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Communication

Molecules and Cells

Insulin-Like Growth Factor-I-Induced Androgen Receptor Activation Is Mediated by the PI3K/Akt Pathway in C2C12 Skeletal Muscle Cells

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Although insulin-like growth factor-I (IGF-I) and androgen receptor (AR) are well known effectors of skeletal muscle, the molecular mechanism by which signaling pathways integrating AR and IGF-I in skeletal muscle cells has not been previously examined. In this study, the role of PI3K/Akt on IGF-I-induced gene expression and activation of AR in skeletal muscle cells was investigated. C2C12 cells were treated with IGF-I in the absence or presence of inhibitors of PI3K/Akt pathway (LY294002 and Wortmannin). Inhibition of the PI3K/Akt pathway with LY294002 or Wortmannin led to a significant decrease in IGF-I-induced AR phosphorylation and total AR protein expression. Furthermore, IGF-I-induced AR mRNA and skeletal α-actin mRNA were blocked by LY294002 or Wortmannin. Confocal images showed that IGF-I-induced AR translocation from cytosol to nucleus was inhibited significantly in response to treatment with LY294002 or Wortmannin. The present results suggest that modulating effect of IGF-I on AR gene expression and activation in C2C12 mouse skeletal muscle cells is mediated at least in part by the PI3K/Akt pathway.

INTRODUCTION

Although androgen receptors (AR) are known as ligand-regulated transcription factors, recent studies suggest that AR can be regulated via a ligand-independent mechanism (Lin et al., 2001; Wen et al., 2000; Wu et al., 2006). AR can be activated in the absence of ligand by growth factors, keratinocyte growth factor (KGF), insulin-like growth factor (IGF-I), and epidermal growth factor (EGF) in prostate cancer cell lines (Culig et al., 1994). One mechanism by which IGF-I could directly affect the function of the AR would be to alter AR phosphorylation. Although such ligand-independent mechanisms of AR have been studied in various cell types, the molecular mechanism by which signaling pathways integrating AR and IGF-I in skeletal muscle cells is poorly understood.

It is well known that IGF-I are 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). Phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) are two key pathways that are known to be activated by IGF-I (Butler et al., 1998; Meng et al., 2007). IGF-I is known to increase skeletal muscle mass via stimulation of the PI3K pathway, which results in the downstream activation of targets that are required for muscle protein synthesis (Bodine et al., 2001). PI3K phosphorylates inositol lipids that act as second messengers for the serine-threonine protein kinase, Akt/protein kinase B (Franke et al., 1997). Overload-induced muscle hypertrophy has the ability to activate the PI3K/Akt pathway by directly inducing IGF-I expression in skeletal muscle (Devol et al., 1990). The addition of IGF-I to differentiated muscle cells promotes myotube hypertrophy, whereas pharmacological blockade of PI3K/Akt activity with the drug LY294002 blocks this IGF-I-induced hypertrophy (Rommel et al., 2001), indicating that skeletal muscle hypertrophy can be mediated by downstream signaling pathways activated by IGF-I.

Although these previous studies suggest a possible interaction between the AR signaling pathway and IGF-I signaling pathways, the modulating effect of the PI3K/Akt pathway on the activation of AR have not been demonstrated in C2C12 skeletal muscle cells. Therefore, the purpose of the present study was to investigate the role of PI3K/Akt on IGF-I-induced gene expression and activation of AR in skeletal muscle cells. It was hypothesized that the modulating effect of IGF-I on AR gene expression and activation in C2C12 cells would be mediated by the PI3K/Akt pathway.

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

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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 IGF-I in the absence or presence of inhibitors of PI3K/Akt (LY294002 and Wortmannin). IGF-I and all inhibitors used in this study were purchased from Sigma (USA).

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. For the detection of Akt, phospho-Akt, the membranes were blocked with 5% skim milk in TBS with 0.1% Tween20 for 1 h at room temperature and subsequently incubated with Akt (polyclonal rabbit antibody, 1:1000), and phospho-Akt$^{(Ser473)}$ (polyclonal rabbit antibody, 1:1000) (Cell Signaling, USA) antibodies overnight at 4°C in 5% BSA in TBS with 0.1% 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′; 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-2-phenylinodole (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.

