell morphology with peripherally located nuclei, with little morphological differences between the WT and ARKO mice. In addition to H&E staining, immunohistochemical staining with an antibody against desmin (lower panel of Fig. 2), a muscle-specific protein,

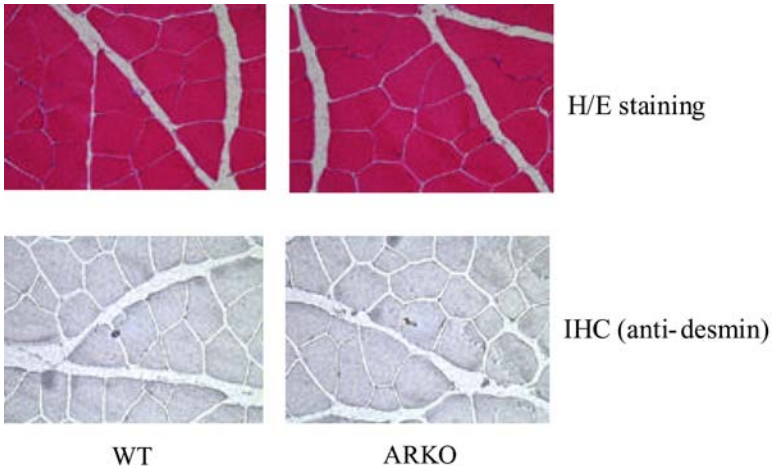

Fig. 2. No morphological difference in quadriceps muscle from the WT and ARKO mice. Analyses of hematoxylin & eosin staining (upper panel) and immunohistochemical staining with anti-desmin antibody (lower panel) of quadriceps muscle from the WT and ARKO mice (age 11-wk-old) suggested that impairment of the AR gene did not change the morphology of skeletal muscle fibers.

showed little morphological/histological difference in quadriceps of hind limb muscle from the WT and ARKO mice. Immunohistochemical staining analysis with an antibody against β-dystroglycan, a muscle-specific membrane protein, also showed little difference between the WT and ARKO mice (data not shown). These results suggest that skeletal muscles in ARKO mice are normally differentiated as those in WT mice.

Protein Levels of Skeletal Muscle–Specific Proteins from the WT and ARKO Mice

In order to analyze the function of the androgen–AR signaling pathway in skeletal muscle development, relative expression levels of skeletal muscle–specific proteins from the WT and ARKO mice were determined using series of Western blotting analyses. Three different samples of quadriceps tissues from age 8 -, 16-, and 20-wk-old male mice were analyzed. As shown in Fig. 3, there was little difference in the amounts of desmin, a muscle-specific structural protein, between the quadriceps obtained from WT and ARKO male mice.

Because skeletal muscle is composed of three different fiber types, expression levels of skeletal muscle–specific proteins from the tissues were determined with antibodies against the specific proteins for slow-twitch or fast-twitch muscle fiber types. In order to analyze if the androgen–AR signaling pathway upregulates slow-twitch fiber type–specific skeletal muscle proteins, the protein levels of slow-twitch fiber type–specific protein, myosin (slow), were determined. As shown in Fig. 3, the amount of myosin (slow) is decreased in quadriceps muscle from the ARKO mice, while the amount of desmin from the same samples is not. Protein levels of troponin T specific for fast-twitch fibers,

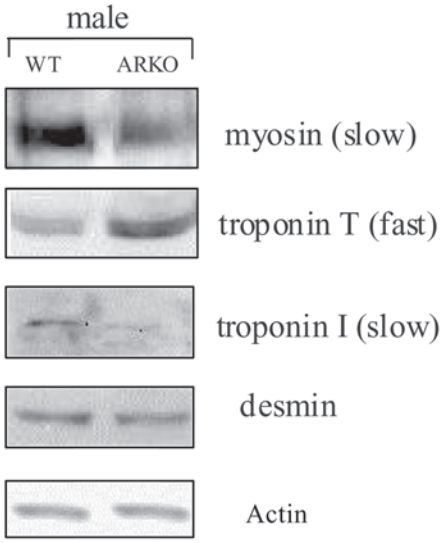

Fig. 3. Expression levels of troponin I and myosin specific for slow-twitch fiber type were upregulated in quadriceps muscle from the AR KO mice. Total proteins prepared from quadriceps muscles were analyzed on SDS gradient polyacrylamide (7–16%) gel, transferred to membranes, and visualized by Western blotting. Expression levels of desmin and actin were used to show that equal amounts of proteins were analyzed.

