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PKC and PTPα participate in Src activation by 1α,25(OH)$_2$ vitamin D$_3$ in C2C12 skeletal muscle cells

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ABSTRACT

We previously demonstrated that 1α,25(OH)$_2$-vitamin D$_3$ [1α,25(OH)$_2$D$_3$] induces Src activation, which mediates the hormone-dependent ERK1/2 and p38 MAPK phosphorylation in skeletal muscle cells. In the present study, we have investigated upstream steps whereby 1α,25(OH)$_2$D$_3$ may act to transmit its signal to Src. Preincubation with the PKC inhibitor Ro318220 demonstrated the participation of PKC in 1α,25(OH)$_2$D$_3$-dependent Src activation. Of interest, the hormone promoted the activation of δ the isoform of PKC. We also explored the role of PTPα in PKC-mediated Src stimulation. Silencing of PTPα with a specific siRNA suppressed Src activation induced by 1α,25(OH)$_2$D$_3$. Hormone treatment increased PTPα (Tyr789) phosphorylation and PKC-dependent phosphatase activity. Accordingly, 1α,25(OH)$_2$D$_3$ promoted serine phosphorylation of PTPα in a PKC-dependent manner. Confocal immunocytochemistry and co-immunoprecipitation assays revealed that the hormone induces the co-localization of Src and PTPα with PKC participation. Computational analysis revealed that the electrostatic interaction between Src and PTPα is favored when PTPα is phosphorylated in Tyr789. These data suggest that 1α,25(OH)$_2$D$_3$ acts in skeletal muscle upstream on MAPK cascades sequentially activating PKC, PTPα and Src.

1. Introduction

1α,25-Dihydroxyvitamin D$_3$ [1α,25(OH)$_2$D$_3$] is a secosteroid hormone whose biological actions extend far beyond its known effects on mineral metabolism (Walters, 1992). Of interest, this hormone acts as a key modulator of skeletal muscle growth and contractility (Smith and Stern, 1969; Boland et al., 1995, 2005). In common with other steroid hormones, 1α,25(OH)$_2$D$_3$ modulates gene expression via its specific intracellular receptor (VDR) (Minghetti and Norman, 1988) and also induces fast non-transcriptional responses through transmembrane signal transduction pathways (de Boland and Nemere, 1992). Although the 1α,25(OH)$_2$D$_3$-dependent rapid responses have been studied in various tissues (Civitelli et al., 1990; Norman et al., 1999; Boland et al., 2002), the non-genomic pathways underlying these actions have not been completely elucidated. Among them, and relevant to the present work, the mitogen-activated protein kinase (MAPK) cascades, specifically ERK1/2 and p38 MAPK, are required to modulate proliferation and differentiation of skeletal muscle cells (Lee et al., 2009). Of relevance, skeletal muscle development is regulated by p38 MAPK (Keren et al., 2006). These signaling routes comprise a cascade of three kinases (MAPKKK, MAPKK, MAPK) that are activated by sequential phosphorylation and by inputs of lateral molecules, like PKC and Src, among others (Kansra et al., 1999). Our previous investigations involved PKC and Src in 1α,25-(OH)$_2$D$_3$ stimulation of ERK1/2 and p38 MAPK in proliferative skeletal muscle cells (Morelli et al., 2001; Buitrago et al., 2006). Moreover, Src-ERK1/2-p38 MAPK pathway activation has been linked to 1α,25(OH)$_2$D$_3$ administration in other tissues as well (Gniadecki, 1998). Altogether, these observations pointed that Src has a relevant role in modulation of MAPKs by the vitamin D hormone.

Src activation comprises first a dephosphorylation on its Tyr530 residue (Tyr527 in chicken), which opens up its folded conformation, and then this kinase undergoes autophosphorylation on Tyr419 (Tyr416 in chicken) to achieve its complete catalytic activity (Piwnica-Worms et al., 1987). Different mechanisms of Src activation have been described. They may involve G proteins and integrins associated complexes (Luttrell and Luttrell, 2004; New and Wong, 2007) or can be related to PKC and PTP (protein tyrosine phosphatase) upstream action (Brandt et al., 2003). PKCα and PKCδ, both responsive to phorbol esters, are expressed in skeletal muscle cells (Sampson and Cooper, 2006) and, of our interest, they have a close relationship with Src in different tissues (Steinberg,

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2004; Shah et al., 2003). It has been shown that PKCδ is involved in Src activation through phosphorylation and stimulation of PTPα in rat aortic smooth muscle cells (Brandt et al., 2003; Harper and Sage, 2006).

