Original Paper

17 β -Estradiol Protects Mitochondrial Functions through Extracellular-Signal-Regulated Kinase in C2C12 Muscle Cells

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Key Words

17 β -estradiol • ERK • COXIV • Mitochondrial membrane potential • Apoptosis • Muscle

Abstract

Background/Aims: We have previously shown that exposure to 17 β -estradiol (E2) prior to induction of apoptosis with H₂O₂ protects skeletal muscle cells against oxidative damage. However, the mechanism involved in the protective action of the hormone is poorly understood. In the present study, we focused on the mechanism by which ERK mediates this survival effect in connection with COXIV activity and mitochondrial membrane potential. **Methods:** Immunocytochemistry, Western blot, cytochrome c oxidase complex IV (COXIV) activity, coimmunoprecipitation and JC-1 dye by flow cytometry were carried out using C2C12 myoblasts as experimental model. **Results:** E2 is able to activate ERK and then induces its translocation to mitochondria. Using the pharmacological inhibitor of ERK activation U0126 we show that E2, through ERK activation, is able to enhance COXIV activity. Moreover, the hormone increases the interaction between COXIV and ERK. Also, we found that hydrogen peroxide decreases COXIV activity and that preincubation of the cells with E2 prior to induction of apoptosis prevents this effect. In addition, we observe that the estrogen inhibits the collapse of mitochondrial membrane potential induced by H₂O₂, involving ERK and COXIV. **Conclusion:** Our data demonstrate that E2 promotes ERK activation and translocation to mitochondria preventing the decline in COXIV activity and in turn, alteration of mitochondrial membrane potential by oxidative stress, in C2C12 myoblasts.

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Introduction

Sarcopenia is the loss of skeletal muscle mass associated with aging in which death of muscular cells by increased apoptosis has been demonstrated as one of the potential etiologic mechanisms [1]. Many factors are associated with this pathology such as estrogen deficiency in menopause [2]. Indeed, skeletal muscle responds to estrogens since both estrogen receptor (ER) isoforms, ER α and ER β were found in this tissue [3-6] and the hormone is required for its normal function [2, 7, 8]. We have previously demonstrated that E2 exerts a protective effect against H₂O₂-induced apoptosis in C2C12 murine skeletal myoblasts. Specifically, we observed an antiapoptotic action of the hormone through ERs with non classical localization involving PI3K/Akt/Bad, HSP27 and MAPKs [9-11]. MAPKs are activated by phosphorylation via different upstream MAPK kinases (MKKs or MEKs) which in turn are upregulated by the MAPK kinase kinases or MEKKs [12]. The ultimate biological consequences of MAPK superfamily activation depend on the specific targets of their activity, and this is in part regulated by the subcellular localization of the kinases [13]. Certainly, it has been shown that a fraction of ERK1/2 is targeted to mitochondria where it modulates specific functions of the organelle [14, 15]. Since MAPKs are involved in the antiapoptotic effects of E2 in skeletal myoblastic cells, and although it has been also observed that the hormone protects mitochondrial membrane integrity through an ERK and p38 MAPK-dependent mechanism [11], additional aspects of the role and localization of MAPKs during the estrogen effect in myoblasts need to be elucidated.

Mitochondria are important targets for estrogen actions [16], and play a fundamental role in cellular respiration involving a series of enzyme complexes and electron carriers. Cytochrome c oxidase (COX) is the terminal enzyme (complex IV) that transfers electrons from cytochrome c to an oxygen molecule, creating a proton gradient across mitochondrial membranes, which results in adenosine triphosphate (ATP) production. Also, COXIV plays a critical role in the organelle integrity and deficiencies of this enzyme are one of the most common defects of the respiratory chain found in mitochondrial diseases [17]. Moreover, mitochondria are targets of Bcl-2 family members which regulate apoptosis, affecting the outer mitochondrial membrane and the electrochemical gradient [18, 19].

The objective of this work was to perform more in-depth investigations on the mechanism by which ERK mediates the survival effects of E2 in skeletal muscle cells in connection with COXIV activity and mitochondrial membrane potential. Here we show that the hormone, at physiological concentrations, induces ERK translocation to mitochondria preventing the decrease in COXIV activity and depolarization of the mitochondrial membrane by H₂O₂.

