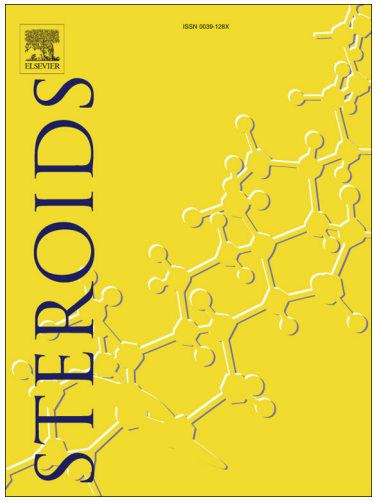

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PII: S0039-128X(15)00305-0
DOI: http://dx.doi.org/10.1016/j.steroids.2015.12.007
Reference: STE 7880

To appear in: *Steroids*

Received Date: 22 April 2015
Revised Date: 10 November 2015
Accepted Date: 10 December 2015

Please cite this article as: Pronsato, L., Milanesi, L., Effect of testosterone on the regulation of p53 and p66shc during oxidative stress damage in c2c12 cells, *Steroids* (2015), doi: http://dx.doi.org/10.1016/j.steroids.2015.12.007

EFFECT OF TESTOSTERONE ON THE REGULATION OF P53 AND P66SHC DURING OXIDATIVE STRESS DAMAGE IN C2C12 CELLS

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Running title: Role of testosterone during oxidative stress damage on p53 and p66Shc.

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Abstract

Accumulating evidence indicates that apoptosis is activated in the aged skeletal muscle, contributing to sarcopenia. We have previously demonstrated that testosterone protects against hydrogen peroxide (H_2O_2)-induced apoptosis in C2C12 muscle cells, at different levels: morphological, physiological, biochemical and molecular. In the present study we observed that H_2O_2 induces the mitochondrial permeability transition pore (mPTP) opening and exerts p53 activation in a time-dependent way, with a maximum response after 1-2 hours of treatment. Testosterone treatment, prior to H_2O_2, reduces not only p53 phosphorylation but also p66Shc expression, activation and its mitochondrial localization, at the same time that it prevents the mPTP opening. Furthermore, testosterone diminishes JNK and PKCβI phosphorylation induced by H_2O_2 and probably contributing thus, to reduce the activation of p66Shc. Thus, the mPTP opening, p53, JNK and PKCβI activation, as well as p66Shc mRNA increase, induced by oxidative stress, were reduced by testosterone pretreatment. The data presented in this work show some of the 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.

Keywords

Apoptosis; skeletal muscle; testosterone; p53; p66Shc.

Introduction

The tumor suppressor protein p53, popularly known as 'the guardian of the genome', has an essential role as damage-control system. It senses a variety of cellular stresses and induces cells to undergo cell cycle arrest, DNA repair, senescence or apoptosis, in order to limit the propagation of damaged cells. P53 induces these responses at least in part through the transcriptional activation of specific target genes [1], in response to many types of cell stress, including DNA damage, energetic deprivation, oncogenic derangement, hypoxia and oxidative stress [2]. 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.

One important effect of the genetic program triggered by p53 during the induction of apoptosis is the increase of the content of mitochondrial reactive oxygen species (ROS) [3]. p53 up-regulates the expression of p66Shc, a mitochondrial generator of hydrogen peroxide. Activated p53 increases p66Shc stability, and it is unable to induce apoptosis in p66Shc-/- fibroblasts, suggesting that p66Shc regulates p53-dependent apoptosis [4]. The stimulation of the β-adrenergic receptors in differentiated H9c2 muscle cells, results in apoptosis and cardiovascular degeneration, with an increase in the levels of p53 and phosphorylated-p66Shc [5]. However, the molecular mechanisms through which p66Shc mediates oxidative stress-induced apoptosis remain largely unknown.

The mammalian adaptor protein ShcA is expressed in three isoforms, p46Shc, p52Shc, and p66Shc, derived from a single gene through the differential use of transcription/translation initiation sites and alternative splicing.

The p66Shc isoform has an additional amino-terminal collagen homology-like domain (CH2), unlike p46 and p52, which contains a serine residue at position 36 (Ser36), that is phosphorylated in response to several stimuli including H_2O_2 and UV irradiation [6]. While p52 and p46 are cytoplasmic signal transducers involved in mitogenic signaling from activated tyrosine kinase receptors to Ras, the p66 isoform regulates ROS metabolism and apoptosis [4, 6-8]. Likewise, p66Shc protein exerts a relevant inhibitory signaling effect on the ERK pathway in skeletal muscle myoblasts, which is required for appropriate regulation of actin cytoskeleton polymerization and normal glucose transport responses, contributing to the changes in glucose uptake [9, 10].

In response to apoptotic stimuli, p66Shc translocates from cytosol to mitochondria, upon phosphorylation on Serine 36 by a number of stress-activated kinases including JNK1 and PKCβ, where it acts as a redox enzyme to amplify oxidative stress, oxidizing cytochrome c, generating H_2O_2, and thus inducing the opening of the mitochondrial permeability transition pore and eventually apoptosis [11-13].

P66Shc has come into focus as a major determinant of cell susceptibility to oxidative damage controlling mammalian life span by regulating the cellular response to oxidative stress [6]. However, the underlying molecular mechanisms are unknown.

The loss of muscle mass and strength with aging, also referred to as sarcopenia, is a prevalent condition among the elderly and predicts several adverse outcomes, is a result of reduction in the size and number of muscle fibers [14] possibly due to a multifactorial process that involves lack of physical activity, reduced nutritional intake, oxidative stress, and hormonal changes [15,

16]. Sarcopenia has been associated with a deficit of sex hormones as the levels of estrogens and androgens decline with aging. Thus, hormone replacement therapies prevent a decline in muscle performance [17, 18]. It is well known that skeletal muscle is a target tissue for androgens and its protective role on it, receives increased attention. Testosterone supplementation increases muscle mass in healthy young and old men, healthy hypogonadal men and in other physiological or pathological conditions with low levels of this steroid [19]. Other studies have demonstrated that testosterone-induced increase in muscle size is associated with hypertrophy of muscle fibers and significant increases in myonuclear and satellite cell numbers [20-22]. 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 be a key mechanism responsible for the impairment of muscle performance [23, 24].

In our laboratory, we have previously demonstrated that testosterone protects against hydrogen peroxide (H_2O_2)-induced apoptosis in the C2C12 murine skeletal muscle cell line [25, 26]. Typical changes of apoptosis induced by 1 mM H_2O_2, such as nuclear fragmentation, mitochondrial redistribution/dysfunction and cytoskeleton disruption, are reduced in presence of testosterone. The steroid also prevents PARP cleavage, cytochrome c release, decreases Bax expression and prevents the loss of mitochondrial membrane potential, associated to apoptosis [26]. The aim of the present study was to evaluate some of the components upstream of the classical apoptotic pathway which could trigger this response, and the role of the hormone during these events, pretending thus to improve the knowledge of the molecular

mechanisms involved in the antiapoptotic action of the steroid. In this work we demonstrated that testosterone, in its protective action, prevents the DNA fragmentation and the opening of the mitochondrial permeability transition pore (mPTP) induced by oxidative stress. We observed that the steroid reduces the activation that arises during apoptosis, of p53 and p66Shc, as well as the stress-activated kinases JNK and PKCβI. This last response of testosterone, on the kinases phosphorylation, probably contributes to regulate p53 and p66Shc activation, modulating thus finally, the apoptotic response. Although further studies are required to establish the molecular basis of sarcopenia associated with states of testosterone deficit, the data presented in this work allow us to begin to elucidate the mechanism by which the hormone regulates apoptosis in skeletal muscle.

Materials and Methods

Materials

Testosterone was purchased from Sigma–Aldrich. Anti-phospho-p53 (Ser15) rabbit polyclonal antibody and SP600125 were from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-SHC (phospho Ser36) mouse monoclonal antibody was from Abcam® (Cambridge, MA, USA). MitoTracker (MitoTracker® Red CMXRos) dye and Alexa Fluor 488-conjugated anti-rabbit secondary antibody were from Molecular Probes (Eugene, OR, USA). Phorbol 12-myristate 13 acetate (PMA) was purchased from Promega (Madison, WI, USA). Anti-Lamin B, anti-phospho-JNK (Thr 183 and Tyr 185), anti-JNK and anti-phospho-PKCβ I antibodies were from Santa Cruz Biotechnology, Inc.

