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Effects of a single dose of menadione on the intestinal calcium absorption and associated variables

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Abstract

The effect of a single large dose of menadione on intestinal calcium absorption and associated variables was investigated in chicks fed a normal diet. The data show that 2.5 μmol of menadione/kg of b.w. causes inhibition of calcium transfer from lumen-to-blood within 30 min. This effect seems to be related to oxidative stress provoked by menadione as judged by glutathione depletion and an increment in the total carbonyl group content produced at the same time. Two enzymes presumably involved in calcium transcellular movement, such as alkaline phosphatase, located in the brush border membrane, and Ca^{2+}- pump ATPase, which sits in the basolateral membrane, were also inhibited. The enzyme inhibition could be due to alterations caused by the appearance of free hydroxyl groups, which are triggered by glutathione depletion. Addition of glutathione monoester to the duodenal loop caused reversion of the menadione effect on both intestinal calcium absorption and alkaline phosphatase activity. In conclusion, menadione shifts the balance of oxidative and reductive processes in the enterocyte towards oxidation causing deleterious effects on intestinal Ca^{2+} absorption and associated variables, which could be prevented by administration of oral glutathione monoester.

Keywords: Menadione; Ca^{2+} absorption; Glutathione; Oxidative stress; Hydroxyl groups

1. Introduction

Intestinal calcium absorption occurs via two pathways: intracellular and paracellular. The first pathway is a saturable process, which is highly regulated by cholecalciferol. It comprises three steps: 1) Ca^{2+} entrance at the apical brush border membrane down a favorable electrochemical gradient, 2) Ca^{2+} movement along the cytoplasm and 3) Ca^{2+} exit through the basolateral membranes [1]. The mechanism of Ca^{2+} entrance to the enterocyte remains unclear although several proteins have been implicated such as intestinal alkaline phosphatase, Ca^{2+} receptor and, lately, the epithelial Ca^{2+} channels [2]. By using an ion microscopy system, Wasserman and Fullmer [3] have observed that Ca^{2+} ions were sequestered in the microvillar terminal web region of the enterocyte, remaining there for over 20 min. in vitamin D-deficient chicks. The authors also showed that previous administration of vitamin D_3 to vitamin D deficient animals caused the release of Ca^{2+} from its binding sites in the apical region and promoted a rapid transcellular transport. Calbindin D_{28k} in avian species and calbindin D_{9k} in mammals are considered responsible for removing Ca^{2+} from the apical sites and accelerating the transcellular movement to the basal region of the cell. The expression of both proteins has been shown to be regulated by $1,25(OH)_2D_3$ through a genomic mechanism [4]. The extrusion of Ca^{2+} out of intestinal cells is against the electrochemical gradient and requires energy. Two proteins can mediate Ca^{2+} exit through the basolateral membranes: the ATP-dependent Ca^{2+} pump and the Na^+/Ca^{2+} exchanger, which is energized by a Na^+ gradient created by the Na^+/K^+-ATPase. Either calcitriol or low Ca diets have been shown to modulate plasma membrane Ca^{2+}-ATPase activity by increas-

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ing the transcription rate of the Ca^{2+} pump gene [5]. In our laboratory, we have recently demonstrated that the intestinal Na^+/Ca^{2+} exchanger activity is also increased by $1,25(OH)_2D_3$ [6].

The enterocyte thiol redox state is an important factor that influences on the optimal vitamin D dependent intestinal Ca^{2+} absorption. As we reported earlier [7], the intestinal Ca^{2+} absorption in vitamin D deficient chicks treated with cholecalciferol is inhibited when intestinal glutathione (GSH) levels are decreased by DL-buthionine-S, R-sulfoximine (BSO) administration. The kinetic properties of intestinal alkaline phosphatase are also altered by GSH depletion in chicks with an adequate vitamin D status, an effect that is was reversed by addition of GSH monoester to the duodenal loop [8].

Quinone compounds such as menadione (MEN) represent a clinically important category of agents with application in anti-tumor therapy [9] and in the treatment of osteoporosis [10]. MEN metabolism is known to involve redox cycling, resulting in the release of various reactive oxygen species including free hydroxyl radicals. It has been reported that toxic concentrations of MEN may mediate cell injury at various sites, producing alteration of intracellular thiols and Ca^{2+} homeostasis, ATP depletion and / or DNA single strand breaks in a concentration dependant manner [11, 12].