troponin T (fast) are increased, and protein levels of troponin I specific for slow-twitch fibers, troponin I (slow), are decreased in the muscle from the ARKO mice.

In summary, results in Fig. 3 indicate that the androgen–AR signaling pathway decreases the expression levels of slow-twitch fiber type–specific proteins and downregulates the expression levels of fast-twitch fiber type–specific proteins.

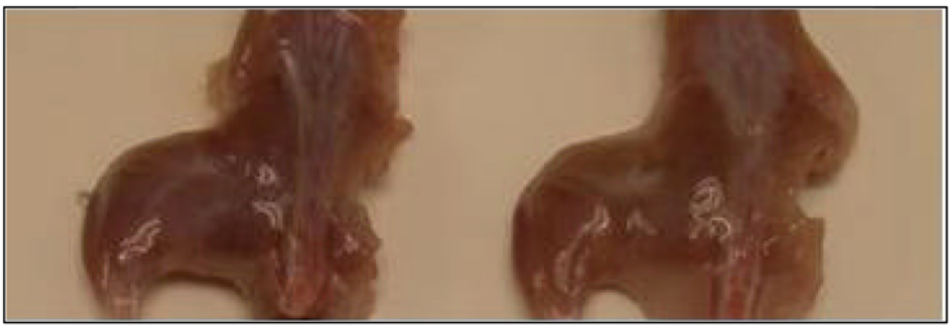

Fig. 4. A dorsal view of the skinned-off WT and ARKO mice (age 16-wk-old). The mice were sacrificed, skinned-off, cleaned in saline solution, and photographed.

Androgen–AR Signaling Pathway Plays a Role in Determination of Skeletal Muscle Fiber Type

Because the androgen–AR signaling pathway appears to upregulate expression levels of slow-twitch fiber–specific proteins and downregulate fast-twitch fiber–specific proteins, it is likely that the androgen–AR signaling pathway may influence the skeletal muscle fiber types toward the slow-twitch fiber type. A gross morphological perspective of the skeletal muscle was analyzed from dorsal views of the skinned-off WT and ARKO mice (Fig. 4). The dorsal view of the WT and ARKO male mice showed that the skeletal muscle color of the WT mouse was more reddish, which is characteristic of skeletal muscles with high ratios of slow-twitch fiber type over fast-twitch fiber type. This result is consistent with upregulated protein levels of myosin specific for slow-twitch fiber type in quadriceps obtained from the WT mice (Fig. 3). Together, our results indicate that the androgen–AR signaling pathway may play important roles in determining the ratios of skeletal muscle fiber types in quadriceps muscles by regulating protein levels of skeletal muscle fiber–specific proteins.

Discussion

In order to analyze the functional roles of AR in skeletal muscle development, we took advantage of cell lines as well as animal model systems. In order to mimic the physiological condition of AR expression in cells, the AR expression in the C_2C_{12} variant stable cells was under the control of 3.6 kb upstream regulatory region of the AR gene. AR mRNA expression in the C_2C_{12} variant stable cells was markedly upregulated during terminal cell differentiation into myotubes (Fig. 1). Clinical and animal studies showed that administration of T increased AR mRNA and protein levels in skeletal muscle, followed by hypertrophy of the muscle cells *(9–12)*. This indicates that upregulation of AR expression might be required for the lean skeletal muscle mass, muscle strength, and muscle protein synthesis.