(Santa Cruz, CA, USA). Anti-β-tubulin and anti-PKCβ rabbit polyclonal antibodies were purchased from Thermo Fisher Scientific (Rockford, IL, 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.

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 ºC) fetal bovine serum, 1% nystatin, and 2% streptomycin. Cells were incubated at 37 ºC in a humid atmosphere of 5% CO_2 in air. Cultures were passaged every 2 days with fresh medium [27, 28]. Under these conditions, C2C12 myoblasts resemble the activated satellite cells that surround the mature myofibers and proliferate and differentiate, participating in the repair of the tissue when a cellular injury exists [29]. 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 30 min. During this preincubation, cells were exposed to 10 µM SP600125 (for 1 hour) or 100 nM PMA (for 2 hours) in the experiments indicated. Treatments were carried out by adding 10^{-9} M testosterone at physiological

concentration [25, 26, 30] or the hormone vehicle, isopropanol during 60 min (the isopropanol percentage in the culture medium assay, of cells treated with the hormone or the hormone vehicle alone, was less than 0.001%) before induction of apoptosis with 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, 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 12,000 x g during 5 min to separate the particulate fraction from the soluble fraction. Protein concentration from the supernatant was estimated by the method of Bradford [31], using bovine serum albumin (BSA) as standard.

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 100 x g for 5 min. The upper fraction was collected and a nuclear pellet was obtained by low speed centrifugation (300 x g, 20 min). The supernatant was further centrifuged at 10,000 x g for 20 min to pellet mitochondria. The remaining supernatant was centrifuged at 120,000 x g for 60 min, to yield the cytosolic fraction and a plasma membrane containing particulate pellet (microsomes). 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.

Western blot analysis

Protein aliquots (25 mg) were combined with sample 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% 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. For tubulin or Lamin B loading control, 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 corresponded 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,) [32].

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 ºC 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 ºC, in the presence or absence (negative control) of primary antibodies (1:50 dilution). The primary antibodies were recognized by fluorophore-conjugated secondary antibodies.

Mitotracker (MTT) was employed for selective stain of active mitochondria. Coverslips with adherent cells were stained with MTT, which was prepared in dimethyl sulfoxide (DMSO) and then added to the cell culture medium at a final concentration of 1 µmol/l. After 15 to 30 min of incubation at 37 ºC, the cells were washed with PBS and fixed with methanol at -20 ºC for 30 min. Finally, the coverslips were analyzed by conventional fluorescence microscopy.

TUNEL assay

After the specific treatments, cells grown over coverslips, were processed for in situ immunocytochemical localization of nuclei exhibiting DNA fragmentation by the technique of terminal deoxynucleotidyl transferase (TdT)-mediated dUTP digoxigenin nick-end labeling (TUNEL) with the use of the

apoptosis detection kit DeadEnd™ Fluorometric TUNEL System (Promega, Madison, USA). The protocols were followed according to the manufacturer's instructions. Then cells were mounted with 95% glycerol and analyzed by conventional fluorescence microscopy. At least 500 cells of each experimental condition were counted and apoptotic cells were identified by nuclei staining (TUNEL-positive cells). The results were expressed as percentage of apoptotic nuclei.

Measurement of mPTP opening

We assessed the mPTP opening using the calcein-AM/cobalt method according to Petronilli et al. (1999) [33]. Briefly, 70–80% confluent cultures in 10 cm (flow cytometry) or 3 cm (microscopy) plates were loaded for 30 min with 1 mM calcein-AM at 37 ºC in DMEM media. We quenched the cytosolic and nuclear calcein fluorescence using 1 mM $CoCl_2$. After attainment of quenching, cells were washed twice with warm DMEM without serum. Following the corresponding treatments, cells were examined by digital fluorescence microscopy. Additionally, the loaded cells were trypsinized, harvested, and analyzed in a FACS Calibur flow cytometer (excitation wavelength of 488 nm).

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 hour 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' TTGATGGCAACAATCTCCAC 3'; p66Shc set: forward 5' ACTACCCTGTGTTCCTTCTTTC 3', reverse 5' TCGGTGGATTCCTGAGATACTGT 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 [34, 35].

Statistical analysis

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

Results

Testosterone prevents nuclear DNA fragmentation induced by H_2O_2 in C2C12 muscle cells

We have previously demonstrated that testosterone exerts an antiapoptotic effect on H_2O_2-induced apoptosis, its actions being mediated by the androgen receptor (AR) [26]. In order to detect *in situ* the nuclear DNA

fragmentation induced by oxidative stress, and to evaluate the protective effect of testosterone on this event, immunocytochemistry assays and immunofluorescence conventional microscopy using TUNEL dye were performed after treatments. C2C12 cells grown on coverslides, were incubated with the steroid hormone (10^{-9} M) for 60 min prior to H_2O_2 treatment (1 mM for 4 h). We also checked the effect of an antagonist of the AR, flutamide. Thus, cells were pretreated with testosterone (10^{-9} M) plus flutamide (10^{-8} M) for 60 min, followed by the incubation with H_2O_2 (1 mM for 4 h) to induce apoptosis. We could observe that after the apoptotic stimuli cells with nuclear DNA fragmentation (TUNEL-positive cells) increased to ~70% respect to control (isopropanol) or respect to cells treated with testosterone alone (1-2% of the cells were stained). The incubation with testosterone (10^{-9} M) for 60 min prior to the apoptotic agent diminished to ~44% the number of TUNEL-positive cells. However, the inhibitory effect of testosterone on H_2O_2-promoted apoptotic DNA fragmentation was almost totally abolished by flutamide, which increased again the TUNEL-positive nucleus to ~72% (Fig. 1). These findings further support previous results of our laboratory [26] suggesting an antiapoptotic action of testosterone against H_2O_2 on nuclear DNA fragmentation and the participation of the AR in this event.

Testosterone prevents the mPTP opening induced by H_2O_2 in skeletal muscle cells

Mitochondria play a crucial role in apoptosis, releasing several apoptosis-inducing factors into the cytoplasm. This process requires the increase of outer mitochondrial membrane permeability, which probably depends on the

activation of mPTP. We have previously demonstrated that testosterone exerts its antiapoptotic action against H_2O_2 by preventing the loss of mitochondrial membrane potential [26]. In order to further study the protective effect of testosterone on mitochondrial integrity, we evaluated the effect of the steroid hormone on mPTP function in C2C12 cells by using a cobalt-quenched calcein-AM method. Calcein-AM is an anionic, esterified fluorochrome that enters to the cells freely and labels cytoplasmic as well as mitochondrial regions following esterase removal of the AM group. Because cobalt ions enter to the cytoplasm but do not pass through mitochondrial membranes, mitochondria can be specifically identified by the cobalt quenching of cytoplasmic, but not mitochondrial calcein fluorescence. In consequence, mPTP opening can be recognized by a decrease of mitochondrial calcein fluorescence [33].

C2C12 cultures, loaded with calcein-AM/$CoCl_2$, were incubated with testosterone or vehicle isopropanol (control) before induction of apoptosis with 1 mM H_2O_2 (4 h). The cells were then analyzed by microscopy and flow cytometry as described in *Materials and Methods*. As seen in Fig. 2A, microscopic analysis showed that the treatment with H_2O_2 resulted in a significant decrease of calcein fluorescence intensity due to $CoCl_2$ quenching as a consequence of the mPTP opening. However, when cells were previously treated with 10^{-9} M testosterone, the fluorescence was diminished to a lesser extent, implying a protective role of the hormone on the prevention of the opening of this pore. These data were confirmed by flow cytometry, which allows for accurate quantification of cell fluorescence labeling. Thus, it was found that incubation with H_2O_2 reduced the mitochondrial calcein fluorescence, evidenced by a decreased FL-1 signal compared with control (from 80% to 63% respectively).

