

# Testosterone exerts antiapoptotic effects against H<sub>2</sub>O<sub>2</sub> in C2C12 skeletal muscle cells through the apoptotic intrinsic pathway

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## Abstract

Experimental data indicate that apoptosis is activated in the aged skeletal muscle, contributing to sarcopenia. We have previously demonstrated that testosterone protects against hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)-induced apoptosis in C2C12 muscle cells. Here we identified molecular events involved in the antiapoptotic effect of testosterone. At short times of exposure to H<sub>2</sub>O<sub>2</sub> cells exhibit a defense response but at longer treatment times cells undergo apoptosis. Incubation with testosterone prior to H<sub>2</sub>O<sub>2</sub> induces BAD inactivation, inhibition of poly(ADP-ribose) polymerase cleavage, and

a decrease in BAX levels, and impedes the loss of mitochondrial membrane potential, suggesting that the hormone participates in the regulation of the apoptotic intrinsic pathway. Simultaneous treatment with testosterone, H<sub>2</sub>O<sub>2</sub>, and the androgen receptor (AR) antagonist, flutamide, reduces the effects of the hormone, pointing to a possible participation of the AR in the antiapoptotic effect. The data presented allow us to begin to elucidate the mechanism by which the hormone prevents apoptosis in skeletal muscle.

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## Introduction

The loss of muscle mass and strength with aging, also referred to as sarcopenia, is a highly prevalent condition among the elderly and predicts several adverse outcomes, including disability, institutionalization, and mortality. Age-related muscle loss is a result of reduction in the size and number of muscle fibers (Lexell 1993), possibly due to a multifactorial process that involves lack of physical activity, reduced nutritional intake, oxidative stress, and hormonal changes (Baumgartner *et al.* 1999, Roubenoff & Hughes 2000). Sarcopenia has been associated with a deficit of sex hormones as the levels of estrogens and/or testosterone decline with aging. Although the exact mechanisms underlying sarcopenia are far from being clarified, accumulating evidence suggests that an age-related acceleration of myocyte loss via apoptosis might represent a key mechanism responsible for impairment of muscle performance (Dirks & Leeuwenburgh 2002, Dupont-Versteegden 2005).

Apoptosis, a process of programmed cell death, is an evolutionarily conserved, tightly regulated, systematic set of events resulting in cellular self-destruction without inflammation or damage to the surrounding tissue (Kerr *et al.* 1972). Apoptosis occurs in response to environmental or developmental cues, cellular stresses, and specific cell death signals, and plays a fundamental role in the development and maintenance of tissue homeostasis.

Androgens are important regulators of reproductive physiology and anabolic biological activities in multiple

tissues (Omwancha & Brown 2006). The protective action of androgens on tissues is currently receiving increased attention. The existence of beneficial effects of androgens on cardiovascular system, pancreas, neurons, testis, and bone has been reported (Erkkilä *et al.* 1997, Morimoto *et al.* 2005, Pike *et al.* 2008, Chen *et al.* 2010, Sánchez-Más *et al.* 2010). It is well known that skeletal muscle is also a target tissue for androgens. Testosterone through its effects on muscle and fat mass is an important determinant of body composition in male mammals, including human. Testosterone supplementation increases muscle mass in healthy young and old men, healthy hypogonadal men, and other pathological or physiological conditions with low levels of this hormone (Bhasin *et al.* 2006). It has also been shown that testosterone-induced increase in muscle size is associated with hypertrophy of muscle fibers and significant increases in myonuclear and satellite cell numbers (Sinha-Hikim *et al.* 2002, 2003, 2006). Available evidence further suggests that exogenous testosterone administration results in faster recovery from hind limb paralysis after sciatic nerve injury in the rat (Brown *et al.* 1999), and completely prevents the castration-induced apoptosis in muscle cells of the rat levator ani muscle (Boissonneault 2001).

In our laboratory, we have recently demonstrated that testosterone protects against hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)-induced apoptosis in the C2C12 muscle cell line (Pronsato *et al.* 2010). Typical changes of apoptosis such as nuclear fragmentation, cytoskeleton disorganization, and mitochondrial reorganization/dysfunction induced by 1 mM H<sub>2</sub>O<sub>2</sub> are

abolished when cells are previously exposed to the hormone. In view of this evidence, the aim of the present work was to identify the molecular events that occur during the anti-apoptotic effect of testosterone on C2C12 muscle cells. We demonstrated that at short times of exposure to the apoptotic agent H<sub>2</sub>O<sub>2</sub>, cells exhibit a defense response showing ERK2, AKT, and BAD phosphorylation and an increase in HSP70 levels. However, at longer treatment times with H<sub>2</sub>O<sub>2</sub>, a decrease in the phosphorylation of the proteins mentioned before, cytochrome *c* release and poly(ADP-ribose) polymerase (PARP) cleavage occur, leading finally to cell apoptosis. But if cells are treated with testosterone prior to H<sub>2</sub>O<sub>2</sub> exposure, a protective effect of the steroid hormone is observed, which involves BAD inactivation (phosphorylation), a decrease in BAX levels, and prevention of loss of mitochondrial membrane potential, suggesting an involvement of testosterone in the regulation of the apoptotic intrinsic pathway.

Although further studies are required to establish the molecular basis of sarcopenia associated with states of testosterone deficit, the data presented in this work allow us to begin to elucidate the mechanism by which the hormone prevents apoptosis in skeletal muscle.

## Materials and Methods

### Materials

Testosterone (T-1500), anti-actin rabbit polyclonal antibody (A-5060), and Cytochrome *c* Oxidase Assay Kit were purchased from Sigma-Aldrich. Anti-phospho-ERK1/2 mouse monoclonal (91065), anti-phospho-Akt (Ser473), anti-phospho-Bad (Ser136), and anti-ERK1/2 (9102) rabbit polyclonal antibodies were from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti- $\beta$ -tubulin (PA1-21153), anti-Bax (PA1-30415), anti-HSP70 (PA1-37842), and anti-PARP (PA3-951) rabbit polyclonal antibodies were purchased from Thermo Fisher Scientific (Rockford, IL, USA). Anti-Akt (sc-8312), anti-Bad (sc-942), anti-myogenin (sc-12732), and anti-14-3-3 (sc-629) rabbit polyclonal antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). DAPI dye was from Molecular Probes (Eugene, OR, USA). 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) was purchased from Becton Dickinson Biosciences (San Jose, CA, USA). All other reagents used were of analytical grade.

