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Testosterone modulates FoxO3a and p53-related genes to protect C2C12 skeletal muscle cells against apoptosis

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ABSTRACT

The loss of muscle mass and strength with aging, sarcopenia, is a prevalent condition among the elderly, associated with skeletal muscle dysfunction and enhanced muscle cell apoptosis. We have previously demonstrated that testosterone protects against H_2O_2-induced apoptosis in C2C12 muscle cells, at different levels: morphological, biochemical and molecular. Since we have observed that testosterone reduces p-p53 and maintains the inactive state of FoxO3a transcription factor, induced by H_2O_2, we analyzed if the hormone was exerting its antiapoptotic effect at transcriptional level, by modulating pro and antiapoptotic genes associated to them. We detected the upregulation of the proapoptotic genes Puma, PERP and Bim, and MDM2 in response to H_2O_2 at different periods of the apoptotic process, and the downregulation of the antiapoptotic gene Bcl-2, whereas testosterone was able to modulate and counteract H_2O_2 effects. Furthermore, ERK and JNK kinases have been demonstrated to be linked to FoxO3a phosphorylation and thus its subcellular distribution. This work show some transcription level components, upstream of the classical apoptotic pathway, that are activated during oxidative stress and that are points where testosterone exerts its protective action against apoptosis, exposing some of the puzzle pieces of the intricate network that aged skeletal muscle apoptosis represents.

1. Introduction

The p53 pathway is promoted by a wide range of stimuli that threaten to affect genomic integrity or the proper cell proliferation. It is p53 the backbone of an intricate network, which integrates and translates these stimuli in the most appropriate response (cell cycle arrest, DNA repair, senescence, apoptosis) in order to prevent the cell damage spread [1,2]. P53 exerts its action essentially as a transcription factor, transcribing a program of a wide range of genes to accomplish its biological responses [2]. Depending on cell type, the stressor and the extracellular environment, the p53 activation may lead to apoptosis rather than induce cell cycle arrest, by transactivating a number of apoptotic genes such as Bax, Bid, Puma, Noxa, AIP-1 p53, PERP and the repression of anti-apoptotic genes such as Bcl-2 and Bcl-XL [3–5]. The clear elucidation of the role of the genes involved in p53-dependent apoptosis requires the analysis in a system suited to the complexity of the p53 apoptotic response, which is implemented through different assemblages of effectors according to the cellular context. Besides the transcriptional well known actions of p53, their transcriptionally independent activities [6] have been more difficult to define, and are much less well understood, with some continuing debate as to their importance or even existence, in the physiological response to p53.

Tight regulation of p53 function is critical for normal cell growth and development and one mechanism by which p53 function is controlled is through interaction with the MDM2 protein. In unstressed cells, p53 was maintained at low levels via its interaction with this protein, an E3 ubiquitin ligase that blocks p53 transcriptional activity directly (sterically) and mediates the proteasome-dependent degradation of the p53 protein. Under cellular stress, post-translational modification of p53, such as Ser15 phosphorylation, disrupts p53/MDM2 interaction, reducing thereby p53 ubiquitinization and degradation, which induces an increase in protein expression levels [7]. Meanwhile, the gene encoding MDM2 is under transcriptional control of p53, generating a feedback loop by which p53 controls its own degradation and return to basal levels after stress response [8].

The forkhead box class O (FoxO) subfamily of transcription factors has been receiving increased attention over the last years. Four different FoxO proteins are encoded by the mammalian genome: FoxO3a, FoxO4, FoxO6, and FoxO1 [9], which are conserved from Homo sapiens to yeast, both structurally and functionally. FoxOs binds to DNA response elements via the forkhead domain and regulates the expression of several genes, mediating oxidative stress by regulating the transcription of antioxidant enzymes such as MnSOD and catalase as well as genes involved in diverse physiological phenomena such as cell

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proliferation, the cell cycle, differentiation, cancer, apoptosis and aging [10–12]. Of relevance, several nodes of interaction have been identified between FoxO3a and p53, such as the binding between the two transcription factors [13], the stabilization of p53 by FoxO3a and the activation of p53-dependant apoptosis [14] and the modulation of the transcriptional activity of FoxO3a by p53, under oxidative stress conditions [15,16].

The major mechanism of FoxO transcription factors regulation in response to external stimuli is by changes in their subcellular localization. In the setting of growth factor stimulation, FoxO1, 3 and 4 are Akt-mediated phosphorylated which result in their nuclear export and the inhibition of their transcriptional activity [10,17,18]. Cytoplasmic FoxO proteins can not only be sequestered in cytosol but also ubiquitinated and degraded in the proteasome [18]. This is the case of FoxO3a, whose phosphorylation at three conserved serine/threonine residues (Thr32, Ser253 and Ser315) by Akt abrogates its access to DNA binding sites, inhibiting its transcription factor activity and thus, its proapoptotic action by avoiding the expression of several proapoptotic factors. The apoptotic effects of FoxO3a can also be jointly regulated by ERK and MDM2 [19]. ERK not only regulates MDM2 at the transcriptional level [20] but also can directly phosphorylate FoxO3a inducing thus, FoxO3a association to MDM2 [19]. So, the FoxO3a phosphorylation by ERK at three serine residues (Ser294, Ser344 and Ser425) would induce its ubiquitin-dependent degradation, reducing its circulating levels. On the other hand, oxidative stress causes the retention of FoxOs in the nucleus and the activation of their transcriptional activity, by promoting FoxO post-translational modifications, including phosphorylation on amino-acid residues distinct from those phosphorylated by Akt [11,21]. Thus, under stress stimuli FoxOs phosphorylation by MST1 and JNK override the sequestration of FoxOs in the cytoplasm by growth factors and trigger the relocalization of FoxOs from the cytoplasm to the nucleus [21,22].

Reactive oxygen species (ROS) are well known to be signaling molecules that regulate growth, differentiation [23,24], proliferation [25] and apoptosis [26,27], at least in physiological concentration. However, excessive ROS generation, defective oxidant scavenging, or both, have been implicated in the aging process and in the pathogenesis of several conditions, including acute muscle atrophy and sarcopenia [28,29]. The age-related muscle loss is a result of reduction in the size and number of muscle fibers [30] and it has been associated with a deficit of sex hormones, as the levels of estrogens and/or testosterone decline with aging. Thus, hormone replacement therapies prevent a decline in muscle performance [31,32]. It is well known that skeletal muscle is a target tissue for androgens and the beneficial effects of them on muscle mass, strength and regeneration are increasing [33–35]. 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 [36,37].

We have previously demonstrated that testosterone protects against hydrogen peroxide (H_2O_2)-induced apoptosis in the C2C12 muscle cell line at morphological, physiological and biochemical level [26,38], modulating p53 transcription factor [39]. 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 [40] having an important role in skeletal muscle recovered [41]. In this work, we postulated the protective effect of testosterone at transcriptional level by regulating the p53 target genes associated to apoptosis: Bcl-2, Puma, PERP, Bim, Noxa, and MDM2; and its relation to another transcription factor, Foxo3a. The data presented in this work allow us to continue elucidating the mechanism by which the hormone regulates apoptosis in skeletal muscle, providing this work an insight of what is happening at transcriptional level during apoptotic aged-skeletal muscle.

2. Materials and methods

2.1. Materials

Testosterone and PD98059 inhibitor were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-phospho-Foxo3a (Ser253), anti-phospho-Akt (Ser473) rabbit polyclonal antibodies and SP600125 inhibitor were from Cell Signaling Technology, Inc. (Danvers, MA, USA). Alexa Fluor 488-conjugated anti-rabbit secondary antibody was from Molecular Probes (Eugene, OR, USA). Anti-β-tubulin rabbit polyclonal antibody was purchased from Thermo Fisher Scientific (Rockford, IL, USA). LY294002 was purchased from Calbiochem (San 134 Diego, CA, USA). The ECL blot detection kit was provided by Perkin-Elmer, Inc (Waltham, MA, 135 USA). High Pure RNA Isolation kit (11828665001) was from Roche Diagnostics (Mannheim, Germany). High Capacity cDNA Reverse Transcription Kit (4368814) was purchased from Applied Biosystems, KAPA SYBR® FAST qPCR Kit Master Mix (2X) Universal (KR0389 – v8.12) was from Kapa Biosystems, Inc. (Woburn, MA, USA) and primer sets were from Invitrogen (Carlsbad, CA, USA). All other reagents used were of analytical grade.