The extremely low or no AR protein level in C_2C_{12} myoblast cells may explain why no AR activity in C_2C_{12} myoblast cells was reported previously *(17)*. Skeletal muscle cells have been reported to express AR *(1,2)*; however, AR expression in C_2C_{12} myoblast cells may be masked. Masked AR expression in prostate cancer DU145 cells has been reported *(24)*. The AR promoter region in prostate cancer DU145 cells is heavily methylated and treating cells with demethylation reagent 5-aza-2'-deoxycytidine restored AR expression in DU145 cells *(24)*. However, treatment of C_2C_{12} myoblast cells with the demethylation reagent did not restore AR expression in C_2C_{12} myoblast cells (data not shown), indicating that AR expression in C_2C_{12} myoblast cells is masked by another mechanism *(15,16)*. The possible mechanism of silencing AR expression in C_2C_{12} myoblast cells needs to be further studied. Nevertheless, the marked upregulation of AR protein in differentiated myotubes shown in this study is consistent with the previous studies demonstrating AR expression in skeletal muscles.

Morphological examination of skeletal muscle fibers in quadriceps from the WT and ARKO mice showed little difference between the mice (Fig. 2). Expression levels of several muscle-specific proteins, such as desmin and b-dystroglycan, were not changed by the impaired AR function in the ARKO mice (Fig. 3). However, expression levels of troponins and myosin that determine muscle fiber types were changed in quadriceps from the ARKO mice (Fig. 3). The results showed decreases of troponin I (slow) and myosin (slow), and an increase of troponin T (fast) in quadriceps of ARKO mice, indicating that AR may play a role in determination of skeletal muscle fiber types toward slow-twitch fiber type.

Studies so far found the maximum muscles contraction velocities to be in the order type IIB > type IIA >> type I *(14–16,26)*. Contractile speed of the fast-twitch fiber types is much faster than that of the slow-twitch fiber types, thus the fast-twitch fiber type muscles may contract faster but get tired quickly owing to lactic acid accumulation in muscle

beds. As expected from the oxidative capacity of the muscle fibers, type I slow-twitch fibers, those dependent on oxidative metabolism from mitochondria, have the greatest endurance, followed by type IIA fibers, and lastly type IIB fibers, those dependent on glycolysis for energy source. Based on our study demonstrating that the androgen–AR signaling pathway plays a role in the determination of muscle fiber types toward slow-twitch fiber type, it is likely that AR plays a role in development of sexually dimorphic skeletal muscles by balancing skeletal muscle fiber type toward higher numbers of slow-twitch muscle fiber types in male mice.

Administration of physiological or near-physiological concentration of T has been shown to increase fat-free skeletal muscle mass and muscle strength in old men or young hypogonadal men (reviewed in refs. *11* and *12*). In addition, T administration to middle-aged men with visceral obesity improved insulin sensitivity, and changed fat metabolism and regional fat distribution *(27)*. Thus, administration of physiological concentration of T may be utilized as a tool to improve the quality of life for certain types of patients.

Materials and Methods

Experimental Animals

WT and ARKO mice were obtained as described elsewhere *(18)*. Animals were housed in the one-way animal room at the University of Rochester Medical Center Vivarium. Animals were maintained and sacrificed according to the regulations of the University Committee of Animal Research of the University of Rochester Medical Center.

Establishment of Stable Cell Lines

Regulation of AR expression from the plasmid pIRESNP-fAR is under the control of the 3.6 kb 5' upstream flanking region of the AR gene. The C_2C_{12} variant stable cell lines were selected and maintained in DMEM supplemented with 10% CD-FBS and 200 μg/mL neomycin. C_2C_{12}/pIRES and C_2C_{12}/pIRESNP-fAR cells were grown and induced into myotube differentiation as described *(15–17,24)*. In our system, charcoal-dextran stripped serum was applied in both GM and DM. All the steroids present in the serum have been removed by the charcoal-dextran treatment.

