Article

17β-ESTRADIOL ABROGATES APOPTOSIS INHIBITING PKCδ, JNK AND p66Shc ACTIVATION IN C2C12 CELLS[†]

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In memory of Ricardo L. Boland, Ph.D. (1942-2014)

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Abstract

17β-Estradiol (E2) protects several non-reproductive tissues from apoptosis, including skeletal muscle. Previously, we showed that E2 at physiological concentrations prevented apoptosis induced by H_2O_2 in skeletal myoblasts. As we have also demonstrated a clear beneficial action of this hormone on skeletal muscle mitochondria, the present work further characterizes the signaling mechanisms modulated by E2 that are involved in mitochondria protection, which ultimately result in antiapoptosis. Here, we report that E2 through estrogen receptors (ERs) inhibited the H_2O_2-induced PKCδ and JNK activation, which results in the inhibition of phosphorylation and translocation to mitochondria of the adaptor protein p66Shc. In conjunction, the inhibition by the hormone of this H_2O_2-triggered signaling pathway results in protection of mitochondrial potential membrane. Our results provide basis for a putative mechanism by which E2 exerts beneficial effects on mitochondria, against oxidative stress, in skeletal muscle cells. This article is protected by copyright. All rights reserved

Introduction

The steroid 17β-estradiol (E2) has been shown to serve as a protective agent in several tissues and organs not involved in reproduction (Wang et al., 2006; Spyridopoulos et al., 1997). There is evidence demonstrating that skeletal muscle is a target tissue for E2 (Milanesi et al., 2008; Vasconsuelo et al., 2008). In agreement with these observations, muscle pathologies such as sarcopenia have been associated to decreased levels of estrogens (Dionne et al., 2000). It has been well established that the hormone acts through genomic mechanisms underlying the regulation of nuclear gene transcription but also nongenomic actions are a common property of steroid hormones and are frequently associated with the activation of various protein-kinase cascades. Previously, it was shown that E2 at physiological concentrations prevented apoptosis in skeletal myoblasts involving MAPKs, HSP27, and the survival PI3K/Akt pathway, which phosphorylates and inactivates proapoptotic members of the Bcl-2 family (Vasconsuelo et al. 2008, 2010, Ronda et al. 2010). Moreover, the steroid was able to abolish the typical changes of apoptosis such as nuclear fragmentation, cytoskeleton disorganization, mitochondrial reorganization/dysfunction and cytochrome c release induced by H_2O_2 (Vasconsuelo et al., 2008). Recently, we observed that E2 regulates mitochondrial functions, resulting in protection of the organelle. Thus, we found that the hormone could elicit an antiapoptotic effect via modulation of the mitochondrial permeability transition pore (mPTP) and the antioxidant enzyme manganese superoxide dismutase (MnSOD) (La Colla et al., 2013). However, a detailed description of the molecular actions triggered by E2 exerting its antiapoptotic effects has not been reported yet. Interestingly, studies indicate that a possible mechanism of regulation of mPTP is through a protein encoded by the SHC gene, p66Shc, which has been proposed to have a central role in apoptosis. In fact, expression and phosphorylation of this adaptor protein has been shown to play an important role in signaling events leading to cell death in response to oxidative stress (Migliaccio et al., 1999; Pellegrini and Baldari, 2009), due to persistent mPTP opening (Almeida et al., 2010). Therefore, misregulation of upstream pathways involved in p66Shc expression and phosphorylation may cause sustained activation of this pore, which ultimately induces cell death.

Signaling molecules, including members of the MAPK family as well as protein kinase C (PKC), have been shown to be involved in the regulation of apoptosis. PKC represents a family of at least 12 serine/threonine kinases that participate having either pro- or antiapoptotic activities depending on the stimulus and cell type (Gutcher et al, 2003). PKCδ is generally associated with apoptotic effects whose regulation by this isoform is rather complex (Blass et al., 2001; Reyland 2007). Numerous apoptotic

stimuli may activate PKCδ by phosphorylation of tyrosine residues (Konishi et al., 1997) and the resulting function depends on various factors, including its localization and the presence of other pro- and antiapoptoic signaling molecules**.** For instance, JNK, which is one of the key mediators of stress signaling (Hibi et al., 1993), is able to be phosphorylated/activated by PKCδ (Yoshida et al., 2002).

JNK is essential for apoptosis that is mediated by mitochondrial dysfunction (Chambers and LoGrasso, 2011). However, it is not clear what events downstream of this kinase activation contribute to the ultimate cellular damage. Of interest, it has been reported that both JNK and PKCδ are targets of E2 regulation (Shanmugam et al., 1999; Yao et al., 2007). Nevertheless, the molecular mechanism modulated by the steroid in which these kinases are involved is not fully established.

In this work, we postulate that E2 protects skeletal myoblasts against apoptosis induced by H_2O_2 preventing PKCδ, JNK and p66Shc expression and phosphorylation, hence avoiding sustained mPTP opening. These studies are of relevance to skeletal muscle physiology as the cell line C2C12 is an appropriate experimental model of satellite cells. C2C12 myoblasts resemble the satellite cells that surround mature myofibers. As differentiated adult skeletal muscle fibers have scarce ability to repair and regenerate themselves when a cellular injury exists, satellite cells have the capacity to proliferate and differentiate vital properties to repair the injured tissue (Yoshida et al., 1998). In this context, satellite cells and their response to oxidative stress are important to mature skeletal muscle performance and function. Of significance for our work, enhanced satellite cell apoptosis has been related to compromised recovery potential in skeletal muscle of aged animals (Jejurikar and Kuzon 2003, Jejurikar et al., 2006). The knowledge of the molecular mechanism underlying the antiapoptotic action of E2 in these cells is relevant to understand the hormone protective effects and could help to further characterize the causes of satellite cells loss in view of a future therapeutic impact.

Materials and methods

Materials

Anti-p-PKCδ Tyr311 antibodies (1:1000), anti-PKCδ (1:1000) and JNK inhibitor SP600125, were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-p-JNK (1:1000) and anti-JNK (1:500) were from Santa Cruz Biotechnology, Inc. Anti-β-tubulin (1:1000) antibody was obtained from Thermo Fisher Scientific, Inc. (Rockford, IL, USA). Anti-p-p66Shc ser36 (1:1000 for Western blot analysis and 1:50 for immunocytochemistry) was purchased from Abcam (Cambridge, MA, USA).

Tetramethylrhodamine methyl ester (TMRM) dye was provided by Molecular Probes (Eugene, OR, USA). The PKC inhibitor chelerythrine was from Alomone (Jerusalem, Israel). Phorbol 12-myristate 13-acetate (PMA) was from Promega. MitoTracker Red (MitoTracker Red CMXRos) dye and Cy2-conjugated anti-mouse secondary antibody (1:200) were provided by Molecular Probes (Eugene, OR, USA). E2 and fulvestrant were from Sigma-Aldrich (St. Louis, MO, USA). The ECL blot detection kit was provided by Perkin-Elmer, Inc (Waltham, MA, USA). The protein molecular weight marker was from Amersham (Buckinghamshire, England). All the other reagents used were of analytical grade. The High Pure RNA Isolation kit was from Roche Diagnostics (Mannheim, Germany). The High Capacity cDNA Reverse Transcription Kit and the KAPA SYBR® FAST qPCR Kit Master Mix Universal were from Applied Biosystems Inc (CA, USA).

Cell culture and treatment

C2C12 murine skeletal myoblasts obtained from American Type Culture Collection (Manassas, VA, USA) were cultured in growth medium (DMEM) supplemented with 10% heat-inactivated (30 min, 56°C) fetal bovine serum, 1% nistatine, and 2% streptomycin. These highly myogenic cells have been widely used to study muscle functions (Burattini et al., 2004, Vasconsuelo et al., 2008, Pronsato et al., 2012). Cells were incubated at 37°C in a humid atmosphere of 5% CO_2 in air. The treatments were performed with 70–80% confluent cultures (120000 cells/cm^2) in medium without serum for 30 min. Then, 10^{-8}M E2, fulvestrant (1 µM, 1h) prior to addition of 10^{-8}M E2, vehicle 0.001% isopropanol (control) or the corresponding inhibitors (SP600125, Chelerythrine) were added 1h before induction of apoptosis with hydrogen peroxide (H_2O_2). H_2O_2 was diluted in culture medium without serum to 0.5 mM in each assay (Jiang et al., 2011; Vasconsuelo et al., 2008). After treatments, cells were lysed using a buffer composed of 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.2 mM Na_2VO_4, 2 mM EDTA, 25 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% NP40, 20 mg/ml leupeptin, and 20 mg/ml aprotinin. Protein concentration was estimated by the method of Bradford (1976).

