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High passage numbers induce resistance to apoptosis in C2C12 muscle cells

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ABSTRACT: Cell lines with high passage numbers exhibit alterations in cell morphology and functions. In the present work, C2C12 skeletal muscle cells with either low (<20) or high (>60) passage numbers (identified as l-C2C12 or h-C2C12, respectively) were used to investigate the apoptotic response to H_2O_2 as a function of culture age h-C2C12. We found that older cultures (h-C2C12 group) were depleted of mitochondrial DNA (mtDNA). When we analyzed the behavior of Bad, Bax, caspase-3 and mitochondrial transmembrane potential, we observed that cells in the h-C2C12 group were resistant to H_2O_2 induction of apoptosis. We propose serially cultured C2C12 cells as a refractory model to H_2O_2-induced apoptosis. In addition, the data obtained in this work suggest that mtDNA is required for apoptotic cell death in skeletal muscle C2C12 cells.

Introduction

Mitochondria are involved in cellular processes such as energy metabolism, generation of reactive oxygen species and regulation of apoptosis. Mitochondria contain their own DNA and machinery for transcription and translation. Nevertheless, the replication, transcription, and translation of mtDNA depend on the nuclear genome (Pagliarini *et al*., 2008). Despite the fact that mtDNA comprises only a minimum percentage of genetic material in animal cells, several lines of evidence suggest that its contribution to cellular physiology could be much greater than that expected from its small size (Alexeyev *et al*., 2004; Hiona and Leeuwenburgh, 2008). Moreover, important pathologies have been associated to mtDNA depletion (Hirano *et al*., 2001; Spinazzola and Zeviani, 2007; Moraes *et al*., 1991). Also, pathologies due to aging are often associated to mitochondrial abnormalities. For instance, human studies suggest an increase in and a correlation between mtDNA mutations and the occurrence of skeletal muscle abnormalities with advancing age (Cormio *et al*., 2005). Sarcopenia encompasses the loss of muscle strength, muscle mass, and muscle quality with advancing age, and even though its causative mechanisms are currently under investigation, several theories of aging focuse on mitochondrial performance in controlling survival as well as apoptosis in myocytes (Dirks *et al*., 2006). Studies show that mtDNA depletion induces cellular apoptosis (Mignotte and Vayssiere, 1998), but others investigations, show that loss of mitochondrial transmembrane potential or defective oxidative phosphorylation may not contribute to apoptosis (Dey and Moraes, 2000; Amuthan *et al*., 2001).

Multiple molecular components such as death receptors, Bcl-2 family proteins, cytochrome c, inhibitor

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of apoptosis proteins (IAP), and many others, are involved in apoptosis signaling that converge on caspase activation, the executers of cell death (Aslan and Thomas, 2009). Loss of mitochondrial membrane potential ($\Delta\Psi m$) is a critical event in apoptosis (Ly *et al*., 2003; Penninger and Kroemer, 2003). In normal cells, $\Delta\Psi m$ is mainly maintained by transmembrane proton pumping occurring during electron transfer or during ATP hydrolysis catalyzed by the ATPase-ATP synthase. In mtDNA depleted cells, these processes could be unsuccessful since the complexes involved in proton pumping lack the mtDNA-coded subunits. In addition, the proapoptotic member of Bcl-2 family, Bax, has been associated to $\Delta\Psi m$ loss (Smaili *et al*., 2001). During apoptosis Bax translocates to mitochondria affecting their function (Hsu *et al*., 1997). It has been shown that 14-3-3 protein binds to Bax and plays a crucial role in the negative regulation of this proapoptotic protein (Nomura *et al*., 2003). Upon apoptotic stimulation, 14-3-3 releases Bax and allows its translocation to mitochondria (Nomura *et al*., 2003).

