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Mitochondrial alterations and apoptosis in smooth muscle from aged rats

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Abstract

We studied changes in mitochondrial morphology and function in the smooth muscle of rat colon. Under confocal microscopy, tissues loaded with potentiometric dye displayed rapid and spontaneous depolarization. Cyclosporin A (CsA), inhibitor of the permeability transition pore (PTP), caused an increase in mitochondrial membrane potential ($\Delta\Psi_m$) in tissues from adult young animals. In aged rats these changes were not observed. This suggests that physiological activation of PTP in aged rats is reduced. Electron microscopy showed alterations of the mitochondrial ultrastructure in tissues from aged rats involving a decreased definition of the cristae and fragmentation of the mitochondrial membranes. We also detected an increase in apoptotic cells in the smooth muscle from aged animals. Our results show that the aging process changes PTP activity, the ability to maintain $\Delta\Psi_m$ and mitochondrial morphology. It is suggested that these can be associated with mitochondrial damage and cell death.

Keywords: Mitochondria; Calcium; Smooth muscle; Aging; Mitochondrial dysfunction

1. Introduction

The aging process has been associated with changes in mitochondrial function as a result of exposure to genotoxic agents [1–3]. It has been reported that this may affect several aspects of mitochondrial function and morphology [4], such as alterations in mitochondrial membrane potential ($\Delta\Psi_m$) and a high rate of reactive oxygen species (ROS) production in rat liver mitochondria [5] and decrease in mitochondrial definition in retinal Muller (glia) cells from aged guinea pigs [6]. Impairment of mitochondria has been associated with the full opening of the permeability transition pore (PTP) in aged mice [7]. The precise mechanisms and functional meanings of the pore are not understood [8–11], although the implication of a mitochondrial cyclophilin (cyclophilin-D) was described [12]. The immunosuppressant cyclosporin A (CsA) is a classical inhibitor of PTP opening and may dissociate the inner membrane protein cyclophilin D (CyP-D) from other proteins of the PTP complex [12,13].

Openings of PTP may occur in two distinct modes, one in a high conductance state with full opening and the other in a low conductance state with transient openings [9]. Under certain conditions, this flickering of the pore is thought to contribute to physiological signaling [14–16] and transient pore openings contribute to regulate mitochondrial Ca^{2+} levels by allowing Ca^{2+} efflux and limiting Ca^{2+} overload [14,16–18]. On the other hand, the increase in mitochondrial Ca^{2+} uptake stimulates ROS production and openings of PTP [19]. Thus, Ca^{2+} overload, oxidative stress and changes in voltage trigger the opening of PTP in the high conductance state [12,13]. This opening can cause mitochondrial dysfunction and damage and the release of proapoptotic proteins from this organelle [9,20,21].

Mitochondrial intermembrane space is a site for several molecules that lead to apoptosis. Upon an apoptotic stimulus these molecules may be released by mechanisms that remain a matter of heated debate. There are several possibilities that include rupture of the mitochondrial membranes, selective permeabilization of the outer mitochondrial mem-

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branes with or without a specific pore formation or PTP opening in the high conductance state [22,23]. In aged animals, oxidative stress, openings of PTP and mitochondrial dysfunction could be associated with degeneration and apoptosis [21–23]. Several authors have proposed the "mitochondrial theory of aging" [24] which postulates that mitochondrial dysfunction may be one of the central points underlying the aging process [21,22]. Recent investigations suggested that aging is associated with mitochondrial damage that induces apoptosis and contributes to degenerative processes [1,24].

In the present study, we investigated $\Delta\Psi_m$ and the transient openings of PTP to determine whether this function is affected by aging. Mitochondria from aged rats also showed changes in ultrastructure. The mitochondria of aged rats displayed a decreased number of well-defined cristae and disruption of membranes, suggesting mitochondrial deterioration. Besides these findings we observed an increased number of apoptotic cells. Our results support the hypothesis that aging, PTP dysfunction and mitochondrial degeneration are associated during apoptosis.

