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PROTECTIVE ROLE OF 17β-ESTRADIOL AND TESTOSTERONE IN APOPTOSIS OF SKELETAL MUSCLE

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Abstract
The loss of muscle mass and strength with aging, also referred to as sarcopenia, is a prevalent condition among the elderly and predicts adverse outcomes, including disability, institutionalization and mortality. Sarcopenia has been associated to a deficit of sex hormones since the levels of estrogens and/or testosterone decline upon ageing. Although the mechanisms underlying sarcopenia are far from being clarified, evidence suggests that an age-related acceleration of myocyte loss via apoptosis might represent a mechanism responsible for muscle loss performance. Furthermore, increased levels of apoptosis have also been reported in old rats undergoing muscle atrophy. We previously demonstrated that 17β-estradiol (E2) inhibits apoptosis in C2C12 murine skeletal muscle cells through estrogen receptors (ERs) with non classical localization involving PI3K/Akt, MAPKs and HSP27. Here, using siRNAs to silence ER isoforms, we show that E2 activates ERK through ERα and p38 MAPK stimulation is independent of ERs. We confirmed that E2 is able to abrogate apoptosis through MAPKs in primary cultures of neonate mouse skeletal muscle. Also, we proved that testosterone blocks apoptosis as E2. 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 C2C12 cells were preincubated with testosterone. Further studies are required to establish whether there is a parallelism between the mechanisms triggered by both hormones which might be involved in muscle pathologies associated to apoptosis. The data presented deepen the knowledge on the molecular basis of sex hormone-dependent sarcopenia.

Key words: *estradiol; testosterone; skeletal muscle; apoptosis; sarcopenia.*

Resumen

PAPEL PROTECTOR DEL 17β-ESTRADIOL Y DE LA TESTOSTERONA EN LA APOPTOSIS DEL MÚSCULO ESQUELÉTICO

La sarcopenia, pérdida de masa y fuerza del músculo esquelético, es una condición frecuente durante el envejecimiento. Conduce a incapacidad motora resultando en internación y mortalidad. Puesto que los niveles de estrógenos y/o testosterona disminuyen con la edad, la sarcopenia se ha asociado al déficit de estas hormonas. Aunque los mecanismos moleculares involucrados en esta patología no están totalmente dilucidados, exis-

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ten evidencias indicando que la apoptosis es en parte responsable de la pérdida de miocitos en la adultez. Previamente demostramos que el 17β-estradiol (E2) inhibe la apoptosis en la línea celular C2C12 de músculo esquelético a través de PI3K/Akt, MAPKs, HSP27 y receptores estrogénicos (ERs) con localización no clásica. Usando *siRNAs* específicos para silenciar las isoformas del ER, comprobamos que el E2 activa ERK involucrando a ERα, mientras que la activación de p38 MAPK es independiente de ERs. Confirmamos que el E2 puede inhibir la apoptosis a través de las MAPKs en cultivos primarios de músculo esquelético de ratón. Al igual que el E2, la testosterona bloquea la apoptosis. Las alteraciones morfológicas típicas de la apoptosis como fragmentación nuclear, desorganización del citoesqueleto, reorganización/disfunción mitocondrial y liberación de citocromo c, inducidos por H_2O_2 fueron suprimidas al preincubar las células con testosterona. Se requieren investigaciones adicionales para establecer un paralelismo entre los mecanismos de acción de ambas hormonas, que podrían estar implicados en patologías musculares asociadas a apoptosis. Los datos presentados en este estudio profundizan el conocimiento de las bases moleculares de la sarcopenia relacionada con estados de déficit de hormonas sexuales.

Palabras clave: estradiol; testosterona; músculo esquelético; apoptosis, sarcopenia.

Introduction

The protective role of estrogens and androgens on tissues is currently receiving increased attention. There is evidence showing that skeletal muscle is a target tissue for both steroid hormones. 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 and testosterone levels. Thus, hormone replacement therapies prevent a decline in muscle performance.[1,2] It has also been shown that estrogens promote proliferation and differentiation of skeletal myoblasts.[3] On the other hand, testosterone through its effects on muscle and fat mass, is an important determinant of body composition in male mammals, including humans. Testosterone supplementation increases muscle mass in healthy young and old men, healthy hypogonadal men and in other pathological or physiological conditions with low levels of this steroid.[4] Other studies have demonstrated that testosterone-induced increase in muscle size is associated with hypertrophy of muscle fibers and significant increases in myonuclear and satellite cell numbers.[5-7] Available evidence further suggests that exogenous testosterone administration results in faster recovery from hind limb paralysis after sciatic nerve injury in the rat [8], and completely prevents the castration induced apoptosis in muscle cells of the rat levator ani muscle.[9]

In agreement with these observations, it has been established that human skeletal muscle contains not only estrogen receptors (ERs) α and β [10,11] but also androgen receptor (AR).[12-16] Although the exact mechanism by which estrogens and androgens prevent sarcopenia remains to be clarified, in previous work, we demonstrated that 17β-estradiol (E2) inhibits apoptosis in C2C12 skeletal muscle cells through ERs with non-classical localization and involving the PI3K/Akt pathway.[17]

Programmed muscle cell death (apoptosis) has been implicated as a potential mechanism of muscle atrophy and wasting in aging, during injury, and in the pathogenesis of many diverse human diseases, as cardiomyopathy, chronic obstructive pulmonary disease (COPD), muscular dystrophies or others neuromuscular disorders.[18-21] Diverse stimuli trigger apoptosis by activating one or more

signal transduction pathways, which converge in the activation of a conserved family of cysteine proteases known as caspases.[22] Caspase activation may be elicited through intrinsic or extrinsic apoptotic pathways. The hallmarks of the intrinsic apoptotic pathway involve mitochondrial proteins and apoptosome formation which results in activation of the caspase cascade. In contrast, the extrinsic pathway is initiated through stimulation of the transmembrane death receptors and generation of the death-inducing signal complex and in turn caspase activation[23,24]. Studies with myoblasts have demonstrated that apoptosis plays an important role in muscle development, by controlling the size of the population of proliferating myoblasts which undergo differentiation into mature myotubes.[25-27] Then, the effects of the steroid hormones on skeletal muscle development could also be regulated, in part, through their effects on apoptosis.