### Cell culture and treatment

C2C12 murine skeletal muscle cells, from the American Type Culture Collection (ATCC number: CRL-1772) at Manassas, VA 20108, USA, were cultured in growth medium (DMEM) supplemented with 10% heat-inactivated (30 min, 56 °C) fetal bovine serum, 1% nistatin, 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 (Vasconsuelo *et al.* 2008, Ronda *et al.* 2010). 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 (Yoshida *et al.* 1998). The treatments were performed with 70–80% confluent cultures (120 000 cells/cm<sup>2</sup>) in medium without serum containing 10<sup>-9</sup> M testosterone (Kerry *et al.* 2004, Pronsato *et al.* 2010) or vehicle (0.001% isopropanol (IPA)), ~45 min before induction of apoptosis with H<sub>2</sub>O<sub>2</sub> during different periods of times. H<sub>2</sub>O<sub>2</sub> was diluted in culture medium without serum at a final concentration of 1 mM in each assay (Aksenova *et al.* 2004, Pronsato *et al.* 2010). After treatments, cells were lysed using a buffer composed of 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 1% Triton X-100, 0.2 mM Na<sub>2</sub>VO<sub>4</sub>, 2 mM EDTA, 25 mM NaF, 1 mM phenylmethylsulphonyl fluoride (PMSF), 20  $\mu$ g/ml leupeptin, and 20  $\mu$ 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 BSA as standard. Cells were cultured in chamber slides for microscopy or in 10 cm plates (Greiner Bio-One GmbH, Frickenhausen, Germany) for western blots, mitochondrial membrane integrity, and mitochondrial transmembrane potential assays.

### Western blot analysis

Protein aliquots (25  $\mu$ g) were combined with sample buffer (400 mM Tris-HCl (pH 6.8), 10% SDS, 50% glycerol, 500 mM dithiothreitol (DTT), and 2  $\mu$ g/ml Bromophenol Blue), boiled for 5 min and resolved by 10% SDS-PAGE. Fractionated proteins were then electrophoretically transferred onto PVDF membranes (Immobilon-P; Millipore, Darmstadt, Germany), using a semi-dry system. Nonspecific sites were blocked with 5% nonfat dry milk in PBS containing 0.1% Tween-20 (PBS-T). Blots were incubated overnight with the appropriate dilution of the primary antibodies. The membranes were repeatedly washed with PBS-T prior incubation with HRP-conjugated secondary antibodies. The 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 or tubulin loading control, membranes were stripped with stripping buffer (62.5 mM Tris-HCl (pH 6.7); 2% SDS; 50 mM  $\beta$ -mercaptoethanol) and then blocked for 1 h with 5% nonfat dry milk in PBS containing 0.1% Tween-20 (PBS-T). The blots were then incubated for 1 h with a 1:10 000 dilution of anti-actin or anti-beta-tubulin polyclonal antibodies. After several washings with PBS-T, membranes were incubated with anti-rabbit (1:10 000) as secondary antibody, conjugated with HRP. The corresponding immunoreactive bands were developed by means of ECL. Relative quantification of western blot signals was performed by ImageJ software (NIH, USA; Abramoff *et al.* 2004).

### Quantitation of apoptotic cells

After treatments, the cells were fixed with methanol at  $-20^{\circ}\text{C}$  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 then washed with PBS. Cells were mounted on glass slides and examined under a fluorescence microscope (NIKON Eclipse E 600) equipped with standard filter sets to capture fluorescent signals. Images were collected by 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 were counted for each treatment from at least three independent experiments.

### 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 *c* release by the determination of cytochrome *c* oxidase activity, according to the manufacturer's instructions. Briefly, C2C12 confluent monolayers were scraped and homogenized in ice-cold TES buffer (50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 250 mM sucrose, 1 mM DTT, 0.5 mM PMSE, 20 mg/ml leupeptin, 20 mg/ml aprotinin, and 20 mg/ml trypsin inhibitor) using a Teflon-glass hand homogenizer. Lysates were centrifuged at 10 000 g for 20 min in order to separate the cytosolic fraction. For cytochrome *c* oxidase activity measurements, 50  $\mu\text{l}$  reduced cytochrome *c* (0.22 mM) were added to 30  $\mu\text{l}$  cytosolic fraction, and changes in absorbance at 550 nm were monitored for 1 min. The percentage of cytochrome *c* activity was calculated as:  $\text{Units/ml} = \Delta\text{Abs}/\text{min} \times \text{dil} \times 1.1 / (\text{vol sample}) \times 21.84$ , where  $\Delta\text{Abs}/\text{min} = \text{Abs}/\text{min}_{\text{sample}} - \text{Abs}/\text{min}_{\text{blank}}$ ; dil, dilution factor of sample; 1.1, reaction volume in ml; and 21.84, extinction coefficient. Differences were calculated by normalizing cytochrome *c* activity of each condition to that of total protein level obtained by Bradford assay in the same sample. The results were expressed as percentage of mitochondria with damaged outer membrane.

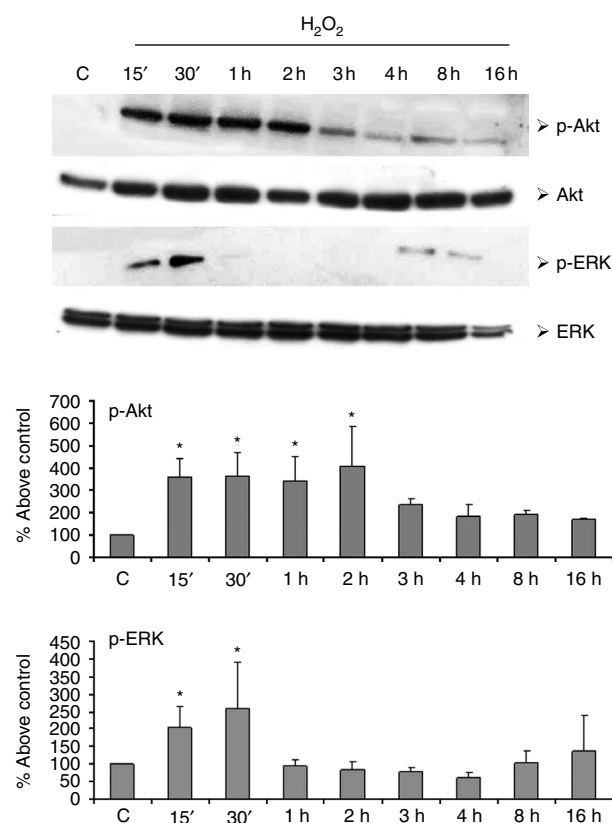
### Flow cytometry

Loss of mitochondrial transmembrane potential was also determined using the JC-1 mitochondrial transmembrane potential ( $\Delta\psi\text{m}$ ) detection kit from Becton Dickinson Biosciences. JC-1 is a cationic fluorescent dye probe (green as monomer) that accumulates in mitochondria in a potential-dependent manner. Cells with functional mitochondria incorporate into JC-1 leading to the formation of JC-1 aggregates, which show a red spectral shift resulting in higher levels of red fluorescence emission measured in the red (FL-2 channel) and green monomers (detectable in FL1 channel). Cells with collapsed mitochondria contain mainly green JC-1 monomers and are detectable in the FL1 channel.