Subcellular fractionation

C2C12 cells were scrapped and homogenized in ice-cold Tris–EDTA-sucrose buffer (50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 250 mM sucrose, 1 mM DTT, 0.5 mM PMSF, 20 mg/ml leupeptin, 20 mg/ml aprotinin, and 20 mg/ml trypsin inhibitor) using a Teflon-glass hand homogenizer. Total homogenate free of debris was used in order to isolate the different fractions. Nuclear pellet was obtained by centrifugation

at 800 g for 15 min at 4°C. The supernatant was further centrifuged at 10000g for 30 min at 4°C to yield the mitochondrial pellet. The remaining supernatant was further centrifuged at 40000g for 1h at 4°C to obtain the cytosolic fraction. Pellets were resuspended in lysis buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.2 mM Na_2VO_4, 2mM EDTA, 25mM NaF, 1mM PMSF, 20 mg/ml leupeptin, and 20 mg/ml aprotinin). Protein concentration of the fractions was estimated by the method of Bradford (1976) and Western blot assays were performed. Cross contamination between fractions was assessed by immunoblot analysis using anti-VDAC as mitochondrial marker.

Western blot analysis

Protein samples (20 µg) were mixed with buffer (400 mM Tris–HCl (pH 6.8), 10% SDS, 50% glycerol, 500 mM dithiothreitol (DTT), and 2 mg/ml bromophenol blue), boiled for 5 min, and resolved by 10–12% SDS–PAGE according to the method of Laemmli (1970). Fractionated proteins were electrotransferred to polyvinylidene fluoride membranes (Immobilon-P; PVDF) and then blocked for 1h at room temperature with 5% nonfat dry milk in PBS containing 0.1% Tween-20 (PBS-T). Blots were incubated overnight with the primary antibodies: anti-p-PKCδ, anti-PKCδ, anti-VDAC and anti-β-tubulin using anti-rabbit secondary antibodies for all of them; anti-p-JNK, anti-JNK and anti-p-p66Shc with anti-mouse secondary antibodies. The membranes were washed with PBS-T before incubation with HRP-conjugated secondary antibodies. The ECL blot detection kit was used. Relative migration of unknown proteins was determined by comparison with molecular weight markers. When needed, membranes were stripped with stripping buffer (62.5 mM Tris–HCl (pH 6.7), 2% SDS, 50 mM β-mercaptoethanol), washed with PBS 1% Tween-20 and blocked for 1h with 5% non-fat dry milk in PBS-T. The blots were then treated as before using other antibodies. Relative quantification of bands was performed using ImageJ software (NIH, USA).

Terminal Transferase dUTP Nick End Labeling (TUNEL) assays

After the corresponding treatments, TUNEL assays were performed according to manufacturer's directions (Promega, Madison, USA). Briefly, cells were fixed with paraformaldehyde (4% in PBS, pH 7.4), washed with PBS and permeabilized with Triton X-100 (0,2% in PBS) and labeled with rTdT incubation solution (60 min, 37°C, in the dark). After washings, samples were analyzed in order to detect green fluorescence of apoptotic cells by fluorescence microscope.

Immunocytochemistry

After the corresponding treatments, coverslips with adherent cells were stained with MitoTracker Red, prepared in DMSO and then added to the cell culture medium at a final concentration of 1 µmol/l. After 30 min of incubation at 37 ºC in darkness, cells were washed with PBS (pH 7.4, 8 g/l NaCl, 0.2 g/l KCl, 0.24 g/l KH_2PO_4, and 1.44 g/l Na_2HPO_4) and fixed with methanol at -20 ºC for 30 min. After fixation, cells were rinsed three times with PBS and then, non-specific sites were blocked for 1 h with PBS 5% BSA. Cells were incubated with appropriate primary antibodies overnight at 4 ºC. The primary antibodies were recognized by fluorophore-conjugated secondary antibodies. Finally, the stained cells were analyzed with a confocal scanning laser microscopy (Leica TCS SP2 AOBS microscope), using a 63x objective. The specificity of the labeling techniques was proven by the absence of fluorescence when the primary or the secondary antibodies were omitted. At least ten fields per slide were examined. Representative photographs are shown.

Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)

Mitochondrial membrane potential was determined using TMRM. After the corresponding treatments, C2C12 cells were loaded with this dye (20 nM, 30 min, 37°C in darkness) in medium without serum and the fluorescence was measured using a fluorescence microscope. A total of 500 cells per treatment were studied for the determination of the percentage (%) of TMRM fluorescence intensity. Quantification of results was performed using ImageJ. Background fluorescent intensity was subtracted.

RNA isolation, reverse transcription, and real-time quantitative PCR

Cells were treated during different periods of time with the vehicle (C), H_2O_2 (0.5 mM) or preincubated with 10^{-8} M E2 during 1h and then exposed to H_2O_2 (0.5 mM) for the times specified. Total RNA was extracted after the corresponding treatments, using the High Pure RNA Isolation kit (Roche Diagnostics, Mannheim, Germany). RNA (2 µg) was reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., CA, USA) according to the manufacturer´s instructions. Quantitative real-time PCR was done using KAPA SYBR® FAST qPCR Kit Master Mix (2X) Universal (KR0389 - v8.12), MicroAmp® Fast 96-Well Reaction Plate, and ABI Prism7500Fast® Sequence Detection System with 96-wells (Applied Biosystems Inc., CA, USA) under the standard conditions recommended by the manufacturer. Primer sets to amplify murine cDNAs used in the analysis were as follows: glycelaldehyde 3-phosphatedehydrogenase (GAPDH) set: forward 5´CGTCCCGTAGACAAAATGGT3´,

reverse 5´TTGATGGCAACAATCTCCAC3´ and p66Shc set: forward 5´ACTACCCTGTGTTCCTTCTTTC 3´, reverse 5´TCGGTGGATTCCTGAGATACTGT3´. The specificity of PCR products was confirmed by melting curve analysis. Levels of the transcripts were normalized to GAPDH, used as housekeeping gene. Relative quantification of gene expression was determined by the comparative CT method (Livak and Schmittgen, 2001).

Statistical analysis

Results are shown as means ± S.D. of not less than three independent experiments. Statistical differences among groups were performed using ANOVA and a multiple comparison post hoc test (Tukey 1953). The statistical significance of data was determined as $p< 0.05$.

Results

E2 inhibits phosphorylation of PKCδ and JNK in skeletal myoblast cells

As mentioned above, PKCδ is considered to be an apoptotic mediator (Blas et al., 2001; Reyland 2007). Thus, we explored whether E2 regulates PKCδ signaling in C2C12 cells. Tyrosine phosphorylation is a relatively specific regulatory mechanism for PKCδ, but not for the others PKC isoforms. To our interest, it has been shown that tyrosine phosphorylation of PKCδ occurs in cells under oxidative stress, affecting its activity (Konishi et al., 1997).

As an initial approach, we explored the activation of PKCδ in C2C12 cells evaluating its phosphorylation in the residue tyrosine 311. C2C12 cells were treated with H_2O_2 (0.5 mM for different periods of time). We observed that H_2O_2 induced the activation of this kinase at 1h of treatment, but began to decay at 4h of treatment, showing that this effect was not maintained over time (Fig. 1A). In order to evaluate if the hormone E2 was able to modulate PKCδ activation, C2C12 cells were incubated with physiological concentrations of E2 (10^{-8}M, 1h) prior addition of the apoptotic inductor H_2O_2. As shown in Fig. 1B, under these conditions, E2 diminished PKCδ phosphorylation at tyrosine 311. However in presence of fulvestrant (1 μM, 1h), an ER antagonist, E2 was unable to diminish the H_2O_2-induced phosphorylation of this kinase.

Another stress-pathway that could act in conjunction with PKCδ is the JNK cascade. Then, we investigated whether JNK is also activated in response to this apoptotic inductor in C2C12 cells. We evidenced that H_2O_2 activated this kinase after 1h of incubation but it behaved differently from PKCδ,

since its activation was maintained over time (Fig. 2A). To explore the effect of E2 on JNK activation, C2C12 cells were incubated with E2 (10^{-8} M, 1h) prior addition of H_2O_2. It was observed that E2 could reverse its phosphorylation. When cells were treated with the ER antagonist before the addition of E2, the H_2O_2-induced phosphorylation of JNK was maintained (Fig. 2B).