Since intestinal Ca^{2+} absorption depends on intracellular GSH levels, it is reasonable to predict that a large dose of MEN could alter the thiol redox state in the enterocyte causing a deleterious effect on intestinal calcium absorption. To examine this possibility, we determined the effect of a single large dose of MEN on the redox state and calcium absorption in duodenum from chicks with a normal vitamin D status. The activities of intestinal alkaline phosphatase and plasma membrane Ca^{2+}-ATPase, two proteins presumably involved in the transcellular Ca^{2+} transport pathway, were also measured.

2. Materials and methods

One day-old Cobb Harding chicks (*Gallus domesticus*) (INDACOR, Córdoba, Argentina) were fed a commercial normal avian diet (Cargill, S.A.C.I., Pilar, Provincia de Córdoba, Argentina). At 4 to 5 weeks of age, the animals were divided into two groups: a) normal chicks (controls), b) normal chicks treated i.p. with 0.25 or 2.5 μmoles of MEN / kg of b.w. at different times as indicated in Results section.

2.1. Ca^{2+} transfer from lumen-to-plasma

At the chosen time after MEN or vehicle administration, the animals were laparotomized under ether anesthesia and a 10 cm segment of duodenum was ligated following the technique described previously [7]. One milliliter of 150 mmol/L NaCl, 1 mmol/L $CaCl_2$ containing 5 μCi $^{45}Ca^{2+}$, pH 7.2, was introduced into the lumen of the ligated intestinal segment. After 30 min, blood was withdrawn by cardiac puncture, centrifuged, and the plasma $^{45}Ca^{2+}$ was determined in a liquid scintillation counter. Results are expressed as nmol of Ca^{2+} transfer/mL of plasma.

2.2. Basolateral membrane vesicles (BLMV) preparation

Duodenal mucosa was scraped with a glass slide, and BLMV were prepared according to the procedure of Scalera et al. [13]. The purification factor of these preparations was around 9 fold in relation to their respective homogenates as assayed by Na^+/K^+ ATPase activity, used as a marker enzyme. The orientation of BLMV was not modified by the different experimental conditions.

2.3. ATP – dependent Ca^{2+} uptake in BLMV

The activity of plasma membrane Ca^{2+}-ATPase was determined by Ca^{2+} uptake experiments, which were done in BLMV at 25°C in the presence or absence of 5mmol/L Tris-ATP by using the rapid Millipore filtration technique, as described elsewhere [14].

2.4. Spectrophotometric procedures

Total GSH content was assayed in supernatants from homogenates of duodenal mucosa diluted in 5 vol of 5% (wt/vol) 5-sulfosalicylic acid. The determinations were carried out by the glutathione disulfide reductase-5,5′-dithiobis (2-nitrobenzoate) recycling procedure [15]. Intestinal alkaline phosphatase (EC 3.1.3.1) (IAP) activity assay was done in a diluted aliquot from water homogenates (1 : 10) of scraped duodenal mucosa using 3 mmol/L p-nitrophenyl phosphate as substrate in 0.5 mol/L diethanolamine buffer pH 9.8, following an adaptation of the method of Walter and Schütt [16]. γ-glutamyl transpeptidase (EC 2.3.2.2) activity was determined in a diluted aliquot from buffered homogenate (1 : 50) at pH 7.4 of scraped duodenal mucosa using 0.1 mol/L glycylglycine and 5 mmol/L Lγ-glutamyl-p-nitroanilide as substrates following the technique of Meister et al. [17]. Sucrase (EC 3.2.1.26) activity was determined in a diluted aliquot from water homogenates (1 : 10) of scraped duodenal mucosa following the technique of Dahlqvist [18]. Na^+/K^+-ATPase (EC 3.6.1.3) activity was determined in a diluted aliquot (1:500) of BLMV using 200 mmol/L ATP as substrate and pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (1.1.1.28) as coupled enzymes, in buffer pH 7.4 according to the method of Scharschmidt et al. [19].

