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Melatonin not only restores but also prevents the inhibition of the intestinal Ca^{2+} absorption caused by glutathione depleting drugs

Vanessa Areco [a], Valeria Rodriguez [a], Ana Marchionatti [a], Agata Carpentieri [a,b], Nori Tolosa de Talamoni [a,*]

[a] Laboratorio "Dr. Fernando Cañas", Cátedra de Bioquímica y Biología Molecular, Facultad de Ciencias Médicas, INICSA (CONICET-Universidad Nacional de Córdoba), Pabellón Argentina, 2do. Piso, Ciudad Universitaria, 5000 Córdoba, Argentina
[b] Química Biológica, Facultad de Odontología, Universidad Nacional de Córdoba, Córdoba, Argentina

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ABSTRACT

We have previously demonstrated that melatonin (MEL) blocks the inhibition of the intestinal Ca^{2+} absorption caused by menadione (MEN). The purpose of this study were to determine whether MEL not only restores but also prevents the intestinal Ca^{2+} absorption inhibited either by MEN or BSO, two drugs that deplete glutathione (GSH) in different ways, and to analyze the mechanisms by which MEN and MEL alter the movement of Ca^{2+} across the duodenum. To know this, chicks were divided into four groups: 1) controls, 2) MEN treated, 3) MEL treated, and 4) treated sequentially with MEN and MEL or with MEN and MEL at the same time. In a set of experiments, chicks treated with BSO or sequentially with BSO and MEL or with BSO and MEL at the same time were used. MEL not only restored but also prevented the inhibition of the chick intestinal Ca^{2+} absorption produced by either MEN or BSO. MEN altered the protein expression of molecules involved in the transcellular as well as in the paracellular pathway of the intestinal Ca^{2+} absorption. MEL restored partially both pathways through normalization of the $\bullet O_2^-$ levels. The nitrergic system was not altered by any treatment. In conclusion, MEL prevents or restores the inhibition of the intestinal Ca^{2+} absorption caused by different GSH depleting drugs. It might become one drug for the treatment of intestinal Ca^{2+} absorption under oxidant conditions having the advantage of low or null side effects.

1. Introduction

Melatonin (MEL) is an ancient and highly conserved indoleamine present in all phyla of multicellular animals (Chu et al., 2015). In mammals and avian species, MEL is mainly synthesized by the pineal gland in a circadian manner and is critical for many physiological functions (Shochat et al., 1997; Salti et al., 2000; Reiter et al., 2009; Li and Cassone, 2015). There are also several extrapineal sites where MEL is synthesized, including gastrointestinal (GI) tract (Mukherjee and Maitra, 2015). The level of MEL in the gut is 400 times larger than that in the pineal gland (Bubenik, 2002). Although its presence in the gut has been discovered more than thirty years ago (Bubenik, 2008), its physiological significance is not completely understood nowadays. Circadian variation of GI MEL seems not to be controlled by photoperiodicity as occurs in the pineal gland, but by eating and food composition (Siah et al., 2014). MEL affects the motility of the intestine; at small doses MEL accelerates the intestinal transit in rats, while high doses reverse this effect (Drago et al., 2002). MEL participates in 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 (Bubenik et al., 1999; Carrillo-Vico et al., 2005). MEL has also been shown to scavenge reactive oxygen species (ROS) and inhibit macrophage by suppressing proinflammatory agents including inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (Crespo et al., 1999; Deng et al., 2006). It has been demonstrated that MEL protects against the deleterious effects of ischemia and reperfusion (I/R) through a stimulation of certain antioxidant enzymes, preserves cellular energy and prevents mitochondrial damage (Carpentieri et al., 2012). Recently, gut MEL has been implicated in the mechanism of response to microbial infections in any fish species (Pal et al., 2015).

We have previously demonstrated that MEL blocks the inhibition of the intestinal Ca^{2+} absorption caused by menadione (MEN) or vitamin K_3, which is due, at least in part, to the increase in the activity of antioxidant enzymes, returning the glutathione (GSH) and protein carbonyl values to control levels, and rescuing the epithelial cells from MEN-induced apoptosis. By itself, MEL does not alter the intestinal cation

Abbreviations: O_2^-, superoxide anion; BSO, DL-buthionine-S,R-sulfoximine; CB D_{28k}, calbindin D_{28k}; CB D_{9K}, calbindin D_{9k}; CLDNs, claudins; DAB, 3,3′-diaminobenzidine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GI, gastrointestinal; GSH, glutathione; GSSG, oxidized glutathione; I/R, Ischemia and reperfusion; iNOS, inducible nitric oxide synthase; MEL, melatonin; MEN, menadione; NBT, nitro blue tetrazolium; NCX1, Na^+/Ca^{2+} exchanger; NO, nitric oxide; $PMCA_{1b}$, plasma membrane Ca^{2+}-ATPase; SS, saline solution; TJ, tight junctions; γ-GCS, γ-glutamylcysteinesynthetase.