However, in presence of the steroid, the loss of mitochondrial fluorescence induced by H_2O_2 was reduced, and percentage of cells with green fluorescence (74%) were near to the control condition (80%) (Fig. 2B) showing, one more time, that testosterone prevented the mPTP activation.

H_2O_2-induced oxidative stress activates p53 in a time-dependent manner in C2C12 muscle cells

As a first approach to evaluate the response of p53 to the apoptotic agent in C2C12, we exposed cultures to different treatment times with 1 mM H_2O_2, ranging from minutes to hours (15 and 30 min, 1, 2, 3 and 4 h), followed by subcellular fractionation and the measurement of activated p53 by immunoblot analysis. Since once activated, p53 acts as transcription factor regulating the expression of target genes, we studied the effect of the apoptotic agent on p53 phosphorylation in the nuclear fraction. Using a specific antibody against phospho-p53 (Ser15), Western blot assay revealed a time-dependent p53 activation in response to H_2O_2, with a maximum after 1-2 hours from the beginning of the treatment (Fig. 3A). From then on, p-p53 mildly decreased.

Also immunocytochemical detection of p-p53 was performed on C2C12 cultures by fluorescence microscopy. After oxidative stress induced by H_2O_2, it was possible to observe the nuclear localization of p-p53, with a maximum intensity of the nuclear fluorescence after 1-2 h of the apoptotic stimuli. From then on, the fluorescence decays significantly, almost to be zero at longer treatment times (Fig. 3B). These results showed a time-dependent activation of p53 after the oxidative stress induction.

Effect of testosterone on H_2O_2-induced p53 activation

Next, the effect of testosterone treatment on the phosphorylation of p53, induced by oxidative stress, was evaluated. C2C12 cultures were preincubated with the hormone 10^{-9} M during 60 min and then exposed to H_2O_2 (1 mM, 2 h). In order to evaluate if the AR was mediating the effect of the hormone in this event, cells were treated in addition with flutamide (10^{-8} M, 60 min) prior to H_2O_2 and testosterone. Then, the nuclear fraction obtained was analyzed by Western blot assay. As shown in Figure 4A, testosterone reduced the activation of p53 induced by H_2O_2, event that was blocked by flutamide, pointing to the participation of the AR in the regulation of p53 phosphorylation.

These effects were also observed by immunocytochemical assays, employing a specific antibody against p-p53 (ser15) to label methanol fixed C2C12 muscle cells, after the specific treatments. As seen in Figure 4B, a high nuclear green fluorescence was observed after 1-2 h of H_2O_2 treatment, which was significantly diminished by the incubation with testosterone prior to the apoptotic stimuli. In presence of flutamide, the effect of testosterone was almost totally blocked, detecting no differences between H_2O_2 condition and the simultaneous treatment of testosterone, flutamide and the apoptotic agent. These results suggest that, in presence of testosterone, environmental stress conditions induced by H_2O_2 are less adverse, so cell damage is not greatly extended and that is the reason why the activation of p53 is not required, neither for interrupting the cell cycle and repairing the damage nor for inducing apoptosis.

Activation of p66Shc by H_2O_2 in C2C12 muscle cells

One way that p53 induces apoptosis is by the up regulation of p66Shc expression, which enhances mitochondrial H_2O_2 production [4]. As a first approach we evaluate changes on p66Shc activation/localization, during oxidative stress in our experimental system, immunocytochemistry assays were performed, after treatment with 1 mM H_2O_2 during different periods of time, and then fixed with methanol and labeled with the specific p-p66Shc (Ser36). Surprisingly, it was observed the almost completely nuclear localization of p-p66Shc in control cells (untreated and maintained in serum free DMEM) (Fig 5). Other groups reported that this protein remains inactivated in cytosol until an apoptotic stimulus, such as oxidative stress, triggers their activation/phosphorylation and translocation to mitochondria, where it amplifies the oxidative signal. However, in C2C12 cell line, nuclear localization of p-p66Shc was observed under basal conditions. When cells were exposed to H_2O_2 a slight general increase in green fluorescence over the cells was observed, at the same time that they acquired the typical apoptotic rounded shape. Furthermore, the location of p66Shc is no longer almost exclusively nuclear, but more homogeneous along the cell. At 1-2 h of H_2O_2 treatment, less nuclear labeling was observed, being located a greater green fluorescence around them. This behavior points to the possibility that apoptotic stimuli induced, in C2C12 cells, the translocation of p66Shc from the nucleus (which could be sequestered by a chaperone or associated with transcription factors, fulfilling a function unknown until now) to mitochondria, and thereby it regulates apoptosis. After 4 hours of treatment with H_2O_2 fluorescence intensity markedly decreased until it became almost imperceptible. The number of cells in this latter condition was significantly reduced. Longer treatments were impossible to

analyze by immunocytochemistry because of the total loss of the adherent cells in the coverslip.

Testosterone prevents the activation and translocation of p66Shc induced by H_2O_2 in C2C12 cells

In order to visualize the effect of the hormone on p66Shc activation/translocation, immunocytochemical analysis of C2C12 cells were carried out. In parallel, we employed a JNK inhibitor (SP600125) and a PKC activator (PMA). Thus, cells grown on coverslips were treated with 10^{-9} M testosterone for 1 hour prior to the oxidative stress induction with 1 mM H_2O_2 for 2 hours, or with 1 mM H_2O_2 alone. The effects were compared with conditions treated with 10^{-9} M testosterone alone or with the vehicle of the hormone (0.001% isopropanol). Furthermore, the cells were also preincubated in serum-free DMEM containing 10 µM SP600125 for 1 hour and then exposed to 1 mM H_2O_2 during 2 hours. PKC activation was induced by 100 nM PMA during 2 hours. After treatments, cells were labeled with p-p66Shc and with the mitochondrial marker Mitotracker, and then analyzed by fluorescence microscopy.

As shown in Figure 6A, a strong nuclear localization of p66Shc in control (C) and testosterone treated cells (T) were detected. Treatment with H_2O_2 not only induced an increase in the number of apoptotic cells with the classic rounded condensed form, but also allowed to see the yellow signal in the *merge* of the individual images, showing the mitochondrial localization of p-p66Shc. Furthermore, it was possible to observe a decrease in the nuclear fluorescence, at the same time that it increased in mitochondria. These effects were reduced

by testosterone treatment prior to oxidative stress. The activation of PKC by PMA induced similar effects to those exerted by H_2O_2, even more markedly. A high percentage of apoptotic cells, with an intense green fluorescence located predominantly in mitochondria (evidenced by the strong yellow signal in *merge*) was detected. Therefore, PKC activation would exert a clear proapoptotic function in C2C12. In addition, H_2O_2 effects were almost completely blocked by JNK inhibition detecting no differences between cells treated with SP600125 + H_2O_2 and controls.

Similar results were obtained when total cell lysates were analyzed, by Western blot. PMA and H_2O_2 enhanced the p-p66Shc levels, whereas pretreatment with testosterone prior H_2O_2 slightly reduce the activation of the protein. Moreover, the inhibition of JNK by SP600125 treatment previous to apoptosis induction, leads p66Shc phosphorylation to control levels (Fig 6B). This effect allows us to assign JNK a fundamental role in the regulation of apoptosis in our experimental model. Thus, the activation/phosphorylation of p66Shc is probably modulated, at least in part, by JNK and some PKCs that respond to PMA.

Testosterone reduces p66Shc expression induced by H_2O_2 in C2C12 cells

In order to evaluate gene expression of p66Shc during H_2O_2-induced apoptosis, in C2C12 cells, and its possible regulation by testosterone, we analyzed, by quantitative PCR, the relative levels of p66Shc using the comparative C_T method described in *Materials and Methods* section. Total RNA from cell cultures exposed to 1 mM H_2O_2 during different times, ranging

between 30 minutes and 4 hours, was isolated and employed to determine the relative levels of p66Shc mRNA expression. The GAPDH gene was used as a reference gene to determine the relative expression levels of p66Shc. As shown in Figure 7, the increase in mRNA levels of the apoptotic protein p66Shc was detected at 1 hour and a half after the beginning of H_2O_2 treatment. From then on, the expression of the mRNA decays significantly. Then, we evaluated if testosterone was able to counteract the H_2O_2 effects on p66Shc gene expression. So, C2C12 cells were treated with 10^{-9} M testosterone during 60 minutes, 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 of p66Shc gene was quantified. Pretreatment with testosterone reduced the p66Shc expression induced by H_2O_2. These results point to a protective effect of testosterone against H_2O_2-induced apoptosis at the gene transcription level.

