17β-Estradiol abrogates apoptosis in murine skeletal muscle cells through estrogen receptors: role of the phosphatidylinositol 3-kinase/Akt pathway

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Abstract

Estrogens can regulate apoptosis in various cellular systems. The present study shows that 17β-estradiol (E_2), at physiological concentrations, abrogates DNA damage, poly (ADP-ribose) polymerase cleavage, and mitochondrial cytochrome *c* release induced by H_2O_2 or etoposide in mouse skeletal muscle C2C12 cells. This protective action, which involved PI3K/Akt activation and Bcl-2 associated death agonist (BAD) phosphorylation, was inhibited by antibodies against the estrogen receptor (ER) α or β isoforms, or transfecting siRNA specific for each isoform. The inhibition of the antiapoptotic action of E_2 at the mitochondrial level was more pronounced when ER-β was immunoneutralized or suppressed by mRNA silencing, whereas transfection of C2C12 cells with either ER-α siRNA or ER-β siRNA blocked the activation of Akt by E_2, suggesting differential involvement of ER isoforms depending on the step of the apoptotic/survival pathway evaluated. These results indicate that E_2 exerts antiapoptotic effects in skeletal muscle cells which are mediated by ER-β and ER-α and involve the PI3K/Akt pathway.

Journal of Endocrinology (2008) **196,** 385–397

Introduction

The estrogen 17β-estradiol (E_2) is a steroid hormone whose actions involve genomic and non-genomic mechanisms (Bjornstrom & Sjoberg 2005). It is generally accepted that the majority of the effects of the hormone are mediated via two estrogen receptors (ERs), namely ER-α and ER-β, which are members of the nuclear receptor superfamily, by regulating nuclear estrogen responsive genes (Evans 1988, Tsai & O'Malley 1994, Beato *et al.* 1996, Pettersson *et al.* 2000, Hall *et al.* 2001, Hewitt & Korach 2002). Also, several investigators have pointed out the possibility that the ER could be non-classically associated with intracellular membranes (Parikh *et al.* 1980, Watson & Muldoon 1985, Muldoon *et al.* 1988, Monje & Boland 1999, Watson *et al.* 1999). Moreover, there is evidence showing that ER-α and ER-β may be located in the plasma membrane (Luconi *et al.* 1999, Norfleet *et al.* 2000, Ropero *et al.* 2002, Monje & Boland 2001, Monje *et al.* 2001, Li *et al.* 2003) and mitochondrial compartments (Zheng & Ramirez 1999, Horvat *et al.* 2001, Monje & Boland 2002, Yang *et al.* 2004, Solakidia *et al.* 2005). In addition, the non-genomic events triggered by E_2 suggest the ability of the hormone to activate extranuclear receptors (Bjornstrom & Sjoberg 2005). Among the rapid non-transcriptional actions of E_2, the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway has been shown in various cellular lines (Fernando & Wimalasena 2004, Guo *et al.* 2006). PI3K regulates phosphoinositide metabolism and is responsible for the generation of phosphatidylinositol-3,4,5-trisphosphate (PIP_3; Vanhaesebroeck *et al.* 2001, Osaki *et al.* 2004). The activation of PI3K results in PIP_3-mediated activation of the serine–threonine kinase Akt by phosphorylation. In turn, phospho-Akt modulates the function of numerous substrates involved in the regulation of cell functions as for example apoptosis (Coffer *et al.* 1998, Vanhaesebroeck *et al.* 2001). There is evidence that E_2 is able to promptly activate the PI3K/Akt pathway by different mechanisms, in an ER-dependent and ER-independent manner, depending on the cellular type (Guo *et al.* 2006). Accordingly, estrogens exert antiapoptotic effects on various cell types such as vascular endothelial, smooth muscle, and breast cancer cells, among others (Spyridopoulos *et al.* 1997, Sudoh *et al.* 1998, Razandi *et al.* 2000).

There is evidence that skeletal muscle is a target tissue for estrogens. Muscle mass and strength diminish during the postmenopausal years leading to sarcopenia which is a risk factor for osteoporosis since it is associated with physical disability and immobility resulting in bone loss. Sarcopenia depends, in part, on estrogen levels. Thus, hormone replacement therapies prevent a decline in muscle performance (Dionne *et al.* 2000). Congruent with these observations, it has been recently established that human skeletal muscle contains ER-α and ER-β (Lemoine *et al.* 2003, Wiik *et al.* 2003), although the exact mechanism by which estrogens prevent sarcopenia remains to be clarified.

It has been shown that estrogens promote proliferation and differentiation of skeletal myoblasts (Kahlert *et al.* 1997). Moreover, studies with myoblasts have demonstrated that

DOI: 10.1677/JOE-07-0250
0022–0795/08/0196–385
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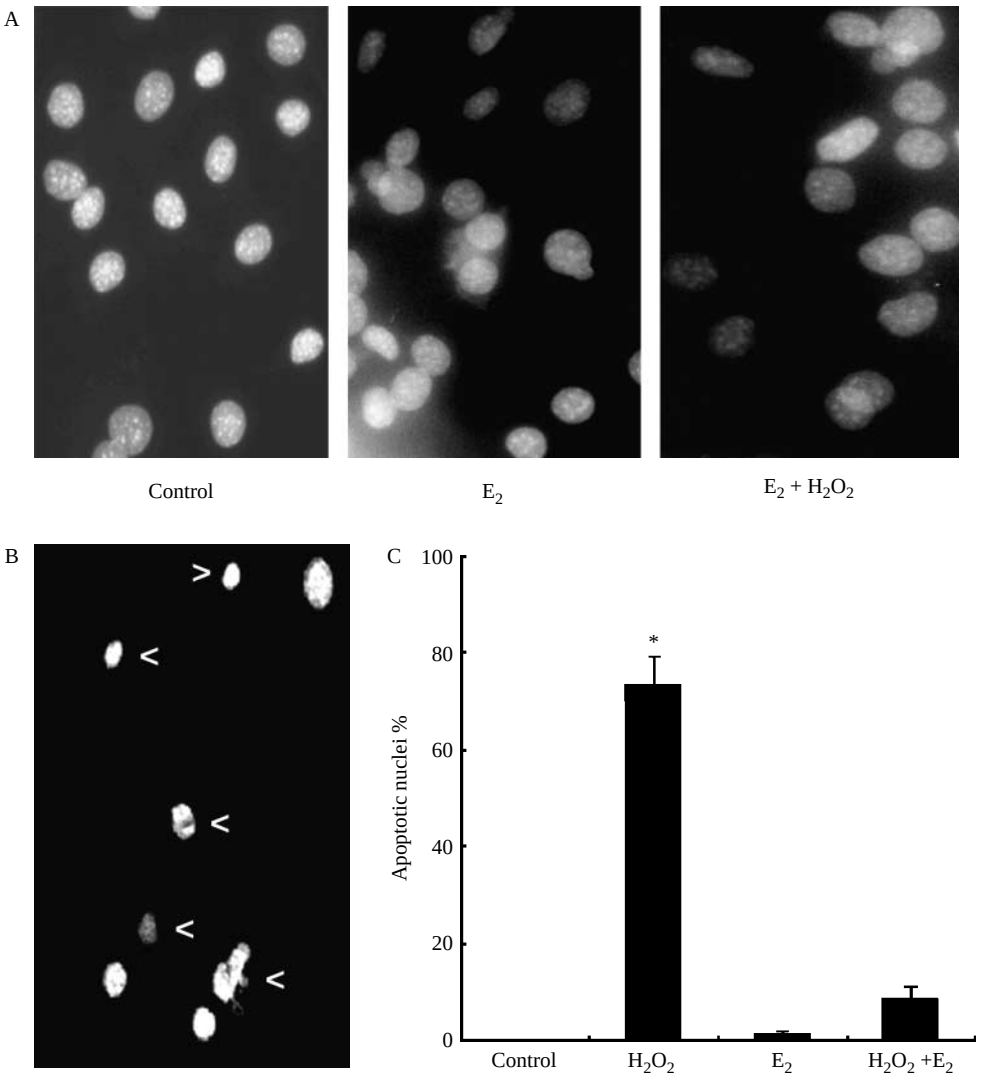