* Corresponding author.
E-mail addresses: ntolosatalamoni@yahoo.com.ar, ntolosa@biomed.fcm.unc.edu.ar (N. Tolosa de Talamoni).

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transport and its mechanism of protection is only switched on in the presence of MEN (Carpentieri et al., 2014). Whether the protective action of MEL also occurs in the presence of other oxidant agents such as DL-buthionine-S,R-sulfoximine (BSO) needs to be clarified. Prior to MEN, BSO has been also demonstrated to reduce the chick intestinal Ca^{2+} absorption (Tolosa de Talamoni et al., 1996). BSO inhibits specifically the activity of γ-glutamylcysteine synthetase (γ-GCS), limiting step enzyme in the GSH synthesis (Akai et al., 2007), depleting intracellular GSH levels and leading to oxidative stress.

The small intestine represents the major site of Ca^{2+} absorption in most species; it is responsible for approximately 90% of the total Ca^{2+} absorption, whereas the rate of absorption in the colon is less than 10% (Wasserman, 2004; Pérez et al., 2008). Intestinal Ca^{2+} absorption occurs *via* both active transport and passive transport (Kuwabara and Tanaka, 2015). The first mechanism implicates Ca^{2+} movement from the mucosal-to-serosal side of the intestinal barrier occurring against a concentration gradient. It is a saturable process, predominates in the duodenum and jejunum mainly under conditions of low Ca intake, and is finely stimulated by vitamin D (Diaz de Barboza et al., 2015). The molecules involved in the transcellular pathway comprise epithelial Ca^{2+} channels, calbindins and two proteins extruding Ca^{2+} out of the cells such as the plasma membrane Ca^{2+-}ATPase ($PMCA_{1b}$ or Ca^{2+} pump) and the Na^{+}/Ca^{2+} exchanger (NCX1) (Areco et al., 2015). The passive transport, also called the paracellular mechanism, is a non saturable process that occurs across the majority of the intestine under moderate or adequate Ca intake (Bronner and Pansu, 1999; Kuwabara and Tanaka, 2015). Ca^{2+} moves through the tight junctions (TJ), which depends on the concentration and the electric gradient across the epithelium. There is some evidence that Claudins (CLDNs) 2, 12 and 15 are transmembrane proteins of TJ structures responsible for transporting Ca^{2+} in the intestine (Inai et al., 2005; Fujita et al., 2006). It remains unknown if the inhibition of intestinal Ca^{2+} absorption provoked by MEN and the protective action of MEL involve one or both mechanisms of Ca^{2+} transport.

Based upon previous considerations, the aims of the present study were as follows: 1) to determine whether MEL could also prevent or restore the intestinal Ca^{2+} absorption inhibited either by MEN or BSO, two drugs that deplete GSH in different ways, 2) to precise the mechanisms by which MEN and MEL alter the movement of Ca^{2+} across the duodenum, and 3) to elucidate if ROS and/or reactive nitrogen species are involved in their actions.

2. Material and methods

2.1. Chemicals

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

2.2. Animals

One-day-old Cobb Harding chicks (*Gallus gallus domesticus*) were purchased in Indacor S.A. (Rio Ceballos, Córdoba, Argentina) and were maintained under constant temperature (24 ± 2 °C), synchronized to a 12:12 light/dark cycle (light from 7:00 a.m. to 7:00 p.m.). They were fed a commercial normal avian diet (GEPSA Feeds, Pilar, Córdoba, Argentina) and water "*ad libitum*". At 4 weeks of age, chicks 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/SS, 4) treated sequentially with MEN and MEL (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). All animals were killed 20 min after the last injection by cervical dislocation and the excised duodena were rinsed with cold 0.15 M NaCl and mucosa was scraped to make homogenates or the enterocytes were isolated, as described below. The chosen dose of MEN was diluted in 0.15 M NaCl and corresponds to that dose capable of inhibiting intestinal Ca^{2+} absorption after 30 min injection, as previously described (Marchionatti et al., 2003). The dose of 10 mg/kg of b.w. MEL was the lowest dose necessary to block the inhibition of MEN on intestinal Ca^{2+} absorption (data not shown) and it is known as a useful dose to overcome oxidative stress caused by oxidants in other tissues (Carpentieri et al., 2012). In some experiments another group of animals was included (MEN–MEL); they received at the same time an i.p. injection of MEN + MEL (identical doses to those described above) for 20 min. In a different set of experiments, some animals received BSO (1 mmol/kg of b.w. for 20 min), a second group was administered with BSO–MEL (1 mmol/kg of b.w. and 10 mg/kg of b.w., respectively, for 20 min), and a third group was treated with BSO for 20 min followed by MEL administration for 20 min more. In all cases, chicks were fasted for 24 h and the sacrifice was made between 10:30 a.m. and 12:30 a.m. 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.