The assays were performed with 70–80% confluent cultures in 10 cm plates. After treatments, cells were trypsinized (2 min), and incubated with JC-1 probe in 5 ml polystyrene round-bottom tubes (Becton Dickinson), according to the manufacturer's instructions, for 15 min at  $37^{\circ}\text{C}$ . Cells were then washed twice and analyzed in a FACSCalibur flow cytometer (excitation wavelength of 488 nm).

### Statistical analysis

Results are shown as means  $\pm$  S.E.M. Statistical differences among groups were determined by ANOVA followed by a multiple comparison *post hoc* test, the Di Rienzo, Guzmán Casanovés (DGC) test (Di Rienzo *et al.* 2002). Data are expressed as significant at  $P < 0.05$ .



**Figure 1** Time course of  $\text{H}_2\text{O}_2$ -induced AKT and ERK activation in muscle cells. C2C12 cells were incubated with 1 mM  $\text{H}_2\text{O}_2$  for the indicated time periods. Cell lysates were prepared and subjected to western blot analysis using anti-phospho-AKT and anti-phospho-ERK antibodies. Treatment of C2C12 cells with  $\text{H}_2\text{O}_2$ -activated AKT and ERKs in a time-dependent manner. At short times of exposure to  $\text{H}_2\text{O}_2$  both AKT and ERK2 MAPK were highly phosphorylated, their maximum activation lasting 30 min for ERK2 and 2 h for AKT. From then on they diminished significantly. No changes in total AKT and ERK were observed. The blots are representative of three independent experiments with comparable results. Densitometric quantification of p-AKT and p-ERK blots is shown. Averages  $\pm$  S.E.M. are given; \* $P < 0.05$  with respect to the control.

## Results

### Activation of AKT and ERK2 MAPK at short times of exposure to H<sub>2</sub>O<sub>2</sub> in C2C12 muscle cells

As a first approach to evaluate the response of C2C12 skeletal muscle cells to the apoptotic agent, we exposed cultured cells to different treatment times with 1 mM H<sub>2</sub>O<sub>2</sub>, ranging from minutes to hours (15 and 30 min, 1, 2, 3, 4, and 16 h), followed by measurement of proteins associated with survival events, by immunoblot analysis. As observed in Fig. 1, using specific antibodies against phospho-AKT, a downstream effector of PI3-K, and phospho-ERK1/2 MAPK, western blots revealed a time-dependent AKT and ERK2 activation (phosphorylation) in response to the apoptotic agent. At short times of exposure to H<sub>2</sub>O<sub>2</sub> both AKT and ERK2 MAPK were highly phosphorylated, their maximum activation lasting for 30 min for ERK2 and 2 h for AKT. From then on p-AKT diminished significantly by twofold and p-ERK by onefold.

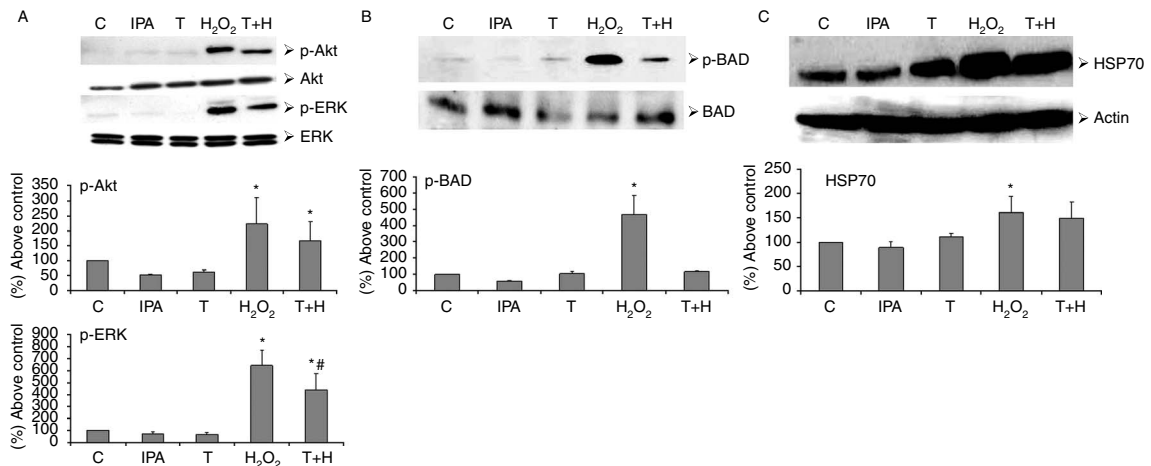
Next, we tested C2C12 cells by simultaneous treatment with H<sub>2</sub>O<sub>2</sub> and testosterone at short times. Cultured cells were preincubated with the hormone (10<sup>-9</sup> M – 45 min) prior to H<sub>2</sub>O<sub>2</sub> (1 mM, 2 h) treatment. Western blot analysis showed again not only AKT and ERK2 activation (Fig. 2A) in the presence of H<sub>2</sub>O<sub>2</sub>, but also the inactivation (phosphorylation) of the apoptotic protein BAD (Fig. 2B), probably due, in part, to AKT activation. So, after short time treatments with H<sub>2</sub>O<sub>2</sub>, cells exert a defense response against the apoptotic agent. The fact that testosterone decreases the BAD phosphorylation/inactivation induced by short-term stimulation with H<sub>2</sub>O<sub>2</sub> is probably due to its protective action against apoptosis. When

cells are pretreated with testosterone prior to the exposure to the apoptotic agent, the conditions are less adverse than the cultures exposed to H<sub>2</sub>O<sub>2</sub> alone. For this reason, it is not essential for cells to activate a defense mechanism, because of the protecting effect of testosterone. Thus, the activation of AKT is not necessary and the levels of p-AKT decrease. As a consequence, lower levels of p-BAD are observed in simultaneous treatments with testosterone and H<sub>2</sub>O<sub>2</sub>. Moreover, Western blots showed that H<sub>2</sub>O<sub>2</sub> induces an increase in the expression of HSP70, a protein associated with survival events (Fig. 2C). Of relevance, no changes in the levels of total ERK and AKT proteins were observed (Fig. 2A).