2.2. Cell culture and treatment

C2C12 murine skeletal muscle cells, from the American Type Culture Collection (ATCC number: CRL-1772™) at Manassas, VA 20108, were cultured in growth medium (Dulbecco's modified Eagle's medium) supplemented with 10% heat-inactivated (30 min, 56 °C) fetal bovine serum, 1% nystatin, and 2% streptomycin. Cells were incubated at 37 °C in a humid atmosphere of 5% CO_2 in air. Cultures were passaged every 2 days with fresh medium. Under these conditions, C2C12 myoblasts resemble the activated satellite cells that surround the mature myofibers and proliferate and differentiate in response to muscle damage, being a proper experimental model to study muscle functions under stress or cell injury [40]. Cells were cultured in chamber slides for microscopy or in 10 cm plates (Greiner Bio-One, Frickenhausen, Germany) for Western blots. The treatments were performed with 70–80% confluent cultures (120,000 cells/cm^2) in medium without serum for 20 min. Then, treatments were carried out by adding 10^{-9} M testosterone at physiological concentration [26,38], the vehicle of the hormone, isopropanol (control) during 60 min (the isopropanol percentage in the culture medium assay of cells treated with the hormone or with the hormone vehicle alone, was less than 0.001%) or the corresponding inhibitors (LY294002, SP600125, PD98059) during 1 h, before induction of apoptosis with hydrogen peroxide (H_2O_2) during the periods of time indicated for each experiment. H_2O_2 was diluted in culture medium without serum at a final concentration of 1 mM. To obtain a total cell lysate after treatments, a buffer composed of 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.2 mM Na_2VO_4, 25 mM NaF, 1 mM PMSF, 20 μg/ml leupeptin, 20 μg/ml aprotinin and 20 μg/ml trypsin inhibitor, was employed. The lysates were collected by aspiration and centrifuged at 12,000g during 5 min to separate the particulate fraction from the soluble fraction. Protein concentration from the supernatant was estimated by the method of Bradford [42], using bovine serum albumin (BSA) as standard.

2.3. Subcellular fractionation

C2C12 confluent monolayers were scrapped and homogenized in ice-cold TES buffer (50 mM Tris/HCl pH 7.4, 1 mM EDTA, 250 mM sucrose, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 20 μg/ml leupeptin, 20 μg/ml aprotinin, 20 μg/ml trypsin inhibitor). The debris was separated by centrifugation at 100g for 5 min. The upper fraction was collected and a nuclear pellet was obtained by low speed centrifugation (300g, 20 min). The supernatant was further centrifuged at 10,000g for 20 min to pellet mitochondria. The remaining supernatant was centrifuged at 120,000g for 60 min, to

yield the cytosolic fraction and a plasma membrane containing particulate pellet (microsomes). Pellets were resuspended in lysis buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.2 mM Na_2VO_4, 2 mM EDTA, 25 mM NaF, 1 mM PMSF, 20 μg/ml leupeptin, 20 μg/ml aprotinin and 20 μg/ml trypsin inhibitor). Protein concentration from each fraction was estimated by the Bradford method as above. Contamination of nuclear, microsomal and cytosolic fractions with mitochondrial components was assessed by immunodetection of the specific mitochondria marker Smac/DIABLO. anti-Lamin B antibody was employed for immunodetection of the nuclear marker Lamin B in the different fractions.

2.4. Western blot analysis

Protein aliquots (~20 μg) were combined with sample buffer (400 mM Tris–HCl (pH 6.8), 10% SDS, 50% glycerol, 500 mM dithiothreitol (DTT), and 2 μg/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 horseradish peroxidase-conjugated secondary antibodies. The enhanced chemiluminescence (ECL) blot detection kit (Amersham, Buckinghamshire, England) was used as described by the manufacturer to visualize reactive products. When it was necessary, membranes were stripped with stripping buffer (62.5 mM Tris–HCl, pH 6.7; 2% SDS; 50 mM β-mercaptoethanol), washed with PBS 1% Tween-20 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 the primary antibody. After several washings with PBS–T, membranes were incubated with the secondary conjugated antibody. The corresponding immunoreactive bands were developed by means of ECL. Relative quantification of Western blot signals was performed using ImageJ software (NIH, USA) [43].

2.5. Immunocytochemistry

After treatments, semi-confluent (60–70%) monolayers were washed with serum-free phenol red-free DMEM, and then fixed and permeabilized during 20 min at −20 °C with methanol to allow intracellular antigen labeling. After fixation, cells were rinsed 3 times with PBS. Non-specific sites were blocked for 30 min in PBS that contained 5% bovine serum albumin. Cells were then incubated overnight at 4 °C, in the presence or absence (negative control) of the primary antibody specified (1:50 dilution). The primary antibodies were recognized by fluorophore-conjugated secondary antibodies.

2.6. Quantitative Real time RT-PCR

C2C12 cells were treated during different periods of time with 1 mM H_2O_2 or preincubated with 10^{-9} M testosterone during 1 h and then exposed to 1 mM H_2O_2 for the times specified. After treatments, total RNA (10^{-6} cells/condition) was extracted using the High Pure RNA Isolation kit (Roche Diagnostics, Mannheim, Germany) and approximately 2 μg of total RNA was reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., CA, USA) according to the manufacturer's instructions. Quantitative measurement of real-time PCR was done using KAPA SYBR® FAST qPCR Kit Master Mix (2X) Universal (KR0389 – v8.12) 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′ CGTCCCGTAGACAAAATGGT 3′, reverse 5′ TTGATGGCAACAA TCTCCAC 3′; Bcl-2 set: forward 5′ CGTCAA CAGGGAGATGTCA 3′, reverse 5′ TTCCACAAAGGCATCCCAGC 3′, Bim set: forward 5′ AATGTCTGACTCTGACTCTCGGAC 3′, reverse 5′ TCTCCGCAGGCTGCAATTGTCTAC 3′, Puma set: forward 5′ TACGAGCGGCGGAGACAAG 3′, reverse 5′ GTGTAGGCA CCTAGTTGGGC 3′, PERP set: forward 5′ ACCACATCCAGACATCGTCG 3′, reverse 5′ CTCGTCCCCATGCGTACTCC 3′, Noxa set: forward 5′ GTCGGAACGCGCCAGTGAACCC 3′, reverse 5′ TCCTTCC TGGAGGTCCCTTCTTGC 3′, MDM2 set: forward 5′ GCCTGGATCAGGATTCAGTTTCTG 3′, reverse 5′ GTGACCCGA TAGACCTCATCATCC 3′. The specificity of PCR products was confirmed by melting curve analysis. Relative quantification of gene expression was determined by the comparative C_T method [44].

2.7. Statistical analysis

Results are shown as means ± S.D. Statistical differences among groups were determined by ANOVA followed by a multiple comparison post hoc test. Data were considered significant at $p < 0.05$.

