

Estradiol exerts antiapoptotic effects in skeletal myoblasts via mitochondrial PTP and MnSOD

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Abstract

17 β -Estradiol (E₂) protects several non-reproductive tissues from apoptosis, including skeletal muscle. We have shown that E₂ at physiological concentrations prevented apoptosis induced by H₂O₂ in C2C12 skeletal myoblasts. As we also demonstrated the presence of estrogen receptors in mitochondria, the present work was focused on the effects of E₂ on this organelle. Specifically, we evaluated the actions of E₂ on the mitochondrial permeability transition pore (MPTP) by the calcein-acetoxymethylester/cobalt method using fluorescence microscopy and flow cytometry. Pretreatment with E₂ prevented MPTP opening induced by H₂O₂, which preceded loss of mitochondrial membrane potential. In addition, it was observed that H₂O₂ induced translocation of Bax to mitochondria; however, in the presence of the steroid this effect was abrogated suggesting that members of the Bcl-2 family may be regulated by E₂ to exert an antiapoptotic effect. Moreover, E₂ increased mitochondrial manganese superoxide dismutase protein expression and activity, as part of a mechanism activated by E₂ that improved mitochondrial performance. Our results suggest a role of E₂ in the regulation of apoptosis with a clear action at the mitochondrial level in C2C12 skeletal myoblast cells.

Key Words

- ▶ 17 β -estradiol
- ▶ C2C12 skeletal myoblasts
- ▶ antiapoptotic effect
- ▶ MPTP
- ▶ MnSOD

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Introduction

The steroid hormone 17 β -estradiol (E₂) plays an important role in development, cell growth, and differentiation, acting through both genomic and non-genomic mechanisms (De Feo 1996, Bjornstrom & Sjöberg 2005). It is generally accepted that the majority of the hormone effects are mediated by two estrogen receptors (ERs), namely ER α and ER β (Evans 1988, Tsai & O'Malley 1994, Beato *et al.* 1996, Pettersson *et al.* 2000, Hall *et al.* 2001, Hewitt & Korach 2002). The actions of E₂ have been typically linked to reproductive functions. However, during the last decade it has been shown that practically

every animal cell/tissue/organ system responds to the hormone to some extent (reviewed in Vasconsuelo *et al.* (2011)). One of the reasons for this opening out in the non-conventional estrogen actions was the finding of ER in non-classical tissues and with non-classical intracellular localizations. For example, the presence of ERs in mitochondria of C2C12 skeletal myoblasts has previously been shown (reviewed in Vasconsuelo *et al.* (2011)). Although the localization of mitochondrial ERs is well established, allowing one to envision an influence of E₂ upon mitochondrial functions, the physiological

role of the steroid at this cellular level remains unclear.

Mitochondria are vital organelles, mainly involved in cellular energy production and regulation of programmed cell death (apoptosis). In previous studies, it was shown that E_2 at physiological concentrations prevented apoptosis in skeletal myoblasts in an ER-dependent fashion. Typical changes of apoptosis such as nuclear fragmentation, cytoskeleton disorganization, mitochondrial reorganization/dysfunction, and cytochrome *c* release induced by H_2O_2 , were abolished when cells were previously exposed to E_2 (Vasconsuelo *et al.* 2008). Likewise, we demonstrated that E_2 exerts its antiapoptotic effect through ERs with non-classical localization involving MAPKs, HSP27, and the survival PI3K/Akt pathway, which phosphorylates and inactivates proapoptotic members of the Bcl-2 family (Vasconsuelo *et al.* 2008, 2010, Ronda *et al.* 2010). The mitochondrial protein cytochrome *c* plays a key role in apoptosis (reviewed by Jiang & Wang (2004)). This soluble protein is localized in the intermembrane space and is loosely attached to the surface of the inner mitochondrial membrane. In response to a variety of apoptosis-inducing agents, cytochrome *c* is released from mitochondria to the cytosol, an event that is considered the key regulatory step irreversibly committing cells to apoptosis (Liu *et al.* 1996, Reed 1997, Kumarswamy & Chandna 2009). Several models have been proposed to explain the mechanism of cytochrome *c* release from mitochondria, such as the loss of integrity or/and an increment of permeability of the outer mitochondrial membrane (Crompton *et al.* 1999, Green & Kroemer 2004). Thus, the increase of outer mitochondrial membrane permeability is a crucial event in apoptosis (Desagher & Martinou 2000, Tsujimoto 2003). Nevertheless, the mechanism(s) responsible for this augmented permeabilization is/are not well established. It is known that members of Bcl-2 family are involved in this process (Antignani & Youle 2006). Indeed, Bax is found in the cytosol of most normal healthy tissues, which indicates that the protein is kept in an inactive form in the cells. In apoptotic cells or when Bax is overexpressed, it is targeted to mitochondrial membranes, where the protein triggers cytochrome *c* release, leading to apoptosis (reviewed in Sharpe *et al.* (2004)). Another mechanism postulated, which results in outer mitochondrial membrane permeabilization, involves the mitochondrial permeability transition pore (MPTP), which generally is associated with mitochondrial membrane potential ($\Delta\Psi_m$) functionality (Armstrong 2006). The exact molecular nature of the MPTP is still a matter of debate, although this pore is

considered a large multiprotein complex, primarily composed of the adenine nucleotide transporter (ANT), cyclophilin D, and voltage-dependent anion channel (VDAC, also called porin) that can interact with several other proteins (Kroemer *et al.* 1998, Crompton 1999). Under normal physiological conditions, the mitochondrial inner membrane is nearly impermeable to all ions. However, under stress conditions, the MPTP can open in the mitochondrial inner membrane allowing the free passage of solutes with molecular masses up to 1500 Da (Halestrap *et al.* 2002). Its opening results in the disruption of the inner membrane permeability barrier and swelling of the matrix space. As the inner mitochondrial membrane has a surface area several times that of the outer mitochondrial membrane, swelling of the matrix space results in rupture of the outer membrane and, in consequence, the release of mitochondrial components such as cytochrome *c* occurs. It has been shown that members of Bcl-2 family could regulate the MPTP (Reed & Kroemer 1998).