Figure 1 Inhibition of H_2O_2-induced apoptosis by 17β-estradiol in C2C12 muscle cells. Cultured C2C12 cells were treated with 0·5 mM H_2O_2 (H_2O_2) for 24 h or preincubated with 10^{-8} M 17β-estradiol before addition of H_2O_2 ($E_2+H_2O_2$). The cells were then stained with DAPI as described under Materials and Methods. Morphological analysis of fluorescence-stained nuclei is shown. (A) Control- (untreated C2C12 cells cultured in regular medium), E_2-, or $E_2+H_2O_2$-treated cells, with normal nuclear morphology. (B) Apoptotic cells ($+H_2O_2$) with pyknotic nuclei, condensed chromatin or DNA fragmentation (arrowheads). (C) Percentages of apoptotic cells at each condition. At least ten fields per dish were examined. Each value represents the mean of three independent experiments ± S.D. $^*P<0·05$ with respect to the control. Representative photographs of apoptotic and normal cells are shown. Experiments were repeated at least three times with essentially identical results.

apoptosis plays an important role in skeletal muscle development, by controlling the size of the population of proliferating myoblasts which undergo differentiation into mature myotubes (Walsh 1997, Sandri & Carraro 1999, Huppertz *et al.* 2001). Then, the effects of the hormone on skeletal muscle development could also be regulated, in part, through its effects on apoptosis.

In view of the above lines of evidence, the objective of the present work was to investigate whether E_2 exerts a regulatory action on apoptosis in skeletal muscle and to obtain information on the mechanism involved therein. To that end, C2C12 murine skeletal muscle cells treated with etoposide or hydrogen peroxide (H_2O_2) were used as experimental model. H_2O_2 and etoposide, a topoisomerase II inhibitor, have been widely used as inducers of apoptosis and a substantial literature details many biochemical events that occur upon apoptotic induction by both agents in a variety of cell types including C2C12 muscle cells (Mizumoto *et al.* 1994, Kavurma & Khachigian 2003, Biswas *et al.* 2005, Jiang *et al.* 2005*a*,*b*). The data obtained demonstrate

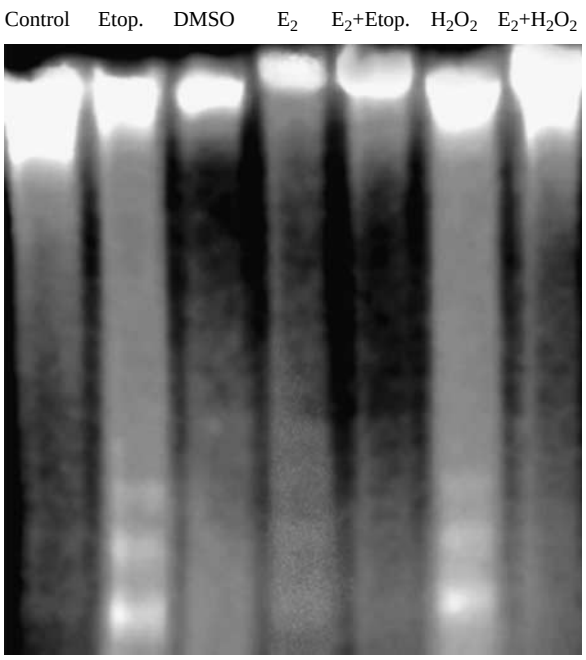


Figure 2 Inhibition of H_2O_2-or etoposide-induced DNA fragmentation by 17β-estradiol in C2C12 muscle cells. After treatments, DNA was extracted from C2C12 cells as described under Materials and Methods, separated by electrophoresis on agarose gels, stained with ethidium bromide and visualized under u.v. light. Control, untreated cells; Etop., cells treated with 25 μg/ml etoposide for 24 h; DMSO, cells treated with etoposide dissolvent; E_2, cells treated with 10^{-8} M 17β-estradiol for 45 min; E_2+ Etop., cells preincubated with 10^{-8} M 17β-estradiol for 45 min and then treated with 25 μg/ml etoposide for 24 h; H_2O_2, cells treated with 0·5 mM H_2O_2 during 24 h; H_2O_2 +E_2, cells preincubated with 10^{-8} M 17β-estradiol for 45 min and then treated with 0·5 mM H_2O_2 during 24 h. Experiments were repeated at least three times with essentially identical results.

an antiapoptotic action of E_2 in C2C12 skeletal muscle cells exposed to etoposide or oxidative stress (H_2O_2), which requires PI3K/Akt activation and is mediated by ER-β and ER-α.

Materials and Methods

Materials

ER-α mouse monoclonal antibody Ab-10/clon TE111.5D11 (anti-ER ligand binding domain) was purchased from NeoMarkers (Fremont, CA, USA). ER-β goat polyclonal antibodies L-20 (mapping near C-terminus) and Y-19 (mapping at the N-terminus), and anti-lamin B were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). PhosphoDetect anti-Bad (Ser136) and anti-poly (ADP-ribose) polymerase (PARP) pAb were from Calbiochem (San Diego, CA, USA). Anti-phospho-Akt (Ser473) was from Cell Signaling Technology Inc (Danvers, MA, USA). DAPI and MitoTracker Red (MitoTracker Red CMXRos) dyes were from Molecular Probes (Eugene, OR, USA). ER-α and ER-β ShortCut siRNA, fluorescein-siRNA transfection control and TransPass R2 transfection reagent were from New England Biolabs (NEB, Beverly, MA, USA). ICI 182 780 was from Tocris (Ellisville, MO, USA). Cytochrome *c* oxidase assay kit, etoposide, E_2-peroxidase and diethylstilbestrol (DES) were purchased from Sigma–Aldrich. DNAzol Reagent was from GIBCO BRL. The PI3K inhibitors wortmannin and LY294002 were obtained from Alomone Labs Ltd (Jerusalem, Israel). All the other reagents used were of analytical grade.

Cell culture and treatment

C2C12 murine skeletal muscle cells, kindly donated by Dr Enrique Jaimovich (Universidad de Chile, Santiago, Chile), were cultured in growth medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated (30 min, 56 °C) fetal bovine serum), 1% nistatine, and 2% streptomycin. 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 in medium without serum by adding E_2, ICI 182 780, or the non-steroidal analog DES, ~45 min before induction of apoptosis with hydrogen peroxide (H_2O_2) or etoposide during 24 h or the time indicated in specific experiments. H_2O_2 was diluted in culture medium without serum at a final concentration of 0·5 mM in each assay and etoposide was prepared in dimethyl sulfoxide (DMSO) at a final concentration of 25 μg/ml in each assay. Unless otherwise noted, cells were cultured in chamber slides for microscopy.