The protein carbonyl content was determined by using the 2,4-dinitrophenylhydrazine in a diluted aliquot from water homogenates (1:10) of scraped duodenal mucosa following the technique of Levine et al. [20].

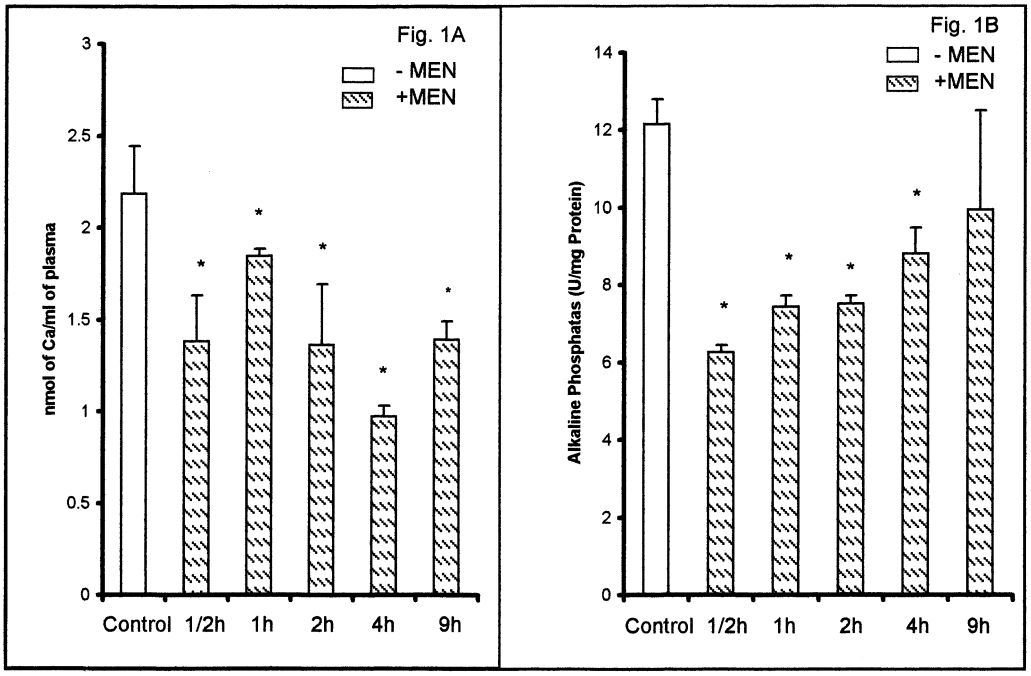

Fig. 1. Time effects of MEN administration A: Ca^{2+} transfer from lumen-to plasma, B: IAP activity. Dose: 2.5 μmol MEN/kg of b.w. Values are means ± S.E. of samples from 4 to 10 animals for each treatment. *$P < 0.05$ for comparison with control.

2.5. Duodenal villus tip cell isolation

The method of Walters and Weiser [21] was used for isolation of villus tip cells from the entire duodenum. After harvesting, the cell suspensions were diluted (1 : 2 vol/vol) in 140 mmol/L KCl, 10 mmol/L HEPES, pH 7.4 (buffer A), centrifuged at 550 g at 4°C for 5 min and the pellet resuspended in the same buffer, procedure that was performed twice. The final pellet was resuspended in a small volume and divided into two parts: one half was incubated in buffer A and the other part was incubated in buffer A plus addition of MEN.

2.6. Determination of free hydroxyl radicals

A spin trapping technique [22] was used in order to identify hydroxyl radicals from villus tip cells previously exposed to 50 or 500 μmol/L MEN concentrations. The incubations were done at 37°C for 15 or 30 min under gentle shaking. The spin trap 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) was employed and the electron spin resonance (ESR) spectra were measured at room temperature by an ESR spectrometer system, Bruker ECS 106, cavity ER4102ST, Germany.

2.7. Statistical methods

All results are expressed as mean ± standard error and were analyzed by analysis of variance and Student "t" test. Duncan's test [23] was performed to make multiple comparisons of the means. Differences were considered significant at the level of $P < 0.05$.