RESULTS AND DISCUSSION

Modulating effect of PI3K/Akt on IGF-I-induced AR activation

It is well known that PI3K/Akt is a major pathway that is activated by IGF-I (5). We examined the PI3K/Akt pathway to determine if this pathway is involved in the induction and activation of AR in IGF-I-stimulated C2C12 cells by treating the cells with the specific PI3K inhibitors (LY294002 and Wortmannin). As shown in Fig. 1A, we evaluated Akt phosphorylation to determine if it was regulated by IGF-I treatment in C2C12 cells. As shown in Fig. 1A, phosphorylated Akt was not readily detectable in the absence of IGF-I in cultured C2C12 cells. As expected, treatment with IGF-I (250 ng/ml) led to significantly increased levels of phosphorylation of Akt (> 13 fold) after 30 min of treatment. Additionally, Akt phosphorylation in C2C12 cells following IGF-I treatment was significantly blocked by PI3K/Akt inhibitors (LY294002 and Wortmannin). However, the level of total Akt protein expression was not affected by IGF-I treatment or PI3K/Akt inhibitors (Fig. 1A). To demonstrate whether PI3K/ Akt mediates the induction and activation of AR, we measured the levels of total AR and phosphorylated AR in the presence of LY294002 or Wortmannin. As shown in Fig. 1B, IGF-I-induced total AR and phosphorylated AR were significantly suppressed by LY294002 or Wortmannin. Figure 2 showed AR and phosphorylated AR (Ser 213) protein expression in cytosol and nuclear fractions of C2C12 cells after treatment with IGF-I in the absence or presence of LY294002 or Wartmannin. IGF-I (250 ng/ml) did not increase cytosol fraction of total AR and phosphorylated AR. However, nuclear fraction of total AR and phosphorylated AR were significantly induced by IGF-I. In addition, IGF-I-mediated nuclear total AR and phophsphorylated AR expression was significantly suppressed by LY294002 or Wartmannin. Taken

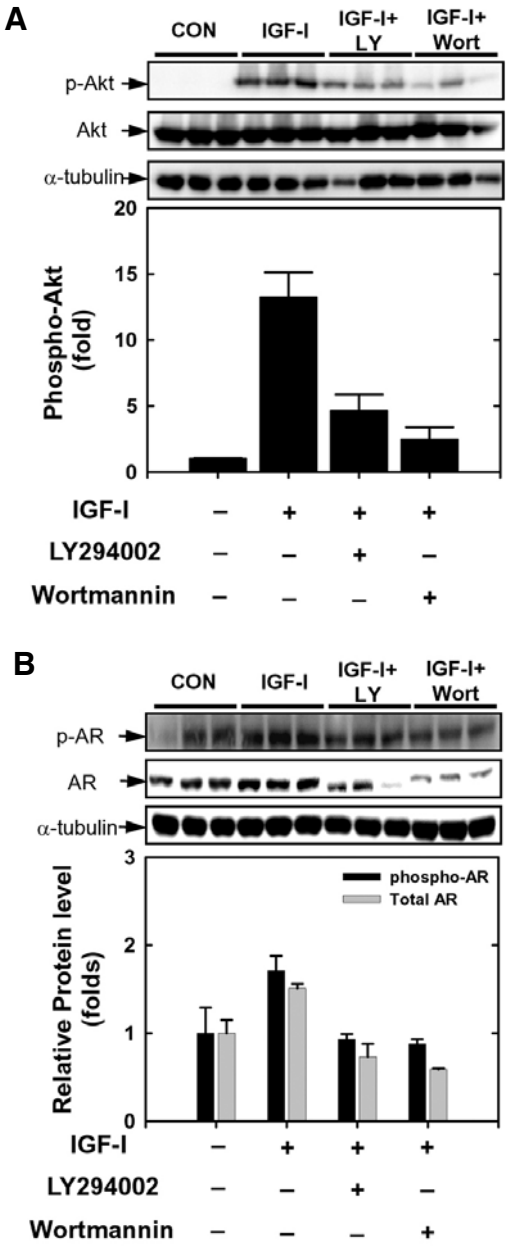

Fig. 1. (A) phosphorylated Akt and total Akt expression in C2C12 cells treated with IGF-I (250 ng/ml) for 30 min in the absence or presence of LY294002 (100 nM) or Wotmannin (50 nM). *Inset*: Western blot image of phospho-Akt, total Akt, and α-tubulin detection. (B) effect of treatment with 250 ng/ml IGF-I for 30 min in the absence or presence of LY294002 (100 nM) or Wotmannin (50 nM) on the level of phospho-AR (Ser 213) and total AR protein expression in C2C12 cells. *Inset*: Western blot image of phospho-AR, total AR, and α-tubulin detection. Values are means ± SE with n = 3 for each condition.