Nuclear Run-On Transcription Assay

C_2C_{12} stable variant cells grown in growth medium or differentiation medium were resuspended in cell lysis buffer (10 m*M* Tris-HCl/pH 7.4, 10 m*M* NaCl, 3 m*M* $MgCl_2$, 0.5% NP40, and protease inhibitor cocktail), and incubated on ice for 10 min. The nuclei pellets were spun down at 500*g* and resuspended in nuclei storage buffer (10 m*M* Tris-HCl/pH 8.0, 40% glycerol, 5 m*M* $MgCl_2$, and 0.1 m*M* EDTA). Nuclei were frozen and stored in liquid nitrogen in portions of 100 mL corresponding to 2×10^7 nuclei. The nuclei were mixed with 100 mL of reaction buffer (10 m*M* Tris-HCl/pH 8.0, 5 m*M* $MgCl_2$, and 300 m*M* KCl, 0.5 m*M* each of ATP, GTP, UTP, and 100 mCi of [α-^{32}P]CTP [800 Ci/mmol]), and incubated for 30 min at 30°C. RNase-free DNase I was added and the incubation was continued for 10 min at 37°C. Protease K was added to a final concentration of 300 mg/mL in 0.1% SDS and the reaction mixture was incubated 30 min at 37°C. The labeled RNA transcripts were isolated by phenol extraction, phenol/chloroform extraction, and ethanol precipitation. Nuclear transcripts were separated from unincorporated nucleotides using Sephadex G-50 columns equilibrated with 10 m*M* Tris-HCl/pH 7.8, 0.5 m*M* EDTA, and 0.3% SDS.

The plasmid containing AR gene was digested with Pst 1 and the DNA fragments containing the AR NH_2-terminal domain were gel-purified. The plasmid containing 7SK gene was digested with Pst 1 and gel-purified. The DNA fragments were separated on agarose gel, denatured, neutralized, and transferred to a membrane as described *(19)*. The membrane was prehybridized as described *(19)* and hybridized with solutions containing 1×10^6 cpm labeled RNA transcripts. The membrane was washed twice in 2X SSC for 30 min at 60°C and then treated in 2X SSC containing 5 mg/mL RNase A for 20 min at 30°C to remove unhybridized regions of RNA. Signals were detected using Molecular Dynamics PhosphorImager.

Western Blot Assay

Cell lysis buffer (1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail in 1X PBS) was added to culture plates, and total cell lysates from C_2C_{12}/pIRES and C_2C_{12}/pIRESNP-fAR cells were collected. Protein concentrations were determined using the Bradford protein assay (Bio-Rad). Equal amounts of proteins were analyzed on SDS-polyacrylamide gels. Proteins were transferred to membranes and analyzed by Western blotting assays with antibody against AR (NH27).

Mouse quadriceps were collected in an Eppendorf tube homogenizer and minced in the cell lysis buffer containing 0.3% SDS. Total proteins from the tissues were obtained by centrifugation for 5 min at 14,000 rpm and analyzed on SDS gradient polyacrylamide (7–16%) gels. Proteins were transferred to membranes for Western blotting analyses with polyclonal antibodies against troponin T (fast) and troponin I (slow) (Santa Cruz), desmin (Sigma), and monoclonal antibody against myosin (slow) (Sigma).

Hematoxylin & Eosin and Immunohistochemical Staining

Quadriceps from the WT and ARKO mice were collected, fixed in 10% formalin in PBS for 4 h, dehydrated in graded ethanol and xylene, embedded in paraffin, and cut into 8-mm sections. The tissue sections on slides were deparaffinized, stained in hematoxylin (Gill's formulation) and 0.5% eosin, dehydrated, and stored in permount (Sigma) with cover slips.

For immunohistochemical staining, deparaffinized tissue sections were treated by antigen unmasking, blocking, antibody, and biotinylated secondary antibody (Vector Labs) solutions, according to the manufacturer's instructions. The tissue sections were visualized by Vectorstain ABC kit, according to the manufacturer's instruction (Vector Labs), and analyzed under a Nikon Eclipse E800 microscope.

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