Materials and Methods

Materials

Anti-ERK1/2 (1:1000 for Western blot analysis, 1:50 for immunocytochemistry and 1:100 for coimmunoprecipitation), anti-pERK1/2 (1:1000 for Western blot analysis and 1:25 for immunocytochemistry) and anti-COX (complex IV) (1:1000) for Western Blot analysis and 1:100 for coimmunoprecipitation) antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-lamin B (1:1000) antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). MitoTracker Red (MitoTracker Red CMXRos) dye and Alexa Fluor 488-conjugated anti-rabbit secondary antibody (1:200) were supplied by Molecular Probes (Eugene, OR, USA). U0126 was purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA, USA). E2, and cobalt chloride was from Sigma-Aldrich (St. Louis, MO, USA). All the other reagents used were of analytical grade.

Cell culture and treatment

C2C12 murine skeletal muscle cells purchased from the American Type Culture Collection (Manassas, VA, USA) were cultured in growth medium DMEM supplemented with 10% inactivated (30 min, 56°C) fetal bovine serum, 1% nystatine, and 2% streptomycin. Cells were incubated at 37°C in a humid atmosphere

of 5% CO₂ in air. Cultures were passaged every 2 days with fresh medium. Under these conditions C2C12 myoblasts resemble the activated satellite cells that surround the mature myofibers and proliferate and differentiate participating in the repair of the tissue when a cellular injury exists [20]. The studies were performed with 70–80% confluent cultures (120,000 cells/cm²) for Western blot and COXIV assays or with 50% confluent cultures in chamber slides for microscopy. Before treatments, cultures were incubated in medium without serum for 30 min (starvation). During this period, cells were exposed to 10 μM U0126 when indicated in the experiments. Compounds were dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 0.1% (v/v). It was verified that DMSO applied to the myoblasts at this concentration did not interfere with E2 action. Treatments were carried out by adding 10⁻⁸ M E2 or vehicle (control: 0.001% isopropanol) during the times specified in each assay.

MitoTracker Red

Coverslips with adherent cells were stained with MitoTracker Red, which was prepared in DMSO and then added to the cell culture medium at a final concentration of 1 μmol/l during starvation. Subsequently, cells were washed with PBS (pH 7.4, 8 g/l NaCl, 0.2 g/l KCl, 0.24 g/l KH₂PO₄, and 1.44 g/l Na₂HPO₄) and fixed with methanol at -20°C for 30 min. Then immunocytochemistry assays were performed.

Immunocytochemistry

Semi-confluent (50% confluence) monolayers were fixed with methanol at -20°C for 30 min. After fixation, non-specific sites were blocked for 1 h in 5% BSA. Cells were incubated with appropriate primary antibodies overnight at 4°C. The primary antibodies were recognized by fluorophore-conjugated secondary antibodies. Finally, the stained cells were analyzed with a conventional fluorescence microscope (NIKON Eclipse Ti-S equipped with standard filter sets to capture fluorescent signals, and images were collected using a digital camera) or confocal scanning laser microscopy (Leica TCS SP2 AOBS). The specificity of the labeling techniques was proven by the absence of fluorescence when the primary or the secondary antibodies were omitted.

Subcellular fractionation

C2C12 monolayers were scrapped and homogenized in ice-cold Tris-EDTA-sucrose (TES) buffer (50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 250 mM sucrose, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride, 20 mg/ml leupeptin, 20 mg/ml aprotinin, 20 mg/ml trypsin inhibitor) using a Teflon-glass hand homogenizer. Total homogenate free of debris was used in order to obtain the different fractions. Nuclear pellet was obtained by centrifugation at 300 g during 15 min. The supernatant was further centrifuged at 10,000 g for 30 min to pellet mitochondria. The remaining solution was called mitochondrial supernatant. Pellets were re-suspended in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2 mM Na₂VO₄, 2 mM EDTA, 25 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 mg/ml leupeptin, and 20 mg/ml aprotinin). Protein concentration of the fractions was estimated by the method of Bradford [21], using BSA as standard and Western blot analysis was performed as described below. Cross contamination between fractions was assessed by immunoblots using antibodies against lamin B and COX (complex IV), nuclear and mitochondrial markers, respectively.