Mitochondria are especially sensitive to reactive oxygen species (ROS). Of importance for our studies, under high levels of ROS MPTP can be activated (Petronilli *et al.* 1994). Generally, cells are equipped with antioxidant enzymes that can prevent these and other effects of ROS. Thus, superoxide anion is dismutated by the enzyme superoxide dismutase (SOD), to yield H_2O_2 , which is eliminated by glutathione peroxidase and catalase (Bhuyan & Bhuyan 1978). There are three isoforms of SOD, the cytosolic-CuZnSOD (SOD1), mitochondrial manganese SOD (MnSOD), and the extracellular SOD (ecSOD). It has been reported that E_2 can preserve mitochondrial function by regulating mitochondrial antioxidant enzymes in human lens and cerebral vessels (Stirone *et al.* 2005, Gottipati & Cammarata 2008) and that MnSOD overexpression prevents apoptosis (Manna *et al.* 1998, Fujimura *et al.* 1999).

Skeletal myoblasts are essential for the normal growth, repair, and regeneration of differentiated adult skeletal fibers. C2C12 cells resemble these activated satellite cells. Moreover, apoptosis increases significantly with age affecting satellite cells and contributing to sarcopenia (Jejurikar & Kuzon 2003). Therefore, knowledge of the mechanism underlying the antiapoptotic action of E_2 in C2C12 myoblasts is relevant to understand the hormone protective effects on satellite skeletal cells. We propose that E_2 protects skeletal myoblasts through a direct mitochondrial action. To test this hypothesis, the present work investigates the mitochondrial effects of E_2 , which in turn regulates apoptosis in C2C12 skeletal myoblasts. It is

shown that E_2 protects myoblasts against apoptosis induced by H_2O_2 , involving MPTP, Bax, and MnSOD. These findings have implications in the prevention and management of sarcopenia, including development of pharmacologic inhibitors of specific mitochondrial targets to block apoptosis and reduce the loss of satellite cells.

Materials and methods

Material

Anti-cytochrome *c* oxidase (COX) IV antibody (1:1000) was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-SOD2 (1:1000) was from Santa Cruz Biotechnology, Inc. Anti-beta tubulin (1:10 000) and anti-Bax (1:1000 for western blot analysis and 1:50 for immunocytochemistry) antibodies were obtained from Thermo Fisher Scientific, Inc. (Rockford, IL, USA). MitoTracker Red (MitoTracker Red CMXRos) dye and Alexa Fluor 488-conjugated anti-rabbit secondary antibody (1:200) were provided by Molecular Probes (Eugene, OR, USA). E_2 and fulvestrant (ICI 182 780) were from Sigma-Aldrich. Superoxide Dismutase Assay Kit II was from Calbiochem Corp. (La Jolla, CA, USA). Calcein-easter-acetoxymethylester (AM) was purchased from Invitrogen. 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) was obtained from Becton Dickinson Biosciences (San Jose, CA, USA). The ECL blot detection kit and protein molecular weight markers were provided by Amersham. All the other reagents used were of analytical grade.

Cell culture and treatment

C2C12 murine skeletal myoblasts obtained from American Type Culture Collection (Manassas, VA, USA) were cultured in growth medium (DMEM) supplemented with 10% heat-inactivated (30 min, 56 °C) fetal bovine serum, 1% nistatine, and 2% streptomycin. These highly myogenic cells have been widely used to study muscle functions (Burattini *et al.* 2004, Vasconsuelo *et al.* 2008, Pronsato *et al.* 2012). Cells were incubated at 37 °C in a humid atmosphere of 5% CO_2 in air. Cultures were passaged every 2 days with fresh medium. The treatments were performed with 70–80% confluent cultures (120 000 cells/cm²) in medium without serum for 30 min. During this preincubation, cells were exposed to 1 μ M fulvestrant in specific experiments. Then 10^{-8} M E_2 or vehicle 0.001% isopropanol (control) was added ~120 min before induction of apoptosis with hydrogen peroxide (H_2O_2) at the times indicated (ranging from

30 min to 4 h). This time and concentration range of the oxidant has been previously used to study apoptosis in C2C12 cells (Jiang *et al.* 2005, Vasconsuelo *et al.* 2008, Siu *et al.* 2009, Pronsato *et al.* 2012). H_2O_2 was diluted in culture medium without serum at a final concentration of 0.5 mM in each assay. After treatments, cells 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 μ g/ml leupeptin, and 20 μ g/ml aprotinin. Protein concentration was estimated by the method of Bradford (1976), using BSA as standard. For microscopical assays, cells were cultured in chamber slides.

