



## ARTÍCULOS ORIGINALES / Originals

# 17 $\beta$ -ESTRADIOL AND TESTOSTERONE PROTECT MITOCHONDRIA AGAINST OXIDATIVE STRESS IN SKELETAL MUSCLE CELLS

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### Summary

We have previously shown that testosterone (T) and 17 $\beta$ -estradiol (E2) protect C2C12 muscle cells against apoptosis induced by hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Since we also showed the presence of estrogen and androgen receptors in mitochondria, this work was focused on the effects of both steroids on this organelle, which result in cellular survival. Specifically, we evaluated the actions of T and E2 on the mitochondrial membrane potential with JC-1 dye and on the mitochondrial permeability transition pore (MPTP) by the calcein-acetoxymethylester (AM)/cobalt method, using fluorescence microscopy and flow cytometry. We demonstrated that T and E2 prevent MPTP opening and the loss of mitochondrial membrane potential induced by H<sub>2</sub>O<sub>2</sub>. In addition, it was observed that H<sub>2</sub>O<sub>2</sub> increase voltage-dependent anion channel (VDAC) protein expression levels and induce translocation of Bax to mitochondria. However, in the presence of the steroids Bax

translocation was abrogated suggesting that members of the Bcl-2 family may be regulated by E2 and T. The observed effects triggered by E2 and T were reflected on mitochondrial morphology. Microscopic analysis of C2C12 cells and primary cultures of mouse skeletal muscle, with Janus Green and Mitotracker staining revealed a protective effect of the steroids against oxidative stress damage which included mitochondrial redistribution and pyknosis of the organelle.

**Keywords:** estradiol; testosterone; skeletal muscle cell; mitochondria

### Resumen

## EL 17 $\beta$ -ESTRADIOL Y LA TESTOSTERONA PROTEGEN A LAS MITOCONDRIAS CONTRA EL ESTRÉS OXIDATIVO EN CÉLULAS DEL MÚSCULO ESQUELÉTICO

En trabajos previos demostramos que la testosterona (T) y el 17 $\beta$ -estradiol (E2) protegen a las células musculares C2C12 de la

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apoptosis inducida por peróxido de hidrógeno ( $H_2O_2$ ). Conjuntamente evidenciamos la existencia de receptores de estrógenos y andrógenos en las mitocondrias. El presente trabajo se ha centrado en caracterizar los efectos de ambos esteroides en esta organela, que conducen a la supervivencia celular. Específicamente, se evaluaron las acciones de T y E2 sobre el potencial de membrana mitocondrial con el colorante JC-1 y sobre el poro de permeabilidad transitoria mitocondrial (MPTP) mediante el método de calceína/acetoximetil éster (AM)/cobalto, utilizando microscopía de fluorescencia y citometría de flujo. Demostramos que T y E2 previenen la apertura del MPTP y la pérdida de potencial de membrana mitocondrial inducidas por  $H_2O_2$ . Además, observamos que el  $H_2O_2$  aumenta los niveles de expresión proteica del canal aniónico dependiente de voltaje (VDAC) e induce la translocación de Bax a mitocondria. Sin embargo, en presencia de las hormonas la translocación de Bax fue inhibida lo cual sugiere que los miembros de la familia Bcl-2 pueden ser regulados por E2 y T. Los eventos moleculares desencadenados por E2 y T a nivel mitocondrial se reflejaron en la morfología de las organelas. El análisis microscópico de las células C2C12 y cultivos primarios de músculo esquelético de ratón, mediante tinciones con verde de Jano y Mitotracker reveló un efecto protector de los esteroides contra el daño por estrés oxidativo inhibiendo la redistribución y picnosis mitocondrial.

## **Introduction**

Sarcopenia refers to the decline in muscle mass and strength with age<sup>1</sup>. This pathology leads to a significant impairment in the ability to carry out normal daily functions, increasing the risk of falls and fractures, resulting in loss of independence.<sup>2</sup> Although the molecular mechanisms responsible for muscle fiber loss and atrophy have not been completely

clarified, oxidative stress, mitochondrial dysfunction and apoptosis play important roles in age-dependent muscle atrophy.<sup>3-5</sup> These cellular events have been associated to the age-related decrease in androgen and estrogen levels, which have also been postulated as a direct cause of sarcopenia.<sup>6</sup> In agreement with these observations, it has been shown that human skeletal muscle contains both estrogen (ERs) and androgen receptors (AR).<sup>7-9</sup>

Although apoptosis may occur via several mechanisms, mitochondria are the most important regulatory centers for programmed cell death<sup>10</sup>. In response to oxidative stress, they are extensively damaged in aged skeletal muscle of humans and rodents, releasing cytochrome c into the cytosol, a key step during apoptosis.<sup>11,12</sup> In previous studies, it was shown that physiological concentrations of 17β-estradiol (E2) and testosterone (T) prevent apoptosis in skeletal muscle, activating signaling cascades and in turn abolishing the typical cytochrome c release.<sup>13-15</sup> Although several models have been proposed to explain cytochrome c release from mitochondria, opening of the mitochondrial permeability transition pore (MPTP) has gained significance. This event is generally associated with the organelle membrane potential ( $\Delta\Psi_m$ ).<sup>16</sup> Under normal physiological conditions, the mitochondrial inner membrane is impermeable to all, except for a few selected metabolites and ions. However, under stress conditions, the MPTP can be opened in the mitochondrial inner membrane allowing the free passage of any molecule smaller than 1.5 kDa.<sup>17,18</sup> MPTP comprises the voltage-dependent anion channel (VDAC), the adenine nucleotide translocator (ANT) and cyclophilin D in association with other proteins.<sup>19-21</sup> It has been shown that Bax and Bak can directly open VDAC to induce cytochrome c release in a liposome system.<sup>22-25</sup> Moreover, it has been proven using VDAC-deficient yeast



mitochondria that this channel is required for apoptotic  $\Delta\psi_m$  loss as well as for the release of cytochrome c.<sup>23</sup> It has also been reported that ANT may be a functional target of Bax.<sup>21,25</sup>