3. Results

Intraperitoneal administration of 2.5 μmoles MEN/kg of b.w. to four-week-old chicks reduced the intestinal Ca^{2+} movement from lumen to blood as compared to the controls. This effect was observed within 30 min and persisted up to 9 hr after MEN injection (Fig. 1A). The activity of IAP was also reduced by the same dose of MEN (Fig. 1B). The inhibition of IAP activity was pronounced half an hour after treatment, remained low for 4 h and returned to the control values 9 hr after dosing (Fig. 2B). The Ca^{2+} transfer from lumen to blood was also inhibited with a lower dose (0.25 μmoles MEN/kg), while the IAP activity was not altered (Fig. 2A and 2B). The addition of GSH monoester to the lumen of the duodenum sac 30 min before sacrifice, reversed the inhibitory effect of MEN on Ca^{2+} absorption (Fig. 3A) and IAP activity (Fig. 3B). The IAP activity and Ca^{2+} absorption were fully recovered by addition of 5 mmol/L GSH monoester to the intestinal lumen sac.

The activities of other intestinal brush border membrane enzymes such as sucrase and γ-glutamyl transpeptidase were not modified by MEN as compared to the control values. With regards to the basolateral membrane enzymes, MEN inhibited the plasma membrane Ca-ATPase but not the Na^+/K^+-ATPase activity (Table 1).

Total carbonyl content from proteins of intestinal homogenates of duodenal mucosa was significantly increased

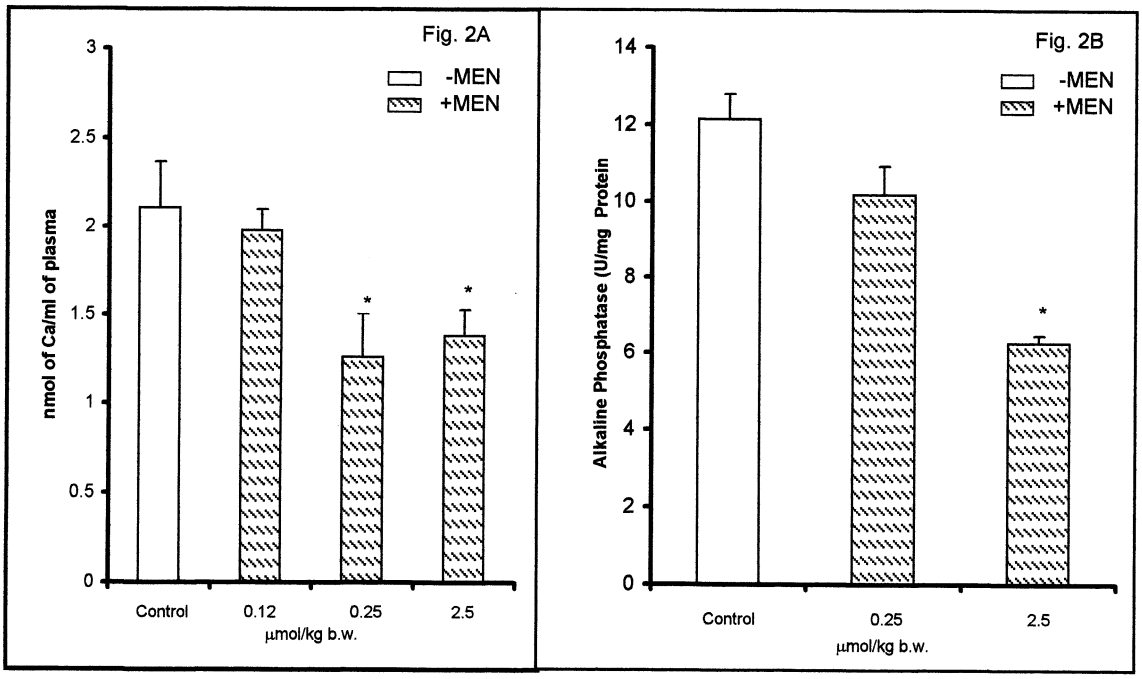

Fig. 2. Dose-dependent effects of MEN administration A: on Ca^{2+} transfer from lumen-to plasma, B: on IAP activity. Time: ½ hour before sacrifice. Values are means ± S.E. of samples from 4 to 6 animals for each treatment. *$P < 0.05$ for comparison with control.