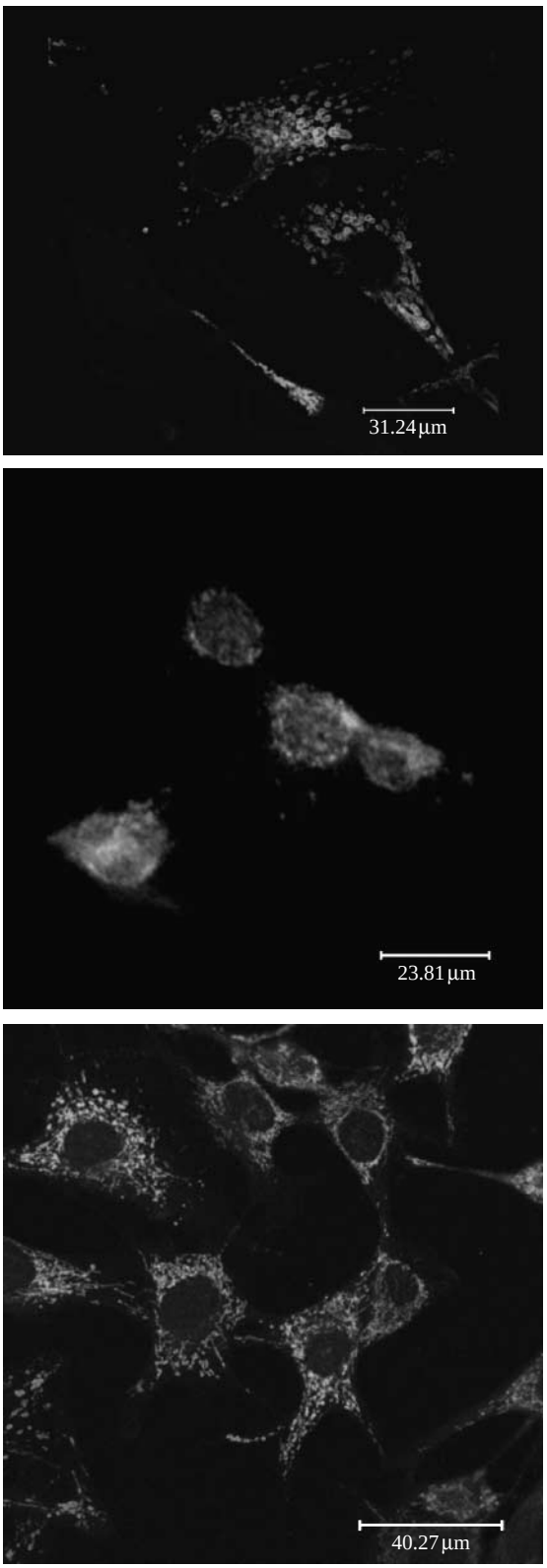
To block the protective effects of hormone on H_2O_2-induced cytochrome *c* release (determined by evaluation of outer mitochondrial membrane integrity; see below) using different monoclonal antibodies against ER-α and ER-β, the cultured muscle cells were first permeabilized with saponin (50 μg/ml; 1 min at 37 °C) and then incubated for 1 h at 37 °C in presence of a 1:100 dilution of the antibodies in DMEM.

Quantitation of apoptotic cells

After treatments, the cells were fixed with methanol at −20 °C for 30 min and then washed with PBS. Fixed cells were incubated for 30 min at room temperature in darkness with 1:500 of a stock solution of DAPI (5 mg/ml) and next washed with PBS. Cells were mounted on glass slides and examined using a fluorescence microscope (NIKON Eclipse E 600) equipped with standard filter sets to capture fluorescent signals. Images were collected using a digital camera. Apoptotic cells were identified by the condensation and/or fragmentation of their nuclei. The results were expressed as percentage of apoptotic cells. A minimum of 500 cells was counted for each treatment from at least three independent experiments.

Detection of DNA fragmentation

DNA was extracted from treated or control cells, using DNAzol Reagent (Gibco-BRL) as described by the manufacturer. Isolated



DNA (30 μg for each condition) was electrophoresed on a 1·5% agarose gel in Tris-acetate-EDTA buffer at 40 mA. The gel was stained with ethidium bromide and visualized under u.v. light.

Subcellular fractionation

C2C12 confluent 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 μg/ml leupeptin, 20 μg/ml aprotinin, 20 μg/ml trypsin inhibitor) using a Teflon-glass hand homogenizer. A nuclear pellet was obtained by low speed centrifugation (800 ***g***, 20 min) of the lysed cell preparation. The supernatant was further centrifuged at 10 000 ***g*** for 15 min to pellet mitochondria. The remaining supernatant was centrifuged at 120 000 ***g*** for 90 min to yield a soluble supernatant (cytosol) and a plasma membrane-containing particulate pellet (microsomes). Contamination of nuclear, microsomal, and cytosolic fractions with mitochondrial components was assessed by measuring the activity of the mitochondrial marker enzyme cytochrome *c* oxidase employing the Cytochrome *c* Oxidase assay kit (Sigma) according to the manufacturer's instructions. Also, anti-lamin B antibody was employed for the immunodetection of the nuclear protein marker lamin B in the different fractions. Negligible cross-contamination of fractions with nuclei and mitochondria was detected (data not shown).

Protein concentration of the fractions was estimated by the method of Bradford (1976), using BSA as standard.

Western blot analysis

Protein samples (25 μg) were mixed with one-fourth of sample buffer (400 mM Tris–HCl (pH 6·8), 10% SDS, 50% glycerol, 500 mM DTT, and 2 mg/ml bromophenol blue), boiled for 5 min, and resolved by 10% sodium dodecyl sulfate-PAGE (SDS-PAGE) according to the method of Laemmli (1970). Fractionated proteins were electrotransferred to polyvinylidene fluoride membranes (Immobilon-P) 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 for 1 h with the appropriate dilution of the primary antibodies: Ab-10/clon

Figure 3 17β-Estrdiol inhibits changes in morphology and localization of mitochondria induced by H_2O_2 in C2C12 muscle cells. C2C12 cells grown on coverslips as 60–70% confluent monolayers were treated (see below), stained with MitoTracker Red, and fixed with methanol as described under Materials and Methods. (A) Untreated cells. (B) Cells treated with 0·5 mM H_2O_2 during 24 h. (C) Cells preincubated with 10^{-8} M 17β-estradiol for 45 min and then treated with 0·5 mM H_2O_2 during 24 h. Cells in A and C present normal mitochondrial morphology and distribution throughout the entire cell distant to the nucleus or display 'spider-web' mitochondria; but cells in B exhibit mitochondria clustered around the nucleus with condensed or pyknotic aspect. At least ten fields per slide and three independent cultures were examined. Representative photographs are shown. Magnification: 63×.

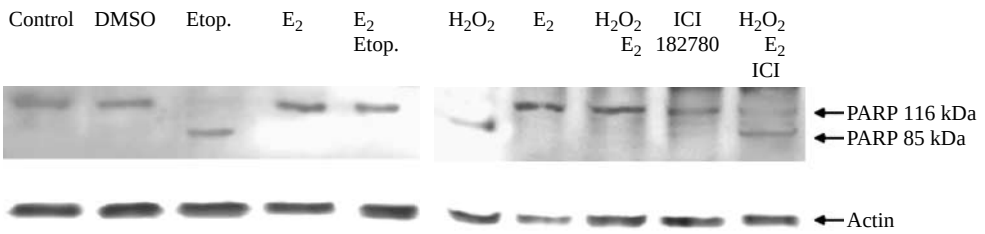


Figure 4 Inhibition of H_2O_2- or etoposide-induced PARP cleavage by 17β-estradiol. C2C12 cells were treated with the indicated stimuli. Control, untreated cells; DMSO, cells treated with etoposide dissolvent 24 h; Etop., cells treated with 25 μg/ml etoposide for 24 h; E_2, cells incubated with 10^{-8} M 17β-estradiol for 24 h; E_2 + Etop., cells preincubated with 10^{-8} M 17β-estradiol for 45 min and then treated with 25 μg/ml etoposide for 24 h; H_2O_2, cells treated with 0·5 mM H_2O_2 during 24 h; H_2O_2 + E_2, cells preincubated with 10^{-8} M 17β-estradiol for 45 min and then treated with 0·5 mM H_2O_2 during 24 h; ICI 182 780, cells incubated with 1 μM ICI 182 780 for 24 h; ICI + E_2 + H_2O_2, cells preincubated with 1 μM ICI 182 780 for 45 min and then with 17β-estradiol and H_2O_2 as before. Cell lysate proteins from each condition containing equivalent protein amounts (25 μg) were fractionated by SDS-PAGE, transferred to PVDF membranes, and western blotted with anti-PARP antibody as described in Materials and Methods. Bands represents uncleaved PARP (116 kDa) and cleavage band at 85 kDa. Actin levels are shown as protein loading control. Immunoblots representative are shown. Experiments were repeated at least three times with essentially identical results.