2.3. Intestinal Ca^{2+} absorption

We used the intestinal loop technique ligated *in situ* as previously described (Tolosa de Talamoni et al., 1996) using $^{45}Ca^{2+}$ as tracer. Briefly, chicks were laparotomized under anesthesia (50 mg/kg b.w. ketamine and 10 mg/kg b.w. xylazine) and a 10 cm segment of duodenum was ligated. One milliliter of 150 mM NaCl, 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 plasma.

2.4. Spectrophotometric procedures

The levels of nitric oxide ($NO^{\bullet}$) were determined as total nitrate/nitrite using Griess reagent as previously described (Miranda et al., 2001) with the modification of replacing zinc sulfate by ethanol for protein precipitation in the homogenate supernatant (Arab et al., 2014). The absorbance was read at 540 nm. A standard curve of sodium nitrate was used (1–10 μM). The data were expressed as nmol NO•/mg of protein. Superoxide anion ($^{\bullet}O_2^-$) measurements were accomplished in mature enterocytes from the duodenal villus tip, which were isolated as previously described (Centeno et al., 2004). Cellular viability was assayed by the Trypan blue exclusion technique. Cells were washed twice with Hanks buffer (137 mM NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4,0 1.3 mM $CaCl_2$, 1 mM $MgSO_4$, 4.2 mM $NaHCO_3$, 6.24 mM glucose, pH 7.4) and incubated with nitroblue tetrazolium (NBT) (1 mg/mL) at 37 °C for 1 h. The formazan precipitates formed were dissolved in dimethylsulfoxide and quantified at 560 nm. OD values are direct indicators of $\bullet O_2^-$ concentration in the samples (Serrander et al., 2007).

2.5. Western blot analysis

Immunoblotting analysis of NCX1, $PMCA_{1b}$, CB D_{28k}, CLDN 2, CLDN 12 and iNOS was performed using mucosal homogenates from chick duodenum. Suspensions were done in RIPA (radio immuno precipitation assay buffer) lysis buffer (1% SDS, 0.5% sodium deoxycholate in PBS, containing 1 mM PMSF and 1 M NaF) and then centrifuged. Afterwards, proteins (100 μg) were denatured for 5 min at 95 °C and separated in 12% (w/v) SDS-polyacrylamide minigels for CB D_{28k}, CLDN 2, CLDN 12 and in 8% (w/v) SDS-polyacrylamide minigels for the other proteins

(Laemmli, 1970). Gels containing the separated proteins were immersed in the transfer buffer (25 mM Tris–HCl, 192 mM glycine, 0.05% w/v SDS and 20% v/v methanol) (Towbin et al., 1979). Nitrocellulose membranes (0.45 μm) were blocked for 1.5 h with 2% w/v nonfat dry milk in 0.5 M Tris-buffered saline solution and incubated overnight at 4 °C with the specific primary antibody at 1:1000 dilution in each case. The antibodies were: anti-NCX 1 monoclonal antibody PPS019 (R&D Systems, Minneapolis, MN, USA), anti-$PMCA_{1b}$ (human erythrocyte clone 5F10 A7952 SIGMA Saint Louis, Missouri, USA), anti-CB D_{28k} (polyclonal antibody produced in rabbit, SIGMA Saint Louis, Missouri, USA), anti-CLDN 2 monoclonal antibody (Invitrogen, Carlsbad, CA, USA), anti-CLDN 12 (polyclonal antibody produced in rabbit, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit anti-NOS2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After three washings, appropriate secondary biotinylated antibodies were incubated at room temperature for 1 h. Then, the blots were washed three times and streptavidin-biotin conjugate (Histostain-SP Broad Spectrum, Invitrogen CA, USA) was added. Detection was performed using 3,3′-diaminobenzidine (DAB) as the 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 the other proteins. The band intensities were quantified using an Image Capturer EC3 Imaging System, Launch VisionWorks LS software (Life Science, Cambridge, UK) in order to obtain the relative expression of proteins.