3. Results

3.1. Testosterone modulates pro and anti apoptotic genes during apoptosis induced by H_2O_2 in C2C12 cells

Apoptosis is a highly conserved and tightly gene regulated event that conduces to a real "smart cell suicide", in which cellular self-destruction occurs in a perfectly controlled manner, without inflammation or damage of neighboring cells [45]. In order to evaluate the gene expression during H_2O_2-induced apoptosis in C2C12 cells, and the possible regulation by testosterone, we analyzed by quantitative PCR, the relative levels of a number of genes associated to apoptosis, using the comparative C_T method described in the *Materials and Methods* section. Total RNA from cell cultures exposed to 1 mM H_2O_2 during different times, ranging between 30 min and 4 h, was isolated and employed to determine the relative levels of mRNA expression. In order to evaluate if testosterone was able to counteract H_2O_2 effect and if it was modulating gene expression, C2C12 cells were treated with 10^{-9} M testosterone during 60 min, and then exposed to 1 mM H_2O_2 for the same periods of time mentioned above. The mRNA was then isolated and the relative expression levels were quantified. The GAPDH was used as a reference gene.

BCL-2

Gene expression that modulate the engagement of apoptosis, such as Bcl-2 which promotes survival or the proapoptotic genes Bax, Bim, or Noxa, play a key role in the determination of this survival threshold and will arbitrate for cell survival or death after damage [46]. Bcl-2 protein was the first intracellular regulator of apoptosis to be identified [47], and it has been widely reported that high levels enhance cell survival under diverse cytotoxic conditions [48,49].

In C2C12 cells, H_2O_2 induced the time dependant downregulation of Bcl-2 gene expression, reaching their lower levels after 4 h of treatment. Testosterone pretreatment during 1 h before the addition of H_2O_2 counteracted the effects of the apoptosis inducer and enhanced the Bcl-2 mRNA levels, downregulated by H_2O_2. These results suggest that the antiapoptotic effect of testosterone in C2C12 cells involves the modulation of Bcl-2 at its transcription level (Fig. 1).

BIM

Bim is a novel p53 and FoxO3a target gene, member of the BH3-only subset of the Bcl-2 family proteins, which plays a key role in promoting apoptosis. It is firmly established that their BH3 domains can bind to some Bcl-2 family members that promote cell survival, and only those

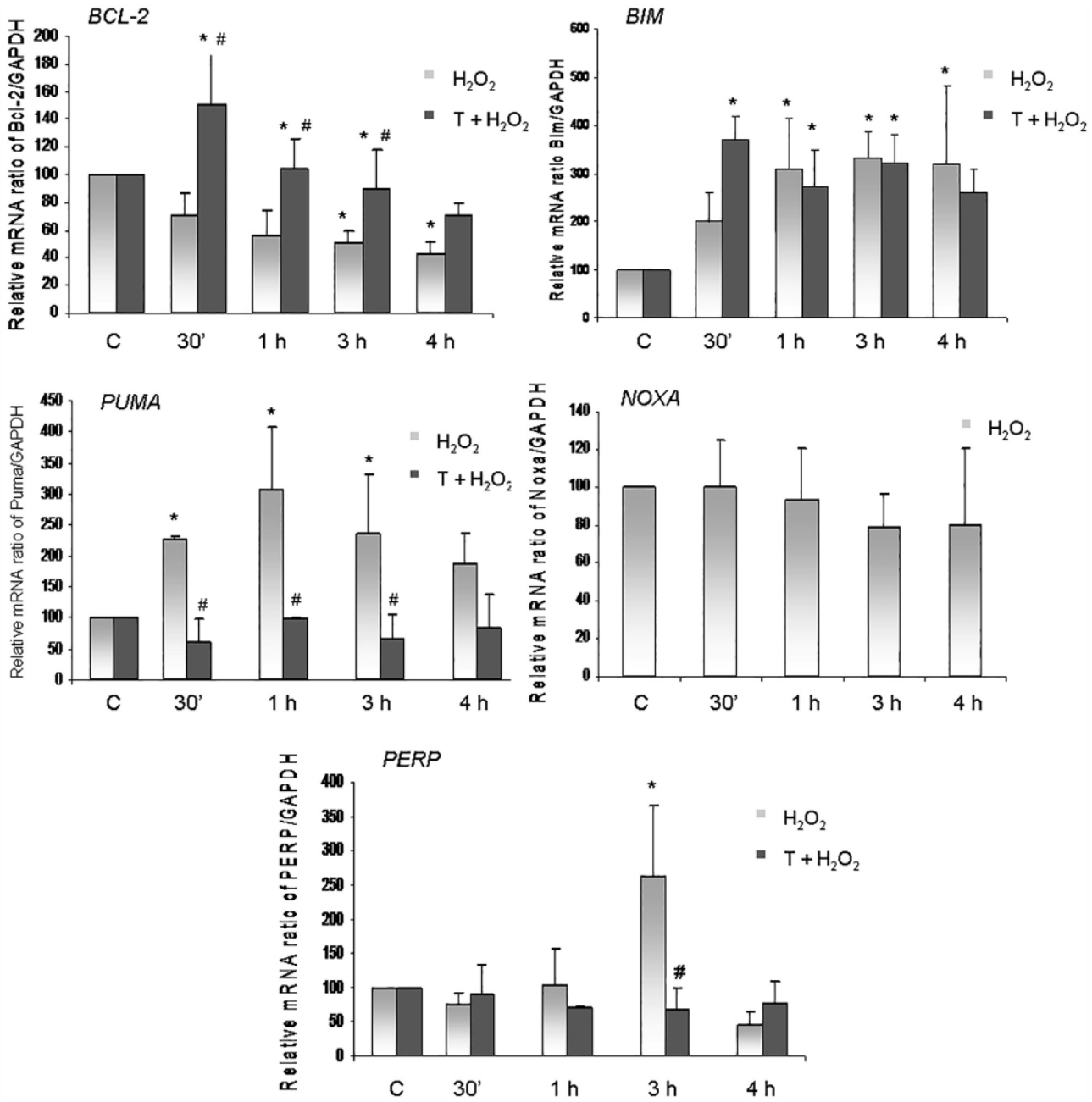

Fig. 1. Testosterone modulates H_2O_2-responsive genes in C2C12 cells. Transcript levels of Bcl-2, Bim, Puma, Noxa and PERP were determined by Real Time PCR as described in *Methods*, using C2C12 cultures preincubated with 10^{-9} M testosterone for 60 min and then exposed to 1 mM H_2O_2 for 30 min (T + H 30), 1 h (T + H 1 h), 3 h (T + H 3 h) and 4 h (T + H 4 h) or treated with 1 mM H_2O_2 alone for the same periods of time; (C) Untreated cells. Transcript levels are expressed in arbitrary units related to the expression of GAPDH gene expression. Averages ± S.D. are given; $^{*}p < 0.05$ respect to Control condition; $^{\#}p < 0.05$ respect to the counterpart H_2O_2 condition.

prosurvival relatives that bind to it, can neutralize its cytotoxicity [50,51].

When we analyzed the effect of the apoptotic agent on Bim transcriptional expression, we observed that H_2O_2 enhanced Bim mRNA levels from 30 min of treatment, with a maximum expression at 3 h of treatment, maintaining this effect for all the periods of time evaluated. Testosterone treatment before the addition of H_2O_2 slightly increased Bim levels at 30 min of treatment, whereas no significant changes were detected respect to H_2O_2 treatment, in the other times evaluated. Thus, with respect to the induction of apoptosis mediated by Bcl-2 protein family interactions, it is probably that the steroid was exerting its protective action at transcriptional level by upregulating antiapoptotic proteins (Bcl-2), more than by downregulating proapoptotic ones (Bim) (Fig. 1).

PUMA

Amongst the numerous genes whose expression is directly stimulated in response to p53 activation, a number of them are involved in the two main apoptotic pathways. Puma (p53 up-regulated modulator of apoptosis) and Noxa are among the proapoptotic p53-target genes whose products can localize at the mitochondria. Both of them share homology with Bcl-2 family proteins, but only within a short stretch of amino acids termed the BH3 domain (Bcl-2 homology 3). Puma is extremely effective in inducing apoptosis: when expressed, it kills cancer cells within a few hours, and gene knockouts in human colorectal cancer cells showed that Puma was required for apoptosis induced by p53, hypoxia and DNA-damaging agents [52,53].