TE111.5D11 anti ER-α (1:400), anti-lamin B (1:200), anti-phospho-BAD (1:400), anti-PARP pAb (1:200), anti-phospho-Akt (1:1000), using anti-rabbit secondary antibodies for all of them. L-20 (1:300) anti-ER-β is an affinity purified goat polyclonal antibody. The membranes were repeatedly washed with PBS-T prior incubation with horseradish peroxidase-conjugated secondary antibodies. The enhanced chemiluminescence (ECL) blot detection kit (Amersham) was used as described by the manufacturer to visualize reactive products. Relative migration of unknown proteins was determined by comparison with molecular weight colored markers (Amersham). For actin loading control, membranes were stripped with stripping buffer (62·5 mM Tris–HCl (pH 6·7); 2% SDS; 50 mM β-mercaptoethanol) and then blocked for 1 h with 5% non-fat dry milk in PBS containing 0·1% Tween-20 (PBS-T). The blots were then incubated 1 h with a 1:20 000 dilution of anti-actin policlonal antibody (A-5060) as primary antibody.

After several washings with PBS-T, membranes were incubated with anti-rabbit (1:10 000) or anti-goat (1:50 000) secondary antibodies, depending on the source of the primary antibody, conjugated to horseradish peroxidase. The corresponding immunoreactive bands were developed by means of ECL.

ER-α recombinant protein and ER-β blocking peptides were used to confirm the specificity of antibodies used in the assays. Secondary antibodies alone were also employed as a negative control in western blots (Milanesi, de Boland and Boland submitted). All the antibodies employed were tested in the MCF-7 cell line and in cytosolic preparations from rabbit uterus and ovary (Monje & Boland 1999, 2001).

Transfection of short interfering RNA (siRNA)

Transfection was performed with a culture cellular density reaching 40–60% confluence with ER-α or ER-β ShortCut siRNA (NEB) according to the manufacturer's instructions. Briefly, TransPass R2 Transfection Reagent was mixed with ER-α or ER-β siRNAs (NEB). The mix was incubated for 20 min at room temperature and diluted with complete culture medium. The culture medium of the cells was aspirated and replaced with the diluted transfection complex mixture. The cells transfected were used in the indicated assays.

To estimate the transfection efficiency of siRNA, 10–30 pmol of fluorescein-siRNA (NEB) were used according to the manufacturer's instructions. Cells were then visualized, 24 and 48 h post transfection, in a conventional fluorescence microscope.

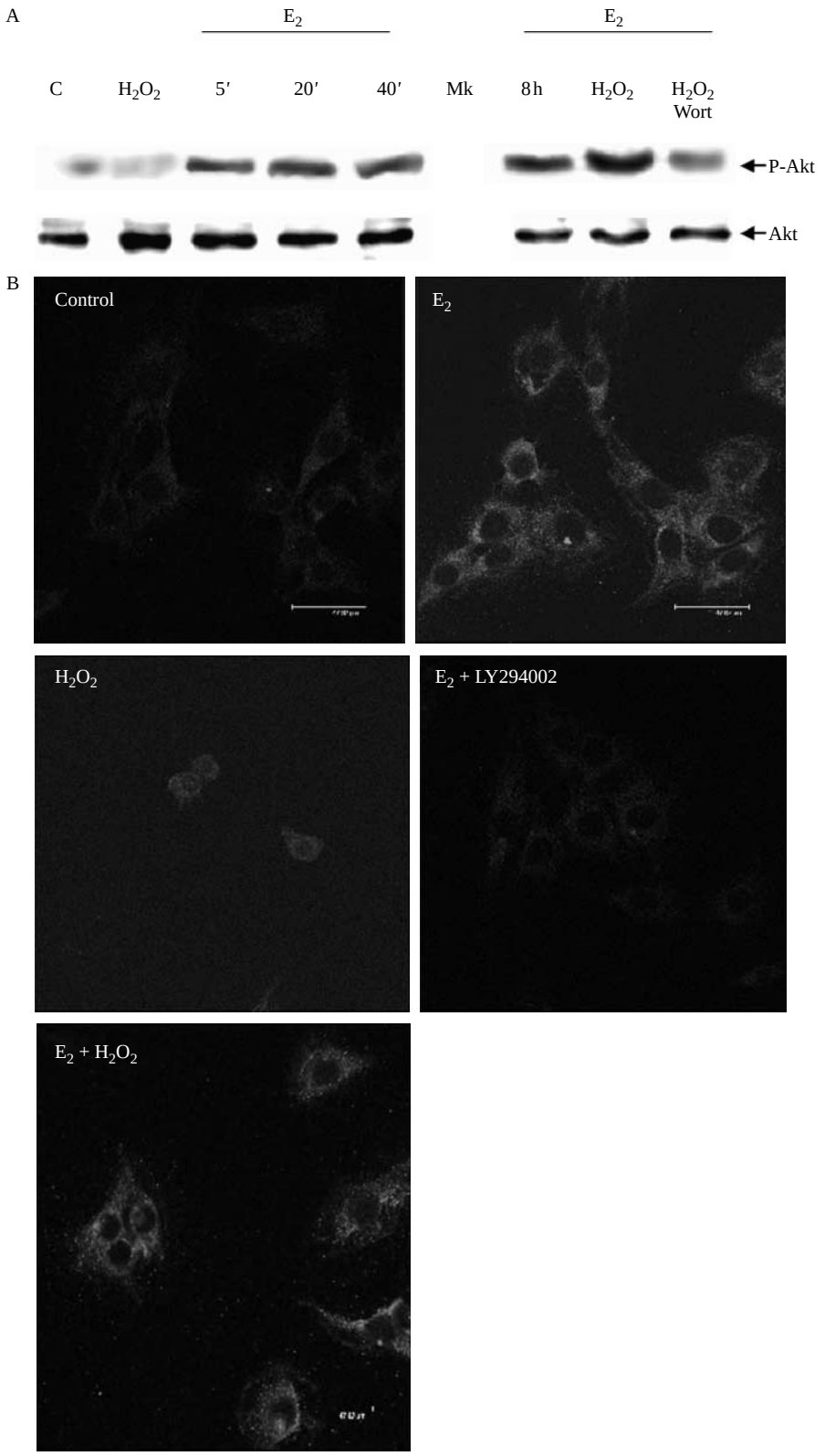
To evaluate the effective silencing of ER-α or ER-β, total proteins from transfected and non-transfected cells (controls) were extracted 24 and 48 h post transfection and ER-α or ER-β expression was tested by western blot analysis as described above using TE111.5D11 specific monoclonal antibody and Y-19 specific polyclonal antibody respectively.

