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Antioxidant and antiapoptotic properties of melatonin restore intestinal calcium absorption altered by menadione

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Abstract The intestinal Ca^{2+} absorption is inhibited by menadione (MEN) through oxidative stress and apoptosis. The aim of this study was to elucidate whether the antioxidant and antiapoptotic properties of melatonin (MEL) could protect the gut against the oxidant MEN. For this purpose, 4-week-old chicks were divided into four groups: (1) controls, (2) treated i.p. with MEN (2.5 μmol/kg of b.w.), (3) treated i.p. with MEL (10 mg/kg of b.w.), and (4) treated with 10 mg MEL/kg of b.w after 2.5 μmol MEN/kg of b.w. Oxidative stress was assessed by determination of glutathione (GSH) and protein carbonyl contents as well as antioxidant enzyme activities. Apoptosis was assayed by the TUNEL technique, protein expression, and activity of caspase 3. The data show that MEL restores the intestinal Ca^{2+} absorption altered by MEN. In addition, MEL reversed the effects caused by MEN such as decrease in GSH levels, increase in the carbonyl content, alteration in mitochondrial membrane permeability, and enhancement of superoxide dismutase and catalase activities. Apoptosis triggered by MEN in the intestinal cells was arrested by MEL, as indicated by normalization of the mitochondrial membrane permeability, caspase 3 activity, and DNA fragmentation. In conclusion, MEL reverses the inhibition of intestinal Ca^{2+} absorption produced by MEN counteracting oxidative stress and apoptosis. These findings suggest that MEL could be a potential drug of choice for the reversal of impaired intestinal Ca^{2+} absorption in certain gut disorders that occur with oxidative stress and apoptosis.

Keywords Melatonin · Calcium absorption · Apoptosis · Oxidative stress · Menadione

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Introduction

Melatonin (MEL) is an ubiquitous hormone synthesized and secreted by the pineal gland in a circadian manner [1]. MEL is also synthesized in other extrapineal tissues like retina [2, 3] and gastrointestinal (GI) tract [4], which are related to paracrine and autocrine functions. In the gut, MEL is secreted by the enteroendocrine cells of mucosa with no circadian pattern, but conditioned by food intake, mainly by food enriched in tryptophan [5]. It is interesting to note that in this tissue MEL level is 400 times larger than that in the pineal gland [6], but its physiological significance is not completely understood. It was attributed several effects such as the regulation of food intake and digestion, stimulation of duodenal HCO_3^- secretion, improvement of the immune system in the gut, and prevention of GI mucosa ulcerations [7], among others. Recently, it was found that in the GI tract this hormone has a protective effect in ischemia and reperfusion experiments, stimulating the activity of certain antioxidant enzymes [8]. The protection of MEL appears to be

mediated by its antioxidant and anti-inflammatory properties. MEL has the ability to act as a "scavenger" of reactive oxygen species (ROS) and nitrogen as nitric oxide (NO), including peroxynitrite, and as an inhibitor of transcription factors that induce proinflammatory cytokines. Besides, MEL preserves cellular energy and prevents mitochondrial damage caused by the nitrooxidative levels [9]. Consequently, MEL for its antioxidant and antinflammatory properties might be useful to prevent or treat pathological conditions such as gastric ulcers, pancreatitis, and some cancers [10].

Intestinal calcium (Ca^{2+}) absorption is the only gate of the cation to the body. It occurs through paracellular and transcellular pathways. The latter pathway has received more attention and is widely known to be regulated by multiple hormonal and dietary factors. Calcitriol, the hormone derived from vitamin D, is the main stimulant regulator [11]. Chicks have been extensively used to study intestinal Ca^{2+} absorption because they show similar molecular mechanisms to those from mammals, have strong response to vitamin D and, besides, have the advantage of a larger duodenum size/body weight size ratio. For many years in our laboratory, we have been studying the mechanisms of different modulators affecting the transcellular pathway of chick intestinal Ca^{2+} absorption. We have shown that a single large dose of vitamin K_3 or menadione (MEN), a drug used in the anticancer therapy and in the treatment of osteoporosis [12, 13], inhibits chick intestinal Ca^{2+} absorption by mitochondrial dysfunction as revealed by GSH depletion and alteration of the permeability triggering the release of cytochrome c and DNA fragmentation [14, 15].