Western blot analysis

Cell cultures were scrapped and resuspended using a lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2 mM Na₂VO₄, 2 mM EDTA, 25 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 mg/ml leupeptin, and 20 mg/ml aprotinin). Lysates were collected by aspiration and centrifuged at 12,000 g for 15 min. The protein content of the supernatant was quantified by the Bradford procedure [21] using BSA as standard. Then, lysate proteins dissolved in Laemmli [22] sample buffer were separated on 10–12% SDS-polyacrylamide gels and electrotransferred to polyvinylidene difluoride membranes. Relative migration of unknown proteins was determined by comparison with molecular weight colored markers (Amersham, Piscataway, NJ, USA). Membranes were blocked 1 h at room temperature in PBS-T buffer (PBS 0.1% Tween-20) containing 5% dry milk. Membranes were incubated with different primary antibodies overnight at 4°C, then washed three times in PBS-T and incubated in PBS-T containing 1% dry milk with peroxidase-conjugated secondary antibodies for 1 h at room temperature. Next, membranes were visualized using an enhanced chemiluminescent technique according to the manufacturer's instructions. For reprobing with

other antibodies, membranes were incubated in stripping buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 50 mM mercaptoethanol) for 30 min at 55°C, washed for 10 min in PBS-T, and then blocked and blotted as described above.

Coimmunoprecipitation

Aliquots of the mitochondrial fraction from lysates of C2C12 cells, containing equal amounts of proteins, were immunoprecipitated with 30 µl of a 30% suspension of protein A-sepharose after incubation with the antibody indicated in each experiment. The immunoprecipitates were washed three times with washing buffer (50 mM Tris-HCl, pH 7.4; 1 mM EDTA; 1% Triton X-100; protease inhibitors: 2 mM PMSF, 20 µg/ml leupeptin, 20 µg/ml aprotinin, and 10 µg/ml of trypsin inhibitor). The final pellets were obtained by centrifugation for 6 min at 10,000 *g*, resuspended then in electrophoresis sample buffer without dithiothreitol, and resolved by SDS-PAGE. Fractionated proteins were electrotransferred to PVDF membranes and then blocked for 1 h with 5% non-fat dry milk in PBS-T. The blots were incubated overnight at 4°C with primary monoclonal antibody against the protein of interest. After several washings with PBS-T, the membranes were incubated with the secondary antibody conjugated to horseradish peroxidase. Immunoreactive proteins were developed by means of enhanced chemiluminescence. The apparent molecular weight of reactive bands was estimated by reference to a wide size range of protein markers.

Measurement of Cytochrome c oxidase activity

The activity of COXIV was measured using a commercially available kit from Sigma (CYTOC-OX1) according to the manufacturer's instructions. Briefly, C2C12 cells were scrapped and homogenized in ice-cold TES buffer (50 mM Tris/HCl, pH 7.4, 1 mM EDTA, 250 mM sucrose, 1 mM dithiothreitol, 0.5 mM PMSF, 20 mg/ml leupeptin, 20 mg/ml aprotinin, and 20 mg/ml trypsin inhibitor) using a Teflon-glass hand homogenizer. Cell lysates were first centrifuged at 300 *g* during 15 min in order to eliminate the debris and nuclear fractions, the supernatant was then centrifuged at 10,000 *g* for 30 min to obtain the mitochondrial fraction. Mitochondria were resuspended in TES buffer and COXIV activity was measured. For this, 50 µl of reduced cytochrome c (0.22 mM) were added to 30 µl of the sample, and changes in absorbance at 550 nm were monitored for 1 min. The percentage of COXIV activity was calculated using: Units/ml = $\Delta\text{Abs}/\text{min} \times \text{dil} \times 1.1/(\text{vol sample}) \times 21.84$, where $\text{Abs}/\text{min} = \text{Abs}/\text{min}_{\text{sample}} - \Delta\text{Abs}/\text{min}_{\text{blank}}$; dil, dilution factor of sample; 1.1, reaction volume in ml; 21.84, extinction coefficient. Differences were calculated by normalizing the activity of the enzyme of each condition to that of total mitochondrial protein level obtained by Bradford assay in the same sample. Results were expressed as percentage of COXIV activity respect to control (100%).

Flow cytometry

Mitochondrial membrane potential ($\Delta\psi_m$) was determined using the JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide) mitochondrial transmembrane potential detection kit from Becton-Dickinson Biosciences (San Jose, CA, USA). JC-1 is a cationic fluorescent dye probe (green as monomer/ red as aggregates) that accumulates in mitochondria in a potential-dependent manner. Cells with functional mitochondria incorporate JC-1 leading to the formation of JC-1 aggregates, which show a red spectral shift resulting in higher levels of red fluorescence emission measured in the red (FL-2 channel) and green monomers (detectable in FL1 channel). Cells with collapsed mitochondria contain mainly green JC-1 monomers. The assays were performed with 70-80% confluent cultures in 10 cm plates. After treatments, cells were trypsinized, harvested, and incubated with JC-1 probe in 5 ml polystyrene round-bottom tubes (Becton-Dickinson Biosciences), according with manufacturer specifications, for 15 min at 37°C. Cells were then washed twice and analyzed in a FACS Calibur flow cytometer (excitation wavelength of 488 nm).

