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Role of estrogen receptors, PKC and Src in ERK2 and p38 MAPK signaling triggered by 17β-estradiol in skeletal muscle cells

Ana C. Ronda, Claudia Buitrago, Ricardo Boland *

Departamento de Biología, Bioquímica & Farmacia, Universidad Nacional del Sur, San Juan 670, 8000 Bahía Blanca, Argentina

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ABSTRACT

We have previously reported in C2C12 murine skeletal muscle cells that 10^{-8} M 17β-estradiol promotes MAPKs stimulation which in turn mediates the activation of CREB and Elk-1 transcription factors. In this work, we demonstrated that the hormone induces ERK2 phosphorylation (without affecting ERK1 activation) and also stimulates p38 MAPK, both in a dose-dependent manner. Moreover, estrogen receptors involvement in MAPKs activation by the estrogen was studied. The use of ICI182780 (1 μM), an antagonist of ERs, and specific siRNAs to block ERα and ERβ expression, demonstrated that ERα mediates ERK2 activation but not p38 MAPK phosphorylation by 17β-estradiol, and that ERβ isoform is not implicated in MAPKs activation by the hormone. Furthermore, Src and PKC contribution in estrogen stimulation of the MAPKs was investigated. Compounds PP2 and Ro318220, Src and PKC family inhibitors, respectively abrogated ERK2 and p38 MAPK phosphorylation by 17β-estradiol. Of interest, the hormone was able to induce Src and PKCδ activation. In addition, Ro318220 decreased estrogen-dependent Src modulation implicating PKC in hormone upregulation of Src. Accordingly, PP2 and Ro318220 suppressed CREB and Elk-1 phosphorylation as well as c-Fos and c-Jun oncoprotein levels induced by 17β-estradiol. Altogether, these data indicate that 17β-estradiol activates ERK2 through ERα and p38 MAPK in an ERα/β-independent manner and that PKC and Src proteins are key upstream components on MAPKs activation in C2C12 skeletal muscle cells.

1. Introduction

The steroid hormone 17β-estradiol (E2) acts in target cells by two different mechanisms. A genomic mode of action is related to long-term responses where E2 binds to the estrogen receptors (ERs) in the nucleus that function as ligand-dependent transcription factors regulating gene expression. The non-genomic mechanism is associated with rapid actions of E2 that regulate signal transduction pathways. In many cases, these events appear to be initiated at plasma membrane level. There is growing evidence that a subpopulation of the traditional ER localized at the cell membrane mediates some of these signaling events [1]. Other reports show that novel membrane receptors, unrelated to the classical ER, are implicated in non-transcriptional effects of the hormone [2]. We have demonstrated in the mouse skeletal muscle cell line C2C12 that ERβ is localized mainly in mitochondria and ERα in endoplasmic reticulum and the perinuclear zone and to lesser extent in mitochondria [3]. Recently, Galluzzo et al. have reported that ERα-mediated signals regulate first steps of skeletal muscle cells differentiation [4]. Of relevance, it has been established that depending on the cellular type, mitogen-activated protein kinase (MAPK) pathways can be stimulated by E2 through α or β isoform of ER [5].

Many rapid responses of E2 are mediated by MAPKs, specifically ERK1/2 and p38 MAPK [6]. These pathways comprise central components of signal transduction cascades which regulate cell growth, survival and differentiation [7]. We have previously shown that short treatment with 10^{-8} M E2 induces stimulation of ERKs and p38 MAPK in C2C12 muscle cells [8]. In E2-dependent activation of MAPKs in mouse neocortex, the participation of Src has been reported [9]. Moreover, there is evidence that PKC also mediates upstream stimulation of MAPKs induced by the estrogen in chondrocytes [10]. Of relevance, it has been shown that PKCδ activates Src through stimulation of the protein tyrosine phosphatase α (PTPα) in aortic smooth muscle cells and in platelets treated with TPA [11,12]. However, the participation of PKC in Src activation by E2 has not been investigated in skeletal muscle or any other cell type. Furthermore, the role of both PKC and Src in E2 modulation of MAPKs in muscle cells has not been established.

After being stimulated, ERK1/2 and p38 MAPK translocate to the nucleus where they activate several transcription factors [13]. We have previously reported in C2C12 muscle cells that E2 phosphorylates the cAMP response element binding protein (CREB) and the ETS-domain transcription factor Elk-1 through activation of ERKs and p38 MAPK [8]. Phosphorylation of CREB and Elk-1 may induce

* Corresponding author. Tel.: +54 291 4595101x2430; fax: +54 291 4595130.
E-mail address: rboland@criba.edu.ar (R. Boland).

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the expression of *c-fos* and *c-jun* early proto-oncogenes whose protein products interact with the AP-1 binding site of DNA regulating distinct biological functions [14]. E2 has been shown to increase c-Fos levels in myoblasts [15] and increases c-Jun expression in uterine tissue [16]. Recently, we demonstrated that E2 modulates c-Fos expression through MAPKs signaling pathways in C2C12 cells [8].

Although estrogens are known as regulators of the development of the skeletal muscle phenotype [17,18], the intracellular mechanisms involved in the rapid effects of E2 on skeletal muscle cells are scarcely known. Therefore, the aim of the present work was to investigate the involvement of ERs, PKC and Src in MAPK activation and downstream in CREB and Elk-1 phosphorylation and c-Fos and c-Jun expression in the signaling triggered by E2 in C2C12 cells.