together, these date suggests that PI3K/ Akt, a downstream signaling pathway of IGF-I, mediates IGF-I-induced AR induction and activation in C2C12 cells. Furthermore, IGF-I and its downstream signaling pathway, PI3K/Akt, modulate AR primarily in nuclear compartment. Although down-stream signaling pathways of IGF-I have been extensively studied in many cell

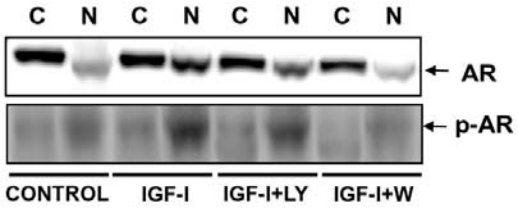

Fig. 2. Phosphorylated AR and total AR in cytosol (C) and nuclear fractions (N) of C2C12 cells after treatment of IGF-I in the absence or presence of LY294002 or Wortmannin.

types, the roles of IGF-I downstream signaling pathways on AR induction and activation in skeletal muscle cells remains poorly understood. Previous studies have shown a functional link between the AR and PI3K/Akt signaling pathways. The pharmacological inhibitor of PI3K, LY294002, suppressed Her-2/neu-induced AR activation, suggesting that the PI3K/Akt pathway is linked to the AR signaling pathway in prostate cancer cells (Wen et al., 2000). Lin et al. (2003) reported that IGF-I increased Akt phosphorylation and reduced AR protein levels in LNCaP cells. They also showed that the PI3K inhibitor, LY 294002, reversed the suppressive effect of IGF-I on AR protein concentration, suggesting that IGF-I-induced down-regulation of AR protein levels is mediated by the PI3K/Akt pathway. AR phosphorylation at serine 213 has been found to stimulate AR to interact with the ubiquitine ligase to increase AR ubiquitylation and degradation (Lin et al., 2002). In contrast to previous studies, the results of the present study clearly show that total AR and AR phosphorylation (Ser 213) was increased by IGF-I treatment in C2C12 cells, and this IGF-I-induced AR activation was tightly regulated by the PI3K/Akt pathway, suggesting that the role of IGF-I-induced PI3K/Akt activation in the regulation of AR gene differs between cell types.

Effects of PI3K/Akt on IGF-I-induced AR mRNA and AR target gene mRNA expression

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., 2003; Lu et al., 1999). However, the modulating effect of PI3K/Akt on IGF-I-induced AR and its target genes in skeletal muscle has not been well studied. To test whether the PI3K/Akt pathway was involved in IGF-I-induced AR mRNA and AR target gene mRNA expression, transcript levels of these genes were determined by real-time PCR. GAPDH mRNA abundance was examined as a correction factor. As shown in Fig. 3A, the induction of AR mRNA following IGF-I treatment in C2C12 cells was significantly blocked by LY 294002 or Wortmannin. To further investigate the modulating effect of the PI3K/Akt pathway on IGF-I-induced AR target gene, skeletal α-actin mRNA expression was evaluated in the presence of LY294002 or Wortmannin. As shown in Fig. 3B, IGF-I-induced skeletal α-actin mRNA expression was significantly suppressed by PI3K/Akt inhibitors. Taken together, these data suggest that IGF-I transiently induces the mRNA level of AR and skeletal α-actin, and the PI3K/Akt pathway is necessary for the regulation of IGF-I on AR and its target gene by demonstrating that its pharmacologic inhibition prevents AR mRNA and skeletal α-actin mRNA.

AR nuclear localization

It is known that AR phosphorylation is necessary for nuclear translocation of AR. Although PI3K/Akt is involved in ligand-