Mitogen-activated protein kinases (MAPKs) comprise a family of serine/threonine kinases that function as critical mediators of a variety of extracellular signals.[28,29] Members of the MAP kinase superfamily include, among others, the extracellular signal-regulated kinases (ERKs) and the p38 MAP kinases (p38 MAPKs). Available data from various cell systems suggest that ERK 1 and ERK 2 are activated in response to growth stimuli and promote cell growth whereas p38 MAPKs are activated in response to a variety of environmental stresses and inflammatory signals and promote apoptosis and growth inhibition.[28,29] However, the regulation of apoptosis by MAPKs is more complex and varies depending on tissues, nature of the apoptotic stimulus, and duration of their activation.[28-32]

Recently, a correlation between the expression of heat shock proteins (HSPs) and increased cell survival was shown, pointing them as regulatory agents of components of apoptotic pathways.[33,34] Heat shock proteins are a family composed of ubiquitous and conserved proteins that according to their molecular weight include high molecular mass HSPs (≥100 kDa), HSP90 (81 to 99 kDa), HSP70 (65 to 80 kDa), HSP60 (55 to 64 kDa), HSP40 (35 to 54 kDa), and small HSPs (≤34 kDa [35]). HSPs are known for controlling cell homeostasis, proper folding of proteins, and translocation through cell membranes acting as molecular chaperones.[36-38] Moreover, this family of proteins supplies an intrinsic mechanism to defend the cell against external physiological stresses. As mentioned before, high expression levels of HSPs imply increased cell survival. Specifically, the HSP involved in cytoprotective actions is the small (24–28 kDa) HSP, referred to as HSP25 or HSP27. HSP27 is expressed constitutively in many mammalian tissues and cell lines.[39] Of relevance for our work, HSP27 also appears to be involved in the suppression of apoptosis.[38,40,41] Interestingly, HSP27 is induced by estrogens in various cells such as platelets [42] as well as breast and endometrial tumors.[43] Furthermore, related to its antiapoptotic role, high levels of HSP27 are a marker for increased malignancy in breast cancer.[44] Regarding skeletal muscle, it has been observed that the expression of HSP27 is induced by a variety of stimuli.[36] However, its functions in muscle cells in connection to the effects of estrogens on this tissue have not been studied yet.

In the present work, we obtained evidence that ERK, p38 and HSP27 are involved in the protective effects of E2 in muscle. We also confirmed that E2 is able to abrogate apoptosis in primary cultures of neonate mouse skeletal muscle. Besides, we demonstrated that testosterone can inhibit apoptosis as E2, preventing nuclear fragmentation, cytoskeleton disorganization and mitochondria outer membrane damage in the C2C12 muscle cell line. These studies deepen the knowledge of the molecular basis of sarcopenia related to deficit states of sexual hormones.

Materials and methods

Materials

Estrogen receptor α mouse monoclonal antibody clone TE111.5D11 (anti-ER ligand binding domain) was purchased from NeoMarkers (Fremont, CA, USA). Estrogen receptor β goat policlonal antibody (Y-19) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cytochrome c Oxidase Assay Kit, anti-actin polyclonal antibody (A-5060), 17β-estradiol and testosterone (T-1500) were purchased from Sigma-Aldrich (St. Louis, MO, USA). ICI182780 was obtained from TOCRIS (Ellisville, MO, USA). The extracellular regulated kinase kinase (MEK1) inhibitor UO126 and the p38 MAPK inhibitor SB203580 were from Calbiochem-Novabiochem Corp.(La Jolla, Ca, USA). Anti-HSP27, anti-p-ERK 1/2 and anti-p-p38 antibodies were from Cell Signaling Technology Inc (Danvers, MA, USA). Anti-rabbit Alexa 488, DAPI and MitoTracker Red (MitoTracker Red CMXRos) dyes were from Molecular Probes (Eugene, OR, USA). Estrogen receptor β (ERβ) ShortCut^{R}siRNA Mix, Estrogen receptor α (ERα) ShortCut^{R}siRNA Mix, Fluorescein-siRNA transfection Control and TransPass™ R2 Transfection Reagent were purchased from New England BioLabs Inc. (Beverly, MA, USA). Other chemicals 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) 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 10^{-8} M E2, testosterone or vehicle (0.001% isopropanol, control), approx. 45 min before induction of apoptosis with hydrogen peroxide (H_2O_2) during 8 h. H_2O_2 was diluted in culture medium without serum at a final concentration of 1 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 PMSF, 1% NP40, 20 g/ml leupeptin and 20 g/ml aprotinin. Lysates were collected by aspiration and centrifuged at 12.000×*g* during 15 min. Protein concentration from the supernatant was estimated by the method of Bradford (1976), using bovine serum albumin (BSA) as standard. Unless otherwise noted, cells were cultured in chamber slides for microscopy.