2. Materials and methods

2.1. Chemicals

17β-Estradiol and fetal bovine serum (FBS) were from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), low glucose, with L-glutamine and HEPES, without phenol red, was from US Biological (Swampscott, MA, USA). Anti-phospho-p38 MAPK, anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-Src (Tyr416), anti-phospho-CREB, anti-phospho-Elk-1, anti-c-Jun, and anti-c-Fos antibodies were from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-active PKCδ [anti-phospho PKCδ (Thr505) antibody], secondary antibodies goat anti-rabbit, goat anti-mouse horse radish peroxidase-conjugated IgG, anti-ERα, anti-ERβ and anti-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Enhanced Chemiluminiscence Plus Western blotting detection reagents were from GE Healthcare (Anaheim, CA, USA). Protein size markers were from Amersham Biosciences (Piscataway, NJ, USA). The C2C12 cell line (American Type Culture Collection, Manassas, VA) was kindly provided by Dr. E. Jaimovich (Universidad de Chile, Santiago, Chile). U0126, SB203580 and ICI182780 were from Tocris Cookson Ltd. (Bristol, UK). PP2 and Ro318220 were from Calbiochem-Novabiochem Corp. (La Jolla, CA, USA). ERα-siRNA and ERβ-siRNA were from New England Biolabs (NEB, Beverly, MA, USA). All other reagents were of analytical grade.

2.2. Cell culture

C2C12 murine skeletal muscle cells were seeded at an appropriate density (120,000 cells/cm^2) in Petri dishes (100 mm diameter) with DMEM without phenol red supplemented with 10% FBS and antibiotic-antimycotic solution at 37 °C under a humidified atmosphere (95% air/5% CO2). Undifferentiated cells cultured for two days were used. Before each treatment, cells were deprived of serum for 30 min. During this preincubation the cells were exposed to inhibitors in the indicated experiments. All cell treatments were carried out in phenol red-free medium without serum.

2.3. SDS-PAGE and immunoblotting

Cells were lysed using a buffer made 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 PMSF, 1% NP40, leupeptin 20 μg/ml and aprotinin 20 μg/ml. Lysates were collected by aspiration and centrifuged at 12,000 × *g* during 15 min. The protein content of the supernatant was quantified by the Bradford procedure [19]. Lysate proteins dissolved in Laemmli sample buffer [20] were separated on SDS-polyacrylamide (10%) gels and electrotransferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked 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 milk. Membranes were subjected to immunoblotting using different primary antibodies overnight at 4 °C. Membranes were then washed three times in TBST, incubated in TBST containing 1% dry milk with peroxidase-conjugated secondary antibody for 1 h at room temperature and washed again three times with TBST. Next, membranes were visualized using an enhanced chemiluminiscent technique (ECL) according to the manufacturer's instructions. Images were obtained with a GS-700 Imaging Densitomer from Bio-Rad (Hercules, CA, USA) by scanning at 600 dpi. 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, incubated in stripping buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS and 50 mM mercapthoethanol) for 30 min at 55 °C, washed 10 min in TBST and then blocked and blotted as described above.

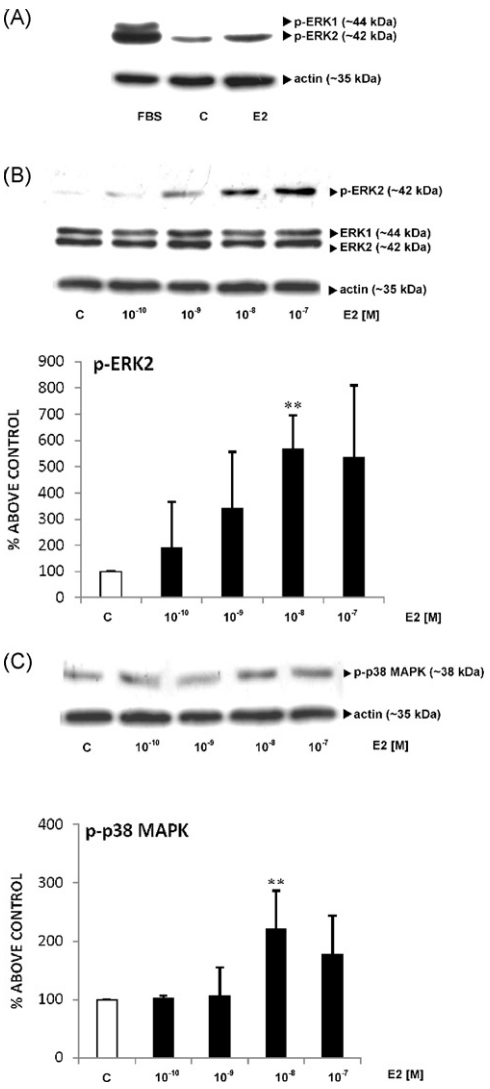

Fig. 1. 17β-Estradiol induces ERK2 and p38 MAPK activation in a dose-dependent manner. C2C12 cells incubated in serum-free medium were treated during 15 min with (A) 10% SFB, 10^{-8} M 17β-estradiol (E2) or vehicle (C: 0.001% isopropanol) or with (B and C) different doses of E2 (10^{-10} to 10^{-7} M) or vehicle. Western blot analyses were performed using anti-phospho-ERK1/2, anti-ERK1/2 and anti-p38 MAPK antibodies. Actin levels are shown as loading control. Immunoblots quantified by scanning volumetric densitometry were normalized with actin levels. Averages ± SD from two independent experiments are given. $^{**}p < 0.01$ respect to the control.

