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Molecules and Cells

Insulin-Like Growth Factor-I Induces Androgen Receptor Activation in Differentiating C2C12 Skeletal Muscle Cells

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The modulating effect of IGF-I on the regulation of AR gene expression and activation in skeletal muscle cells remains poorly understood. In this study, the effects of IGF-I treatment on AR induction and activation in the absence of AR ligands were examined. Differentiating C2C12 cells were treated with different concentrations (0-250 ng/ml) of IGF-I or for various periods of time (0-60 min) of 250 ng/ml IGF-I. Treatment of C2C12 cells with IGF-I resulted in a dose- and time-dependent increase in total AR and phosphorylated AR (Ser 213). IGF-I treatment also led to significantly increased AR mRNA expression when compared with the control. The levels of skeletal α-actin and myogenin mRNA, known target genes of AR, were also significantly upregulated after 5 or 10 min of treatment with IGF-I. Confocal images revealed that IGF-I stimulated nuclear localization of AR in the absence of ligands. In addition, an electrophoretic mobility shift assay indicated that IGF-I stimulated the AR DNA binding activity in a time-dependent manner. The present results suggest that IGF-I stimulates the expression and activation of AR by ligand-independent mechanism in differentiating C2C12 mouse skeletal muscle cells.

INTRODUCTION

Androgens have anabolic effects on skeletal muscles. Androgen actions are mediated by binding to androgen receptor (AR) (Lee et al., 2003a; Tyagi et al., 2000), which is a transcription factor that belongs to a member of the subfamily of steroid receptors and regulates target genes (Lin et al., 2001; MacLean et al., 2008). Classically, it is considered that AR regulates gene expression through a ligand-dependent mechanism. After binding of its ligand, AR changes its conformation and AR is then dimerized and phosphorylated. Dimerization and phosphorylation facilitate the translocation of AR from the cytosol to the nucleus (Roy et al., 2001). In the nucleus, AR can bind to its specific DNA element and interact with the transcription initiation complex (Xu et al., 1999). Previous studies have shown that skeletal muscle hypertrophy resulted from an increase in AR expression in humans and animals that were administered testosterone followed by increasing muscle protein synthesis (Lee et al., 2003a; 2003b; Urban et al., 1995; Wade, 1972). Although AR is known to be a ligand-regulated transcription factor, there is growing evidence that steroid receptors are regulated via a ligand-independent mechanism. Culig et al. (1994) found that AR can be activated in the absence of ligand by keratinocyte growth factor (KGF), insulin-like growth factor (IGF-I), and epidermal growth factor (EGF) in prostate cancer cell lines. Even though such ligand-independent mechanisms of AR have been studied in various cell types, little is known about its molecular regulation in skeletal muscle cells.

It is well known that IGF-I is a potent regulator of skeletal muscle mass in humans and animals. Indeed, transgenic mice that over-express IGF-I exhibit substantially enlarged skeletal muscle mass (Musaro et al., 1999) because IGF-I exerts positive effects on protein balance by increasing protein synthesis and decreasing protein degradation (Frost and Lang, 1999). Although IGF-I and AR play pivotal roles in skeletal muscle in terms of proliferation, differentiation, development, and hypertrophy (Adams and Haddad, 1996; Chen et al., 2005; Fanzani et al., 2006; Lee et al., 2003a; 2003b; McLellan et al., 2006; Wannenes et al., 2008), it is not known whether there is an interaction between IGF-I and AR in skeletal muscle cells.

Many studies demonstrating the effects of IGF-I on AR function have been conducted in cells other than skeletal muscle cells. However, the results of previous studies showing the modulating effect of IGF-I on AR are controversial. One mechanism by which IGF-I could directly affect the function of the AR would be to alter AR phosphorylation. Some studies have reported that IGF-I and its downstream signaling pathways lead to increased AR phosphorylation in the absence of ligand (Lin et al., 2001; Wen et al., 2000), whereas other studies have shown that IGF-I had no effect or led to decreased AR phosphorylation depending on cell types or treatment conditions (Taneja et al., 2005; Wu et al., 2006). Therefore, there is still a lack of information regarding the molecular mechanisms associated with IGF-I regulation of AR induction and activation, especially in skeletal muscle cells.