2. Materials and methods

2.1. Isolation and preparation of colon rat

Female Wistar 2BWA adult (4–6 months) and aged rats (24–30 months) were anesthetized by inhalation of ether and a segment of the terminal colon was removed and dissected. The smooth muscle layer was freed of the adjacent mucosa and washed in Tyrode solution of the following composition: 145 mM/l NaCl, 5.5 mM/l KCl, 2.5 mM/l $CaCl_2$, 1 mM/l $MgCl_2$, 10 mM/l HEPES and 10 mM/l glucose. Smooth muscle was cut longitudinally into slices that were fixed to a coverslip with acrylic glue and bathed with Tyrode solution.

2.2. Confocal microscopy

For confocal measurements, coverslips with tissues were washed in Tyrode solution, placed in a Leiden coverslip chamber and transferred to a thermostatically regulated microscope chamber (37 °C) (Harvard Instruments, Holliston, MA, USA). Experiments were performed using an inverted confocal microscope LSM510 (Carl Zeiss, Heidelberg, Germany) equipped with ArKr 488/568, HeNe543 lasers with 20× and 40× Apochromat objectives.

2.3. $\Delta\Psi_m$ measurements

$\Delta\Psi_m$ was measured using tetramethylrhodamine ethyl ester (TMRE), a cationic potentiometric indicator, which accumulates preferentially into energized mitochondria driven by membrane potential. TMRE is regularly and reversibly taken up by live cells and provides qualitative measurements of $\Delta\Psi_m$ [15]. The membrane potential controls the average environment of the dye, since the changes in fluorescence reflect fluctuations of the membrane potential. The fluorescent cationic dye is distributed according to the Nernst equation [25]. In intact tissues TMRE (50 nM) was incubated for 60 min for complete and stable loading of the dye in mitochondria.

Images were acquired in the presence of TMRE during all experiments to compensate for the loss of dye caused by photobleaching. In addition, imaging was carried out under low illumination conditions to avoid photodynamic effects related to excessive light exposure [15,18]. After incubation with TMRE, images were acquired at 543-nm excitation and 568-nm emission wavelengths at 1 frame/0.5 s in a total of 30 frames for each scan, acquired every 5 min. Control fluorescence images were acquired in resting condition against time. To investigate the contribution of PTP function, samples were incubated with CsA (20 μM) for 30 min and scans were again performed at 5-min intervals. Experiments were also done with FK506, which is an immunosuppressant that does not bind to CyP-D and has not shown any effect on PTP (16). For calibration, at the end of each experiment tissues were incubated with carbonyl cyanide *p*-trifluoro-methoxyphenylhydrazone (FCCP, 5 μM), a protonophore that collapses $\Delta\Psi_m$. FCCP was used with the mitochondrial ATPase inhibitor, oligomycin (1 μg/ml) for 10–15 min to avoid rapid ATP consumption caused by FCCP (data not shown). Experiments were performed in the presence of verapamil (0.1 μM) to inhibit the participation of the MDR (multidrug resistance) pump [26]. The MDR pump promotes active efflux of the drugs from cells to the outer medium and this process could induce resistance to the drugs. However, it can pump out not only drugs but also various fluorescent dyes such as TMRE. The process of dye efflux was found to be sensitive to the calcium channel blocker verapamil, which reverses P-glycoprotein associated MDR [26,27]. Some experiments were also performed in the absence of verapamil and since no differences were found between the two conditions, MDR inhibitor was present in all experiments.

Fluorescence intensity was measured in apparently individual mitochondria identified by TMRE staining and selected using the region of interest (ROI) tool. ROIs were drawn around mitochondria that did not overlap with others. Fluorescence intensity in the non-zero pixels within the ROIs were averaged (F) and plotted as normalized fluorescence ($\Delta F/F_0$) against time. ΔF was calculated as the difference between the mean value of the acquisition prior to stimulation of the tissue (F_0) and F. Fluorescence intensity was measured in arbitrary units.