Although we have demonstrated that E2 and T exert antiapoptotic effects in the C2C12 murine skeletal muscle cell line,<sup>13-15,26</sup> the knowledge of the molecular mechanisms underlying the antiapoptotic action of both hormones has not been fully elucidated. Here, we propose that these steroids protect skeletal myoblasts regulating mitochondrial events. To test this hypothesis, in the present work, we investigate the effects of E2 and T on specific mitochondrial parameters, which in turn regulate apoptosis in C2C12 skeletal myoblasts.

### **Materials and methods**

Alexa Fluor 488-conjugated anti-rabbit secondary antibody (1:200) was obtained from Molecular Probes (Eugene, OR, USA). Testosterone and 17 $\beta$ -estradiol were purchased from Sigma-Aldrich (St Louis, MO, USA). Anti-beta tubulin (1:1000) and anti-Bax (1:1000) antibodies were obtained from Thermo Fisher Scientific, Inc. (Rockford, IL, USA). VDAC antibody (1:1000) was obtained from Cell Signalling Technology, Inc. (Danvers, MA, USA). Calcein AM (Calcein acetoxymethyl ester) and MitoTracker Red were purchased from Invitrogen. 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylben-zimidazolylcarbocyanine 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 GE Healthcare Lifescience- Amersham (Pittsburgh, PA; USA). All the other reagents used were of analytical grade.

### **Cell culture and treatment**

C2C12 murine skeletal muscle cells were cultured in growth medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated

(30 min, 56°C) fetal bovine serum (FBS), 1% nystatin and 2% streptomycin. Cells were incubated at 37°C in a humid atmosphere of 5% CO<sub>2</sub> in air. Cultures were passaged every 2 days with fresh medium. Under these conditions C2C12 myoblasts resemble the activated satellite cells that surround the mature myofibers and proliferate and differentiate participating in the repair of the tissue when a cellular injury exists. The assays were performed with 70-80% confluent cultures (120,000 cells/cm<sup>2</sup>) which were previously starved with medium without serum for 30 min. Treatments were carried out adding 10<sup>-8</sup> M 17 $\beta$ -estradiol, 10<sup>-9</sup> M testosterone or vehicle (control: 0.001% isopropanol) during 60 min before induction of apoptosis with 0.5 mM hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) for 4 h. Unless otherwise noted, cells were cultured to 50% of confluence in chamber-slides for microscopy.

### **Janus Green staining**

The supravital Janus Green colorant is a water-soluble dye that is absorbed by the whole cell, staining it in a blue-greenish coloration (oxidative state). Nevertheless, this tint disappears in the cytoplasm a few minutes later due to the reductive properties of the cytosol, except in the mitochondria, where it is seen as little dots, as a consequence of the oxidative environment of the organelle which maintains the Janus Green colorant in its oxidative state (blue-greenish coloration).<sup>27,28</sup> Janus Green staining can indirectly indicate mitochondrial dysfunction, since any rupture/disruption of the mitochondrial membrane cause release of their content to the cytosol inducing colorant oxidation in the cytoplasm. After treatments, the cells were incubated with 0.1 % Janus Green in serum-free medium (1:2, v/v) during 30 min at 37°C. Cells were examined by bright field microscopy.

### **MitoTracker Red staining**

After treatments, coverslips with adherent cells were stained with MitoTracker Red,

which was prepared in dimethyl sulfoxide and then added to the cell culture medium at a final concentration of 1  $\mu$ mol/L. After 15 to 30 min incubation at 37°C, the cells were washed with PBS (pH 7.4, 8 g/L NaCl, 0.2 g/L KCl, 0.24 g/L KH<sub>2</sub>PO<sub>4</sub>, 1.44 g/L Na<sub>2</sub>HPO<sub>4</sub>) and fixed with methanol at -20 °C for 30 min.

Cells were examined using a fluorescence microscope (NIKON Eclipse Ti-S) equipped with standard filter sets to capture fluorescent signals. Images were collected using a digital camera. Additionally, samples were analyzed with a confocal scanning laser microscopy (Leica TCS SP2 AOBS microscope), using a 63X objective.

### **Immunocytochemistry**

Semi-confluent monolayers were fixed as before. After fixation, cells were rinsed three times with PBS and non-specific sites were blocked for 1 h in PBS 2% 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 or confocal scanning laser microscopy. The specificity of the labeling techniques was proven by the absence of labeling when the primary or the secondary antibodies were omitted.