These data suggest that short-term stimulation with H<sub>2</sub>O<sub>2</sub> activates signaling pathways that are thought to be a protective response against oxidative stress, in order to avoid apoptosis. Moreover, myogenin expression levels were analyzed to exclude any effect of 1 mM H<sub>2</sub>O<sub>2</sub> on myogenic differentiation. No changes in the levels of this protein during the different treatments were observed (data not shown), indicating that under this condition, H<sub>2</sub>O<sub>2</sub> does not promote C2C12 differentiation.

### Protective effect of testosterone in apoptosis induced by long-term stimulation with H<sub>2</sub>O<sub>2</sub> of C2C12 muscle cells

In order to characterize the apoptotic response pattern of muscle cells when simultaneously treated with testosterone and H<sub>2</sub>O<sub>2</sub> for longer periods of time, C2C12 cell cultures were incubated with the steroid hormone (10<sup>-9</sup> M) for 45 min prior to the long-term stimulation (4 h) with 1 mM H<sub>2</sub>O<sub>2</sub>. Moreover, we checked the effect of an antagonist of the



**Figure 2** Short-term effects of simultaneous treatments with H<sub>2</sub>O<sub>2</sub> and testosterone in C2C12 muscle cells. Activation of a defense mechanism. C2C12 cells were not treated (C) or treated with IPA (0.001%) for 45 min, 10<sup>-9</sup> M testosterone for 45 min (T), 1 mM H<sub>2</sub>O<sub>2</sub> for 2 h (H<sub>2</sub>O<sub>2</sub>) or pretreated with 10<sup>-9</sup> M testosterone for 45 min prior to the exposure of 1 mM (H<sub>2</sub>O<sub>2</sub>) for 2 h (T+H). Western blot analyses were performed using (A) anti-phospho-ERK1/2 and anti-phospho-AKT, (B) anti-phospho-BAD, and (C) anti-HSP70 antibodies. Actin levels are shown as loading control. Short-term stimulation with H<sub>2</sub>O<sub>2</sub> phosphorylates ERK1/2, AKT, and BAD that are thought to be involved in a protective response against oxidative stress, in order to avoid apoptosis. No changes in total ERK and AKT proteins levels were observed. The blots are representative of three independent experiments with comparable results. Densitometric quantification of p-AKT, p-ERK, p-BAD, and HSP70 blots are shown. Averages ± s.e.m. are given; \**P* < 0.05 with respect to the control or #*P* < 0.05 with respect to H<sub>2</sub>O<sub>2</sub>.

androgen receptor (AR), flutamide ( $10^{-8}$  M), in our experimental system. Thus, cells were pretreated with testosterone ( $10^{-9}$  M) plus flutamide ( $10^{-8}$  M) for 45 min, followed by incubation with  $H_2O_2$  (1 mM for 4 h) to induce apoptosis.

As seen in Fig. 1, at longer treatment times with the apoptotic agent a decrease in both AKT and ERK2 MAPK phosphorylation/activation occurs. In agreement with the results observed before, and probably as a consequence of AKT inactivation, BAD dephosphorylation is also observed here, at long-term stimulation with  $H_2O_2$  (Fig. 3A). No changes in total AKT and total ERK proteins were observed (Fig. 1).

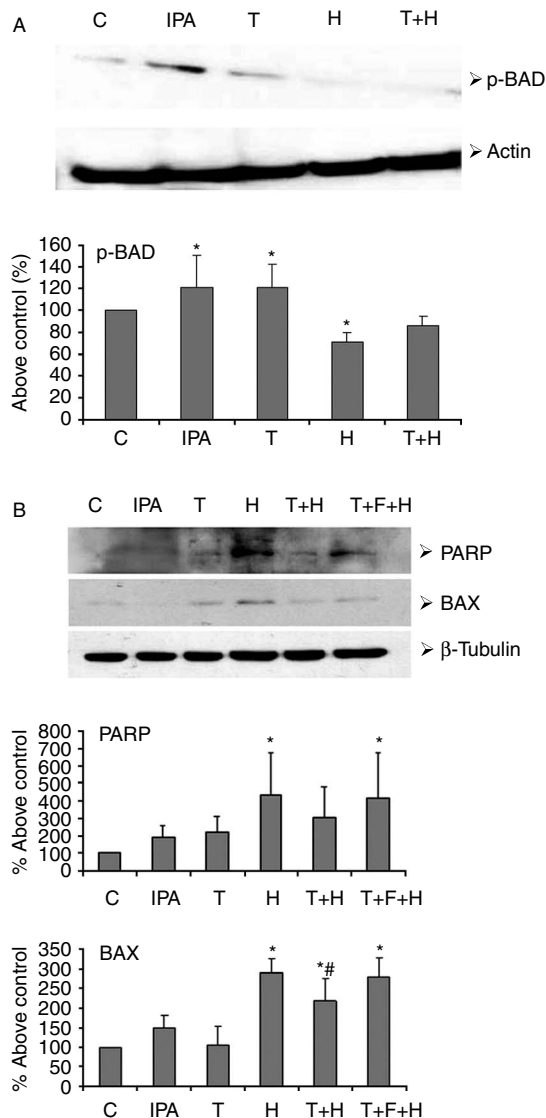
Besides, in Fig. 3B it is also observed that  $H_2O_2$  at longer treatment times induced the cleavage of PARP fourfold with respect to the controls, which is a common event during programmed cell death induced by a variety of apoptotic stimuli. However, incubation with testosterone ( $10^{-9}$  M) for 45 min prior to the apoptotic stimulus diminished onefold the cleavage of PARP induced by  $H_2O_2$ . In addition, the inhibitory effect of testosterone on  $H_2O_2$ -promoted apoptotic cleavage of PARP was almost totally abolished by flutamide. Congruent results were obtained when changes in the expression of the proapoptotic protein BAX were measured. From a western blot analysis it was observed that testosterone appears to have decreased BAX expression, an effect that was abolished by the addition of flutamide, which increased the levels of BAX again (Fig. 3B).