In C2C12 cells, oxidative stress induced by 1 mM H_2O_2 increased

Puma expression from 30 min of treatment, with a maximum activation at 1 h. From then on, Puma mRNA decreased but maintained its levels over the control. Testosterone pretreatment was able to reverse the upregulation of Puma induced by H_2O_2, showing the protective effect of the steroid by negative modulating the expression of this proapoptotic gene (Fig. 1).

NOXA

As it was previously mentioned, Noxa is a "BH3-only" member of the Bcl-2 family that was shown to be a target of p53-mediated transactivation and functions as a mediator of p53-dependent apoptosis through mitochondrial dysfunction. Although Noxa is a primary p53-response gene, in certain tissues, induction of Noxa occurred efficiently also in the absence of p53 indicating that, Noxa induction in response to DNA damage can also occur in a p53-independent manner [54]. When we studied the effects of H_2O_2 on Noxa mRNA expression in our experimental model, we observed no significant changes on gene levels, suggesting that this proapoptotic protein would not be involved in H_2O_2-induced apoptosis in C2C12 cells (Fig. 1).

PERP

PERP (p53 apoptosis effector related to PMP-22) is expressed in a p53-dependent manner and its transcriptional activation by p53 appears essential for PERP's ability to induce cell death and its overexpression is sufficient to induce apoptosis in fibroblasts, thymocytes and neurons. Furthermore, compelling evidence indicates that PERP functions only to induce apoptosis and not cell cycle arrest being its function cell type specific [55,56].

When we analyzed the effect of the apoptotic agent on PERP expression at transcription level, we observed that H_2O_2 induced a maximum peak of expression at 3 h of treatment, from then on the levels of PERP mRNA decreased up to control, suggesting the participation of PERP in H_2O_2-induced apoptosis in C2C12 cells. In order to evaluate if testosterone was exerting its antiapoptotic action by reducing this proapoptotic gene expression, cells where pretreated with the steroid for 1 h and then exposed to H_2O_2. The protective effect of testosterone against apoptosis was evidenced by counteracting the effect of the oxidative stress on the upregulation of PERP (Fig. 1).

3.2. FoxO3a involvement in H_2O_2-induced apoptosis in C2C12 cells

The *Forkhead box O* (FoxO) transcription factor subfamily has been widely described as regulators of homeostasis mainly under stress, such as oxidative stress damage and apoptosis. The FoxOs are phosphorylated in response to a variety of stimuli conducing to their subcellular relocalization in order to induce or suppress their action. With the aim of study the effect of the apoptotic agent on the transcription factor FoxO3a activation in C2C12 cells, cultures were exposed to 1 mM H_2O_2 during different periods of time (30 min, 1 h y 3 h). In total homogenates, Western blot assay showed that H_2O_2 induced FoxO3a phosphorylation on Ser253 at 30 min and 1 h treatment times (Fig 2A). When cell cultures are preincubated with the PI3 K inhibitor, LY294002 for 1 h, the phosphorylation of FoxO3a, induced by H_2O_2 is significantly reduced, showing that PI3 K/Akt signaling pathway is actually upregulating FoxO3a phosphorylation in C2C12 cells. In accordance, it was observed that Akt was activated/phosphorylated at the same periods of time of p-FoxO3a detection, in response to H_2O_2 treatment (Fig. 2A). This effect was also observed by fluorescence microscopy. An increase in green fluorescence was detected after 30 min–1 h of treatment with H_2O_2 while pretreatment with the Akt inhibitor reduced FoxO3a phosphorylation, involving thus the PI3K/Akt pathway in the serine 253 FoxO3a phosphorylation induced by oxidative stress (Fig. 2B).

When we examined the subcellular fractions by Western blot, we detected cytosolic p-FoxO3a under basal conditions (Control) and at short time of exposure to H_2O_2. Then, the levels slightly decreased in cytosol after longer H_2O_2 treatment times, being almost absent after 3 h of H_2O_2. Moreover, phosphorylated FoxO3a was highly detected in nucleus after 1–2 h with H_2O_2, suggesting the possibility that the transcription factor translocates to the nucleus even if it is phosphorylated in Ser253 or that FoxO3a was phosphorylated inside the nucleus. After 2 h treatment with the apoptotic agent, nuclear p-FoxO3a detection was reduced, suggesting a possible dephosphorylation and, in consequence, FoxO3a activation in order to regulate gene expression (Fig. 3A).

Similar results were obtained when immunocytochemical detection of p-FoxO3a was performed in C2C12 cultures by fluorescence microscopy. It was observed not only an increase in the fluorescence after oxidative stress induced by H_2O_2, with a maximum intensity of the nuclear fluorescence after 1 h of the apoptotic stimuli, but also a change in the subcellular distribution of the transcription factor. Under basal conditions (Control) the fluorescence is homogeneously distributed among the cell, whereas H_2O_2 treatment enhances the nuclear localization of the phosphorylated protein (Fig. 3B).

Given that FoxOs have been reported to be phosphorylated by JNK and that previous results of our laboratory showed the activation of the kinase after 30 min–1 h of H_2O_2 treatment in C2C12, we study if JNK could be modulating FoxO3a activity in response to the apoptotic agent. We observed that cell incubation with SP600125 before H_2O_2 treatment diminishes FoxO3a phosphorylation, whereas Akt remained phosphorylated, at short term of H_2O_2 stimulation, in the presence of the JNK inhibitor. These results suggest that both PI3K/Akt pathway and JNK participates in the phosphorylation of FoxO3a in serine 253 independently during the apoptotic process (Fig. 4).

3.3. Testosterone maintains inactivated levels of FoxO3a during the apoptotic process in C2C12 cells

In order to evaluate if testosterone exerts any effect on the activation/inactivation of FoxO3a in response to H_2O_2, cells were preincubated with testosterone before the addition of 1 mM H_2O_2 during 30 min and 1 h (maximum FoxO3a phosphorylation). No significant changes in FoxO3a phosphorylation were detected after testosterone treatment in total homogenates, suggesting that the hormone maintains the levels of the inactive mode (Ser253 phosphorylated) of the transcription factor (Fig. 5).

3.4. Testosterone and ERK modulate the transcription of MDM2 induced by H_2O_2 in C2C12 cells

The human homologue of the mouse double-minute gene MDM2 is the main regulator of p53, by limiting the p53 tumor suppressor function [8]. Transcription of the MDM2 gene is regulated by two distinct promoters (P1 and P2). The P1 promoter controls the basal constitutive expression of MDM2 [57] whereas the P2 promoter is highly regulated and responsible for the inducible expression of MDM2. MDM2 itself is the product of a p53-inducible gene [8] so p53 stimulates the expression of the ubiquitin ligase inducing its own degradation. Thus, the two molecules are linked to each other through an autoregulatory negative feedback loop which maintains low cellular p53 levels in the absence of stress.

We analyzed MDM2 gene expression in C2C12 after H_2O_2-induced oxidative stress and we observed an increase over control in MDM2 mRNA levels from 30 min of treatment to 1 h. However, long term treatment times, conduced to a decrease in MDM2 expression up to control levels. Testosterone preincubation before the addition of the apoptotic agent led to an increase in MDM2 expression over the effect of H_2O_2, being even more markedly at 3 h of treatment (Fig. 6).