### **Western blot analysis**

Cells from 70-80% confluent cultures (120,000 cells/cm<sup>2</sup>) were lysed using a buffer made of 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.2 mM Na<sub>2</sub>VO<sub>4</sub>, 2 mM EDTA, 25 mM NaF, 1 mM PMSF, 20  $\mu$ g/ml leupeptin and 20  $\mu$ g/ml aprotinin. Lysates were collected by aspiration and centrifuged at 12,000 x g during 15 min. The protein content of the supernatant was quantified by the Bradford procedure<sup>29</sup>. Then lysate proteins dissolved in Laemmli<sup>30</sup> sample buffer (30  $\mu$ g; ca. 2,000,000 cells) were separated on 10% SDS-polyacrylamide gels and

electrotransferred to polyvinylidene difluoride (PVDF) membranes. Relative migration of unknown proteins was determined by comparison with molecular weight colored markers (Amersham). Membranes were blocked 1 h at room temperature in PBS-T buffer (PBS 0.1% Tween-20) containing 5% dry milk. Membranes were incubated with different primary antibodies overnight at 4°C, and then repeatedly washed with PBS-T prior incubation with PBS-T containing 1% dry milk with peroxidase-conjugated secondary antibodies for 1 h at room temperature. Next, membranes were visualized using an enhanced chemiluminiscent technique (ECL) according to the manufacturer's instructions. Relative migration of unknown proteins was determined by comparison with molecular weight colored markers (Amersham). Relative quantification of Western blot signals was performed using Image J software (NIH, USA). For reprobing with other antibodies, the membranes were incubated in stripping buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS and 50 mM mercaptoethanol) for 30 min at 55°C, washed 10 min in PBS-T and then blocked and blotted as described above.

### **Subcellular fractionation**

C2C12 monolayers were scrapped and homogenized in ice-cold Tris-EDTA-sucrose (TES) buffer (50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 250 mM sucrose, 1 mM DTT, 0.5 mM PMSF, 20 mg/ml leupeptin, 20 mg/ml aprotinin, 20 mg/ml trypsin inhibitor) using a Teflon-glass hand homogenizer. Total homogenate free of debris was used in order to obtain the different fractions. The nuclear pellet was obtained by centrifugation at 300 g during 15 min. The supernatant was further centrifuged at 10,000 g for 30 min to pellet mitochondria. The remaining solution was called mitochondrial supernatant. Pellets were re-suspended in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2 mM Na<sub>2</sub>VO<sub>4</sub>, 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<sup>29</sup>, using BSA as standard and Western blot analysis were performed as described above. Cross contamination between fractions was assessed by immunoblots using antibodies against lamin B and COX (complex IV), nuclear and mitochondrial markers, respectively.

### Flow cytometry

Mitochondria are critical organelles involved in cell death.<sup>31</sup> During this process, the mitochondrial transmembrane potential ( $\Delta\psi_m$ ) collapses.<sup>32</sup> Therefore, to determine the role of T and E2 against mitochondrial dysfunction induced by  $H_2O_2$ , we evaluated the  $\Delta\psi_m$  in 10,000 C2C12 cells using the JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide) mitochondrial transmembrane potential detection kit from Becton-Dickinson Biosciences (San Jose, CA, USA) with flow cytometry. 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 (FL-2 channel), and green monomers (detectable in FL1 channel). Cells with collapsed mitochondria contain mainly green JC-1 monomers and exhibit fluorescence in the green end of the spectrum<sup>33</sup>. Then, apoptotic cells, showing primarily green fluorescence, are easily differentiated from healthy cells that show red and green fluorescence. The assays were performed with 70-80% confluent cultures in 10 cm plates. After treatments, cells were trypsinized, harvested, and incubated with JC-1 probe in 5 ml polystyrene round-bottom tubes (Becton-Dickinson Biosciences), according with manufacturer'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).

### Measurement of MPTP opening

We assessed the MPTP opening using the calcein-AM/cobalt method.<sup>34</sup> Calcein-AM is an anionic, esterified fluorochrome that enters to 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<sup>34</sup>. Briefly, 70–80% confluent cultures in 10 cm (flow cytometry) or 3 cm (microscopy) plates were loaded for 30 min with 1 mM calcein-AM at 37°C in DMEM medium. The cytosolic and nuclear calcein fluorescence was quenched using 1 mM  $CoCl_2$ . After attainment of quenching, cells were washed twice with warm DMEM without serum. Following the corresponding treatments, cells were examined by fluorescence microscopy. Image quantification was performed by measuring the fluorescence intensity profiles (in arbitrary units) using the Image J software 1.41.

### Statistical analysis

Statistical treatment of the data was performed using the Student's t-test.<sup>35</sup> Data are shown as means  $\pm$  standard deviation (SD) from three independent experiments. The data were considered statistically significant when  $p < 0.05$ .

### Results

#### **$17\beta$ -estradiol and testosterone preserve mitochondrial membrane potential ( $\Delta\psi_m$ ) in C2C12 muscle cells exposed to $H_2O_2$**

We investigated the role of T and E2 against mitochondrial dysfunction induced by  $H_2O_2$ , evaluating the  $\Delta\psi_m$  in C2C12 cells. Myoblasts were starved during 30 min, incubated with  $10^{-8}$  M E2,  $10^{-9}$  M T or vehicle during 1 h prior to induction of apoptosis with 0.5 mM  $H_2O_2$  during 4 h. Figure 1 shows that the percentage

of depolarized cells increased in cultures treated with H<sub>2</sub>O<sub>2</sub> compared with control/T or E2-treated cultures, while the incubation with T or E2 prior to H<sub>2</sub>O<sub>2</sub> exposure was capable of reducing the percentage of depolarized cells.