2.6. RNA isolation and analysis of iNOS gene expression by RT-qPCR

Total RNA isolation was performed with TRIZOL reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). RNA concentration and purity were determined by spectrophotometry. RT-qPCR amplification was performed in a thermocycler (Quantitative PCR thermocycler Stratagene Mx 3000P, Agilent Technologies, Inc., Santa Clara, USA). Primer sequences for the *iNOS* gene were as follows: forward 5′-GAACAGCCAGCTCATCCGATA-3′and reverse 5′-CCCAAGCT CAATGCACAACTT-3′, which amplify a 64 pb long sequence. Amplification mixture (total volume: 20 μL) contained 0.5 μg RNA, 0.3 μM each primer, 0.4 μL of the diluted reference dye, 0.4 μL of reverse transcriptase (RT)/RNase block enzyme mixture and 10 μL of 2X Brilliant II SYBR Green QRT-PCR master mix (Stratagene, Agilent Technologies, Inc., Santa Clara, CA, USA). The following protocol was used: 1 cycle at 50 °C for 30 min, 1 cycle at 95 °C for 10 min, 40 cycles as follows: denaturation at 95 °C for 30 s, annealing at 60 °C for 60 s and extension at 72 °C for 30 s. The amount of PCR products formed in each cycle was evaluated on the basis of SYBR Green fluorescence. Cycle-to-cycle fluorescence emission readings were monitored and quantified using the DDCt method (Kenneth and Schmittgen, 2001). The copy numbers of *iNOS* mRNA was normalized relative to that of 18 S.

2.7. Statistics

Data are expressed as means ± SE. Results were evaluated by one-way analysis of variance (ANOVA) and the Bonferroni's test as a *post hoc* test. Differences were considered statistically significant at $p < 0.05$. All the analyses were carried out by using SPSS software (version 22.0) for Windows 8.1 (SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. MEL prevents/restores the inhibition of the intestinal Ca^{2+} absorption caused by MEN or BSO

As previously observed (17, 28), either MEN or BSO caused a significant decrease in the chick intestinal Ca^{2+} absorption after 30 and 20 min treatment, respectively. MEL not only restored but also prevented this inhibition produced by each drug. However, MEL *per se* did not affect the intestinal Ca^{2+} absorption (Fig. 1).

3.2. MEL restores partially the intestinal transcellular and paracellular Ca^{2+} pathways altered by MEN

MEN decreased the protein expression of CB D_{28K} and $PMCA_{1b}$, molecules involved in the transport from the apical to the basolateral pole of the enterocytes and in the extrusion of Ca^{2+}, respectively. MEL was capable of returning the protein expression of CB D_{28K} to the control values, but it was unable to normalize the protein expression of $PMCA_{1b}$. MEL by itself did not change the protein expression of any protein involved in the transcellular Ca^{2+} transport. The protein expression of NCX1, also involved in the Ca^{2+} extrusion from the enterocytes, was not altered by any treatment (Fig. 2). In relation to the proteins presumably involved in the paracellular pathway of intestinal Ca^{2+} absorption, MEN decreased the protein expression of CLDN 2, but it did not affect that of CLDN 12. Each protein expression was not altered by MEL; however, MEL blocked the inhibition of CLDN 2 protein expression caused by MEN (Fig. 3).

