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17β-Estradiol Protects Skeletal Myoblasts From Apoptosis Through p53, Bcl-2, and FoxO Families

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ABSTRACT

17β-Estradiol (E_2) protects several nonreproductive tissues from apoptosis, including skeletal muscle. Previously, we showed that E_2 at physiological concentrations prevented apoptosis induced by H_2O_2 in skeletal myoblasts, reverting PKCδ, JNK, and p66Shc activation and exerting a beneficial action over mitochondria. Since genomic actions underlying the regulation of nuclear gene transcription are a common property of this steroid, the present work characterizes the transcriptional activity modulated by E_2 to exert its antiapoptotic effect. We report that E_2 protects skeletal myoblasts against apoptosis induced by H_2O_2 modulating p53 and FoxO transcription factors and then their target genes Bcl-2, Bim, Puma, PERP, and MDM2, without affecting Noxa gene. The results presented in this work support the notion that the transcription factors FoxO and p53 coordinate apoptosis in C2C12 cells, and deepens our knowledge about a putative molecular mechanism by which E_2 exerts beneficial effects against oxidative stress in skeletal myoblasts. J. Cell. Biochem. 9999: 1–12, 2016. © 2016 Wiley Periodicals, Inc.

KEY WORDS: 17β-ESTRADIOL; C2C12 MUSCLE CELLS; p53; Bcl-2; FoxOs

The steroid hormone 17β-estradiol (E_2) plays an important role in development, cell growth, and differentiation. It acts through genomic mechanisms underlying the regulation of nuclear gene transcription but also nongenomic actions are a common property of this steroid. Although E_2 has been shown to serve as a protective agent in several tissues and organs not involved in reproduction [Wang et al., 2006], the mechanisms underlying this beneficial role have not been fully elucidated. There is evidence demonstrating that skeletal muscle is a target tissue for E_2 [Milanesi et al., 2008; Vasconsuelo et al., 2008]. In agreement with these observations, the decline of estrogens that occurs during aging has been closely related to muscle pathologies, such as sarcopenia which is characterized by loss of skeletal muscle mass and strength (reviewed in Morley and Malmstrom [2013]). Since differentiated adult skeletal muscle fibers have scarce ability to repair and regenerate themselves in response to a cellular injury, satellite cells that surround mature myofibers have the capacity to proliferate and differentiate to repair this injured tissue. Of significance for our work, enhanced satellite cell apoptosis has been related to compromised recovery potential in skeletal muscle of aged animals [Jejurikar et al., 2006].

One of the principal functions of the transcription factor p53 is the control of apoptosis. In response to numerous stress signals, p53 accumulates in the nucleus to exert its proapoptotic activity [Vousden and Lu, 2002]. Its phosphorylation at various sites, including Ser^{15}, Ser^{20}, and Ser^{46}, enhances its transcriptional as well as proapoptotic capacity. Therefore, the activation of p53 can upregulate or downregulate several target genes, whose promoters have consensus p53 response elements, including Bcl-2 (B-cell lymphoma 2) family members, other transcription factors, and various molecules which, then, can initiate a series of events resulting in apoptosis [Prives and Hall, 1999]. Certainly, a significant link between p53-mediated activation and apoptosis comes from its capacity to control the transcription of proapoptotic members of the Bcl-2 family, which includes the multidomain member Bax [Miyashita et al., 1994] and the BH3-only members Bid, Noxa, and Puma [Oda et al., 2000; Nakano and Vousden, 2001; Sax et al., 2002]. Conversely, the promoter of the antiapoptotic member Bcl-2 contains a p53-negative response element, raising the possibility that Bcl-2 may be a direct target of p53-mediated repression [Miyashita et al., 1994]. Additionally, p53 transcriptionally activates other genes that have been linked to apoptosis, such as PERP (p53 apoptosis effector related to PMP-22) [Attardi et al., 2000]. In spite of these transcriptional actions, some studies suggested that p53 may promote transcription-independent apoptosis [Caelles et al., 1994]. However, it has not been completely elucidated how these members

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act downstream p53 to modulate apoptosis and contribute to a net effect that favors cell death in skeletal muscle cells.

Under normal conditions, p53 is a short-lived protein whose expression levels are kept extremely low. Certainly, cellular p53 levels are mainly determined by the rate of degradation, mostly associated with the E3 ubiquitin protein ligase MDM2 (murine double minute-2) [Harris and Levine, 2005]. In response to stress signals, the increase of p53 stability is related to decreased MDM2 protein levels and reduced p53-MDM2 interaction, while the ensuing elevated levels of transcriptionally active p53 result in increased MDM2 transcription [Piette et al., 1997]. Interestingly, it has been identified a mechanism that involves the phosphorylation of p53 in serine residues, such as Ser^{15}, which interferes with the interaction between p53 and its negative regulator MDM2 [Ryan et al., 2001].

During the last years, it has been given significance to the family of Forkhead Box O (FoxO) transcription factors. Of importance, there have been shown connections between p53 and members of FoxO family [Renault et al., 2011]. FoxOs have been involved in multiple physiological and pathological processes, including apoptosis, aging, proliferation, and metabolism [Storz, 2011]. They modulate the expression of different target genes depending on the cell type and cellular context. In addition, subcellular localization of these factors plays an important role in the regulation of their functions, which are closely controlled by multiple signaling pathways, including PI3 K/Akt, ERK, and JNK. One of the members of this family, FoxO3a, is a key downstream effector of Akt. It has been evidenced that phosphorylation of FoxO3a at three conserved serine/threonine residues (Thr^{32}, Ser^{253}, and Ser^{315}) by Akt abrogates its access to DNA binding sites. On the contrary, in its nonphosphorylated form, FoxO3a can promote cell death by inducing the expression of various proapoptotic factors. With respect to ERK signaling, it has been shown to downregulate FoxO3a by directly phosphorylating it at three serine residues (Ser^{294}, Ser^{344}, and Ser^{425}), and to induce its degradation via MDM2, which results in an antiapoptotic effect [Yang et al., 2008]. In contrast with the inhibitory activities of Akt and ERK, JNK phosphorylation induces FoxO3a nuclear localization and an increment in its transcriptional activity. With regard to FoxO4, other member of this family, it has been evidenced that its phosphorylation mediated by JNK at Thr^{447} and Thr^{451} induces FoxO4 activation, retaining it in the nucleus [Essers et al., 2004]. However, although there is much research on the role of FoxOs in the homeostasis of various experimental models, it is still unclear whether estradiol prevents apoptosis in skeletal muscle cells involving these transcription factors.

In this work, we postulate that E_2 protects skeletal muscle cells against apoptosis induced by H_2O_2 modulating p53 and FoxO transcription factors and, in consequence, their target genes Bcl-2, Bim, Puma, Noxa, PERP, and MDM2. These studies are of relevance to skeletal muscle physiology as the cell line C2C12 is an appropriate experimental model of satellite cells. C2C12 myoblasts resemble the satellite cells that surround mature myofibers. Satellite cells and their response to oxidative stress are important to mature skeletal muscle performance and function. As a consequence, the knowledge of the molecular mechanism underlying the antiapoptotic action of E_2 in these cells is relevant to understand the hormone protective effects and could help to further characterize the causes of satellite cells loss in view of a future therapeutic impact.

MATERIALS AND METHODS

MATERIALS

SP600125, anti-p-p53 Ser^{15}, anti-p-FoxO3a Ser^{253}, anti-FoxO3a, anti-FoxO4, anti-p-Akt Ser^{473}, and anti-Akt antibodies were from Cell Signaling Technology, Inc. (Danvers, MA). Anti-p-FoxO4 $Thr^{447/451}$ antibody was from Abcam (Cambridge, MA). Anti-β-tubulin antibody was obtained from Thermo Fisher Scientific, Inc. (Rockford, IL). Anti-lamin B and HRP-conjugated anti-rabbit secondary antibodies were from Santa Cruz Biotechnology, Inc. (CA). Alexa 488-conjugated rabbit secondary antibody was from Molecular Probes (Eugene, OR). E_2 and PD98059 inhibitor were from Sigma–Aldrich (St. Louis, MO). LY294002 was purchased from Calbiochem (San Diego, CA). The ECL blot detection kit was provided by Perkin-Elmer, Inc. (Waltham, MA). The protein molecular weight marker was from Amersham (Buckinghamshire, England). All the other reagents used were of analytical grade. The High Pure RNA Isolation kit was from Roche Diagnostics (Mannheim, Germany). The High Capacity cDNA Reverse Transcription Kit and the KAPA SYBR® FAST qPCR Kit Master Mix Universal were from Applied Biosystems, Inc. (CA).