In addition to p53, MDM2 can be transcriptionally regulated by several oncogenic and tumor suppressive pathways. As it was

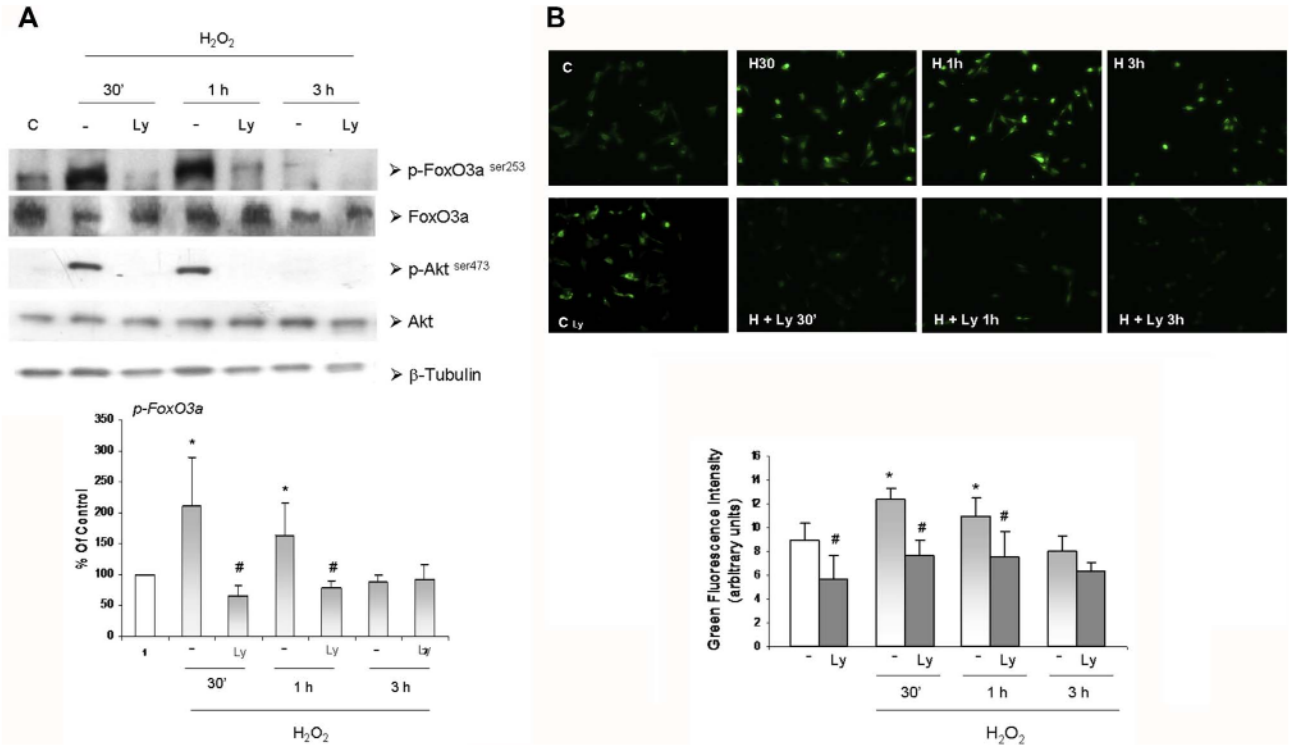

Fig. 2. H_2O_2 induces FoxO3a phosphorylation through Akt, at short time of exposure, in C2C12 cells. A) C2C12 were incubated with DMEM without serum (C), H_2O_2 (1 mM) during different periods of time (30 min, 1 h and 3 h) or LY294002 (25 μM, 1 h) before the addition of H_2O_2 (1 mM for 30 min, 1 h and 3 h). After treatments, total cell homogenates were subjected to Western blot assays using anti-p-Foxo3a (Ser 253), anti-FoxO3a, anti-p-Akt Ser473 and anti-Akt antibodies. β-Tubulin levels are shown as protein loading control. The bands were quantified by densitometry. The blot is representative of three independent experiments with comparable results. Averages ± S.D are given. $^{*}p < 0.05$ respect to the control (C); $^{\#}p < 0.05$ respect to the counterpart H_2O_2 condition. B) Cells grown on coverslips were incubated with DMEM without serum (C), treated with 1 mM H_2O_2 for the specified periods of time or incubated with LY294002 (25 μM, 1 h) before the addition of H_2O_2 (1 mM for 30 min, 1 h and 3 h). Then cells were labeled with anti-p-FoxO3a (Ser 253) as described in *Methods*. At least ten fields per slide were examined by fluorescence microscopy. Representative photographs are shown. Magnification: 20X. A fluorescence intensity graphic is shown, $^{*}p < 0.05$ respect to control; $^{\#}p < 0.05$ respect to H_2O_2 condition.

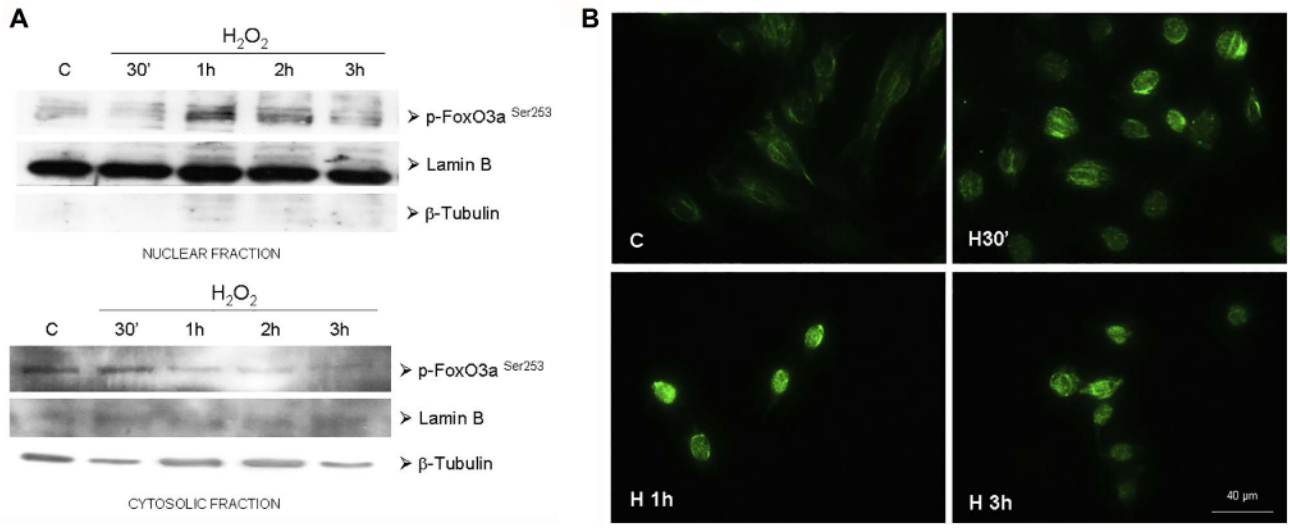

Fig. 3. H_2O_2 induces FoxO3a redistribution in C2C12 cells. A) Cell cultures were incubated with DMEM without serum (C) or with H_2O_2 (1 mM) for the indicated times. Then, the nuclear and cytosolic fractions were isolated as described in *Methods* and analyzed by Western blot using a specific anti-p-FoxO3a (Ser 253) antibody. Lamin B was used as nuclear marker and β-Tubulin as cytosolic marker. B) Cells grown on coverslips were treated with DMEM without serum (C) or with 1 mM H_2O_2 for different periods of time (30 min, 1 h and 3 h) and then labeled with anti-p-FoxO3a (Ser 253) as described in *Methods*. At least ten fields per slide were examined by fluorescence microscopy. Representative photographs are shown. Magnification: 63×.

mentioned in the Introduction section, the Ras–MEK–ERK mitogen-activated protein kinase (MAPK) pathway has been shown to upregulate MDM2 expression. Besides, a further connection between ERK and MDM2 has been drawn, showing that these two oncoproteins coordinately regulate FoxO3a, favoring its degradation [19].