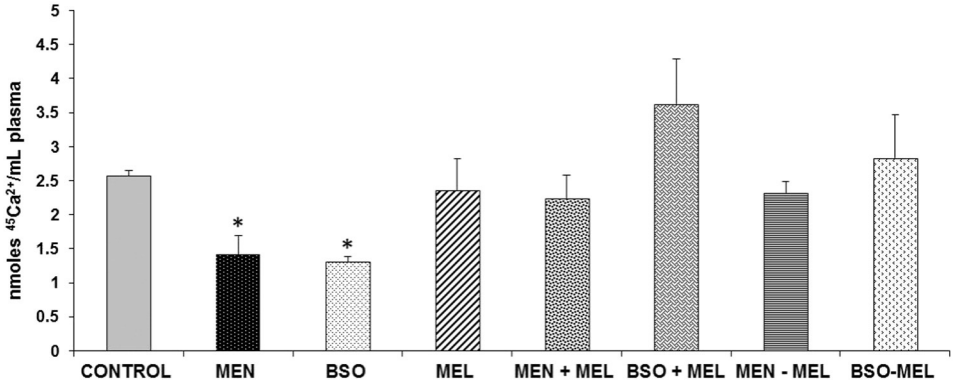

Fig. 1. Effects of MEN, BSO and MEL on chick intestinal Ca^{2+} absorption. MEN: chicks first received an i. p. injection of 2.5 μmol of MEN/kg of b.w. and 30 min later they received a second i. p. injection of SS for 20 min; BSO: chicks first received an i. p. injection of 1 mmol BSO/kg of b.w. and 20 min later they received a second i. p. injection of SS for 20 min; 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.for 20 min; MEN + MEL: chicks treated sequentially with MEN (2.5 μmol/kg of b.w.) for 30 min and MEL(10 mg/kg of b.w.) for 20 min; BSO + MEL: chicks treated sequentially with BSO (1 mmol/kg of b.w.) for 20 min and MEL (10 mg/kg of b.w.) for 20 min; MEN-MEL: chicks received at the same time an i.p. injection of MEN (2.5 μmol/kg of b.w.) and MEL (10 mg/kg of b.w.) for 20 min; BSO-MEL: chicks received at the same time an i.p. injection BSO (1 mmol/kg of b.w.) and MEL (10 mg/kg of b.w.) for 20 min. Absorption was defined as appearance of $^{45}Ca^{2+}$ in plasma. Values represent means ± SE from six chicks for each experimental condition. *$p < 0.05$ *vs* control, MEL, MEN + MEL, BSO + MEL, MEN − MEL and BSO − MEL.

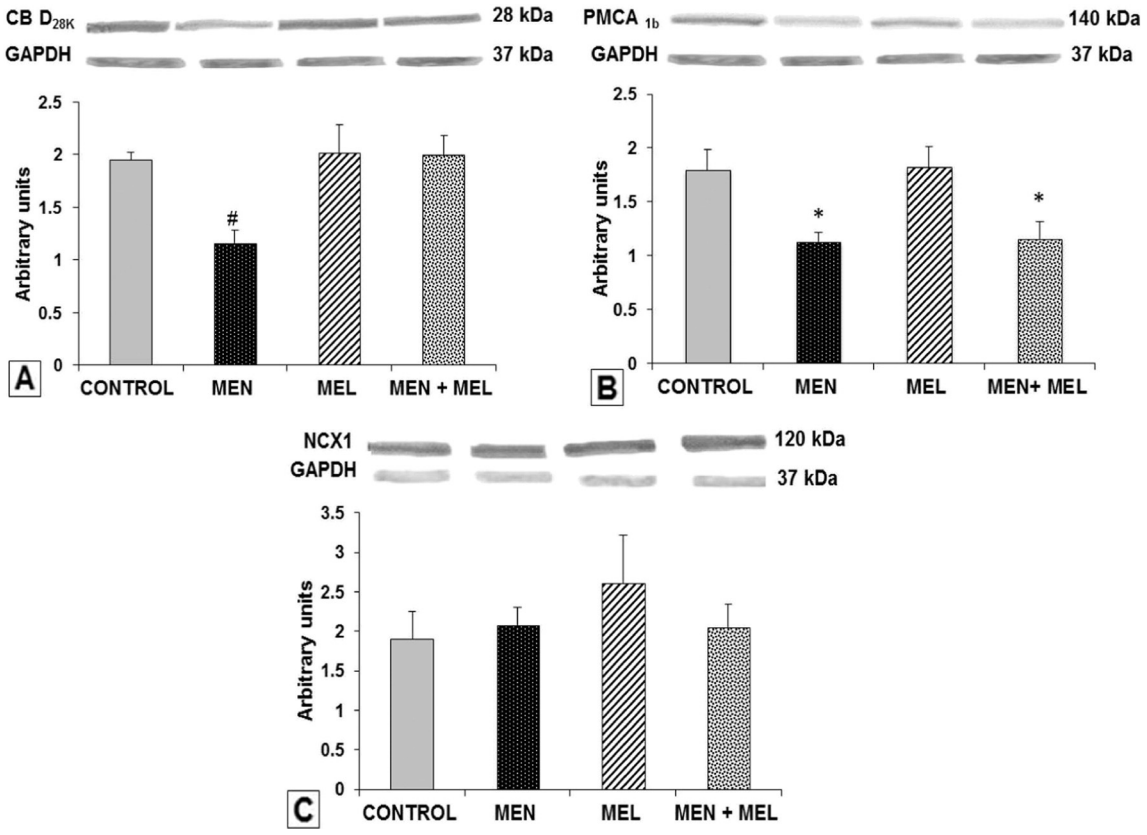

Fig. 2. Protein expression of CB D_{28k}, $PMCA_{1b}$ and NCX1 was analyzed by Western blot in mucosa from chick duodena. CB D_{28k} and $PMCA_{1b}$ were analyzed simultaneously on the same blot. One hundred micrograms of duodenal mucosa homogenate suspension was separated by SDS-PAGE and immunoblotted with the corresponding antibodies followed by detection with DAB as the chromogen. Five independent experiments were accomplished. Values are expressed as means ± S.E. $^{*}p < 0.05$ *vs* control and MEL. $^{\#}p < 0.05$ *vs* control, MEL and MEN + MEL.

3.3. MEL normalizes the $\bullet O_2^-$ levels altered by MEN in the chick duodenum

The duodenal content of $\bullet O_2^-$ was increased by MEN. In contrast, MEL did not affect it. However, MEL returned the $\bullet O_2^-$ levels altered by MEN to the control values (Fig. 4).