by 2.5 µmol of MEN injection/kg of b.w. half an hour after treatment (Control chicks: *n* 6, 1.14 ± 0.16 nmol of carbonyl content/mg protein vs. MEN treated: n = 6, 1.75 ± 0.11* nmol of carbonyl content/mg protein, * $P < 0.05$ vs. control). On the contrary, the GSH content of intestinal mucosa was decreased by MEN treatment (Control chicks:

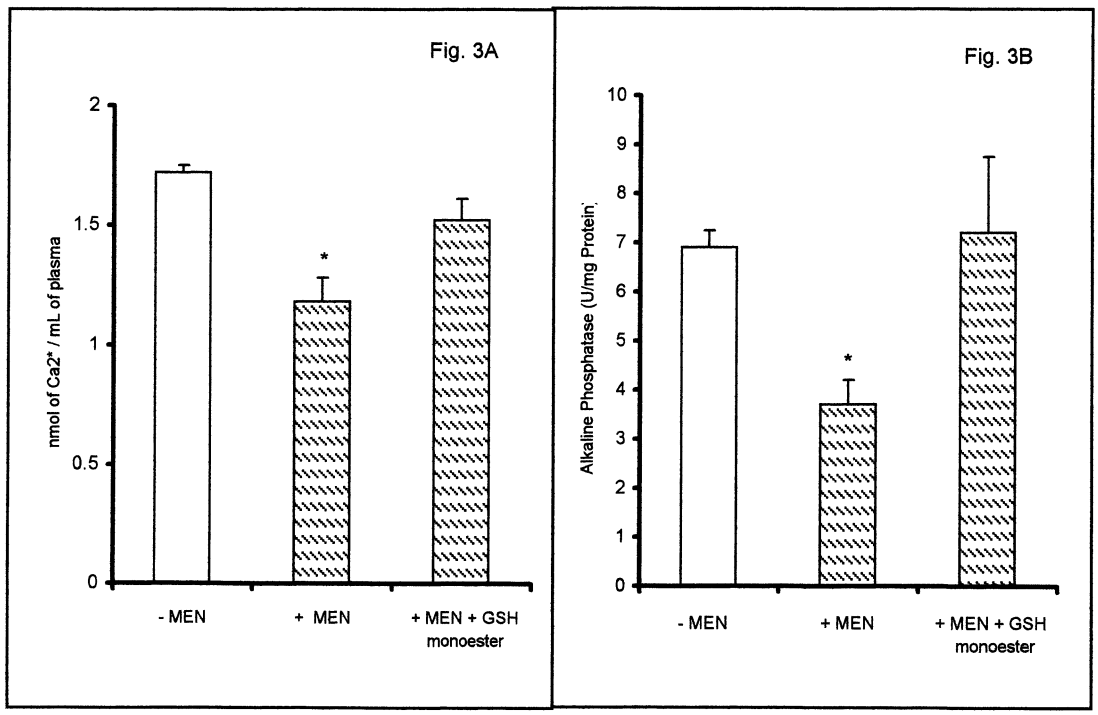

Fig. 3. A Reversibility of MEN effect on Ca^{2+} transfer from lumen-to plasma and B on IAP activity. Chicks were injected i.p. with 2.5 µmol MEN/kg of b.w. 1 h before sacrifice. Addition of 5 mol/L GSH monoester to the intestinal sac was done 30 min before killing. Values are means ± S.E. of samples from 3 to 6 animals for each treatment. * $P < 0.05$ for comparison with control and +MEN+GSH monoester.

Table 1
Effect of MEN administration on the enzyme activities of intestinal brush border and basolateral membranes

	Control	+MEN
	Brush border membrane enzyme activities	
Sucrase[a]	30.06 ± 5.32 (n=6)	28.21 ± 4.34 (n=6)
γ-Glutamyl transpeptidase[a]	26.23 ± 3.09 (n=3)	35.43 ± 8.38 (n=3)
	Basolateral membrane enzyme activities	
Ca-ATPase[b]	0.64 ± 1.11 (n=5)	0.34* ± 0.09 (n=5)
Na^+-ATPase[a]	55.64 ± 6.82 (n=3)	47.47 ± 7.58 (n=3)

The chicks were injected i.p. with 2.5 μmol MEN/kg of b.w. 30 min before sacrifice. Values are means ± S.E.