In order to evaluate the involvement of ERK MAPK in the regulation of MDM2 gene expression under stress conditions in our experimental model, C2C12 cells were preincubated with 10 μM PD98059 during 1 h before the treatment with 1 mM H_2O_2 for 30 min, 1 h, 3 h and 4 h. At short times of exposure to the apoptotic agent (30 min and 1 h) the inhibition of ERK reduced the levels of the MDM2 mRNA upregulated by H_2O_2, to control level, whereas at 3 and 4 h of treatment, ERK

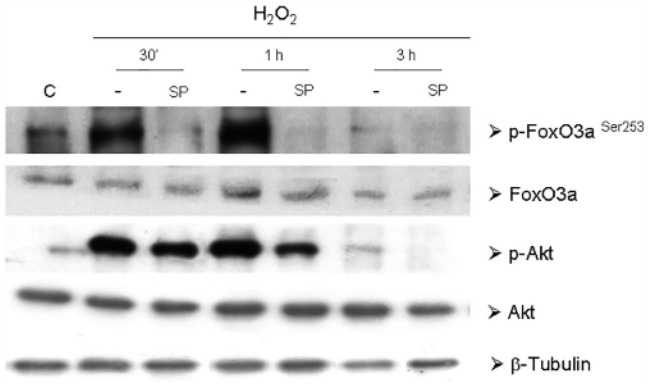

Fig. 4. JNK participates in FoxO3a Ser 253 phosphorylation in C2C12 cells. C2C12 were incubated with DMEM without serum (C), H_2O_2 (1 mM) during different periods of time (30 min, 1 h and 3 h) or SP600125 (10 μM, 1 h) before the addition of H_2O_2 (1 mM for 30 min, 1 h and 3 h). After treatments, cell lysates were subjected to Western blot assays using anti-p-Foxo3a (Ser 253), anti-p-Akt (Ser 473), anti-FoxO3a, and anti-Akt antibodies. β-Tubulin is shown as loading protein control. Blots are representative of three independent experiments with comparable results.

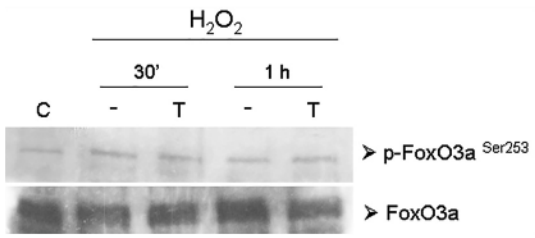

Fig. 5. Testosterone maintains FoxO3a in its inactivated mode. C2C12 cultures were incubated with DMEM without serum (C), H_2O_2 (1 mM) during 30 min and 1 h or preincubated with 10^{-9} M testosterone for 60 min and then exposed to 1 mM H_2O_2 for 30 min and 1 h. After treatments, total cell homogenates were subjected to Western blot assays using anti-p-Foxo3a (Ser 253) and anti-FoxO3a. The blot is representative of three independent experiments with comparable results.

inhibition enhanced the levels of MDM2 mRNA over H_2O_2 treatment, with values similar to those obtained with testosterone treatment (Fig. 6). These results suggest the participation of ERK in the modulation of the transcription of MDM2 gene.

4. Discussion

The protective and beneficial actions of androgens have been reported in a wide range of tissues such as cardiovascular system, pancreas, neurons, testis, and bone [58–62]. It is well known that skeletal muscle is one of the greatest target tissues of androgens, being the androgen-AR signaling pathway required for skeletal muscle development and for sustaining muscle mass, strength and protein synthesis, having thus an important role in determining body composition [63]. We have previously reported that testosterone protects skeletal muscle C2C12 cells against apoptosis through a mechanism involving intermediates of the apoptotic intrinsic pathway and the androgen receptor (AR), preventing the loss of mitochondrial membrane potential and the opening of the mitochondrial permeability transition pore (mPTP) [26,38,39]. Furthermore, testosterone negatively regulates the activation of p53 in response to the apoptosis inducer H_2O_2, being probably through this transcription factor that the steroid further exerts its protective action at the transcriptional level [39]. In this work and in order to deep the knowledge of the antiapoptotic mechanism activated by testosterone in C2C12 cells, we analyzed the expression of genes that promote or antagonize cell survival, inducible by transcription factors modulated by testosterone.

The p53 tumor suppressor protein exerts an essential role as damage-control system, in order to limit the propagation of damaged cells, being apoptosis one of the most important antiproliferative responses, translated in response to a range of stimuli that threaten to alter the genomic integrity or to affect the proper cell proliferation [1,2]. We have previously observed that oxidative stress caused by the exposure to H_2O_2, induces p53 activation in C2C12 cells in a time-dependent manner with a maximum level of phosphorylation at 1–2 h of treatment. However, this activation of p53 induced by oxidative stress stimulus is reduced by the preincubation with testosterone. Probably, the stressful oxidative condition to which C2C12 are subjected with H_2O_2 treatment, are attenuated in the presence of the hormone and the environmental conditions become less adverse, being the stress stimulus that induces p53 activation, less intense [39].

P53 is activated following stress and initiates a heterogeneous response in a cell-, tissue- and (type and intensity) stress-dependent manner. The precise combination of the signals received, or the marks that they can leave on the protein, by post-translational modifications, dictates the behavior of p53 in any given situation. Thus, the specific set of circumstances, dictates whether the p53 response is dependent or independent of transcription, the timing and range of gene expression altered, and so governs the fate of the cell [1]. The induction of certain sets of target genes is one of the mechanisms by which p53 integrates stress signals into a cellular response. The genes involved in cell-cycle arrest were found to be the first to have high expression levels, being the apoptotic ones expressed at intermediate and later stages [64]. Low p53 levels activate genes with high-affinity promoters that tend to be associated with cell-cycle arrest, and high p53 levels activate low-affinity promoters that tend to be involved in the apoptotic response [65].

Works of our laboratory have shown that the apoptotic agent H_2O_2 induces apoptosis in C2C12 cells in a time-dependent manner. At short times of exposure, H_2O_2 triggers the activation of a cell defense

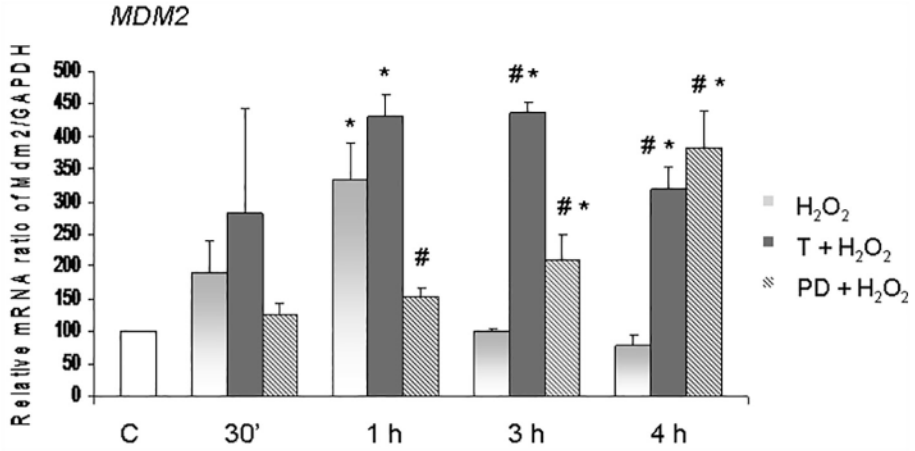

Fig. 6. Testosterone and ERK modulate MDM2 mRNA transcript level in response to H_2O_2 in C2C12 cells. Transcript levels of MDM2 were determined by Real Time PCR as described in *Materials and Methods* section, using C2C12 cultures preincubated with 10^{-9} M testosterone for 60 min and then exposed to 1 mM H_2O_2 for the times specified (T + H_2O_2); preicubated with PD PD98059 (10 μM, 1 h) before addition of H_2O_2 (1 mM) during the indicated times (PD + H_2O_2) or treated with 1 mM H_2O_2 alone for the same periods of time (H_2O_2); (C) Untreated cells. Transcript levels are expressed in arbitrary units related to the expression of GAPDH gene expression. Averages ± S.D. are given; $^{*}p < 0.05$ respect to Control condition; $^{\#}p < 0.05$ respect to the counterpart H_2O_2 condition.

response, whereas at long treatment times, the programmed cell death finally starts, and it is at this time when the protective action of testosterone against apoptosis is more evident [38]. In parallel, nuclear localization of p53 significantly increases in C2C12 cells after 1–2 h of H_2O_2 treatment [39], probably modulating target genes that mediate these apoptotic processes.

