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Effects of quercetin and menadione on intestinal calcium absorption and the underlying mechanisms

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ABSTRACT

Quercetin (QT) could be considered as a potential therapeutic agent for different diseases due to its antioxidant, anti-inflammatory, antiviral and anticancer properties. This study was designed to investigate the ability of QT to protect the chick intestine against menadione (MEN) induced injury *in vivo* and *in vitro*. Four-week old chicks (*Gallus gallus*) were treated i.p. with 2.5 μmol of MEN/kg b.w. or with i.l. 50 μM QT or both. QT protected the intestinal Ca^{2+} absorption against the inhibition caused by MEN, but QT alone did not modify. Glutathione (GSH) depletion provoked by MEN in chick enterocytes was abolished by QT treatment, whereas QT alone did not modify the intestinal GSH content. The enhancement of GSH peroxidase activity produced by MEN was blocked by QT treatment. In contrast, superoxide dismutase activity remained high after simultaneous treatment of enterocytes with MEN and QT. The flavonol also avoided changes in the mitochondrial membrane permeability (swelling) produced by MEN. The FasL/Fas/caspase-3 pathway was activated by MEN, effect that was abrogated by QT. In conclusion, QT may be useful in preventing inhibition of chick intestinal Ca^{2+} absorption caused by MEN or other substances that deplete GSH, by blocking the oxidative stress and the FasL/Fas/caspase-3 pathway activation.

1. Introduction

Quercetin (QT) is a flavonol ingested by man and animals with their regular diets and is considered beneficial for health (Mi et al., 2010) It is largely present in fruits, vegetables, aromatic plants, tea and red wine (Rice-Evans, 2001). QT exhibits various biological effects such as antioxidant, anti-inflammatory, antiviral and anticancer activities (Suzuki and Hara, 2009). These biological properties have made that QT could be considered as a potential therapeutic agent for different diseases, including cancer, viral infection, inflammation/allergy, hypertension and atherosclerosis (Park et al., 2003). QT promotes apoptosis of tumor cells, in part through depression of the heat shock protein 70, but may inhibit apoptosis in some nontumorigenic cells, such as in H_2O_2-induced apoptosis of mesangial cells, fibroblasts and epithelial cells (Ishikawa and Kitamura, 2000).

The gastrointestinal tract (GIT) is the first target after oral ingestion of QT (Trischitta and Faggio, 2006). There is not much information about the intraluminal concentration of QT in the GIT. It is assumed that its glycosides reach the intestine, where they are hydrolyzed by the enteric microflora, and then, the aglycone is conjugated intracellularly and released into the blood (Graf et al., 2006; Barnes et al., 2011). Natsume et al. (2009a) have observed that a low dose of QT produces changes in the gene expression profile of the mouse intestine such as an up-regulation of glutathione-S-transferases. The same group has also shown that QT suppresses the endoplasmic reticulum stress caused by calcium dynamics dysregulation by the inhibition of PI3K (Natsume et al., 2009b). The anti-inflammatory effects of QT on murine intestinal epithelial cells have been demonstrated to occur through mechanisms that inhibit cofactor recruitment at the chromatin of proinflammatory genes (Ruiz et al., 2007). The modulation of transport properties of intestine by QT is also possible as judged by the enhancement of the barrier function caused by the flavonol in the Caco-2 cell line via an increase in claudin-4 expression and in the assembly of zonnula occludens-2, occludin and claudin-1 (Amasheh et al., 2008; Suzuki and Hara, 2009). Barrenetxe et al. (2006) have shown that QT alters the intestinal enzymatic activity and nutrient uptake in healthy mice without impairing normal development, serum biochemical parameters or body weight. Sucrose and maltase activities were decreased by QT, whereas the activities of aminopeptidase N and alkaline phosphatase were increased. Recently, it has been observed that QT attenuates fasting and postprandial hyperglycemia in animal models of *Diabetes mellitus* by enhancing insulin sensitivity via α-glucosidase inhibition and enhanced insulin signaling (Kim et al.,

Abbreviations: QT, Quercetin; GIT, Gastrointestinal tract; MEN, Menadione; GSH, Glutathione; intraperitoneal, i.p.; intraluminal, i.l.; body weight, b.w.; Cu-Zn-SOD, Superoxide dismutase; GPX, Glutathione peroxidase; EDTA, Ethylendiaminetetracetic acid; NBT, Nitro blue tetrazolium; O_2^-, Superoxide anion.

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2011). It has been also suggested that QT may improve Ca^{2+} absorption from epithelia considering that QT is a weak activator of the vitamin D receptor and stimulates TRPV6 gene expression in Caco-2 cells (Inoue et al., 2010).