Additionally, in order to quantify apoptotic cells after long-term stimulation with  $H_2O_2$  and to evaluate the protective effect of testosterone, morphological changes of nucleus, characteristic of apoptosis, were studied. Immunocytochemistry assays and immunofluorescence conventional microscopy using DAPI dye were performed after treatments. Images of Fig. 4 show intact/normal nuclei of muscle cells in the control condition (IPA) and treated with  $10^{-9}$  M testosterone. C2C12 cell cultures exposed to 1 mM  $H_2O_2$  for 4 h exhibited morphological changes typical of apoptosis such as nuclear fragmentation or condensation (pyknotic nuclei) which represented  $\sim 75\%$  of the cultured muscle cells (indicated with white arrows). These effects were markedly reduced in cells incubated with the hormone during 45 min prior to the exposure to  $H_2O_2$  (from 75% when the cells were treated with  $H_2O_2$  alone to  $\sim 25\%$  when cultures were preincubated with testosterone before addition of  $H_2O_2$ ).

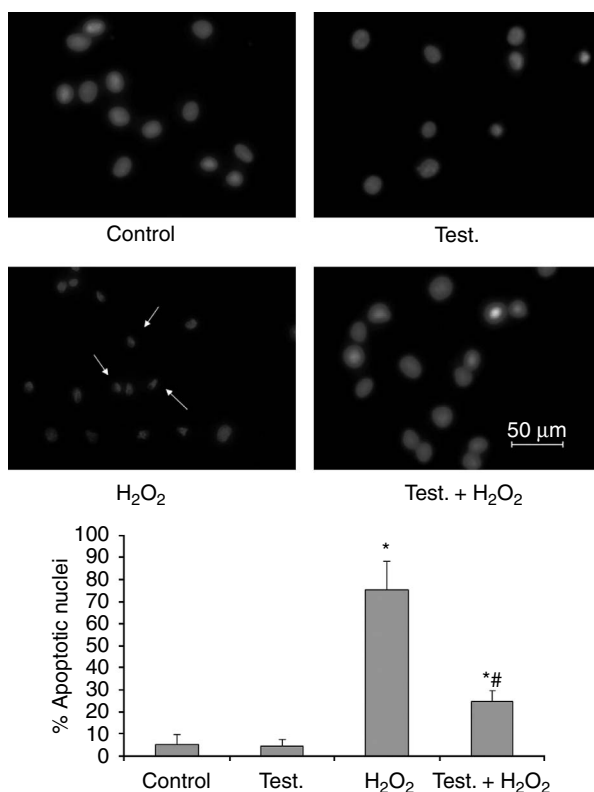
These data revealed that at longer incubation times with  $H_2O_2$ , the muscle cells finally initiate the apoptotic program, effect that is reversed by testosterone. The fact that simultaneous treatments with testosterone,  $H_2O_2$ , and flutamide reduce the action of the hormone on BAX and PARP proteins points to a possible participation of the AR in its antiapoptotic effect.

#### Effect of testosterone on muscle cell BAX levels

With the purpose of evaluating the direct effect of testosterone on BAX expression, C2C12 cell cultures were



**Figure 3** Protective effect of testosterone in apoptosis induced by long-term stimulation with  $H_2O_2$  (H) in C2C12 muscle cells. C2C12 cells were treated with the indicated stimuli, (C) untreated cells, (IPA) cells treated with IPA (0.001%) for 45 min, (T) cells incubated with  $10^{-9}$  M testosterone for 45 min, ( $H_2O_2$ ) cells treated with 1 mM  $H_2O_2$  for 4 h, (T+H) cells pretreated with  $10^{-9}$  M testosterone for 45 min prior to the exposure to 1 mM  $H_2O_2$  for 4 h, (T+F+H) cells pretreated with  $10^{-8}$  M flutamide and  $10^{-9}$  M testosterone for 45 min prior to the exposure to 1 mM  $H_2O_2$  for 4 h. Western blot analyses were performed using (A) anti-phospho-BAD, (B) anti-PARP, and anti-BAX antibodies. Actin and  $\beta$ -tubulin levels are shown as loading control. The data revealed that at longer incubation times with  $H_2O_2$ , the muscle cells finally initiate the apoptotic program showing BAD dephosphorylation, PARP cleavage, and increased BAX levels, an effect that is reversed by testosterone. The blots are representative of three independent experiments with comparable results. Densitometric quantification of p-BAD, BAX, and the cleavage of PARP blots is shown. Averages  $\pm$  s.e.m. are given; \* $P < 0.05$  with respect to the control or # $P < 0.05$  with respect to  $H_2O_2$ .



**Figure 4** Inhibition of H<sub>2</sub>O<sub>2</sub>-induced apoptosis by testosterone (Test.) in C2C12 muscle cells. Cultured C2C12 cells were treated with 1 mM H<sub>2</sub>O<sub>2</sub> (H<sub>2</sub>O<sub>2</sub>) for 4 h or preincubated with 10<sup>-9</sup> M testosterone before addition of H<sub>2</sub>O<sub>2</sub> (Test. + H<sub>2</sub>O<sub>2</sub>), and with testosterone 10<sup>-9</sup> M alone (testosterone) or vehicle (IPA 0.001%) (C) for 45 min, as controls. The cells were then stained with DAPI as described in Materials and Methods. Morphological analysis of fluorescence-stained nuclei and percentages of apoptotic cells at each condition are shown. At least ten fields per dish were examined. Each value represents the mean of three independent experiments. Averages ± S.E.M. are given; \**P* < 0.05 with respect to the control or #*P* < 0.05 with respect to H<sub>2</sub>O<sub>2</sub>. Representative photographs of apoptotic and normal cells are shown. Arrows indicate apoptotic nuclei. Original magnification 400×.

incubated with different concentrations of the steroid hormone (10<sup>-6</sup>, 10<sup>-7</sup>, 10<sup>-8</sup>, 10<sup>-9</sup>, 10<sup>-10</sup>, and 10<sup>-11</sup> M) or with the vehicle alone as controls for 10<sup>-6</sup> and 10<sup>-9</sup> M of testosterone. From a western blot analysis and using the specific antibody against the proapoptotic protein BAX, we observed that testosterone was able to decrease the expression of BAX by more than threefold (Fig. 5). We noticed that testosterone does not alter the levels of 14-3-3 protein, total ERK, and AKT (data not shown).