Numerous p53 target genes have been identified to be involved in the induction of apoptosis instead of promoting cell cycle arrest, at a given stress signal. By the transactivation of a number of apoptotic genes such as Bax, Bid, Puma, Noxa, AIP-1 p53, PERP [3] and repression of antiapoptotic genes such as Bcl-2 and Bcl-XL [4,5], p53-dependant apoptosis can be exerted through multiple genes acting in concert. It is possible that not all of these target genes are induced in all tissues or in response to all signals.

Although p53 has been implicated in both pathways, it predominantly seems to influence the intrinsic pathway. Thus, an important role of Bcl-2 family proteins in p53 dependant cell death is not unexpected given the vital role of the mitochondria in apoptosis and the vital role of these proteins in regulating mitochondrial integrity. The proapoptotic member Bax was the first Bcl-2 protein identified as a direct target of p53 [66]. On the other hand, the antiapoptotic member, Bcl-2, was the first member of the family to be discovered, and it is another p53 target gene. It has been demonstrated the existence of a negative response element in the Bcl-2 gene through which p53 may either directly or indirectly transcriptionally downregulate expression of this gene involved in programmed cell death [66]. In this research, it was shown that C2C12 treatment with the apoptotic agent H_2O_2 conduces to the downregulation of the transcription level of Bcl-2, whereas pretreatment with testosterone, counteracts the effect of H_2O_2 enhancing the Bcl-2 mRNA. This effect confers the steroid another protective action by the upregulation of the antiapoptotic protein at transcriptional level.

Initiation of apoptosis requires not only pro-apoptotic Bcl-2 family members, but also distant cousins that are related only by the small BH3 protein-interaction domain. The BH3-only proteins are sentinels that detect developmental death cues or intracellular damage. The BH3-only proteins are expressed and activated following cellular stress scenarios and are hypothesized to promote the mitochondrial outer membrane permeabilization by interacting with the anti-apoptotic Bcl-2 proteins or inducing the oligomerization and pore forming function of the pro-apoptotic Bcl-2 effector proteins, Bak and Bax [67,68]. Some of these proteins, including Puma and Noxa, were identified as transcriptional targets of p53. Unlike Noxa, that generally has less intense activity, the dramatic effect that the loss of Puma has on the sensitivity of different cell types to p53-induced cell death has been described [69] indicating that Puma is a crucial mediator of apoptosis in response to p53. In this work, it was demonstrated that H_2O_2 conduces to the upregulation of Puma mRNA levels in C2C12 skeletal muscle cells, whereas no significant difference respect to control were detected in the transcript levels of Noxa gene. Testosterone pretreatment decreased Puma mRNA levels, counteracting thus, the proapoptotic role of this member of the Bcl-2 family. Given that our previous reports showed a coordinately pattern of p53 activation respect to the induction of the transcription of Puma [39], we could suggest that Puma is under the transcriptional regulation of p53 during H_2O_2 treatment, contributing to C2C12 cell death. However, further studies need to be done to confirm this. The binding of Puma to the inhibitory members of the Bcl-2 family (Bcl-2-like proteins) via its BH3 domain seems to be a critical regulatory step in the induction of apoptosis. It results in the displacement of the proteins Bax and/or Bak [52], followed by their activation and the formation of pore-like structures on the mitochondrial membrane, which permeabilizes the outer mitochondrial membrane, leading to mitochondrial dysfunction and caspase activation. Given that our previous works showed that testosterone reduces the levels of Bax expression and prevents the loss of mitochondrial membrane potential [26], these results further assign another mitochondrial protective effect to the steroid, by reducing the transcription levels of Puma mRNA.

Bim is another BH3-only member of the Bcl-2 family. Unlike Noxa and Puma, it can interact with the antiapoptotic repertoire as well as the effectors, and can directly induce Bak and Bax oligomerization and mitochondrial outer membrane permeabilization. Here, we demonstrated that H_2O_2-induced apoptosis conduces to the upregulation of Bim mRNA in C2C12 cells. As it was mentioned before, H_2O_2 downregulates Bcl-2 gene expression, at the same time that enhances the levels of Bim mRNA. Given the capacity of Bim to both interact with antiapoptotic Bcl-2 proteins and act as a direct activator of proapoptotic effectors, the Bim apoptotic role would prevail, under oxidative stress conditions, against protective action of Bcl-2. The Bcl-2 levels would not be enough to neutralized Bim action, being promoted cell death. Contrary to what it was expected, testosterone treatment previous H_2O_2 could not reduce Bim transcription levels, and even slightly increased them during the first 30 min of treatment. However, as mentioned above, the hormone transcriptionally increased Bcl-2 level, so due to the net levels and the interaction of these proteins, the apoptotic action of Bim as direct inducer of Bak and Bax oligomerization, could be inhibited by Bcl-2 during testosterone treatment.

PERP (p53 apoptosis effector related to PMP-22), as a tetraspan protein, represents a novel type of effector involved in p53-dependent apoptosis. The transcriptional activation of PERP by p53 appears essential for PERP's ability to induce cell death and its overexpression is sufficient to induce apoptosis in fibroblasts, thymocytes and neurons. Furthermore, PERP gene is highly expressed in cells undergoing p53-dependent apoptosis as compared to cells undergoing p53-dependent G1 arrest. Although its exact mechanism of action has not been elucidated, it is known that it functions only to induce apoptosis and not cell cycle arrest [55,56], being its requirement dictated by cellular type and context [55]. In this work, it has been shown that PERP gene is upregulated by H_2O_2-induced apoptosis in C2C12, whereas testosterone pretreatment reduced the levels of PERP mRNA. This effect further support the idea of the antiapoptotic role of the hormone, by transcriptionally downregulating this proapoptotic factor which is mediating apoptosis in C2C12. Testosterone is probably modulating this effect by blocking the p53 transcription activity, previously reported [39].

FoxO factors have been implicated in a wide range of cellular functions, including regulation of cell cycle, apoptosis, atrophy, DNA repair, energy metabolism, and defense against oxidative stress [11,12]. In skeletal muscle, FoxOs contribute to processes, such as myocyte fusion, metabolism regulation and atrophy [70–72]. Their activity is tightly controlled by signaling pathways through post-translational modifications, namely phosphorylation, acetylation, ubiquitination, and protein interactions [11]. Particularly, FoxO transcription factors are important downstream targets of the PI3K/Akt signaling pathway. Phosphorylation by PI3K/Akt controls a shuttling system that modulates FoxO activity [17]. Inhibition of PI3K/Akt allows dephosphorylation and nuclear translocation of Foxo3a leading to the activation of its transcriptional activity [17].

In this work, it has been demonstrated that FoxO3a, during the first hour of apoptosis induction, is maintained phosphorylated/inactive (in serine 253 residue) due to the downregulation mediated by Akt. The stay of p-FoxO3a in the cytosol in the onset of H_2O_2-induced apoptosis, avoid the activation of proapoptotic genes, limiting the propagation of the apoptotic signal and the execution of apoptosis. Accordingly with our previous research, this effect could be part of the defense response activated by C2C12 cells in response to the apoptotic agent [26]. At short time of exposure to H_2O_2, cells activate survivor pathway (such as PI3K/Akt activation up to 2 h) in order to avoid apoptosis. If the apoptosis inducer persists over this time, this mechanism cannot be sustained, and skeletal muscle cells finally get into apoptosis. Although PI3K/Akt could be promoting cell survival phosphorylating FoxO3a, in our experimental model, after 1 h of H_2O_2 treatment, phosphorylated

FoxO3a was located mainly in the nucleus.