MtDNA-depleted mammalian cells lines have been generated (Czarnecka *et al*., 2006; Hofmann *et al*., 1997; Rose *et al*., 2001, 2002) in order to investigate the molecular mechanism activated in the pathologies mentioned before, as well as in certain types of cancer and aging. Commonly, mtDNA-depleted cells have been produced by long-term culture with compounds like ethidium bromide that damage mtDNA and inhibit the replication of the mitochondrial genes (King and Attardi, 1996). When mammalian cells are cultured in presence of ethidium bromide, the synthesis of mtDNA and RNA encoded by mtDNA is inhibited and closed-circular mtDNA is broken down. Since the repair machinery of mtDNA is less efficient than the nuclear DNA one, after long-term culture in presence of sublethal ethidium bromide concentrations, the cells lack mtDNA (Miller *et al*., 1996). Although the ethidium bromide treatment is an effective approach to obtain mtDNA-depleted cells, possible adverse effects have not been thoroughly investigated. For instance, it is well documented that ethidium bromide induces in cultured vertebrate cells the formation of morphologically atypical mitochondria and reduces substantially their cytochrome b and aa_3 content (Soslau and Nass, 1971).

C2C12, a murine muscle cell line, provides a good *in vitro* system for studying the major steps of myoblast proliferation and differentiation (Yaffe and Saxel, 1977). We have used C2C12 cells as well as primary cultures of mouse skeletal muscle, as an experimental model to investigate the molecular mechanisms of apoptosis induced by treatment with hydrogen peroxide (H_2O_2) or etoposide, (Ronda *et al*., 2010; Vasconsuelo *et al*., 2008). The purpose of the present study was to evaluate the apoptotic response of C2C12 cells to H_2O_2 when they were serially cultured (high passage numbers).

Materials and Methods

Materials

Anti-actin rabbit polyclonal antibody (A-5060) was purchased from Sigma-Aldrich (St Louis, MO, USA). Anti-caspase-3 rabbit polyclonal antibody, anti-phospho-Bad (Ser112) and (Ser136) rabbit polyclonal antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-Bax (PA1-30415) rabbit polyclonal antibody was purchased from Thermo Fisher Scientific (Rockford, IL, USA). Anti-14-3-3 (sc-629) rabbit polyclonal was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). 5,5',6,6'-tetrachloro-1,1',3-3'-tetraethylben-zimidazolylcarbocyanine iodide (JC-1) was purchased from Becton-Dickinson Biosciences (San Jose, CA, USA). All the other reagents used were of analytical grade.

Cell culture and treatment

C2C12 murine skeletal muscle cells were purchased from American Type Culture Collection (ATCC). The cells were cultured in growth medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated (30 min, 56ºC) fetal bovine serum), 1% nistatine and 2% streptomycin. Cells were incubated at 37ºC in a humid atmosphere of 5% CO_2 in air. Cultures were passaged every 2 days with fresh medium.

Two different groups of C2C12 cells were used for the experiments. One of them was obtained from low passage numbers (l-C2C12 cells) (PN<20) and the other group, from high passage numbers (h-C2C12 cells) (PN>60). The treatments were performed, in each group of cells, with 70–80% confluent cultures in medium without serum by adding H_2O_2 at the times indicated. H_2O_2 was diluted in culture medium without serum at a final concentration of 1 mM in each assay.

Fluorescence microscopy

Attached cells were incubated for 5 min at 37ºC in darkness with 2 μg/ml ethidium bromide and then washed with phosphate buffer saline (PBS). Cells were

mounted on glass slides and examined using a fluorescence microscope (Nikon Eclipse Ti-S) equipped with standard filter sets to capture fluorescent signals, and the images were collected using a digital camera. The results were expressed as percentage of cells with detectable mtDNA. A minimum of 300 cells was counted for each group from at least three independent experiments.