PKCδ and JNK are involved in skeletal myoblast cell apoptosis

Since the role of PKCδ and JNK in the apoptotic process depends on the cell type, nature of the death stimulus, duration of its activation and the activity of other signaling pathways, we next investigated whether inhibition of PKC and JNK affects the ability of C2C12 cells to undergo apoptosis. Cells were treated with H_2O_2 (0.5 mM, 1h) in the presence and absence of the PKC specific inhibitor chelerythrine (2 μM, 1h) or the JNK inhibitor SP600125 (10 μM, 1h). Cells exhibited a large increase in DNA fragmentation (74.01% ± 12.09 of TUNEL positive cells above the control) in response to H_2O_2. Pre-treatment of cells with chelerythrine diminished DNA fragmentation caused by H_2O_2 (from 88.05% to 46.3%). Similar effects were obtained when cells were treated with SP600125 (from 88.05% to 46.9%) (Fig. 3A-B). Moreover, since it is known that diacylglycerol (DAG) activates PKC (Nishizuka, 1992) and that DAG induced activation of PKC can be mimicked by phorbol esters (PMA) (Newton, 1997), cells were treated with PMA (1 μM, 1h). This treatment increased the percentage of TUNEL positive cells (69.01% above the control) to the same extent as H_2O_2 (Fig. 3A-B). These results show that both kinases are involved in the apoptosis triggered by H_2O_2 in skeletal myoblast cells. Additionally, treatment of cells with E2 before the induction of apoptosis showed similar results (53.04%) as those obtained in presence of both inhibitors. The presence of fulvestrant prior addition of E2 and H_2O_2 increased the percentage of TUNEL positive cells (85.04 %) as H_2O_2 did.

PKCδ can act upstream JNK in the apoptotic pathway in skeletal myoblast cells

In the present study it was shown that both PKCδ and JNK were activated in response to H_2O_2 exerting a pro-apoptotic role. To further elucidate the mechanism of action of these kinases, we investigated whether PKCδ and JNK might function connectedly in skeletal muscle cells. To that end, PKC was downregulated by treating C2C12 cells with a high concentration of PMA (10 μM) for a prolonged time (48h) (Takuwa et al., 1988; Rodriguez-Pena et al., 1984). After downregulation of PKCδ, cells were exposed to H_2O_2 and the phosphorylation of JNK was assessed. As observed in Fig. 4, PMA reduced the H_2O_2-induced phosphorylation of JNK. In contrast, when muscle cells were treated with the

JNK inhibitor and then probed for the H_2O_2-induced phosphorylation of PKCδ, no changes were observed. These results strongly suggest that PKCδ acts upstream JNK in the apoptotic pathway triggered by H_2O_2.

E2 decreases p66Shc mRNA expression and inhibits its phosphorylation in skeletal myoblast cells

p66Shc expression and its phosphorylation at serine 36 is linked to cell death in response to oxidative stress (Migliaccio et al, 1999; Pellegrini and Baldari, 2009). Initially, to gauge the effect of H_2O_2 (0.5 mM) over p66Shc mRNA level in skeletal muscle, cells were incubated with the apoptotic inducer for the periods of time specified in Fig. 5A. We found that expression levels of p66Shc mRNA were upregulated after H_2O_2 treatments. In addition, when p66Shc protein phosphorylation was evaluated in response to H_2O_2, it was found that after 1h of treatment with H_2O_2 (0.5 mM), p66Shc was phosphorylated in serine 36 residue. This effect was maintained up to 3 h of incubation (Fig. 5B). Since we found that JNK was activated in response to H_2O_2, we further studied if this kinase can act upstream p66Shc. Consequently, cells were exposed to the JNK inhibitor SP600125 (10 μM, 1h), prior to incubation with H_2O_2 and probed for the phosphorylation of p66Shc. The data obtained support the notion that JNK is involved in the phosphorylation of p66Shc. However, when PKCδ was inhibited, the phosphorylation of this protein was not affected (Fig. 5C).

Then, we investigated whether E2 could abrogate the expression and phosphorylation of p66Shc in response to H_2O_2. p66Shc mRNA levels induced by H_2O_2 (0.5 mM) were diminished by preincubation with the hormone (10^{-8} M, 1h) (Fig. 5A) at 1h of treatment, effect that was maintained. When immunoblot assays were performed, it was observed that E2 could abolish p66Shc phosphorylation on serine 36 residue. These results taken together suggest that both p66Shc expression and phosphorylation by H_2O_2 through JNK activation are important to conduce cells to death, and that E2 protected muscle cells from apoptosis by preventing these effects.

E2 affects the mitochondrial translocation of p66Shc triggered by JNK

As shown, p66Shc increased its expression and became phosphorylated through JNK in response to H_2O_2 in C2C12 cells. In order to further investigate putative targets of the adaptor protein in the stress response, we analyzed its intracellular localization once it is phoshorylated.

To address this issue, cells were treated with the apoptotic inducer and then cytosolic and mitochondrial fractions were obtained. Immunoblots of fractions showed that the amount of p66Shc localized in mitochondria is higher than its content in the cytosolic fraction (Fig. 6). Moreover, since JNK was activated in response to H_2O_2 inducing serine 36-phosphorylation of p66Shc, it was further studied whether this kinase is involved in its translocation to mitochondria. The cells were treated with the JNK inhibitor SP600125 (10 μM, 1h) prior to incubation with H_2O_2 followed by subcellular fractionation of lysates. Fractions were probed for p66Shc by immunoblot assays. Under this condition, p66Shc was unable to translocate to mitochondria, demonstrating that JNK activation is a crucial step in this translocation. Furthermore, when we studied whether E2 could prevent its translocation, it was observed that addition of physiological concentrations of the hormone (10^{-8} M, 1h) prior to addition of H_2O_2 (0.5 mM, 1h) decrease p66Shc migration. Moreover, immunocytochemistry studies were performed using an anti-p-p66Shc antibody (green fluorescence) and Mitotracker (red fluorescence). In the control condition, p66Shc is not phosphorylated (no fluorescence was detected) while H_2O_2 treatment induced its phosphorylation (high green fluorescence) and translocation to mitochondria, evidenced by the yellow fluorescence in mitochondria from the merged image. In presence of the hormone, a slight green fluorescence appeared diffuse in all the cytosol, showing inhibition of p-p66Shc translocation to mitochondria. When cells were treated with fulvestrant prior addition of E2 and H_2O_2, the inhibitory effect of E2 was not observed. These data support again the notion that the estrogen protects skeletal myoblast cells from apoptosis inhibiting JNK, which in turn cannot phosphorylate p66Shc maintaining it mostly in the cytosol.

E2 prevents loss of mitochondrial membrane potential affecting translocation of p66Shc

In previous studies, we found that E2 could develop an antiapoptotic effect via the modulation of the mPTP, resulting in the prevention of its sustained H_2O_2-induced opening (La Colla et al., 2013). Considering that E2 inhibits the mitochondrial translocation of p66Shc mediated by JNK, a possible relationship between the protection of $\Delta\psi m$ by the hormone and this adaptor protein was investigated. To that end, cells were treated with SP600125 (10 μM, 1h), E2 (10^{-8}M, 1h) or E2 (10^{-8}M, 1h) and fulvestrant (1 μM, 1h), prior to incubation with H_2O_2 (0.5 mM, 1h). After these treatments, live cells were incubated with TMRM (20 nM, 30 min, 37°C) and membrane potential was estimated analysing the levels of fluorescence by microscopy. We observed that the apoptotic inducer lead to a decrease in the

percentage of fluorescent intensity (Fig. 7A-B), indicative of Δψm loss, which is associated to mPTP opening in C2C12 cells (La Colla et al., 2013). The pretreatment with the JNK inhibitor, SP600125, restored the Δψm, since it was observed an increase in the percentage of fluorescent intensity (from 36.54 ± 9.6 (H_2O_2) to 151.39 ± 3.1). This result suggests that JNK is involved in the sustained opening of the mPTP. Moreover, given that the mitochondrial translocation of p66Shc requires JNK activation and that p66Shc phosphorylation has been linked to persistent mPTP opening (Almeida et al., 2010), these results strongly suggest that p66Shc modulates mPTP opening following JNK activation by H_2O_2 in skeletal muscle cells. When cells were pretreated with 10^{-8} M E2 and then induced to apoptosis, the level of fluorescence intensity was maintained as in control condition (104.09 ± 20.1) demonstrating that E2 could prevent mPTP opening and the subsequent Δψm loss by the inhibition of p66Shc translocation to mitochondria, showing the protective action of the steroid (Fig 7A-B). Additionally, the incubation with fulvestrant and E2 prior to induction of apoptosis, conduce to a decrease in the percentage of fluorescent intensity similar to that observed in H_2O_2 condition, involving the ERs in this effect.