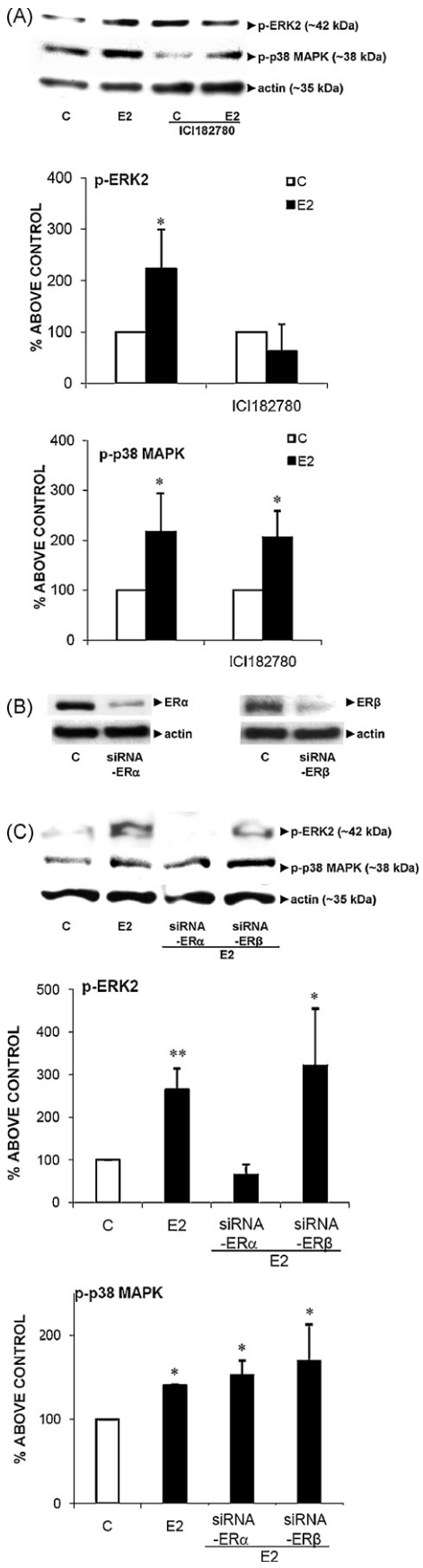

Fig. 2. ERs involvement in ERK2 and p38 MAPK phosphorylation induced by 17β-estradiol. (A) C2C12 cells incubated in presence or absence of ICI172780 1 μM were treated with 10^{-8} M E2 or its vehicle during 15 min. (B and C) Cells were transfected with ERα-siRNA or ERβ-siRNA as described in Materials and Methods. (C) 24 h later cells were exposed to 10^{-8} M E2 or its vehicle during 15 min. Western blot analyses were performed using anti-ERα, anti-ERβ, anti-phospho-ERK1/2 and anti-p38 MAPK antibodies. Immunoblots quantified by scanning volumetric densitometry were normalized with actin levels. Averages ± SD from three independents experiments are given. $^{*}p < 0.05$ and $^{**}p < 0.01$ respect to the control.

2.4. Transfection of short interfering RNA (siRNA)

Transfection was performed with a culture cellular density reaching 40–60% confluence with ERα-siRNA (NEB catalog number 2010S) or ERβ-siRNA (NEB catalog number 2021S) according to the manufacturer's instructions. These siRNAs are derived from human cDNAs and induce effective silencing of both estrogen receptor isoforms in mammalian cell lines. Briefly, TransPass^{TM} R2 Transfection Reagent was mixed with each siRNA (20 pmol) in antibiotic and serum-free medium. The mix was incubated for 20 min at room temperature. The culture medium of the cells was aspirated and replaced with the diluted transfection complex mixture and the cells incubated 5 h at 37 °C in a CO_2 incubator. The transfection mixture was removed and replaced with normal growth medium. Cells were incubated for an additional 24 h until used for treatments.

2.5. Statistical analysis

Statistical significance of the data was evaluated using Student's *t*-test [21] and probability values below 0.05 ($p < 0.05$) were considered significant. Results are expressed as means ± standard deviation (SD) from the indicated set of experiments.

3. Results

3.1. ERK2 and p38 MAPK activation by 17β-estradiol: dose–response study

We have previously shown that treatment of C2C12 cells with 10^{-8} M E2 rapidly stimulates one of the ERK isoforms as the phosphorylation of only one band was detected using an antibody that reacts against phospho-ERK1/2 [8]. However, the isoform of ERK (ERK1 or ERK2) activated by the hormone could not be identified. To verify that ERK1 and ERK2 can be phosphorylated in our experimental model and to test the selectivity of E2 effect, we used fetal bovine serum (FBS), which has been shown to activate both isoforms in smooth muscle cells [22]. To that end, C2C12 cells were incubated with 10% FBS or 10^{-8} M E2 during 15 min followed by immunoblot analysis. Fig. 1A shows that FBS induced the phosphorylation of the two isoforms of ERK, whereas the hormone only caused ERK2 activation. To further confirm this observation, we investigated ERKs stimulation induced by the estrogen at different doses (10^{-10} to 10^{-7} M) or its vehicle (isopropanol 0.001%) during 15 min. Western blot analysis in Fig. 1B shows that although equivalent ERK1 and ERK2 protein levels are expressed in C2C12 cells, E2 promoted only ERK2 phosphorylation. Similar results were obtained when the cells were treated with the estrogen for 30 min (data not shown). We have also reported p38 MAPK stimulation by 10^{-8} M E2 during 15 min [8]. In addition, we analyzed changes in p38 MAPK phosphorylation when cells were treated

with 10^{-10} to 10^{-7} M E2 or its vehicle (isopropanol 0.001%) during 15 min. As observed for ERK2, maximal p38 MAPK activation was at 10^{-8} M (Fig. 1C). We have previously determined that the effects of E2 on ERK and p38 MAPK phosphorylation in C2C12 cells are not significant at very short treatment times (0.5–5 min; unpublished), but within the 10–60 min interval, maximal activation of both kinases at 15 min was observed in response to estrogen [8].