Coimmunoprecipitation

Total homogenates from the LC2C12 and h-C2C12 cells containing 100 µg of protein were immunoprecipitated with 10 µl of a 50% suspension of protein A–agarose after incubating the extracts with the antibody. The immunoprecipitates were washed three times with buffer (50 mM Tris–HCl, pH7.4; 1 mM EDTA; 1% Triton X-100; protease inhibitors: 2 mM phenylmethylsulfonyl fluoride, 20 µg/ml leupeptin, 20 µg/ml aprotinin, and 10 µg/ml of trypsin inhibitor). The final pellets were obtained by centrifugation for 3 min at 10,000 ∞ g, resuspended in electrophoresis sample buffer without dithiothreitol, boiled for 5 min, and resolved by SDS-PAGE. Fractionated proteins were electrotransferred to polyvinylidene difluoride membranes and then blocked for 1 h with 5% non-fat dry milk in PBS-Tween 20. The blots were incubated overnight at 4ºC with primary monoclonal antibody against the protein of interest. After several washings with the buffer, the membranes were incubated with the secondary antibody conjugated to horseradish peroxidase. Immunoreactive proteins were developed by means of enhanced chemiluminescence. The apparent molecular weight of reactive bands was estimated by reference to a wide size range of protein markers.

Determination of caspase-3 activity

For estimation of this apoptosis marker in cell cultures, we have used the Caspase-Glo 3 assay (Promega Corp., Madison, WI), according to the manufacturer's instructions. The assay provides a proluminescent caspase-3 substrate (containing the tetrapeptide sequence DEVD) that is cleaved to aminoluciferin. The released aminoluciferin is a substrate that is consumed by luciferase, generating a luminescent signal. The signal is proportional to caspase-3 activity. Luminescence was measured with a microplate luminometer/fluorimeter (PerkinElmer Victor 3), and caspase activity was normalized to equal cell numbers.

Western blot analysis

Protein samples (25 µg) were mixed with one fourth of the sample buffer (400 mM Tris/HCl pH 6.8, 10% sodium dodecyl sulfate (SDS), 50% glycerol, 500 mM dithiotreitol, and 2 mg/ml bromophenol blue), boiled for 5 min, and resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE) according to Laemmli (1970). Fractionated proteins were electrotransferred to polyvinylidene fluoride membranes (Immobilon-P; PVDF) and then blocked for 1 h at room temperature with 5% non-fat dry milk in PBS containing 0.1% Tween-20. Blots were incubated for 1 h with the appropriate dilution of the primary antibodies: anti-caspase-3 (1:1,000), anti-phospho-Bad (Ser136 or Ser112) (1:1,000) and anti-Bax (1:1,000) using, for all of them, anti-rabbit secondary antibodies. The membranes were repeatedly washed with PBS-Tween 20 prior to incubation with horseradish peroxidase-conjugated secondary antibodies. The enhanced chemiluminescence blot detection kit (Amersham, Buckinghamshire, England) was used as described by the manufacturer to visualize reactive products. Relative migration of unknown proteins was determined by comparison with molecular weight markers (Amersham). For loading control, membranes were stripped with stripping buffer (62.5 mM Tris–HCl pH6.7, 2% SDS, 50 mM β-mercaptoethanol) and then blocked for 1 h with 5% non-fat dry milk in PBS-T. The blots were then incubated 1 h with a 1:20,000 dilution of anti-actin polyclonal antibody (A-5060) as primary antibody. After several washings with PBS-T, membranes were incubated with anti-rabbit (1:10,000) conjugated to horseradish peroxidase. The corresponding immunoreactive bands were developed by means of enhanced chemiluminescence and the bands were semiquantified using a GS-700 Imaging Densitomer (Bio-Rad).

Measurement of mitochondrial membrane potential ($\Delta\Psi m$)

Cellular $\Delta\Psi m$ was determined using the JC-1 mitochondrial transmembrane potential detection kit from Becton-Dickinson Biosciences. JC-1 is a cationic fluorescent dye probe (green as monomer) that accumulates in mitochondria in a potential-dependent manner. Cells with functional mitochondria incorporate JC-1 leading to the formation of JC-1 aggregates, which show a red spectral shift resulting in higher levels of red fluorescence emission measured in the red (FL-2

channel) and green monomers (detectable in FL1 channel). Cells with collapsed mitochondria contain mainly green JC-1 monomers.