Although these previous studies suggest a possible interaction between the AR and IGF-I signaling pathways, the effects of IGF-I on AR induction and activation have not been demonstrated in differentiating C2C12 skeletal muscle cells. Therefore,

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the purpose of the present study was to investigate whether IGF-I induces gene expression and activation of AR in skeletal muscle cells.

MATERIALS AND METHODS

Cell cultures and IGF-I treatment

C2C12 mouse skeletal muscle cells were obtained from the American Type of Culture Collection (ATCC, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Welgene, Korea) supplemented with 10% fetal bovine serum (FBS) (Hyclone, USA) and antibiotics (100 U/ml of penicillin G and 100 μg/ml streptomycin) (Welgene, Korea) in an atmosphere composed of 95% air and 5% CO_2 at 95% humidity and 37°C. The cells used in all experiments conducted for this study were at passage 4 to 7. For the experiments, C2C12 myoblasts were plated in six-well culture plates at a density of 5×10^5 cells/well in growth medium (DMEM, 10% FBS). For all experiments, cells at 90% confluence were treated with DMEM (Welgene, Korea) supplemented with 2% horse serum (Hyclone, USA), antibiotics (100 U/ml of penicillin G and 100 μg/ml streptomycin) (Welgene, Korea), and various concentrations of IGF-I (0-250 ng/ml) for 0 to 60 min.

Western blot

Cells were lysed and scraped in ice-cold lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, and complete protease inhibitor cocktail). The cell extracts were then centrifuged at 13,000 rpm for 15 min at 4°C. Next, the protein in the supernatant was quantified using a Bradford protein assay kit (Bio-Rad, USA). Sixty micrograms of total protein were resolved on 7% SDS-PAGE gel (150 V, 25°C, 1 h) and then transferred to PVDF membranes (12 V, 25°C, 1 h). All of the blots were then incubated with Ponceau S (Sigma, USA) to ensure equal loading in all lanes (data not shown). For the detection of AR, phospho-AR, and α-tubulin protein, the membranes were probed with AR (polyclonal rabbit antibody, 1:2000), phospho-AR^{ser213} (monoclonal mouse antibody, 1:1000) (Santa Cruz Biotechnology, USA), or α-tubulin (monoclonal mouse antibody, 1:2500) (Calbiochem, USA) antibodies overnight at 4°C in 1% skim milk in Tris-buffered saline (TBS) with 0.05% Tween20. The membranes were then washed three times for 5 min each in TBST, after which they were incubated for 1 h with anti-rabbit or mouse IgG horse-radish peroxidase-linked secondary antibody (1:2500) (Cell signaling, USA). The membranes were then washed as described above, after which enhanced chemiluminescent (ECL) or ECL Advance reagent (GE Healthcare UK Ltd., UK) was applied according to the manufacturer's instructions to develop a signal that was subsequently detected using the LAS-3000 imaging system (Fuji Film, Japan) and quantified by densitometry. The target protein levels were then normalized against the α-tubulin protein levels.