Although gut is well preserved from oxidative stress, physiological or pathological conditions such as aging [16], inflammatory bowel disease [17], celiac disease [18], and gut cancer [19] can produce enhancement of ROS and reactive nitrogen species, which in turn would cause alteration in the intestinal Ca^{2+} absorption. Therefore, it becomes necessary to know strategies in order to protect or prevent the oxidative stress, which probably could deteriorate not only the cation absorption but also the uptake of other nutrients and minerals. Recently, we have shown that the quercetin protects the intestinal Ca^{2+} absorption against the inhibition caused by MEN, but the flavonol alone does not affect the process [20]. It would be interesting to know whether an endogenous antioxidant with low side effects as MEL could reverse the oxidative stress provoked by MEN, and hence, to overcome the intestinal Ca^{2+} absorption.

Based upon previous considerations, the aim of this study was to test the use of MEL as a candidate drug to restore the intestinal Ca^{2+} absorption inhibited by MEN trying to elucidate the molecular mechanisms underlying the gut response.

Materials and methods

Animals

One-day-old Cobb Harding chicks (*Gallus gallus domesticus*) were obtained from Indacor S.A. (Rio Ceballos, Córdoba, Argentina) and were kept under constant temperature (24 ± 2 °C), synchronized to a 12:12 light/dark cycle (light from 07:00 to 19:00 h) and with food (commercial normal avian diet, Cargill, S.A.C.I., Pilar, Córdoba, Argentina) and water "ad libitum." At 4 weeks of age, the animals were divided into four groups: (1) Controls, which received 2 i. p. injections of 0.15 M NaCl or saline solution (SS) with an interval of 30 min between each other. (2) Treated with MEN. Chicks first received an i. p. injection of 2.5 μmol of MEN/kg of b.w. and 30 min later they received a second i.p. injection of SS. (3) Treated with MEL. Chicks first received an i.p. injection of SS and 30 min later a second i.p. injection of 10 mg/kg of b.w. MEL dissolved in 0.1 % ethanol/saline solution. (4) Treated with MEN+MEL. First, chicks received and i.p. injection with MEN and 30 min later a second i.p. injection of MEL (at the same doses than the groups 2 and 3). They were killed 20 min after the last injection by cervical dislocation and the excised duodenae were rinsed with cold 0.15 M NaCl and mucosa was scrapped to make homogenates or the enterocytes/the intestinal mitochondria were isolated, as described below. The chosen dose of MEN was diluted in 0.15 M NaCl and corresponds to that dose capable to inhibit intestinal Ca^{2+} absorption after 30-min injection, according to Marchionatti et al. [14]. The dose of 10 mg/kg of b.w. MEL was selected as the lowest dose necessary to block the inhibition of MEN on intestinal Ca^{2+} absorption (data not shown) and it is well documented as a useful dose to overcome oxidative stress caused by oxidants in other tissues [9]. In all cases, chicks were fasted for 24 h and the sacrifice was made in the morning between 10:30 and 12:30 h. The protocol was conducted according to the guide for care and use of laboratory animals. All efforts were made to minimize the number of animals used and their suffering.

Chemicals

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Mature enterocytes and intestinal mitochondria isolation

Mature enterocytes were isolated from intestinal mucosa of the four groups of animals as previously described [21]. Cellular viability was assayed by the Trypan blue exclusion

technique. Mitochondria were isolated from intestinal mucosa of the four groups of animals by differential centrifugations, as previously reported [22].

Intestinal Ca^{2+} absorption

We used the intestinal loop technique ligated in situ as Tolosa de Talamoni et al. [23] previously described using ^{45}Ca as tracer. Briefly, chicks were laparotomized under anesthesia and a 10-cm segment of duodenum was ligated. One milliliter of 150 mM NaCl, and 1 mM $CaCl_2$, containing 1.85×10^5 Bq^{45} Ca^{2+}, pH 7.2, was introduced into the lumen of the ligated intestinal segment. Half an hour later, blood was withdrawn by cardiac puncture, centrifuged and the plasma $^{45}Ca^{2+}$ was measured in a liquid scintillation counter. Absorption was defined as appearance of $^{45}Ca^{2+}$ in blood.