Statistical analysis

Results are shown as means $\pm$ standard deviation (SD). Statistical differences among groups were determined by analysis of variance (ANOVA) followed by a multiple comparison *post hoc* test [23]. Data are expressed as significant at $p < 0.05$.

Fig. 1. 17β -estradiol induces ERK activation in mitochondria of C2C12 muscle cells. C2C12 cell cultures were serum-starved during 30 min and then treated with 10^{-8} M E2 for 15 min. (A) Activated ERK was labeled using an anti-phospho-ERK antibody and then an Alexa Fluor 488-conjugated secondary antibody (green fluorescence). Mitochondria were stained with MitoTracker Red (red fluorescence) as described under Materials and Methods. At least 10 fields per slide of independent experiments were examined. Representative photographs are shown. Magnification 400X. (B) After treatments, cells were subject to subcellular fractionation as described in Materials and Methods and then Western blot analysis were performed using anti-phospho-ERK1/2, anti-lamin B and anti-COX (complex IV). Immunoblots from three independent experiments were quantified. Averages $\pm$ SD are given. Different letters indicate significant differences among groups (ANOVA $p < 0.05$). Nuc: nuclear fraction; Mit: mitochondrial fraction; MitSP: post-mitochondrial supernatant.

Results

17 β -estradiol induces ERK translocation to mitochondria in C2C12 myoblasts.

We have previously observed that E2 through ER α stimulates ERK phosphorylation in C2C12 cells [24, 25]. We have now investigated where this intracellular phosphorylation takes place and whether the hormone induces mobilization of the MAPK. To this end, C2C12 cells were incubated with the specific mitochondrial probe Mitotracker (red fluorescence). Then myoblasts were treated with E2 or its vehicle for 15 min when maximal phosphorylation of ERK by the estrogen has been shown to occur [24, 25]. As shown in Fig. 1 A, immunocytochemistry assays demonstrate that the estrogen caused MAPK activation (strong green fluorescence vs weak/absent green fluorescence in controls). Of interest, merge images reveal that E2 is able to induce ERK phosphorylation in mitochondria (yellow fluorescence which results from overlap of green and red fluorescences). To confirm this result, serum-starved cells were treated as before and mitochondria were isolated by differential centrifugation followed by Western blot assays. Blots shown in Fig. 1 B, corroborate that E2 stimulates ERK in the mitochondrial fraction.

To study if the hormone induces translocation of the kinase to mitochondria, cells stained with Mitotracker as before were exposed to the estrogen for various time intervals (10, 15, 30 and 40 min). Immunocytochemistry studies, using an antibody that recognizes the phosphorylated and nonphosphorylated forms of ERK1/2 did not reveal significant changes in MAPK levels implying that the hormone does not affect ERK1/2 expression at the times analyzed (Fig. 2 A). These results were confirmed by Western blots assays (Fig. 2 B). However, merge images from Fig. 2 A show differences in the degree of superposition (yellow fluorescence) indicating changes in the extent of localization of the kinase in mitochondria

Fig. 2. 17 β -estradiol induces ERK activation and then translocation to mitochondria in C2C12 muscle cells. Cells were serum-starved in absence (A and B) and presence (C) of 10 μ M U0126 and then incubated with 0.001% isopropanol (C) or 10⁻⁸ M E2 as indicated in the graphs. (A and C). Total ERK was labeled using an anti-ERK antibody and then an Alexa Fluor 488-conjugated secondary antibody (green fluorescence). Mitochondria were stained with MitoTracker Red (red fluorescence) as described under Materials and Methods. Fluorescence intensities monitored along the cells (white arrows in merge images) are shown. At least 10 fields per slide of independent experiments were examined. Magnification 400X. Representative photographs are shown. (B) Western blot analysis was performed using anti-ERK1/2 and anti-actin as a loading control. Blots shown are representative of independent experiments.

as a function of time in response to E2. Analysis of fluorescence intensity profiles monitored along the cells (white arrow in merge images) showed that in control conditions (C10 and C40) intensity profiles recorded in green and red channels do not follow exactly each other suggesting that ERK1/2 is localized mostly in cytosol and to a lesser extent in mitochondria. After E2 treatment, green fluorescence intensity profile matches up with red intensity profile demonstrating that ERK1/2 increased in mitochondria. A maximal localization of the kinase in the organelle was observed after 15-30 min of treatment.