Western blot analysis

Protein samples (25 μ g) were mixed with sample buffer (400 mM Tris-HCl (pH 6.8), 10% SDS, 50% glycerol, 500 mM dithiothreitol (DTT), and 2 mg/ml bromophenol blue), boiled for 5 min, and resolved by 10–12% SDS-PAGE according to the method of Laemmli (1970). Fractionated proteins were electrotransferred to polyvinylidene fluoride membranes (Immobilon-P; PVDF) and then blocked for 1 h at room temperature with 5% non-fat dry milk in PBS containing 0.1% Tween-20 (PBS-T). Blots were incubated overnight with the appropriate dilution of the primary antibodies: anti-Bax, anti-COX IV, and anti- β -tubulin using anti-rabbit secondary antibodies for all of them; anti-SOD2 (MnSOD) with anti-mouse secondary antibody. The membranes were repeatedly washed with PBS-T before incubation with HRP-conjugated secondary antibodies. The ECL blot detection kit was used as described by the manufacturer to visualize reactive products. Relative migration of unknown proteins was determined by comparison with molecular weight markers. When needed, membranes were stripped with stripping buffer (62.5 mM Tris-HCl (pH 6.7), 2% SDS, 50 mM β -mercaptoethanol), washed with PBS containing 1% Tween-20 and then blocked for 1 h with 5% non-fat dry milk in PBS-T. The blots were then incubated with the corresponding primary antibody. After several washings with PBS-T, membranes were incubated with secondary antibodies. The corresponding immunoreactive bands were developed as before.

Subcellular fractionation

C2C12 cells were scrapped and homogenized in ice-cold Tris-EDTA-sucrose buffer (50 mM Tris-HCl (pH 7.4),

1 mM EDTA, 250 mM sucrose, 1 mM DTT, 0.5 mM PMSF, 20 mg/ml leupeptin, 20 mg/ml aprotinin, and 20 mg/ml trypsin inhibitor) using a Teflon-glass hand homogenizer. Total homogenate free of debris was used in order to isolate the different fractions. Nuclear pellet was obtained by centrifugation at 800 *g* for 15 min at 4 °C. The supernatant was further centrifuged at 10 000 *g* for 30 min at 4 °C to yield the mitochondrial pellet. The remaining supernatant was called cytosolic supernatant. Pellets were resuspended 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 PMSF, 20 mg/ml leupeptin, and 20 mg/ml aprotinin). Protein concentration of the fractions was estimated by the method of Bradford (1976), using BSA as standard and western blot assays were performed as described before. Cross contamination between fractions was assessed by western blot analysis using anti-COX IV as mitochondrial marker.

Measurement of SOD activity

Two million cells were lysed in 20 mmol/l HEPES buffer containing 1 mmol/l EGTA, 210 mmol/l mannitol, and 70 mmol/l sucrose to measure the enzyme activity using Superoxide Dismutase Assay Kit II according to the manufacturer's directions. To obtain mitochondrial SOD, the lysates were centrifugated at 800 *g* for 15 min at 4 °C and the supernatant at 10 000 *g* for 30 min at 4 °C. Then, the mitochondrial pellets were suspended in the cold buffer used before. This suspension (10 µl) was mixed with 200 µl of radical detector (tetrazolium salt). The reaction was incubated at room temperature for 20 min and read at 450 nm using a Genius plate reader from Tecan (Mannedorf, Switzerland). One unit of SOD activity is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

Immunocytochemistry

Coverslips with adherent cells were stained with Mito-Tracker Red, prepared in DMSO and then added to the cell culture medium at a final concentration of 1 µmol/l. After 15–30 min of incubation at 37 °C in darkness, 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. After fixation, non-specific sites were blocked for 1 h with PBS 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). The specificity of the labeling techniques was proven by the absence of fluorescence when the primary or secondary antibodies were omitted.

Measurement of MPTP opening

We assessed the MPTP opening using the calcein-AM/cobalt method according to Petronilli *et al.* (1999). Briefly, 70–80% confluent cultures in 10 cm (flow cytometry) or 3 cm (microscopy) plates were loaded for 30 min with 1 µM calcein-AM at 37 °C in DMEM media. We quenched the cytosolic and nuclear calcein fluorescence using 1 mM CoCl₂. After attainment of quenching, cells were washed twice with warm DMEM without serum. Following the corresponding treatments, cells were examined by digital fluorescence microscopy. Image quantification was performed by measuring the fluorescence intensity profiles (in arbitrary units) using the IMAGEJ program 1.46. Additionally, the loaded cells were trypsinized, harvested, and analyzed in a FACS Calibur flow cytometer (excitation wavelength of 488 nm).

Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)

Cellular $\Delta\Psi_m$ was determined using the JC-1 mitochondrial transmembrane potential detection kit from Becton-Dickinson Biosciences. 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 according to the manufacturer's 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 s.d. of not less than three independent experiments. Statistical differences among groups were performed using ANOVA followed by a multiple comparison *post hoc* test (Tukey 1953). The statistical significance of data was determined as $P < 0.05$.

Results

E₂ inhibits MPTP opening in C2C12 skeletal myoblasts

Mitochondria, which play a crucial role in apoptosis, release several apoptosis-inducing factors into the cytoplasm. This process requires the increase of outer mitochondrial membrane permeability, which probably depends on the activation of MPTP. To evaluate the effects of E₂ on MPTP function in C2C12 cells, we used a cobalt-quenched calcein-AM method. Calcein-AM is an anionic, esterified fluorochrome that enters the cells freely and labels cytoplasmic as well as mitochondrial regions following esterase removal of the AM group. Because cobalt ions enter to the cytoplasm but do not readily pass through the mitochondrial membrane, mitochondria can be specifically identified by the cobalt quenching of cytoplasmic, but not mitochondrial, calcein fluorescence. In consequence, MPTP opening can be recognized by a decrease of mitochondrial calcein fluorescence (Petronilli *et al.* 1999).