The assays were performed with 70-80% confluent cultures in 10 cm plates. After treatments, cells were tripsinized, harvested, and incubated with JC-1 probe in 5 ml polystyrene round-bottom tubes (Becton-Dickinson Biosciences), according with manufacturer specifications, for 15 min at 37ºC. Cells were then washed twice and analyzed in a FACS Calibur flow cytometer (excitation wavelength of 488 nm).

Statistical analysis

Results are shown as means +/- standard error of not less than three independent experiments. Statistical treatment of the data was performed using the Student's t-test (Snedecor & Cochran, 1967) or by ANOVA followed by Fisher LSD test. The level of significance was set at $p < 0.05$.

Results

Loss of mtDNA by high passage numbers in C2C12 cells

To investigate whether aged C2C12 cultures (high passage numbers, h-C2C12) lose mtDNA, the presence of mtDNA in h-C2C12 was compared with that in young, low passage numbers, l-C2C12 cells. To that end, the cells were stained with ethidium bromide as described in Methods and fluorescence was evaluated by microscopy. Ethidium bromide is known to stain mtDNA in living cells (Hayashi *et al*., 1994; Kasashima *et al*., 2008). Normal mtDNA stained with ethidium bromide appears as fine-grained fluorescence spots in the cytoplasm of l-C2C12 cells (77% ± 6.12) whereas the lack of this punctuate fluorescence in h-C2C12 cells (17% ± 5) suggests mtDNA depletion (Fig. 1). Using the mitochondrial fluorescent dye Mitotracker we confirmed the localization of ethidium bromide spots within mitochondria (not shown).

Resistance to H_2O_2-induced apoptosis in aged C2C12 cells

We observed Bad activation by the apoptotic stimulus in l-C2C12 cells (Fig. 2A and B, left panel). Conversely, it was observed Bad phosphorylation (inactivation), in serine 112 and serine 136, in h-C2C12 cells after H_2O_2 treatment (Fig. 2A and B, right panel). Likewise, Western blot analysis with anti-caspase-3 antibody (C-3FL), which recognizes full-length of the enzyme (35 kDa), showed that H_2O_2 was unable to induce activation of caspase-3 in h-C2C12 cells (Fig. 2C, right panel). In l-C2C12 cells, the band detected showed the decrease of full length caspase-3 induced by H_2O_2. Moreover, we evaluated the caspase-3 activity by means of a bioluminescent assay in h-C2C12 cells. In agreement with Western blot results, H_2O_2-induced caspase-3 activity was not detected in h-C2C12 cells (Fig. 2D).

Mitochondrial membrane potential ($\Delta\Psi m$) is refractory to H_2O_2 treatment in h-C2C12 cells

We investigated $\Delta\Psi m$ by means of the cationic fluorescent dye probe JC-1 in aged and l-C2C12 cultures, after treatment with 1 mM H_2O_2 during 4 h. JC-1 cation fluoresces differently in apoptotic and healthy cells. In healthy cells, it aggregates in mitochondria and exhibits red and green fluorescence.