PTPα is a transmembrane protein of 130 kDa activated in muscle cells by different extracellular stimuli and it is a recognized physiological regulator of Src Tyr530 dephosphorylation (Zheng et al., 2002; Pallen, 2003). The complete mechanism of PTPα activation is unclear. Some reports indicate that PTPα becomes activated after phosphorylation of serine residues by PKC (den Hertog et al., 1995; Zheng et al., 2002). However, the relationship between PTPα and PKC-Src activation has not been investigated for any steroid hormone. In view of the above evidence, the aim of this work was to study the participation of PKC and PTPα as mediators of Src stimulation induced by $1\alpha,25(OH)_2D_3$ in skeletal muscle cells. Specifically, we investigated whether the hormone sequentially promotes PKC–PTPα–Src activation in C2C12 skeletal muscle cells.

2. Materials and methods

2.1. Chemicals

$1\alpha,25(OH)_2D_3$ was kindly provided by Dr. Jan-Paul van de Velde from Solvay Pharmaceuticals (Weesp, The Netherlands). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Protein A Sepharose (PAS) and OAG (1-oleoyl-2-acetyl-sn glycerol), the analog of diacylglycerol used in this work as a PKC activator, were from Sigma–Aldrich Co. (St. Louis, MO, USA). Anti-phospho p38 MAPK, anti-PTPα, anti phospho (Tyr530) Src, anti-phospho serine, PTPα siRNA, transfection reagents and secondary antibodies (goat anti-rabbit and goat anti-mouse horse radish peroxidase-conjugated IgG) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho ERK1/2, anti Src, anti-phospho (Tyr416) Src, anti-PKC, anti-phospho (Thr505) PKCδ and anti-phospho (Tyr789) PTPα antibodies were acquired in Cell Signaling Technology, Inc. (Beverly, MA, USA). Alexa Fluor 488 goat anti-rabbit antibody and anti-mouse antibody conjugated with rhodamine were from Invitrogen Corporation (Carlsbad, CA, USA). The Super Signal CL-HRP substrate system for enhanced chemiluminiscence (ECL) was from Perkin Elmer (Boston, MA, USA). The C2C12 cell line (American Type Culture Collection, Manassas, VA) was kindly provided by Dr. E. Jaimovich (Universidad de Chile, Santiago, Chile). The compounds TPA (12-O-tetradecanoylphorbol-13-acetate, a phorbol ester activator of some isoforms of the PKC family), PP2, a Src inhibitor, Ro318220 and Calphostin C (both inhibitors of PKC) were from Calbiochem-Novabiochem Corp. (La Jolla, CA, USA). All other reagents were of analytical grade.

2.2. Cell culture

The murine skeletal myoblastic cell line C2C12 was seeded at an appropriate density (120,000 cells/cm^2) in Petri dishes (100 mm diameter) with DMEM supplemented with 10% FBS and 1% antibiotic–antimycotic solution. Cells were cultured at 37 °C in a humidified atmosphere (95% air/5% CO_2). Under these conditions, myoblasts divide within the first 48 h and at day sixth become differentiated into myotubes expressing morphological characteristics of adult skeletal muscle fibers (Burattini et al., 2004). Undifferentiated cells cultured for two days (at 70% confluence) were used. Before each treatment, cultures were deprived of serum during 60 min to stabilize the cells.

Treatments of cultures were performed in the absence and presence of inhibitors, using 1 nM $1\alpha,25(OH)_2D_3$ or its vehicle, isopropanol (0.001%). Dose–response studies have previously shown that this hormone concentration induces maximal activation of ERK1/2 and p38 MAPK in cultured proliferating avian and murine skeletal muscle cells (Morelli et al., 2000; Buitrago et al., 2006).

2.3. Preparation of cell lysates

Cell cultures were lysed using a buffer composed of 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.2 mM Na_2VO_4, 2 mM EDTA, 25 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% NP40, 20 μg/ml leupeptin and 20 μg/ml aprotinin. The lysate was collected by aspiration and centrifuged at 12,000 × *g* during 15 min. The protein content of the supernatant was quantified by the Bradford procedure (Bradford, 1976).

2.4. SDS-PAGE and immunoblotting

Lysate proteins dissolved in Laemmli sample buffer (Laemmli et al., 1970) were separated on SDS-polyacrylamide (10%) gels, and electrotransferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked for 1 h at room temperature in TBST buffer (50 mM Tris–HCl, pH 7.4, 200 mM NaCl, 0.1% Tween-20) containing 5% dry non-fat milk. Membranes were subjected to immunoblotting using different primary antibodies. Next, the membranes were washed three times with TBST, incubated in TBST containing 1% dry milk with a 1:5000 or 1:10,000 dilution of peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies for 1 h at room temperature and washed three additional times with TBST. The membranes were then visualized using an enhanced chemiluminiscent technique (ECL) and KODAK films, according to the manufacturer's instructions. Images were obtained with a model GS-700 Imaging Densitomer from Bio-Rad (Hercules, CA, USA) by scanning at 600 dpi and printing at the same resolution. Bands were quantified using the Molecular Analyst program (Bio-Rad).