Optimal calcium absorption is an important function of the intestine not only to maintain Ca^{2+} homeostasis, but also for proper mineralization of bone in the prevention of osteoporosis and osteoporotic fractures (Kumari et al., 2010). We have widely reported that normal intracellular glutathione (GSH) levels are essential to maintain a proper intestinal Ca^{2+} absorption (Tolosa de Talamoni et al., 1996; Marchionatti et al., 2001). We have shown that menadione (MEN) or vitamin K_3, which is used in the anticancer therapy and in the treatment of osteoporosis (Hattori et al., 2001; Graciani and Ximenes, 2012), inhibits transiently the intestinal Ca^{2+} absorption in normal chicks. This inhibitory effect is caused, at least in part, by GSH depletion leading to oxidative stress, mitochondrial dysfunction and apoptosis via the intrinsic pathway (Marchionatti et al., 2003; Marchionatti et al., 2008). Probably, MEN affects essential thiol groups or other groups from proteins involved in the intestinal transcellular Ca^{2+} pathway by enhanced ROS production, which was previously demonstrated (Marchionatti et al., 2003). Whether intestinal apoptosis via the extrinsic pathway is induced by MEN in intestine has not been investigated. Although enzymes of the antioxidant system enhance their activities to attenuate the oxidant effects of MEN, the transcellular Ca^{2+} movement is reduced affecting the global process of intestinal Ca^{2+} absorption (Marchionatti et al., 2008).

The aim of this study was to investigate the ability of QT to protect the chick intestine against MEN-induced injury *in vivo* and *in vitro* and to explore whether the modulation of the FasL/Fas/caspase-3 signalling pathway is involved. In addition, the total GSH content and the enzyme activities of the antioxidant system were also investigated in chick enterocytes treated with MEN, QT or both.

2. Experimental methods

2.1. Animals

One-day-old Cobb Harding chicks (*Gallus gallus domesticus*) were obtained from Indacor S.A. (Rio Ceballos, Cordoba, Argentina) and were fed a commercial normal avian diet (Cargill, S.A.C.I., Pilar, Provincia de Cordoba, Argentina). At 4 weeks of age, they were killed by cervical dislocation and the excised duodenae were rinsed with cold 0.15 M NaCl and enterocytes or intestinal mitochondria were isolated as described below. The protocols were 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.

2.2. Chemicals

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. MEN and QT were always diluted in 0.15 M NaCl.

2.3. Intestinal Ca^{2+}absorption

Four week old chicks were divided into four groups: 1) normal chicks injected i.p. with 0.15 M NaCl for 30 min (controls), 2) normal chicks treated i.p. with 2.5 μmol of MEN/kg of b.w. for 30 min, 3) normal chicks treated i.l. with 1 mL of 50 μM QT for 30 min, and 4) normal chicks treated i.p. with 2.5 μmol of MEN/kg of b.w. simultaneously with 1 mL i.l. of 50 μM QT for 30 min. Chicks were laparotomized under anesthesia and a 10 cm segment of duodenum was ligated following the technique previously described (Tolosa de Talamoni et al., 1996). One mL 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. After half an hour, blood was withdrawn by cardiac puncture, centrifuged and the plasma ^{45}Ca was measured in a liquid scintillation counter. Absorption was defined as appearance of $^{45}Ca^{2+}$ in the blood.

2.4. Duodenal villus tip cell isolation

Mature enterocytes were isolated as previously described (Centeno et al., 2004). Cellular viability was assayed by the Trypan blue exclusion technique. Enterocytes were divided into four groups: 1) controls, 2) treated with 500 μM MEN, 3) treated with 50 μM QT, and 4) treated simultaneously with 500 μM MEN and 50 μM QT. Incubation time was 30 min. Protein was determined by the method of Gornall et al. (1949).

2.5. Mitochondrial isolation

Mitochondria were isolated from intestinal mucosa of the four groups of animals by differential centrifugation as previously described (Tolosa de Talamoni et al., 1985).

2.6. Determination of GSH content

Total GSH content was assayed in supernates from homogenates of enterocytes. The determinations were carried out by the glutathione disulfide reductase-5,5′-dithiobis (2-nitrobenzoate) recycling procedure (Anderson, 1985).

2.7. Enzyme assays

Superoxide dismutase (Cu-Zn-SOD, EC 1.15.1.1), and glutathione peroxidase (GPX, EC 1.11.1.9) activities were performed in diluted aliquots of the supernates from homogenates of enterocytes. Cu-Zn-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. Cu-Zn-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 (Beauchamp and Fridovich, 1973). GPX activity was determined in 50 mM potassium phosphate buffer pH 7.8, 1 mM EDTA, 1 mM NaN_3, 1 mM GSH and 1 U GSH reductase. The activity was measured by following NADPH oxidation after addition of 1 mM NADPH (Cheng et al., 1999). Caspase-3 measurements were also accomplished in supernatants 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) (Garcia-Calvo et al., 1998).

2.8. Western blots

Fas and FasL expressions were analyzed by Western blot procedure using supernates from homogenates of control and treated enterocytes. Homogenate suspensions were done in RIPA lysis buffer (1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate in PBS, containing 1 mM PMSF and 1 mM 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 (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 h with 2% w/v nonfat dry milk in 0.5 M Tris-buffered saline solution and incubated overnight at 4 °C with the following primary antibodies: mouse anti-CD95 monoclonal antibody (BD Pharmigen Biosciences, San José, CA, USA) at 1:1000 dilution or rabbit anti-FasL polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:1000 dilution. After three washings, appropriate biotinylated secondary antibodies were incubated at room temperature for 1 h. Then, the blots were washed three times and streptavidin–biotin 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 other proteins. The band intensities were quantified using an