3.4. Neither MEN nor MEL affect the nitric oxide system in the chick duodenum

NO• content as well as gene and protein expression of iNOS was not modified by MEN or MEL. This occurred either with the single treatment of each drug or the consecutive combination of both drugs (Fig. 5).

4. Discussion

This study clearly shows that MEL protects the intestinal Ca^{2+} absorption from the inhibition caused by GSH depleting drugs. Previously, we have demonstrated that MEL could restore the intestinal cation transport altered by MEN (Carpentieri et al., 2014). In the present work we have demonstrated that MEL not only restores but also prevents the inhibition provoked by MEN. One of the mechanisms involved in the action of MEN is the oxidative stress derived from a decrease of GSH, which is a result of the GSH consumption in the redox cycle of this quinone (Ross et al., 1985). MEL also restores or prevents the inhibition of the intestinal Ca^{2+} absorption produced by BSO, a drug that selectively inhibits the activity of γ-GCS, limiting step enzyme in GSH

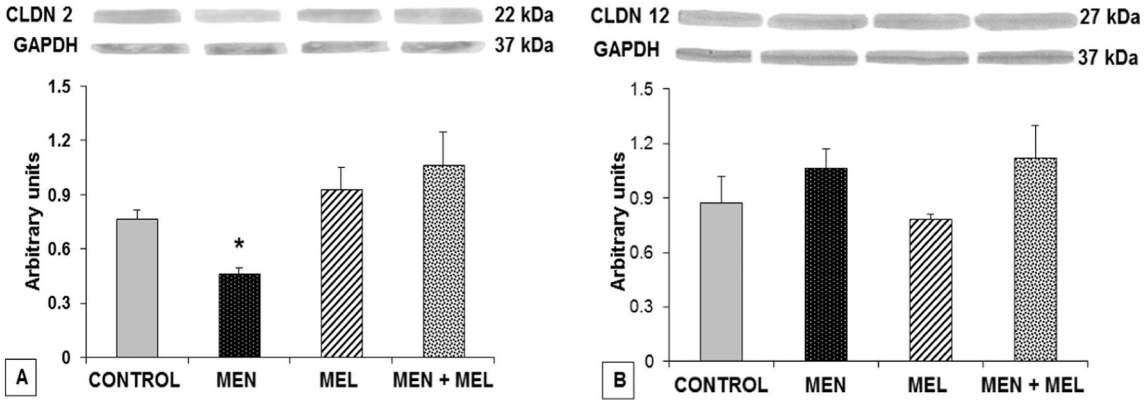

Fig. 3. Protein expression of CLDN 2 and CLDN 12 was analyzed by Western blot in mucosa from chick duodena. One hundred micrograms of duodenal mucosa homogenate suspension was separated by SDS-PAGE and immunoblotted with the corresponding antibodies followed by detection with DAB as a chromogen. Five independent experiments were accomplished. Values are expressed as means ± S.E. $^{*}p < 0.05$ *vs* control, MEL and MEN + MEL.

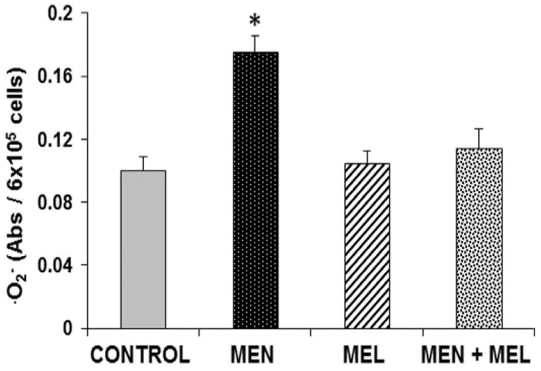

Fig. 4. Effects of MEN, MEL and MEN ± MEL on superoxide anion content in isolated enterocytes. Values represent means ± S.E. from four chicks for each experimental condition. $^{*}p < 0.05$ *vs* control, MEL and MEN + MEL.

synthesis (Akai et al., 2007). Since MEL alone does not affect the intestinal Ca^{2+} absorption, its protective role on this process is apparently triggered when the intestine is exposed to oxidant conditions provoked by any GSH depleting drug.

GSH is the main antioxidant of intestinal epithelial cells (van Ampting et al., 2009). It is produced endogenously and serves as electron donor in the GSH peroxidase reaction, which catalyzes the conversion of peroxides and H_2O_2 and reduced glutathione (GSH) to oxidized glutathione (GSSG), thereby reducing oxidative damage. When GSH is depleted, the GSH:GSSG ratio is decreased causing an oxidative shift in the cellular redox state (Smith et al., 2015). In our laboratory, we have largely demonstrated that the GSH depletion leads to an inhibition of intestinal Ca^{2+} transport. Not only GSH depleting drugs, but altered metabolic conditions that deplete intestinal GSH as occurs in the type I Diabetes mellitus (Rivoira et al., 2015) are associated with inhibition of intestinal Ca^{2+} absorption.