Effect of testosterone on JNK and PKCβI activation induced by H_2O_2 in C2C12 cells

One of the signaling pathways involved in cell death mediated by oxidative stress is the activation of MAP kinase JNK pathway. JNK activation induces several biological responses, which are mainly characterized by the activation of programmed cell death [36-38]. c-Jun was the first JNK substrate described [39], but later other JNK targets were identified such as p53, Elk-1 and c-Myc [36, 40]. As mentioned above, depending on the cellular context and the apoptotic stimulus, phosphorylation/activation of p66Shc serine 36 can be exerted by various kinases, in which JNK has been identified [41].

It was recently observed by researchers of our group that H_2O_2-induced oxidative stress leads to the phosphorylation/activation of JNK from 1 h from the starting of H_2O_2 treatment remaining activated during all the time of exposure to H_2O_2 evaluated (4 h) in C2C12 cells [42].

Given that we previously observed not only that testosterone was able to reduce the phosphorylation of p53 and p66Shc induced by H_2O_2, but also that the JNK inhibition conduces to a reduction of H_2O_2-induced p66Shc phosphorylation, we then evaluate if testosterone exerts any effect on this kinase modulating thus, p66Shc activation. So C2C12 cultures were preincubated with 10^{-9} M testosterone for 60 min and then treated with 1 mM H_2O_2 for 1-2 hours. Results were compared with untreated cells (C), cells treated with 10^{-9} M testosterone (T) or with the vehicle 0.001% isopropanol (IPA). By Western blot assays, and employing specific antibodies against phosphorylated JNK1/2 (Tyr 185 and Thr 183), we observed that pretreatment with testosterone reduced JNK phosphorylation induced by H_2O_2 (Fig 8A), whereas no significant changes of total JNK were detected.

Given that our previous immunocytochemical observations suggest a proapoptotic effect of PKC in C2C12, we analyzed if oxidative stress induced by H_2O_2 was activating PKCβ, kinase reported as a p66Shc activator. Thus, C2C12 cultures were preincubated with 10^{-9} M testosterone for 60 min and then treated with 1 mM H_2O_2 for 1-2 hours, comparing the effects with cultures treated with the apoptotic agent alone (H_2O_2), with 10^{-9} M testosterone (T) or the vehicle 0.001% isopropanol (C). By Western blot assays, and employing a specific antibody against p-PKC βI (Thr 641), we observed that pretreatment with

testosterone reduced the PKCβI phosphorylation, slightly increased by H_2O_2. No significant changes in total PKCβ were observed (Fig 8B).

Discussion

Several *in vivo* and *in vitro* reports demonstrate that the androgen-AR signaling pathway is required for skeletal muscle development and for sustaining muscle mass, strength and protein synthesis [43]. 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 AR [26]. In this work, it was also shown the protective effect of testosterone and its receptor in the prevention of the nuclear DNA fragmentation induced by the apoptotic agent H_2O_2. It is well known that mitochondria play a major role in apoptosis triggered by many stimuli [44]. The collapse in the mitochondrial membrane potential is one of the earliest events of programmed cell death. Previous reports of our group have demonstrated that after H_2O_2-induced oxidative stress the collapse of the mitochondrial membrane potential and the release of the proapoptotic factor cytochrome c, occur, whereas testosterone prevents these events [26]. The decrease in the mitochondrial membrane potential has been related to mPTP opening [45]. Focusing our work on mitochondria as a key organelle in the regulation of apoptosis, it was further evaluated the mPTP opening, showing the H_2O_2-induced pore aperture. However, the preincubation with testosterone reduces the effects of the apoptotic agent on the mPTP opening, resulting in mitochondrial protection.

In this research it has been also demonstrated 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 hours of treatment. The p53 pathway is promoted by a range of stimuli that threaten to alter the genomic integrity or to affect the proper cell proliferation. It is p53 the backbone of this intricate network and which integrates and translates these stimuli in the most appropriate response (cell cycle arrest, DNA repair, senescence or apoptosis) [46-49]. We have observed that the activation of p53 induced by H_2O_2 stimulus is reduced by the preincubation with testosterone. We speculate that the stressful oxidative condition to which C2C12 cultures are subjected with H_2O_2 treatment, are attenuated in the presence of the hormone and the environmental conditions probably become less adverse, being the stress stimulus, that induces p53 activation, less intense.

Depending on the cell type, the stress stimuli and the extracellular environment, activation of p53 can lead to apoptosis instead of inducing cell cycle arrest, by the induction of the transactivation of a number of apoptotic genes such as Bax, Bid, Puma, Noxa , AIP-1 p53, PERP [50-54] and the repression of antiapoptotic genes such as Bcl-2 and Bcl-XL [55, 56]. One of the ways that p53 induces apoptosis is by the upregulation of p66Shc expression. In this work, it has been demonstrated that, at transcriptional level, testosterone is capable of reversing the H_2O_2-induced upregulation of p66Shc.

Phosphorylation in serine 36 is an essential step in the p66Shc response to oxidative stress and its apoptotic action. Depending on cellular context and the nature of the stimulus, p66Shc can be phosphorylated by different kinases, such us PKCβ and JNK [13, 41], and translocate to mitochondria where it

provokes changes in the structure and function of the organelle, generating ROS, inducing the mPTP opening and the oxidation and release of cytochrome c [11, 13].

In this work it was shown that testosterone diminishes the activation/phosphorylation of p66Shc protein, induced by H_2O_2. The hormonal treatment prior to the apoptotic stimulus was able to reduce the levels of p-p66Shc and its mitochondrial localization, increased after H_2O_2 treatment. Oxidative stress provokes, in C2C12 cells, the activation of p53, probably contributing to the increase of circulating levels of p66Shc. Furthermore, it has been shown that H_2O_2 induces JNK activation, which could act as an activator kinase of p66Shc pool inducing its translocation to mitochondria to exert its apoptotic action [41], or activate and stabilize p53 [36, 40].

In embryonic stem cells, it has been reported that H_2O_2 causes JNK phosphorylation while DHT via AR, reverts the H_2O_2 effects and reduces ROS levels enhancing the catalase activity [57]. In muscle of aged rats, highly phosphorylated JNK was detected, being its levels reduced by testosterone [58]. Consistent with the observations of these authors, we observed that testosterone treatment prior to the apoptosis induction reverses the JNK phosphorylation/activation induced by H_2O_2. Thus, the decrease in JNK activation could be responsible, in part, of the decrease in p53 and p66Shc phosphorylation observed after testosterone preincubation.

Studies of the subcellular distribution of p66Shc in mouse embryonic fibroblasts (MEFs) showed that about 32% of the protein is located in the cytoplasm, 24% in the endoplasmic reticulum and 44% in mitochondria [59]. Differences in the levels of its subcellular distribution depend on the external

influences that induce p66Shc translocation. Increased mitochondrial p-p66Shc is detected in aged mice respect to the young mice levels [60]. Considering the theory of free radicals of aging [61], it can be assumed that p66Shc is involved in the cellular response to oxidative stress in adulthood, explaining the increased production of free radicals and the damage produced in aged mice, as well as the resistance to oxidative stress in animals deficient in p66Shc.