In view that we found both, the presence of active ERK in mitochondria in response to the hormone and an E2-induced migration of the MAPK to these organelles, we studied whether phosphorylation of the kinase is a necessary step for its translocation. To this end, C2C12 cells were stained with Mitotracker and preincubated with U0126 to inhibit ERK activation. Then, cultures were treated with the estrogen or its vehicle during 15 min. Immunocytochemistry assays were carried out as before. Results show that in presence of U0126, the hormone was unable to increase yellow fluorescence in merge images (Fig. 2 C). Similar studies at different times (10, 30 and 40 min) were performed and no significant differences between conditions were found (data not shown). Altogether, these results suggest that phosphorylation is a requirement for ERK translocation to mitochondria.

Fig. 3. 17 β -estradiol induces COXIV activity through ERK in C2C12 muscle cells. Cells serum-starved in absence (A, B and D) or presence (C) of 10 μ M U0126 were incubated with 0.001% isopropanol (C) or 10 $^{-8}$ M 17 β -estradiol (E2) at the times indicated in the graphs. Then, mitochondria were obtained by differential centrifugation followed by measurement of cytochrome c oxidase (COX) activity as described in Materials and Methods. Averages $\pm$ SD are given. Different letters indicate significant differences among groups (ANOVA $p < 0.05$) (A and C). Western blot analysis were performed using anti-COXIV (complex IV) antibody and membrane staining with Coomassie Blue is shown as loading control (B). Mitochondrial lysates were immunoprecipitated using anti-COXIV or anti-ERK antibodies and immunoblotted with anti-ERK and anti-COXIV antibodies, respectively (D). Results from three independent experiments are shown.

17 β -estradiol increases COXIV activity in mitochondria involving ERK in C2C12 myoblasts.

The enzyme cytochrome c oxidase complex IV (COXIV) plays a critical role in mitochondrial energy generation and it has been demonstrated that E2 can regulate its activity [26, 27]. To elucidate if the hormone modulates COXIV activity in C2C12 cells, the myoblasts were treated with E2 or vehicle during different time intervals (1 – 4 h). Then, mitochondrial fractions were obtained and COXIV activity was determined as described in Materials and Methods. Results in Fig. 3 A show that E2 treatment induced a significant increase of COXIV activity upon 4 h of estrogen action (53.41%). Similar experiments as before were carried out and COXIV protein levels were analyzed by Western blot in mitochondrial fractions. No changes in COXIV protein amounts were observed in response to the estrogen (1 – 4 h) (Fig. 3 B). To study if ERK activation is necessary for hormonal regulation of COXIV, myoblasts were preincubated with the ERK inhibitor U0126 and treated with E2 or vehicle during 4 h, then COXIV activity was determined. Fig. 3 C shows that E2 was not able to stimulate COXIV in presence of U0126 involving ERK in this event. Similar results were obtained using PD98059, another ERK inhibitor (data not shown).

Since E2 was able to both induce ERK translocation to mitochondria and modulate COXIV activity through the MAPK, we then investigated the interaction between these proteins by coimmunoprecipitation assays. To this end, cells were treated with E2 or vehicle during 4 h and mitochondrial fractions were immunoprecipitated with anti-COXIV or anti-ERK antibodies and then analyzed by Western blot using anti-ERK and anti-COXIV antibodies, respectively. We found that COXIV and ERK proteins interact weakly under basal conditions (C) and treatment with the hormone increases this interaction (Fig. 3 D).

Fig. 4. 17 β -estradiol protects COXIV activity against H₂O₂ through ERK activation in C2C12 muscle cells. Myoblasts were serum-starved in absence (A and B) or presence (C) of 10 μ M U0126 and then treated as follows: A and B: Cells were incubated with H₂O (C) or 0.5 mM H₂O₂ at different times (1 – 4 h). C: C: 0.001% isopropanol; E2: 10⁻⁸ M 17 β -estradiol; H₂O₂: 0.5 mM H₂O₂; E2+H₂O₂: 10⁻⁸ M 17 β -estradiol + 0.5 mM H₂O₂; U0+E2+H₂O₂: 10 μ M U0126 + 10⁻⁸ M 17 β -estradiol + 0.5 mM H₂O₂. Then, mitochondria were obtained by differential centrifugation. Cytochrome c oxidase (COX) activity was measured as described in Materials and Methods. Averages $\pm$ SD are given. Different letters indicate significant differences among groups (ANOVA p <0.05) (A and C) Western blot analysis were performed using anti-COX (complex IV) antibody. Membrane staining with Coomassie Blue is shown as loading control (B). Results from three independent experiments are shown.