2.4. Electron microscopy

Tissues were fixed in phosphate-buffered 2% glutaraldehyde buffered at pH 7.2 with 0.2 M sodium phosphate for 4 h at 4 °C. They were then postfixed in phosphate-buffered

1% osmium tetroxide for 1 h at 4 °C, dehydrated in graded ethanol, treated with propylene oxide and embedded in Araldite. Ultrathin sections were cut with an ultramicrotome Sorvall, Porter-Blum MT-1 and stained with uranyl acetate and lead citrate for transmission electron microscopy. Electron microphotographs were taken with a Carl Zeiss EM-900, 80-kV (Carl Zeiss) electron microscope.

2.5. TUNEL method

Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling (TUNEL) experiments, based on the specific binding of terminal deoxynucleotidyl transferase (TdT) to 3′ -OH ends of DNA [28], were performed with the Apop Tag plus Kit (Intergen Discovery products, USA). The colon was removed and fixed in 4% formaldehyde buffered at pH 7.2 with 0.1 M sodium phosphate at room temperature. After dehydration, the specimens were embedded in paraffin and 6-μm sections were attached to silanized slides. Deparaffinized sections were washed in PBS (50 mM sodium phosphate, pH 7.4, 200 mM NaCl) and pre treated with 20 μg/ml proteinase K (Sigma-Protein Digesting Enzyme) for 15 min at room temperature. Sections were washed in distilled water, immersed in 8% H_2O_2 to inactivate endogenous peroxidase and immersed in buffer for 20–30 min. Subsequently, the sections were incubated in TdT in a humidified chamber at 37 °C for 1 h. The stop/wash buffer and antidigoxigenin-peroxidase were applied in a humid chamber at 37 °C for 30 min and the sections were then treated with 0.06% diaminobenzidine (Sigma, St Louis, MO, USA) for 3–6 min at room temperature. Sections were counterstained with Carazzi's hematoxylin. Slices of the involuting mammary gland were used as positive controls and omission of TdT of the labeling mixture used as negative controls. Cells were counted using an image analysis system Leica Quantimete 500IW connected to a Nikon Optiphot II light microscope with a final magnification on the video display of 860× (40× objective lens). Several fields of the circular and longitudinal layers (9–10 of each) and the myenteric plexus of the smooth muscle of the colon were counted. Results are expressed as number of stained cells per μm^2 of tissue and as percentage of dead cells in relation to the total cell count. Student's *t* test was used to compare the normalized fluorescence of TMRE and the apoptotic cell counts between aged and adult young groups.

2.6. Materials

FCCP, oligomycin and verapamil and other chemicals were purchased from Sigma. TMRE was from Molecular Probes. CsA was a gift from Novartis (Switzerland) and FK506 was a gift from Department of Nephrology of State University of São Paulo and Apoptag Plus kit was purchased from Intergen.

3. Results

3.1. $\Delta\Psi_m$ measurements in aged and adult young animals

To measure $\Delta\Psi_m$ variations in smooth muscle of adult young rats, samples were incubated with TMRE to evaluate $\Delta\Psi_m$ in the resting condition. In these conditions, mitochondria appear regularly distributed as cylindrical and elongated "threads" along the smooth muscle fibers from adult young animals (Fig. 1A). In a time series experiments, analysis of mitochondrial fluorescence revealed spontaneous fluctuations of $\Delta\Psi_m$ (Fig. 1B). In another series of experiments, smooth muscles of adult rats were exposed to CsA (20 μM) for 30 min which induced an increase in TMRE fluorescence in at least 80% of the analyzed mitochondria (Fig. 1C and D).

In contrast, TMRE fluorescence was less intense and not homogeneously distributed in mitochondria from aged animals when compared with those from younger rats (Figs. 1A and 2A). After incubation with CsA for 30 min, there was little increase in TMRE fluorescence in relation to the control before CsA; (Fig. 2C and D); thus, these results were significantly different from those of adult young animals (Figs. 1D and 2D). In addition, the fluorescence extracted from individual mitochondria showed little fluctuation of $\Delta\Psi_m$ in aged animals (Fig. 2B) when compared with that of adult young rats (Fig. 1B).