For primary cultures, neonate (4-5 days) CF-1 mice were used to isolate myoblasts according to previously described techniques.[45] The animals were anesthetized and skeletal muscle from limbs was dissected and enzymatically disrupted by shaking in saline solution with 0.15% trypsin at 37°C in a humid atmosphere of 5% CO_2 in air, during 30 min. Dissociated cells were collected by centrifugation and cultured in DMEM medium to 80-90% confluence.

Measurement of outer mitochondrial membrane integrity

The integrity of outer mitochondrial membranes was evaluated using a commercially available kit from Sigma (CYTOC-OX1) which measures cytochrome release by the determination of cytochrome c oxidase activity, according to manufacturer's instructions. Briefly, C2C12 confluent monolayers 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 (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 20 mg/ml leupeptin, 20 mg/ml aprotinin, 20 mg/ml trypsin inhibitor) using a Teflon-glass hand homogenizer. Lysates were centrifuged at 10.000 x *g* for 20 min in order

to separate the cytosolic fraction. To these samples, 50 µl of 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.

Western blot analysis

Protein aliquots (25 µg) were combined with 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-polyacrylamide gel electrophoresis (SDS-PAGE). Fractionated proteins were then electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Millipore), using a semi-dry system. Non-specific sites were blocked 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. The membranes were repeatedly washed with PBS-T prior incubation with horseradish peroxidase-conjugated secondary antibodies. The enhanced chemiluminescence blot detection kit (Amersham, Buckinghamshire, England) 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 treated 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 1:20000 dilution of anti-actin polyclonal antibody (A-5060) as primary antibody. After several washings with PBS-T, membranes were incubated with anti-rabbit (1:10000) conjugated to horseradish peroxidase. The corresponding immunoreactive bands were developed by means of ECL.

Coimmunoprecipitation

Total homogenates from the C2C12 cell line containing 100 g of protein were immunoprecipitated with 10 l of a 50% suspension of protein A–agarose after incubating the extracts with the antibody indicated in each experiment. The immunoprecipitates were washed three times with buffer containing 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1% Triton X-100, protease inhibitors: 2 mM PMSF, 20 g/ml leupeptin, 20 g/ml aprotinin, and 10 g/ml of trypsin inhibitor. The final pellets were obtained by centrifugation for 3 min at 10000 × g, resuspended then in electrophoresis sample buffer without dithiothreitol, boiled for 5 min, 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.

Immunocytochemistry

Semi-confluent (60-70%) monolayers were washed with serum-free phenol red-free DMEM, incubated 1 h in the same medium and then fixed and permebilized during 20 min at -20 °C with methanol to allow intracellular antigen labeling. After fixation, cells were rinsed 3 times with PBS. Non-specific sites were blocked for 30 min in PBS that contained 5% bovine serum albumin. Cells were then incubated for 60 min in the presence or absence (negative control) of primary antibodies (anti-p-ERK 1/2, anti-p-p38, anti-HSP27 and anti β-ER Y19; 1:50 dilution).

MitoTracker® Red CMXRos was employed for selective stain of active mitochondria. Coverslips with adherent cells were stained with MitoTracker Red, which was prepared in

dimethyl sulfoxide (DMSO) and then added to the cell culture medium at a final concentration of 1 mol/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 confocal and conventional microscopy. Images were collected using a digital camera.

In order to evaluate the nuclear morphology, 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.

Janus Green staining

After treatments, the cells were incubated with 0.1 % Janus Green in serum-free medium (1:2, v/v) during 30 min at 37ºC. Cells were examined by bright field microscopy.

Confocal microscopy

Images were acquired on a Leica TCS SP2 AOBS confocal laser-scanning microscope in an epifluorescence mode. The 488 nm line of an argon ion laser and the 543 nm line of a helium-neon laser were used to excite the samples. A DD 488/543 filter was used to separate red/green fluorescence signals. A 50 mm pinhole was generally used. Cells were imaged through a 63X, 1.3 numerical aperture water immersion objective. Images were collected and saved using the software Meridian, and exported to Adobe PhotoShop for digital processing.

Transfection of short interfering RNA (siRNA)

Transfection was performed with a culture cellular density reaching 40–60% confluence with ERα or ERβ ShortCut siRNA according to the manufacturer's instructions. Briefly, TransPass R2 Transfection Reagent was mixed with ERα or ERβ siRNAs. 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 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 ERα or ERβ, total proteins from transfected and non-transfected cells (controls) were extracted 24 and 48 h post transfection and ERα or ERβ expression was tested by Western blot analysis as described above using TE111.5D11 specific monoclonal antibody and Y-19 specific polyclonal antibody respectively.

Statistical analysis

Statistical treatment of the data was performed using the Student's t-test (Snedecor & Cochran 1967). Data are means±SD of not less than three independent experiments. The data were considered statistical significant when $p < 0.05$.

Results

17β-estradiol increases HSP27 levels in C2C12 cells

As a first approach to evaluate the role of HSP27 in the regulation of apoptosis by E2 in skeletal muscle cells, we investigated the chaperone expression levels in response to the hormone. C2C12 cell cultures were incu-

bated with the steroid hormone (10^{-8} M) during different times (5, 20, 40, and 60 min and 4 h) followed by measurement of HSP27 levels by immunoblot analysis. As observed in Fig. 1a, Western blots using an anti-HSP27 monoclonal antibody revealed a time-dependent increase in the expression of HSP27 in response to E2, the effects being very marked after estrogen treatment for 20 min (+167%) and 40 min (+271%). In agreement with these results, immunocytochemistry studies using confocal microscopy and the same antibody showed that after 20-40 min of treatment with E2 (10^{-8}M), the fluorescence intensity was higher than control conditions (Fig. 1b).