Discussion

Rapid signaling cascades triggered by E2 regulate various cellular processes such as differentiation, proliferation, and even apoptosis (Review in Boland et al., 2008). In previous studies, we presented a molecular link between E2 and apoptosis in skeletal muscle cells. Indeed, we showed an antiapoptotic role of the steroid (Vasconsuelo et al., 2008; La Colla et al., 2013) involving ERs, PI3K/Akt/Bad, HSP27 and MAPKs (Vasconsuelo et al. 2008, 2010; Ronda et al., 2010), exerting an important effect over mitochondria (La Colla et al., 2013). In this work, we deepen the molecular pathways modulated by E2 that conduct to mitochondrial protection and that, in conjunction, lead to an antiapoptotic effect.

The family of intracellular signaling molecules that are regulated during apoptosis is increasing and includes a variety of protein kinases (Bokoch, 1998). Several studies have demonstrated that PKCδ has multiple targets in response to apoptotic stimuli, acting either as pro-apoptotic or anti-apoptotic depending on the cell type and stimuli (Brodie and Blumberg, 2003; Jackson and Foster, 2004). Even in the same cell type, relying on the physiological state, PKCδ may accomplish multiple diverse functions. This flexibility in PKCδ signaling may be due to the complex regulation mechanisms of the kinase

(Steinberg, 2004; Corbalan-Garcia and Gomez-Fernandez, 2006). In addition to the traditional model of PKCδ activation that requires lipid cofactors and anchoring proteins that localize the active conformation of the kinase to membranes, a distinct mode for PKCδ activation that involves tyrosine phosphorylation has been identified (reviewed in Kikkawa et al., 2002). This particular phosphorylation on tyrosine residues is an early response to many apoptotic stimuli and it is generally accepted that it conduces to its activation (Konishi et al., 1997). Here, we found that H_2O_2 induced PKCδ activation, probed by its phosphorylation in residue tyrosine 311, exerting a pro-apoptotic role in muscle cells which was prevented by E2. The involvement of ERs in this role was also demonstrated, in agreement with our previus work which showed that the protective effects of this hormone in skeletal myoblasts are through ERs (Vasconsuelo et al., 2008; La Colla et al., 2013). In addition, we corroborated these results using the PKC activator PMA. PKC isozymes, including PKCδ, can be up-regulated as a result of cell treatment with pharmacological concentrations of PMA (Assert et al., 1996). Since we found that PMA treatment lead to similar results as H_2O_2, it can be additionally demonstrated the apoptotic role of this PKC in C2C12 cells under H_2O_2 stimuli. Although here is clearly demonstrated the participation of PKCδ in H_2O_2-induced apoptosis, it cannot be excluded the involvement of other PKC isotypes responsive to PMA.

Even though the apoptotic role of JNK has been established, it has also been reported that the early transient activation of JNK is associated with proliferation, while sustained activation of JNK correlates with the induction of apoptosis by a variety of agents (Chen et al., 1998). Accordingly, here we demonstrated that JNK was activated by H_2O_2 in C2C12 skeletal myoblast cells and afterwards its activation was sustained during the time studied. Even though both kinases, PKCδ and JNK, were activated by the apoptotic stimuli at the same time, they differ in the duration of their activation. Additionally, we found that JNK and PKCδ act in conjunction mediating the apoptotic signal. It is known that long-term exposure of cells to high concentrations of phorbol esters such as PMA cause downregulation of PKCδ (Ahnadi et al., 2000). However, the mechanism whereby PMA induces downregulation of PKC is still unclear. Since the downregulation of PKCδ with PMA inhibited JNK activation, we showed that JNK acts downstream to this PKC in H_2O_2-treated C2C12 cells. It has been reported that E2 prevented breast cancer cell death induced by UV radiation by inhibiting JNK activity (Razandi et al., 2000). Accordingly, we found that JNK activation was prevented by E2. Apoptosis assays further support this link between JNK and PKCδ, since comparable results were obtained with the apoptotic inducer plus each of the kinase inhibitors, SP600125 and chelerythrine. The current data

strongly suggest a connection between JNK and PKCδ in the apoptotic process in skeletal muscle cells. We found that the inhibition of H_2O_2 induced-JNK activation, by the downregulation of PKC, was more evident on the p46 isoform than p54 isoform. This different behavior between PKCδ and each of the JNK isoforms could be due to different localization of these kinases or interaction with different intermediates in the apoptotic process, the p46 isoform being a more suitable target for PKCδ activation. Therefore, in this work we provide more details of the complex molecular mechanism responsible of the protective role exerted by E2 on myoblasts against apoptosis.

p66Shc is a key protein involved in oxidative stress signaling whose expression changes between different cell types and even is not present in some cells (Migliaccio et al., 1997; Pelicci et al., 1992). Of our interest, it was previously demonstrated that expression and phosphorylation of this adaptor protein at serine 36 is important for the death response upon oxidative damage during aging (Migliaccio et al., 1999; Chen et al., 2014). In view of these observations, it was studied whether p66Shc participates in the apoptotic response in skeletal muscle cells and if JNK and/or PKCδ, shown in this work to be activated in response to H_2O_2, are involved in phosphorylation of p66Shc. It was first demonstrated that p66Shc mRNA level was augmented in response to the apoptotic inducer and that E2 could revert this effect. Accordingly, p66Shc was phosphorylated in serine 36 upon H_2O_2 treatment. This activation was not observed when cells were pretreated with the hormone. Afterwards, the JNK inhibitor SP600125 prevented the H_2O_2-induced p66Shc phosphorylation, suggesting that this adaptor protein is a substrate of JNK. Moreover, the results suggest that p66Shc phosphorylation is independent of PKC, since it was not affected when this kinase was downregulated with PMA. Then, one may envision that p54 JNK is the isoform mainly involved in the phosphorylation of this adaptor protein, since it was affected to a lesser extent by PKC downregulation.

Another issue for understanding the molecular mechanism of p66Shc action is its intracellular localization after being phosphorylated. However p66Shc has a mitochondrial targeting signal which allows translocation to this organelle (Ventura et al., 2004), the mechanism underlying this event is not fully elucidated. The results obtained in the present work further support the notion that p66Shc changes its localization upon its phosphorylation from the cytosol to the mitochondrial compartment, and that E2 through ER prevents its translocation. This is in concordance with the fact that the hormone inhibits JNK activation, and subsequently p66Shc phosphorylation/translocation is suppressed. In previous studies, we found that E2 protected C2C12 cells from apoptosis by inhibiting the sustained opening of mPTP (La

Colla et al., 2013), an event which was directly related to the loss of mitochondrial membrane potential (Armstrong 2006). Furthermore, we demonstrated that this opening precede the $\Delta\psi m$ loss (La Colla et al., 2013). We then hypothesize that E2 can regulate p66Shc to avoid the loss of mitochondrial membrane potential, and in turn preserves mitochondrial function. Indeed, it was found that E2 through ERs restored the loss of mitochondrial membrane potential, showing that in presence of the steroid, p66Shc is not phosphorylated nor it can translocate to mitochondria, and as a consequence the mitochondrial membrane potential is maintained indicating that p66Shc is not able to alter mitochondrial permeability when acting from the outside of the organelle.

Altogether these data with our previous findings support the relevant role of E2 in the inhibition of multiple cellular pathways in skeletal myoblast cells that acting in concert lead to apoptosis (Fig. 8), confirming that mitochondria are influenced by E2 signalling. As it was found that E2 cannot protect mitochondrial functions during apoptosis when ERs were inhibited in C2C12 cells, in concordance with previous studies (Vasconsuelo et al., 2008; La Colla et al., 2013), then we demonstrate that ERs participate in the molecular pathways studied herein.

As was mentioned, it is known that adult skeletal muscle fibers have scarce capacity to repair and regenerate themselves in response to an injury such as oxidative stress. Satellite cells are responsible of the repair of this injured muscle. Since the C2C12 cell line is a proper experimental model to study satellite cells (Yoshida et al. 1998), this work could contribute with molecular details to further understand the response of myoblasts to oxidative injury and the effects of estradiol in the regulation of apoptosis in skeletal muscle.

Acknowledgments

The authors declare that there is no conflict of interest that would prejudice the impartiality of this work.