The histogram in Fig. 3 shows the effect of CsA on adult and aged rats. Results show that CsA induced a significantly greater increase in $\Delta\Psi_m$ in adult young animals when compared with aged animals. In addition, experiments performed with FK506 (500 nM) showed that this immunosuppressant did not induce a significant increase in TMRE fluorescence as observed with CsA in adult young animals. As pointed out before, these experiments were done in the presence of verapamil (0.1 μM) to avoid the influence of the MDR pump [26,27]; however, similar results were obtained in the absence of this drug. At the end of all experiments, FCCP (5 μM) plus oligomycin (1 μg/ml) were added for calibration. The uncoupler induced a collapse of $\Delta\Psi_m$ suggesting that the fluorescence was a result of TMRE accumulation within the mitochondria (data not shown). Fluorescence data in arbitrary unities were normalized as described above (see Materials and methods) using the $\Delta F/F_0$ calculation. Results are presented as average of increases in fluorescence in relation to baseline.

3.2. Ultrastructure of smooth muscle of aged and adult young animals

The circular layer from smooth muscle segments of adult rats showed longitudinally arranged fibers. Mitochondria were well defined and distributed along the muscle with a cylindrical morphology. The cristae and the membranes were well defined as shown in Fig. 4A. In aged animals, we verified intramitochondrial inclusion as a myelin-like

