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Communication

Molecules and Cells

Ligand-Independent Activation of the Androgen Receptor by Insulin-Like Growth Factor-I and the Role of the MAPK Pathway in Skeletal Muscle Cells

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In this study, the roles of the p38 MAPK, ERK1/2 and JNK signaling pathway in IGF-I-induced AR induction and activation were examined. C2C12 cells were treated with IGF-I in the absence or presence of various inhibitors of p38 MAPK (SB203580), ERK1/2 (PD98059), and JNK (SP600125). Inhibition of the MAPK pathway with SB203580, PD98059, or SP600125 significantly decreased IGF-I-induced AR phosphorylation and total AR protein expression. IGF-I-induced nuclear fraction of total AR and phosphorylated AR were significantly inhibited by SB203580, PD98059, or SP600125. Furthermore, IGF-I-induced AR mRNA and skeletal α-actin mRNA were blocked by those inhibitors in dose-dependent manner. Confocal images showed that IGF-I-induced AR nuclear translocation from cytosol was significantly blocked by SB203580, PD98059, or SP600125, suggesting that the MAPK pathway regulates IGF-I-induced AR nuclear localization in skeletal muscle cells. The present results suggest that the MAPK pathways are required for the ligand-independent activation of AR by IGF-I in C2C12 skeletal muscle cells.

INTRODUCTION

Androgens are required for the development, growth, and function of skeletal muscle cells. Androgen action is mediated by the androgen receptor (AR), which is a transcription factor known to play an important role in the regulation of target genes in skeletal muscle cells. Although AR is regarded as a ligand-dependent activated transcription factor, it has been widely studied that AR can also be activated by ligand-independent mechanisms in various cell types (Culig et al., 1994). The AR can be activated by several growth factors, modulation of protein kinase pathways, Rho GTPase, and interleukin-6 (Ueda et al., 2002).

Insulin-like growth factor-I (IGF-I) is well known regulator of skeletal muscle cells in terms of protein synthesis, satellite cells activation, development, and differentiation. Mitogen-activated protein kinase (MAPK) is one of the major downstream signaling pathways activated by IGF-I (Butler et al., 1998; Meng et al., 2007). The MAPK family of kinases transmits various stimuli from the extracellular and cytoplasmic compartments to the nucleus. The MAPK family mainly consists of three subfamilies, including extracellular regulated kinase 1 and 2 (ERK1/2), p38 MAPK, and Jun NH_2-terminal kinase (JNK) pathways. IGF-I leads to the development of skeletal muscle hypertrophy through activation of the ERK1/2 pathways (Haddad et al., 2004). Additionally, p38 MAPK activity has been found to be sustained at elevated levels in response to overload-induced skeletal muscle hypertrophy (Carlson et al., 2001). These pathways are known to be activated by IGF-I and play an important role in the development of skeletal muscle (Carlson et al., 2001; Haddad et al., 2004; Keren et al., 2006; Wen et al., 2000). Because both 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; McLallen et al., 2006; Wannenes et al., 2008), it is speculated that there may be an interaction between IGF-I and AR in skeletal muscle cells.

Previously, we showed that IGF-I stimulates the expression and activation of AR by ligand-independent mechanism in differentiating C2C12 mouse skeletal muscle cells (Kim and Lee, 2009). However, the modulating effect of MAPK signaling pathways on the ligand-independent regulation of AR gene expression and activation in skeletal muscle remains poorly understood. Therefore, the role of the p38 MAPK, ERK1/2, and JNK signaling pathways in IGF-I-induced AR activation in cultured C2C12 cells was examined in this study.

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 experi-

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ments, 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 p38 MAPK (SB203580), ERK1/2 (PD98059), and JNK (SP600125). 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: 2,000), phospho-AR^{ser213} (monoclonal mouse antibody, 1:1,000) (Santa Cruz Biotechnology, USA), or α-tubulin (monoclonal mouse antibody, 1:2,500) (Calbiochem, USA) antibodies overnight at 4°C in 1% skim milk in Tris-buffered saline (TBS) with 0.05% Tween20. For the detection of ERK1/2, phosphor-ERK1/2, p38 MAPK, phospho-p38 MAPK, the membranes were blocked with 5% skim milk in TBS with 0.1% Tween20 for 1 h at room temperature and subsequently incubated with ERK1/2 (polyclonal rabbit antibody, 1:1,000), and phospho-ERK1/2$^{(Thr202/Tyr204)}$ (polyclonal rabbit antibody, 1:4,000), p38 MAPK (polyclonal rabbit antibody, 1:2,000), phospho-p38 MAPK$^{(Thr180/Tyr182)}$ (polyclonal rabbit antibody, 1:1,000) (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 (1:2,500) (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