Measurement of outer mitochondrial membrane integrity

The integrity of outer mitochondrial membranes was evaluated using a commercially available kit from Sigma (CYTOC-OX1) according to the manufacturer's instructions. Briefly, mitochondrial fractions (2 μg protein) were added to the assay buffer (10 mM Tris–HCl (pH 7·0) and 120 mM KCl), in presence and absence of the detergent *n*-dodecyl β-D-maltoside. To these samples, 50 μl reduced cytochrome *c* (0·22 mM) were added and changes in absorbance at 550 nm were monitored for 1 min. An extinction coefficient of 21·84 was used. The results were expressed as percentage of mitochondria with damaged outer membrane.

MitoTracker red staining

Coverslips with adherent cells were stained with MitoTracker red (Molecular Probes), which was prepared in dimethyl sulfoxide and then added to the cell culture medium at a final



concentration of 1 μmol/l. After 15- to 30-min incubation at 37 °C, the cells were washed with PBS and fixed with methanol at −20 °C for 30 min. Finally, the coverslips were analyzed by conventional and confocal fluorescence microscopy as described previously. Images were collected using a digital camera.

Confocal microscopy

The samples used for confocal microscopy were processed as described above and confocal scanning laser microscopy was performed with a Leica TCS SP2 AOBS microscope, using a 63× objective. The specificity of the labeling techniques was proven by the absence of labeling when the primary or the secondary antibodies were omitted.

Statistical analysis

Statistical treatment of the data was performed using the Student's *t*-test (Snedecor & Cochran 1967). Data are means ± S.D. of not less than three independent experiments. The data were considered statistical significant when $P<0{\cdot}05$.

Results

E_2 effects on H_2O_2- or etoposide-induced apoptosis in C2C12 muscle cells

C2C12 myogenic cells were challenged with 0·5 mM H_2O_2 for 24 h and apoptotic events were investigated. The nuclear dye DAPI showed morphological changes typical of apoptosis such as nuclear fragmentation/condensation (pyknotic nuclei) after treatment with inducers, which represented ∼70% of the cultured muscle cells (Fig. 1). The same results were obtained by treating the C2C12 cells with the semi-synthetic derivative of the podophyllotoxin etoposide (25 μg/ml) during 24 h (data no shown). Furthermore, H_2O_2 treatment resulted in DNA fragmentation in C2C12 cells, as evidenced by the formation of a DNA ladder in agarose gels (Fig. 2), also providing evidence that exposure to H_2O_2 induces apoptosis in the muscle cells. Comparable results were obtained using etoposide (Fig. 2). Trypan blue staining excluded the possibility that these treatments induced necrosis of the cells (data not shown). On the other hand, we observed that treating C2C12 cells with E_2 (10^{-8} M) or with the synthetic non-steroidal analog DES (10^{-8} M) for 45 min prior to the apoptotic stimulus of H_2O_2, the percentage of apoptotic nuclei was significantly diminished (from 73% when the cells were treated with H_2O_2 alone to ∼8% when the cultures were preincubated with E_2 before addition of H_2O_2). Similar values were obtained with etoposide (data not shown). In addition, DNA fragmentation induced by H_2O_2 or etoposide was abolished by the hormone treatment (Figs 1 and 2). The same results were obtained when the assays were performed in presence of DES (results not given).

The effects of E_2 and DES on the release of cytochrome *c* due to loss of outer mitochondrial membrane integrity induced by H_2O_2 were evaluated by means of CYTOC-OX1 assays (see Materials and Methods). We observed that 70 ± 1·5% of the cells presented damaged mitochondria after H_2O_2 treatment, whereas when the cultures were preincubated with E_2 or DES before addition of H_2O_2, only 21 ± 1·2% or 10 ± 0·4% respectively, of the mitochondria were affected ($P<0{\cdot}05$; three independent experiments). In addition, morphological changes and cellular redistribution of mitochondria could be detected in C2C12 cells treated with H_2O_2 as described above and stained with the fluorescent mitochondrial probe MitoTracker red. Thus, Fig. 3 shows that cells treated with vehicle (control) or E_2 display 'spider-web' or uniform distribution of mitochondria through the cytosol. On the other hand, when apoptosis was induced with H_2O_2, the cells showed reduced size 'pyknotic' mitochondria and characteristic clustering of the organelle around the nucleus (which represented ∼70% of the cultured muscle cells). These modifications could be prevented when the C2C12 cells were incubated with E_2 prior to treatment with H_2O_2 (Fig. 3). Likewise, Fig. 4 shows by western blot assays that both H_2O_2 and etoposide, under the same conditions as indicated before, induced the cleavage of PARP, whereas incubation with E_2 (10^{-8} M) for 45 min prior to the apoptotic stimuli abolished the cleavage of PARP.

Activation of the PI3K/Akt pathway by E_2 in C2C12 muscle cells

The ability of E_2 to regulate the PI3K/Akt pathway in muscle cells was evaluated. This signaling pathway influences cell death through its direct effects on the phosphorylation state of BAD. C2C12 cell cultures were incubated with the steroid hormone

Figure 5 17β-Estradiol induces phosphorylation of Akt. (A) C2C12 cells were incubated with 10^{-8} M E_2 for the indicated times or treated with H_2O_2 in the absence and presence of E_2 with and without wortmannin as follows. Cell lysates were prepared and subjected to western blot analysis using an anti-phospho-Akt antibody. Total Akt levels were measured as protein loading control. C, untreated cells; H_2O_2, cells treated with 0·5 mM H_2O_2 during 24 h; E_2, cells incubated with 10^{-8} M 17β-estradiol for 5, 20, and 40 min or 8 h; H_2O_2 + E_2, cells preincubated with 10^{-8} M 17β-estradiol for 45 min and then treated with H_2O_2 during 24 h; Wort + E_2 + H_2O_2, cells were preincubated with wortmannin (0·1 μM during 20 min) and then with 17β-estradiol and H_2O_2 as before. Mk, molecular weight colored marker. The blot is representative of three independent experiments with comparable results. (B) Confocal microscopy of Akt activation (phosphorylation). Phosphorylated Akt (green fluorescence) was stained by using anti-phospho-Akt antibody. Control, untreated cells; E_2, cells incubated with 10^{-8} M 17β-estradiol for 40 min; H_2O_2, cells treated with 0·5 mM H_2O_2 during 24 h; E_2 + Ly294002, cells were preincubated with Ly294002 (25 μM during 20–30 min) and then treated whit E_2 for 40 min. H_2O_2 + E_2, cells preincubated with 10^{-8} M 17β-estradiol for 45 min and then treated with H_2O_2 during 24 h. Magnification: 63×. Images are representative of at least three independent experiments.