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Figure Legends

Fig. 1. Inhibition of H_2O_2 induced-PKCδ activation by 17β-estradiol in C2C12 muscle cells. C2C12 cells were incubated under different experimental conditions: **A)** Control (C) or 0.5 mM H_2O_2 during the times indicated (H_2O_2); **B)** vehicle (C); 10^{-8} M 17β-estradiol for 1 h (E2); 0.5 mM H_2O_2 for 1 h (H_2O_2), ~~or~~ 10^{-8} M 17β-estradiol for 1 h followed by 0.5 mM H_2O_2 during 1 h (H_2O_2 + E2) or 1μM fulvestrant (F) for 1 h followed by 10^{-8} M 17β-estradiol for 1 h and 0.5 mM H_2O_2 during 1 h (E2 + H_2O_2 + F). Cell lysates were prepared and subjected to Western blot analysis using anti-p-PKCδ Y311 and anti-PKCδ antibodies. β-tubulin levels were measured as protein loading controls. The blots are representative of three independent experiments with comparable results. They were quantified by scanning volumetric densitometry and normalized with PKCδ level. Averages ± S.D. are given. * $p < 0.05$, with respect to the control (C); $^{\#}$ $p < 0.05$, with respect to H_2O_2; ** $p < 0.05$, with respect to E2 + H_2O_2

Fig. 2. 17β-estradiol abrogates H_2O_2 induced-JNK activation in C2C12 muscle cells. C2C12 cells were incubated with: **A)** 0.5 mM H_2O_2 for the times indicated (H_2O_2), and **B)** vehicle (C), 10^{-8} M 17β-estradiol for 1 h (E2), 0.5 mM H_2O_2 for 1 h (H_2O_2), ~~or~~ 10^{-8} M 17β-estradiol for 1 h followed by treatment with 0.5 mM H_2O_2 during 1 h (H_2O_2 + E2) or 1μM fulvestrant (F) for 1 h followed by 10^{-8} M 17β-estradiol for 1 h and 0.5 mM H_2O_2 during 1 h (E2 + H_2O_2 + F). Cell lysates were prepared and subjected to Western blot analysis using an anti-p-JNK and anti-JNK antibodies. β-tubulin levels were measured as protein loading controls. Blots representative of three independent experiments with comparable results. They were quantified by scanning volumetric densitometry and normalized with JNK level. Averages ± S.D are given. * $p < 0.05$, with respect to the control (C); $^{\#}$ $p < 0.05$, with respect to H_2O_2. ** $p < 0.05$, with respect to E2 + H_2O_2.

Fig. 3. H_2O_2 induces apoptosis through PKC and JNK in C2C12 cells. A) and **B)** C2C12 cells were left untreated (C) or incubated with 10^{-8} M 17β-estradiol for 1 h (E2), 10^{-8} M 17β-estradiol for 1 h (E2) followed by treatment with H_2O_2 (0.5 mM, 1h) (H_2O_2 + E2), 1μM fulvestrant (F) for 1 h followed by 10^{-8} M 17β-estradiol for 1 h and 0.5 mM H_2O_2 during 1 h (E2 + H_2O_2 + F), the apoptotic inducer H_2O_2 (0.5 mM, 1h) in the presence and absence of 2 μM chelerythrine during 1 h (Chel), 10 μM SP600125 during 1 h (SP), or with 1 μM Phorbol 12-myristate 13-acetate for 1 h (PMA). Then, apoptosis was determined by TUNEL assays as described under Materials and Methods and expressed as the percentage of TUNEL

positive cells in the coverslips. Each value represents the mean of three independent determinations ± S.D.; * p <0.05, with respect to the control; # p< 0.05, with respect to H_2O_2, ** p < 0.05, with respect to E2 + H_2O_2. **B)** Representative images of each assayed condition are shown. Magnification 20x.

Fig.4. Downregulation of PKC inhibits H_2O_2 induced JNK activation. Cells without treatment (C) or treated with 0.5 mM H_2O_2 for 1 h (H_2O_2), or preincubated with PMA (10 μM, 48 h) and then treated with 0.5 mM H_2O_2 for 1 h (H_2O_2) or 10 μM SP600125 (SP) for 1 h followed by incubation with 0.5 mM H_2O_2 during 1 h (H_2O_2 + SP). Cell lysates were obtained following the different treatments and subjected to Western blot analysis using antibodies specific for p-PKC δ Y311 and p-JNK and their corresponding unphosphorylated total proteins. β-tubulin levels were measured as protein loading controls. The blots are representative of three independent experiments with comparable results. Densitometric quantification is shown, normalizing with PKC δ and JNK, respectively. In particular, in the condition PMA + H_2O_2, β-tubulin level was used to normalize. Averages ± S.D are given. * p < 0.05 with respect to the control (C). # p< 0.05 with respect to H_2O_2.

Fig. 5. 17β-estradiol diminishes p66Shc mRNA levels and inhibits p66Shc activation through JNK. A) Cells were treated with 0.5 mM H_2O_2 for the indicated times (H_2O_2) or preincubated with 10^{-8} M 17β-estradiol for 1h, followed by incubation with 0.5 mM H_2O_2 during the indicated times (E2 + H_2O_2). Under the designated conditions, quantification by real-time PCR of p66Shc mRNA levels was carried out. Transcript levels were normalized to the expression level of GAPDH. Syber green runs were performed on duplicate samples of cDNAs from 2 independent reverse transcription reactions. The CT method was applied as a comparative method of quantification. Averages ± S.D are given. * p < 0.05 with respect to the control (C); # p < 0.05 with respect to the corresponding H_2O_2 treatment without E2. **B)** Cell were incubated under different experimental conditions: 0.5 mM H_2O_2 for the indicated times (H_2O_2) or **C)** vehicle (C), 10^{-8} M 17β-estradiol for 1 h (E2), 0.5 mM H_2O_2 for 1 h (H_2O_2), 10 μM SP600125 (SP) for 1 h followed by incubation with 0.5 mM H_2O_2 during 1 h (H_2O_2 + SP) or 10^{-8} M 17β-estradiol for 1h prior addition of 0,5 mM H_2O_2 during 1h (H_2O_2 + E2). Under the designated conditions, cells were preincubated with PMA (10 μM, 48 h). Lysates were used to probe for serine 36-phosphorylation of p66Shc by Western blot assays. The blots are representative of three independent experiments with comparable results.

Densitometric quantification of blots is shown. Averages ± S.D are given. * $p < 0.05$, with respect to the control (C); # $p < 0.05$, with respect to H_2O_2.

Fig. 6. E2 affects the mitochondrial translocation of p66Shc triggered by JNK. A) C2C12 cell lysates were obtained following the different treatments and then subjected to subcellular fractionation. Enriched mitochondrial and cytosolic fractions were used to perform Western blots assays and probed for p66Shc serine 36. Experiments were repeated at least three times with essentially identical results. VDAC was used as mitochondrial marker. **B)** Cells were treated with vehicle (C), H_2O_2 for 1 h (H_2O_2), 10^{-8} M 17β-estradiol for 1 h (E2) followed by treatment with H_2O_2 (0.5 mM, 1h) (H_2O_2 + E2) or 1μM fulvestrant (F) for 1 h followed by 10^{-8} M 17β-estradiol for 1 h and 0.5 mM H_2O_2 during 1 h (E2 + H_2O_2 + F). Then, cells were incubated with Mitotracker (red fluorescence) and anti-p-p66Shc followed by a fluorophore-conjugated secondary antibody (green fluorescence). Representative images by confocal microscopy are shown. Magnification 60x.

Fig. 7. E2 prevents loss of mitochondrial membrane potential. A) C2C12 cells were grown on glass coverslips. After the different treatments indicated in the graph, coverslips were incubated with the red fluorescent probe TMRM at a final concentration of 20 nM in serum-free DMEM medium for 30 min at 37 °C in darkness. Afterwards, samples were examined under a fluorescence microscope. Experiments were repeated at least three times with essentially identical results. Images were captured with a digital camera and their quantification was performed using ImageJ software (NIH). Averages ± S.D. are given. * $p < 0.05$ with respect to the control. # $p < 0.05$, with respect to H_2O_2, ** $p < 0.05$, with respect to E2 + H_2O_2. **B)** Representative images are shown. Magnification 40x.

Fig. 8. Schematic diagram showing the participation of PKCδ, JNK and p66Shc in the protective action of 17β-estradiol in skeletal muscle myoblasts. E2 prevents PKCδ, JNK and p66Shc phosphorylation involving ERs. These events are implicated in the antiapoptotic action of the hormone that in turn abrogate the collapse of mitochondrial membrane potential induced by hydrogen peroxide stimulus.