METHODS

Cell culture and treatments. C2C12 murine skeletal myoblasts obtained from American Type Culture Collection (Manassas, VA) were cultured in growth medium Dulbecco's modified Eagle's medium (DMEM) without Phenol Red supplemented with 10% heat-inactivated (30 min, 56°C) fetal bovine serum, 1% nistatine, and 2% streptomycin. These highly myogenic cells have been widely used to study muscle functions [Burattini et al., 2004]. Cells were incubated at 37°C in a humid atmosphere of 5% CO_2 in air. The treatments were performed with 70–80% confluent cultures (120,000 cells/cm^2) in medium without serum for 20 min. Then, 10^{-8} M E_2, vehicle 0.001% isopropanol (control), or the corresponding inhibitors (LY294002, SP600125, PD98059) were added 1 h before induction of apoptosis with hydrogen peroxide (H_2O_2). H_2O_2 was diluted in culture medium without serum to 0.5 mM in each assay. Different times of treatment with H_2O_2 were used (30 min, 1 h, 3 h, and 4 h) to evaluate the progression of apoptosis, previously characterized as early/initial (30 min, 1 h) or late/advanced apoptosis (3 and 4 h) [Samejima and Earnshaw, 2005; Pronsato et al., 2012]. After treatments, cells were lysed using a buffer composed of 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.2 mM Na_2VO_4, 2 mM EDTA, 25 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% NP40, 20 mg/ml leupeptin, and 20 mg/ml aprotinin. Protein concentration was estimated by the method of Bradford [1976].

Subcellular fractionation. C2C12 cells were scrapped and homogenized in ice-cold Tris–EDTA-sucrose buffer (50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 250 mM sucrose, 1 mM DTT, 0.5 mM PMSF, 20 mg/ml leupeptin, 20 mg/ml aprotinin, and 20 mg/ml trypsin inhibitor) using a Teflon-glass hand homogenizer. Total homogenate was centrifugated at 300*g* for 5 min at 4°C to clear of unbroken

cells, partially disrupted cells and other debris. Then, this supernatant free of debris was used in order to isolate the different fractions. Nuclear pellet was obtained by centrifugation at 800*g* for 15 min at 4°C. The supernatant was further centrifuged at 10,000*g* for 30 min at 4°C to yield the mitochondrial pellet. The remaining supernatant was further centrifuged at 40,000*g* for 1 h at 4°C to obtain the cytosolic fraction. Pellets were resuspended in lysis buffer (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, 20 mg/ml leupeptin, and 20 mg/ml aprotinin). Protein concentration of the fractions was estimated by the method of Bradford [1976] and Western blot assays were performed. Cross contamination between fractions was assessed by immunoblot assays using anti-lamin B as nuclear marker.

Western blot assays. Protein samples (20 μg) were mixed with buffer (400 mM Tris–HCl [pH 6.8], 10% SDS, 50% glycerol, 500 mM dithiothreitol [DTT], and 2 mg/ml bromophenol blue), boiled for 5 min, and resolved by 10–12% SDS–PAGE according to the method of Laemmli [1970]. Fractionated proteins were electrotransferred to polyvinylidene fluoride membranes (Immobilon-P; PVDF) and then blocked for 1 h at room temperature with 5% nonfat dry milk in PBS containing 0.1% Tween-20 (PBS-T). Blots were incubated overnight with the primary antibodies: anti-p-p53, anti-p-FoxO3a, anti-p-FoxO4, anti-p-Akt, anti-Akt, anti-lamin B, and anti-β-tubulin using anti-rabbit secondary antibodies for all of them. The membranes were washed with PBS-T before incubation with HRP-conjugated secondary antibodies. The ECL blot detection kit was used. Relative migration of unknown proteins was determined by comparison with molecular weight markers. When needed, membranes were stripped with stripping buffer (62.5 mM Tris–HCl [pH 6.7], 2% SDS w/v, 50 mM β-mercaptoethanol), washed with PBS 1% Tween-20 and blocked for 1 h with 5% nonfat dry milk in PBS-T. The blots were then treated as before using other antibodies. Relative quantification of bands was performed using ImageJ software (NIH, USA).

Immunocytochemistry. After the corresponding treatments, coverslips with adherent cells were washed with PBS (pH 7.4, 8 g/l NaCl, 0.2 g/l KCl, 0.24 g/l KH_2PO_4, and 1.44 g/l Na_2HPO_4) and fixed with methanol at −20°C for 30 min. After fixation, cells were rinsed three times with PBS and then, nonspecific sites were blocked for 1 h with PBS 5% BSA. Cells were incubated with appropriate primary antibodies overnight at 4°C. The primary antibodies were recognized by fluorophore-conjugated secondary antibodies. Finally, the stained cells were analyzed with a confocal scanning laser microscopy (Leica TCS SP2 AOBS microscope) or a conventional fluorescence microscope (NIKON Eclipse Ti-S). The specificity of the labeling techniques was proven by the absence of fluorescence when the primary or the secondary antibodies were omitted. At least 10 fields per slide were examined. Representative photographs are shown.

RNA isolation, reverse transcription, and quantitative PCR. Cells were treated during different periods of time with the vehicle or DMEM without serum (C), H_2O_2 (0.5 mM), or preincubated with 10^{-8} M E_2 during 1 h and then exposed to H_2O_2 (0.5 mM) for the times specified. Total RNA was extracted after the corresponding treatments, using the High Pure RNA Isolation kit (Roche Diagnostics). RNA (2 μg) was reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Inc.) according to the manufacturer's instructions. Quantitative real-time PCR was done using KAPA SYBR® FAST qPCR Kit Master Mix (2X) Universal (KR0389—v8.12), MicroAmp® Fast 96-Well Reaction Plate, and ABI Prism7500Fast® Sequence Detection System with 96-wells (Applied Biosystems, Inc.) under the standard conditions recommended by the manufacturer. Primer sets to amplify murine cDNAs used in the analysis were as follows: glycelaldehyde 3-phosphate-dehydrogenase (GAPDH) set: forward 5′CGTCCCGTAGA-CAAAATGGT3′, reverse 5′TTGATGGCAACAATCTCCAC3′, Bcl-2 set: forward 5′GCGTCAACAGGGAGATGTCA3′, reverse 5′TTCC-ACAAAGGCATCCCAGC3′, Bim set: forward 5′AATGTCTGA-CTCTGACTCTCGGAC3′, reverse 5′TCTCCGCAGGCTGCAATTG-TCTAC3′, Puma set: forward 5′TACGAGCGGCGGAGACAAG3′, reverse 5′GTGTAGGCACCTAGTTGGGC3′, PERP set: forward 5′ACCACATCCAGACATCGTCG3′, reverse 5′CTCGTCCCCATGCGT-ACTCC3′, Noxa set: forward 5′GTCGGAACGCGCCAGTGAACCC3′, reverse 5′TCCTTCCTGGAGGTCCCTTCTTGC3′, MDM2 set: forward 5′GCCTGGATCAGGATTCAGTTTCTG3′, reverse 5′GTGACCCGAT-AGACCTCATCATCC3′. The specificity of PCR products was confirmed by melting curve analysis. Levels of the transcripts were normalized to GAPDH, used as housekeeping gene. Relative quantification of gene expression was determined by the comparative CT method.

Statistical analysis. Results are shown as means ± SD of not less than three independent experiments. Statistical differences among groups were performed using ANOVA and a multiple comparison post hoc test [Tukey, 1953]. The statistical significance of data was determined as $P < 0.05$.

RESULTS

17β-ESTRADIOL PREVENTS H_2O_2-INDUCED p53 ACTIVATION IN SKELETAL MUSCLE CELLS

As it was described, p53 has been extensively related to apoptotic cell death. Therefore, to examine the involvement of p53 in this process in skeletal muscle, we explored the activation of p53 in C2C12 cells evaluating its phosphorylation in serine 15 residue. To our interest, it has been shown that serine phosphorylation of p53 in response to oxidative stress enhances its proapoptotic activity.