To strip the membranes for reprobing with other antibodies, the membranes were washed 10 min in TBST and then incubated in stripping buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS and 50 mM β-mercaptoethanol) for 30 min at 55 °C. The membranes were again blocked and blotted as described above.

2.4.1. Time-dependence of $1\alpha,25(OH)_2D_3$-induced Src activation

Previously, by Western blot analysis, we have reported Src activation by $1\alpha,25(OH)_2D_3$ in C2C12 muscle cells at 1–2 min (Buitrago et al., 2006) and at 60 min of treatment (Buitrago et al., 2010). The time-dependence of Src activation by the hormone showed that $1\alpha,25(OH)_2D_3$ increases Tyr416 phosphorylation of Src at 1 min, then the effect was either less pronounced or absent between 3 and 30 min, followed by a highest response at 60 min. Therefore, we performed the successive experiments in this work exposing the muscle cells to $1\alpha,25(OH)_2D_3$ during 60 min.

2.5. Short interfering RNA

Specific knockdown of PTPα expression was conducted using a commercial pool of short interfering RNAs (PTPα siRNA; Santa Cruz Biotechnology). C2C12 cells were transfected with 50 pmol PTPα siRNA or control siRNA (scrambled sequence that will not lead to the specific degradation of any known cellular mRNA) for 6 h using Transfection Medium and Reagent according to the manufacturer's specifications. Transfected cells were incubated for an additional 24 h in fresh medium until treatments were performed.

2.6. Co-immunoprecipitation assays

Co-immunoprecipitation assays were performed using total C2C12 muscle cell extracts according to a protocol for the analysis of putative protein–protein interactions in eukaryotic cells. After treatments, cells were lysed at 4 °C in 50 mM Tris–HCl pH 7.4, 3 mM KCl, 150 mM NaCl, 0.5 mM EDTA, 1% Tween 20, 0.1% SDS and 1% NP-40 with freshly added 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF, 10 μg/ml aprotinin and 10 μg/ml leupeptin. Lysates were clarified by centrifugation (14,000 × *g*, 10 min, 4 °C) and the amount of proteins in the supernatants was measured by the Bradford procedure (Bradford, 1976). One microgram of anti-Src antibody in lysis buffer was added to 10% (v/v) protein A Sepharose and incubated for 2 h at 4 °C on a rotary mixer. Lysates containing 200 μg of protein were added to the above mixture and incubated overnight at 4 °C with slow agitation. As a negative control, protein A Sepharose was incubated with 200 μg protein in the absence of antibody. The precipitated immunocomplexes were washed four times with cold lysis buffer and then resuspended in Laemmli sample buffer followed by SDS-PAGE.

2.7. Immunocytochemistry

C2C12 cells grown onto glass coverslips were permeabilized and fixed in methanol (at −20 °C) for 20 min. Non-specific sites were blocked with 5% BSA in PBS for 1 h. Samples were then incubated with the appropriate primary antibody prepared in 2% BSA in PBS (1:50, 1 h, room temperature). After washing with PBS, the samples were incubated with secondary Alexa Fluor 488 goat anti-rabbit IgG or rhodamine goat anti-mouse conjugated antibodies (1:200, 1 h, room temperature). The samples were examined using a Zeiss LSM 5 Pascal confocal laser microscope.

2.8. PTPα activity assay

The method described by Brandt et al. (2003) was used. Cells treated with the hormone (with or without PKC inhibitor or activators) were lysed on ice in RIPA buffer containing 50 mM Tris pH 7.4, 150 mM NaCl, 10 mM MgCl, 1% Triton, 0.1% SDS, 0.5% deoxycholate, 1 mM EDTA, 1 mM NaF, 1 μg/ml leupeptin and 1 mM PMSF. Lysates were clarified by centrifugation for 10 min at 14,000 × *g*. Cleared lysates with 200 μg of total proteins were incubated overnight at 4 °C with Protein A Sepharose (10%, v/v) and anti-PTPα antibody (1–2 μg). Precipitated PTPα immunocomplexes were washed once with RIPA buffer (now containing 500 mM NaCl) and twice with assay buffer (100 mM Tris pH 7.0, 150 mM NaCl, 1 mM EDTA) followed by incubation for 30 min at room temperature in 200 μl of assay buffer containing 10 μM p-nitrophenyl phosphate (pNPP). PTPα immunocomplexes were pelleted by centrifugation, 15 μl of sample buffer were added, and the amount of PTPα was estimated by immunoblotting using anti-PTPα antibody. The supernatant was mixed with 400 μl of 2 N NaOH and the absorbance at 415 nm was measured to determine PTPα activity using a standard curve made with p-nitrophenol.