In order to determine the subcellular localization of p66Shc in C2C12 cells after H_2O_2 treatment and the influence of hormonal pretreatment on the effects of the apoptotic agent, immunocytochemical assays were performed. Surprisingly, cell controls treated with the vehicle of the hormone or with testosterone, showed a clear nuclear localization of p-p66Shc. So far, there are no reports describing a non-classical localization of this protein in the nucleus, or the role that could be exerting at this level. There was detected only in bovine embryos, a perinuclear and nuclear localization of p66Shc [62]. Our speculations point to the regulation of the activity of transcription factors, such us the Forkhead family, by p66Shc, as it has been already described [63], or to p66Shc binding to a chaperone or a transcription gene coregulator. After H_2O_2 treatment we have also noticed, not only the increment in the number of cells with the classical apoptotic morphology, but also the increase in the mitochondrial localization of p-p66Shc. Moreover, these apoptotic cells seem to have “empty” p-p66Shc nuclei, suggesting a possible translocation of the protein from the nucleus to the mitochondria to exert its apoptotic action there. Testosterone treatment prior to the apoptosis induction reversed the H_2O_2 effects, showing the nuclear location of p-p66Shc again. The activation of PKC by PMA, showed the same effects triggered by H_2O_2, even more markedly. So,

an apoptotic role of PKC in C2C12 cells is evidenced as well as the modulation of p66Shc activation by these kinases.

It has been recently reported that the PKCβII suppression protects against apoptosis and remote organ injury induced by intestinal ischemia and reperfusion in mice lung and liver, which may be partially attributed to the inhibition of the p66Shc-cytochrome-c axis [64]. In this work, by Western blot assay we showed that H_2O_2 induces the phosphorylation of the βI isoform of PKC in skeletal muscle cells and that testosterone reverses this effect, so it is possible that the steroid also exerts its antiapoptotic effect by reducing this kinase activation and thus, p66Shc phosphorylation and mitochondrial localization.

The employment of a JNK inhibitor prior to the induction of apoptosis, almost completely blocked the effects of H_2O_2 in C2C12 cells, which showed its classical star shape, without the presence of pyknotic cells and the same pattern in p-p66Shc location observed in controls. The levels of p-p66Shc detected by Western blot after oxidative stress (H_2O_2 treatment), were reduced when JNK is inhibited, pointing the kinase as a p66Shc activator.

Although further studies are necessary to clarify the role of testosterone in the signaling pathways involving p53 and p66Shc in C2C12 cells, in this work it has been noticed the antiapoptotic action of the steroid, by attenuating the activation of these proteins induced by H_2O_2. It was also shown that the hormone is able to reduce p66Shc RNAm, upregulated by H_2O_2, suggesting a protective effect of the hormone against apoptosis at the gene expression level. Furthermore, the steroid reduces PKCβI and JNK activation probably

contributing thus, to the reduction of p66Shc and possibly p53 activation, observed after H_2O_2-induced oxidative stress.

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 the C2C12 skeletal muscle cell line. Additional studies in this line of evidence are being developed by our group, to further elucidate the signaling mechanisms which mediate the antiapoptotic action of testosterone in skeletal muscle cells and its relationship with myopathies associated with hormonal deregulation.

Funding

This work was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), and Universidad Nacional del Sur, Argentina.

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Figure legends

Figure 1: Effect of testosterone on nuclear DNA fragmentation induced by H_2O_2 in C2C12 muscle cells. C2C12 cultures were treated with 1 mM H_2O_2 for 4 h (H_2O_2) or preincubated with 10^{-9} M testosterone (T+H) or with 10^{-9} M testosterone and 10^{-8} M flutamide (T+F+H) during 60 minutes, before the exposure to the apoptotic agent for 4 h. Treatment with 10^{-9} M testosterone alone (T) or isopropanol 0.001% (hormone vehicle) (IPA) for 60 min were used as controls. After treatments, cells were stained by the TUNEL method as described in *Methods*. Results are expressed as percentage of apoptotic nuclei. At least ten fields per dish were examined. Each value represents the mean of three independent experiments. Averages ± S.D. are given; *$p<0.05$ respect to the control or #$p<0.05$ respect to H_2O_2. Magnification: 20X.

Figure 2: Testosterone prevents H_2O_2-induced mPTP opening in C2C12 skeletal muscle myoblasts. Cells loaded with calcein-AM/$CoCl_2$ were treated with 1 mM H_2O_2 (H_2O_2) for 4 h, with 10^{-9} M testosterone before the addition of H_2O_2 (T+H), with testosterone 10^{-9} M alone (T) or with isopropanol 0.001% (C), as described in *Materials and Methods*. mPTP opening was analyzed by: A) Fluorescence microscopy. Mitochondria of C2C12 cells were also stained with Mitotracker (red fluorescence) after treatments. Representative photographs of each condition are shown. Magnification: 63X. Green fluorescence quantification graphic is shown, *$p<0.05$ respect to the control; B) Flow cytometry. Averages ± S.D. are given; *$p<0.05$ respect to the control. FL-1: FL-1 channel that detects green fluorescence.

Figure 3: Activation and nuclear translocation of p53 by H_2O_2 in C2C12 cells. A) Cell cultures were incubated for the indicated times with 1 mM H_2O_2 or maintained in DMEM (C). Then, the nuclear fraction was isolated as described in *Methods* and analyzed by Western blot using a specific anti-p-p53 (Ser 15). Lamin B levels are shown as a control for nuclear fraction. The blot is representative of three independent experiments with comparable results. Densitometric quantification of p-p53 blots is shown. Averages ± S.D. are given; *P<0.05 with respect to the control. B) Cells grown on coverslips were treated with 1 mM H_2O_2 for the specified periods of time and then labeled with anti-p-p53 (Ser 15) as described in *Methods*. At least ten fields per slide were examined by fluorescence microscopy. Representative photographs are shown. Magnification: 63X. A fluorescence intensity graphic is shown, *p<0.05 respect to the control; #p<0.05 respect to H15.

Figure 4: Effect of testosterone on the activation of p53 induced by H_2O_2 in C2C12 muscle cells. A) C2C12 cells were treated with the indicated stimuli: (C) cells treated with isopropanol 0.001% for 60 min, (T) cells incubated with 10^{-9} M testosterone for 60 min, (H_2O_2) cells treated with 1 mM H_2O_2 for 2 h, (T+H) cells pretreated with 10^{-9} M testosterone for 60 min prior to the exposure to 1 mM H_2O_2 for 2 h, (T+F+H) cells pretreated with 10^{-8} M flutamide and 10^{-9} M testosterone for 60 min prior to the exposure to 1 mM H_2O_2 for 2 h. Nuclear fraction was isolated as described in *Methods* and analyzed by Western blot using an anti-p-p53 (Ser15). The blot is representative of three independent experiments with comparable results. Lamin B is shown as nuclear fraction marker. Densitometric quantification of p-p53 blots is shown. Averages ± S.D.

are given; *P<0.05 with respect to the control. B) Cells grown on coverslips were subjected to the same treatments mentioned above. They were then labeled with anti-p-p53 (Ser 15) as described in *Methods*, and analyzed by conventional fluorescence microscopy. Representative photographs and green fluorescence quantification graphic are shown. Magnification: 63X; *p<0.05 respect to the control.

Figure 5: Activation of p66Shc induced by H_2O_2 in C2C12 cells. Cells grown on coverslips were treated with 1 mM H_2O_2 during the periods of time specified, ranging between 15 minutes and 4 hours, or maintained in DMEM without serum for 2 hours (C). Then, cells were fixed with methanol and labeled with anti-p-p66Shc (Ser 36) as described in *Methods*. At least ten fields per condition were analyzed by conventional fluorescence microscopy. Representative photographs are shown. Magnification: 63X. Green fluorescence quantification graphic is shown; *p<0.05 respect to the control.

Figure 6: Effects of the different treatments on the activation and subcellular localization of p66Shc. (C) Cells treated with 0.001% isopropanol for 60 min; (T) Cells incubated with 10^{-9} M testosterone for 60 min; (H_2O_2) Cells exposed to 1 mM H_2O_2 for 2 h; (T + H) Cells incubated with 10^{-9} M testosterone for 60 min and then subjected to 1 mM H_2O_2 for 2 hours; (PMA) Cells treated with 100 nM PMA during 2 h; (SP) Cells treated with 10 µM SP600125 for 1 hour; (SP + H_2O_2), Cells treated with 10 µM SP600125 for 1 hour and then exposed to 1 mM H_2O_2 for 2 hours. A) After treatments, cells were stained with Mitotracker and then labeled with anti-p-p66Shc as described in *Methods*.