Spectrophotometric procedures

All the enzymes activities were assayed in supernates of duodenal homogenates. Superoxide dismutase (Mg^{2+}-SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), and glutathione peroxidase (GPx; EC 1.11.1.9) activities were performed in diluted aliquots of the supernates (1:5). Mg^{2+}–SOD activity was determined in 1 μM EDTA, 50 mM potassium phosphate buffer, pH 7.8, 13 mM methionine, 75 μM nitro blue tetrazolium (NBT), and 40 μM riboflavine. Mg^{2+}–SOD activity was defined in terms of its ability of inhibiting the superoxide anion (O_2^-) dependent reaction due to the competition between SOD and NBT [24]. CAT activity was assayed in 50 mM potassium phosphate buffer pH 7.4 and 0.3 M H_2O_2. The H_2O_2 decomposition rate was directly proportional to enzyme activity [25]. GPx activity was determined in 50 mM potassium phosphate buffer pH 7.8, 1 mM EDTA, 1 mM NaN_3, 1 mM glutathione (GSH), and 1 U GSH reductase. The activity was measured by following NADPH oxidation after addition of 1 mM NADPH [26]. Total GSH content was also assayed in supernates from intestinal homogenates. The determinations were carried out by the glutathione disulfide reductase-5,5′-dithiobis(2-nitrobenzoate) recycling procedure [27]. The protein carbonyl content was determined by using 2,4-dinitrophenylhydrazine in a aliquot from homogenates of scraped duodenal mucosa diluted in a isolation buffer (50.3 mM HEPES, 127 mM KCl, 1.36 mM EDTA, 0.5 mM MgSO4, and 0.183 mM PMSF, pH 7.4) following the procedure of Levine et al. [28].

TUNEL assay

Intestines of chicks were fixed in 4 % paraformaldehyde in 0.01 M sodium phosphate buffer pH 7.3 and sections of 5 μm were obtained. Tissue morphology was visualized and analyzed after hematoxylin–eosin staining. DNA fragmentation was measured by the terminal transferase-mediated dUTP nick-end labeling procedure (TUNEL) employing ApopTag Plus peroxidase in situ Apoptosis Detection Kit (Chemicon International, Temecula, CA, USA). The apoptotic index, expressed as the percentage of TUNEL positive cells in relation to the total number of cells, was calculated in order to determine the magnitude of the apoptotic process. The apoptotic index was assessed by counting at 400× magnification at least ten villi from three chicks for each treatment, which was accomplished by two independent researchers in a blinded fashion.

Caspase 3 protein expression and activity

Caspase 3 expression was analyzed by Western blot procedure, using supernates of mucosa from chick duodenae. Suspensions were done in RIPA lysis buffer and then centrifuged. Afterward, proteins (100 μg) were denatured for 5 min at 95 °C and separated in 12 % (w/v) SDS–polyacrylamide minigels [29]. Gels containing the separated proteins were immersed in the transfer buffer [30]. Nitrocellulose membranes (0.45 μm) were blocked for 1.5 h with 2 % w/v non-fat dry milk in 0.5 M Tris-buffered saline solution and incubated overnight at 4 °C with the purified rabbit anti-active caspase 3 (BD Biosciences Pharmingen, San Jose, CA, USA). After three washings, an appropriate secondary biotinylated antibody was incubated at room temperature for 1 h. Then, the blots were washed three times and streptavidin–peroxidase conjugate (Zymed Laboratories Inc., Invitrogen, Carlsbad, CA, USA) was added. Detection was performed using DAB as a chromogen. Monoclonal antibody anti-GAPDH (clone GAPDH-71.1) from Sigma-Aldrich, St. Louis, MO, USA was used to detect GAPDH as a marker to normalize the relative expression of caspase 3. The band intensities were quantified using an Image Capturer UVP EC3 Imaging System, Launch Visionworks Ls software in order to obtain the relative expression of protein.

Caspase 3 activity was accomplished in supernates from homogenates of enterocytes following in a plate reader at 405 nm the absorbance of *p*-nitroaniline obtained from a caspase 3-substrate I (Calbiochem, San Diego, CA, USA) [31].

Mitochondrial membrane permeability transition (swelling)

Isolated intestinal mitochondria (3 mg protein) were incubated in 3 ml of respiratory buffer (0.1 M NaCl, 10 mM MOPS, 1 mM glutamate, and 1 mM malate, pH 7.4) for 10 min at 25 °C and monitored at 540 nm in a Beckman Coulter DU 640 spectrophotometer (USA). Basal values of