As an initial approach, C2C12 cells were incubated with the apoptotic agent H_2O_2 (0.5 mM) during different periods of time, followed by subcellular fractionation of lysates to obtain nuclear fractions, since active p53 translocates from the cytosol to the nucleus to regulate its target genes. Immunoblots of these fractions showed that H_2O_2 induced the activation of p53 at 1 h of treatment, effect that was maintained up to 3 h of incubation (Fig. 1). To evaluate if the hormone E_2 can modulate p53 activation, cells were treated with physiological concentrations of E_2 (10^{-8} M, 1 h) prior addition of the apoptotic inductor H_2O_2. Under these conditions, E_2 reverted p53 phosphorylation at serine 15 residue (Fig. 1). These data support the notion that the estrogen protects skeletal myoblasts from apoptosis inhibiting the activation of the transcription factor p53 at the nucleus.

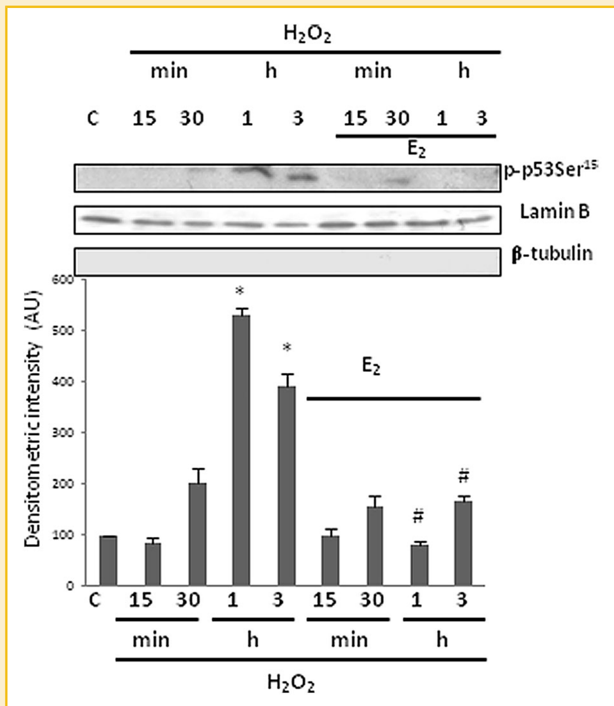

Fig. 1. 17β-Estradiol inhibits H_2O_2-induced p53 phosphorylation in C2C12 cells. Cells were incubated with the vehicle of the hormone or DMEM without serum (C), H_2O_2 (0.5 mM) during different periods of time (15 min, 30 min, 1 h, and 3 h) or E_2 (10^{-8} M, 1 h) before treatment with H_2O_2 (0.5 mM for 15 min, 30 min, 1 h, and 3h). Cell lysates were used to obtain the corresponding nuclear fractions by differential centrifugation which, then, were subjected to Western blot assays using an anti-p-p53 Ser^{15} antibody. Lamin B was used as nuclear marker. Blots are representative of three independent experiments with comparable results. Densitometric quantification of blots is shown. Averages ± SD are given. $^{*}P < 0.05$ with respect to the control (C); $^{\#}P < 0.05$ with respect to H_2O_2 condition.

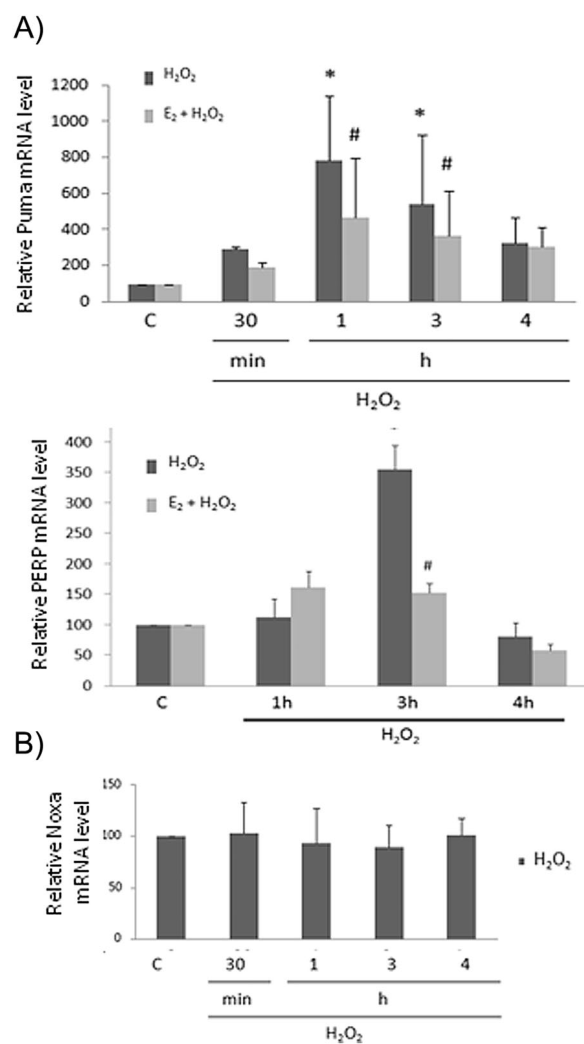

Fig. 2. 17β-Estradiol reduces H_2O_2-upregulation of Puma and PERP mRNA transcript levels in C2C12 cells. C2C12 cells were incubated under different experimental conditions: (A) Vehicle of the hormone or DMEM without serum (C), H_2O_2 (0.5 mM) for the indicated times or preincubated with E_2 (10^{-8} M, 1 h) followed by treatment with H_2O_2 (0.5 mM) during the indicated times ($E_2 + H_2O_2$); (B) DMEM without serum (C) or H_2O_2 (0.5 mM) for the indicated times. Under the designated conditions, quantification by real-time PCR of Puma, PERP, and Noxa mRNA transcript levels was carried out, which were normalized to the expression level of GAPDH. SYBR Green runs were performed on duplicate samples of cDNAs from three independent reverse transcription reactions. The comparative CT method was used for quantification. Averages ± SD are given. $^{*}P < 0.05$ with respect to the control (C); $^{\#}P < 0.05$ with respect to the corresponding H_2O_2 treatment without E_2.

17β-ESTRADIOL DOWNREGULATES H_2O_2-INDUCED mRNA TRANSCRIPT LEVELS OF THE PROAPOPTOTIC Puma AND PERP IN SKELETAL MUSCLE CELLS

Several genes have been identified as targets for activation by p53, including the proapoptotic factors Puma, Noxa, and PERP. As it has been described above, Puma and Noxa are BH3-only members of the Bcl-2 family that can act upstream of Bax and play a role in mediating p53-induced cell death through the intrinsic pathway while PERP was identified to be distinctively induced during p53-dependent apoptotic pathway, and not cell cycle arrest.

To gauge the effect of H_2O_2 (0.5 mM) over Puma, Noxa, and PERP mRNA transcript levels in skeletal muscle, C2C12 cells were treated with the apoptotic inductor for the periods of time specified in Figure 2. We observed that Puma and PERP mRNA levels were upregulated after H_2O_2 treatment, while Noxa mRNA levels were not affected by the apoptosis inducer. Moreover, whereas the augment of PERP mRNA level was evidenced at 3 h of incubation with H_2O_2 (255.56% above control) and then it decays to control level, the increment of Puma mRNA level was maximum at 1 h of treatment with H_2O_2 (682.09% above control) and, in the following times, the increase was less pronounced but also significant (3 h: 443.03% and 4 h: 227.94% above control).

Then, we investigated whether E_2 could affect the H_2O_2-induced increments of PERP and Puma mRNA transcript levels. To that end, C2C12 cells were preincubated with E_2 (10^{-8} M, 1 h) prior induction of apoptosis with H_2O_2 (0.5 mM) during the periods studied above. We evidenced that their mRNA levels were diminished by

pretreatment with the hormone, suggesting that E_2 exerts its protective effect through the negative regulation of these apoptotic factors at the transcriptional level in C2C12 cells (Fig. 2).