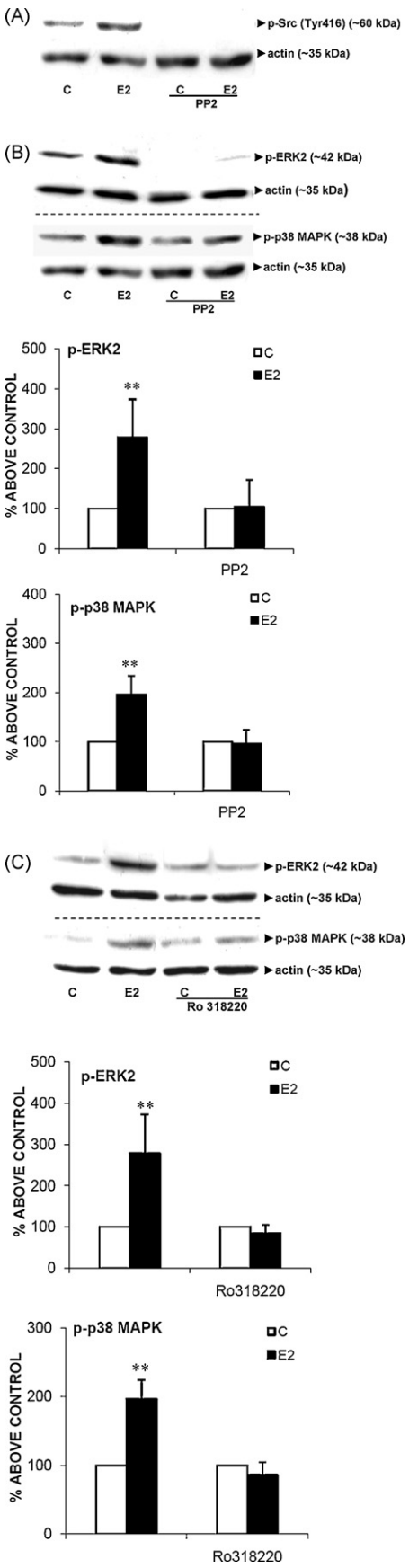

Fig. 3. 17β-Estradiol modulates ERK2 and p38 MAPK activation in a PKC and Src -dependent manner. Cultures of C2C12 muscle cells were incubated in presence or absence of 25 μM PP2 (A and B) or 5 μM Ro318220 (C) for 30 min. Then, cells were treated with 10^{-8} M E2 or vehicle during15 min. Western blot analysis was carried out using anti-phospho-Src (Tyr416) (A), anti-phospho-ERK1/2 or anti-phospho-p38 MAPK antibodies (B and C). Immunoblots quantified by scanning volumetric densitometry were normalized with actin levels. Averages ± SD from three independents experiments are given. $^{**}p < 0.01$ respect to the control.

Of relevance, in other cellular types E2 has been shown to activate ERK at comparable times through a non-genomic mechanism (not suppressed by protein synthesis inhibitors) [10].

3.2. ERα and β contribution to 17β-estradiol-induced MAPKs activation

It is well known that many of the rapid responses of E2 involve the participation of ERs. It has been reported that ERK1/2 and p38 MAPK activation induced by the hormone can occur through α or β ER isoforms in endothelial cells [6]. In view of this, we investigated the role of ERs in MAPKs activation by E2 using 1 μM ICI182780, which antagonizes both classical estrogen receptors in C2C12 cells [23]. As shown in Fig. 2A, a significant ERK2 phosphorylation was detected in the control condition with the antagonist. In agreement with this observation, it has been previously reported that ICI182780 treatment increases ERK phosphorylation in other cell types [24,25]. However, the hormone was not able to stimulate ERK2 above such control. On the other hand, E2-induced p38 MAPK activation in presence of 1 μM ICI182780. The decrease in p38 phosphorylation observed in the ICI182780 control condition of Fig. 2A can be explained by the variability in basal control (C) levels (compare its C blot with that of other figures) as this MAPK is sensitive to stress. These results suggest that ERα and/or ERβ are implicated in hormone-dependent ERK2 stimulation but not in p38 MAPK phosphorylation. To confirm these results, we next used a siRNA transfection protocol developed in our laboratory that successfully blocks the expression of α and β ER isoforms in C2C12 cells [23]. Then, we evaluated the ERα and ERβ contribution in ERK2 and p38 MAPK hormone-dependent activation. Fig. 2B demonstrates that both siRNAs effectively blocked the expression of ERα and ERβ. The results showed in Fig. 2C indicate that ERK2 activation induced by E2 was abrogated when ERα expression was abolished. However, the estrogen could significantly induce ERK2 phosphorylation when ERβ protein levels were suppressed. On the other hand, the hormone was able to promote p38 MAPK stimulation both when ERα or ERβ expression was blocked. These results show that ERK2 activation modulated by E2 is mediated by ERα and confirm that neither ERα nor ERβ is implicated in p38 MAPK activation by estrogen.