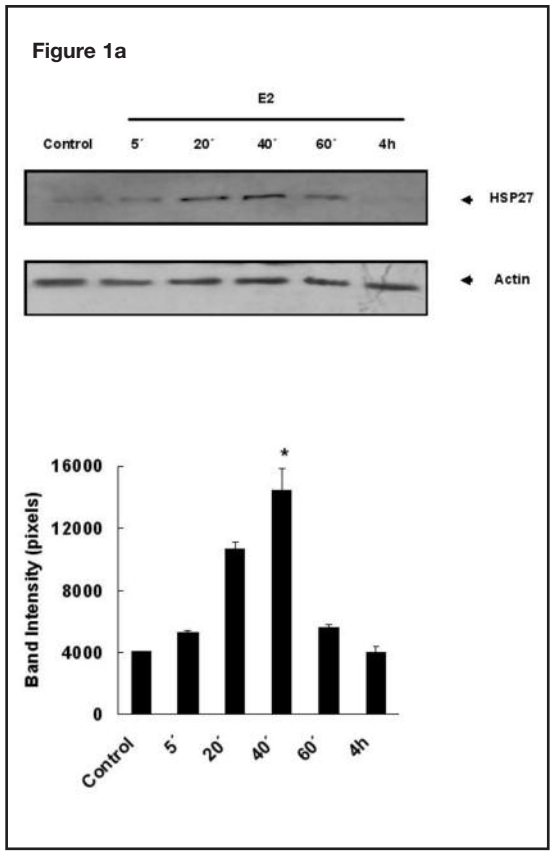
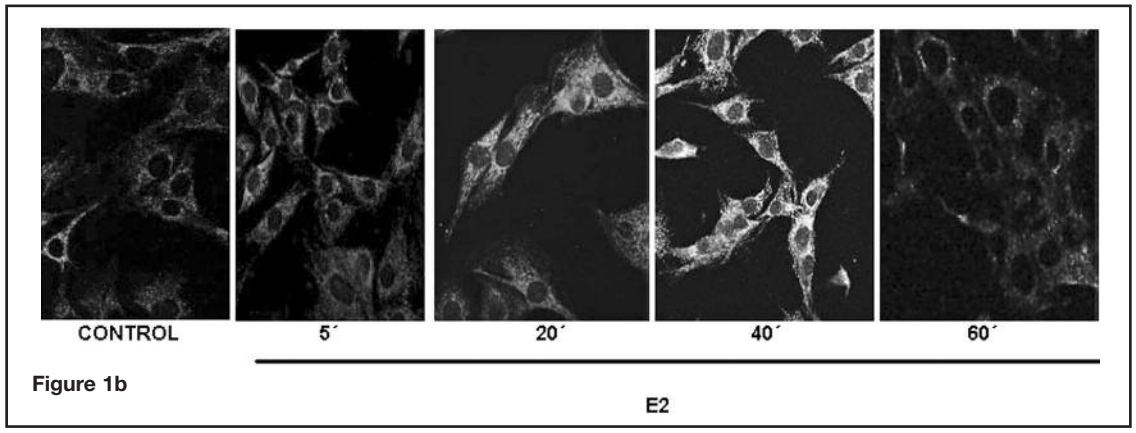


Figure 1. 17β-estradiol increases the expression of HSP27 in C2C12 skeletal muscle cells. C2C12 cells were treated with 10^{-8} M E2 for the times indicated. Controls were exposed to vehicle isopropanol (0.001%). The cells were harvested and lysed as described in Materials and Methods. The lysates were then subjected to SDS-PAGE and blotted with anti-HSP27 antibody. Actin levels were measured as protein loading controls. **a.** Representative immunoblot (upper panel) of three independent experiments with comparable results and the corresponding densitometric analysis (bottom panel) showing the increase of total HSP27 during E2 treatment. Bars are means ± SD; *p<0.05 and **p<0.01 with respect to the control. **b.** Confocal microscopy of HSP27 expression. HSP27 was stained using anti-HSP27 primary antibody and Alexa 488-conjugated secondary antibody. Control cells were treated with vehicle and E2 cells incubated with 10^{-8} M 17β-estradiol for the times indicated. Magnification 63X. Images are representative of at least three independent experiments.



HSP27 interacts with ERβ in C2C12 muscle cells

In order to study whether HSP27 interacts with the ERs, confocal microscopy was first performed to detect colocalization of these proteins. In a previous work, we demonstrated that in C2C12 muscle cells, ERα localizes in the cytosol and perinuclear region[46], whereas ERβ is mainly associated to mitochondria[47]. C2C12 cells were treated with 10^{-8} M E2 during 40 min. The ERs and HSP27 were recognized by immunofluorescence using specific antibodies for each ER isoform and a highly selective monoclonal antibody for HSP27. Immunocytochemistry assays demonstrated colocalization of HSP27 with ERβ in the mitochondria, shown in the merged images as yellow fluorescence due to combination of each antibody fluorescence (red for ERβ and green for HSP27) , which was more intense in cells exposed to E2 (Fig. 2a shows the colocalization of both proteins as brighter fluorescence). To assess whether this colocalization may imply physical interaction between ERs and HSP27, coimmunoprecipitation assays using both anti-ERβ and anti-ERα antibodies with lysates from control and estrogen-treated (10^{-8} M, 40 min) C2C12 cells were performed. The precipitates were then analyzed by Western blot with the anti-HSP27 antibody. As shown in Fig. 2b, the anti-ERβ antibody immunoprecipitated ERβ associated to HSP27, indicating the association between both proteins. This interaction was also observed when the coimmunoprecipitation assay was performed using the same antibodies in reverse order (data not shown). On the other hand, the association of the chaperone with ERα was not significant (Fig. 2b).