17 β -estradiol inhibits the effects of H₂O₂ on COXIV activity through ERK activation in C2C12 myoblasts.

Hydrogen peroxide has been widely used as apoptotic stimulus in various cell types. In fact, we have previously demonstrated that E2 exerts a protective effect against H₂O₂-induced apoptosis in C2C12 murine skeletal myoblasts [9-11]. H₂O₂ decreases the activity of COXIV in cardiomyocytes [28], therefore, we investigated the effect of H₂O₂ (0.5 mM) on the enzyme activity at different time intervals (1 – 4 h) in C2C12 skeletal myoblasts. Mitochondrial fractions were then isolated and COXIV activity and protein levels measured as before. Fig. 4 A shows that the apoptotic agent impaired the catalytic function of the enzyme in mitochondria at all the times studied, the effect being considerably greater at 2 h and 4 h of treatment (52.3% and 56.3% decrease, respectively). Western blot assays revealed that the amounts of COXIV protein in mitochondria were not affected by H₂O₂ (Fig. 4 B), indicating that the apoptotic stimulus diminishes COXIV activity without changing the expression levels of the enzyme.

In view that ERK mediates COXIV activity induced by E2 and given that it is also involved in the antiapoptotic effects of E2 [11], we next examined if E2 counteracts the decline of COXIV activity by H₂O₂ in presence of U0126. For this purpose, cells were starved with or without U0126, and then treated with E2 for 1 h or vehicle prior to induction of apoptosis with H₂O₂ during 4 h. Finally, mitochondrial fractions were isolated and COXIV activity measured as before. Results from Fig. 4 C show, in addition to the already observed reduction in COXIV activity by H₂O₂ (57.4% decrease respect to control), that E2 stimulates COXIV both in absence and presence of the apoptotic stimulus (73.6% and 167.5% increase above control, respectively). However, when cells were preincubated with U0126 and then treated with E2 and H₂O₂, the hormone was unable to avoid the effect of the oxidative stress (Fig.

Fig. 5. 17β -estradiol inhibits the mitochondrial membrane potential loss involving ERK and COXIV in C2C12 muscle cells. (A) Cultures were serum-starved during 30 min in the absence or presence of $10\ \mu\text{M}$ U0126 or $0.5\ \text{mM}$ CoCl_2 and then treated as described in the text. C: 0.001% isopropanol; E2: $10^{-8}\ \text{M}$ 17β -estradiol; H_2O_2 : $0.5\ \text{mM}$ H_2O_2 ; E2+ H_2O_2 : $10^{-8}\ \text{M}$ 17β -estradiol + $0.5\ \text{mM}$ H_2O_2 ; U0+E2+ H_2O_2 : $10\ \mu\text{M}$ U0126 + $10^{-8}\ \text{M}$ 17β -estradiol + $0.5\ \text{mM}$ H_2O_2 ; CoCl_2 : $0.5\ \text{mM}$ CoCl_2 (Co). Then cells were stained *in vivo* with JC-1 followed by flow cytometry analysis (Materials and Methods). Representative images and percentages in each gate from independent experiments are shown. (B) Percentages of depolarized cells under different conditions are shown. Averages $\pm$ SD are given. Different letters indicate significant differences among groups (ANOVA $p < 0.05$).

4 C), suggesting a role for ERK in this E2-protective action over COXIV. Cultures incubated with U0126 alone showed similar COXIV activity than control conditions (data not shown).

17 β -estradiol protects mitochondrial membrane potential ($\Delta\psi_m$) through ERK and COXIV in C2C12 myoblasts.