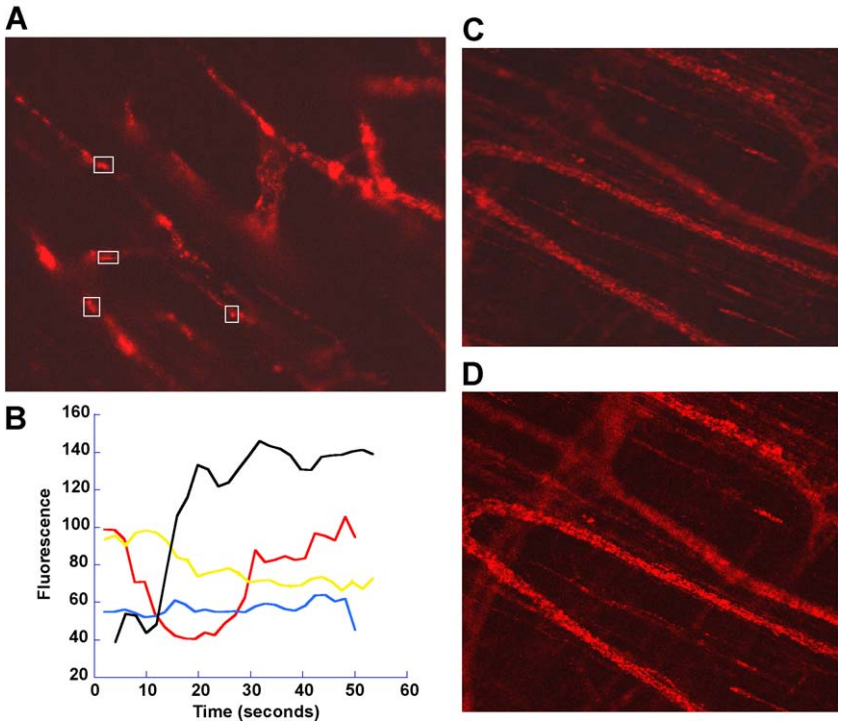

Fig. 1. Mitochondrial membrane potential ($\Delta\Psi_m$) in smooth muscle of colon from adult young rats. (A) Representative image of $\Delta\Psi_m$ in resting condition after incubation with TMRE (50 nM, 60 min). The figure shows one of 30 similar confocal images that were acquired at 1 frame/0.5 s with 40× objective. Mitochondria appear distributed along the smooth muscle fibers. The squares show how mitochondrial regions of interest (ROIs) were delimited for further analysis, as described in Materials and methods. (B) Representative traces of changes in TMRE fluorescence fluctuations extracted from ROIs. The different colors represent measurements from four different mitochondria of adult young rats during a scan time of 50 s. (C) Image of mitochondria loaded with TMRE to evaluate $\Delta\Psi_m$ fluctuations in colon smooth muscle of an adult young rat before addition of CsA. The tissue was previously incubated with TMRE (50 nM, 60 min) and 30 similar confocal images were acquired at 1 frame/0.5 s with 20× objective. (D) Image from the tissue shown in (C) after incubation with CsA (20 μM, 30 min). The fluorescence increased significantly in the presence of CsA. Results are representative of at least five experiments.

structure. The results also presented numerous mitochondria from old animals with a visible rupture of outer mitochondrial membrane and degeneration of the cristae (Fig. 4B).

Table 1 shows the number of mitochondria analyzed in both groups (adult young (A) and aged (O) animals) and the percentage of organelles with different appearances described as: (I) morphologically intact mitochondria; (II) mitochondria with inclusions (myelin-like structure), (III) mitochondria with rupture of membrane(s) or crista degeneration. These data show that old smooth muscles from aged animals present a significant increase number of mitochondria with alterations, especially cristae degeneration.

3.3. Apoptosis

We investigated apoptosis in muscular (circular and longitudinal) layers of the colon from young adults and aged animals. TUNEL-positive cells are shown in Fig. 5, where a significant increase in the number of apoptotic nuclei in both (longitudinal and circular) smooth muscle layers from aged animals is observed when compared to the control adult young rats (Fig. 5A and B). Furthermore, apoptotic nuclei were significantly increased in the longitudinal muscle layer when compared to the circular muscle layer in adult young and aged animals (Table 2). Aged animals presented a significant increase in apoptotic nuclei from the myenteric plexus (Table 2).

4. Discussion

In the present study we show that aged animals present changes in the transient openings of PTP, alterations of $\Delta\Psi_m$, morphological alterations of mitochondria with mitochondrial damage and an increase in cell death. We presented evidence that in colon smooth muscles the aging process affects mitochondrial function and morphology. However, it is not clear if these events are correlated and if they culminate in cell death. Herein, we evaluated mitochondrial fluctuations of $\Delta\Psi_m$ that may be a representation of the repetitive openings of PTP [5,14,18,29]. It is shown that the aged animals showed a decrease in spontaneous fluctuations of $\Delta\Psi_m$ with lower amplitudes in relation to the controls. This difference between the two groups may be due to a decrease in the flickering activity of PTP in aged animals that was corroborated by the fact that CsA caused a significantly lower increase in fluorescence of mitochondrial