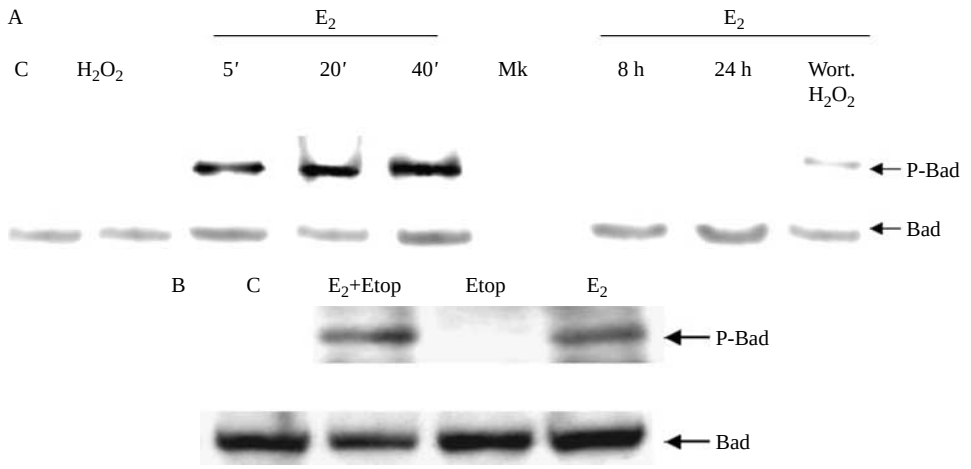


Figure 6 17β-Estradiol induces phosphorylation of BAD. C2C12 cells were treated with the indicated stimuli. (A) C, untreated cells; H_2O_2, cells treated with 0·5 mM H_2O_2 during 40 min; E_2, cells incubated with 10^{-8} M 17β-estradiol for 5, 20, and 40 min or 8 and 24 h; Wort $+E_2+H_2O_2$, cells were preincubated with wortmannin (0·1 μM during 20 min) and then with 17β-estradiol and H_2O_2 for 40 min. Mk, molecular weight colored marker. (B) C, untreated cells; E_2+ Etop., cells were preincubated with 10^{-8} M 17β-estradiol for 40 min and then with etoposide (25 μg/ml) during 1 h; Etop., cells treated with etoposide during 1 h; E_2, cells were preincubated with 17β-estradiol for 40 min. The cells were harvested and used to prepare cell lysates. The lysates were subjected to SDS-PAGE and blotted with anti-phospho-BAD (Ser 136) antibody. Actin levels were measured as protein loading control. The blot shown is representative of three independent experiments with comparable results.

(10^{-8} M) for various time intervals (5, 20, and 40 min, 8 h) followed by measurement of phospho-Akt levels. As shown in Fig. 5A, western blot analysis using anti-phospho-Akt (Ser 473) polyclonal antibody revealed Akt activation (phosphorylation) in response to E_2. No appreciable changes in phosphorylation of Akt were induced by treatment with 0·5 mM H_2O_2 for 24 h as in the previous experiments. Of relevance, activation of Akt by the hormone was blocked when the cells were preincubated with the PI3K specific inhibitor wortmannin (0·1 μM during 20 min). Immunocytochemistry studies using confocal microscopy and the same antibody were congruent with the western blot results. Moreover, when the C2C12 cells were preincubated with Ly294002 (25 μM during 20–30 min), another PI3K inhibitor, and then treated with E_2 as before, slight fluorescence was detected (Fig. 5B). These results demonstrate then that E_2 is able to promptly activate Akt acting through PI3K.

E_2 induces BAD phosphorylation in C2C12 muscle cells

Next, we investigated the action of E_2 on BAD phosphorylation, an event related to cell survival via the PI3K/Akt pathway. Figure 6 shows a marked increase in phosphorylation of BAD after 5–40 min treatment with E_2, the effects being no longer detectable at 8 and 24 h. Total BAD levels were unchanged during this treatment interval. As expected, in the presence of inducers of apoptosis, H_2O_2, or etoposide, phosphorylation of BAD was not observed (Fig. 6A and B). Co-treatments with E_2 and H_2O_2 or etoposide restored BAD phosphorylation (data not shown for H_2O_2). Wortmannin (0·1 μM) partially inhibited this recovery of the steroid hormone phosphorylation effect (Fig. 6A).

Role of ERs in the antiapoptotic effects of E_2

To address whether the antiapoptotic action of E_2 on C2C12 muscle cells is exerted through ERs, the above experiments on the effects of E_2 on H_2O_2-induced cytochrome *c* release were performed in presence of 1 μM ICI 182 780, an ER antagonist. Figure 7 illustrates that the antagonist blocked the protective effect of the hormone. In addition, western blot assays showed that the inhibitory effect of E_2 on H_2O_2-promoted apoptotic cleavage of PARP was almost totally abolished by ICI 182 780 (Fig. 4). Also, we evaluated cytochrome *c* release in C2C12 cells preincubated with specific antibodies against the ER isoforms α and (see Materials and Methods and legend to Fig. 7) followed by treatment with 10^{-8} M E_2 for 45 min and finally challenged with 0·5 mM H_2O_2 during 24 h. Under these conditions, inhibition of H_2O_2-induced cytochrome *c* release by E_2 (or DES) was reduced (Fig. 7). Thus, polyclonal anti-ER-β antibodies L-20 (mapping near C-terminus) and Y-19 (mapping at the N-terminus) abolished it by 66% the antiapoptotic effect of E_2, whereas monoclonal ER-α antibody Ab-10 (against ER ligand binding domain) suppressed it by 40%. Appropriate controls allowed to exclude nonspecific effects due to the permeabilization treatment and/or antibody incubation (see condition 'ANTIBODY' in Fig. 7).

To strengthen the above evidence involving ER-β and possibly ER−, we evaluated cytochrome *c* release in C2C12 cells transfected with specific siRNAs to induce silencing of

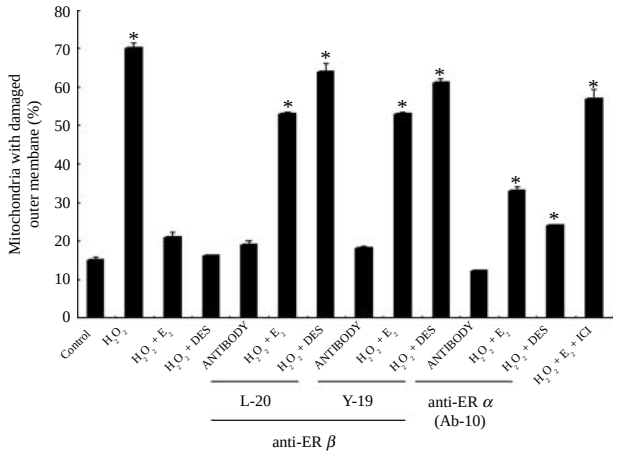


Figure 7 Antibodies against the estrogen receptor (ER) isoforms α and β block 17β-estradiol inhibition of H_2O_2-induced cytochrome *c* release in muscle cells. C2C12 muscle cells were permeabilized and then incubated with specific antibodies against the isoforms (L-20, 1:200 or Y-19, 1:100) or (Ab-10/clone TE111.5D11, 1:400) of ER as described under Materials and Methods, then treated with 10^{-8} M 17β-estradiol or DES for 45 min and finally challenged with 0·5 mM H_2O_2 during 24 h. Cytochrome *c* release was measured as described under Materials and Methods. Control, untreated cells; H_2O_2, cells treated with 0·5 mM H_2O_2 during 24 h; H_2O_2 +E_2, cells preincubated with 10^{-8} M 17β-estradiol for 45 min and then treated with 0·5 mM H_2O_2 during 24 h; H_2O_2+DES, cells preincubated with 10^{-8} M DES for 45 min and then treated with 0·5 mM H_2O_2 during 24 h; ANTIBODY, cells were only permeabilized in presence of the indicated antibody without further treatment. ICI+E_2+H_2O_2, cells preincubated with 1 μM ICI 182 780 for 45 min and then with 17β-estradiol and H_2O_2 as before. Each value represents the mean of three independent determinations ± S.D. *$P<0·05$ with respect to the control.

each ER isoform. Optimum transfection conditions were established using fluorescein-siRNA (NEB; ≥70% after 24-h incubation with 20 pmol of fluorescent probe; Fig. 8A). To verify the silencing efficiency and specificity of siRNA effects, we examined by western blot analysis the expression levels of ER-α and ER-β after transfection with isoform-selective siRNAs (24/48 h with 10/20 pmol of ER-α or ER-β siRNA (NEB)). Figure 8B indicates that each siRNA probe led to a significant suppression in the levels of the corresponding protein. Under these conditions, ER-β silencing caused a significant blockade (~78%±2·5) of E_2 effects on cytochrome *c* liberation, whereas ER-α silencing induced a minor reduction of E_2 effects (~30%±0·7). The siRNAs used are highly specific. We used the fluorescent probe of the kit (chemically synthesized 21 bp RNA which has no sequence identity to any mammalian sequences) and differently to siRNA α or β no blockage was observed (not shown). These pieces of evidence show that E_2 inhibition of cytochrome *c* release is mediated mainly by ER-β (Fig. 8C). On the other hand, transfection of C2C12 cells with either ER-α siRNA or ER-β siRNA blocked the activation of Akt by E_2 shown before (Fig. 5A), revealing that both receptor isoforms mediate this step (Fig. 8D).