Effects of IGF-I-induced downstream signaling pathways on AR induction and activation

Steroid receptors are generally considered as ligand dependent transcription factors. However, the AR can be activated by several growth factors, modulation of protein kinase pathways, and interleukin-6 (Ueda et al., 2002). Previous reports suggest that IGF-I has an ability to modulate AR gene in several other cell types (Lin et al., 2001; Taneja et al., 2005; Wen et al., 2000; Wu et al., 2006). However, the results of previous studies showing the modulating effect of IGF-I on AR are controversial. Some studies report that IGF-I activates AR in the absence of ligand (Lin et al., 2001; Wen et al., 2000), whereas other studies fail to show ligand-independent activation of AR by IGF-I depending on cell types (Taneja et al., 2005; Wu et al., 2006). Previously, we demonstrate that IGF-I mediates the activation of AR in the absence of ligand in differentiating C2C12 cells (Kim and Lee, 2009). However, the molecular mechanisms associated with IGF-I regulation of AR induction and activation in skeletal muscle cells has not been studied. Therefore, in the present study, we examined the MAPK pathways to determine if they are involved in the induction and activation of AR in IGF-I-stimulated C2C12 cells by treating the cells with the specific the p38 MAPK inhibitor SB203580, ERK1/2 inhibitor PD98059, or JNK inhibitor SP600125. Although previous study reports that AR activation by interleukin-6 in the absence of ligand depends on the MAPK pathways and is enhanced by MAPK-directed phosphorylation of the steroid receptor coactivator-1 in prostate cancer cells (Ueda et al., 2002), the role of the MAPK pathway on IGF-I-induced AR activation in skeletal muscle cells are unknown. As shown in Fig. 1A, IGF-I-induced total AR and AR phosphorylation were significantly blocked by the specific p38 MAPK inhibitor SB203580 in a dose-dependent manner, suggesting that p38 MAPK signaling pathway at least in part regulates IGF-I-induced AR induction and activation in C2C12 cells.

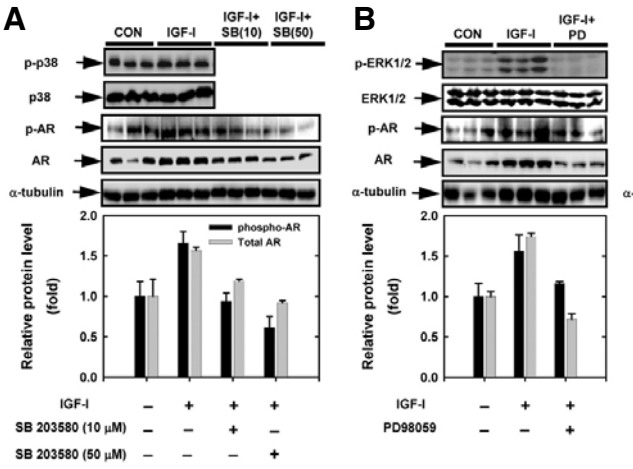

Fig. 1. Effect of treatment with 250 ng/ml IGF-I for 30 min in the absence or presence of 10 μM or 50 μM SB203580 (A), 30 μM of PD98059 (B), or 5 μM or 10 μM of SP600125 on the phospho-AR (Ser 213) and total AR protein levels in C2C12 cells. Sixty micrograms of protein were fractionated by 7% SDS-PAGE, after which they were immunobloted with phospho-AR (Ser 213) or AR antibodies. Values are means ± SE with n = 3 for each condition.

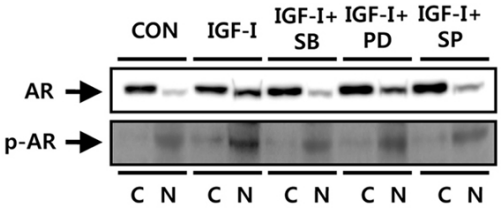

Fig. 2. Phosphorylated AR and total AR in cytosol (C) and nuclear fractions (N) of C2C12 cells after treatment of IGF-I (250 ng/ml) for 30 min in the absence or presence of SB203580 (10 μM), PD98059 (30 μM), or SP600125 (10 μM).