To gain more insight into the mechanisms of action of MEL, we have analyzed whether MEL protects the intestinal Ca^{2+} absorption affecting molecules involved in the transcellular and/or paracellular pathways of the intestinal Ca^{2+} absorption. In this regard, we have demonstrated that MEL was able to return the decreased protein expression of CB D_{28K} caused by MEN to the control values. However, MEN decreased the protein levels of $PMCA_{1b}$, one molecule involved in the Ca^{2+} extrusion, effect that could not be restored by MEL. The protein expression of NCX1, another transporter also located in the basolateral membrane from enterocytes, was not altered by any treatment. The selective enhancing effect of MEL on CB D_{28K} could be a matter of timing; it is not possible to discard that the other two proteins involved in the transcellular Ca^{2+} absorption could also be increased at longer times. The protein expression of CLDN 2, molecule that forms part of the TJ structures presumably involved in the paracellular pathway, was also inhibited by MEN. However, the CLDN 12 protein expression was not altered by the quinone. Since the sensitivity of TJ proteins to the redox state is variable (Molina-Jijón et al., 2014), one possible explanation could be that CLDN 2 is more sensitive to MEN-triggered ROS than CLDN 12.

Therefore, MEL protects the intestinal Ca^{2+} absorption against the inhibition caused by MEN normalizing some molecules involved either in the transcellular or in the paracellular pathway. The modulation of transporters of Ca^{2+} by MEL has also been reported in other tissues. Huai et al. (2012) have demonstrated that MEL can reduce pancreatic damage *via* the up-regulation of SERCA and NCX expression, alleviating calcium overload in pancreatic acinar cells. Yoo and Jeung (2010) have shown that MEL regulates CB D_{9K} expression during hydrogen peroxide-induced cell death in rat pituitary GH3 cells.