Different oxidative stress stimuli affect the permeability of mitochondrial membranes and promote a collapse of the $\Delta\psi_m$, event known as mitochondrial depolarization. H_2O_2 causes loss of $\Delta\psi_m$ in many cell types including C2C12 muscle cells [29, 30]. In addition, it has been demonstrated that E2 protects against H_2O_2 -induced depolarization in other tissues [31, 32]. Thus, we investigated whether E2 preserves mitochondrial membrane potential against H_2O_2 damage and the role of ERK and COXIV in this process in skeletal muscle cells. To this end, cultures were starved in presence or absence of U0126 and then incubated with estrogen or vehicle for 1 h prior to induction of apoptosis with H_2O_2 during 4 h. Mitochondrial membrane potential ($\Delta\psi_m$) was determined by flow cytometry in 10,000 cells using the JC-1 dye. In Fig. 5 A, dot plot analysis revealed that only one set of cells with normal $\Delta\psi_m$ is present, both in control and E2 conditions (normal $\Delta\psi_m \sim 92.11\%$ and $\sim 84.34\%$, respectively), the number of depolarized cells being non significant (low $\Delta\psi_m \sim 1.73\%$ and $\sim 2.60\%$, respectively). However, after hydrogen peroxide exposure, dot plot analysis showed that C2C12 cells turned into the diffuse green color (green-low $\Delta\psi_m \sim 41.68\%$) indicating that H_2O_2 induced depolarization. Incubation of the cells with E2 ($10^{-8}\ \text{M}$ – 60 min) prior to treatment with the oxidative stimulus revealed that the estrogen partially reversed H_2O_2 -induced depolarization (Fig. 5 B). Thus, cells tend to localize in the

higher mitochondrial membrane potential region (normal $\Delta\psi_m \sim 65.96\%$) decreasing the number of depolarized cells (low $\Delta\psi_m \sim 17.63\%$). Interestingly, in presence of U0126, E2 was unable to prevent mitochondrial membrane depolarization by hydrogen peroxide. Cultures incubated with DMSO or U0126 alone showed similar dot plots than control and E2 conditions (data not shown). The role of COXIV in the mitochondrial membrane potential of C2C12 cells was also investigated, since it has been reported that this enzyme exerts a tight control on $\Delta\psi_m$ in other cell types [33, 34]. Cobalt chloride (CoCl_2), previously shown to inhibit the expression and activity of COXIV from kidney, liver and brain [35], was used. Dose-response studies demonstrated that treatment with 0.50 mM CoCl_2 for 4 h reduces the activity of the enzyme to a similar extent as H_2O_2 in C2C12 myoblasts (data not shown). To investigate if COXIV inhibition affects myoblast mitochondrial membrane potential, C2C12 cells were exposed to 0.50 mM CoCl_2 and then $\Delta\psi_m$ analyzed as before. Results from Fig. 5 A show that in presence of CoCl_2 , cells depolarized (low $\Delta\psi_m \sim 58.55\%$) indicating that inhibition of COXIV activity causes mitochondrial membrane depolarization in the skeletal myoblasts.

Discussion

17 β -estradiol (E2) regulates cellular processes such as differentiation, proliferation, and even apoptosis activating rapid signaling cascades [36, 37]. It is well established that mitochondria play a central role in apoptosis and there is increasing evidence suggesting that these organelles are targets for estrogen actions [16]. However, the molecular mechanisms activated by E2 in these organelles are not fully elucidated. Here we show that the estrogen induces activation and then translocation of ERK to mitochondria. This event prevents both the decrease in COXIV activity and the loss of mitochondrial membrane potential induced by hydrogen peroxide in C2C12 myoblasts. We have previously demonstrated that E2 induces a fast stimulation of ERK through ER α [24, 25]. We also showed that the hormone protects the cells against apoptosis induced by H_2O_2 through the mitochondrial pathway and involving ERK activation [9-11]. Specifically, using the inhibitor U0126 and confirming with a siRNA to knock out ERK we demonstrated that E2 through the ERK2 isoform preserves mitochondrial membrane integrity and abrogates Smac/Diablo release from mitochondria to cytosol [11]. In this paper, we furnish relevant information on the mechanism of action of ERK in estradiol protection of C2C12 myoblasts. We show that stimulation of the muscle cells with the hormone induces phosphorylation and then translocation of the kinase to mitochondria. The observation that the ERK inhibitor U0126 prevented the translocation of the kinase to mitochondria after E2 treatment, implies that phosphorylation of the MAP kinase is a necessary step for its mobilization. In fact, it is well known that one of the mechanisms proposed for translocation of MAPKs is the activation and subsequent dimerization of the protein [12]. ERK translocation to mitochondria induced by E2 could be in part responsible for C2C12 myoblast survival against programmed cellular death promoted by H_2O_2 . Of interest, there are evidences showing that ERK forms signaling complexes with PKC in mitochondria in cardiac cells to promote protective effects in part through phosphorylation and inactivation of Bad [38]. Thus, E2-induced ERK translocation to mitochondria demonstrated in this work could be associated with our previous work where we found that Bad inactivation elicited by the hormone depends on ERK phosphorylation [11] and also with our findings showing that PKC inhibition affects E2-induced ERK activation [25]. Likewise, several ERK downstream effectors have been detected in mitochondria [14, 15, 39]. ERK activated in this organelle by E2 might then provide a key advantage for myoblast survival. In this paper we propose cytochrome c-oxidase complex IV (COXIV) as a possible substrate for ERK activated by E2 in mitochondria. Results from this work demonstrated that the estrogen induces COXIV activity. The fact that the use of U0126 abrogates COXIV stimulation by E2, suggests that ERK activation is involved in hormonal regulation of the enzyme. Of relevance, a basal interaction between COXIV and ERK was found, and the estrogen increased this interaction. To our knowledge, this observation has not been made in muscle cells or other cellular