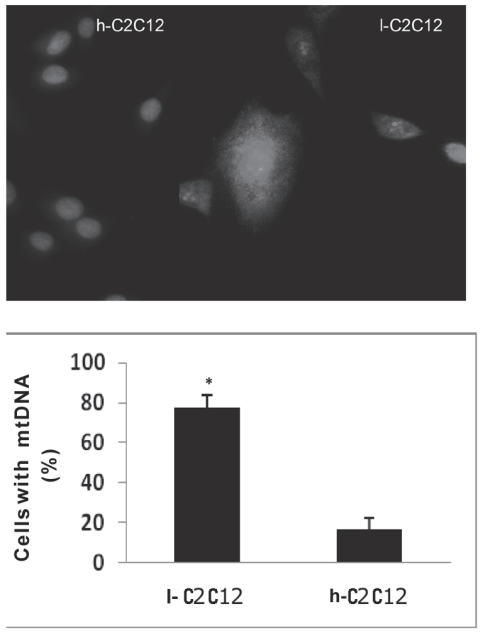

FIGURE 1. High passage numbers lead to mtDNA depletion in C2C12 cells. C2C12 cells were stained with ethidium bromide. l-C2C12 cells present normal nuclear fluorescence and fine-grained fluorescent spots in cytoplasm corresponding to mtDNA. In contrast, only the nuclear fluorescence is observed in h-C2C12 cells. At least ten fields per dish were examined. Experiments were repeated three times with similar results. Percentages of cells with mtDNA at each group are shown (bottom). Each value represents the mean of three independent experiments ± S.E.M. *p<0.05. Representative photographs are shown.

In apoptotic cells, it fails to accumulate in mitochondrial matrix because of altered membrane potential, instead it is found in cytosol as monomers and exhibits fluorescence in the green end of the spectrum (Reers *et al*., 1995). H_2O_2 treatment that led to apoptosis strongly induced ΔΨm loss in l-C2C12 cells. Flow cytometry assays showed that the percentage of cells with loss of ΔΨm increased from 8% in control cultures to 66% in treated cells (Fig. 3A). Conversely, in h-C2C12 cells, their ΔΨm was not altered in response to apoptosis induction, maintaining a ΔΨm similar to untreated cells (0.31% and 1.82%, respectively) (Fig. 3B).

Abnormal Bax response to H2O2 in h-C2C12 cells

Induction of apoptosis did not change Bax expression in aged cultures (h-C2C12 cells). These cultures were treated with 1 mM H_2O_2 for various time intervals (30 min,45 min,1 h to 5 h), followed by measurement of Bax levels. As shown in figure 4A, no statistically significant changes were observed by Western blot analysis using anti-Bax polyclonal antibody in response to H_2O_2. As mentioned before, apoptotic stimulation induced Bax dissociation from cytosolic protein 14-3-3, resulting in Bax translocation