RNA extract and real-time PCR

Total RNA was extracted from C2C12 cells using the phenol-chloroform extraction method with TRIzol Reagent (Invitrogen Life Technologies, USA) according to the manufacturer's instructions, after which the RNA was quantified using a spectrophotometer. Next, cDNA was synthesized from 1 μg of total RNA in the presence of random primer, 2.5 mM dNTP, Rnase inhibitor, and reverse transcriptase (Invitrogen Life Technologies, USA) in a final volume of 20 μg at 25°C for 10 min, followed by 42°C for 60 min and 95°C for 5 min. The sequences of the primers were as follows: AR, forward (F) 5′-CGCTCCCTCTTCCTCCAA-3′, and reverse (R) 5′-ATGCTTCCACACCCAATCC-3′; skeletal muscle α-actin (F) 5′-GCGCAAGTACTCAGTGTGGA-3′, (R) 5′-CACGATTGTCGATTGTCGTC-3′; myogenin (F) 5′-CATCCAGTACATTGAGCGCCTA-3′, (R) 5′-GAGCAAATGATCTCCTGGGTTG-3′; GAPDH (F) 5′-ATGACAATGAATACGGCTACAGCAA-3′, (R) 5′-GCAGCGAACTTTATTGATGGTATT-3′. The primers were purchased from Cosmo (Cosmo Genetech, Korea). Real-time PCR was performed in duplicate using the SYBR Green PCR master mix (Finnzyme, Finland) according to the manufacturer's instructions. All PCR amplifications were conducted using an ABI PRISM 7700 system (Applied Biosystems Inc., USA). The expression of the target genes was then normalized against the expression of glyceraldehydes 3-phosphate dehydrogenase (GAPDH).

Immunocytofluorescence staining

C2C12 cells were seeded on a slide in a 6-well plate at a density of 5×10^5. The cultured and treated slides were then fixed in 4% formaldehyde for 20 min at room temperature. Next, the slides were washed two times each in TBS, after which they were permeabilized with TBS containing 0.2% triton X-100 (0.2% TBST) for 5 min at room temperature. The slides were then washed three times for 5 min each in 0.1% TBST and then blocked with 5% BSA in 0.1% TBST for 1 h at room temperature. Next, the slides were washed once with TBS, after which they were probed with AR (N-20) polyclonal rabbit antibody (Santa Cruz Biotechnology, USA) at a dilution of 1:500 overnight at 4°C in 3% BSA in TBS. The slides were then washed three times for 5 min each in 0.1% TBST, after which they were incubated with Alexa594-conjugated goat anti-rabbit IgG secondary antibody (Invitrogen Life Technologies, USA) diluted 1:200 for 20 min at room temperature in TBS that contained 3% BSA. Next, the cells were washed three times with 0.1% TBST, after which they were mounted with mounting media containing 4′,6-diamidino-2phenylinodole (DAPI) at a concentration of 1.5 μg/ml to localize the nuclei. The slides were then viewed and photographed using a confocal microscope LSM-510 Meta (Carl Zeiss, Germany) equipped with a digital imaging system.

Electrophoretic mobility shift assay (EMSA)

Control and IGF-I treated cells were scraped and centrifuged at 13,000 rpm for 1 min at 4°C. The pellets were then resuspended in 400 μl of buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 0.25 mM EGTA, 1 mM DTT, 0.5 mM PMSF, and complete inhibitor cocktail) and incubated on ice for 15 min, after which 25 μl of 10% NP-40 was added. Next, the cells were vortexed for 10 sec and centrifuged at 12,000 rpm for 30 s at 4°C, after which the pellet was resuspended in 100 μl of cold buffer C [20 mM HEPES (pH 7.9), 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 10% glycerol, and complete inhibitor cocktail]. The nuclei were then rocked on 4°C for 25 min, and then centrifuged at 13,000 rpm for 5 min at 4°C. The protein concentration of the supernatant was then determined using the Bradford protein assay kit (Bio-Rad, USA) according to the manufacturer's instructions. Next, 10 pmoles of upper and lower strand DNA were annealed by heating at 94°C for 10 min, followed by ramping to 37°C for 60 min and then 37°C for 15 min. The annealed oligonucleotides were then end-labeled with ^{32}P-ATP (3000 Ci/mmol) (GE Healthcare UK Ltd., UK). Reactions mixtures containing 15 pmoles of the annealed DNA, 10 pmoles of ^{32}P-ATP, 1.5 μl of 10× PNK buffer, and 1 μl of T4 polynucleotide kinase diluted to a volume of 15 μl with sterile water were then incubated at 37°C for 30 min. The labeled DNA was then separated from the free label using probequant G-50 micro columns (GE Healthcare UK Ltd., UK). Next, the nuclear extract (10 μg) was incu-