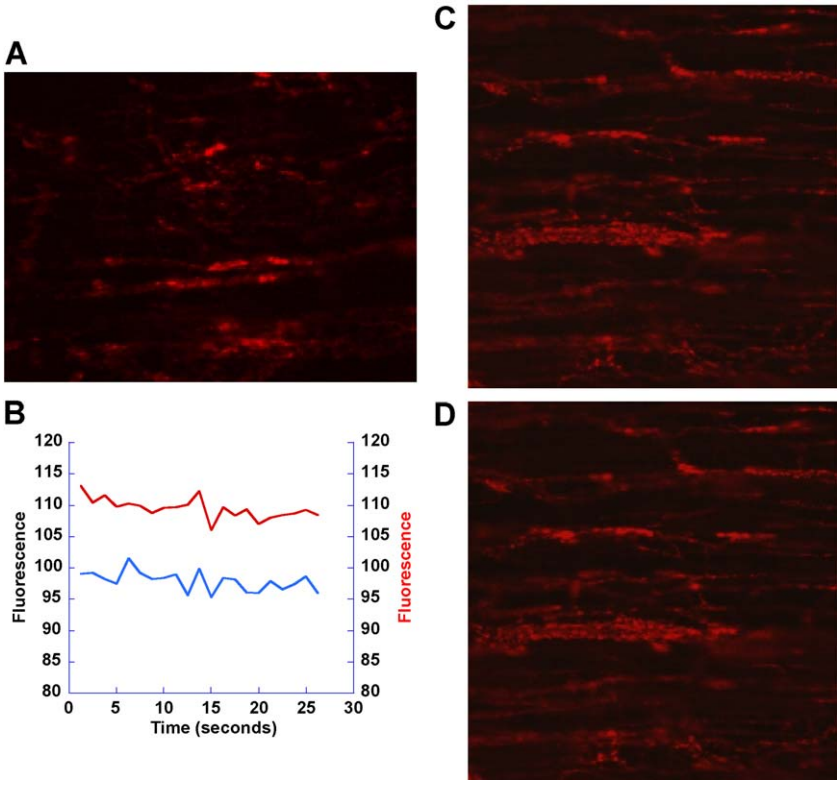

Fig. 2. $\Delta\Psi_m$ in colon smooth muscle from aged rats. (A) Representative image of $\Delta\Psi_m$ in resting condition after incubation with TMRE (50 nM, 60 min). The experimental procedure was similar to that described in Fig. 1A and Materials and methods. In this case, mitochondria appeared ''granular'' when compared to those in tissues from adult young animals (Fig. 1). (B) Representative traces of changes in TMRE fluorescence fluctuations extracted from ROIs. The different colors represent measurements from two mitochondria during about 50 s in resting condition. These mitochondria showed little fluctuation of the $\Delta\Psi_m$, with smaller amplitude when compared with adult young rat. (C) Image of mitochondria loaded with TMRE to evaluate $\Delta\Psi_m$ fluctuations in colon smooth muscle of adult young rat before incubation with CsA. (D) Image from the tissue shown in (C) after incubation with CsA (20 μM, 30 min). Contrary to the results in adult young rats (Fig. 1D) the fluorescence did not change in the presence of CsA. Results are representative of at least five experiments.

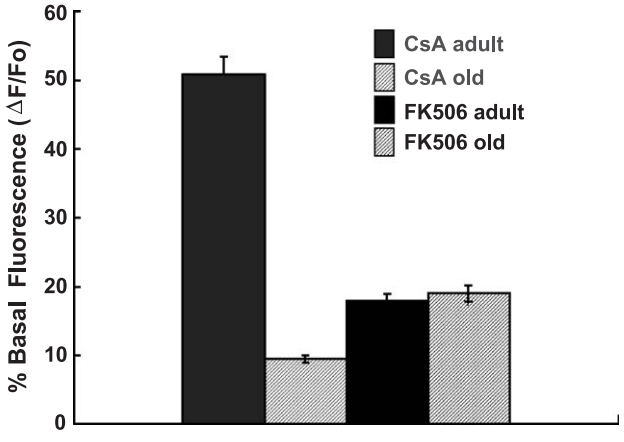

Fig. 3. Effect of CsA on $\Delta\Psi_m$ of colon smooth muscle from adult young and aged rats. Fluorescence intensities (arbitrary units) within mitochondria from six individual experiments were extracted and normalized ($\Delta F/F_0$) for comparison as described in Materials and methods. After 30-min incubation, CsA caused an increase in $\Delta\Psi_m$ in adult young animals. This effect was not observed in aged rats. Normalized fluorescence from more than 400 mitochondria was averaged and presented as average ± standard error of the mean (S.E.).

TMRE in aged rats than in young animals. Although the concentration is four times higher than that used in isolated cells [15,16], it should be considered that a slower diffusion occurs in intact tissues.