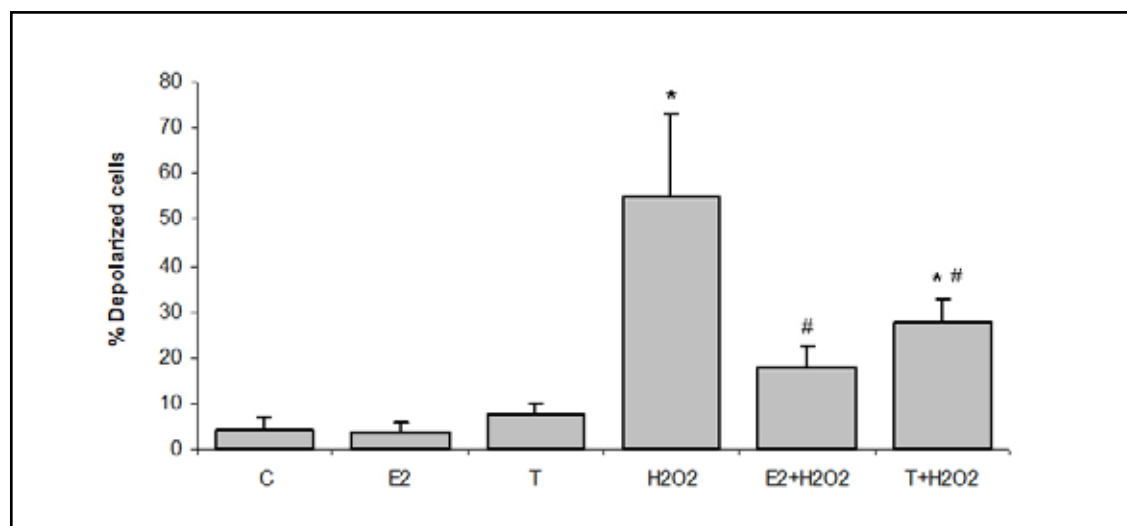
#### 17 $\beta$ -estradiol and testosterone regulate MPTP opening in C2C12 muscle cells

As mentioned before, mitochondria play a crucial role in apoptosis, releasing 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. This activation can be associated to  $\Delta\psi_m$  loss<sup>16</sup>. To evaluate the effects of E2 and T on MPTP function in C2C12 cells, we used a cobalt quenched calcein-AM method. C2C12 cell cultures, loaded with calcein-AM/CoCl<sub>2</sub>, were incubated with E2, T or vehicle isopropanol (control) before induction of

apoptosis with H<sub>2</sub>O<sub>2</sub> (4 h). The cells were then analyzed by microscopy as described in Materials and Methods. As shown in Figure 2 by microscopical analysis, the treatment with H<sub>2</sub>O<sub>2</sub> resulted in a significant decrease on the calcein fluorescence intensity due to CoCl<sub>2</sub> quenching as a result of MPTP opening (from  $\sim 87.5 \pm 12.6\%$  in control, to  $\sim 28 \pm 10.2\%$  in H<sub>2</sub>O<sub>2</sub> condition). However, when the cells were previously treated with T (Figure 2A) or E2 (Figure 2B), the fluorescence was diminished to a lesser extent (remaining  $\sim 70\%$  of fluorescent cells for both hormones), implying a protective role of the steroids over this pore.

#### VDAC and Bax are involved in H<sub>2</sub>O<sub>2</sub>-induced apoptosis in C2C12 muscle cells

The MPTP pore is a large multiprotein complex, primarily composed of the adenine nucleotide transporter (ANT), cyclophilin D, and VDAC.<sup>18,36,37,38</sup> The VDAC has been



**Figure 1. 17 $\beta$ -estradiol and testosterone inhibit the mitochondrial membrane potential loss elicited by H<sub>2</sub>O<sub>2</sub> in C2C12 muscle cells.** Cultures were serum-starved during 30 min and then treated as described in Materials and Methods. C: 0.001% isopropanol; E2: 10<sup>-8</sup> M 17 $\beta$ -estradiol; T: 10<sup>-9</sup> M testosterone; H<sub>2</sub>O<sub>2</sub>: 0.5 mM H<sub>2</sub>O<sub>2</sub>; E2+H<sub>2</sub>O<sub>2</sub>: 10<sup>-8</sup> M 17 $\beta$ -estradiol + 0.5 mM H<sub>2</sub>O<sub>2</sub>; T+H<sub>2</sub>O<sub>2</sub>: 10<sup>-9</sup> M testosterone + 0.5 mM H<sub>2</sub>O<sub>2</sub>. Then, cells were stained *in vivo* with JC-1 followed by flow cytometry analysis (Materials and Methods). Percentages of depolarized cells from three independent experiments are shown. Averages  $\pm$  S.D. are shown. \*p<0.05 with respect to the control. #p<0.05 with respect to the H<sub>2</sub>O<sub>2</sub>.