There were analyzed by conventional fluorescence microscopy, at least ten fields per condition. Representative photographs are shown. Magnification: 63X. B) After treatments, cells were lysated and analyzed by Western blot using a specific anti-phospho-p66Shc. Tubulin levels are shown as loading control. The bands were quantified by densitometry. The blot is representative of three different experiments with comparable results. Results are expressed as percent stimulation over control; *p<0.05.

Figure 7: Testosterone reduce p66Shc gene expression induced by H_2O_2 in C2C12 cells. Transcript levels of p66Shc 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 minutes (T + H 30), 1 hour 30 min (T + H 1:30), 3 hours (T + H 3h) and 4 hours (T + H 4h) or treated with 1 mM H_2O_2 alone for the same periods of time; (C) Untreated cells. Levels of transcripts are expressed in arbitrary units related to the expression of GAPDH gene expression. Averages ± S.D. are given; *p<0.05 respect to the counterpart H_2O_2 condition.

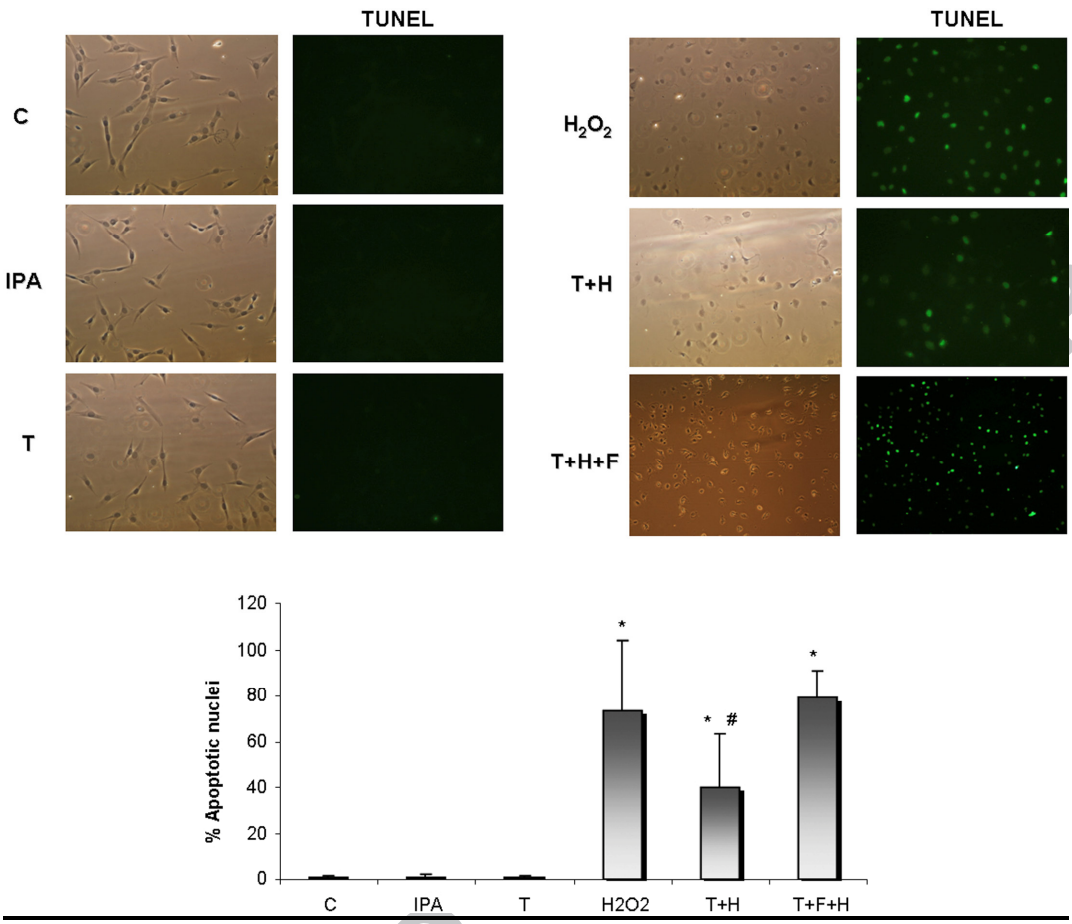
Figure 8: Effect of testosterone on JNK and PKCβ activation induced by H_2O_2. A) (C) Cells maintained in serum free DMEM; (IPA) Cells treated with 0.001% isopropanol for 60 min; (T) Cells incubated with 10^{-9} M testosterone for 60 min; (H_2O_2) Cells treated with 1 mM H_2O_2 for 1-2 h; (T+H) Cells incubated with 10^{-9} M testosterone for 60 min and then subjected to 1 mM H_2O_2 for 1-2 hours. Cell lysates were analyzed by Western blot using specific anti-phospho-JNK1/2 (Thr183 and Tyr185) and anti-JNK. Tubulin levels are shown as loading

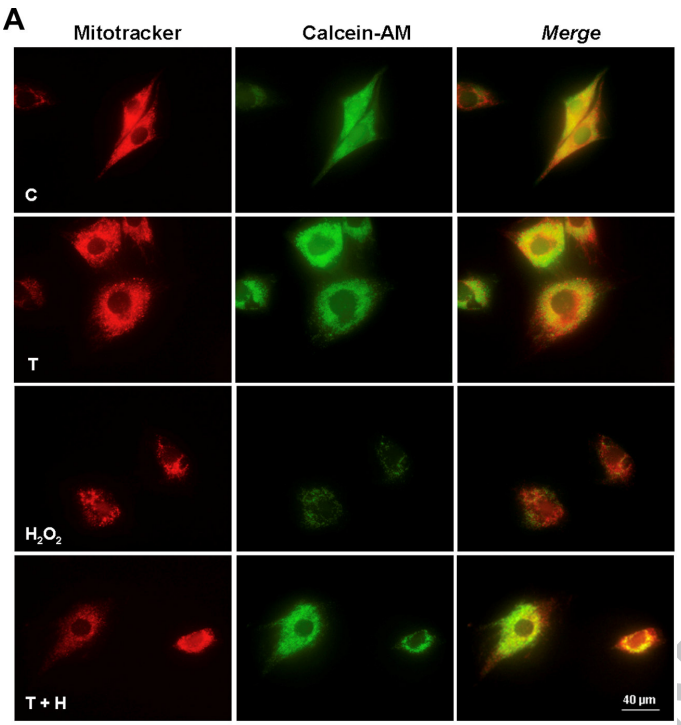
control. The bands were quantified by densitometry. The blot is representative of three different experiments with comparable results. Results are expressed as percent stimulation over control; *p<0.05. B) C2C12 cells were treated with the indicated stimuli, (C) cells treated with the vehicle of the hormone isopropanol 0.001% for 60 min, (T) cells incubated with 10^{-9} M testosterone for 60 min, (H_2O_2) cells treated with 1 mM H_2O_2 for 1-2 h, (T+H) cells pretreated with 10^{-9} M testosterone for 60 min prior to the exposure to 1 mM H_2O_2 for 1-2 h. Cell lysates were analyzed by Western blot using specific anti-phospho-PKCβI (Thr 641) and anti-PKCβ. Tubulin levels are shown as loading control. The bands were quantified by densitometry. The blot is representative of three different experiments with comparable results. Averages ± S.D. are given; *p<0.05 with respect to the control.

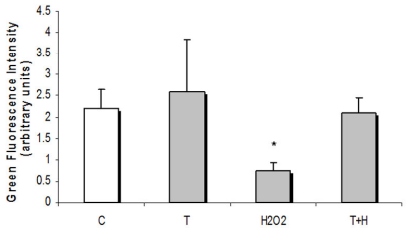
Figure 9: Protective action of testosterone through p53 and p66Shc in C2C12 cells during oxidative stress. Oxidative stress induced by H_2O_2, conduces to mitochondrial permeability transition pore (mPTP) opening, p53, JNK, and PKCβI activation as well as p66Shc RNAm increase. Testosterone treatment, prior to H_2O_2, reduces not only p53 phosphorylation but also p66Shc expression, activation and its mitochondrial localization (organelle where the protein acts as a redox enzyme to amplify oxidative stress, oxidizing cytochrome c, generating H_2O_2, and thus inducing the opening of the mPTP), at the same time that it prevents the mPTP opening. Testosterone also diminishes JNK and PKCβ phosphorylation contributing thus, to reduce the activation of p66Shc, observed after H_2O_2-induced oxidative stress.