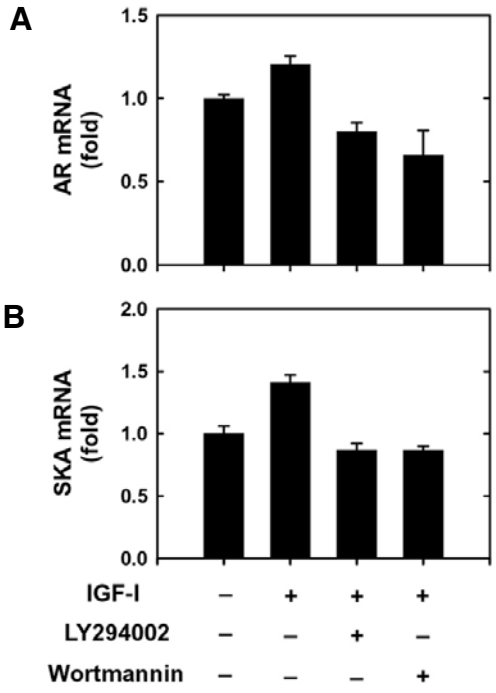

Fig. 3. AR (A) and skeletal α-actin (B) mRNA levels determined by real-time PCR in C2C12 cells cultured for 1 h in the absence (control) or presence of IGF-I (250 ng/ml). Target mRNA values are shown normalized to the GAPDH mRNA level for each sample. Samples were analyzed in duplicate in parallel with GAPDH. Values are means $\pm$ SE of three independent experiments.

independent activation of AR by IGF-I it is not known whether PI3K/Akt regulates AR nuclear translocation. Therefore, we evaluated the modulating effect of PI3K/Akt on IGF-I-induced AR nuclear localization determined by immunofluorescent staining. Confocal images from C2C12 cells were acquired at 30 min after treatment with 250 ng/ml of IGF-I with or without LY294002 or Wortmannin (Fig. 4). As shown in Fig. 4B, IGF-I increased AR nuclear translocation from cytosol compartment. This IGF-I-induced AR nuclear translocation was significantly blocked by LY294002 or Wartmannin (Figs. 4C and 4D). Taken together, these data indicate that ligand-independent induction of AR nuclear localization is mediated by the PI3K/Akt pathway in skeletal muscle cells.

In summary, the results of the present study suggest that ligand-independent AR induction and activation by IGF-I seems to be crucially regulated by activation of the PI3K/Akt pathway in C2C12 skeletal muscle cells. Although further work is needed to determine whether other IGF-I downstream signaling pathways other than PI3K/Akt contribute to the induction and activation of AR, the results of the present study clearly shows that the ligand-independent activation of AR by IGF-I is mediated at least in part by the PI3K/Akt pathway.

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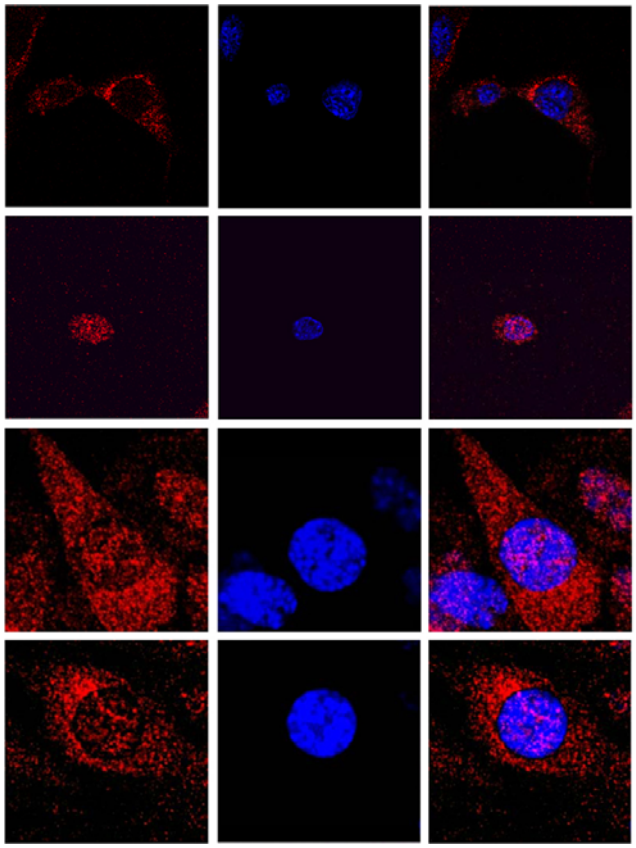

Fig. 4. Confocal image showing IGF-I-induced AR translocation into the nucleus in C2C12 cells in the absence or presence of pharmacological inhibitors. All images were acquired at 30 min after treatment. (A) C2C12 cells in IGF-I free medium. (B) C2C12 cells in medium containing 250 ng/ml IGF-I. (C) C2C12 cells treated with 250 ng/ml IGF-I + 100 nM LY 294002. (D) medium containing IGF-I + 50 nM Wortmannin.

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