Activation of ERK and p38 MAPK by E2 and role of ER in the antiapoptotic effects of 17β-estradiol in muscle cells

The ability of E2 to activate ERK and p38 MAPK in muscle cells was evaluated. These anti-apoptotic proteins become activated by phosphorylation and play an important role in cell proliferation and survival, and in the nuclear genomic response to mitogens and cellular stresses. C2C12 cell cultures were incubated with the steroid hormone (10^{-8} M) followed by measurement of phospho-ERK and phospho-p38 MAPK levels. As shown in Fig. 3, Western blot analysis using anti-phospho-ERK and anti-phospho-p38 polyclonal antibodies revealed ERK and p38 activation (phosphorylation) in response to E2. Of relevance, activation of ERK by the hormone was blocked when the cells were preincubated with the ER specific antagonist ICI182780 (1 μM). However, no appreciable changes in phosphorylation of p38 were observed. Therefore, the estrogen receptor participates in ERK but not in p38 MAPK phosphorylation.

ERK and p38 MAPK mediate the antiapoptotic effects of 17β-estradiol in muscle cells

The protective action of E2 involving ERK and p38 MAPK was evaluated in primary cultures of mouse skeletal muscle, to validate the use of C2C12 cells. Morphological changes of the cytoskeleton, mitochondria and nucleus typical of apoptosis were studied.

Alterations in the cytoskeleton after treatments were evaluated by immunocytochemistry assays using an anti-actin antibody. The images of Fig. 4 show a usual actin filament organization in the control condition (panel a) and in cells treated with the hormone (panel b) whereas in cultures exposed to H_2O_2, disorganization of the cytoskeleton was observed (panel c). Incubation with E2 prior to induction of apoptosis revealed actin arrangement as in controls (panel d). Disruption of actin filaments was not reversed to normal by the estrogen in cells preincubated 30 min with inhibitors of ERK (U0126) or p38MAPK (SB203580) and then treated with E2 and H_2O_2 (panels e and f).

During apoptosis, mitochondria also undergo structural and localization changes. The abnormalities frequently observed include

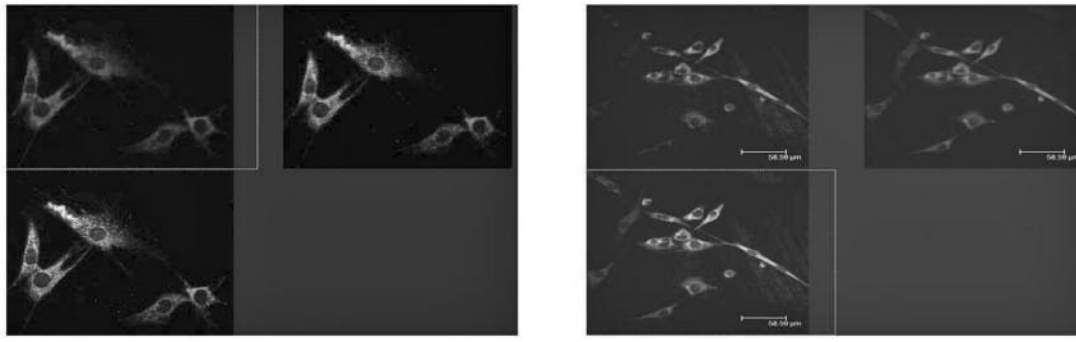


Figure 2a

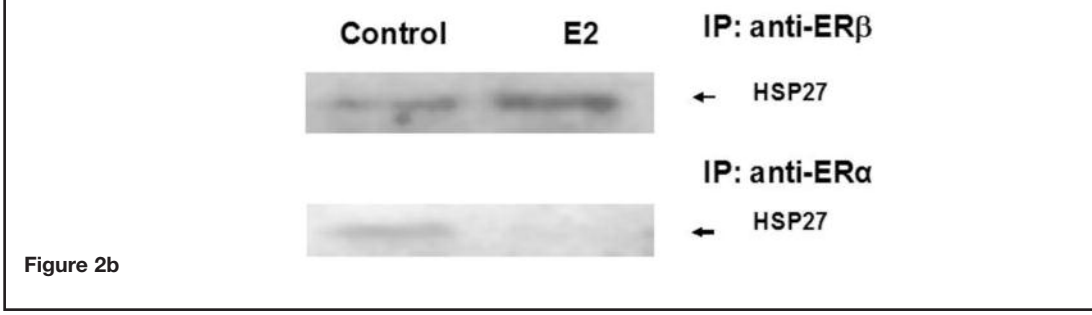


Figure 2b

Figure 2. HSP27 interacts with ERβ in C2C12 skeletal muscle cells.
a. *Immunocytochemistry.* C2C12 cells treated with 0.001% isopropanol (Control) or 10^{-8} M E2 during 40 min were double labeled using polyclonal antibodies anti-ERβ Y19 and anti-HSP27 as described in Materials and Methods. Laser confocal microscopy shows merge of staining with the two antibodies as brighter fluorescence. The photograph is representative of at least three independent experiments. Magnification 63X. **b.** *Coimmunoprecipitation.* Lysates from C2C12 cells treated as indicated above were immunoprecipitated using anti-ERβ or anti-ERα antibody and immunoblotted with anti-HSP27 antibody. Control: cells treated with vehicle, E2: cells treated with 10^{-8} M E2 during 40 min. The blot is representative of three independent experiments.

reduction in size, "mitochondrial picnosis", and grouping of these organelles in the perinuclear zone[16,48]. Fig. 4 shows that mitochondria of mouse skeletal muscle cells exposed to H_2O_2 were clustered around the nucleus (panel c) having lost its characteristic cytoplasmic distribution observed in control or in cells treated with the hormone (panel a and b, respectively). These modifications were reversed when the muscle cells were incubated with E2 (10^{-8} M - 45 min) (panel d), in accord with results previously reported[16]. When cultures were exposed to U0126 or SB203580 and then treated with E2 and H_2O_2, mitochondria showed similar characteristics to those of apoptotic skeletal muscle cells (panel e and f).