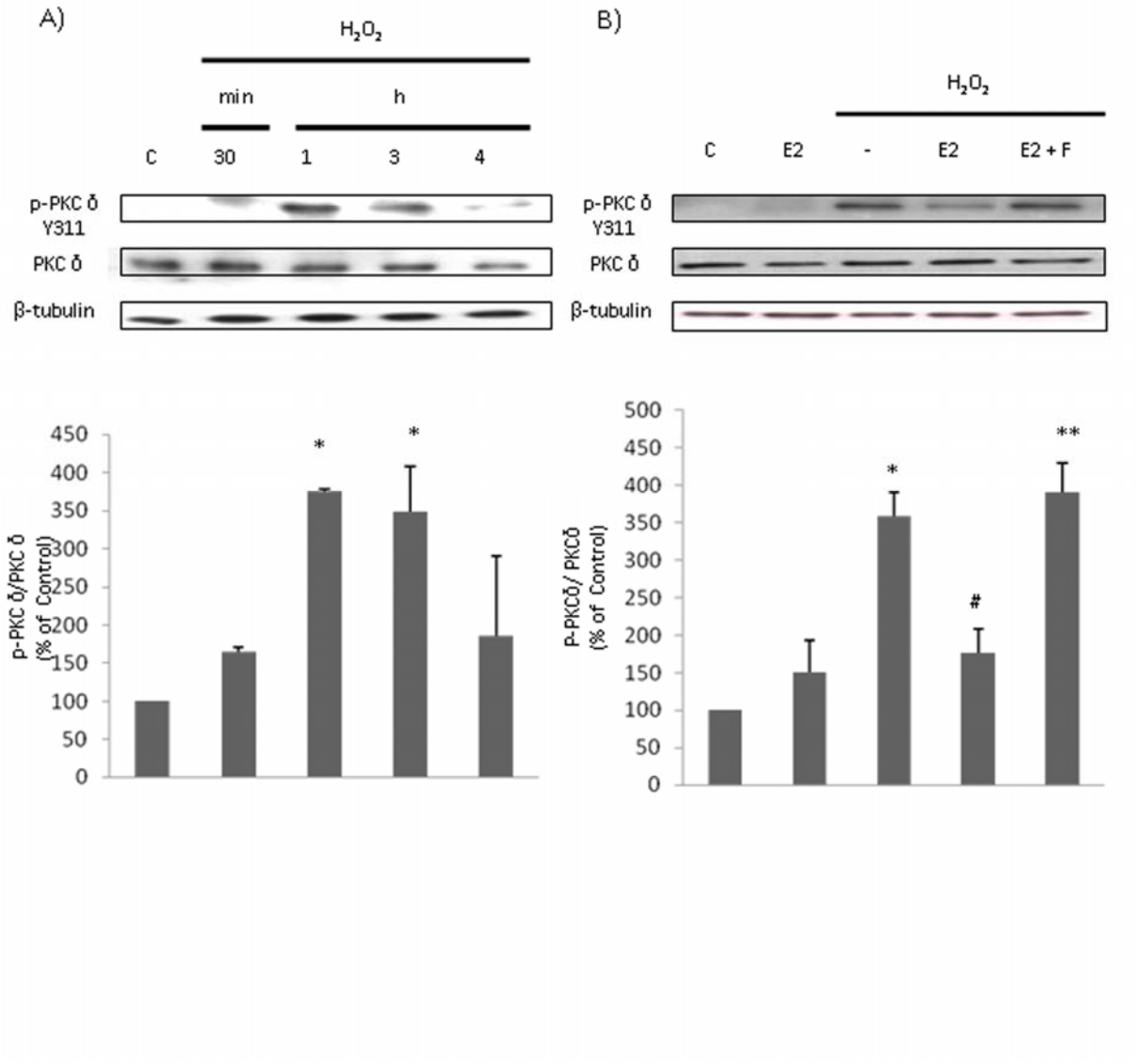

Figure 1

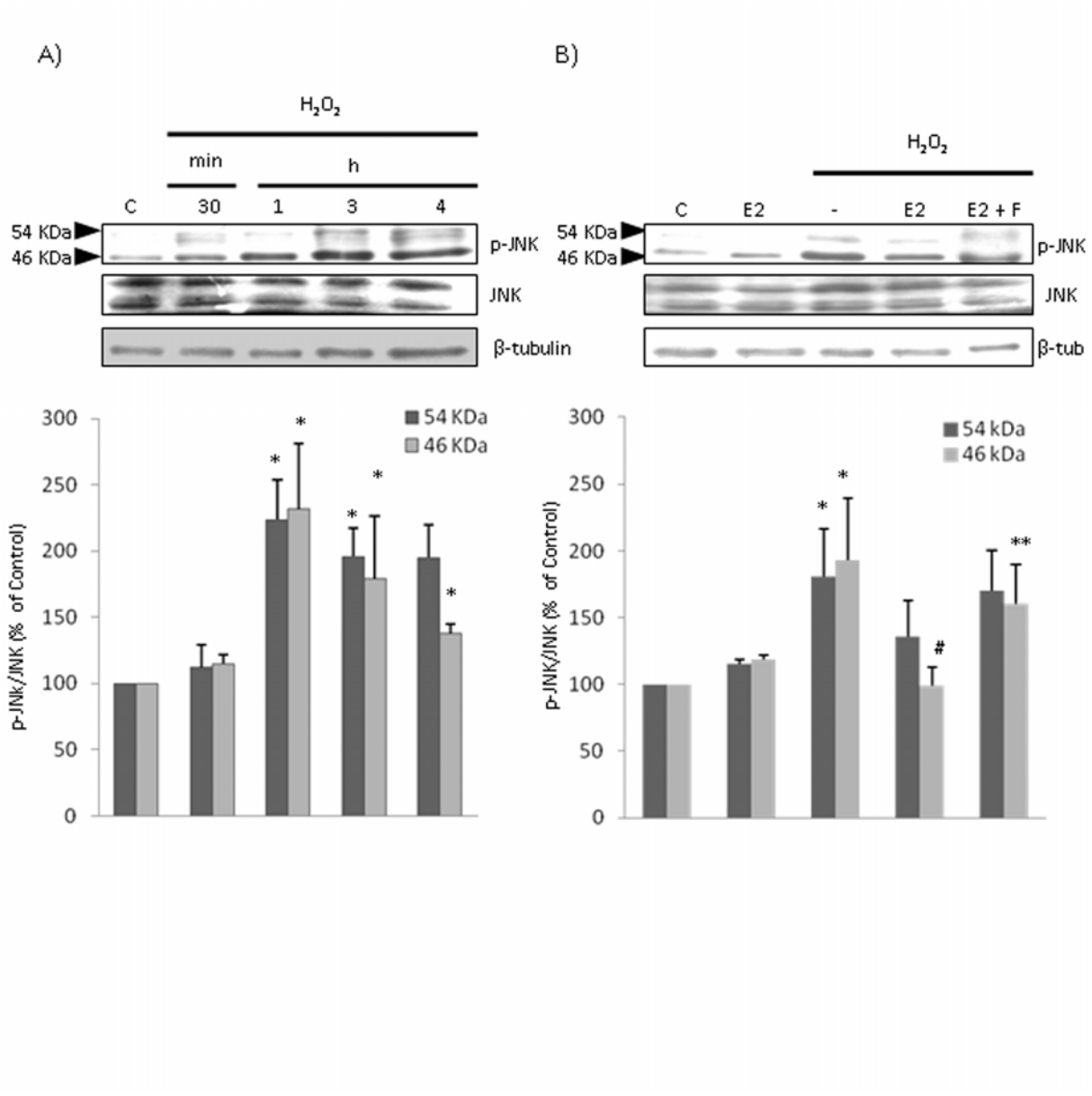

Figure 2

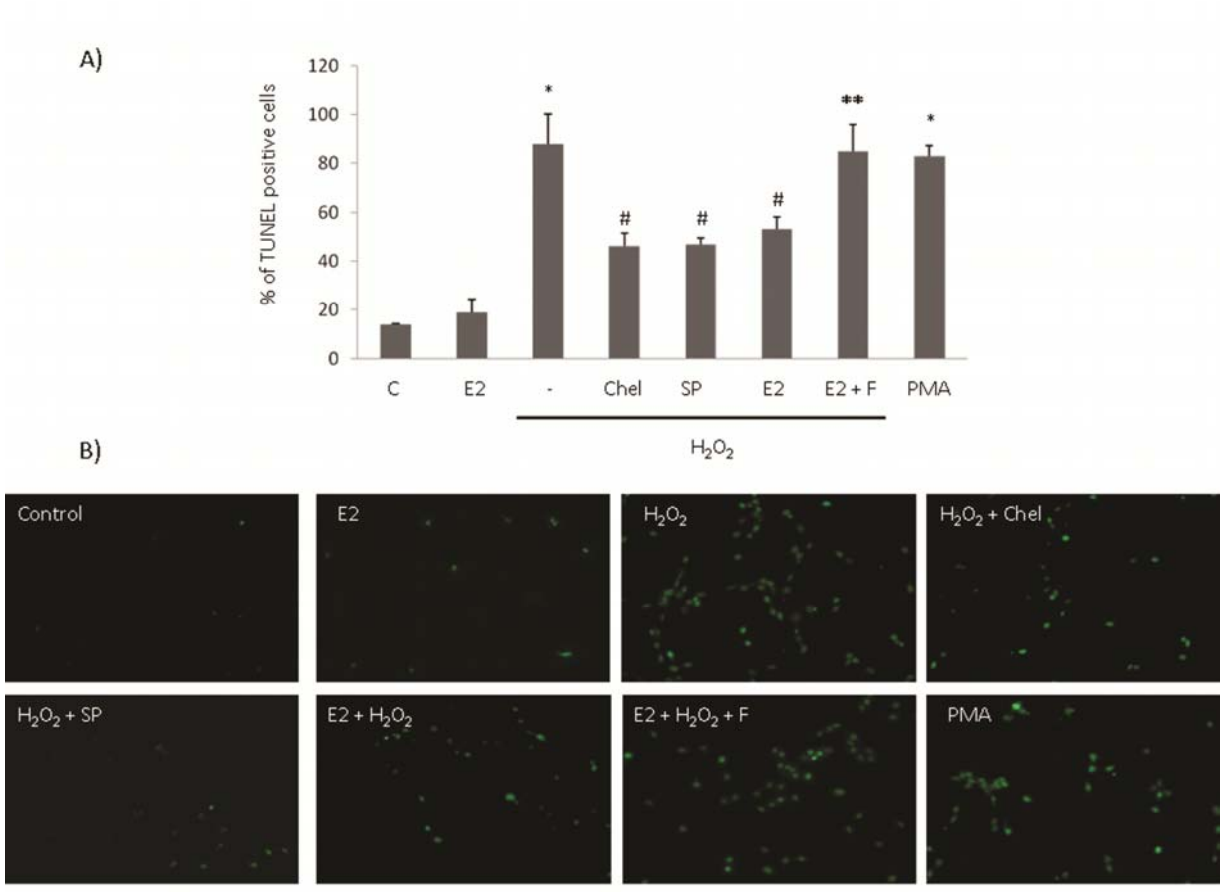