bated in a reaction mixture with a final volume of 20 μl that contained 10× DNA-binding buffer [100 mM HEPES (pH 7.9), 25 mM $MgCl_2$, 0.5 mM EDTA (pH 8.0), 0.5% Triton X-100, 10 mM DTT, 500 mM NaCl, and 20% glycerol] and 1 μg of poly (dIdC)-poly (dIdC) (Sigma Aldrich, USA) for 10 min at room temperature, after which double-stranded ^{32}P-labeled androgen response element (ARE) was added and the mixture was subsequently incubated for 30 min at room temperature. Protein-DNA complexes were then resolved on 5% EMSA gel (5× TBE, 40% acrylamide, 50% glycerol, ADW, TEMED, 10% APS) in 0.5× TBE buffer (100 V, 1 h). Analytical gels were dried, fixed, on 3 M paper (Whatman, USA) and exposed to film for 12-24 h. The films were then scanned using the BAS-3000 image leader (Fuji Film, Japan).

Data analysis

All values are reported as means ± SE. Statistical significance was determined by one-way ANOVA with post hoc Tukey's test using SPSS 12.0. Differences between groups were considered significant at $P < 0.05$.

RESULTS

IGF-I-induced AR protein expression and activation

As shown in Fig. 1A, Western blot analysis revealed that phosphorylated AR and total AR protein expression varied in response to treatment with 250 ng/ml of IGF-I treatment for various lengths of time. Specifically, treatment of C2C12 cells with IGF-I for various lengths of time resulted in a time-dependent increase in phosphorylated AR (Ser 213) protein expression, with the maximal effect being observed after 30 min (223%; $P < 0.05$). Total AR protein expression also increased by up to 89% in response to treatment with 250 ng/ml of IGF-I for 60 min, and these increases occurred in a time-dependent manner (Fig. 1A). In additional experiments, treatment of cultured C2C12 cells for 30 min with various concentrations of IGF-I resulted in a dose-dependent increase in phosphorylated AR of up to 200% ($P < 0.05$) and in total AR protein expression of up to 115% ($P < 0.05$), with the maximal induction occurring at an IGF-I concentration of 200 ng/ml (Fig. 1B). These data suggest that IGF-I have a modulating effect on AR induction and activation in the absence of ligand in C2C12 skeletal muscle cells.

IGF-I-induced AR mRNA and AR target gene mRNA expression

To test whether the increase in AR protein expression was associated with increased expression of the AR gene, AR mRNA levels were determined by real-time PCR. GAPDH mRNA abundance was examined as a correction factor. The results from that experiment revealed an 80% increase in AR mRNA levels in C2C12 cells after treatment with 250 ng/ml of IGF-I for 5 min ($P < 0.05$; Fig. 2A). This time-course experiment showed that the increase in AR mRNA that was induced by IGF-I treatment was nearly saturated after a very short length of time (5 or 10 min). To further investigate the modulating effect of IGF-I on AR target genes, skeletal α-actin and myogenin mRNA expression was evaluated. The skeletal α-actin mRNA expression was increased by 88% and 57% after 5 and 10 min of IGF-I treatment, respectively ($P < 0.05$; Fig. 2B). In addition, the myogenin mRNA expression increased significantly after 5 min of IGF-I treatment ($P < 0.05$; Fig. 2C). Taken together, these data suggest that IGF-I transiently induces the mRNA level of AR and its target genes within a short time following exposure.