Fig. 6. Schematic diagram depicting the role of ERK in the mitochondrial effects of 17 β -estradiol in C2C12 skeletal muscle cells. In myoblastic cells, E2 induces ERK phosphorylation and in turn its translocation to mitochondria, abrogating the decrease of COXIV activity and the collapse of mitochondrial membrane potential induced by hydrogen peroxide stimulus.

systems, identifying COXIV as another target for ERK in mitochondria. Congruent with our findings there are evidences showing that various signaling intermediates connected to ERK modulate COXIV. It has been reported that PKC ϵ interacts with COXIV and enhances its activity in neonatal cardiac myocytes [40]. Moreover, we studied the effects of H₂O₂ on COXIV activity and whether estradiol prevents them via ERK. The decline in COXIV activity observed upon exposure of C2C12 cells to H₂O₂ was comparable to that previously reported for cardiomyocytes [28]. The biochemical mechanism responsible for the decrease in COXIV activity was not established. The constant enzyme protein levels in mitochondria rule out changes due to diminished synthesis or proteolysis. Our data showed that the steroid hormone not only increases COXIV activity but also blocks the inhibitory effects of H₂O₂ on the enzyme, furnishing further information on the protective role of E2 in mitochondria of C2C12 cells. Consistent with this observation, E2 pretreatment prevents the loss of the enzymatic activity of ageing in rats [27]. Noteworthy, we have found that ERK is involved in estradiol protection of COXIV activity since the use of U0126 abolished hormonal modulation of the enzyme.

The increase of the outer mitochondrial membrane permeability is a crucial event in apoptosis [18] which is associated with $\Delta\psi_m$ loss, mitochondrial swelling, and rupture of the outer mitochondrial membrane [41]. In order to investigate whether the hormone stabilizes mitochondrial membrane potential against mitochondrial collapse induced by H₂O₂ we used the specific cationic dye JC-1 and flow cytometry. Our experiments demonstrate that E2 preserves the mitochondrial membrane potential of C2C12 muscle cells from depolarization promoted by the oxidative stress. Likewise, E2 has been previously shown to exert mitochondrial membrane protection in human neuroblastoma and lens epithelial cells [31, 32]. In addition, using U0126, we found that in myoblasts there is also a correlation between ERK activation and hormonal $\Delta\psi_m$ stabilization against damage induced by H₂O₂. However, a specific feature observed in C2C12 muscle cells was that in presence of U0126, E2 did not completely prevent mitochondrial membrane depolarization in response to H₂O₂, suggesting that ERK activation is not the unique mechanism involved in this process in myoblasts. COXIV exerts a tight control on the mitochondrial membrane potential [33, 34]. Accordingly, here we demonstrated that inhibition of COXIV with CoCl₂ decreases $\Delta\psi_m$ in C2C12 myoblasts. It has been proposed that reduced cytochrome c oxidase complex activity promotes depolarization of the mitochondrial membrane, which in turn leads, to abnormal ATP production, and could then affect mitochondrial calcium uptake and the regulation of reactive oxygen species, two important cell signals [34].

In summary, our findings suggest that in C2C12 myoblasts, E2 promotes activation and translocation of ERK to mitochondria and, in turn, stabilizes the organelle against oxidative stress through a mechanism that involves stimulation of COXIV and protection of the mitochondrial membrane potential (Fig. 6). These remarks point out ERK signaling

as an important target for estrogen during its antiapoptotic effects on muscle cells. The results presented in this work contribute to the knowledge about the survival action of E2 on satellite myoblasts and its relationship to age-dependent sarcopenia.

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