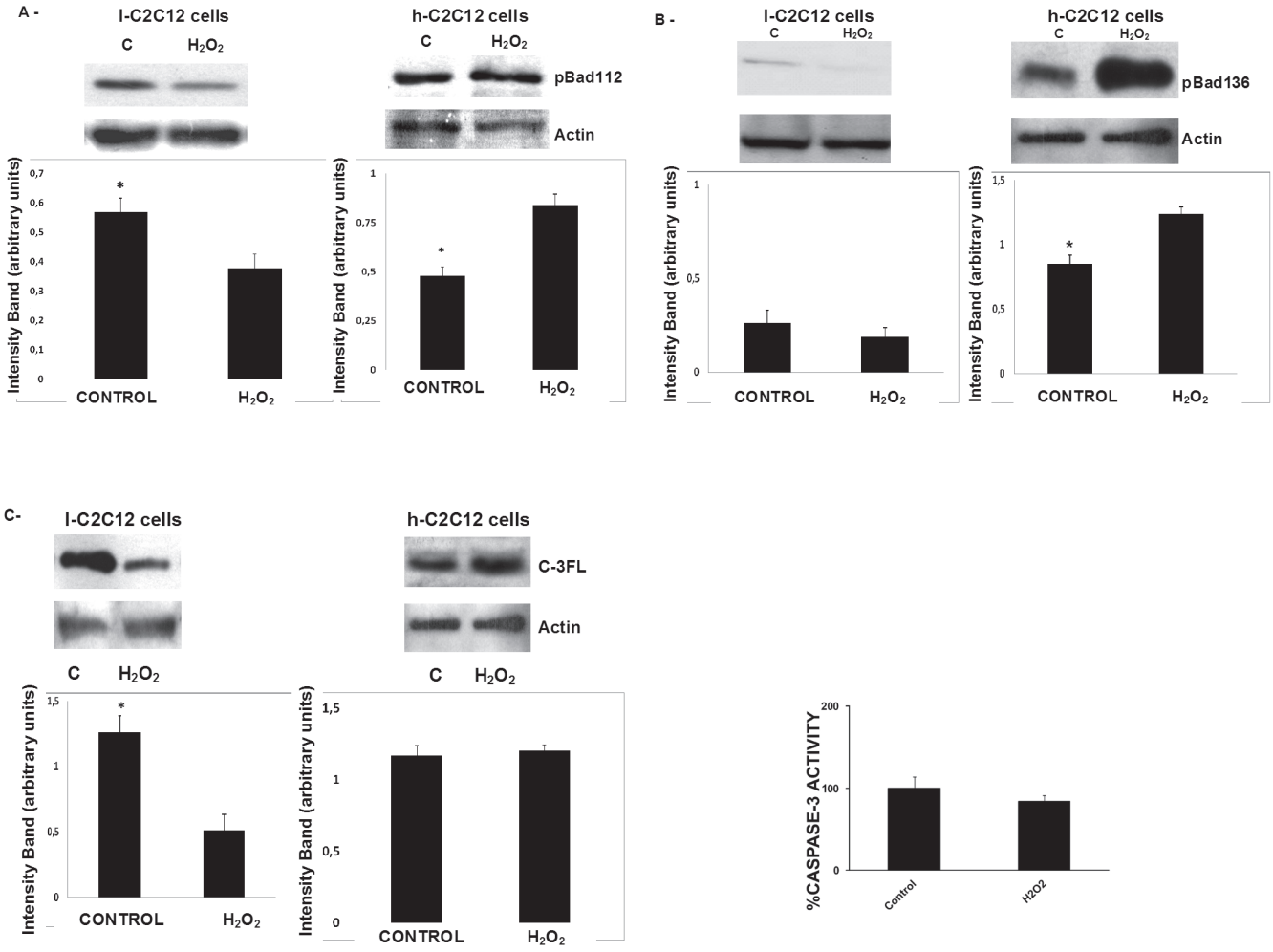

FIGURE 2. Resistance to apoptosis of h-C2C12 cells. l-C2C12 and h-C2C12 cells were treated with 1 mM H_2O_2 during 4h. The cells were harvested and lysed. The lysates were subjected to SDS-PAGE and blotted with anti-phospho-BAD (Ser112) antibody **(A)**, anti-phospho-BAD (Ser136) antibody **(B)** and with anti-caspase-3 full-length (C-3FL) antibody **(C)**. Actin levels were measured as protein loading control. The blot showed is representative of three independent experiments with comparable results. **(D)** h-C2C12 cells were treated with 1 mM H_2O_2 during 4h, and used to quantify caspase-3 activity. Results are presented as means ± S.E.M. of three independent experiments. *p<0.05.