The absence of a CsA effect in the aged animals could be explained by low affinity binding for CyP-D under Ca^{2+} overload or under oxidative stress condition and a loss of adenine nucleotide translocase (ANT) [17,21,22,30–32]. Our results are in agreement with some findings in isolated hepatocytes [5], intact hepatocytes [4] and lymphocytes [33] that presented significantly lower fluorescence in the mitochondria from aged rats suggesting that there is an age-associated alteration in $\Delta\Psi_m$ [5]. Since the transient openings of the pore have been implicated in Ca^{2+} signaling or in preventing mitochondrial Ca^{2+} overload, oxidative stress and excitotoxicity [5,14–16,19,24,29], a dysfunction in the flickering activity could also be related to the aging process. Thus, if this mechanism is activated to maintain physiologically energized mitochondria, aging could lead to altered and less functional organelles. This hypothesis is reinforced by the presence of the myelin-like intramitochondrial inclusion, ruptured mitochondrial membranes and degenerated

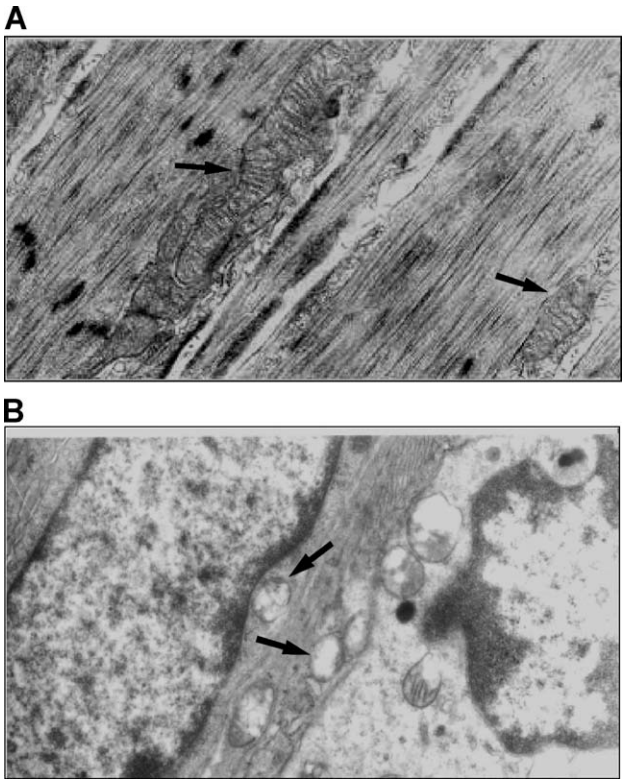

Fig. 4. Representative electron micrograph of colon smooth muscle from adult young and aged rat. (A) Fibers from adult young rats were arranged longitudinally. Arrows point to mitochondria that presented typical morphology with intact membranes, defined structure and cristae. Magnification 30,000 ×. (B) Representative electron micrograph of colon smooth muscle from aged rat. In this condition, mitochondria (arrows) appear with a spherical shape, rupture of membranes and undefined cristae. Magnification 30,000 ×. Images are representative of at least three experiments.

cristae observed in the present study. In senescent rat myocardium, intramitochondrial inclusions were also shown [34].

It is possible that mitochondrial Ca^{2+} accumulation promotes increases in ROS production and, consequently, the full opening of PTP may lead to loss of Ca^{2+} homeostasis [17,19,30]. Sustained oxidative damage of nucleic acids, proteins and lipids caused by oxidative stress is considered one of the major factors in the general functional decline of tissues associated with aging [1,17,19,30].

Table 1
Analysis of the ultrastructural alterations in mitochondria from smooth muscles

	I	II	III
	Intact mitochondria	Inclusions	Rupture of membranes and degenerated cristae
Adult	92.2 ± 2.9	5.4 ± 1.2	2.3 ± 0.7
Old	79.9 ± 3.6*	8.7 ± 0.3*	11.4 ± 0.8*

Percentage of intact mitochondria (I), mitochondria with inclusions (II) and mitochondria with ruptured membranes and degenerated cristae (III).
Data represent mean ± S.D., *$P < 0.05$, $n = 5$.