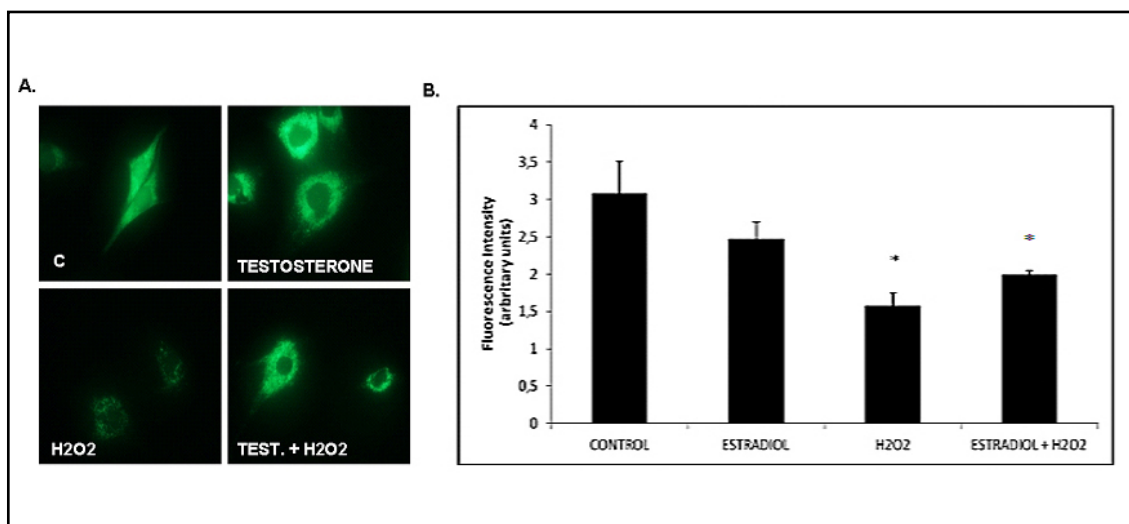


involved in mitochondrial-dependent cell death by forming part of the permeability pore.<sup>39,40</sup> In the present work we investigated the expression of VDAC protein after treatment with H<sub>2</sub>O<sub>2</sub> during different time intervals (15 min – 4 h). As shown in Figure 3, VDAC protein levels increased in a time-dependent fashion. It has been shown that Bax translocates to mitochondria and interacts with VDAC.<sup>24</sup> Moreover, apoptosis can be associated with translocation of cytosolic Bax to mitochondria.<sup>41</sup> In order to evaluate the participation of Bax in the mitochondrial effects studied above, we analyzed by Western blot its localization in skeletal muscle cells after treatments. C2C12 muscle cells were starved for 30 min, followed by incubation with 0.5 mM H<sub>2</sub>O<sub>2</sub> at different times (1, 3, and 4 h) or with 10<sup>-8</sup> M E2 or vehicle during 1 h prior to the induction of apoptosis with 0.5 mM H<sub>2</sub>O<sub>2</sub>

during 4 h. Then mitochondrial and cytosolic fractions were obtained to perform immunoblot assays. It was observed that H<sub>2</sub>O<sub>2</sub> treatment induced time-dependent Bax translocation to mitochondria in C2C12 cells (Figure 4 A), which was more evident after 3 h of induction of apoptosis. However, pretreatment with E2 inhibited Bax translocation (Figure 4 B). A similar protective effect was detected when cells were pretreated with T (data not shown).

### 17 $\beta$ -estradiol and testosterone protect mitochondrial morphology in skeletal muscle cells

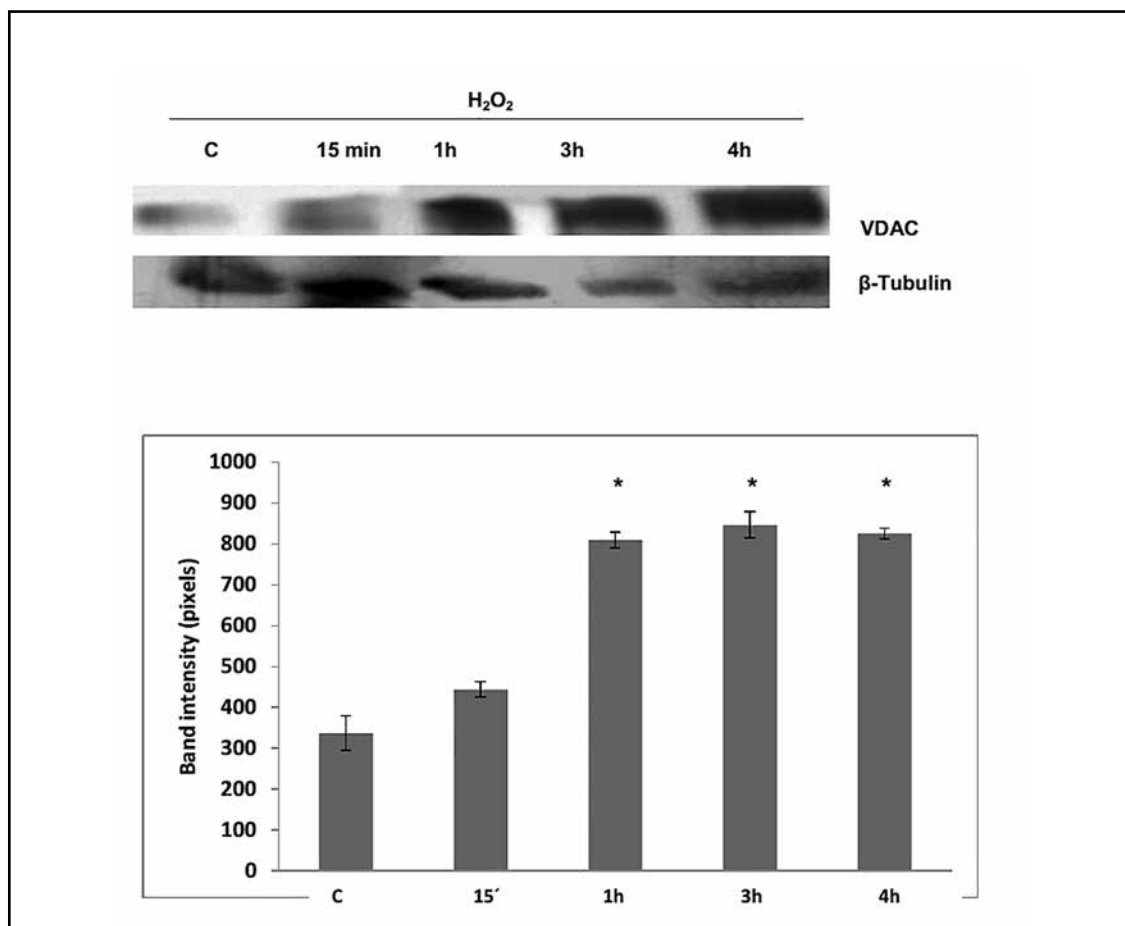
Mitochondrial morphology was evaluated to ascertain whether the molecular events described above affect it. To this end, microscopic analysis was performed with Janus Green and Mitotracker staining of C2C12 cells and primary cultures of mouse