Highlights

- ✓ We identified molecular events involved in the antiapoptotic effect of testosterone.
- ✓ Testosterone prevents nuclear DNA fragmentation and mPTP opening, induced by H_2O_2.
- ✓ H_2O_2 activates p53 but testosterone pretreatment reduces the protein phosphorylation.
- ✓ Testosterone reduces p66Shc activation, gene expression and mitochondrial location.
- ✓ Testosterone diminishes p-JNK and p-PKCβI, probably reducing thus p53/p66Shc activation.




A
Mitotracker
Calcein-AM
Merge
C
T
H2O2
T + H
40 µm


Green Fluorescence Intensity (arbitrary units)
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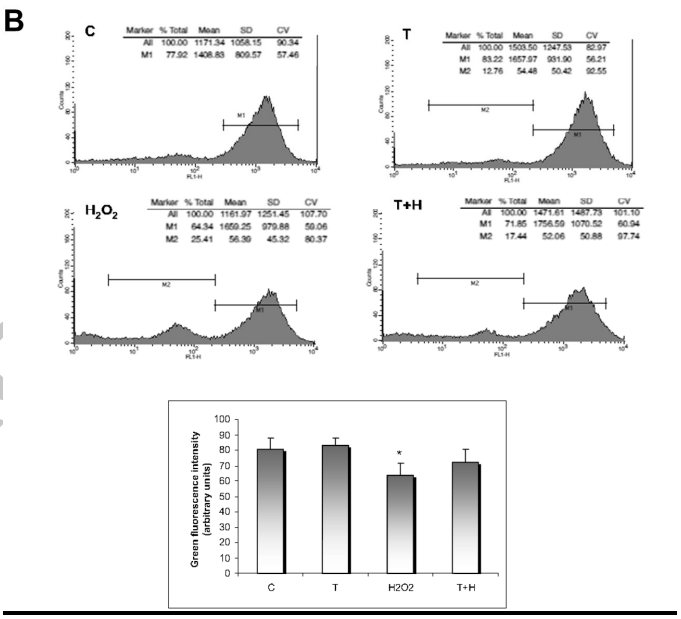
4
3.5
3
2.5
2
1.5
1
0.5
0
*
C
T
H2O2
T+H