In addition, variations in the morphology of nuclei in response to treatments of muscle cells were studied using the nuclear dye DAPI.

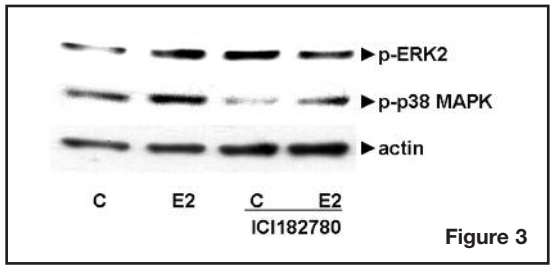


Figure 3

Figure 3. Estrogen receptor participates in ERK but not in p38 MAPK phosphorylation. C2C12 cells were treated with 0.001% isopropanol (C) or 10^{-8} M E2 during 15 min (E2) in absence or presence of 1 M of ER antagonist ICI182780. Western blot analysis were performed using anti-phospho-ERK1/2 and anti-phospho-p38 MAPK antibodies. Actin levels are shown as loading control.

Apoptotic cells were identified by the condensation and/or fragmentation of their nuclei. Fig. 4 shows intact/normal nuclei of cells under the control condition (panel a) and treated with 10^{-8} M E2 (panel b). The cultures exposed to 1 mM H_2O_2 exhibited morphological changes typical of apoptosis (panel c). This effect was observed to a lesser extent in cells incubated with the hormone during 45 min prior to exposure to H_2O_2 which mainly presented normal nuclear morphology (panel d). On the other hand, muscle cell cultures treated with the MAPK inhibitors U0126 or SB203580 as before, clearly showed nuclear fragmentation or condensation (panel e and f).

Activation of ERK by 17β-estradiol through ERα

In order to elucidate which isoform of ER mediates activation of ERK, C2C12 cells were transfected with specific siRNAs to induce silencing of ERα and ERβ. Optimum transfection conditions were established using fluorescein-siRNA. The silencing efficiency and specificity of siRNA effects on ERα and ERβ levels were verified by Western blot analysis after transfection with the isoform selective

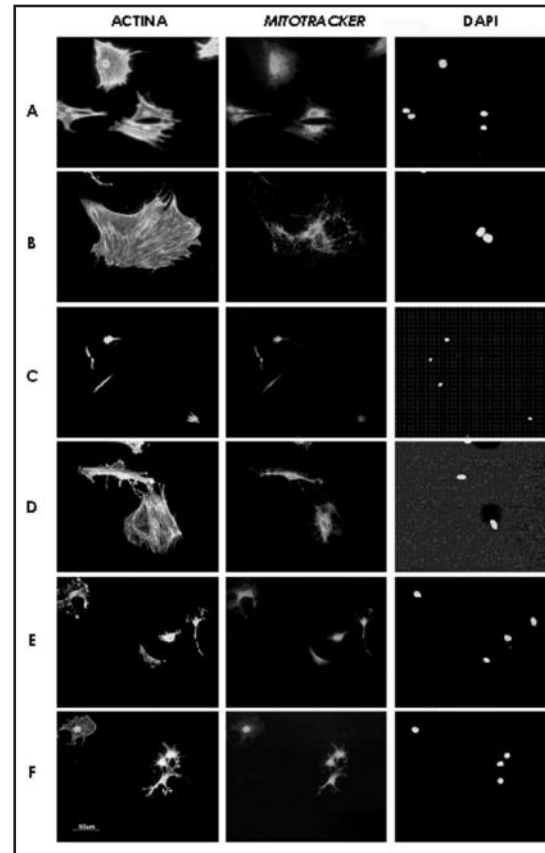


Figure 4

Figure 4. Protective action of 17β-estradiol involving ERK and p38 MAPK in primary cultures of mouse skeletal muscle. A: 0.001% isopropanol; **B:** 10^{-8} M E2 for 40 min; **C:** 0.5 mM H_2O_2 for 2 h; **D:** 10^{-8} M E2 for 40 min and then exposed to 0.5 mM H_2O_2 for 2 h, **E:** 20 M SB203580 (specific p38 MAPK inhibitor) for 30 min and then with 10^{-8} M E2 + 0.5 mM H_2O_2 as before; **F:** 10 M U0126 (specific ERK1/2 inhibitor) for 30 min and then with 10^{-8} M E2 + 0.5 mM H_2O_2 as before. Cells were stained with anti-actin antibody, mitotracker and DAPI dyes. Magnification 20X.

siRNAs (data not shown). Under these conditions, ERα silencing caused a significant blockade of ERK phosphorylation whereas ERβ silencing did not reduce ERK activation (Fig. 5). Thus, these results reveal that ERα is involved in the stimulation of ERK by E2.

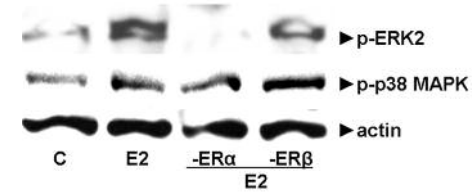


Figure 5

Figure 5. 17β-estradiol activates ERK through ERα. C2C12 cells were transfected with siRNA-ERα (-ERα) and siRNA-ERβ (-ERβ). Then, cells were treated with 0.001% isopropanol (C) or 10^{-8} M E2 (E2) during 15 min. Western blot analysis was performed using anti-phospho-ERK1/2 and anti-phospho-p38 MAPK antibodies. Actin levels are shown as loading control.