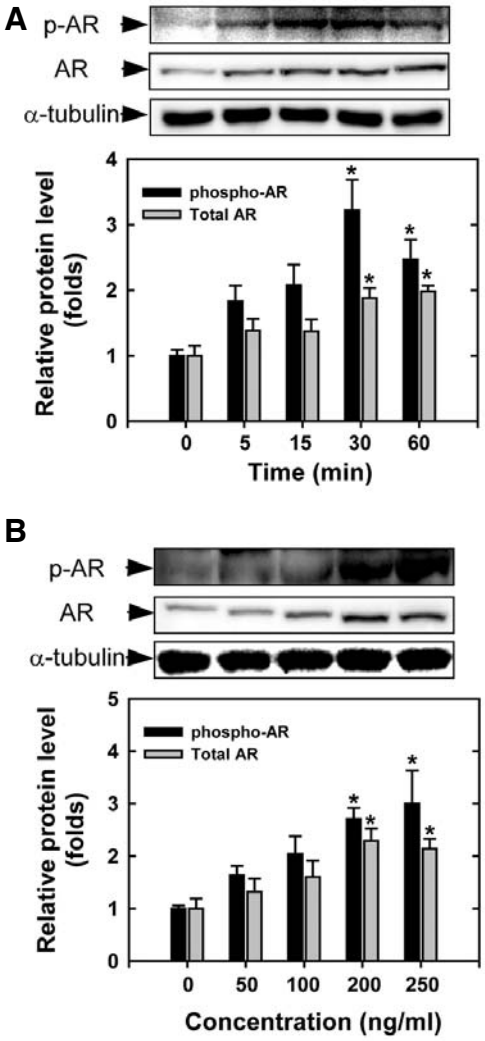

Fig. 1. (A) Influence of treatment with IGF-I for different lengths of time on phosphorylated AR (Ser 213) and total AR protein expression in C2C12 cells. C2C12 cells were treated with 250 ng/ml of IGF-I for up to 1 h. (B) Effect of different concentrations of IGF-I on phosphorylated AR and total AR protein expression in C2C12 cells. C2C12 cells were treated with IGF-I for 30 min. Sixty micrograms of protein were fractionated by 7% SDS-PAGE, after which they were immunobloted with phospho-AR (Ser 213) or AR antibodies. *Inset*: Western blot image of phospho-AR, total AR, and α-tubulin detection. Values are means ± SE with n = 3 for each condition. *$P < 0.05$ vs. control.

AR nuclear localization

Our results showed that IGF-I led to increased AR phosphorylation, which is generally believed to be necessary for nuclear translocation. Therefore, we evaluated IGF-I-induced AR phosphorylation (Ser 213) to determine if it stimulated nuclear translocation of AR in C2C12 cells. Confocal images from C2C12 cells were acquired at 30 min after treatment with 250 ng/ml of IGF-I. As shown in Fig. 3A, AR is primarily located in the cytosolic compartment in the absence of IGF-I. However, IGF-I treatment led to marked enhancement of AR nuclear translocation (Fig. 3B). Taken together, these data indicate that, even in the absence of ligand, IGF-I can induce translocation of the AR in skeletal muscle cells.