C2C12 cell cultures, loaded with calcein-AM/CoCl₂, were incubated with E₂ or vehicle isopropanol (control) before induction of apoptosis with H₂O₂ (4 h), following the experimental protocols indicated in each set of experiments. The cells were then analyzed by microscopy and flow cytometry as described in Materials and methods. As shown in Fig. 1A and B by microscopical analysis, the treatment with H₂O₂ resulted in a significant decrease of calcein fluorescence intensity due to CoCl₂ quenching as a result of MPTP opening. However, when the cells were previously treated with E₂, the fluorescence was diminished to a lesser extent, implying a protective role of estrogen over this pore. These data were confirmed by flow

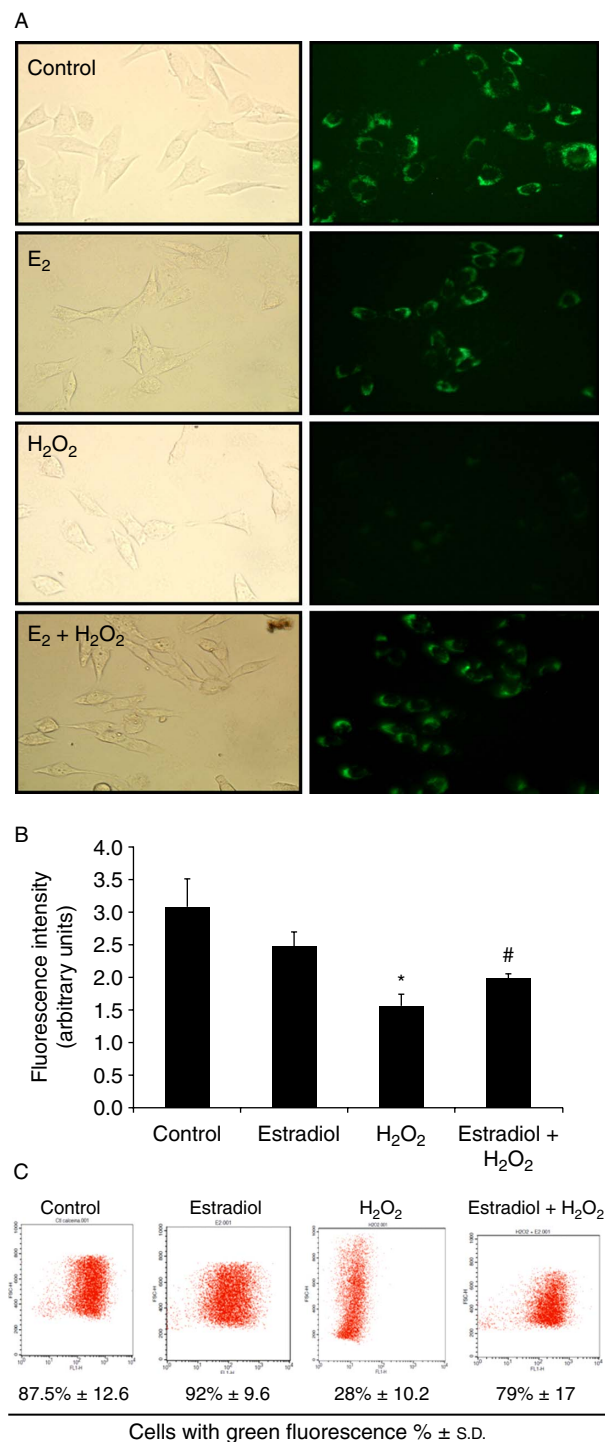


Figure 1

E₂ prevents H₂O₂-induced MPTP opening in C2C12 skeletal myoblasts. Cells loaded with calcein-AM/CoCl₂ were incubated with 10⁻⁸ M E₂ or vehicle, before the induction of apoptosis with 0.5 mM H₂O₂ for 4 h, as described in Materials and methods. MPTP opening was analyzed using a fluorescence microscope and flow cytometry. (A and B) Microscopy analysis. (C) Flow cytometry analysis. Treatment with H₂O₂ leads to a pronounced decrease in mitochondrial calcein fluorescence while pretreatment with E₂ conduces to a recovery of this signal, indicating the prevention of MPTP aperture. Representative photographs of each condition are shown. Experiments were repeated at least three times with essentially identical results. Image quantification was performed using the ImageJ 1.46 program. Averages \pm s.d. are given. * $P < 0.05$ with respect to the control and # $P < 0.05$ with respect to H₂O₂. FL-1: FL-1 channel that detects green fluorescence. Full colour version of this figure available via <http://dx.doi.org/10.1530/JOE-12-0486>.

cytometry, which allows for accurate quantification of cell fluorescence labeling. Thus, it was found that incubation with H_2O_2 led to a reduction in mitochondrial calcein fluorescence, evidenced by a decreased FL-1 signal compared with control (from 87.5 ± 12.6 vs $28 \pm 10.2\%$ respectively). However, in the presence of the steroid, H_2O_2 was unable to induce this loss of mitochondrial fluorescence implying that E_2 prevented MPTP activation. Accordingly, when the cells were pretreated with E_2 and then with the apoptotic agent, the percentage of cells with green fluorescence ($79 \pm 17\%$) was near to the control condition ($87 \pm 12.6\%$) (Fig. 1C).