**Figure 2.** 17 $\beta$ -estradiol and testosterone prevents H<sub>2</sub>O<sub>2</sub>-induced MPTP opening in C2C12 skeletal myoblasts. Cells loaded with calcein-AM/CoCl<sub>2</sub> (Methods) were incubated with C: 0.001% isopropanol; E2: 10<sup>-8</sup> M 17 $\beta$ -estradiol; T: 10<sup>-9</sup> M testosterone; H<sub>2</sub>O<sub>2</sub>: 0.5 mM H<sub>2</sub>O<sub>2</sub>; E2+H<sub>2</sub>O<sub>2</sub>: 10<sup>-8</sup> M 17 $\beta$ -estradiol + 0.5 mM H<sub>2</sub>O<sub>2</sub>; T+H<sub>2</sub>O<sub>2</sub>: 10<sup>-9</sup> M testosterone + 0.5 mM H<sub>2</sub>O<sub>2</sub> as described in Materials and Methods. MPTP opening was analyzed using a fluorescence microscope. **(A)** Representative image of experiments performed with T. **(B)** Quantification of the fluorescence from the experiments performed with E2. Experiments were repeated at least three times with essentially identical results. Averages  $\pm$  S.D. are given. Image quantification was performed using the Image J 1.46 software.

skeletal muscle, as described in Materials and Methods. C2C12 cell line and cells from primary cultures were incubated with H<sub>2</sub>O<sub>2</sub>, E2, T or vehicle isopropanol (control) or treated with each hormone prior to induction of apoptosis with 0.5 mM H<sub>2</sub>O<sub>2</sub> (4h). Figure 5 shows the results obtained with E2, which were similar to those obtained with T (data not shown). C2C12 cells and primary cultures (columns 1 and 2, respectively) were stained with Mitotracker. In panel B, cells exposed to H<sub>2</sub>O<sub>2</sub> (4h) showed mitochondria that were clustered

around the nucleus losing its characteristic “spider web” or uniform cytosolic distribution while in panel A cells treated with the hormone showed a uniform cytoplasmic distribution. In addition, mitochondria of cells treated with the apoptosis inducer were piknotic and smaller. These alterations could be prevented when the skeletal muscle cells were incubated with E2 (10<sup>-8</sup>M, 1h) prior to induction of apoptosis. Moreover, functional changes of mitochondria were evaluated using supravital Janus Green staining. Column 3 shows that C2C12 cells



**Figure 3. H<sub>2</sub>O<sub>2</sub> increases VDAC expression in C2C12 skeletal myoblasts.** C2C12 cells were incubated with vehicle (control) or 0.5 mM H<sub>2</sub>O<sub>2</sub> during the times indicated, as described in Materials and Methods. Lysates were subject to SDS-PAGE and probed for the expression of VDAC by Western blot analysis. The blots are representative of three independent experiments with comparable results. \*p<0.05 with respect to the control.