Discussion

E_2 can sustain survival or alternatively induce apoptosis of cells depending on their biological context (Choi *et al.* 2001, Okasha *et al.* 2001, Florian & Magder 2008, Seli *et al.* 2007). The data obtained in this work using the well-characterized myogenic C2C12 murine cell line provides evidence that the estrogen at physiological concentrations inhibits apoptosis of skeletal muscle cells. As experimental approach, the C2C12 cells were led into apoptosis by exposure to H_2O_2 or etoposide, state which was first evidenced by the results of DAPI staining and DNA laddering. Under these conditions, we observed that the cells preincubated with E_2 (10^{-8} M), similarly as the synthetic non-steroidal analog DES (10^{-8} M), block the effects of hydrogen peroxide or etoposide on the number of apoptotic nuclei and DNA fragmentation. This protective action of the hormone was dose dependent between 10^{-6} and 10^{-10} M, maximal effects being detected at 10^{-8} M (not shown), in agreement with saturation binding analysis data of the ER in C2C12 cells (Milanesi, de Boland and Boland submitted), an observation which may be related to the participation of ER as mediator of the antiapoptotic action E_2 in muscle cells demonstrated in the present investigations (see below). This concentration of estrogen has been shown to inhibit apoptosis in other cell types (Fernando & Wimalasena 2004). Although E_2 is known to induce mitogenic effects, the fact that no difference in the number of apoptotic nuclei between the E_2 and control conditions was observed indicates that the steroid exerts mainly an apoptotic action rather than a mitogenic one in C2C12 cells.

The mitochondria play a central role in apoptosis. Within the past few years, their participation in the control of apoptosis has been well documented. Morphological changes and cellular redistribution of mitochondria in apoptotic cells are known to occur (Desagher & Martinou 2000). 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 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 (Liu *et al.* 1996, Reed 1997). The necessary event for cytochrome *c* release to take place is the loss of integrity of the outer mitochondrial membrane (Crompton *et al.* 1998, Green & Kroemer 2004). Evaluation of the outer membrane state by the determination of cytochrome *c* oxidase activity represents a useful indicator of cytochrome *c* release. The inhibition of H_2O_2-induced cytochrome *c* release by E_2 or DES observed in this study, like the effects of hormone on the size and cytosolic distribution of mitochondria, suggests a protective effect of E_2 and its analog on this organelle.

Several interpretations may apply regarding the physiological relevance of the morphological changes and redistribution of the organelle in response to H_2O_2 treatment. It is possible that clustering of mitochondria near the nucleus generates high energy levels required to maintain the machinery triggered by the apoptotic stimulus active.

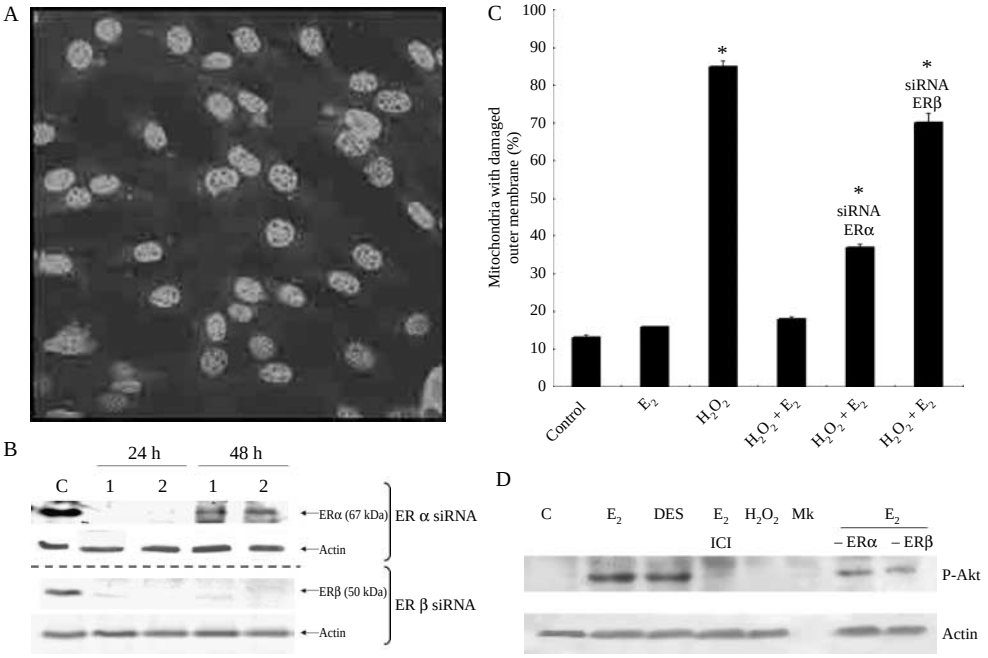


Figure 8 Silencing of ER isoforms inhibits the effects of 17β-estradiol on cytochrome *c* release and Akt activation. Transfections of C2C12 cells with siRNAs were performed as described under Materials and Methods. (A) Transfection efficiency of siRNA was estimated using fluorescein-siRNA transfection control and TransPass R2 transfection reagent. Cells were then visualized in a conventional microscope employing an adequate filter for green fluorescence. A typical pattern of fluorescence with fluorescein-siRNA is shown (more of 70% cells present green fluorescence). Original magnification=400×. (B) C2C12 cells were transiently transfected with two concentrations of ER-α siRNA (NEB; upper panel) or ER-β siRNA (NEB; lower panel). Expression of ER isoforms was analyzed 24 and 48 h post transfection by western blot analysis as described in Materials and Methods. Immunoblots of total cell lysates employing TE111.5D11 monoclonal antibody anti-ER-α or Y-19 specific polyclonal antibody anti-ER-β are shown. C, control; 1, 10 pmol of siRNA; 2, 20 pmol of siRNA. Actin loading control was detected using anti-actin polyclonal antibody. A representative blot from two independent experiments is shown. (C) C2C12 cells were transfected with ER-α siRNA (NEB) or ER-β siRNA (NEB) and then cytochrome *c* release was measured as Fig. 7. Control, untreated cells; E_2, cells treated with 10^{-8} M 17β-estradiol for 24 h; H_2O_2, cells treated with 0·5 mM H_2O_2 during 24 h; $H_2O_2+E_2$, cells preincubated with 10^{-8} M 17β-estradiol for 45 min and then treated with 0·5 mM H_2O_2 during 24 h; H_2O_2 Each value represents the mean of three independent determinations ± S.D. *$P<0·05$ with respect to the control. (D) C2C12 cells were transfected with ER-α siRNA (NEB) or ER-β siRNA (NEB) and Akt activation was evaluated as in Fig. 5A. C, untreated cells; E_2, cells incubated with 10^{-8} M 17β-estradiol for 40 min; DES, cells incubated with 10^{-8} M DES for 40 min; ICI+E_2, cells preincubated with 1 μM ICI 182 780 for 45 min and then treated with 10^{-8} M 17β-estradiol for 40 min; H_2O_2, cells treated with 0·5 mM H_2O_2 during 24 h; Mk, molecular weight colored marker; ER-α, cells transfected with ER-α siRNA and treated with 10^{-8} M 17β-estradiol for 40 min; ER-β, cells transfected with ER-β siRNA and treated with 10^{-8} M 17β-estradiol for 40 min. The blot is representative of three independent experiments with comparable results.