*$P < 0.05$ *vs.* control.

[a] Expressed as mU/mg of protein.

[b] Expressed as nmolCa^{2+}/mg of protein/min.

n 8, 1.24 ± 0.12 μmol GSH/g tissue vs. MEN treated: *n* 8, 0.96 ± 0.07* μmol GSH/g tissue, * $P < 0.05$ vs. control).

ESR spectra analyses indicate that in vitro addition of MEN to incubation medium of mature enterocytes generated a signal that was characteristic of hydroxyl radical groups (Fig. 4). This signal was obtained either 15 (data not shown) or 30 min after MEN (Fig. 4) addition into the incubation medium of enterocytes.

4. Discussion

The present study shows that a single large dose of MEN injected i.p. to normal chicks causes inhibition of Ca^{2+} movements from lumen-to-blood within 30 min. This response is accompanied by GSH depletion, which seems to be a prerequisite for cytotoxicity with pure redox cycling compounds [24].

The alteration in the intestinal Ca^{2+} absorption caused by MEN affects the Ca^{2+} transcellular pathway as indicated by the reduction in the activity of basolateral membrane Ca-ATPase, which is the main protein involved in the Ca^{2+} extrusion from the cell to the lamina propria. The activity of IAP was highly inhibited by MEN but with higher doses than the intestinal Ca^{2+} absorption and the inhibitory effect was reversed 9 hr after MEN treatment, response that did not occur with the Ca^{2+} transfer from lumen-to-blood. Although the exact role of IAP remains unknown, it has been proposed that IAP might regulate intestinal Ca^{2+} absorption [25]. On the contrary, the activity of γGT showed a tendency to increase by MEN treatment but it was not significantly altered, as Mikkelsen et al. demonstrated in carcinoma cells under different experimental conditions [26]. These effects of MEN suggest some degree of specificity in the action of MEN on proteins related to Ca^{2+} movements. However, the increment in the carbonyl content of proteins, which is a marker for protein oxidation, could not only be attributed to oxidation of those two minor membrane proteins, but also to other intestinal proteins.

No histological changes were detected by light microscopy in the intestine under the conditions of this study (not shown). Nevertheless, lesions in kidney, heart, liver and lung have been reported in rats being those lesions more severe after one dose of a single injection of MEN than five doses of multiple injection performed every other day [27]. The reason of this discrepancy could be due to the lower

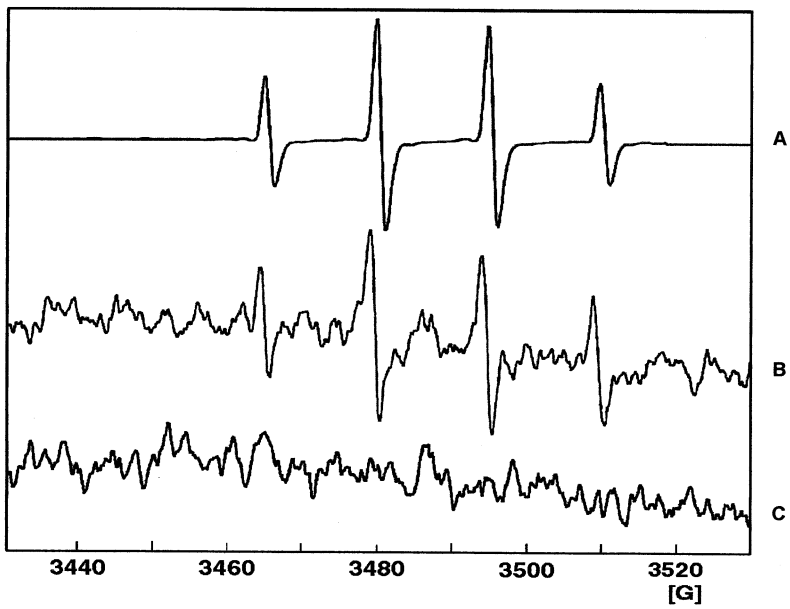

Fig. 4. A ESR spectrum of hydroxyl radicals generating from 10 mmol/L hydrogen peroxide in PBS buffer plus 10 mmol/L glucose pH 7.4 containing 0.1 M DMPO. B ESR spectrum of mature enterocytes (1×10^6 cells) incubated in the same buffer with 500 μmol/L MEN for 30 min and immediately treated with 0.1 mol/L DMPO. C Same as in B but in the absence de MEN. Spectrometer conditions were: microwave power, 20 mW; modulation frequency, 50 kHz; modulation amplitude, 0.5 G, time constant, 655.36 ms; 10 spectra were accumulated. $A_h = A_n = 15.0$ G.