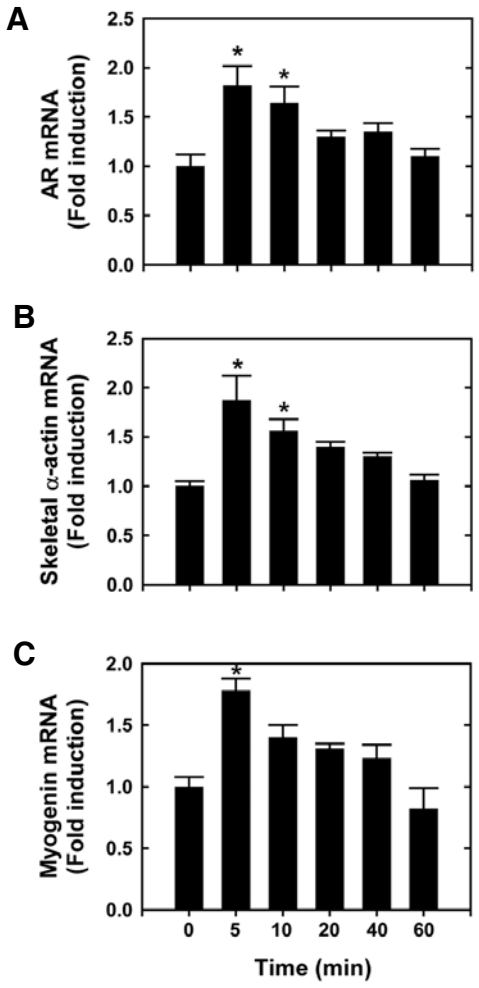

Fig. 2. AR (A), skeletal α-actin (B), and myogenin (C) mRNA levels determined by real-time PCR in C2C12 cells cultured for 1 h in the absence (control) or presence of IGF-I (250 ng/ml). Target mRNA values are shown normalized to the GAPDH mRNA level for each sample. Samples were analyzed in duplicate in parallel with GAPDH. Values are means ± SE of three independent experiments. *$P < 0.05$ vs. control.

AR DNA binding activity

To determine whether IGF-I-induced increase in AR nuclear localization was associated with an increase in AR binding to ARE, the DNA binding activity of AR was determined by EMSA (Fig. 4). In this experiment, the AR DNA binding activity was measured at various time points (0-90 min) after the addition of 50 ng/ml (Fig. 4A) or 250 ng/ml of IGF-I (Fig. 4B). As shown in Fig. 4, IGF-I led to increased AR DNA binding activity in the absence of ligand in a time-dependent manner. Although there was a non-specific band below AR-ARE complex band, a slower mobility band (upper band) was not presented in probe only. Therefore, it is very likely that a slower mobility band is AR-ARE complex.

DISCUSSION

In this study, we investigated a possible interaction between AR and IGF-I signaling in C2C12 cells. The primary finding of this study is that IGF-I treatment led to increased total AR, AR phosphorylation (Ser 213), AR mRNA, DNA binding activity, and AR nuclear localization in the absence of its cognate ligand. This IGF-I-induced AR gene expression and activation appears to be cell-type specific because previous studies conducted using different cell types have shown contrasting results (Lin et al., 2001; 2003; Taneja et al., 2005; Wen et al., 2000).

Androgens act in target cells via an interaction with AR, which results in modulation of gene expression. It is generally accepted that AR regulates gene expression through a ligand-dependent mechanism. In skeletal muscle, AR expression is sensitive to circulating androgen levels (Antonio et al., 1996). Anabolic steroid injection significantly increased AR protein expression in rat skeletal muscles (Lee et al., 2003a; 2003b). In addition to the classical regulation of AR by androgens, AR is also regulated by a ligand-independent mechanism (Culig et al., 1994; Nazareth and Weigel, 1996; Reinikainen et al., 1996; Sadar, 1999; Seaton et al., 2008). Culig et al. (1994) investigated the effect of growth factors on AR-mediated transcription and reported that IGF-I, epidermal growth factor, and keratinocyte growth factor were able to stimulate AR activation in prostatic tumor cell lines. This study suggests that AR can be activated by IGF-I in the absence of ligand.