Figure 3

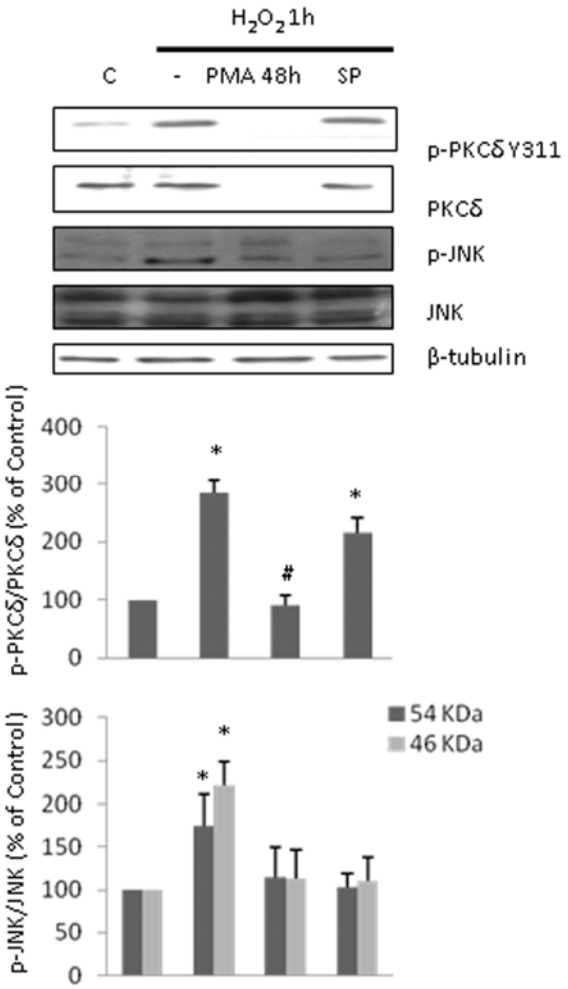

Figure 4

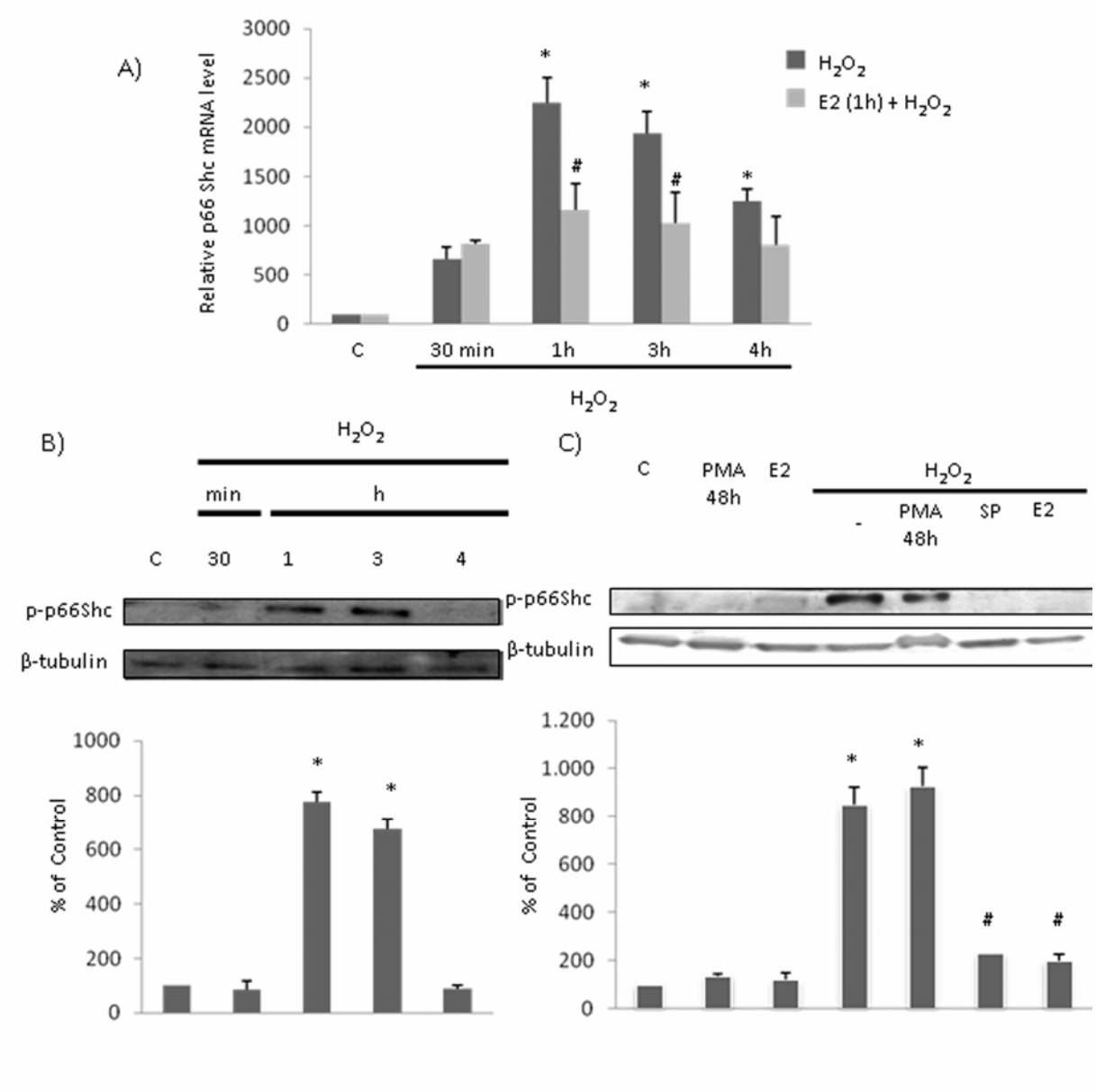

Figure 5