Furthermore, pharmacological inhibitors of ERK1/2 significantly blocked IGF-I-induced AR induction and activation (Fig. 1B). JNK inhibitor SP600125 also suppressed IGF-I-induced phosphorylated AR and total AR protein expression in dose-dependent manner. Taken together, these results suggest that the induction and activation of AR in skeletal muscle cells is regulated by IGF-I and that this effect of IGF-I is at least in part by coactivation of the p38 MAPK, ERK1/2, and JNK pathways.

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 SB203580, PD98059, or SP600125. Inactive AR is normally localized to the cytoplasmic compartment of target cells (Tyagi et al., 2000). However, when AR is activated by ligand-dependent or ligand-independent mechanism, translocation of activated AR to the nucleus is induced by the nuclear localization signal binding to importin proteins (Roy et al., 2001). In the nucleus, AR can bind to its specific DNA element and interact with the transcription initiation complex to induce the expression of target gene (Xu et al., 1999). 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. Our data clearly showed that nuclear fraction of total AR and phosphorylated AR were significantly induced by IGF-I, suggesting that IGF-I stimulates AR activation in C2C12 cells. IGF-I-mediated nuclear total AR and phophsphorylated AR expression was significantly suppressed by SB203580, PD98059, or SP600125 (Fig. 2). Taken together, these data suggest that the MAPK pathway, a downstream signaling pathway of IGF-I, modulates AR in the absence of ligand primarily in nuclear compartment and requires IGF-I-induced AR induction and activation in C2C12 cells.

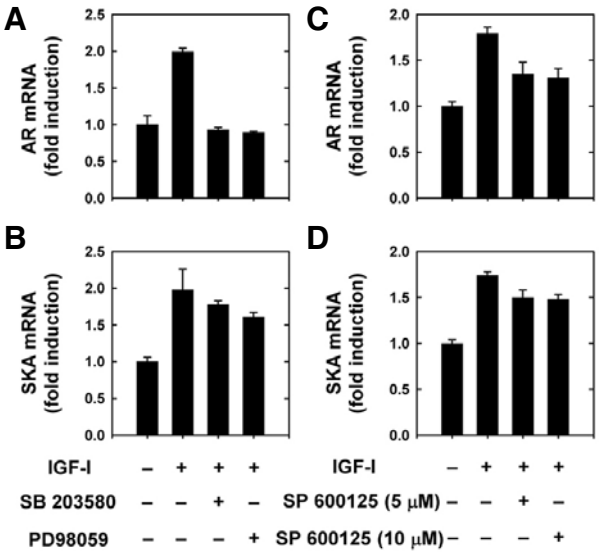

Fig. 3. (A) effect of SB203580 (10 μM) and PD98059 (30 μM) on IGF-I-induced AR mRNA. (B) effect of SB203580 (10 μM) and PD98059 (30 μM) on IGF-I-skeletal α-actin mRNA. C2C12 cells were treated with 250 ng/ml of IGF-I for 5 min. Effect of SP600125 (5 μM or 10 μM) on AR mRNA (C) and skeletal α-actin (D) mRNA levels in C2C12 cells cultured for 5min with 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.

Effects of the MAPK pathway on IGF-I-induced AR mRNA and skeletal α-actin mRNA expression

Previous study reports that testosterone increases AR mRNA accumulation in rat ventral prostate, suggesting pretranslational regulation of AR by its ligand (Mora and Mahesh, 1999). Recently, we examined ligand-independent regulation of AR mRNA by IGF-I in skeletal muscle cells (Kim and Lee, 2009). The data of the present study confirms the result of previous finding, suggesting pretranslational regulation of AR by IGF-I in the absence of ligand. As shown in Figs. 3A and 3C, IGF-I significantly increases AR mRNA expression in the absence of ligand. Furthermore, we examined the role of MAPK pathways on IGF-I-induced AR mRNA expression. The induction of AR mRNA following IGF-I treatment in C2C12 cells was significantly blocked