3.3. Src and PKC mediate ERK2 and p38 MAPK phosphorylation induced by 17β-estradiol in skeletal muscle cells

To investigate Src and PKC participation in ERK2 and p38 MAPK activation by E2 in C2C12 murine skeletal muscle cells, PP2 and Ro318220, specific inhibitors for all members of Src and PKC families, respectively, were used. Various doses of each inhibitor were tested to establish its most effective concentration (data not shown). As observed in Fig. 3A, the estrogen significantly stimulated Src (studied by changes in Tyr416 residue phosphorylation) and PP2 (25 μM) effectively blocked Src activation. ERK2 and p38 MAPK phosphorylation induced by the hormone was inhibited when the cells were incubated with PP2 (Fig. 3 B). Ro318220 (5 μM) also abolished estrogen-dependent ERK2 and p38 MAPK phosphorylation (Fig. 3C) and similar results were found using calphostine C (50 nM), other PKC inhibitor (data not shown). These results suggest that Src and PKC are key upstream components in the activation of MAPKs by E2.

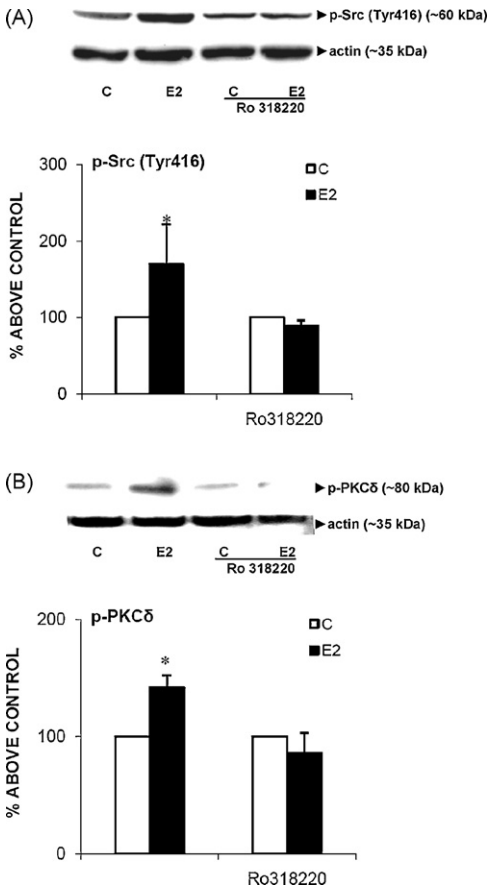

Fig. 4. Src and PKCδ activation induced by 17β-estradiol. C2C12 muscle cells were incubated with or without 5 μM Ro318220 for 30 min. Then, were treated with 10^{-8} M E2 or vehicle during15 min. Western blot assays were carried out using anti-phospho-Src (Tyr416) (A), or anti-phospho-PKCδ (Thr505) antibodies (B). Immunoblots quantified by scanning volumetric densitometry were normalized with actin levels. Averages ± SD from three independents experiments are given. $^{*}p < 0.05$ respect to the control.

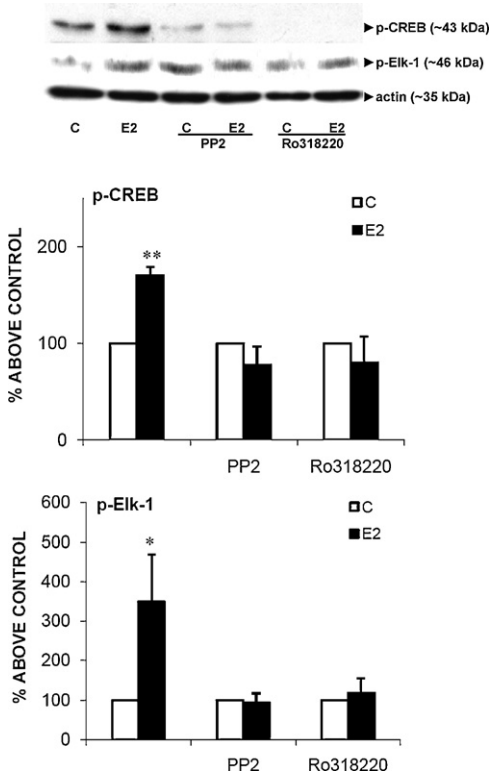

Fig. 5. PKC and Src mediate CREB and Elk-1 phosphorylation induced by 17β-estradiol. C2C12 cells were preincubated with 25 μM PP2 or 5 μM Ro318220. Then, cells were treated with 10^{-8} M E2 or vehicle for 15 min. Western blot assays were performed with anti-phospho-CREB or anti-phospho-Elk-1 antibodies. Immunoblots quantified by scanning volumetric densitometry were normalized with actin levels. Averages ± SD from three independents experiments are given. $^{*}p < 0.05$ and $^{**}p < 0.01$ respect to the control.

Additionally, we found that PMA, a phorbol ester, and *m*-sodium arsenite, exogen activators of PKC and Src families, respectively [26,27], induced ERK1/2 and p38 MAPK phosphorylation demonstrating that PKC and Src are involved in MAPKs modulation (data not shown).