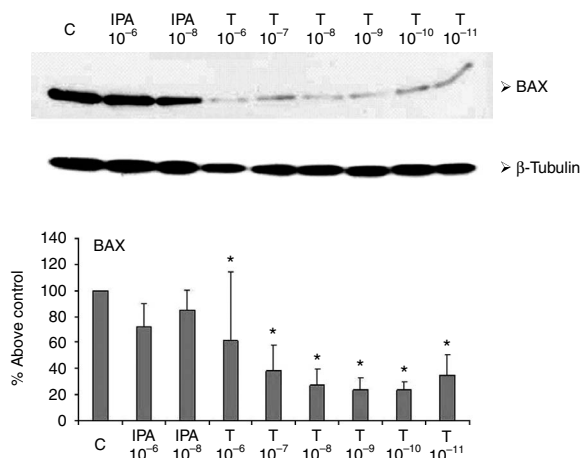
#### Maintenance of the mitochondrial membrane potential by testosterone in muscle cells treated with the apoptotic agent H<sub>2</sub>O<sub>2</sub>

Loss of mitochondrial membrane potential provides an early indication of the initiation of cellular apoptosis, and is a marker for commitment to programmed cell death

(Penninger & Kroemer 2003). To determine the level of mitochondrial dysfunction and the effects of testosterone during oxidative stress, we measured the release of cytochrome *c* (Fig. 6) due to loss of the outer mitochondrial membrane integrity induced by H<sub>2</sub>O<sub>2</sub> by means of CYTOC-OX1 assays (see Materials and Methods). We observed that ~44% of the cells presented mitochondrial damage after H<sub>2</sub>O<sub>2</sub> treatment, whereas in cultures preincubated with testosterone before addition of H<sub>2</sub>O<sub>2</sub> only ~29% of the mitochondria were affected. To further support these results, we also used a mitochondrial membrane sensor kit to perform a JC-1 assay. JC-1 cation fluoresces differently in apoptotic and healthy cells. In healthy cells, it aggregates into mitochondria and exhibits red and green fluorescence. In apoptotic cells, it fails to accumulate in mitochondrial matrix because of altered membrane potential; instead it is found in cytosol as monomers and exhibits fluorescence in the green end of the spectrum (Reers *et al.* 1995). Using a JC-1 mitochondrial membrane potential detection kit, mitochondrial membrane potential was measured in C2C12 cells after treatment with 1 mM H<sub>2</sub>O<sub>2</sub> for 4 h, testosterone (10<sup>-9</sup> M), and flutamide (10<sup>-8</sup> M). We found that H<sub>2</sub>O<sub>2</sub> strongly induced loss of mitochondrial membrane potential. The percentage of cells with loss of mitochondrial membrane potential increased from 8% in control cultures (treated with testosterone vehicle or with hormone alone) to 66% in cultures treated with H<sub>2</sub>O<sub>2</sub>, while the incubation with testosterone prior to H<sub>2</sub>O<sub>2</sub> exposure was capable of reducing the percentage of depolarized cells to 25%. Otherwise, prior addition of flutamide to this treatment almost totally blocked the protective effect of the hormone on H<sub>2</sub>O<sub>2</sub>-induced loss of mitochondrial membrane potential, increasing the percentage of depolarized cells to 53% (Fig. 7). These findings further support previous results of our laboratory (Pronsato *et al.* 2010) suggesting an antiapoptotic action of testosterone against H<sub>2</sub>O<sub>2</sub> by preventing the loss of mitochondria integrity.

## Discussion

Evidence showing that testosterone can sustain survival or alternatively induce apoptosis of cells depending on their biological context has been reported (Morimoto *et al.* 2005, Nguyen *et al.* 2005, Estrada *et al.* 2006, Jin *et al.* 2006). In earlier experiments, using the well-characterized myogenic C2C12 murine cell line, we obtained data implying that testosterone at physiological concentrations, abrogates H<sub>2</sub>O<sub>2</sub>-induced apoptosis of skeletal muscle cells (Pronsato *et al.* 2010). The results from this work indicate that the apoptotic agent H<sub>2</sub>O<sub>2</sub> induces apoptosis in our experimental system in a time-dependent manner. At short times of exposure, H<sub>2</sub>O<sub>2</sub> triggers the activation of a defense response, whereas at long treatment times, the programmed cell death finally starts, and it is at this time that testosterone carries out its protective action against apoptosis.



**Figure 5** Testosterone reduces the expression of BAX in muscle cells in a concentration-dependent manner. C2C12 cells were treated with the indicated concentrations of testosterone or IPA for 45 min. Western blot analyses were performed using anti-BAX antibody.  $\beta$ -Tubulin level is shown as loading control. Testosterone was able to decrease BAX expression. The blots are representative of three independent experiments with comparable results. Densitometric quantification of the blot is shown. Averages  $\pm$  S.E.M. are given; \* $P < 0.05$  with respect to the control.

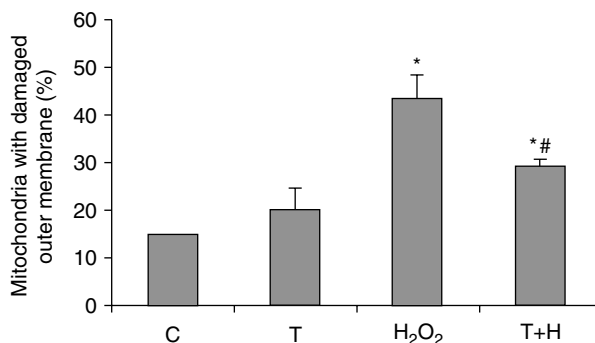
$H_2O_2$  has been shown to act as a signaling molecule involved in many cellular functions such as apoptosis (Singh & Singh 2008, Vasconsuelo *et al.* 2008), differentiation (Steinbeck *et al.* 1998, Orzechowski *et al.* 2002), and proliferation (Sigaud *et al.* 2005) in a dose- and time-dependent manner. In contrast with the wide belief that  $H_2O_2$  has only harmful functions due to its relentless production with a damaging nature, it has been shown that  $H_2O_2$  stimulates cell growth/proliferation and DNA synthesis in different types of cells, indicating that  $H_2O_2$  acts as an important signaling molecule (Rao & Berk 1992, Fiorani *et al.* 1995). Exposure of cells to exogenous  $H_2O_2$  has been used to investigate multiple signaling pathways related to smooth muscle contraction (Jin & Rhoades 1997) and proliferation (Baas & Berk 1995) as well as apoptosis (Li *et al.* 1997).