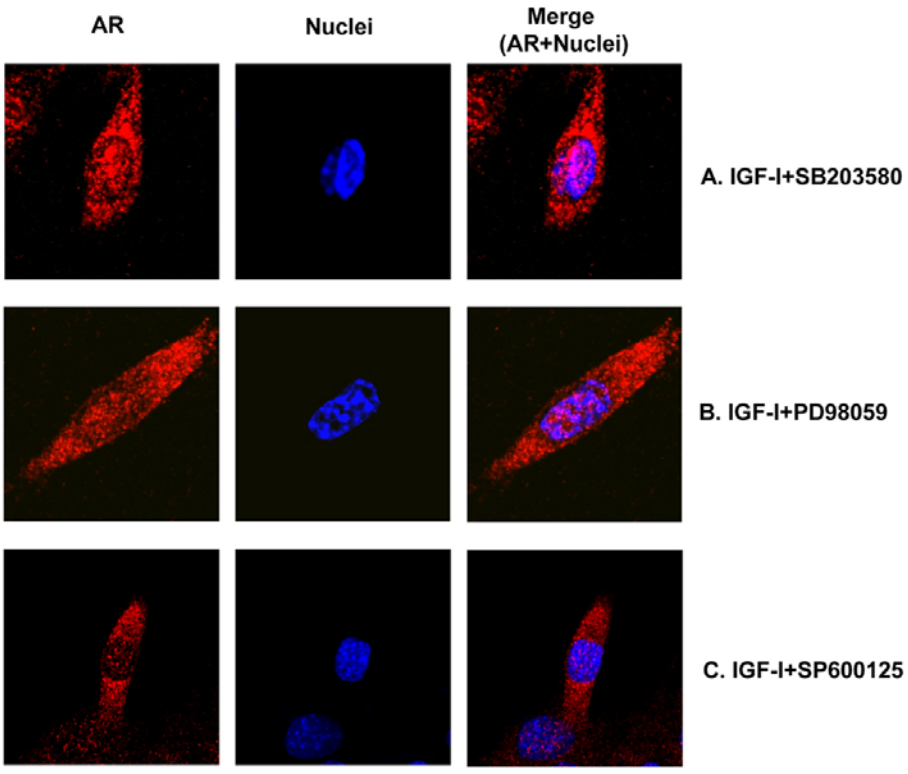

Fig. 4. Confocal image showing the effect of inhibitors of the MAPK pathway on IGF-I-induced AR translocation into the nucleus in C2C12 cells. All images were acquired at 30 min after treatment. (A) C2C12 cells treated 250 ng/ml of IGF-I+SB203580 (10 μM). (B) Medium containing 250 ng/ml of IGF-I+PD98059 (30 μM). (C) Medium containing 250 ng/ml of IGF-I+ SP 600125 (10 μM).

by SB203580, PD98059, or SP600125. 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 modulating effect of the MAPK pathway on IGF-I-induced AR target genes in skeletal muscle has not been well described. As shown in Fig. 3B, the induction of skeletal α-actin mRNA following IGF-I treatment in C2C12 cells was significantly blocked by SB203580 or PD98059. Inhibition of JNK pathway by SP600125 also suppressed IGF-I-induced skeletal α-actin mRNA expression in dose-dependent manner (Fig. 3D). Taken together, these data suggest that the MAPK pathway is required for the pretranlational regulation of AR and its target gene by IGF-I by demonstrating that pharmacologic inhibition of the MAPK pathway suppresses IGF-I-induced AR mRNA and skeletal α-actin mRNA.

The modulating effect of the MAPK pathway on AR nuclear localization

AR phosphorylation is necessary for nuclear translocation of AR. At steady state the AR is predominantly cytoplasmic in the absence of IGF-I and nulear in the presence of IGF-I (Kim and Lee, 2009). Although our data clearly demonstrate that the MAPK pathways is involved in AR activation in the absence of ligand by IGF-I, it is not known whether the MAPK pathway regulates AR nuclear shuttling. Therefore, we evaluated the modulating effect of the MAPK pathway 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 SB203580, PD98059, or SP600125 (Fig. 4). IGF-I-induced AR nuclear translocation from cytosol was significantly blocked by SB203580, PD98059, or SP600125 (Figs. 4A, 4B, and 4C). Taken together, these data indicate that the MAPK pathway regulates IGF-I-induced AR nuclear localization in skeletal muscle cells.

The data in the present study suggest that multiple downstream signaling pathways of IGF-I may play a significant role in IGF-I-induced AR induction and activation. To our knowledge this is the first report showing that the MAPK pathways are required for the ligand-independent activation of AR by IGF-I in C2C12 skeletal muscle cells. Although whether a possible interaction between the MAPK pathways and PI3K/Akt pathway in IGF-I-induced AR activation remains to be determined, the data of present study clearly demonstrate that suppression of the MAPK signaling pathways reduces the activation of AR gene under IGF-I-induced conditions, suggesting that these three pathways converge at a downstream point. It is possible that three MAPK signaling pathways are required to activate a common transcription factor complex to induce AR activation in IGF-I treated C2C12 cells.

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