IGF-I is synthesized primarily in the liver, but is also produced locally in skeletal muscle where they can exert autocrine or paracrine effects (Jones and Clemmons, 1995). It has been well known that IGF-I is essential for muscle differentiation and hypertrophy (Galvin et al., 2003; McLellan et al., 2006; Rommel et al., 2001). IGF-I-induced hypertrophy of myotubes is known to be dependent on a pathway initiated by PI3K/Akt, resulting in increased protein synthesis through increases in the initiation of translation and elongation (Brunn et al., 1997). Our present data clearly demonstrated that IGF-I increased AR protein and mRNA expression in the absence of ligand, indicating that IGF-I had a modulating effect on AR induction and activation in skeletal muscle cells. Androgen-induced expression of AR regulates AR target genes such as myogenic regulatory factors (MRFs), cell cycle regulators, and skeletal α-actin (Lee, 2002; Lee et al., 2003b; Lu et al., 1999). However, the effect of ligand-independent induction of AR by IGF-I on the expression level of its target genes in skeletal muscle has not been well studied. Our current data demonstrate that AR can be influenced by IGF-I to induce AR target gene expression. Specifically, treatment with IGF-I increased skeletal α-actin mRNA expression in the absence of ligand. Myogenin mRNA expression was also induced in IGF-I-treated C2C12 muscle cells, suggesting that IGF-I accelerates myoblast differentiation in the absence of ligand.

A number of studies have suggested that AR is regulated directly or indirectly by phosphorylation. Thus, one mechanism by which the IGF-I signaling pathway can affect AR function is to alter the level of AR phosphorylation. Several earlier studies reported the role of IGF-I signaling pathways on AR activity and phosphorylation in different cell types (Lin et al., 2001; 2003; Taneja et al., 2005; Wen et al., 2000). However, the results of previous studies showing IGF-I-induced phosphorylation of AR are controversial. For example, studies by Lin et al. (2001) demonstrated that IGF-I signaling played a role in the function of AR. They went on to state that IGF-I phosphorylates AR at Ser 213 and 790 in prostate cancer DU145 cells, and that activation of the PI3K/Akt pathway suppressed AR activity. In addition, it has been reported that downstream signaling of IGF-I, Akt, specifically binds to AR and phosphorylates Ser 213 and Ser 791 of AR (Wen et al., 2000). However, these findings have been contradicted by other studies. Wu et al. (2006) found that AR phosphorylation was decreased in the presence of IGF-I, and that this effect was completely blocked by the inhibitory

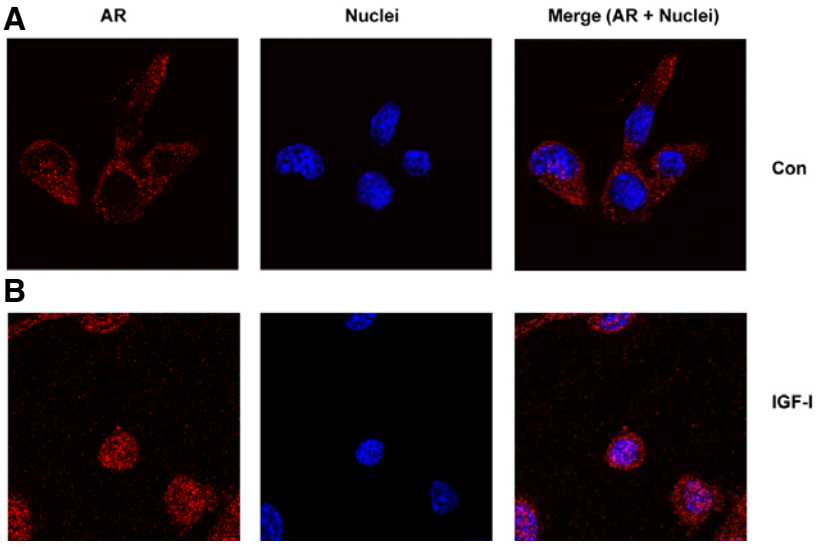

Fig. 3. Confocal image showing IGF-I-induced AR translocation into the nucleus in C2C12 cells in the absence or presence of pharmacological inhibitors. All images were acquired at 30 min after treatment. (A) C2C12 cells in IGF-I free medium. (B) C2C12 cells in medium containing 250 ng/ml IGF-I. AR is stained fluorescent red and nuclei are stained fluorescent blue.