17β-ESTRADIOL UPREGULATES H_2O_2-DECREASED mRNA TRANSCRIPT LEVEL OF THE ANTIAPOPTOTIC Bcl-2 IN SKELETAL MUSCLE CELLS

It has been extensively reported that overexpression of the antiapoptotic member Bcl-2 of Bcl-2 family induces the inhibition of apoptotic cell death [Korsmeyer, 1999] and that p53 can act to repress Bcl-2 transcription in some cells [Haldar et al., 1994].

To determine the effect of H_2O_2 (0.5 mM) over Bcl-2 mRNA transcript level in skeletal muscle, C2C12 cells were incubated with the apoptosis inducer for the times specified in Figure 3A. We evidenced that Bcl-2 mRNA level was downregulated after H_2O_2 treatment, in a time-dependent fashion, showing its most pronounced decrease at 4 h of incubation with the apoptotic inductor (52.12% below control). When cells were pretreated with E_2 (10^{-8} M, 1 h) before the addition of H_2O_2 (0.5 mM) during the periods studied above, we observed that the hormone increased Bcl-2 mRNA levels above each corresponding apoptotic condition (Fig. 3A). These results suggest that the hormone positively regulates Bcl-2 mRNA transcript levels to exert its antiapoptotic effect in C2C12 cells.

17β-ESTRADIOL DOES NOT AFFECT H_2O_2-INDUCED mRNA TRANSCRIPT LEVEL OF THE PROAPOPTOTIC Bim IN SKELETAL MUSCLE CELLS

The BH3-only member Bim is another proapoptotic target of p53. It can interact with some but not all Bcl-2 family proteins that promote cell survival, and only those antiapoptotic components that bind to it, can neutralize its proapoptotic activity. Certainly, it has been evidenced that Bim can interact with Bcl-2, inhibiting cell death (O'Connor et al., 1998). However, it is controversial the function of the interaction between antiapoptotic and BH3-only proapoptotic members of this family. It has been suggested that the net result would depend on the cellular type and stimulus involved.

When it was evaluated the role of the apoptotic agent over the transcriptional activity of Bim, it was evidenced that H_2O_2 increments the mRNA transcript level of Bim from 30 min of treatment (99.42% over control), effect that was maintained in the subsequent periods of time studied, with a peak at 3 h of incubation (232.42% over control) (Fig. 3B). Then, we investigated whether E_2 could affect the H_2O_2-induced increments of Bim mRNA transcript level. To that end, C2C12 cells were preincubated with E_2 (10^{-8}M, 1 h) and then with H_2O_2 (0.5 mM) during the times specified in Figure 3B. We evidenced that its mRNA level was slightly increased (not significant) by pretreatment with the hormone in all the times studied over its respective H_2O_2 condition.

ROLE OF 17β-ESTRADIOL IN H_2O_2-INDUCED REGULATION OF FoxO3a IN SKELETAL MUSCLE CELLS

The FoxO family of transcription factors has been implicated in the regulation of oxidative stress and several other diverse physiologic processes including stress resistance. In response to oxidative stress, FoxOs change their subcellular localization and become phosphorylated at different residues, depending on the cellular type and stimulus.

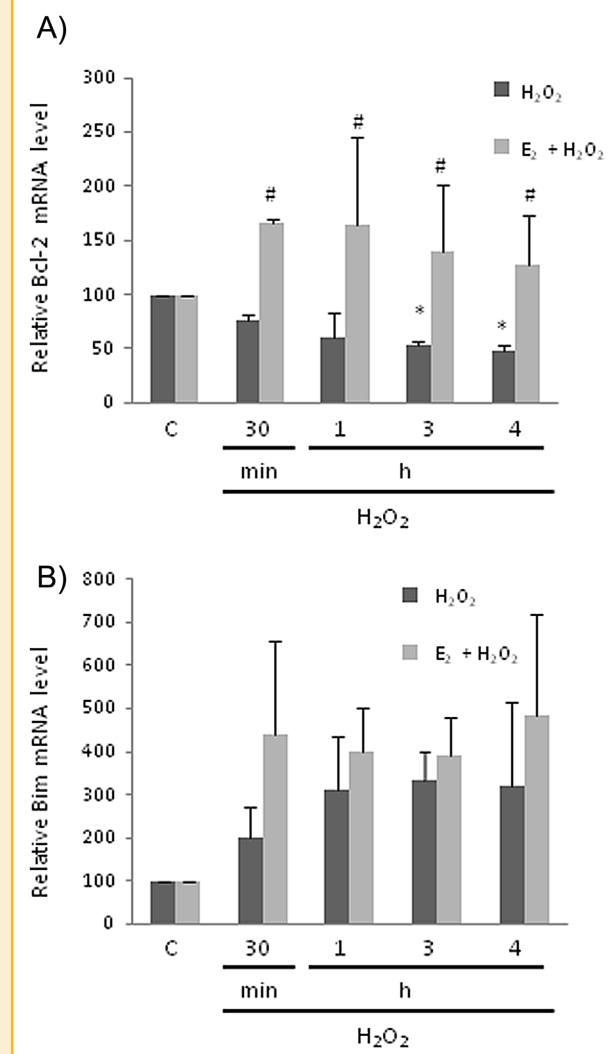

Fig. 3. 17β-Estradiol regulates the balance between Bcl-2 and Bim at the transcriptional level in C2C12 cells. Cells were treated with the vehicle of the hormone or DMEM without serum (C), H_2O_2 (0.5 mM) for the indicated times or preincubated with E_2 (10^{-8}M, 1 h), followed by treatment with H_2O_2 (0.5 mM) during the indicated times ($E_2 + H_2O_2$). Under the designated conditions, quantification by real-time PCR of Bcl-2 (A) and Bim (B) mRNA transcript levels was carried out, which were normalized to the expression level of GAPDH. SYBR Green runs were performed on duplicate samples of cDNAs from three independent reverse transcription reactions. The comparative CT method was used for quantification. Averages ± SD are given. $^{*}P < 0.05$ with respect to the control (C); $^{\#}P < 0.05$ with respect to the corresponding H_2O_2 treatment without E_2.

To study the effect of the apoptotic agent over the transcription factor FoxO3a in C2C12 cells, they were exposed to H_2O_2 (0.5 mM) during different times. It was evidenced that the apoptosis inducer conduces to the phosphorylation of FoxO3a in serine 253 residue at 30 min and 1 h of treatment, showing that under these conditions this transcription factor remains inhibited (344.97%

and 450.20% over control, respectively) (Fig. 4A). As some members of this family could be negatively regulated by PI3 K/Akt pathway, it was evaluated if this via modulates the previously observed H_2O_2-induced effect in C2C12 cells. To that end, cells