In this study, we demonstrated that short-term treatment of C2C12 cells with 1 mM  $H_2O_2$  causes the phosphorylation of ERK2. Moreover, we observed the activation/phosphorylation of AKT and, as a consequence, the inactivation/phosphorylation of the proapoptotic protein BAD. 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). Phosphorylated BAD is devoid of its apoptotic activity, as it is sequestered away from the site of action in the mitochondria by binding to cytosolic 14-3-3 proteins (Datta *et al.* 1997, Del Peso *et al.* 1997, Yano *et al.* 1998). BAD can be phosphorylated by AKT (Datta *et al.* 1997), and since short treatment times with  $H_2O_2$  activated AKT in C2C12 cells, the ability to induce this

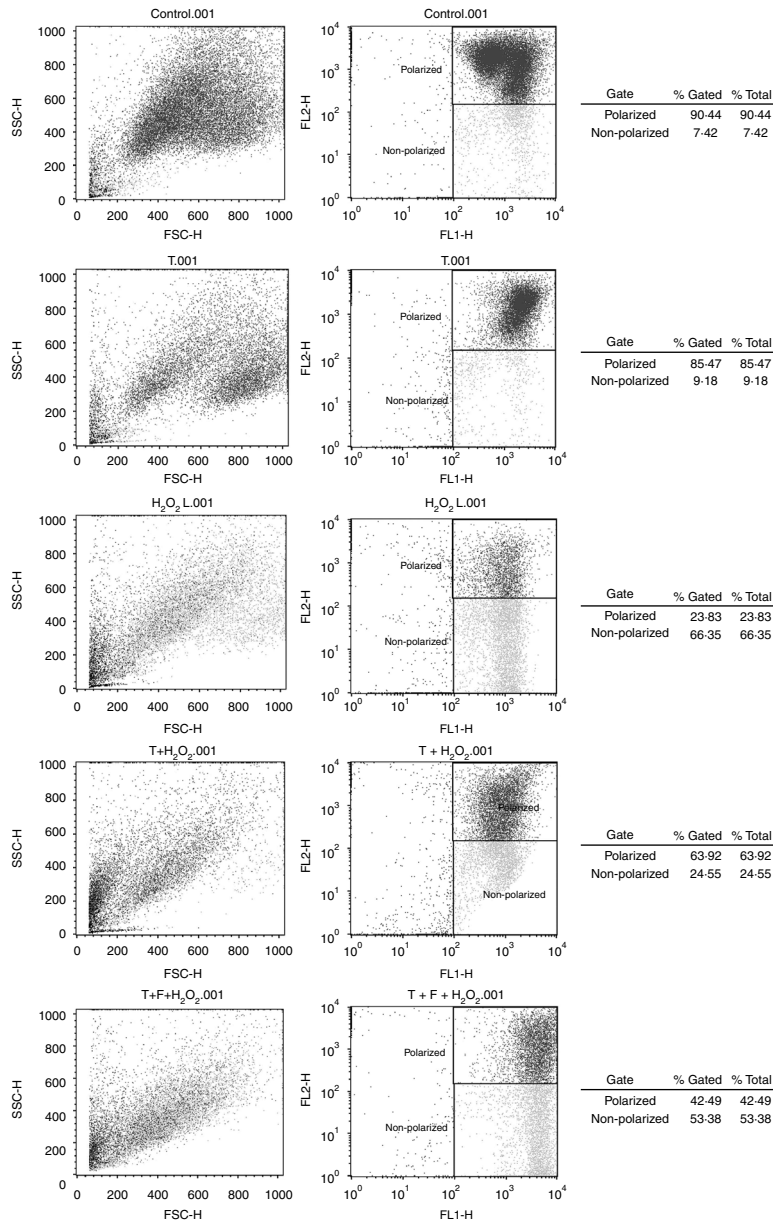
phosphorylation was investigated using a phosphospecific antibody. Thus, our data indicate that short-term stimulation with  $H_2O_2$  activates the signaling pathways of cell mitogenic responses to promote cell survival as has been reported by others (Crossthwaite *et al.* 2002).

However, long-term exposure to  $H_2O_2$  decreases both AKT and ERK2 phosphorylation, and at the same time it activates BAD by dephosphorylation. Convincing evidence has been presented to show the opposing effects of  $H_2O_2$  on cell growth by stimulating proliferation (Guyton *et al.* 1996, Goldkorn *et al.* 1998) and triggering apoptosis (Li *et al.* 1997). These dual effects might depend on the time of  $H_2O_2$  treatment, the concentration of  $H_2O_2$ , and the cell type. Cell viability in the C2C12 cell line treated with 1 mM  $H_2O_2$  showed no difference from controls up to a 2-h time point (data not shown), whereas treatment of C2C12 cells with 1 mM  $H_2O_2$  for 4 h finally induces a significant decrease in the cell viability. Thus, it is possible that induction of PI3K/AKT and ERK signaling during short time of exposure to  $H_2O_2$  treatment may be critical in the regulation of cellular protection in the early stage of cell response to oxidative stress.

However, if C2C12 cells are exposed to longer treatment times with 1 mM  $H_2O_2$ , they are finally led to apoptosis. This state was first evidenced by the selective cleavage of PARP by caspases (Lazebnik *et al.* 1994), which is common and one of the latest events observed during programmed cell death induced by a variety of apoptotic stimuli. Here, we showed using a western blot analysis that the exposure of C2C12 cells to 1 mM  $H_2O_2$  for 4 h induces PARP cleavage, and that testosterone inhibits  $H_2O_2$ -promoted apoptotic cleavage of PARP. This effect was almost totally abolished by flutamide, an antagonist of the AR, suggesting that the antiapoptotic action of testosterone is probably mediated by the AR. Similar



**Figure 6** Inhibition of  $H_2O_2$ -induced cytochrome *c* release by testosterone. C2C12 muscle cells were exposed to different treatments and then cytochrome *c* release was measured as described in Materials and Methods. (C) Cells treated with the hormone vehicle, IPA, for 45 min, (T) cells incubated with  $10^{-9}$  M testosterone for 45 min, ( $H_2O_2$ ) cells treated with 1 mM  $H_2O_2$  for 4 h, (T+H) cells pretreated with  $10^{-9}$  M testosterone for 45 min prior to the exposure to 1 mM  $H_2O_2$  for 4 h. Each value represents the mean of three independent determinations. Averages  $\pm$  S.E.M. are given; \* $P < 0.05$  with respect to the control or # $P < 0.05$  with respect to  $H_2O_2$ .