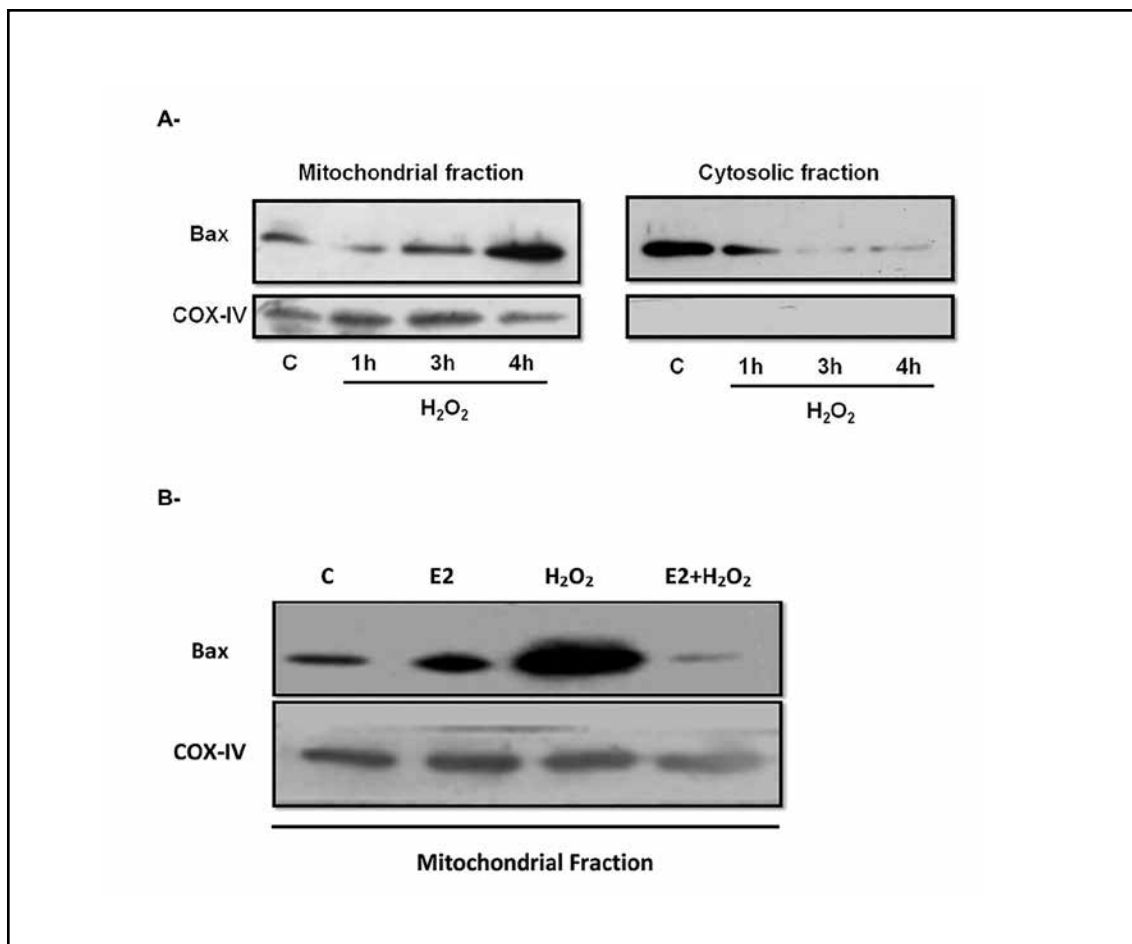


exposed to H<sub>2</sub>O<sub>2</sub> (panel B) exhibited a darker blue-greenish coloration in all the cytoplasm when compared to E2-treated cells (panel A) whereas mitochondria appear as little blue-greenish dots with cytoplasmic distribution and around the nucleus. These results indicate that the rupture of mitochondrial membrane induced by H<sub>2</sub>O<sub>2</sub> caused the release of its content into the cytoplasm, inducing the oxidation of the colorant.<sup>28</sup> This effect was not observed when C2C12 cells

were preincubated with the hormone prior to H<sub>2</sub>O<sub>2</sub> treatment (panel C).

### Discussion

Although the classical site of action of estrogens and androgens is the nucleus, it has been demonstrated that these hormones also target at mitochondria.<sup>42,43</sup> This organelle is not only the powerhouse of the cell, but also participates in a large number of signal transduction pathways for a wide variety of

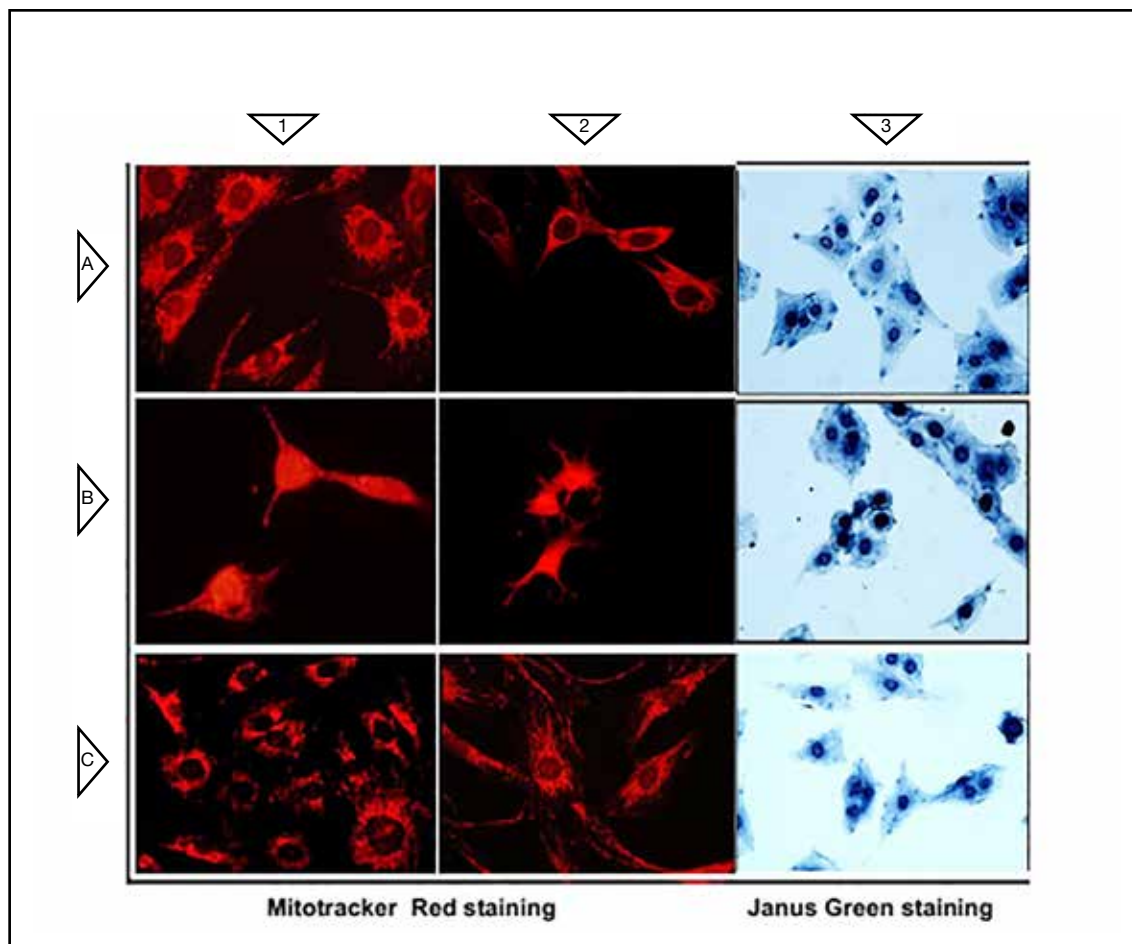


**Figure 4. E2 inhibits H<sub>2</sub>O<sub>2</sub>-induced Bax translocation to mitochondria in C2C12 muscle cells.**

(A) C2C12 cells were incubated with vehicle (control) or 0.5 mM H<sub>2</sub>O<sub>2</sub> during the times indicated, or (B) with E2: 10<sup>-8</sup> M 17 $\beta$ -estradiol; H<sub>2</sub>O<sub>2</sub>: 0.5 mM H<sub>2</sub>O<sub>2</sub>; E2+H<sub>2</sub>O<sub>2</sub>: 10<sup>-8</sup> M 17 $\beta$ -estradiol + 0.5 mM H<sub>2</sub>O<sub>2</sub>; as described in Materials and Methods. Lysates were obtained following different treatments and then subject to subcellular fractionation. Enriched mitochondrial and cytosolic fractions were used to perform Western blots assays and probed for Bax translocation. Experiments were repeated at least three times with essentially identical results.