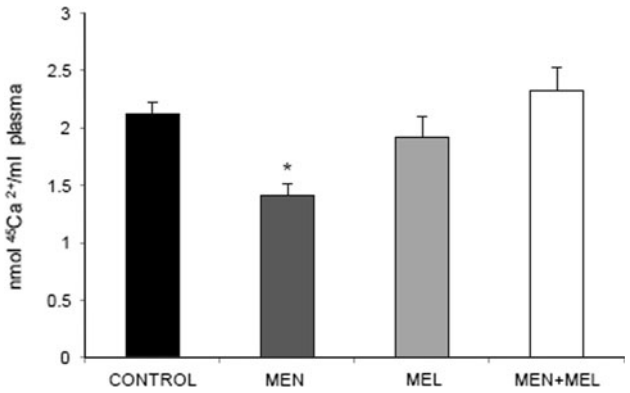

Fig. 1 Effect of *MEN*, *MEL*, and *MEN+MEL* on chick intestinal calcium absorption. 4-week-old chicks were divided into four groups: (1) Controls received 2 i.p. injections of saline solution (SS) with an interval of 30 min between each other. (2) Treated with *MEN*. Chicks first received an i.p. injection of 2.5 µmol of *MEN*/kg of b.w. and 30 min later a second i.p. injection of SS. (3) Treated with *MEL*. Chicks first received an i.p. injection of SS and 30 min later a second i.p. injection of 10 mg *MEL*/kg of b.w. (4) Treated with *MEN+MEL*. First, chicks received and i.p. injection with MEN and 30 min later a second i.p. injection of *MEL* (at the same doses than the groups 2 and 3). 20 min after the last injection, one milliliter of 150 mM NaCl, and 1 mM $CaCl_2$, containing 1.85×10^5 Bq^{45} Ca^{2+}, pH 7.2, was introduced into the lumen of the ligated intestinal segment. Half an hour later, blood of each animal was withdrawn by cardiac puncture, centrifuged, and the plasma $^{45}Ca^{2+}$ was measured in a liquid scintillation counter. Values are expressed as mean ± SE of 4 determinations. $*p < 0.05$ versus *CONTROL*, *MEL*, and *MEN+MEL*

mitochondrial absorbance were measured for 5 min and the optical density was followed for 5 more minutes, after the addition of MEN, MEL, or both [32].

Statistics

Data are given as mean ± SE. Comparisons between multiple groups were performed with one-way analysis of variance (ANOVA) followed by Bonferroni test as a post-hoc test. Differences were considered statistically significant at $p < 0.05$. All the analyses were carried out by using SPSS 11.5 software for Windows (SPSS, Inc., Chicago, IL, USA).

Results

MEL restored intestinal Ca^{2+} absorption diminished by MEN in chicks

The intestinal Ca^{2+} absorption was inhibited by the oxidant MEN and restored by MEL to the control values. After 30 min of treatment, 2.5 µmol of MEN/kg of b.w decreased the intestinal Ca^{2+} absorption in chicks, as previously reported Marchionatti et al. [14]. The administration of 10 mg MEL/kg of b.w 30 min after MEN injection was able to counteract the MEN effect and returned the intestinal Ca^{2+} absorption to the control values (Fig. 1). Lower doses of MEL were unable to restore the intestinal Ca^{2+} absorption altered by the quinone (data not shown). Surprisingly, the usage of 10 mg MEL/kg of b.w. alone produced no significant differences on the intestinal Ca^{2+} absorption as compared to the control group.

MEL reversed alterations in the GSH and carbonyl contents and in the activity of the antioxidant enzymes caused by MEN in chick duodenum

As expected, MEN provoked GSH depletion (Fig. 2a) and increase in the protein carbonyl content (Fig. 2b) in chick duodenae after 30-min injection, an indication that the quinone triggered oxidative stress. These effects were totally abolished by MEL administration. However, MEL alone was not able to modify the tripeptide levels or the carbonyl content (Fig. 2a, b, respectively). SOD and CAT, whose activities had increased by MEN treatment as a compensatory response, returned to control values after MEL injection. MEL alone did not change the SOD and CAT activities. By contrast, the GPx activity remained unaltered in the chick mucosa after any treatment employed (Fig. 3).

MEL blocked intestinal apoptosis triggered by MEN in chicks

In order to know if MEL treatment could block apoptosis triggered by MEN in intestinal villi, the detection of DNA fragmentation (TUNEL), and the activity and protein expression of caspase 3 were studied. As expected, MEN increased the number of TUNEL-positive cells, which can be observed in intestinal sections as compared to control villi (Fig. 4a). Furthermore, the apoptotic index shows that MEN caused approximately a 50 % increase in the number of apoptotic cells (Fig. 4b). MEL alone did not modify the DNA fragmentation as compared to that of the control, but blocked the enhancement of DNA fragmentation caused by MEN (Fig. 4b). The protein expression of caspase 3, a typical executioner of apoptosis [33], was also enhanced by MEN. The arbitrary units of optical density from the protein bands indicate that MEN increased about 40 % the protein expression of caspase 3 (Fig. 5a). The activity of this enzyme was also augmented by MEN treatment (Fig. 5b). These responses were blocked or arrested by MEL treatment (Fig. 5a, b). Again, MEL alone did not modify the protein expression and activity of caspase 3, as compared to those from the control group (Figs. 5a, b).