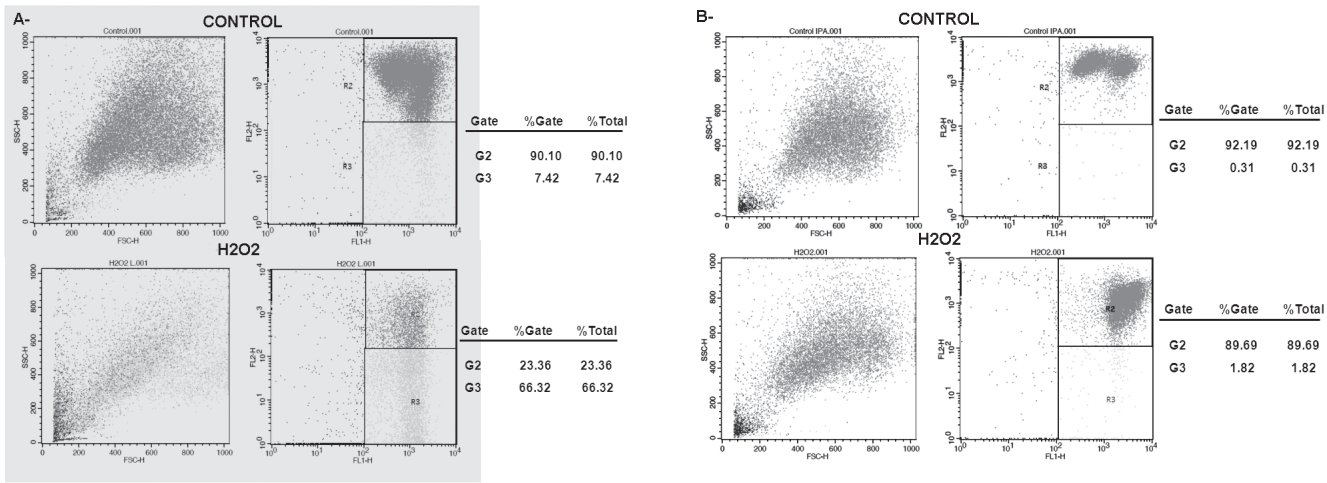

FIGURE 3. h-C2C12 cells are resistant to mitochondrial membrane potential loss in response to H_2O_2 stimulus. l-C2C12 **(A)** and h-C2C12 **(B)** cells were treated with 1 mM H_2O_2 during 4h. Then cells were stained with JC-1 as described in Materials and Methods, and the mitochondrial membrane potential was analyzed in a FACS Calibur flow cytometer. JC-1 aggregates emit simultaneously fluorescence in red (FL-2 channel) and green (FL-1 channel). This is visualized as a population that appears in the upper right quadrant of the graph. After loss of $\Delta\Psi$ (apoptotic cells), JC-1 is not accumulated in mitochondria and remains in the cytoplasm as monomers. These monomers do not have the red spectral shift and consequently have lowered fluorescence in the FL-2 channel, but fluorescence in FL-1 channel remains invariable. This is visualized as a population in the lower right quadrant. FL-1: FL-1 channel that detects green fluorescence, FL-2: FL-2 channel that detects red fluorescence. Experiments were repeated at least three times with essentially identical results.

to mitochondria. In order to study whether Bax dissociates from 14-3-3 during apoptosis induction, coimmunoprecipitation assays using both anti-Bax and anti-14-3-3 antibodies with lysates from control and H_2O_2-treated (1mM, 4h) h-C2C12 cells were performed. The treatment with H_2O_2 was unable to induce Bax-14-3-3 dissociation in h-C2C12 cells (Fig. 4B).

Discussion

Mitochondria have emerged as important players in the integration of the intrinsic apoptotic pathway in mammalian cells (Rutter and Rizzuto, 2000; Wang, 2001). In fact, mitochondria not only are involved in the integration and amplification of both extrinsic and intrinsic apoptotic cascades, but also execute cell death by releasing several proteins including cytochrome c, Smac/DIABLO and procaspases to the cytoplasm (Wang *et al*., 2001). Thus, cells with mitochondrial abnormalities could result in altered apoptosis and several pathologies. In recent years, growing attention has been paid to mitochondrial DNA-depleted cells, which are an important model for studying the function of mitochondria during cell death. Numerous studies using mtDNA depleted cells have shown deregulation of apoptosis (Biswas *et al*., 2005; Ferraresi *et al*., 2008;