concentrations of MEN and/or shorter times of administration that we have used in our experiments and/or the selective uptake of MEN and its GSH conjugates by different tissues [28].

Lately, it has been recognized that the metabolic aberrations caused by oxidants are mediated by either redox imbalance and/or regulation of gene expression [29]. The depletion of intestinal GSH content, the increment in the carbonyl groups from proteins and the appearance of free hydroxyl radicals found in our experiments indicate that MEN shifts the balance of oxidative and reductive processes in the enterocyte towards oxidation, causing deleterious consequences on the intestinal Ca^{2+} absorption. A link between oxidative stress and oxidative DNA damage with up-regulation of p53 gene and p21 gene expression has been reported [29]. Franco et al. [30] have also demonstrated that MEN increases the transcript levels of genes encoding the antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase in mouse myotube cultures. Although the reduction in the activity of alkaline phosphatase and Ca-ATPase could also be produced by regulation of gene expression by MEN, it is more likely that both proteins are inhibited by the attack of free hydroxyl radicals generated by MEN. Rohn et al. [31] have shown inhibition of Ca^{2+}-pump ATPase and Na^{+}/K^{+}-pump ATPase by iron-generated free radicals in red blood cell membranes. We have also previously found that administration of DL-buthionine-S,R-sulfoximine, an inhibitor of GSH synthesis, provokes a reduction in the activity of Ca^{2+} pump [7]. Recently, we have demonstrated that BSO depletes intestinal GSH content and inhibits the IAP activity; this effect was fully reversed by addition of GSH monoester into the intestinal lumen sac [8]. Similarly, MEN effect on IAP, which is dose and time dependent, has been reversed by replenishing of intestinal tripeptide after addition of GSH monoester to the intestinal lumen sac. However, the mechanism of action of these drugs is different; BSO depletes GSH by inhibition of γ-glutamylcystein synthetase, the key enzyme in the GSH synthesis [32], while MEN decreases GSH as a result of its redox cycling. During MEN metabolism in isolated rat hepatocytes, 75% of GSH has been found to be transformed in GSSG, 15% forms MEN-SG conjugates and 10% forms mixed disulfides with protein cysteinyl residues [33].

MEN and other quinones may undergo one or two-electron reduction. When MEN suffers one-electron reduction, there is formation of semiquinone radicals which are very unstable; they react rapidly with molecular oxygen resulting in regeneration of the parent compound and production of superoxide anions that yields H_2O_2 via enzymatic or spontaneous dismutation. Two-electron reduction of MEN by DT-diaphorase produces hydroquinone, a pathway that represents a detoxification mechanism [9]. In both cases, GSH is the electron donor, which explains, at least in part, the depletion of the tripeptide after MEN administration.

MEN is a synthetic hydrosoluble compound capable of inhibition of growth of a variety of animal and human tumor cell lines both in vivo and in vitro. Cell growth inhibition is achieved by induction of apoptosis or necrosis using low or excessive dose, respectively [11]. Clinical trial results are encouraging the use of MEN because of low toxicity, low multiple drug resistance independence and few side effects. Data from this paper indicate that one single large dose of MEN could produce undesirable effects on the intestinal function, mainly on calcium absorption and associated variables, such as the activity of IAP.

To conclude, a large single dose of MEN inhibits intestinal Ca^{2+} absorption by decreasing the activities of proteins presumably involved in the Ca^{2+} transport. This effect is related to the production of reactive oxygen species triggered by GSH depletion since addition of GSH monoester into the lumen, normalizes the intestinal GSH levels and prevents the effects of MEN.

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