The release of proapoptotic molecules from the intermembrane space of mitochondria to the cytoplasm during apoptosis is facilitated by the alteration of the

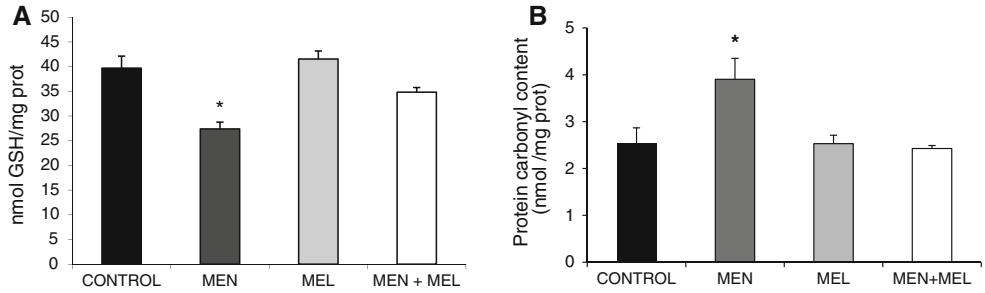

Fig. 2 Effect of *MEN*, *MEL*, and *MEN+MEL* on **a** GSH levels and **b** protein carbonyl content of chick intestine. Treatments were identical to those described in Fig. 1. Total GSH and protein carbonyl contents were assayed in supernates from intestinal mucosa homogenates. Values are expressed as mean ± SE of 4 determinations. *$p < 0.05$ versus *CONTROL*, *MEL*, and *MEN+MEL*

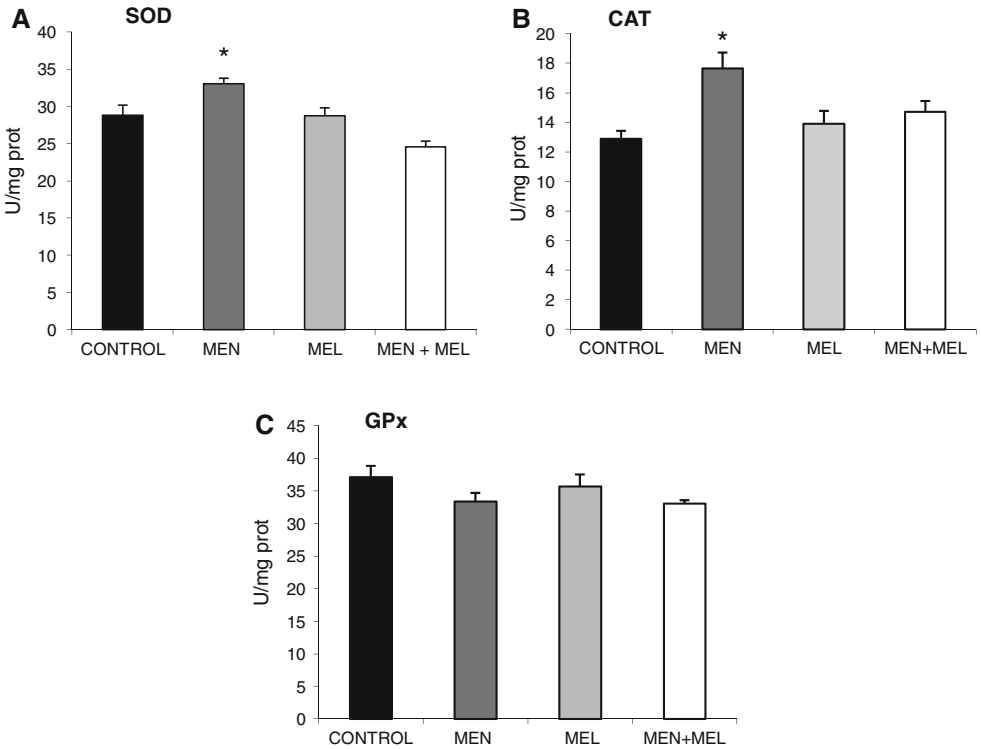

Fig. 3 Effect of *MEN*, *MEL*, and *MEN+MEL* on the activity of antioxidant enzymes. Treatments were identical to those described in Fig. 1. **a** SOD; **b** CAT, and **c** GPx. Enzymatic activity was assayed in supernates from homogenates of chick intestinal mucosa. Values are expressed as mean ± SE of 4 determinations. *$p < 0.05$ versus *CONTROL*, *MEL*, and *MEN+MEL*

mitochondrial membrane permeability. Changes in the absorbance of mitochondrial suspensions at 540 nm were used to monitor mitochondrial swelling due to changes in the mitochondrial permeability. Fresh isolated mitochondria from enterocytes of control chicks were exposed to MEN (0.5 mM), MEL (0.5 mM), or to both drugs at the same time (0.5 mM MEN and 0.5 mM MEL). After 5 min following the baseline (control), a decrease in the absorbance was observed after MEN addition to the mitochondrial incubation, showing this effect dose dependency (data not shown). MEL by itself showed a similar absorbance to the baseline (control). However, the co-treatment avoided the decrease in the absorbance caused by MEN (Fig. 6).