$\Delta\Psi_m$ loss can be associated with the opening of MPTP (Armstrong 2006). In agreement with the operation of this mechanism, we found by flow cytometry and using the JC-1 probe that after 4 h of treatment with

H_2O_2 the $\Delta\Psi_m$ loss was highly pronounced (Fig. 2A). Recent studies have demonstrated that the loss in $\Delta\Psi_m$ induced by H_2O_2 can be prevented by pretreatment with E_2 (AC Ronda, A Vasconsuelo & R Boland, submitted for publication). Moreover, as shown in Fig. 2B, experiments measuring calcein-AM by flow cytometry indicated that the opening of the pore was observed even at 30 min of treatment with H_2O_2 and increased in a time-dependent fashion, persisting for 4 h. However, at 30 min of treatment with H_2O_2 , $\Delta\Psi_m$ loss was not evidenced (data not presented).

These results are in general agreement with data previously obtained using Janus Green stain, an indicator of the status of the oxido-reduction mitochondrial system (Lazarow & Cooperstein 1953, Ernster & Schatz 1981, Ronda *et al.* 2010).

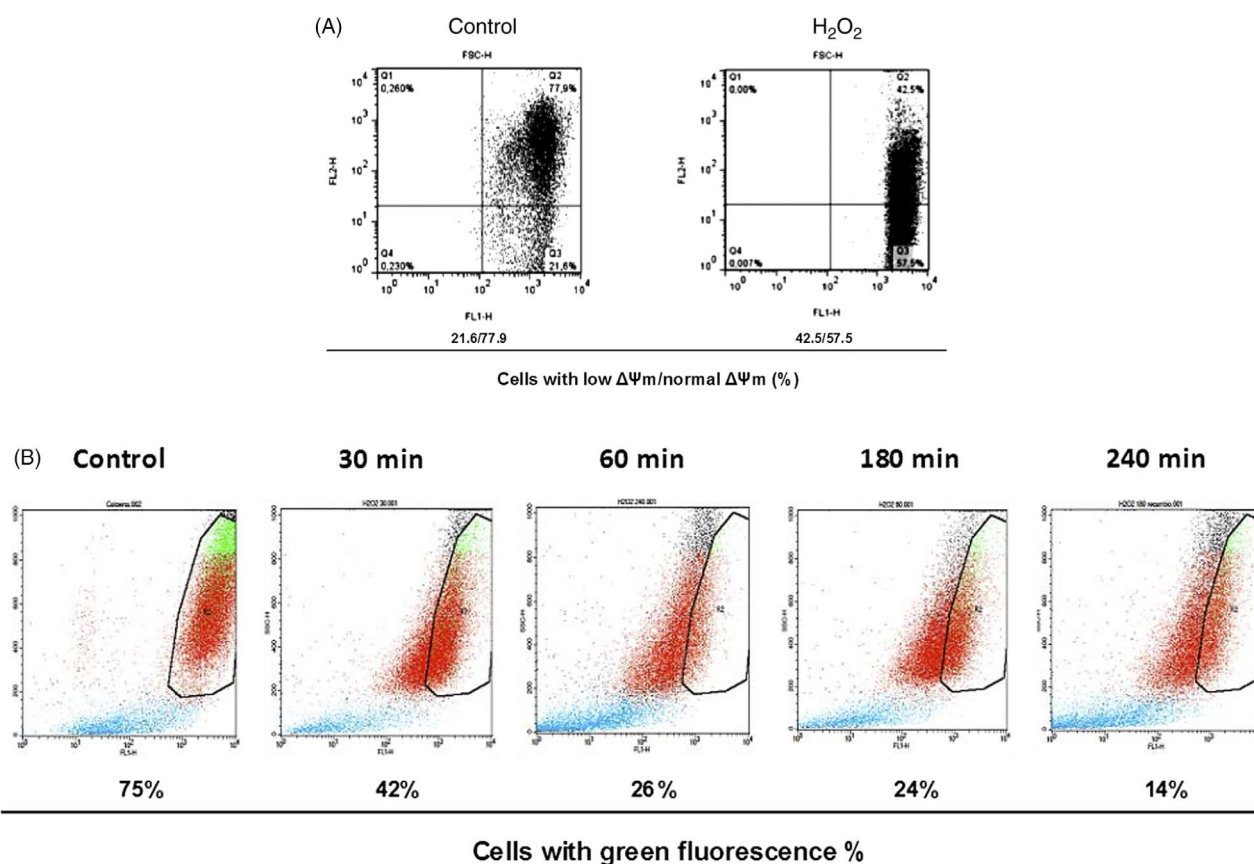


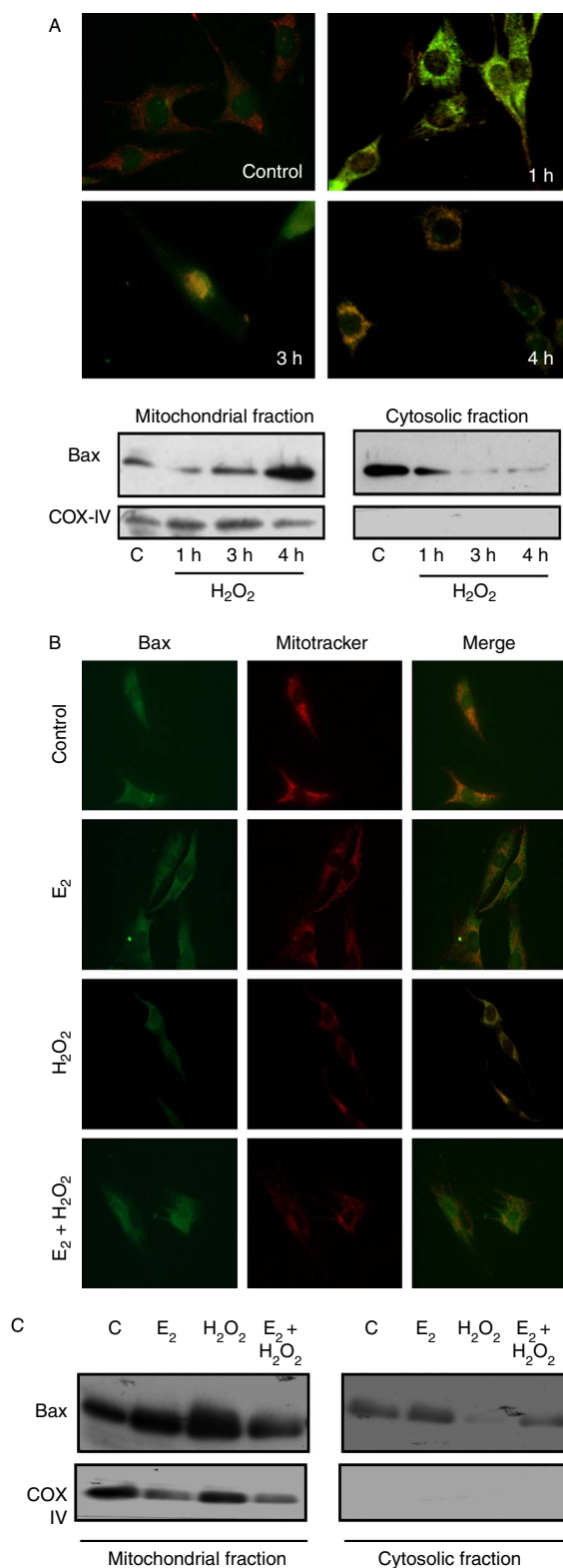
Figure 2

MPTP opening precedes mitochondrial membrane potential loss in C2C12 skeletal myoblasts. C2C12 cells loaded with JC-1 or calcein-AM/CoCl₂ were incubated with 0.5 mM H_2O_2 during the time periods indicated and then analyzed by flow cytometry (10 000 events). (A) $\Delta\Psi_m$ was measured in cells incubated under control conditions for 4 h with H_2O_2 . (B) MPTP opening

was analyzed at different treatment intervals with H_2O_2 (30, 60, 180, and 240 min). Experiments were repeated at least three times with essentially identical results. FL-1: FL-1 channel that detects green fluorescence. Full colour version of this figure available via <http://dx.doi.org/10.1530/JOE-12-0486>.