biologically active molecules. Moreover, it could be considered as a cellular arsenal enclosing a mixture of pro-apoptotic proteins. In fact, mitochondria represent a control point of apoptosis regulated by Bcl-2 family members at two different levels: through the maintenance of both ATP production linked to mitochondrial membrane potential ( $\Delta\psi_m$ )<sup>46</sup> and mitochondrial membrane integrity.<sup>45</sup> Therefore, if mitochondria are target of the sexual hormones, the protection observed by E2 and T against H<sub>2</sub>O<sub>2</sub>-induced

apoptosis in C2C12 cells<sup>13-15,26</sup> could be due to hormonal regulation of the above mentioned mitochondrial events. Accordingly, the data obtained in this work using the C2C12 murine cell line provide evidence that mitochondria play a key role in the antiapoptotic effect, previously observed, of E2 and T in skeletal muscle cells. By means of an established method to evaluate the operational status of the mitochondrial permeability transition pore (MPTP) employing calcein-AM/CoCl<sub>2</sub> loaded cells, it was found that



**Figure 5. Protective role of 17 $\beta$ -estradiol and testosterone on mitochondrial morphology in skeletal muscle cells exposed to H<sub>2</sub>O<sub>2</sub>.** First and third columns correspond to C2C12 cells; the second column stands for cells from primary cultures of mouse skeletal muscle. Panel A: 10<sup>-8</sup> M E2 for 1 h; Panel B: 0.5 mM H<sub>2</sub>O<sub>2</sub> for 4h; Panel C: 10<sup>-8</sup> M E2 for 1h followed by exposure to 0.5 mM H<sub>2</sub>O<sub>2</sub> for 4h. Cells were stained with Janus Green dye (column 3) or Mitotracker dye (columns 1 and 2). Experiments were repeated at least three times with essentially identical results. Magnification: 40X.



H<sub>2</sub>O<sub>2</sub> treatment induced its opening. However, pretreatment of the cells with E2 or T abolished this response. Bax is likely to play a role in these effects since in the present work it is shown that H<sub>2</sub>O<sub>2</sub> induces its translocation to mitochondria. In mitochondria, Bax can interact with the MPTP components VDAC or ANT.<sup>21,46</sup> In agreement with this observation, we found that after induction of apoptosis, Bax as well as VDAC levels augment in mitochondria, increasing thereby the probability of interaction between both proteins in C2C12 cells. Hence, Bax may regulate MPTP function via direct molecular interactions with these pore components. Further investigations of protein associations are necessary to evaluate whether these events occur in skeletal muscle cells in response to H<sub>2</sub>O<sub>2</sub> treatment and whether the steroids can regulate them. Thus, the effects of E2 on MPTP could be in part due to the hormonal action on Bax, since the hormone inhibits its H<sub>2</sub>O<sub>2</sub>-induced translocation to mitochondria.

As mentioned before, the mitochondrial inner membrane is impermeable except for a few selected metabolites and ions. However, under stress conditions, the MPTP can open in the inner membrane allowing the free passage of mitochondrial proteins to the cytosol.<sup>17,18</sup> The induction of apoptosis with hydrogen peroxide in C2C12 cells triggers the loss of mitochondrial components rendering morphological changes of mitochondria. Microscopic analysis with Janus Green and Mitotracker staining revealed a protective effect of the steroids against oxidative stress damage, which included mitochondrial redistribution and pyknosis of the organelle.<sup>13</sup> Several interpretations can be made of the physiological relevance of these changes in response to H<sub>2</sub>O<sub>2</sub> treatment. It is possible that clustering of mitochondria near the nucleus could generate the high energy levels required to maintain the machinery activated by the apoptotic stimulus. Also, displacement of the organelle could facilitate translocation of mitochondrial endonucleases to the nucleus.<sup>47</sup>

Moreover, the mitochondrial modifications such as size reduction could be related to the release of mitochondrial proteins (e.g., AIF, cytochrome c) observed in apoptosis.<sup>48</sup>

In addition to the role of E2 and T on MPTP, in this work, we also demonstrate that E2 as well as T preserve the mitochondrial membrane potential of C2C12 muscle cells from depolarization promoted by H<sub>2</sub>O<sub>2</sub>. Since an increase of the outer mitochondrial membrane permeability is a crucial event in apoptosis,<sup>49</sup> these results potentiate the survival role of these sexual hormones acting on mitochondria.

Finally, these studies are relevant to skeletal muscle physiology since 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 occurs, satellite cells have the capacity to proliferate and differentiate, vital properties to repair the injured tissue.<sup>50</sup> In this context, satellite cells and their response to 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.<sup>51,52</sup> Additional studies will allow deepening the knowledge of the mechanisms by which T and E2 exert the antiapoptotic effect in skeletal muscle cells and its relationship with myopathies associated to hormonal dysregulation.

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#### **Declaration of interest**

The authors declare that they have no conflict of interest.

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