Also, displacement of the organelle could facilitate translocation of mitochondrial proteins, such as apoptosis-inducing factor (AIF) which binds to DNA and triggers its destruction, to the nucleus (Susin *et al.* 2000). Moreover, the modifications that undergo mitochondria like size reduction or pyknosis could be related to the release of mitochondrial proteins (e.g., AIF, cytochrome *c*) observed in apoptosis (Granville *et al.* 2001). Further studies are necessary to elucidate the relative role of these events and the mechanisms by which estrogens exert an inhibitory effect upon them.

Another prominent episode during apoptosis is the selective cleavage of PARP by caspases (Lazebnik *et al.* 1994), which is a universal event observed during programed cell death induced by a variety of apoptotic stimuli. Here, we evidenced that the hormone inhibits H_2O_2-promoted apoptotic cleavage of PARP. This effect was almost totally abolished by ICI 182 780 indicating that the antiapoptotic action of E_2 is mediated by the ER.

As mentioned in Introduction, the lipid kinase PI3K as well as its downstream target Akt regulates a diverse array of cellular events (Cross *et al.* 2000, Brazil & Hemmings 2001) and both have been implicated in cellular survival and apoptosis (O'Groman *et al.* 2000, Xu *et al.* 2003, Grutzner *et al.* 2006). In addition, it has been reported that E_2 modulates the PI3K/Akt

signaling pathway in various cell types (Simoncini *et al.* 2000, Lee *et al.* 2005, Guo *et al.* 2006). Mediation by PI3K and Akt of the antiapoptotic action of estrogen in C2C12 cells was first shown by experiments with Mitotracker and DAPI stains to observe mitochondrial morphology and apoptotic nuclei respectively, which showed that PI3K inhibitors wortmannin or LY294002 abolished the effects of E_2 on cell survival under apoptotic conditions (data not presented in Results). The participation of Akt in the effects of E_2 was further evidenced by western blot and immunocytochemistry assays, revealing a rapid and sustained activation (phosphorylation) of Akt in response to E_2, the latter in accord with the fact that prolonged activity of Akt is required to maintain BAD inactive (Fernando & Wimalasena 2004). Suppression of E_2 effects on Akt by wortmannin and LY940022 implies a role for PI3K/Akt in the antiapoptotic effects of the hormone in C2C12 cells. Additional studies are required to identify the targets of this pathway in C2C12 cells and then clarify the significance of estrogen action on apoptosis of skeletal muscle cells.

Phosphorylated BAD is devoid of its apoptotic activity, since it is sequestered away from the site of action in the mitochondria by binding to cytosolic 14-3-3 proteins (Datta *et al.* 1997, Yano *et al.* 1998). BAD can be phosphorylated on serine 136 by Akt (Datta *et al.* 1997) and since the hormone activated Akt in C2C12 cells, the ability of the steroid to induce this phosphorylation was investigated using a phosphospecific antibody. We found that E_2 rapidly induces phosphorylation of BAD without altering BAD protein levels, suggestive of activation of non-genomic signal pathways. Also we observed that phosphorylation of BAD is partially affected when the cells were preincubated with the PI3K inhibitor wortmannin. Since the effects of E_2 on Akt persist for longer time intervals, it is then possible that PI3K/Akt and BAD could be involved as genomic as well as non-genomic mediators of the antiapoptotic actions of the steroid. Further investigations are required to clarify this aspect.

Altogether these data strongly suggest that E_2 exerts antiapoptotic actions in skeletal muscle cells through inactivation of proapoptotic BAD protein as a consequence of Akt activation by PI3K. Since wortmannin was unable to totally inhibit the effect of E_2, the possibility that more than one kinase participates in E_2-induced BAD phosphorylation cannot be excluded.

As the protective effect of E_2 at the mitochondrial level (cytochrome *c* release) was inhibited in presence of specific anti ER− or ER− antibodies or using siRNA for each isoform, we conclude that it is dependent on ER activation. Regarding the use of antibodies, equivalent concentrations were used for each isoform, and well in excess. These conditions as well as the time of incubation used ensure that all binding sites were blocked. The antibodies are highly specific and preclude cross-reaction between the two isoforms. The fact that inhibition of the hormone protective action on mitochondria was more evident immunoblocking or suppressing ER-β than ER− suggests that the β isoform of the receptor mediates the antiapoptotic effects of E_2 at this site of action to a greater extent than the α isoform. This could be the consequence of greater abundance of this isoform in our cell system or due to the fact that ER-β localizes in mitochondria in C2C12 cells (data not shown). This hypothesis is in agreement with evidence showing that relative receptor abundance of each isoform varies depending on the cellular type; e.g., in MCF-7 ER-β is considerably less abundant than ER-α and, interestingly, this small amount of ER-β is concentrated in mitochondria (Pedram *et al.* 2006).

In summary, this study shows that E_2 inhibits H_2O_2 or etoposide-induced apoptosis in skeletal muscle cells acting at least at two different levels. One of them is inducing PI3K/Akt activation and then BAD phosphorylation, process in which both isoforms of the ER participate. The other relates to a protective effect of mitochondria integrity and involves mainly ER-β, suggested by the inhibition of E_2 effects on cytochrome *c* release upon ER isoform silencing.

Estrogen signaling and cell survival have been investigated in other cell types, revealing similar and/or additional features with respect to the mechanism of signal transduction shown here for the antiapoptotic action of E_2 in C2C12 skeletal muscle cells. Thus, there are data demonstrating that E_2 increases activation of Akt improving survival and decreasing apoptosis in murine cardiomyocytes both *in vivo* and *in vitro* by ER− and PI3K–Akt-dependent pathways (Patten *et al.* 2004), similarly to C2C12 cells. Moreover, E_2 abrogates apoptosis in MCF-7 breast cancer cells (ER+) through inactivation of BAD: Ras-dependent non-genomic pathways requiring signaling through ERK and Akt (Fernando & Wimalasena 2004); however, the participation of the ER was not studied. ER-α and ER-β have been detected in the mitochondria of MCF-7 breast cancer cells and endothelial cells and involved in E_2-induced direct inhibition of mitochondrial ROS formation and cytochrome *c* release, events leading to apoptotic cell death (Pedram *et al.* 2006). Interestingly, we have found ER-β predominantly in mitochondria of C2C12 cells (data not shown). On the other hand, it has been reported that E_2 prevents chemotherapy or radiation-induced apoptosis in these tumorogenic breast cells through plasma membrane-associated ERs (Razandi *et al.* 2000). In cancer cell lines containing transfected ERs, there is evidence that the E_2–ER-α complex rapidly activates multiple signal transduction pathways (i.e., PI3K/Akt, ERK/MAPK) committed to apoptotic cascade prevention (Acconcia *et al.* 2005), whereas, on the contrary, E_2 can enhance antiapoptotic activity via ER-β during oxidative damage in hepatocytes (Inoue *et al.* 2003). Also, in osteocyte bone cells estrogens attenuate apoptosis by activating ERKs through extranuclear ERs (Plotkin *et al.* 2005).

Clearly, additional studies are then necessary to further elucidate the signaling mechanisms which mediate the antiapoptotic action of E_2 in skeletal muscle cells. This knowledge may be of relevance to develop therapies for prevention and treatment of sarcopenia associated with estrogen-deficit states.

Acknowledgements

This research was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT)

and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina. Andrea Vasconsuelo is a recipient of a postdoctoral research fellowship from ANPCyT. Lorena Milanesi and Ricardo Boland are members of CONICET Investigator Career. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 29 October 2007
Accepted 14 November 2007
Made available online as an Accepted Preprint
14 November 2007