Discussion

This work clearly demonstrates that MEL through its antioxidant and antiapoptotic properties restores the chick intestinal Ca^{2+} absorption inhibited by MEN. It is interesting to note that MEL alone does not modify the intestinal Ca^{2+} absorption and other variables influencing on that process. The protective mechanisms triggered by MEL seem to be switched on under oxidative stress conditions, leading cells to the normalization of redox status.

We have previously demonstrated that the intestinal levels of GSH are essential to have an optimal Ca^{2+} absorption [14, 23]. As reported, a single MEN injection

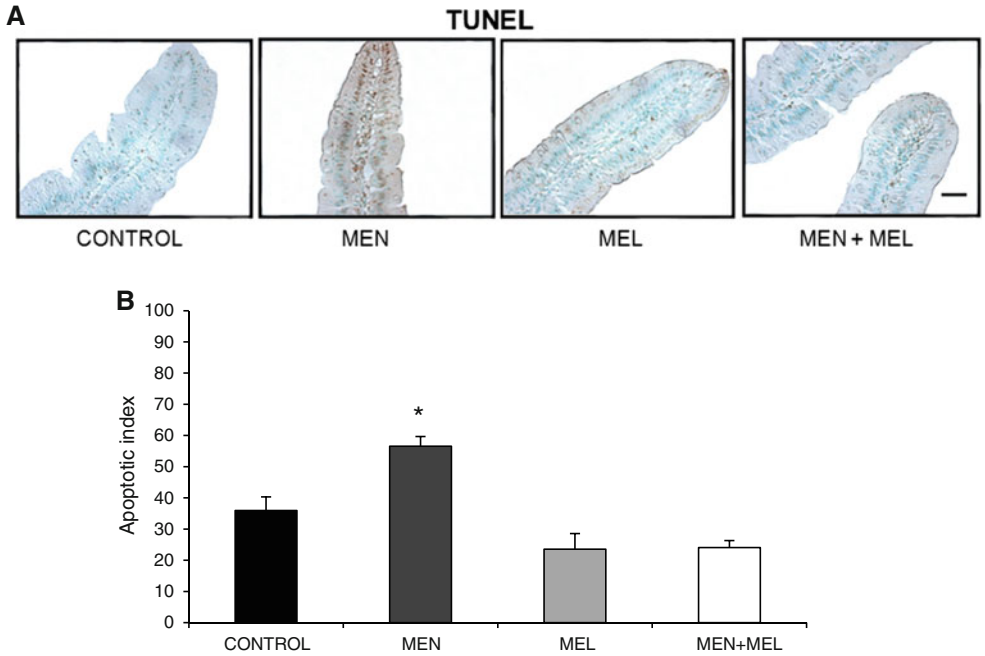

Fig. 4 **a** Effect of *MEN*, *MEL*, and *MEN+MEL* on DNA fragmentation of duodenal villous tip cells from chick intestinal sections (5 μm) analyzed by the *TUNEL* assay. Magnification bars are equivalent to 20 μm. **b** Apoptotic index is expressed as the percentage of *TUNEL*-positive cells in relation to the total number of cells. Treatments were identical to those described in Fig. 1. Values are mean ± SE of 4 total villi from three animals each group. $*p < 0.05$ versus *CONTROL*, *MEL*, and MEN+MEL