Testosterone prevents apoptosis in C2C12 cells

As a first approach to investigate the protective role of testosterone, functional changes of mitochondria were evaluated using supravital Janus Green staining. The images of Fig. 6 show that cells exposed to 1 mM H_2O_2 during 2 h (panel c) exhibited a darker cytoplasmic region near mitochondria than the control with isopropanol (panel a) or the cells treated with 10^{-8} M testosterone (panel b). These results indicate that rupture of the outer mitochondrial membrane due to H_2O_2 caused liberation of its content to the cytoplasm, inducing the oxidation of the colorant (blue-greenish coloration shown in the corresponding figure panel as the dark cytoplasmic region near mitochondria, described above)[49]. This effect was not observed when the cells were preincubated with the hormone (10^{-8} M - 40 min) prior to H_2O_2 (1 mM, 2 h) treatment (panel d). Additionally, morphological changes of the cytoskeleton, mitochondria and nucleus characteristic of apoptosis were studied by immunocytochemistry assays and immunofluorescence conventional microscopy using anti-actin antibody, Mitotracker and DAPI dyes respectively, which were performed after treatments. Images of Fig. 7 show intact/normal nuclei of muscle cells in the control condition (isopropanol) and treated with 10^{-8} M testosterone. C2C12 cultures exposed to 1 mM H_2O_2 exhibited morphological changes typical of apoptosis such as nuclear fragmentation or condensation, i.e. pyknotic nuclei (~40%). These effects were markedly reduced in cells incubated with the hormone during 40 min prior to the addition H_2O_2 (≥ 85% normal nuclear morphology). It was also observed that mitochondria of a great proportion (~70%) of the C2C12 cells exposed to H_2O_2

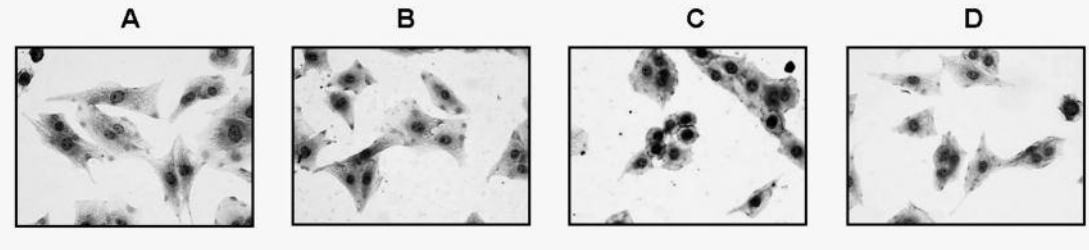


Figure 6

Figure 6. Protective role of testosterone in C2C12 skeletal muscle cells shown by Janus Green staining. A. 10^{-8} M isopropanol. **B:** 10^{-8} M testosterone for 40 min; **C:** 1 mM H_2O_2 for 2 h; **D:** 10^{-8} M testosterone for 40 min followed by exposure to 1 mM H_2O_2 for 2 h. Cells were stained with Janus Green dye. Magnification 40X.

were clustered around the nucleus losing its characteristic "spider web" or uniform cytoplasmic distribution present in control or in cells treated with the hormone. These alterations could be prevented when the muscle cells were preincubated with testosterone (10^{-8} M, 40 min). In addition, the image of Fig. 7 shows a typical organization of actin filaments in control (+ isopropanol) and in hormone-treated cells. When the cultures were exposed to H_2O_2, disorganization of the cytoskeleton was seen as described before. Incubation with testosterone previous to induction of apoptosis, maintained the arrangement of actin filaments observed in control conditions. The data obtained demonstrate an antiapoptotic action of testosterone in C2C12 skeletal muscle cells exposed to oxidative stress (H_2O_2).

The effects of testosterone on the release of cytochrome c (Table 1) due to loss of the outer mitochondrial membrane integrity induced by H_2O_2 were evaluated by means of CYTOC-OX1 assays (see Methods). We observed that 55% of the cells presented mitochondria damaged after H_2O_2 treatment whereas in cultures preincubated with testosterone before addition of H_2O_2 only 9% of the mitochondria were affected.

Discussion

17β-estradiol (E2) and testosterone exert antiapoptotic actions in skeletal muscle. In earlier