In regards with the phosphorylation location, besides the classical knowledge that describes that Akt activation occurs at the plasma membrane followed by translocation of active Akt to the nucleus, lines of evidence indicates that Akt can be also directly activated in the nucleus by nuclear pools of PI3K [73,74]. Thus, it is possible that FoxO proteins can be phosphorylated both in the cytoplasm and in the nucleus and that in different conditions different pools of Akt may target FoxO proteins at different locations. Because of the urgent need of protection, it is possible that Akt phosphorylates FoxO3a inside the nucleus given that in its phosphorylated state FoxO3a cannot bind DNA, so it cannot regulate apoptotic gene expression. It is possible that if apoptotic signal endures, nuclear p-FoxO3a starts to be dephosphorylated to exert its apoptotic action (Fig. 2B and 3A).

In accordance with the antiapoptotic effect of testosterone observed by our group in skeletal muscle, here it was evidenced that preincubation with the hormone prior to the induction of apoptosis, maintains FoxO3a phosphorylation levels at short time of exposure to H_2O_2, probably blocking thus its transcription action and sustaining the defense response of C2C12 cells.

As it was mentioned, the nuclear localization of FoxO3a is a prerequisite for transcriptional transactivation of apoptotic target genes and, accordingly, the majority of FoxO3a was located in the cytoplasm under control conditions. However, it is possible that cells remain healthy, despite the apparent high levels of FoxO3a found in their nucleus at short time of exposure to H_2O_2 (1 h). This observation would suggest that there is a threshold for the amount of FoxO3a present in the nucleus that is required to induce cell cycle arrest and apoptosis [75,76]. The apoptosis induced by H_2O_2 in C2C12 cells could be in part mediated by increased nuclear translocation of FoxO3a to a level at which exceeds this threshold thereby allowing transcription of FoxO target genes. However, it is likely that there are other factors, which potentiate the activity of FoxOs once they are within the nucleus, such as direct phosphorylation by JNK1/2 [77] and other kinases.

It has been demonstrated that JNK is capable of modulating FoxO3a nuclear translocation in invertebrates and in breast cancer mammalian cells [78]. In this work, the employment of the JNK inhibitor SP600125, led us to observe that phosphorylation of FoxO3a triggered by the apoptotic agent is regulated not only by Akt, but also by JNK in C2C12 cells. These results are in accordance with the H_2O_2-mediated activation of JNK that we have previously reported [39,79], which is slightly evidenced at 30 min, enhances its activation at 1 h of treatment and persists on time of exposure to H_2O_2. To our knowledge, no JNK phosphorylation site on Foxo3a has been identified yet. Thus, a direct role for JNK on Foxo3a seems unlikely, so it is probably that this kinase exerts its effect by an indirect way. Since we have previously reported Akt activation at short time of exposure to H_2O_2 (until 2 h of treatment) and the simultaneous activation of JNK from 1 h of H_2O_2 remaining activated until 4 h [26,39,79], JNK could be participating in FoxO3a nuclear relocation through JNK-dependent inhibition of the PI3K/Akt signaling pathway, as it was described in human breast carcinoma cells [78], at long term H_2O_2 treatment times. Furthermore, it appears that, in eukaryotic cells, JNK could exert a role on FoxO3a through phosphorylation of 14-3-3 protein and subsequent release of FoxO3a [80] or by phosphorylation of p66Shc, which in turn indirectly stimulates FoxO phosphorylation [81]. Of relevance, we previously demonstrated that JNK mediates p66Shc phosphorylation and its nuclear-mitochondrial translocation in response to H_2O_2-induced apoptosis in C2C12 cells [39], so it is possible that JNK-p66Shc pathway could also be modulating FoxO3a phosphorylation/inactivation in the nucleus, probably to avoid the transcription of antioxidant enzymes associated to FoxO transcription factors, and thus let p66Shc to exert its proapoptotic oxidative function.

Cellular p53 protein levels are the single most important determinant of its function, being through the stability and degradation of the protein, the principal way in which p53 levels are controlled. In normal unstressed cells, p53 is a very unstable protein with a short half-life ranging from 5 to 30 min, which is present at very low cellular levels owing to continuous degradation largely mediated by MDM2 [8], while under stress conditions its half-life is considerably prolonged. MDM2 is the main antagonist of p53 function, that both blocks p53 transcriptional activity directly (sterically) and mediates its degradation through an ubiquitin dependent pathway on nuclear and cytoplasmic 26S proteasomes [8]. MDM2 itself is also a transcriptional target of p53, which creates an autoregulatory negative loop whereby p53 controls the expression of its own negative regulator, maintaining low cellular p53 levels in the absence of stress. Furthermore, there are additional controls that modulate p53 activity through post-translational modifications, such as phosphorylation in serine 15, which conduce to the disruption of the p53-MDM2 complex, leading to the accumulation of active p53 in the cell [82]. Moreover MDM2 ubiquitin-proteasome pathway has been implicated in ERK-phosphorylated FoxO3a degradation [19]. Here, we have observed that at the beginning of apoptosis induction (30 min–1 h treatment with H_2O_2) MDM2 transcription level is upregulated, in order to enhance protein levels for the interaction with both p53 and FoxO3a and thus, the promotion of their degradation. The elevation of MDM2 mRNA levels in the first phase of apoptosis (short term of H_2O_2 exposure) in C2C12 cells could be part of the defense response, previously reported [26] to protect cells from apoptosis, by promoting the degradation of p53 and FoxO3a. However, at longer treatment times (3–4 h, H_2O_2) the decrease in MDM2 mRNA transcript to control condition, occurs. These results suggest that in advanced stages of apoptosis, the prevalent action of p53 and FoxO3a proapoptotic transcription factors have taken place and gotten the cells into the apoptotic program, conducing the MDM2 levels to basal condition and, as a consequence, to the decrease in the rate of p53 and FoxO3a degradation. Another point of view leads us to think about the time of the outcome apoptosis. The period of incubation of 1 h with the apoptotic agent appears to be a key point in the apoptosis of C2C12 cells. Is at this time when the activation of several kinases involved in the apoptotic process [39,79], and nuclear p53 and FoxO3a are detected. At 3 h and 4 h of treatment with H_2O_2, cells are already committed to apoptosis, and thus the levels of MDM2 mRNA decrease. As it was mentioned before, ERK has been proposed as a promoter kinase of FoxO3a ubiquitination. By the employment of a specific ERK pathway inhibitor, it has been demonstrated in this work, that ERK participates in the upregulation induced by the apoptotic agent of the transcriptional level of MDM2 at the beginning of apoptosis. Besides Akt, ERK would exert a survival effect by acting on FoxO3a, favoring the degradation of FoxO3a and preventing the transcription of proapoptotic genes modulated by it. We have also noticed that testosterone positively regulates MDM2 transcript level, promoting by this way the ubiquitination and degradation of p53, showing its antiapoptotic role.

5. Conclusion

The results presented in this work involve p53 and FoxO3a transcription factors in the apoptotic response of C2C12 against H_2O_2, by the activation of different conjugated and simultaneous mechanisms that promote the modulation of apoptotic genes. We have provided evidence that supports the protective role of testosterone previously reported, this time by counteracting the effect of H_2O_2 in the modulation of the transcription of genes involved in different steps of the apoptotic process. Thus, 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_2O_2 in C2C12 skeletal muscle cell line. This experimental model resembles the activated satellite cells that surround the mature myofibers that participate in the repair of skeletal muscle when a cellular injury exists. Thus, this work could provide details of the molecular mechanisms activated by the hormone during myopathies that lead to the loss of muscle mass, associated with hormonal deregulation.

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