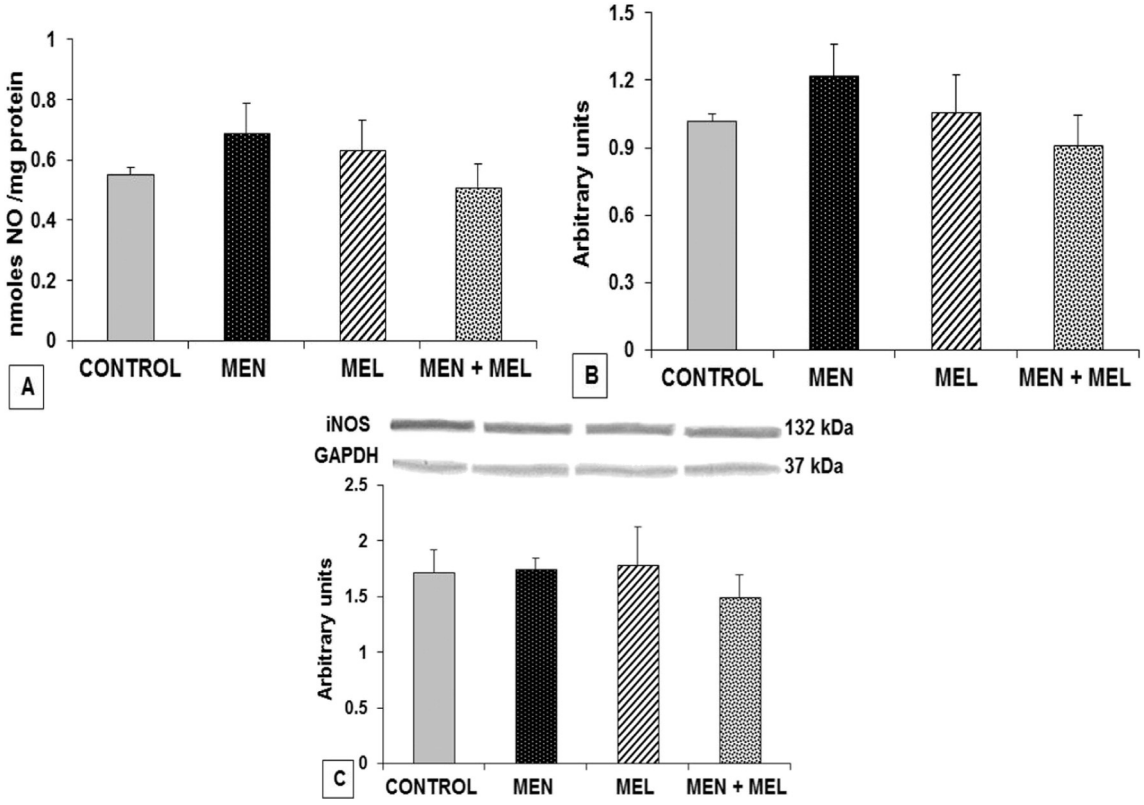

Fig. 5. Effects of MEN, MEL and MEN ± MEL on the NO• system. A) Quantitative PCR analysis of *iNOS* mRNA expression from chick duodena. Gene expression represents the relative mRNA expression compared with that of the *18S*, B) protein expression of iNOS was analyzed by Western blot in mucosa from chick duodena. One hundred micrograms of homogenate suspensions from duodenal mucosa were separated by SDS-PAGE and immunoblotted with the corresponding antibodies followed by detection with DAB as the chromogen.; C) NO• levels, determined by Griess assay. Three independent experiments were accomplished. Values are expressed as means ± S.E.

It has been previously observed that MEL preserves the transient mitochondrial permeability transition (MPT) (Jou, 2011; Carpentieri et al., 2014). This action not only preserves the MPT pore, but also maintains the mitochondrial membrane potential and retains the ATP formation during disturbed Ca^{2+} homeostasis (Jou, 2011). Taking into account that MEN causes mitochondrial dysfunction in chick intestine (Marchionatti et al., 2008), which could contribute to inhibit the intestinal Ca^{2+} absorption, the protective effect of MEL might also occur by preservation of the MPT pore, which could lead to maintain the levels of ATP necessary to provide the enough energy to motorize the intestinal Ca^{2+} absorption. The inhibition in the cardiolipin peroxidation caused by MEL (Petrosillo et al., 2009) might contribute to the prevention of the MPT and cytochrome c release, decreasing apoptosis of mature enterocytes, which are mainly involved in the intestinal Ca^{2+} transport. The attenuation of the mitochondrial dysfunction by MEL has been reported in several conditions such as in I/R of skeletal muscle (Wang et al., 2011), apoptosis of U937 cell line (Luchetti et al., 2007), and autophagy dependent on the SIRT3/SOD2 pathway (Pi et al., 2015).

The enhancement of $\bullet O_2^-$ by MEN is a direct proof that MEN-dependent inhibition of intestinal Ca^{2+} absorption is associated with oxidative stress, as previously reported (Carpentieri et al., 2014). MEL returns the $\bullet O_2^-$ levels to control values. However, MEL by itself does not alter the intestinal $\bullet O_2^-$ levels reinforcing the idea that the protective mechanisms of MEL are switched under oxidative stress conditions (Carpentieri et al., 2014).

In the present study, neither MEN nor MEL alter the intestinal iNOS gene and protein expression and the NO• levels. This is noticeable since one of the mechanisms used by MEL to produce its effects is through the modulation of NO• system. NO• is a short-lived bioactive molecule that participates in the many physiological and pathophysiological conditions. NO• is produced by three nitric oxide synthases (NOS), neuronal NOS (nNOS), endothelial NOS (eNOS), and iNOS. MEL has been shown to markedly dampen the hypobaric hypoxia-induced increases in the expressions of nNOS, eNOS and iNOS, NO• production and Ntyr formation (Huang et al., 2015). The beneficial effects of MEL on heart mitochondrial impairment during sepsis are related to inhibition of iNOS/i-mtNOS induction, restoration of mitochondrial homeostasis, and preservation of the activity of nNOS/mtNOS (Ortiz et al., 2014). However, MEL has been also demonstrated to accelerate healing of chronic gastric ulcers due to increase in the activity of NOS-NO• system resulting in the increase of mucosal blood flow and mucosal integrity (Konturek et al., 2006). As noticed, the interplay MEL-NO is rather complex and the final MEL effect on different NOS may vary according to the pathophysiological conditions, dose or strain tested (Tain et al., 2014).

To conclude, MEL prevents or restores the inhibition of the intestinal Ca^{2+} absorption caused by GSH depleting drugs. The indolamine affects the protein expression of molecules involved in the transcellular and paracellular mechanisms, through dampening the $\bullet O_2^-$ levels without affecting the NO• system. Since MEL has beneficial effects on both Ca^{2+} transport mechanisms, it might improve the intestinal Ca^{2+} absorption under conditions of low or adequate Ca intake. Therefore, it might become one drug for the treatment of deteriorated intestinal Ca^{2+} absorption in certain pathophysiological conditions such as aging, diabetes, celiac disease, cancer and other disorders associated with intestinal oxidative stress having the advantage of low or null side effects.

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