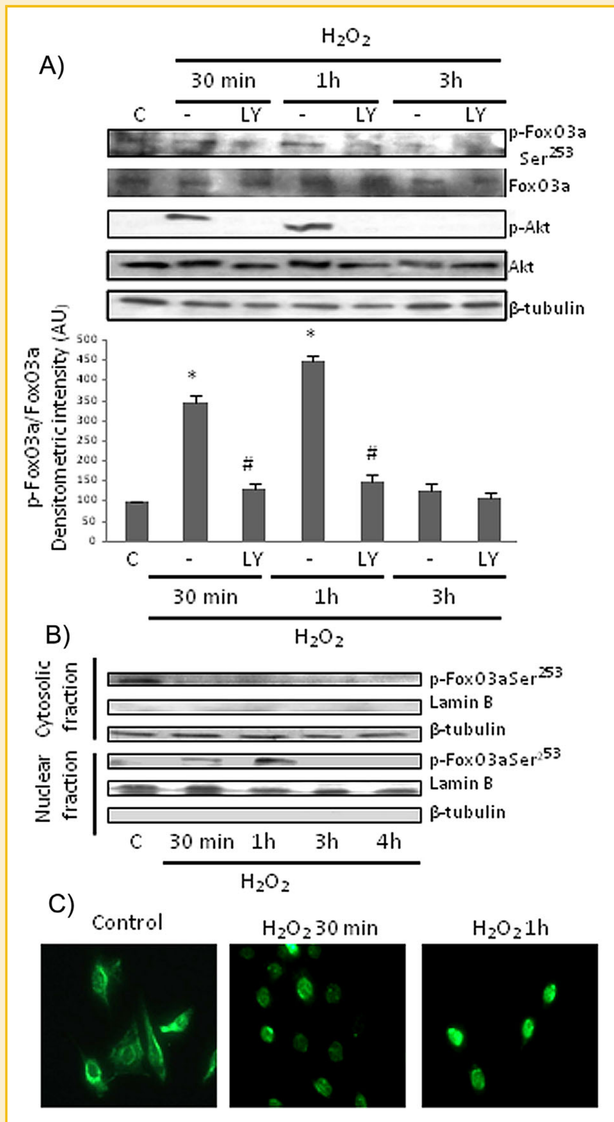

Fig. 4. Early apoptosis induces FoxO3a phosphorylation through Akt and its nuclear translocation in C2C12 cells. (A) Cells were incubated with DMEM without serum (C), H_2O_2 (0.5 mM) during different periods of time (30 min, 1 h, and 3 h) or LY294002 (25 μM, 1 h) before treatment with H_2O_2 (0.5 mM for 30 min, 1 h, and 3 h). Cell lysates were subjected to Western blot assays using anti-p-Foxo3a Ser^{253}, anti-FoxO3a, anti-p-Akt, and anti-Akt antibodies. β-tubulin was used as protein loading control. (B) Cells were incubated with DMEM without serum (C) or H_2O_2 (0.5 mM) during different periods of time (30 min, 1 h, and 3 h). Cell lysates were used to obtain the corresponding nuclear and cytosolic fractions by differential centrifugation and, then, were subjected to Western blot assays using anti-p-FoxO3a antibody Ser^{253}. Lamin B was used as nuclear marker and β-tubulin as cytosolic marker. (C) Cells were incubated with DMEM without serum (C) or H_2O_2 (0.5 mM) during different periods of time (30 min and 1 h). Then, they were incubated with anti-p-FoxO3a Ser^{253}, followed by addition of Alexa Fluor-488 conjugated secondary antibody (green fluorescence). Images are representative of three representative experiments with comparable results. Objective 60X. Blots are representative of three independent experiments with comparable results. Densitometric quantification of blots is shown. Averages ± SD are given. $^*P < 0.05$ with respect to the control (C); $^\#P < 0.05$ with respect to H_2O_2 condition.

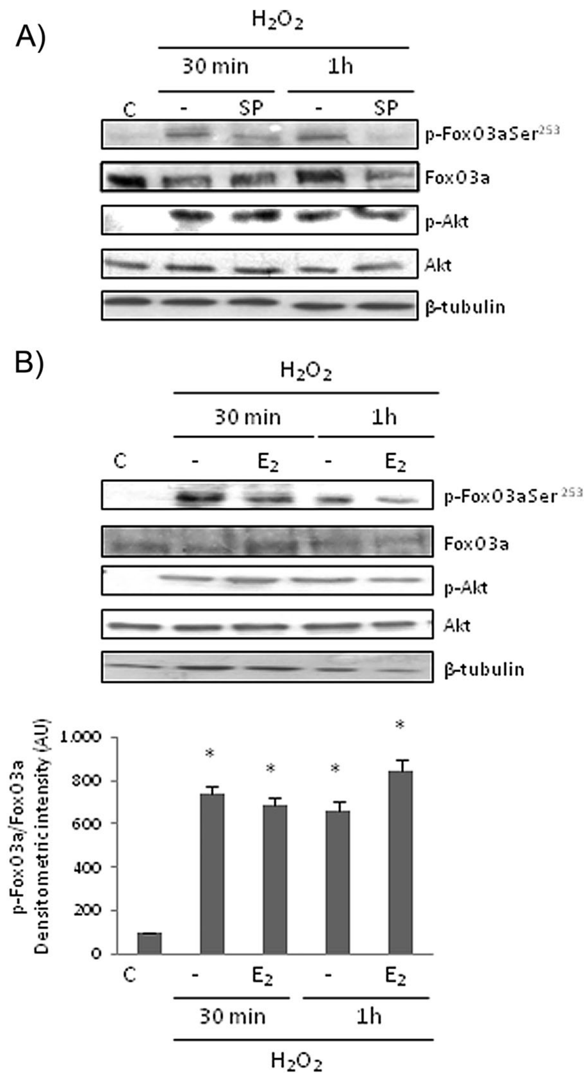

Fig. 5. (A) H_2O_2-induced FoxO3a phosphorylation involves JNK in C2C12 cells. Cells were incubated with DMEM without serum (C), H_2O_2 (0.5 mM, 1 h), or treated with SP600125 (10 μM, 1 h) before addition of H_2O_2 (0.5 mM, 1 h). Cell lysates were used to perform Western blot assays using anti-p-Foxo3a Ser^{253}, anti-FoxO3a, anti-Akt, and anti-p-Akt antibodies. β-tubulin was used as protein loading control. Blots are representative of three independent experiments with comparable results. (B) 17β-Estradiol sustains H_2O_2-induced FoxO3a phosphorylation in C2C12 cells. Cells were incubated with the vehicle of the hormone (C), H_2O_2 (0.5 mM, 30 min and 1 h) or E_2 (10^{-8}M, 1 h) before treatment with H_2O_2 (0.5 mM for 30 min and 1 h). Cell lysates were used to perform Western blot assays using an anti-p-FoxO3a Ser^{253}, anti-FoxO3a, anti-Akt, and anti-p-Akt antibodies. β-Tubulin was used as protein loading control. Blots are representative of three independent experiments with comparable results. Densitometric quantification of blots is shown. Averages ± SD are given. $^*P < 0.05$ with respect to the control (C).

were incubated with the PI3K inhibitor LY294002 (25 μM, 1 h) prior treatment with H_2O_2. It was evidenced that under these conditions, H_2O_2-induced phosphorylation of FoxO3a was inhibited, suggesting that this pathway is the one that negatively regulates this transcription factor in C2C12 cells. In concordance, it was observed that Akt was phosphorylated (active) in the same periods of time that FoxO3a was inactivated in response to H_2O_2 (Fig. 4A). Additionally, it was evidenced that inactive (phosphorylated) FoxO3a was present in cytosolic fraction under basal conditions (control), while it was absent in this fraction when cells were exposed to the apoptotic agent. On the other hand, phosphorylated FoxO3a was visualized at 30 min and 1 h of treatment with H_2O_2 in nuclear fractions, while it was absent in the following periods of time (3 and 4 h), suggesting that this factor translocates to the nucleus in its phosphorylated state and therein it is dephosphorylated to regulate gene expression (Fig. 4B). These observations were corroborated by immunocytochemistry assays. It was evidenced that p-FoxO3a localizes homogenously distributed in the cytosol under basal conditions, while treatment with H_2O_2 (30 min and 1 h) changes its localization from the cytosol to the nucleus (Fig. 4C).

As members of this family can be regulated by JNK, and we have shown in previous works that this kinase is activated in response to the apoptotic stimulus (from 1 to 4 h of treatment) in C2C12 cells [La Colla et al., 2013], it was studied whether JNK modulates the effect of the apoptotic agent over FoxO3a. It was observed that preincubation with the JNK inhibitor SP600125 (10 μM, 1 h) prior addition of H_2O_2 did not induced significant changes in the phosphorylation state of FoxO3a at 30 min of treatment, while led to the inhibition of its phosphorylation at 1 h of incubation. When it was evaluated whether JNK exerted this action through Akt, it was evidenced that Akt remained in its active state even though in presence of the JNK inhibitor (Fig. 5A).

To assess whether the hormone exerts an effect on the observed H_2O_2-induced FoxO3a phosphorylation, cells were preincubated with E_2 before induction of apoptosis with H_2O_2 during 30 min and 1 h, where it was observed the phosphorylation of this factor. This preincubation did not induce a significant change in the level of phosphorylation of FoxO3a, suggesting that the hormone keeps this factor in its phosphorylated inactive state. Interestingly, at the same time, it was observed that Akt was active (phosphorylated) under these conditions (Fig. 5B).