causes a transient inhibitory effect on intestinal Ca^{2+} absorption, at least in part, as a result of oxidative stress as shown by decrease in the GSH content and increase in the protein carbonyl levels. MEN in its redox cycle depletes GSH and increases ROS production [34]. The balance oxidation–reduction is altered and the oxidative stress is triggered causing mitochondrial dysfunction [15]. The present data reveal that MEL administration after MEN injection returns rapidly the intestinal GSH and protein carbonyl levels to control values. Simultaneously, the intestinal Ca^{2+} absorption goes up to the control values, which suggests that the restoration of the redox status of the gut by MEL allows the recovering of the intestinal capacity to absorb the cation properly. Maity et al. [35] have also demonstrated that MEL blocks a decrease of mitochondrial GSH content induced by indomethacin in rat gastric mucosal cells and attenuates the mitochondrial oxidative stress. Tahan et al. [36] found that the acetic acid administration in rats caused colonic mucosal injury accompanied by decreased GSH and SOD levels, whereas MEL injection reversed these effects. Altogether, these studies offer a strong insight in the dual action of MEL as an effective antioxidant and anti-inflammatory drug. However, MEL alone does not modify GSH levels in chick intestine, at least after 30-min injection. In contrast, at a dose 20 mg/kg b.w., MEL was able to increase GSH levels and the activity of GPx as well as GSH transferase (GST) in the mouse liver and kidney in vivo after 5 and 10 days of administration [37]. These discrepancies may be a matter of dose or timing of MEL treatment, although differences among species could not be discarded.

As known, there are intrinsic mechanisms to resist the oxidative damage in organisms. They comprise non-enzymatic and enzymatic antioxidant defense mechanisms. GSH belongs to the non-enzymatic mechanism; it acts as a direct scavenger of free radicals. SOD, CAT, and GPx form the group of enzymatic antioxidant defenses. SOD constitutes the first line of defense to eliminate superoxide anions, which are transformed into H_2O_2, a substrate of CAT and GPx. In this study, we show that MEN causes increment of SOD and CAT activities in duodenal mucosa, effect that is reversed by MEL. In other words, MEL normalizes the antioxidant enzymatic defenses altered by MEN. The increase in the activities of SOD, CAT, GPx, and GST caused by atrazine toxicity in rat erythrocytes was also attenuated by daily MEL administration for 21 days [38]. Similarly, MEL ameliorated doxorubicin (DXR)-induced testicular toxicity and the activity of SOD, glutathione reductase, and GST were not affected when MEL was administered in conjunction with DXR [39]. Since 1993, MEL has been recognized as a direct scavenger of free radicals with the ability to remove singlet oxygen, superoxide anion radical, and hydroperoxide. In addition, MEL has indirect antioxidant action through regulation of antioxidant enzyme activities. The mechanisms involved in the regulation of antioxidant enzymes by MEL in vivo have

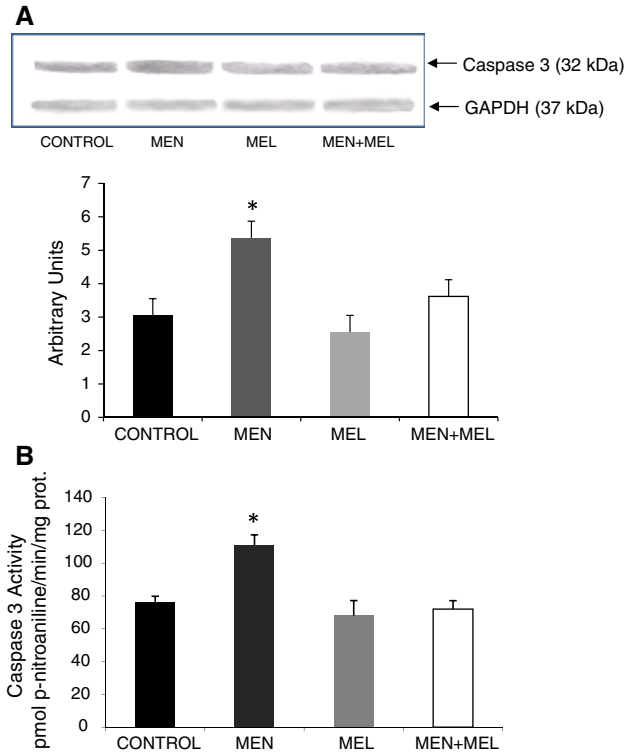

Fig. 5 **a** *Upper panel* effect of *MEN*, *MEL*, and *MEN+MEL* on chick intestine caspase 3 expression analyzed by Western blot. *Lower panel* optical densities of caspase 3 protein bands under the same treatments (expressed in arbitrary units). **b** Effect of *MEN*, *MEL*, and *MEN+MEL* on caspase 3 activity assayed in supernates from homogenates of enterocytes. Treatments were identical to those described in Fig. 1. Values are expressed as mean ± SE of three determinations. *$p < 0.05$ versus *CONTROL*, *MEL*, and *MEN+MEL* groups

