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 N-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% CO₂ 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 x 10⁵ cells/well in growth medium (DMEM, 10% FBS). For all experi...
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 (polycional rabbit antibody, 1:2,000), phospho-ARpSer513 (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, phospho-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 (polycional rabbit antibody, 1:1,000), and phospho-ERK1/2pThr202/Tyr204 (polycional rabbit antibody, 1:4,000), p38 MAPK (polycional rabbit antibody, 1:2,000), phospho-p38 MAPKpThr180/Tyr182 (polycional 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 they were treated with ECL/Advance reagent (GE Healthcare UK Ltd., UK) as 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, reverse inhibitor, and reaction 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′-CGCTCCTCTTCTCTCCA3′; and reverse (R) 5′-ATGTTCCCGAACAAAACTC-3′; skeletal muscle α-actin (F) 5′-GCCCAAGATCTCAGTGCTGA-3′, (R) 5′-CAGATTGTTGATGTGGCTC-3′; GAPDH (F) 5′-ATGACA-ATGATACCGCTACAGCAA-3′, (R) 5′-GCAAGGGAATTTAT-TGATGATT-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 glyceraldehyde 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-phenylindole (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; Tanet 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 (Tan 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 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.