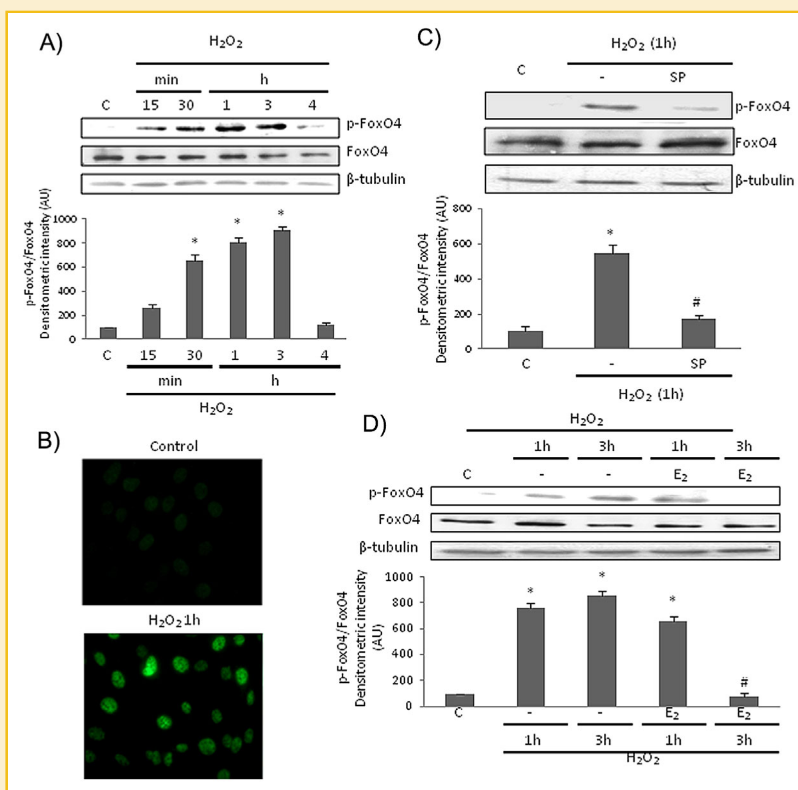

Fig. 6. 17β-Estradiol modulates H_2O_2-induced FoxO4 phosphorylation in C2C12 cells. Role of JNK. C2C12 cells were incubated under different experimental conditions: (A) and B) DMEM without serum (C) or H_2O_2 (0.5 mM) during different periods of time (15 min, 30 min, 1 h, 3 h, and 4 h). (C) DMEM without serum (C), H_2O_2 (0.5 mM, 1 h), or treated with SP600125 (10 μM, 1 h) before addition of H_2O_2 (0.5 mM, 1 h). (D) Vehicle of the hormone (C), H_2O_2 (0.5 mM, 1 and 3 h) or E_2 (10^{-8} M, 1 h) before treatment with H_2O_2 (0.5 mM, 1 and 3 h). Then, cells were incubated with anti-p-FoxO4, followed by addition of Alexa Fluor-488 conjugated secondary antibody (green fluorescence) (B) or cell lysates were subjected to Western blot assays using anti-p-FoxO4 and anti-FoxO4 antibodies (A, C, D). β-Tubulin was used as protein loading control. Blots are representative of three independent experiments with comparable results. Densitometric quantification of blots is shown. Images are representative of three representative experiments with comparable results. Objective 60X. Averages ± SD are given. $^{*}P < 0.05$ with respect to the control (C). $^{\#}P < 0.05$ with respect to H_2O_2 condition.

ROLE OF 17β-ESTRADIOL IN H_2O_2-INDUCED REGULATION OF FoxO4 IN SKELETAL MUSCLE CELLS

To study the effect of the apoptotic agent over the transcription factor FoxO4, C2C12 cells were exposed to H_2O_2 during the different times specified in Figure 6A. It was observed that the apoptotic agent induced the phosphorylation/activation of FoxO4 in threonine 447–451 residue at 30 min of incubation, effect that was maintained up to 3 h of treatment (Fig. 6A). This result was corroborated by immunocytochemistry assays where it was evidenced an increase of the fluorescence intensity of p-FoxO4 under apoptotic conditions (H_2O_2, 1 h), respect to the control (Fig. 6B). Moreover, it was visualized in this assay that the localization of active FoxO4 is nuclear (Fig. 6B).

To evaluate if JNK modulates the action of H_2O_2 over FoxO4, C2C12 cells were preincubated with the inhibitor SP600125 (10 μM, 1 h) before induction of apoptosis. It was observed that in presence of the inhibitor, the H_2O_2-induced phosphorylation of FoxO4 diminished (from 552 ± 45% to 175 ± 22%), suggesting that this kinase regulates FoxO4 in C2C12 cells (Fig. 6C).

To explore the role of the hormone over the response of FoxO4 to the apoptotic agent, cells were preincubated with E_2 (10^{-8}M, 1 h) prior induction of apoptosis with H_2O_2 during the times in which it had been observed FoxO4 activation (1 and 3 h of treatment). The preincubation with the hormone inhibited the phosphorylation of this factor only at 3 h of treatment, but not at 1 h, suggesting that E_2 regulates this factor in advanced phases of apoptosis (Fig. 6D).

ROLE OF 17β-ESTRADIOL IN H_2O_2-INDUCED REGULATION OF MDM2 TRANSCRIPTION IN SKELETAL MUSCLE CELLS

The ubiquitin ligase MDM2 is an important protein in the regulation of p53 abundance, and consequently, in the p53-dependent apoptosis. The inducible expression of MDM2 is determined by P2 promoter which presents two p53 response elements. Thus, p53 can stimulate the expression of MDM2 by an autoregulatory mechanism, and at the same time, induce its own degradation. However, P2 presents additional response elements that allow the p53-independent induction of MDM2 [Ries et al., 2000]. Certainly, ERK kinase promotes MDM2 transcriptional activity to regulate FoxO3a [Yang et al., 2008].

When it was evaluated the effect of the apoptotic agent over the expression of MDM2 mRNA, we observed that H_2O_2 induced an increase in its mRNA transcript level from 30 min (171.23% above control) to 1 h of incubation (331.92% above control) (Fig. 7). However, in the following periods of treatment with H_2O_2 (3 and 4 h), mRNA transcript levels showed a decrease, leading to values similar to control condition. Conversely, while it was studied the effect of the hormone, it was evidenced that the preincubation with E_2 before induction of apoptosis conduces to an augment even more pronounced than that induced by the apoptotic agent alone, effect that was more evidenciable at 3 h of incubation (433.1% above H_2O_2 3 h condition) (Fig. 7).

As it was described above, FoxO3a can be regulated both by ERK and MDM2, thus favoring its degradation. To study the involvement of ERK in the regulation of MDM2 transcription levels after apoptotic induction, C2C12 cells were preincubated with the inhibitor PD98059 (10 μM, 1 h) and then with H_2O_2 during the following times: 30 min, 1 h, 3 h, and 4 h. From 30 min to 1 h of incubation with H_2O_2, ERK inhibition downregulated MDM2 transcript levels, leading to values comparable to control. On the contrary, in the following periods of incubation with the apoptotic agent (3 and 4 h), ERK inhibition positively affected MDM2 transcript level, achieving values similar to those obtained in presence of the hormone (Fig. 7).

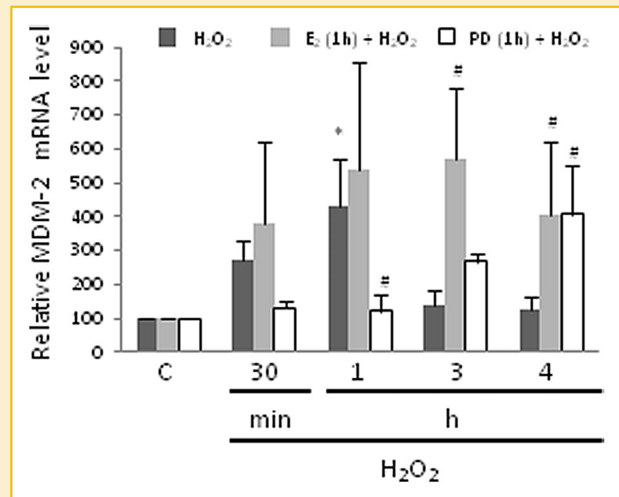

Fig. 7. 17β-Estradiol modulates MDM2 mRNA transcript level differently in response to H_2O_2 in C2C12 cells. Role of ERK. Cells were incubated with the vehicle of the hormone or DMEM without serum (C), treated with H_2O_2 (0.5 mM) for the indicated times, preincubated with E_2 (10^{-8}M, 1 h) followed by treatment with H_2O_2 (0.5 mM) during the specified times ($E_2 + H_2O_2$) or preincubated with PD98059 (10 μM, 1 h) before addition of H_2O_2 (0.5 mM) during the indicated times (PD + H_2O_2). Under the designated conditions, quantification by real-time PCR of MDM2 mRNA transcript levels was carried out, which were normalized to the expression level of GAPDH. SYBR Green runs were performed on duplicate samples of cDNAs from three independent reverse transcription reactions. The comparative CT method was used for quantification. Averages ± SD are given. $^*P < 0.05$ with respect to the control (C); $^{\#}P < 0.05$ with respect to the corresponding H_2O_2 treatment without E_2.