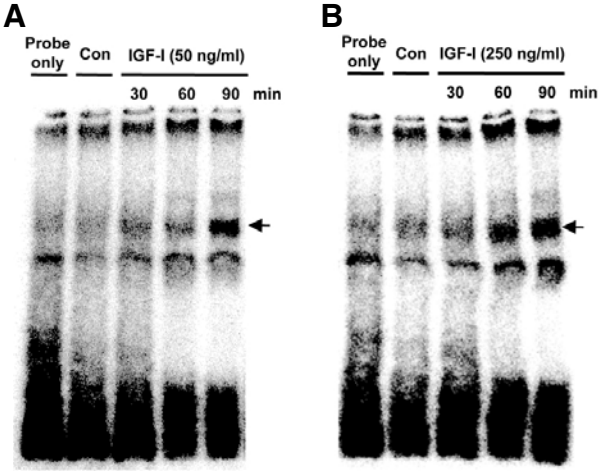

Fig. 4. Influence of IGF-I treatment [(A) 50 ng/ml IGF-I; (B) 250 ng/ml IGF-I] for different lengths of time on AR DNA binding activity determined by electrophoretic mobility shift assay (EMSA). Probe only, labeled ARE probe with no sample; Control, labeled ARE probe with untreated sample.

IGF-I receptor antibody A12 in AR-transfected M12 cells. Taneja et al. (2005) evaluated growth factor to determine if it resulted in AR phosphorylation in the absence of AR ligand in human embryonic kidney 293 cell lines. They found that AR was phosphorylated at Ser 213 in the presence of its ligand (R1881), but not following treatment with IGF-I for up to 2 h in the absence of ligand, suggesting that agonist binding is necessary for cellular kinases to phosphorylate AR. Furthermore, they showed that, although IGF-I did not phosphorylate AR in the absence of ligands, downstream signaling pathways of IGF-I such as Akt and MAPK were activated by IGF-I. Our data clearly showed that IGF-I increased AR phosphorylation at Ser 213 in the absence of ligand in C2C12 cells. Although the reasons for these discrepancies between studies are not clear, we can speculate that IGF-I-induced AR phosphorylation is tightly regulated in a cell-type specific manner. One possible reason for these differences in AR phosphorylation by IGF-I would be differential expression of protein phosphatase 2A in different cell types (Wu et al., 2006). Another possible reason for this discrepancy may have been the difference in cell types or treatment conditions.

The results of previous studies designed to determine whether growth factor exerts a modulating effect on the nuclear localization of AR are controversial (Orio et al., 2002; Wu et al., 2006). In terms of AR nuclear localization, Orio et al. (2002) reported that IGF-I and epidermal growth factor were unable to initiate the nuclear translocation of AR in the absence of androgens in human prostate cell lines (PNT1A and DU-145). However, the results of another study indicated that IGF-I enhanced the nuclear translocation of AR in the absence of androgens, and that this effect was blocked by an IGF-I receptor inhibitory antibody in AR-transfected M12 cells (Wu et al., 2006). Our findings showed that IGF-I enhanced AR trafficking from cytosol to the nucleus in the absence of androgens in C2C12 skeletal muscle cells. Whether the discrepancy in IGF-I-induced AR nuclear localization is attributed to differences in phosphorylation of AR in different cell types or to the recruitment of AR coactivators has yet to be investigated. Nevertheless, our present data suggest that IGF-I-induced AR phosphorylation at Ser 213 stimulates AR translocation to the nucleus in C2C12 skeletal muscle cells via coordinative activation of IGF-I downstream signaling pathways.

The results of the present study suggest that increased gene expression and activation of AR after stimulation of C2C12 skeletal muscle cells by treatment with IGF-I raises the possibility that, even in the absence of its cognate ligand, AR can be activated by IGF-I in skeletal muscle cells. Although further work is needed to determine how detailed molecular signaling pathways contribute to the activation of AR, the results of the present study may provide a better understanding of the ligand-independent activation of AR and signaling pathways integrating IGF-I and AR in skeletal muscle cells.

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