B
C
T
H2O2
T+H

Green fluorescence intensity (arbitrary units)
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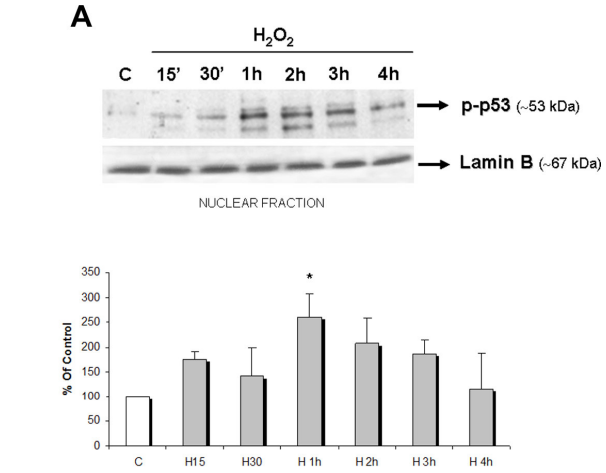
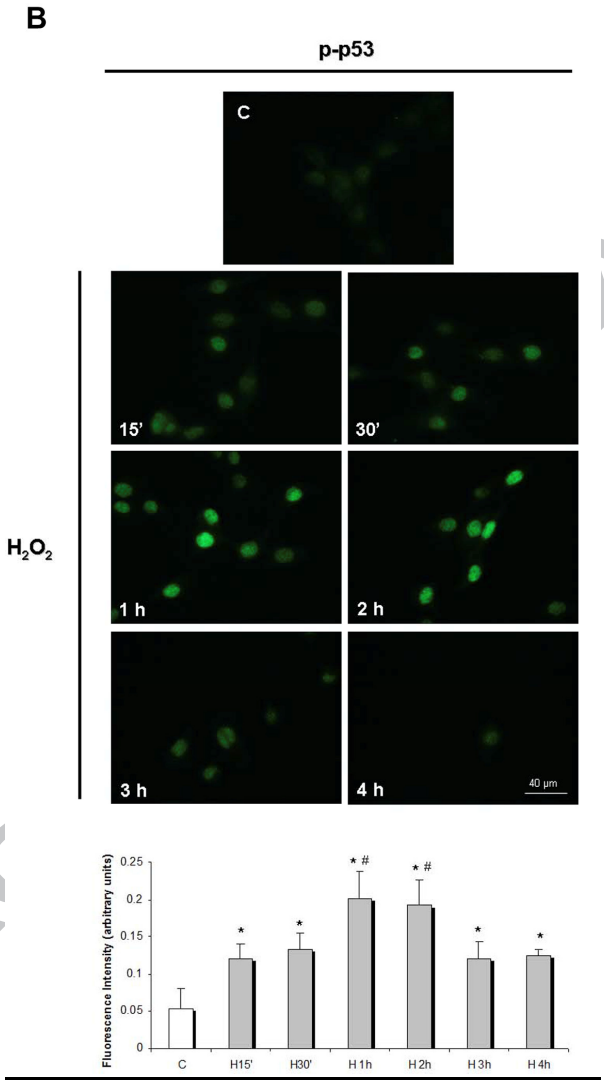
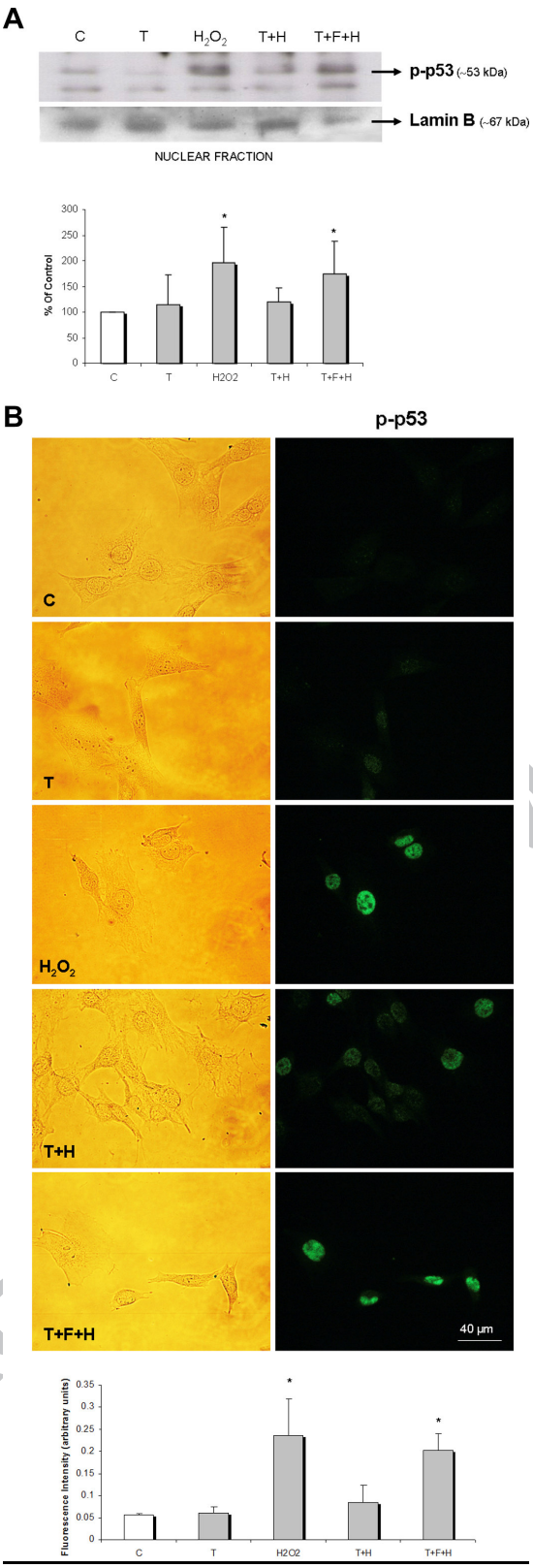
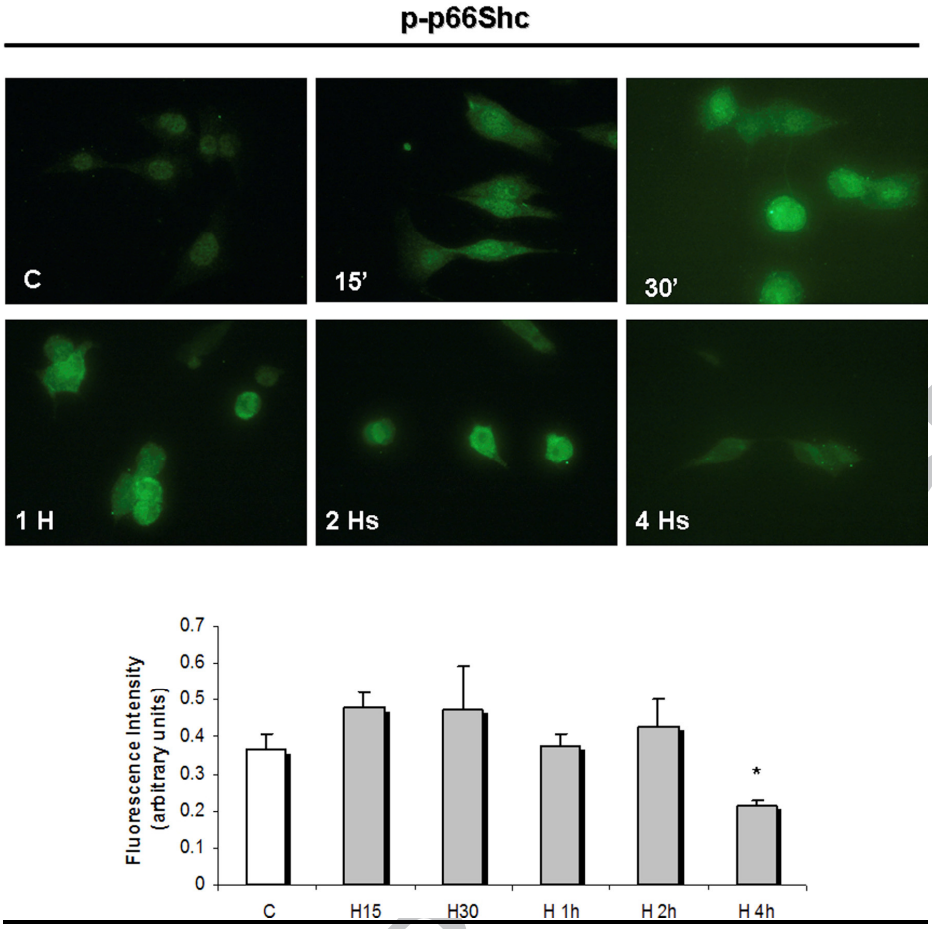
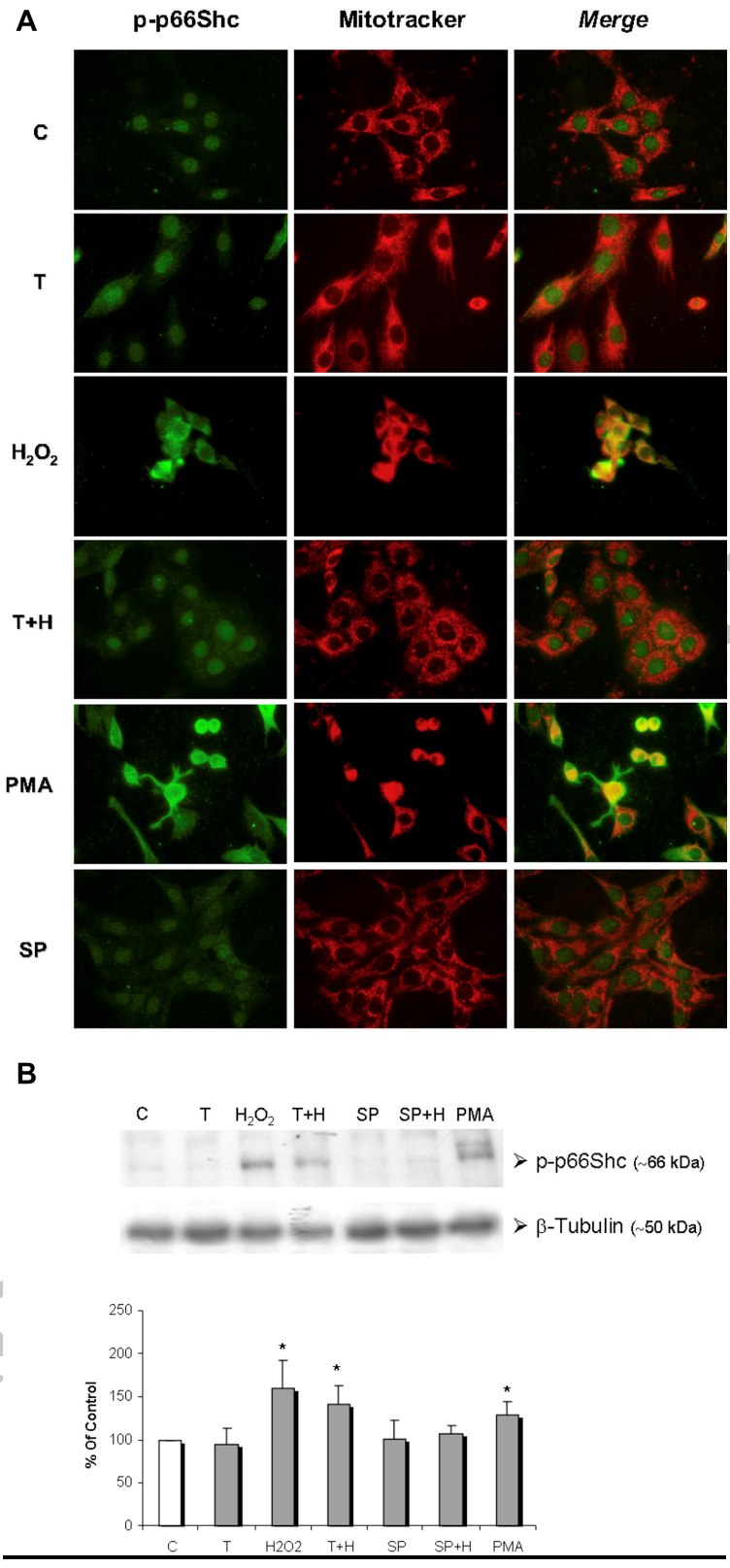
90
80
70
60
50
40
30
20
10
0
*
C
T
H2O2
T+H


A
H_2O_2
C
15'
30'
1h
2h
3h
4h
p-p53 (~53 kDa)
Lamin B (~67 kDa)
NUCLEAR FRACTION
% Of Control
C
H15
H30
H 1h
H 2h
H 3h
H 4h


B
p-p53
C
15'
30'
1 h
2 h
3 h
4 h
40 µm
H_2O_2
Fluorescence Intensity (arbitrary units)
C
H15'
H30'
H 1h
H 2h
H 3h
H 4h






A
p-p66Shc
Mitotracker
Merge
C
T
H2O2
T+H
PMA
SP
B
C
T
H2O2
T+H
SP
SP+H
PMA
p-p66Shc (~66 kDa)
β-Tubulin (~50 kDa)
% Of Control
250
200
150
100
50
0
C
T
H2O2
T+H
SP
SP+H
PMA