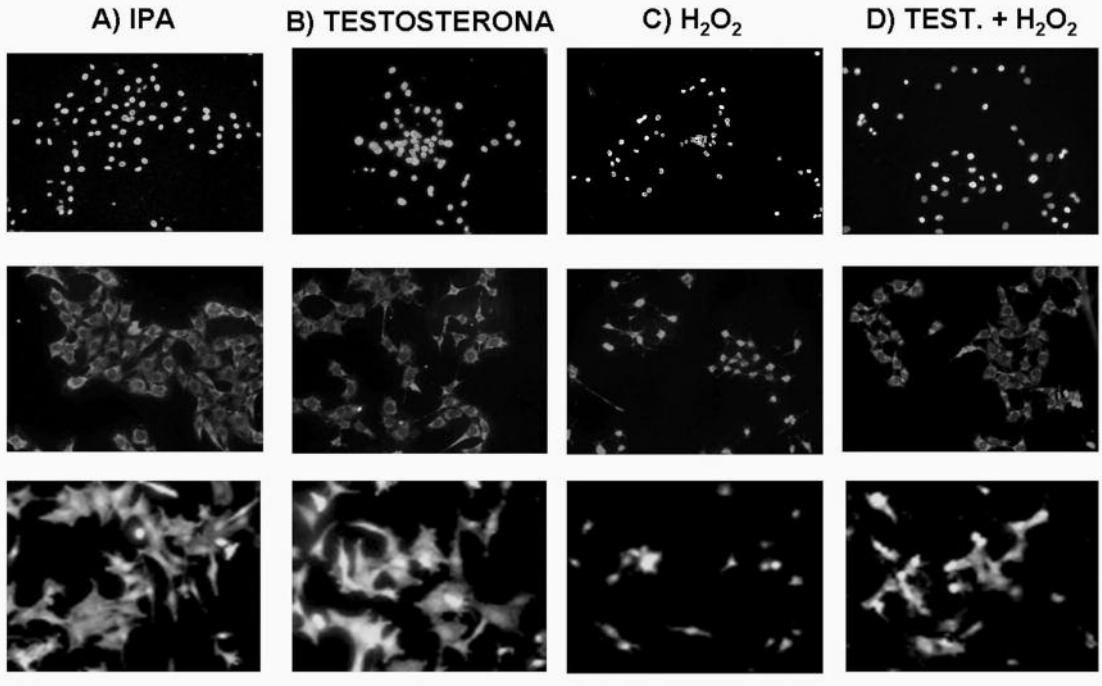


Figure 7

Figure 7. Testosterone prevents morphological alterations of cytoskeleton, mitochondria and nucleus induced by H_2O_2 in C2C12 skeletal muscle cells. A. 10^{-8} M isopropanol; **B:** 10^{-8} M testosterone for 40 min; **C:** 1 mM H_2O_2 for 2 h; **D:** 10^{-8} M testosterone for 40 min followed by exposure to 1 mM H_2O_2 for 2 h. Cells were stained with DAPI dye, Mitotracker dye and anti-actin antibody. Magnification 20X.

experiments we demonstrated that E2, at physiological concentrations, abrogates H_2O_2 induced-apoptosis in C2C12 skeletal muscle cells involving both ERα and ERβ and acting at least at two different levels. One of them relates to PI3K/Akt activation and hence BAD phosphorylation, a process in which both ER isoforms participate. The other consists in a protective effect on mitochondria integrity and mainly involves ERβ.[16]

In this study we found that HSP27 and the ERK and p38 MAPKs are other mediators in the protective action of E2.

E2, at physiological concentrations, increased HSP27 protein levels and induced the interaction of this chaperone with ERβ in the C2C12 cell line. The association detected between the chaperone and ERβ in mitochondria might confer greater stability to this receptor isoform and thus higher efficiency in stress conditions and/or regulation of the estrogen signal. According to this interpretation, it has been reported that the relationship between HSP27 and ERβ leads to estrogen signaling in human coronary arteries[50]. In the present work, the interaction shown in C2C12 muscle cells appears to be specific for ERβ and HSP27, since it was not observed for the chaperone and the α isoform of the estrogen receptor. This may explain the fact that the protective action of E2 is primarily mediated by ERβ [6]

In parallel we demonstrated rapid non-genomic events regulated by E2 in the inhibition of apoptosis. These protective actions involved stimulation of ERK and p38 MAPK and were detected not only in the C2C12 cell line but also in primary cultures of mouse skeletal muscle. Fluorescence microscopy studies of morphological changes of cytoskeleton, mitochondria and nucleus, characteristic of apoptosis, employing MAPK specific inhibitors supported these observations, as the images showed high percentage of cultured cells with actin and mitochondria disorganization and nuclear fragmentation when they were incubated with ERK or p38 MAPK inhibitors before adding the hormone, as in H_2O_2 treatment.

In addition, experiments with the ER specific antagonist ICI182780 indicated that the ER mediated ERK but not MAPK p38 phosphorylation. Furthermore, using specific siRNAs to induce silencing of each ER isoform, evidence was obtained that ERK was selectively activated through ERα.

Table 1. Evaluation of cytochrome C release

Condition	Mitochondria with damaged outer membrane (%)
IPA	15 ± 6.8
Testosterone	15 ± 3.6
H_2O_2	55 ± 3.1
Test. + H_2O_2	9 ± 2.6

Cytochrome c release was evaluated as described under Methods. IPA: cells treated with hormone vehicle (isopropanol); Testosterone: cells treated with 10^{-8} M testosterone; H_2O_2: cells treated with 1 mM H_2O_2; Test. + H_2O_2: cells preincubated with 10^{-8} M testosterone and then treated with 1 mM H_2O_2.

Likewise, testosterone participation in anti-apoptotic events was also observed in the C2C12 cell line. Microscopic analysis with Janus Green, DAPI, Mitotracker and actin staining revealed a protective effect of the steroid hormone by which it reverts the damage caused by oxidative stress (H_2O_2). Testosterone could also reduce cytochrome c release from mitochondria. This protective effect on mitochondrial membrane damage due to stress conditions might be associated with involvement of the androgen in inhibition of the apoptotic intrinsic pathway as observed with E2. However, further studies are necessary to determine whether testosterone acts in addition on the extrinsic apoptotic pathway.

The data presented in this work unravels in part the antiapoptotic molecular mechanism activated by E2 and testosterone, underlying the survival action of both hormones against the oxidative stress damage caused by H_2O_2 in the C2C12 skeletal muscle cell line. These studies also sustain an essential concept regarding the integration of rapid signaling and genomic actions involved in the protective action of these steroids. Additional investigations are necessary to elucidate in depth the mechanism by which testosterone and E2 exert an antiapoptotic effect in skeletal muscle cells and its relationship with myopathies associated to hormonal disregulation.

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