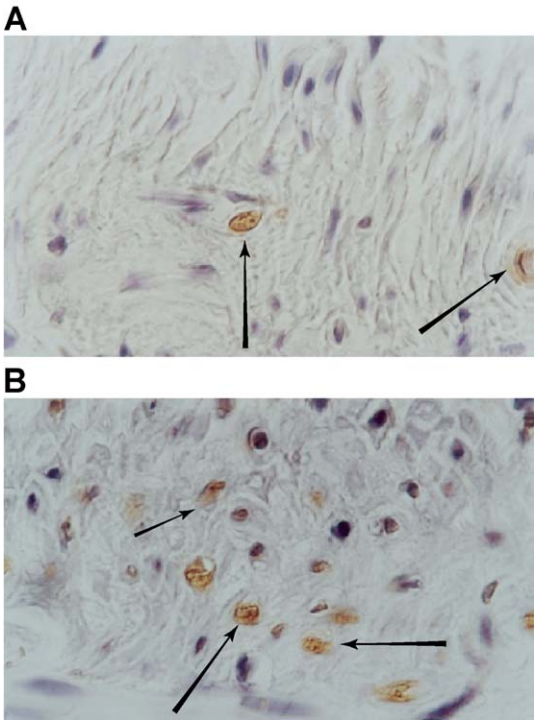

Fig. 5. (A) Representative photomicrograph of TUNEL detection in longitudinal layer of smooth muscle of adult young rat. Arrows indicate the cells showing typical staining and apoptotic morphology. Magnification 1560 ×. (B) Representative photomicrograph of TUNEL detection in longitudinal layer of colon smooth muscle of aged rat. Arrows point to some cells showing typical TUNEL staining and apoptotic morphology. Note a larger number of apoptotic cells in aged, in contrast to adult young rats.

Results from our laboratory have shown that mitochondria of colon smooth muscle from aged rats contain more Ca^{2+} than those from adult young animals. In this case, tissues were loaded with fura-2 and maintained in the presence of thapsigargin, to inhibit ER Ca^{2+}-ATPase. Upon stimulation with FCCP, a greater increase in cytosolic Ca^{2+} was observed in smooth muscle from aged animals when compared with tissues from young adults (Lopes et al., submitted for publication). In the present study the tissues from aged animals showed a loss in PTP flickering. This decrease in PTP activity may increase mitochondrial Ca^{2+} accumulation, and reduce the electron transport which may lead to an increase in superoxide formation [35].

Table 2
Number of TUNEL-positive cells in circular, longitudinal layers and myenteric plexus of smooth muscle of rat colon (from adult young and aged animals)

	Cell number/$\mu m^2 \times 10^{-4}$		% TUNEL-positive cells	
	Adult	Aged	Adult	Aged
Longitudinal layer	5.7 ± 2.2	22.6 ± 5.5*	14.6 ± 5	42.8 ± 7*
Circular layer	1.1 ± 0.4	8.2 ± 2.5*	6.5 ± 2	25.2 ± 4*
Myenteric plexus	2.7 ± 1.9	40.0 ± 8.5*	7.5 ± 1	27.7 ± 2*

Data represent mean ± S.D., *$P < 0.05$, $n = 7$.

Changes in the PTP activity may induce apoptotic cascade [21,36]. In the present study, we observed an increase in cell death of smooth muscle and myenteric cells from aged rats in agreement with other studies performed in rat and human gastrointestinal tract [37–39]. The TUNEL method detects the broken ends of DNA within a cell that could be present in necrosis and apoptosis [40–42]. However, TUNEL method and electron microscopy together validate the cell death investigation in intact tissues [43].

In the light of these results, one could suggest that the increase in apoptosis in the colon smooth muscle and myenteric cells could be associated with the changes in colonic motor functions, since there are clinical alterations frequently observed in the elderly [44]. The present investigation supports the view that the aging process affects the PTP transient openings and mitochondrial functions, leading to cell death.

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