3.4. Role of PKC in Src stimulation. PKCδ activation by 17β-estradiol

Src activation requires first dephosphorylation at Tyr527 followed by autophosphorylation at Tyr416 [26]. It has been demonstrated that PKC stimulates protein tyrosine phosphatase α (PTPα) which once activated dephosphorylates Src in Tyr527 [11]. Next, we investigated if PKC participates in E2-dependent Src activation. To this end, C2C12 cells were stimulated with E2 in presence or absence of Ro318220 (5 μM). Cell lysates were analyzed by Western Blot using an antibody against phospho-Src (Tyr416). As expected, Fig. 4A shows again that E2 stimulates Src activation and that the estrogen could not promote Src phosphorylation in Tyr416 residue in presence of Ro318220. These results suggest that the hormone induces Src activation in a PKC-dependent manner. Comparable results were found using calphostine C (data not shown).

Since PMA activates classic and novel PKCs in the C2C12 cell line [26] and Brandt et al. reported that the δ PKC isoform is required for PTPα-dependent Src stimulation [11], we next investigated whether E2 was able to induce PKCδ activation. To this end, we carried out Western blot assays to study changes in PKCδ-Thr505 phosphorylation. Fig. 4B shows that the hormone modulates PKCδ phosphorylation, which is sensitive to inhibition by 5 μM Ro318220.

3.5. Src and PKC are involved in 17β-estradiol-induced CREB and Elk-1 phosphorylation

As mentioned before, we have previously shown that activation of the ERK1/2 and p38 MAPK pathways by E2 leads to CREB and Elk-1activation [8]. In view of the present results, we investigated the role of PKC and Src in CREB and Elk-1 phosphorylation in response to the hormone. As seen in Fig. 5, PP2 and Ro318220 strongly blocked both basal and estrogen-induced CREB phosphorylation. Although a substantial phosphorylation of Elk-1 in the PP2 control condition was observed, the hormone did not significantly stimulate Elk-1 above such control. Furthermore, an increase of p-Elk-1 after E2 treatment in presence of Ro318820 was also evident in the immunoblot; however, such augment was not statistically significant respect to the control without inhibitor. These data suggest that Src and PKC mediate estrogen activation of CREB and Elk-1.

3.6. 17β-Estradiol increases c-Jun and c-Fos protein levels through Src- and PKC-dependent activation of the ERK1/2 and p38 MAPK pathways

Similarly as we observed before for c-Fos oncoprotein [8], we evaluated whether E2 induces c-Jun in C2C12 muscle cells through

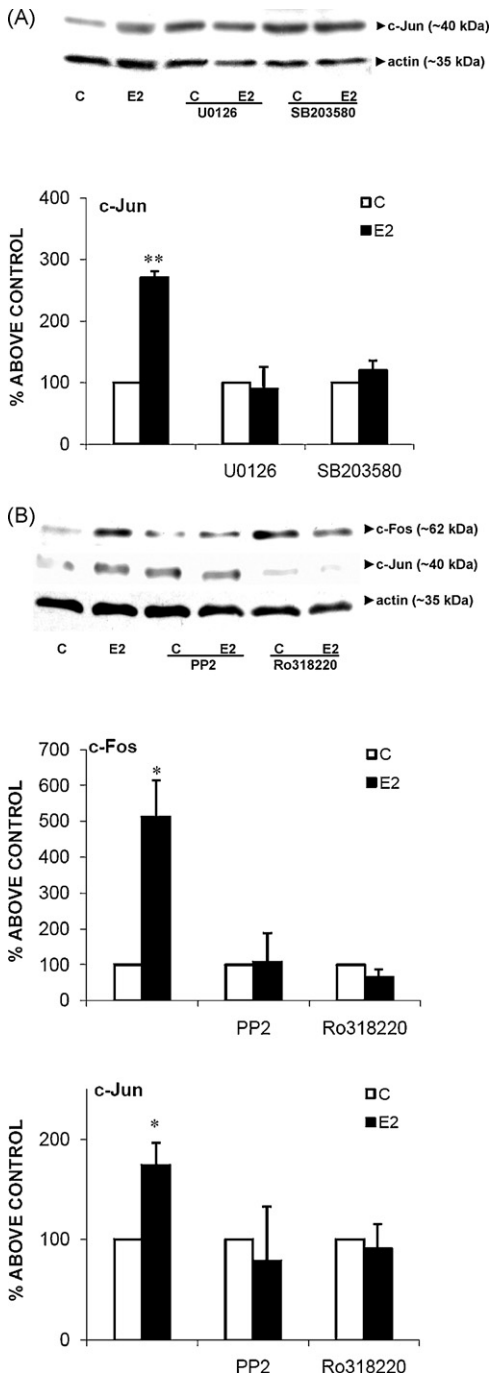

Fig. 6. MAPK involvement in c-Jun expression by 17β-estradiol. Role of PKC and Src in estrogen-dependent c-Jun and c-Fos enhancement in C2C12 cells. C2C12 cells were incubated with 10 μM U0126 or 20 μM SB203580 (A), 25 μM PP2 or 5 μM Ro318220 (B). Then, cultures were treated with 10^{-8} M E2 or vehicle for 15 min. Western blot assays were carried out using anti-c-Jun (A and B) or c-Fos antibodies (B). Immunoblots quantified by scanning volumetric densitometry were normalized with actin levels. Averages ± SD from three independents experiments are given. $^{*}p < 0.05$ and $^{**}p < 0.01$ respect to the control.