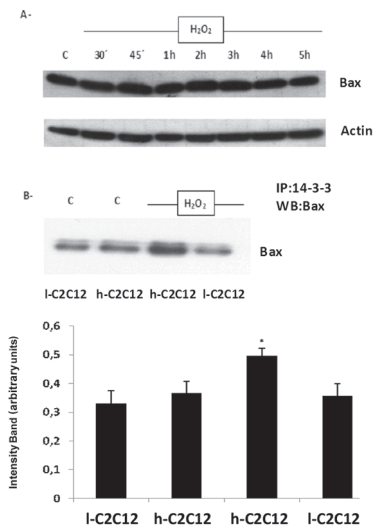

FIGURE 4. H_2O_2 is unable to increase Bax expression and activation in h-C2C12 cells. (A) h-C2C12 cells were treated with 1 mM H_2O_2 during the periods of time indicated or not treated, control (C). The cells were harvested and lysed as described in Methods. The lysates were then subjected to SDS-PAGE and blotted with anti-Bax antibody. Actin levels were measured as protein loading controls. **(B)** Lysates from h-C2C12 and l-C2C12 cells treated with 1 mM H_2O_2 for 4 h or not treated, control (C). Then the lysates were immunoprecipitated using anti-14-3-3 antibody and immunoblotted with anti-Bax antibody. Representative immunoblots of three independent experiments with comparable results are shown. Results are presented as means ± S.E.M. of three independent experiments. ANOVA followed by Fisher LSD test was performed *p<0.05.

Higuchi *et al*., 1997). These cellular models are relevant tools to understand the molecular basis of cancer and to develop new approaches for diagnosis and treatment, since mitochondrial dysfunction does appear to be a factor in cancer etiology, invasive behavior and resistance to chemotherapies (Amuthan *et al*., 2002; Moro *et al*., 2008). In general, mtDNA depletion can be achieved in cultured cell lines by treating with low concentration of ethidium bromide (Ferraresi *et al*., 2008; King and Attardi, 1996).

In the present work, we have studied the response to an apoptotic inductor (H_2O_2) of C2C12 cells with different passage numbers. Interestingly, we found that the older cultures (h-C2C12 cells) were depleted of mtDNA even though they are retained in normal numbers. Since it has been shown that C2C12 cells are able to release micro-vesicles containing mtDNA (Guescini *et al*., 2010), we postulated that the loss of mtDNA in h-C2C12 cells could be consequence of the continuous release of microvesicles during passages.

Substantial literature details biochemical events triggered by H_2O_2 in a variety of cell types including C2C12 (Jiang *et al*., 2005a, b). We have previously shown that H_2O_2 induces apoptosis without necrosis in C2C12 cells, and that this is correlated with induction of Bad and caspase-3 activation (Vasconsuelo *et al*., 2008, 2010). Hence, we investigated the status of Bad and caspase-3 after H_2O_2 treatment as correlated with the loss of mtDNA in h-C2C12 cells, and we found Bad and caspase-3 inactivation in such conditions. We also observed an unusual Bax behavior in response to H_2O_2, since the apoptosis inducer was unable to increase its expression levels.

Bax is associated to $\Delta\Psi$m loss, since it moves to mitochondria during apoptosis, changes its conformation and inserts into the mitochondrial membranes altering their functions. To allow this happens, it is necessary that Bax dissociates from cytosolic 14-3-3. Coimmunoprecipitation assays showed that H_2O_2 was not able to induce Bax-14-3-3 dissociation in h-C2C12 cells and in consequence did not alter $\Delta\Psi$m in response to induction of apoptosis. In general, apoptosis resistance of cancer cells has been correlated with cell differentiation (Gil *et al*., 2008; Melet *et al*., 2008). In muscle, increased apoptosis resistance has also been observed in differentiated myotubes as compared to proliferating myoblasts (Xiao *et al*., 2011). h-C2C12 cells are aged proliferating myoblasts which lack mtDNA, thus we postulate that the molecular mechanism involved in apoptosis-resistance could be different from that observed in differentiated myotubes. Indeed, in myotubes it has been observed elevation of inhibitors of cell death, such as caspase inhibitors (Xiao *et al*., 2011). In h-C2C12 cells, other apoptotic components such as Bad and Bax may be involved in addition to the lack of caspase-3 activity. So, the resistance to apoptosis observed may not only due to higher levels of caspase inhibitors. This hypothesis needs further investigation to understand the mechanism activated by mtDNA alterations in aged muscle as well as in certain muscle pathologies.

The data obtained in this work provides evidence in favor of mitochondrial DNA being required for apoptotic cell death. The reduced susceptibility to apoptosis observed in mtDNA-depleted cells may provide a tool to understand the behavior of cells with impaired mitochondria against apoptotic stimuli.

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