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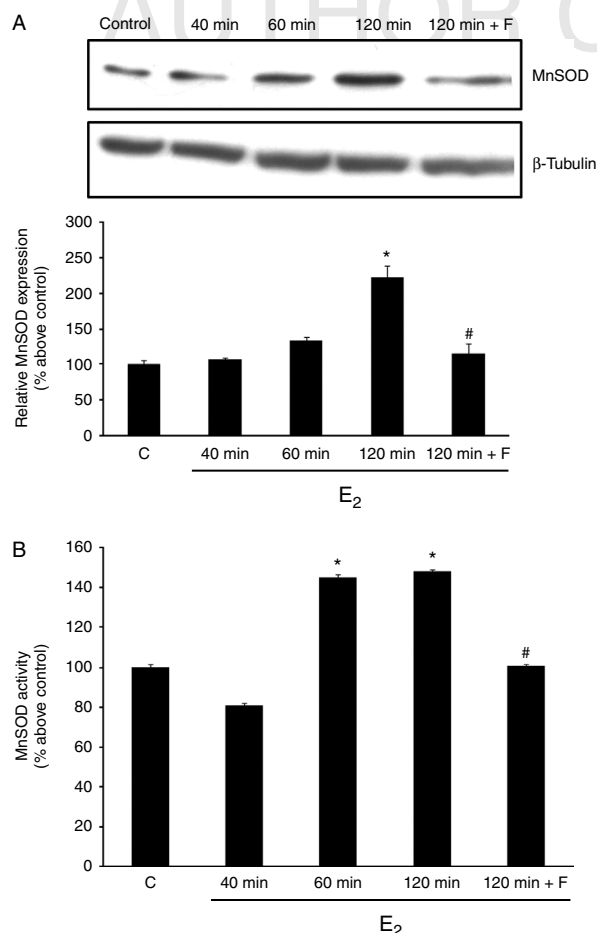
E₂ inhibits Bax translocation to mitochondria in C2C12 skeletal myoblasts



As mentioned in the Introduction, in healthy cells Bax exists as a monomer either in the cytosol or loosely attached to the outer mitochondrial membrane. Upon induction of apoptosis, the cytosolic Bax translocates to mitochondria and is inserted into the organelle's membrane, affecting its function. To correlate the results described above with the participation of Bax, cells were treated with 0.5 mM H₂O₂ during different time intervals. Immunocytochemistry studies were performed using an anti-Bax polyclonal antibody (green fluorescence) and Mitotracker (red fluorescence). We observed that H₂O₂ treatment induced time-dependent (1, 3, and 4 h) Bax translocation to mitochondria in C2C12 cells, which was more evident after 3 h of apoptosis induction. As shown in Fig. 3A (upper panel), control conditions showed a fine-grained fluorescence, homogeneously dispersed in the cytoplasm. However, in presence of H₂O₂ the fluorescence appeared focalized in mitochondria (yellow fluorescence). These results were corroborated by performing western blot assays (Fig. 3A, bottom panel). To evaluate the effect of E₂ over Bax translocation, cells were stained with Mitotracker and then treated with 10⁻⁸ M E₂ or vehicle 0.001% isopropanol (control) ~120 min before induction of apoptosis (0.5 mM H₂O₂, 4 h). Immunocytochemistry studies were performed using an anti-Bax antibody as before. In presence of the hormone, the green fluorescence appeared diffuse in all the cytosol as in the control condition, showing inhibition of Bax translocation to mitochondria