MAPKs. To prevent ERK1/2 and p38 MAPK activation, cells were preincubated with 10 μM U0126 and 20 μM SB203580, potent inhibitors of ERK1/2 and p38 MAPK, respectively. As shown in Fig. 6A, c-Jun levels were significantly increased by E2 treatment. Again, in control conditions with each inhibitor considerable c-Jun levels are observed; however, the hormone was not able to significantly increase further c-Jun amounts, indicating that ERK2 and p38 MAPK mediate E2-induced c-Jun expression. On the basis of previous data, Src and PKC involvement in estrogen regulation of c-Fos and c-Jun expression was then studied. In spite of high levels of the oncoproteins observed in the controls for PP2 and Ro318220 inhibitors, the hormone could not increase c-Fos and c-Jun protein levels beyond those with the inhibitors (Fig. 6B). These results suggest that Src and PKC activation are required for estrogen modulation of these oncoproteins.

4. Discussion

Within the non-genomic actions of E2 in different cellular types is the activation of mitogen-activated protein kinases (MAPKs) ERK1/2, JNK and p38 MAPK [5,6,29]. We have previously observed that in C2C12 murine skeletal muscle cells E2 rapidly induce phosphorylation of p38 MAPK and one of the ERK1/2 isoforms [8]. Regarding ERKs, evidences obtained in this work (see Section 3) indicate that E2 only activates ERK2. The possibility that the hormone can regulate ERK1 at long treatment intervals cannot be definitely excluded. Our results agree with previous studies demonstrating that although ERK1 and ERK2 have approximately 85% homology [7], according to the stimulus, they can be activated selectively mediating diverse responses [30,31]. Of relevance, it has been reported that ERK1 and ERK2 play different roles during the process of differentiation in C2C12 cells [32], which suggest that ERK2 activation (and not ERK1) by E2 could condition these cells to generate a specific response.

ERs have been postulated as key molecules of E2 non-genomic responses [1]. Specifically, it has been demonstrated that activation of MAPKs by the hormone is dependent on estrogen receptors in vascular cells [6,33]. In this work, using the selective antagonist for classical ERs, ICI182780 (1 μM), we demonstrated that such estrogen receptors are involved in ERK2 but not in p38 MAPK activation induced by E2. These observations were further confirmed when ERα was silenced and this abrogated ERK2 stimulation without affecting p38 MAPK activation by the estrogen, in agreement with various reports which demonstrated that ERK1/2 modulation by E2 is mediated by ERα in different cellular types [34–36]. When ERβ expression was blocked, ERK2 and p38 MAPK activation by E2 was observed, showing that the β isoform of ER is not involved in MAPKs signaling induced by the hormone in this cell line. These data lead us to hypothesize that the hormone promotes ERK2 stimulation via ERα and, on the other hand, p38 MAPK phosphorylation could occur through other mechanisms (not addressed in these investigations) where ERα and ERβ are not involved. In other studies, it has been shown that 17β-estradiol induces in C2C12 cells the expression of ERs through the ERK1/2 and p38 MAPK pathways [37]. It has been also reported that resveratrol, an estrogen receptor agonist, stimulates in C2C12 myotubes glucose uptake dependent on ERK/p38 activation [38]. In contrast to our observations, Galluzzo et al. provided evidence that 17β-estradiol regulates the first steps of L6 myoblast differentiation through modulation of Akt and p38 MAPK via ERα [5]. Moreover, Cheng et al. showed that the hormone induces in rat vascular smooth muscle cells ERK and p38 MAPK activation via ERα and β, respectively, which leads in turn to cell proliferation or apoptosis [35]. Clearly, further studies are required to understand the role of ERs in E2-dependent MAPK signaling in myoblasts.

In skeletal muscle cells, two upstream components of MAPKs pathways are Src and PKC [39,40]. Moreover, both kinases have been shown to participate in E2 modulation of MAPK cascades in rat adipocytes [41]. Therefore, in the present work we investigated the role of PKC and Src in ERK2 and p38 MAPK activation by the hormone in C2C12 cells. PKC is a family of serine/threonine kinases involved in different intracellular pathways [42]. Ro318220 and calphostine C, specific inhibitors of PKC family, blocked E2-promoted ERK2 and p38 MAPK phosphorylation indicating that

PKC is necessary in estrogen stimulation of MAPKs in muscle cells. PKC isoforms which mediate the regulation of the MAPK pathways in the C2C12 cell line were not identified. Of interest, the expression of PKCα, PKCβ, PKCδ, PKCθ, PKCη and PKCζ in these cells has been demonstrated [43]. The fact that the hormone promotes PKCδ activation and PMA induced phosphorylation of MAPKs; lead us to suggest that at least this isoform of PKC is involved in MAPKs activation by E2 in C2C12 cells. Of relevance, Qiu et al. reported that E2 induces PKCδ activation by ERα-dependent stimulation of PLC in neurons [44]. Also, it has been demonstrated that ERα interacts with PKC and Src in osteoblast [45]. On the other hand, the non-receptor tyrosine kinases Src has been involved in MAPKs regulation by the hormone in many tissues [9,46–48]. The observation that PP2, a selective inhibitor of the entire Src family, completely blocked ERK2 and p38 MAPK phosphorylation induced by E2 suggests a key role for Src in estrogen-induced stimulation of MAPKs in C2C12 cells. It is well known that activation of Src requires first dephosphorylation at Tyr527 followed by autophosphorylation at Tyr416 [28]. Our results showed that the estrogen activates Src inducing its phosphorylation at Tyr416. Of interest, Ro318220 and calphostine C diminished Src stimulation promoted by E2 in muscle cells, suggesting that the hormone activates Src through PKC. According, there is evidence showing that PKCδ isoform regulates Src via activation of the protein tyrosine phosphatase α (PTPα) that dephosphorylates the Tyr527 residue of Src and then induce its Tyr416 autophosphorylation [11]. In preliminary investigations (data not shown) we observed that E2 promotes PTPα (Tyr789) phosphorylation, which was reported as a crucial event for inducing the specific dephosphorylation of Src at Tyr527 [49,50]. Clearly, more studies are necessary to ascertain whether this mechanism operates in E2 activation of PKC/Src linked to regulation of MAPK cascades in skeletal muscle cells.