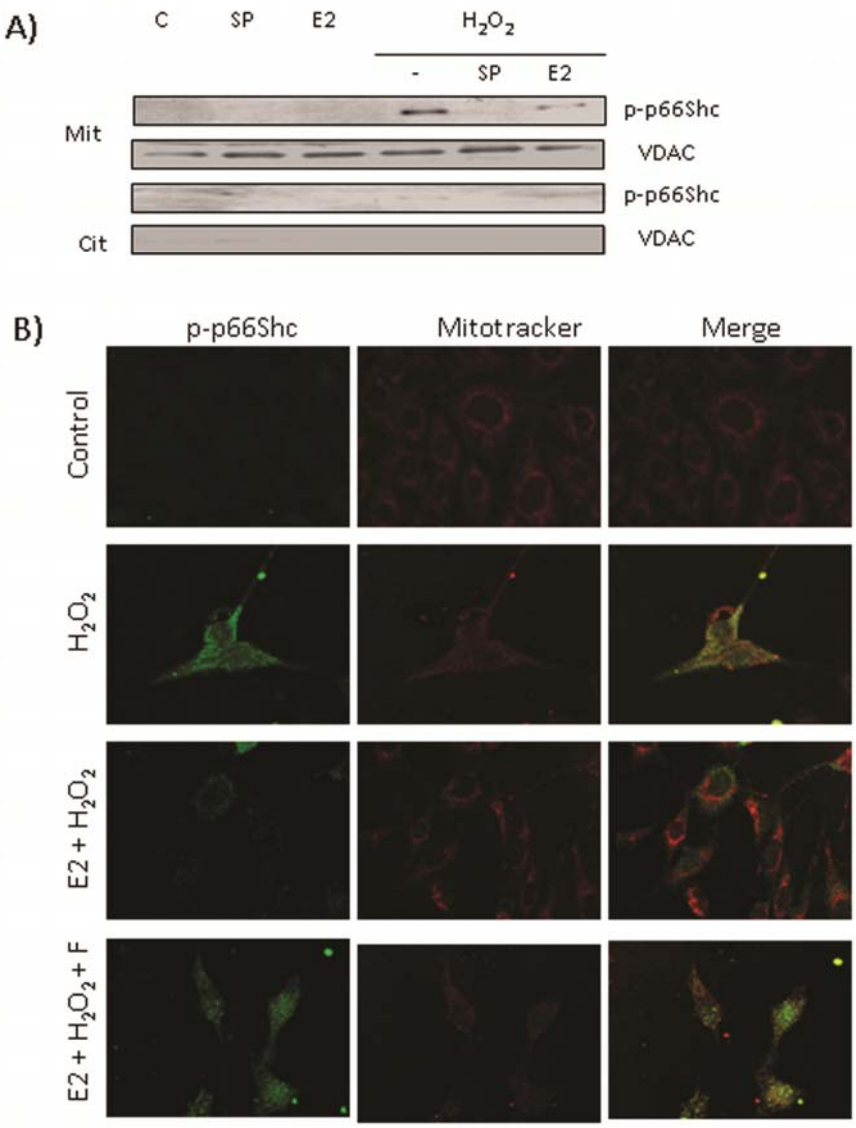

Figure 6

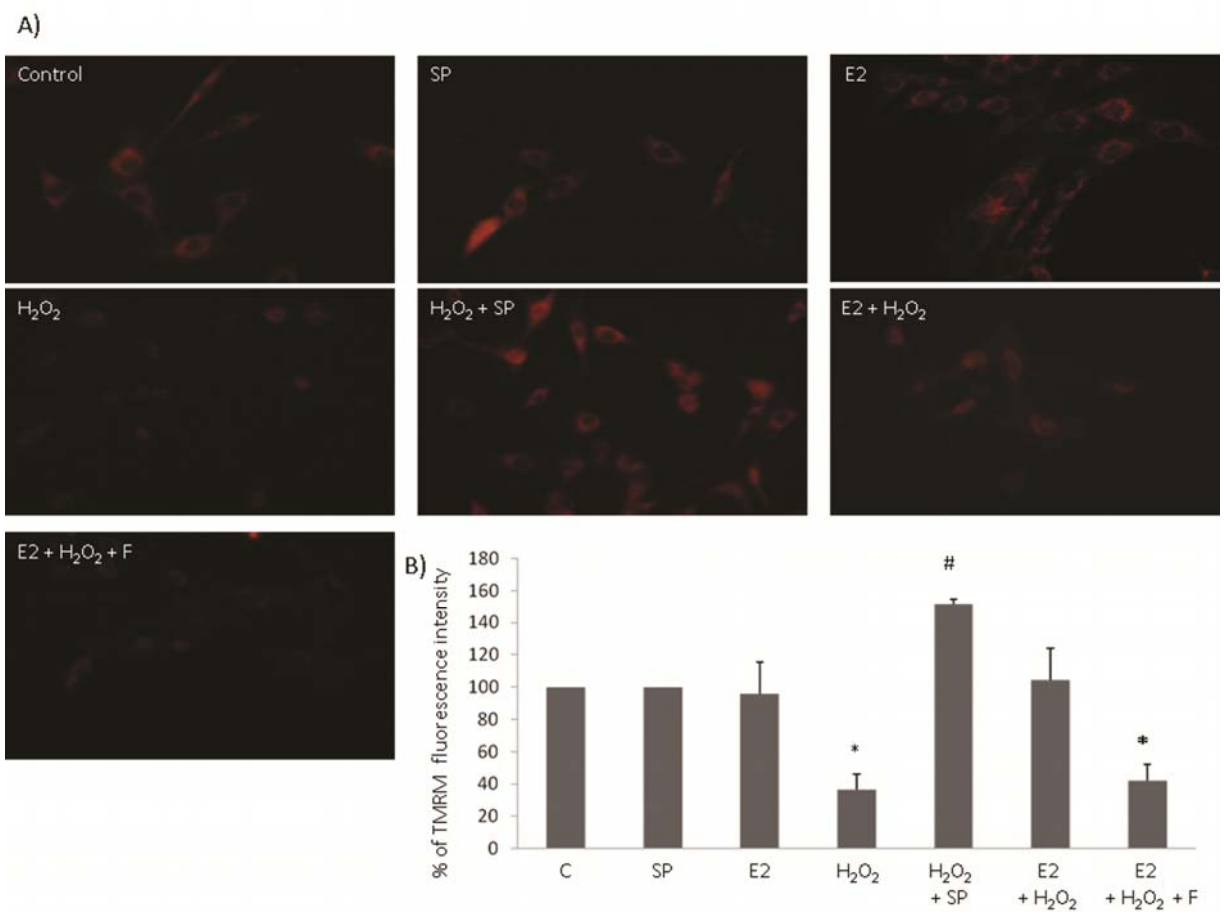

Figure 7

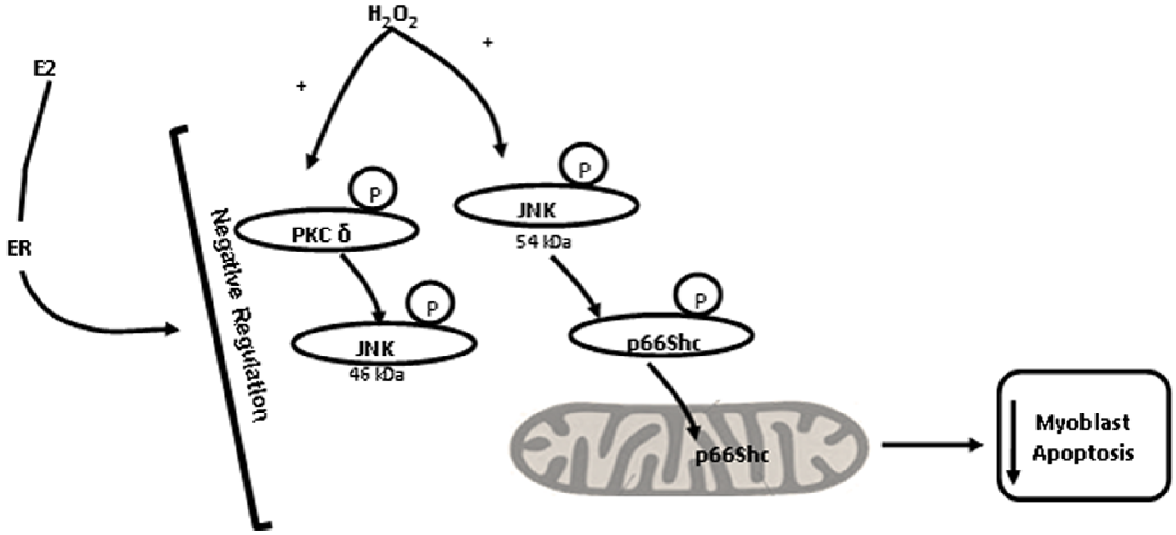

Figure 8