Figure 3

E₂ inhibits H₂O₂-induced Bax translocation to mitochondria in C2C12 skeletal myoblasts. (A) Cells were treated with 0.5 mM H₂O₂ for 1, 3, and 4 h. (Upper panel) Cells were preincubated with Mitotracker (red fluorescence) and then with anti-Bax, and fluorophore-conjugated secondary antibodies (green fluorescence) were used as described in Materials and methods. In the control condition, Bax is visualized as fine-grained fluorescence homogeneously dispersed in the cytoplasm while H₂O₂ treatment induced its translocation to mitochondria (yellow fluorescence). (Bottom panel) Mitochondrial and cytosolic fractions were used to perform western blot assays and probed for Bax localization. (B) Cells were incubated with vehicle (control) or 10⁻⁸ M E₂ before the addition of H₂O₂ for immunocytochemistry studies as described above. Representative images are shown. Magnification 60×. (C) Lysates were obtained following different treatments and then subjected to subcellular fractionation. Enriched mitochondrial and cytosolic fractions were used to perform western blot assays and probed for Bax translocation. Experiments were repeated at least three times with essentially identical results. Full colour version of this figure available via <http://dx.doi.org/10.1530/JOE-12-0486>.

**Figure 4**

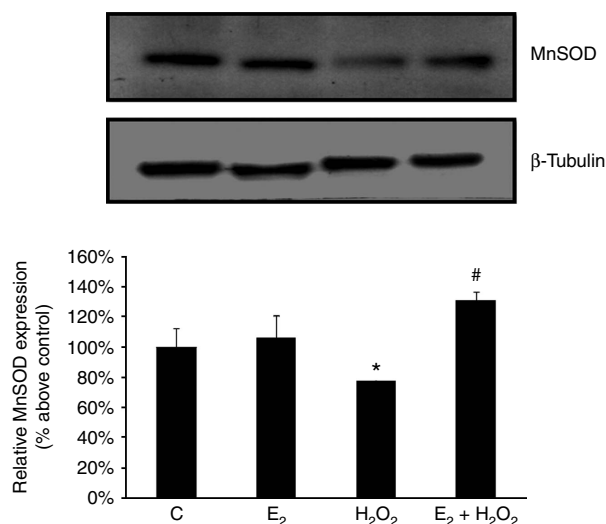
E_2 increases MnSOD expression and activity in C2C12 skeletal myoblasts. (A) Cells were incubated with 10^{-8} M E_2 , 1 μ M fulvestrant (F), or vehicle (control) during the times indicated, as described in Materials and methods. Lysates were subjected to SDS-PAGE and probed for the expression of MnSOD by western blot analysis. The blots are representative of three independent experiments with comparable results. (B) Cells were treated as above. Mitochondrial fractions were obtained and used to estimate MnSOD activity with a commercial kit. Experiments were repeated at least three times with essentially identical results. Densitometric quantification of blots is shown; averages \pm s.d. are given. * $P < 0.05$ with respect to the control and # $P < 0.05$ with respect to E_2 120 min.

(Fig. 3B). We confirmed these results by performing western blots assays using the same antibody (Fig. 3C).

E_2 modulates MnSOD in C2C12 skeletal myoblasts cells

As it is known that ROS levels affect MPTP performance, and that they are regulated in part by mitochondrial MnSOD (Van Remmen *et al.* 2001), we investigated whether E_2 exerts any effects on this antioxidant mitochondrial enzyme. We evaluated MnSOD expression levels in response to the hormone. C2C12 cell cultures

were incubated with 10^{-8} M E_2 during different time intervals (40, 60, and 120 min) followed by measurement of MnSOD protein expression by immunoblot analysis of cell lysates. Figure 4A shows that MnSOD protein level was increased by E_2 time dependently (7 ± 2 , 34 ± 4.7 , and $122 \pm 16.8\%$ over control after 40, 60, and 120 min of treatment respectively). In addition, we observed that in presence of 1 μ M fulvestrant, an ER antagonist, the E_2 -induced MnSOD expression was significantly inhibited ($107 \pm 14\%$). We also investigated the effects of E_2 on enzyme activity. To that end, C2C12 cells were treated with the hormone as before and lysates were centrifugated to obtain the mitochondrial fraction. Then, the activity of MnSOD was evaluated using a commercially available Superoxide Dismutase Assay Kit II as described in Materials and methods. As shown in Fig. 4B, the steroid increased MnSOD activity (45 ± 1.7 and $48 \pm 1.1\%$ above the control after 60 and 120 min of treatment respectively). Moreover, when cells were preincubated with fulvestrant and then treated with the hormone, the E_2 -induced increase in MnSOD activity was prevented ($47 \pm 0.6\%$). We also observed (Fig. 5) that H_2O_2 treatment reduced the levels of MnSOD ($61 \pm 4.6\%$ with respect to control), thus indicating that the enzyme could

**Figure 5**

E_2 restores MnSOD expression in C2C12 skeletal myoblasts treated with H_2O_2 . C2C12 cells were incubated under different experimental conditions. C and E_2 : vehicle or 10^{-8} E_2 for 6 h. H_2O_2 : 0.5 mM H_2O_2 for 4 h. $E_2 + H_2O_2$: 10^{-8} E_2 for 2 h followed by 0.5 mM H_2O_2 for 4 h in the presence of the steroid, as indicated in Materials and methods. Lysates were used to probe for the expression of MnSOD by western blot assays. The blots are representative of three independent experiments with comparable results. Densitometric quantification of blots is shown; averages \pm s.d. are given. * $P < 0.05$ with respect to the control and # $P < 0.05$ with respect to H_2O_2 .

be involved in mitochondrial dysfunction induced by the oxidant. Moreover, E_2 was able to diminish this deleterious effect over the mitochondrial enzyme.