One of the well documented actions occurring when MAPKs are stimulated, is the regulation of a plethora of transcription factors among them CREB, Elk-1 and AP-1 [13,14,51,52]. We have recently reported that E2 activates CREB and Elk-1 via MAPKs in C2C12 cells [8]. In this study, experiments with PP2 and Ro318220 inhibitors blocked E2-induced CREB and Elk-1 phosphorylation reflecting the role of Src and PKC in estrogen modulation of MAPKs, in agreement with previous observation in other tissues [53,54]. The AP-1 transcription factor, a dimeric complex of c-Fos and/or c-Jun, regulates the expression of multiple genes involved in cell growth, differentiation and transformation [14]. We have recently demonstrated in C2C12 cells that E2 modulates c-Fos expression through MAPK activation [8]. In the present work we showed the upregulation of c-Jun protein levels by the hormone in an ERK2 and p38 MAPK -dependent manner. In accord with our results, it has been demonstrated that E2 stimulates both oncoproteins jointly in different cellular types [55,56]. It is likely that ERK2- and p38 MAPK-dependent CREB and Elk-1 activation by the hormone regulates the expression of c-Fos and c-Jun in muscle cells. However, the possibility that the estrogen regulates oncoproteins expression through MAPKs independently of these transcription factors cannot be definitely excluded by the present experiments. There is evidence indicating that PKC regulates expression of c-Fos and c-Jun oncoproteins via MAPK stimulation [57] and other reports show that Src is also involved in this mechanism [58]. In keeping with this information, our studies revealed that Src and PKC participate in the increase of c-Fos and c-Jun protein levels in response to E2.

We can conclude that E2 stimulates PKC and Src, which are key molecules in E2-dependent ERK2 and p38 MAPK activation, where ERK2 phosphorylation is dependent on ERα. Once stimulated, the MAPKs promote CREB and Elk-1 activation and induce upregulation of c-Fos and c-Jun oncoproteins in C2C12 myoblast cells. This mechanism is depicted in Fig. 7. However, further studies are required to identify the proteins which act upstream of PKC and Src in the initiation of estrogen signaling and thereby understand how the steroid hormone elicits rapid non-genomic responses in skeletal muscle. Previously, in our laboratory the role of E2 in skeletal muscle was investigated. Thus, we established a protective action of the hormone in C2C12 cells exposed to an apoptotic stimulus [48] and involvement of MAPKs in this estrogen effect was observed (Ronda et al., unpublished). On the other hand, it has been reported that E2 failed to promote proliferation in undifferentiated C2C12 cells [15,49]. Because of this, we suggest that the signaling mechanism triggered by E2 described in this work is related to cellular maintenance and survival of skeletal muscle cells.

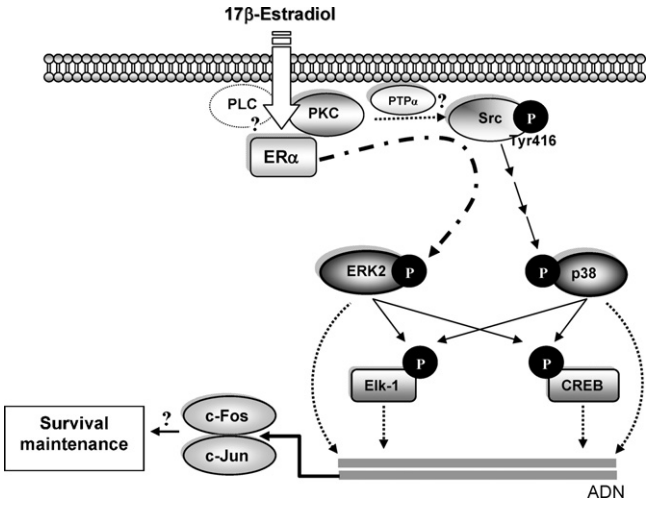

Fig. 7. Intracellular mechanism proposed for 17β-estradiol rapid action in C2C12 skeletal muscle cells. E2 may bind to a plasma membrane-located receptor or to a classical ERα. Possible outcomes include PLC stimulation through ERα followed by the activation of PKCδ [45] or that ERα up regulates PKC directly [46]. Subsequently, PKC triggers signaling cascades including activation of PTPα/Src/MAPKs followed by CREB and Elk-1 phosphorylation which in turn could increase c-Fos and c-Jun protein levels. An alternative mechanism of regulation of c-Fos and c-Jun expression via MAPKs is also considered.

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