**Figure 7** Protective effect of testosterone on  $\text{H}_2\text{O}_2$ -induced loss of mitochondrial membrane potential in C2C12 cells. (C) Untreated cells, (T) cells incubated with  $10^{-9}$  M testosterone for 45 min, ( $\text{H}_2\text{O}_2$ ) cells treated with 1 mM  $\text{H}_2\text{O}_2$  for 4 h, (T + H) cells pretreated with  $10^{-9}$  M testosterone for 45 min prior to the exposure to 1 mM  $\text{H}_2\text{O}_2$  for 4 h, (T + F + H) cells pretreated with  $10^{-8}$  M flutamide and  $10^{-9}$  M testosterone for 45 min prior to the exposure to 1 mM  $\text{H}_2\text{O}_2$  for 4 h. Then cells were stained with JC-1 as described in Materials and Methods, and the mitochondrial membrane potential was analyzed by FACSCalibur flow cytometer. JC-1 aggregates emit simultaneously fluorescence in red (FL-2 channel) and green (FL-1 channel). This is visualized as a population that appears in the upper right quadrant of the graph. After loss of  $\Delta\psi$  (apoptotic cells), JC-1 does not accumulate in mitochondria and remains in the cytoplasm as monomers. These monomers do not have the red spectral shift and consequently have lowered fluorescence in the FL-2 channel, but fluorescence in FL-1 channel remains invariable. This is visualized as a population in the lower right quadrant. FL-1: FL-1 channel that detects green fluorescence, FL-2: FL-2 channel that detects red fluorescence. Experiments were repeated at least three times with essentially identical results.



results were obtained when we evaluated BAX. BAX is a member of the expanding Bcl-2 family that plays a key role in the regulation of apoptosis. BAX is localized mostly in the cytoplasm, but redistributes to mitochondria in response to stress stimuli (Hsu *et al.* 1997, Wolter *et al.* 1997). After translocation to mitochondria, BAX induces cytochrome *c* release either by forming a pore by oligomerization in the outer mitochondrial membrane or by opening other channels (Shimizu *et al.* 1999, Saito *et al.* 2000, Kuwana *et al.* 2002). This proapoptotic protein appears to decrease its expression after testosterone treatment, an effect that was almost totally blocked in the presence of flutamide. Besides, we demonstrated that this effect of the steroid hormone on BAX expression was dose dependent between  $10^{-6}$  and  $10^{-10}$  M, with a maximal effect at  $10^{-9}$  M. These results are consistent with our recent reports (Pronsato *et al.* 2010) and greatly support the notion that testosterone, at physiological concentrations, inhibits apoptosis of skeletal muscle 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). Through loss of their membrane potential, mitochondria have a prominent role in the apoptotic process, signaled by increased membrane permeability and release of proapoptotic factors such as cytochrome *c*, from their intermembrane space into the cytoplasmic compartment. This triggers activation of downstream events that lead to apoptosis (Zimmermann *et al.* 2001). Mitochondrial membrane potential is generated through the electrochemical gradient of membrane, which is created during the processes of mitochondrial electron transport chain. A collapse in the mitochondrial membrane potential is one of the early events of programmed cell death. For this reason, we evaluated the status of mitochondrial membrane potential in C2C12 cells and checked if testosterone was exerting its antiapoptotic effect by the prevention of mitochondrial integrity. Following collapse of the mitochondrial membrane potential and failure of mitochondrial function, the proapoptotic factor, cytochrome *c*, is leaked from mitochondria, and the population of apoptosis increases significantly after H<sub>2</sub>O<sub>2</sub>-induced oxidative stress. 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<sub>2</sub>O<sub>2</sub>-induced cytochrome *c* release by testosterone observed in this study and the effects of hormone on the size and cytosolic distribution of mitochondria previously reported (Pronsato

*et al.* 2010) suggests a protective effect of the steroid hormone on this organelle. Moreover, we tested the  $\Delta\psi_m$  in C2C12 treated cells by using the fluorescent dye, JC-1, that aggregates into healthy mitochondria and fluoresces red and green. Upon mitochondrial collapse in apoptotic cells, JC-1 dye no longer accumulates but instead it is distributed throughout the cell resulting in decrease in red fluorescence. In accordance with this, we found that long-term H<sub>2</sub>O<sub>2</sub> treatment indeed disrupted the mitochondrial membrane potential as observed by a decrease in the intensity of red fluorescence, and testosterone was able to strongly block the effect of the apoptotic agent. However, when C2C12 cells were simultaneously treated with testosterone, flutamide, and H<sub>2</sub>O<sub>2</sub>, mitochondrial membrane potential decreased again. These results further suggest a protective effect of testosterone on this organelle and a possible participation of the AR in this event, at this level.

To summarize, the results of this work indicate that C2C12 cells exhibit a defense response to avoid apoptosis, showing ERK2, AKT, and BAD phosphorylation and an increase in HSP70 levels when exposed to short treatment times with H<sub>2</sub>O<sub>2</sub>. However, long-term stimulation with the apoptotic agent induces a decrease in the phosphorylation of the proteins mentioned before, PARP cleavage, the loss of the mitochondrial membrane potential and the release of cytochrome *c*, finally leading to cell apoptosis. At this time testosterone exerts its protective action against apoptosis. When cells are treated with testosterone prior to H<sub>2</sub>O<sub>2</sub>, BAD inactivation (phosphorylation), inhibition of PARP cleavage, decrease in BAX levels, and prevention of the loss of mitochondrial membrane potential are observed, revealing that the steroid hormone is probably regulating the apoptotic intrinsic pathway. The fact that simultaneous treatments with testosterone, H<sub>2</sub>O<sub>2</sub>, and flutamide reduce the protective effects of the hormone described before indicates a possible participation of the AR in the antiapoptotic effect of testosterone. The data presented in this work unravel in part the antiapoptotic molecular mechanism activated by testosterone, underlying the survival action of the hormone against the oxidative stress damage caused by H<sub>2</sub>O<sub>2</sub> in the C2C12 skeletal muscle cell line. Clearly, additional studies are thus necessary to further elucidate the signaling mechanisms which mediate the antiapoptotic action of testosterone in skeletal muscle cells and its relationship with myopathies associated with hormonal dysregulation.

#### Declaration of interest

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

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