Discussion

Although the classical target of estrogens is the nucleus, it has been demonstrated in the last years that E_2 can act at different cellular sites including mitochondria (reviewed in Vasconsuelo *et al.* (2011)). In previous studies, we showed that E_2 abrogates H_2O_2 -induced apoptosis in skeletal muscle cells involving both $ER\alpha$ and $ER\beta$, PI3K/Akt/Bad, HSP27, and MAPKs (Vasconsuelo *et al.* 2008, 2010, Ronda *et al.* 2010). Accordingly, a protective effect of the steroid on mitochondria integrity was evidenced, as cytochrome *c* release by H_2O_2 was inhibited by E_2 through $ER\beta$ (Vasconsuelo *et al.* 2008). The data obtained in this work, using the C2C12 murine skeletal myoblasts line, deepen the knowledge of molecular actions triggered by E_2 , which result in mitochondrial protection. Although the exact nature of cytochrome *c* release during apoptosis remains to be completely understood, it is believed that multiple mechanisms may coexist in a cell and the activation of one of them under a certain condition may be influenced by several factors such as the nature of the apoptotic inducer (Gogvadze *et al.* 2006). In this work, by means of an established method to evaluate MPTP opening with calcein-AM/ $CoCl_2$ loaded cells, it was found that H_2O_2 treatment induced MPTP aperture. The participation of Bcl-2 proteins, specifically Bax, should be taken into consideration, as the present studies demonstrated that H_2O_2 treatment induced its translocation to mitochondria. In mitochondria from other cell types, Bax can interact with MPTP components: VDAC or ANT (Marzo *et al.* 1998, Narita *et al.* 1998). Therefore, Bax may regulate MPTP function via direct molecular interactions with these pore components. Further investigations of protein interactions are necessary to evaluate whether these events occur in skeletal muscle cells in response to H_2O_2 treatment. In this study it was shown that when cells were preincubated with E_2 , both Bax translocation to mitochondria and MPTP aperture were abolished. These processes could be part of the molecular mechanism activated by the hormone, resulting in mitochondrial protection as a consequence of an antiapoptotic effect.

It has been reported that during apoptosis, the mitochondrial membrane potential ($\Delta\Psi_m$) decreases and that there exists a connection between the functions

of $\Delta\Psi_m$ and of MPTP (Armstrong 2006). However, there are controversies about which event occurs initially, wondering if membrane depolarization is the consequence rather than the cause of pore opening. In the current study, contributing with more data to elucidate this unresolved subject, it was evidenced that opening of MPTP is achieved before $\Delta\Psi_m$ loss in C2C12 skeletal myoblasts cells. In this context, additional studies are necessary to determine the exact point of action of Bcl-2 family members (e.g. Bax might interact with VDAC or other MPTP protein components), regulating MPTP opening and $\Delta\Psi_m$ loss.

We also showed that E_2 modulates the mitochondrial enzyme MnSOD, suggesting a possible role in E_2 protective action. ROS can activate the MPTP (Petronilli *et al.* 1994) but cells are equipped with antioxidant enzymes that can prevent these and other effects of ROS. We demonstrated that the hormone was able to increase MnSOD protein levels and accordingly its activity in skeletal muscle cells. Therefore, it can be postulated a genomic rather than a rapid effect of E_2 at this level. Our results indicate that the hormone prepares the cell to resist to an apoptotic stimulus. H_2O_2 treatment reduced the levels of MnSOD suggesting that this could be a factor contributing to muscle apoptosis. Preincubation with E_2 diminished the loss of MnSOD in H_2O_2 -treated C2C12 cells. In view that the ER antagonist fulvestrant blocked the estrogen effects on MnSOD in the muscle cell system, we propose that an ER is involved in this antiapoptotic mechanism. Other antioxidant enzymes such as catalase or glutathione peroxidase could also mediate the protective effect. Studies are currently in progress to elucidate whether the hormone regulates these enzymes.

In conclusion, the results presented herein, linked to our previously published data, demonstrate that physiological levels of E_2 regulate several muscle cell signaling intermediates that by acting in concert, both at the genomic and non-genomic level, exert antiapoptotic effects in C2C12 skeletal myoblasts.

Finally, these studies are of relevance to skeletal muscle physiology as C2C12 myoblasts resemble the activated satellite cells that surround mature myofibers. As differentiated adult skeletal muscle fibers have scarce ability to repair and regenerate themselves when a cellular injury exists, satellite cells have the capacity to proliferate and differentiate, vital properties to repair the injured tissue (Yoshida *et al.* 1998). In this context, satellite cells and their response to stress such as oxidative stress are important to mature skeletal muscle performance and function. Of significance for our work, enhanced satellite

cell apoptosis has been related to compromised recovery potential in skeletal muscle of aged animals (Jejurikar & Kuzon 2003, Jejurikar *et al.* 2006).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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