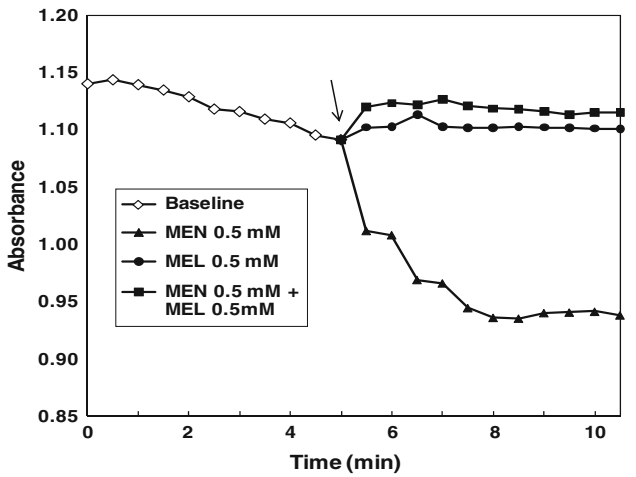

Fig. 6 A representative experiment on the optical density changes (swelling assay) in isolated mitochondria from control chick enterocytes incubated in vitro with *MEN* (0.5 mM), MEL (0.5 mM), or *MEN+MEL*. *Arrow* treatment addition. The experiment was repeated three times

not been precisely determined [40]. It has been proposed that MEL stimulates antioxidant enzymes via its interaction with calmodulin-inactivating nuclear RORα MEL receptor, which is a transcription factor that downregulates NF-κB-induced antioxidant enzyme expression [41]. In addition, MEL was found to increase the antioxidant enzyme mRNAs through Nrf2 activation in rat pancreas [42]. Nevertheless, MEL alone does not affect SOD and CAT activities from chick intestinal mucosa. Neither MEN nor MEL change GPx activity in the chick duodenal mucosa. We have previously demonstrated that MEN does not affect the GPx activity from chick intestinal mucosa or mitochondria [15, 43]. The lack of effect of MEL on GPx activity after 30-min treatment is not surprising because no increase in GPx activity in the first 45 min after MEL injection has been found in time course studies accomplished in chick tissues [44]. In addition, this organ is the least responsive to the hormone in chicks (22 % increase as compared to 125 % in heart and 100 % in brain) after 90-min MEL treatment [45].

Another finding of this study is that MEL not only normalizes the redox state of enterocytes altered by MEN, but also rescues the epithelial cells from MEN-induced apoptosis. An important feature of MEN-induced apoptosis is that it is very rapid. Cytochrome c release has been detected within 2 min and caspase 3 activation after 30 min of drug application [4]. As reported, MEN causes apoptosis of chick enterocytes after 30-min treatment as judged by DNA fragmentation, cytochrome c release from mitochondria, and caspase 3 activation [15]. Mitochondrial membrane permeabilization is considered an event of early apoptosis. This study shows that MEN induces alteration in the mitochondrial membrane permeability (swelling) [46], which agrees with previous data showing alteration in the mitochondrial membrane potential [15]. MEL treatment blocks permeability changes, activation of caspase 3, and DNA fragmentation caused by MEN. Consequently, apoptosis occurs at the same rate than in control guts. Similarly, Wang et al. [47] found that MEL inhibits hepatic caspase 3 activity and attenuates DNA fragmentation in D-galactosamine-lipopolysaccharide-treated mice. Pascua et al. [16] also observed that MEL treatment normalized the oxidative, inflammatory, and apoptotic patterns of colonic smooth muscle in aged rats. In addition, MEL administration was able to prevent apoptosis in rat pancreas before or after an ischemia/reperfusion injury [48]. The antiapoptotic effect of MEL could be a result of a decrease in the Bax/Bcl-2 ratio as reported to occur in rat peripheral blood lymphocytes from gamma irradiation-induced apoptosis [49].

To conclude, this work demonstrates that MEL blocks the oxidative stress and apoptosis produced by MEN in

chick intestinal mucosa, which contributes to restore the capacity of the epithelial cells to absorb calcium. This process requires ATP, which is mainly provided by the mitochondria [50]. Therefore, the preservation of the bioenergetic function of these organelles becomes important for Ca^{2+} transport. It is quite possible that MEL maintains intact the mitochondrial capacity to produce ATP in chick enterocytes and, consequently, the intestinal properties to absorb Ca^{2+}. The beneficial effects of MEL on the mitochondrial physiology have been largely reported [51–53].

Taken together, these findings suggest that MEL could be a potential drug of choice for the reversal of impaired intestinal Ca^{2+} absorption produced by oxidative stress and exacerbated apoptosis that occurs after the administration of drugs promoting the oxidative stress in gut or under certain pathophysiological conditions such as aging, celiac disease, intestinal bowel disease, cancer, and other disorders.

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