DISCUSSION

The relative levels of pro- and antiapoptotic factors reflect the interrelationship of multiple signals and different molecular pathways within a cell. Indeed, the combination of the effects of upregulation of antiapoptotic members, downregulation of proapoptotic ones, and the consequences of their interactions can contribute to the protective effect of E_2. In previous studies, we presented a molecular link between E_2 and apoptosis in skeletal muscle cells. Certainly, we showed an antiapoptotic role of the steroid involving ERs, PI3 K/Akt/Bad, Bax, HSP27, MAPKs, and various other kinases [Vasconsuelo et al., 2008, 2010; La Colla et al., 2015], exerting an important effect over mitochondria of myoblasts [La Colla et al., 2013]. In this work, we evaluated whether the mechanism activated by E_2 to exert its protective role in C2C12 cells includes the regulation of factors at the transcriptional level, studying both, those that promote and antagonize cell survival.

The transcription factor p53 exerts antiproliferative effects, including apoptosis, in response to various types of stress. In accordance to the reported evidence in other mammalian cells [Yamamoto et al., 2007], in the present work, we observed that p53 is involved in H_2O_2-induced skeletal muscle cell death. Moreover, our results showed that the preincubation with the hormone inhibited the activation/phosphorylation of p53 mediated by the apoptotic inductor, as it has been observed by others in cardiomyocytes [Liu et al., 2011].

The identification of several proapoptotic p53 targets, some of them that inhibit antiapoptotic Bcl-2 family members, suggests that it is only through the combined transcriptional activation of numerous proapoptotic targets that p53 exerts its completely apoptotic potential. The first mechanistic connection between p53 and Bcl-2 family came from the identification that p53 directly induces the transcription of the proapoptotic member Bax [Chipuk et al., 2004]. However, since this p53-mediated transactivation does not totally explain the capacity of p53 to inhibit Bcl-2 antiapoptotic members and thus promote apoptosis, this can be explained by the action of proapoptotic BH3-only Bcl-2 family members. Several of these proteins, including Puma and Noxa, were identified as transcriptional targets of p53. While Noxa has generally a minor activity, Puma is considered a key member for the proapoptotic role of p53, whose expression can be induced rapidly in response to various types of stress in different tissues [Yu and Zhang, 2008]. In this work, it was shown that the mRNA transcript level of Puma was upregulated after induction of apoptosis while the mRNA transcript level of Noxa was not affected under these conditions, implying that the BH3-only member Puma, but not Noxa, is transcriptionally regulated in H_2O_2-induced skeletal muscle cell death. In addition, since p53 and Puma showed the same pattern of activation/increased expression, it could be assumed that Puma is under the transcriptional control of p53, contributing to C2C12 cell death. Although this suggestion is very likely to occur, further studies are necessary to confirm this hypothesis. Some studies have shown that BH3-only proteins potentiate Bax dependent apoptotic death [Hemann et al., 2004]. Of significance for this work, we have already demonstrated that Bax is involved in H_2O_2-induced skeletal muscle cell death [La Colla et al., 2013]. Thus, these results suggest that Puma may be responsible of the H_2O_2-induced cell death through Bax in these cells. Moreover, this apoptotic effect was reverted by treatment with the hormone, suggesting that E_2 negatively regulates Puma at the transcriptional level to exert its antiapoptotic action.

Bcl-2 belongs to the subgroup of antiapoptotic members of Bcl-2 family. In addition to p53 faculty to promote the transcription of Bcl-2 antagonists, this transcription factor uses other strategies to regulate Bcl-2. It has been shown that p53 can repress its transcription in some cells [Miyashita et al., 1994], although the mechanism for such repression has not been completely elucidated. However, it has been reported that the Bcl-2 promoter has a p53-negative response element [Miyashita et al., 1994], emerging the possibility that Bcl-2 is a direct target of p53-induced repression. In this work, it was proven that the mRNA transcript level of Bcl-2 was downregulated in response to the apoptotic inductor, whereas preincubation with the hormone reversed this effect, demonstrating that E_2 not only modulates proapoptotic but also antiapoptotic members of Bcl-2 family to protect C2C12 cells. Of interest, the transcription of the gene encoding this protein is controlled by promoters P1 and P2 [Seto et al., 1988], which do not have estrogen response elements (EREs) [Perillo et al., 2000]. As EREs, generally located in the promoters, have also been identified in other regions [Hyder et al., 1995], it could be suggested that the hormone exerts its antiapoptotic effect through its interaction with these atypical EREs. Indeed, it has been reported that E_2 induces the transcription of Bcl-2 via EREs not present in the promoters in MCF-7 human breast cancer cells [Perillo et al., 2000].

As it was described above, Bim is another BH3-only member of the Bcl-2 family. Its proapoptotic activity depends on its capacity to bind prosurvival members of the Bcl-2 family [Strasser et al., 2000]. Even though several models have been proposed to explain the cellular consequences of the interactions between antiapoptotic and BH3-only members of this family, none of them clarify one confusing aspect: the net result of their interactions, such as occurs between Bim and Bcl-2 [Chen et al., 2005]. It has been suggested that the final result would be strictly related to the cellular type and the apoptotic stimulus. In this work, it was observed that Bim is transcriptionally upregulated in H_2O_2-induced apoptosis in C2C12 cells. In view of the capacity of Bcl-2 to interact with Bim, the results obtained herein where it was observed that the apoptotic inductor increased the mRNA transcript level of Bim while decreased the transcript level of Bcl-2, support the notion that it would prevail the apoptotic action of Bim. Since under these conditions, Bim could not be entirely sequestered by Bcl-2 to abrogate its apoptotic action, the net result would be the promotion of cell death. On the contrary, even though treatment with the hormone prior induction of apoptosis conduced to a slight but not significant augment in mRNA transcript levels of Bim, at the same time it induced an increment in mRNA transcript levels of Bcl-2, suggesting that due to the interaction of the resultant proteins, it would be inhibited the apoptotic action of Bim.

As it was mentioned, p53 employs many proapoptotic transcriptional targets to promote cell death, some of which are not Bcl-2 family members. Indeed, PERP is another target gene of p53 that has been exclusively related to apoptosis [Attardi et al., 2000]. Even though it has been confirmed its proapoptotic role, its precise function in eliciting an apoptotic response has not been completely elucidated. In this work, it was shown that the gene encoding PERP is H_2O_2-upregulated in C2C12 cells. In addition, treatment with the hormone prior induction of apoptosis leads to decreased levels of its mRNA, showing that E_2 may exert its protective effect negatively regulating this apoptotic factor at the transcriptional level and reinforcing the concept of the antiapoptotic role of the hormone. Moreover, it could be suggested that p53 would be responsible of PERP transcription, since they share the pattern of activation/increased expression.

As mentioned above, members of the Forkhead family are also involved in oxidative stress response. FoxO factors can change their subcellular localization and become phosphorylated in different residues, according to the cell type and intensity/type of stimulus involved, thus exerting various cellular functions such as the regulation of apoptosis and/or cell cycle, through the transcription of their target genes [Salih and Brunet, 2008]. In this work, it was

evidenced that the FoxO3a member is maintained in its phosphorylated/inactive state (serine 253 residue) due to the negative regulation mediated by Akt in the onset of apoptosis. Thus, FoxO3a prevents activation of proapoptotic genes in the initial stage of apoptosis and, therefore, exerts an early defense response to limit the progress of the apoptotic signal. This behavior, which is characterized by the initial activation of defense mechanisms (at short times of treatment with H_2O_2) which cannot be sustained due to the continued presence of the apoptotic inductor (long times of treatment with H_2O_2), has been observed by others in skeletal muscle cells [Pronsato et al., 2012]. However, to our knowledge, the involvement of FoxO3a in this defense response has not been reported before in C2C12 cells. Indeed, one major pathway by which PI3 K/Akt promotes cell survival is through sequestering FoxOs away from the promoters of apoptotic genes. Even though we observed that under basal conditions, FoxO3a is inactive in the cytosol, then, in contrast to other cell types [Brunet et al., 1999], phosphorylated FoxO3a is visualized in the nucleus in C2C12 cells in the initial phase of apoptosis. Since in its phosphorylated state FoxO3a cannot bind DNA, it cannot regulate apoptotic gene expression. Thus, it could be suggested that this observation may be due to the urgency to respond to the oxidative stimulus, exceeding the requirement of dephosphorylation to enter this organelle, as a consequence of the nature of muscle which is generally exposed to high oxidative stress conditions. Moreover, it could be hypothesized that FoxO3a may require exceeding a threshold level at the nucleus to be susceptible of dephosphorylation, thus capable of regulating gene transcription. Then, in advanced stages of the apoptotic process, active/unphosphorylated FoxO3a will be able to regulate the expression of its target genes. In accordance to the previously observed antiapoptotic effect of the hormone by our group, here it was evidenced that preincubation with E_2 prior induction of apoptosis retains the level of phosphorylation/inactivation of FoxO3a, thus showing a novel pathway by which the hormone prevents the transcription of apoptotic genes and maintains this defense response of C2C12 cells.

It has been shown that there are other factors that can regulate FoxO3a. Indeed, JNK is capable of modulating its nuclear translocation in invertebrates [Oh et al., 2005]. In this work, by the use of the JNK inhibitor SP600125, it was observed that phosphorylation of FoxO3a triggered by the apoptotic agent is regulated by JNK in C2C12 cells. However, this modulation was not evidenced at 30 min of treatment, suggesting that phosphorylation of FoxO3a is initially regulated by Akt. These results are consistent with the H_2O_2-mediated activation of JNK that we reported previously [La Colla et al., 2015], which is evidenced from 1 h of treatment. In contrast to what has been reported in other cell types, data exposed herein suggest that Akt and JNK act independently in the dual-regulation of FoxO3a in C2C12 cells. As there have not been recognized direct JNK-phosphorylation sites on FoxO3a, it could be suggested that this kinase exerts its effect indirectly. Indeed, it has been evidenced that there exists a link between p66Shc and FoxO3a [Guo et al., 2009]. Previously, we showed JNK-mediated p66Shc phosphorylation in C2C12 cells [La Colla et al., 2015], thus it could be postulated that this may be the activated pathway that regulates Akt-independent FoxO3a phosphorylation. Moreover, since it has been evidenced that JNK plays an apoptotic role once activated in C2C12 cells [La Colla et al., 2015], these data suggest that inactive FoxO3a, as a result of the indirect action of JNK-p66Shc pathway, antagonize the PI3K-Akt survival pathway. One could envision that, as a result, at this period (1 h) it would be inhibited the transcription of protective genes. Certainly, some studies have reported that p66Shc is capable of negatively regulating, through FoxO3a, the transcription of antioxidant enzymes catalase and MnSOD, thus decreasing ROS detoxification [Guo et al., 2009].

Other members of the Forkhead family, such as FoxO4, can be activated during apoptosis. Here, it has been evidenced that active/phosphorylated FoxO4 is upregulated by JNK in response to apoptotic induction at the nuclear compartment. Treatment with the hormone prevented this effect in advanced apoptosis (3 h). These data suggest that E_2 exerts its protective action in C2C12 cells inhibiting the activation of FoxO4, thus impeding the transcription of its proapoptotic target genes.

It has been established that p53 function is tightly controlled by its protein stability. In normal cells, this transcription factor has a short half-life, while its half-life is considerably prolonged under stress conditions [Maltzman and Czyzyk, 1984]. Certainly, p53 cellular levels are mainly determined by their rate of degradation rather than their synthesis [Harris and Levine, 2005]. The ubiquitin ligase MDM2 is the most important protein that directly associates with p53, resulting in its ubiquitination and subsequent degradation [Kubbutat et al., 1997]. Even though there exist a regulatory mechanism between p53 and MDM2 to maintain strict p53 levels in stressed and unstressed cells, there are also additional controls through posttranslational modifications, mainly by phosphorylation in serine 15 residue [Appella and Anderson, 2001]. It has been suggested that this modification would affect p53-MDM2 interaction and, consequently, the nature of the transcribed target genes [Mayo et al., 2005]. In addition, it has been reported that MDM2, through ERK, could be involved in the degradation by ubiquitination of FoxO3a [Yang et al., 2008]. Moreover, it has been evidenced that Akt-dependent phosphorylation of this transcription factor may also lead to degradation by the proteasome [Plas and Thompson, 2003]. In this work, it has been observed that the regulation of mRNA transcript levels of MDM2 depends on the period of the apoptotic process evaluated in C2C12 cells. At the beginning of apoptosis (30 min and 1 h), treatment with H_2O_2 positively modulates MDM2 at the transcriptional level, thus to promote its new protein synthesis, its interaction with p53 and/or p-Foxo3a, and the subsequent degradation of the transcription factors. The observed H_2O_2-mediated upregulation of MDM2 in the first phase of apoptosis in C2C12 cells could be a first cellular response to protect them from apoptosis, thus promoting the degradation of p53 and p-FoxO3a (inactive). The period of incubation of 1 h with the apoptotic agent appears to be a key point in the apoptosis related to p53 and FoxO3a. In C2C12 cells, in this period (1 h), the activation of several kinases involved in the apoptotic process is initiated [La Colla et al., 2015], and at the same time, we observed p53 phosphorylation at serine 15 residue and FoxO3a regulation mediated by JNK (discussed above). These events, in conjunction, would be related to the decrease in the MDM2 mRNA transcript level as to control condition in the following periods of time studied (3 and 4 h of treatment with H_2O_2).

These results suggest that in advanced stages of apoptosis, the p53 posttraductional modification and the different activation of molecular pathways that affect FoxO3a would influence the transcriptional control of MDM2, thus conducing to MDM2 levels similar to the basal condition and, as a consequence, would decrease the rate of degradation of p53 and inactive FoxO3a, inclining the balance towards apoptosis. In addition, although it has not been well described the mechanism by which MDM2 mediates the degradation of FoxO3a, ERK has been proposed as the promoter kinase of its ubiquitination. Accordingly, the results obtained in this work using a specific inhibitor suggests that ERK is involved in the positive regulation mediated by the apoptotic agent over the transcriptional level of MDM2 at the beginning of apoptosis. Thus, not only Akt, but also ERK, by acting on FoxO3a would exert a survival effect, thus favoring the degradation of inactive FoxO3a and preventing the transcription of proapoptotic genes. With respect to the effect of E_2, the hormone upregulates MDM2 transcript level, thus promoting ubiquitination and p53 degradation, showing its antiapoptotic role. With respect to FoxO3a, the hormone would not exert any effect over its degradation in this period of time, since active/nonphosphorylated FoxO3a is unable of interacting with MDM2.

In conjunction, the results presented in this work support the notion that the transcription factors FoxO and p53 coordinate apoptosis in C2C12 cells, activating multiple mechanisms which favor the modulation of proapoptotic genes in response to stress, while the hormone exerts its antiapoptotic effect reverting some of these actions or maintaining others depending on the phase of the apoptotic process. Altogether, these data with our previous findings support the relevant role of E_2 in the inhibition of multiple cellular pathways in skeletal myoblast cells that acting in concert lead to apoptosis.

Since satellite cells are responsible of the repair of the injured muscle and the C2C12 cell line is a proper experimental model to study satellite cells, this work could contribute with molecular details to further understand the response of myoblasts to oxidative injury and the effects of estradiol in the regulation of apoptosis in skeletal muscle.

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