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Sodium deoxycholate inhibits chick duodenal calcium absorption through oxidative stress and apoptosis

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ABSTRACT

High concentrations of sodium deoxycholate (NaDOC) produce toxic effects. This study explores the effect of a single high concentration of NaDOC on the intestinal Ca^{2+} absorption and the underlying mechanisms. Chicks were divided into two groups: 1) controls and 2) treated with different concentrations of NaDOC in the duodenal loop for variable times. Intestinal Ca^{2+} absorption was measured as well as the gene and protein expressions of molecules involved in the Ca^{2+} transcellular pathway. NaDOC inhibited the intestinal Ca^{2+} absorption, which was concentration dependent. Ca^{2+}-ATPase mRNA decreased by the bile salt and the same occurred with the protein expression of Ca^{2+}-ATPase, calbindin $D_{28\,k}$ and Na^{+}/Ca^{2+} exchanger. NaDOC produced oxidative stress as judged by ROS generation, mitochondrial swelling and glutathione depletion. Furthermore, the antioxidant quercetin blocked the inhibitory effect of NaDOC on the intestinal Ca^{2+} absorption. Apoptosis was also triggered by the bile salt, as indicated by the TUNEL staining and the cytochrome c release from the mitochondria. As a compensatory mechanism, enzyme activities of the antioxidant system were all increased. In conclusion, a single high concentration of NaDOC inhibits intestinal Ca^{2+} absorption through downregulation of proteins involved in the transcellular pathway, as a consequence of oxidative stress and mitochondria mediated apoptosis.

1. Introduction

Bile is essential for lipid digestion and absorption. Bile acids (BAs) are the major components. Primary BAs are synthesized from cholesterol in liver and transferred to the intestine, where they are transformed in secondary BAs by enzymes of the intestinal flora. Most of BAs are efficiently reabsorbed in the distal part of the small intestine and reach the liver through portal blood, where they are avidly taken up by hepatocytes. Deoxycholic acid or its salt, sodium deoxycholate (NaDOC), is the major secondary BA in humans. High concentrations of NaDOC cause liver damage during cholestasis and act as promoters of colon cancer in experimental animals (Lamireau et al., 2003). Oxidative stress triggered by NaDOC produces mitochondria and endoplasmic reticulum alterations, which could lead to apoptosis (Payne et al., 2005). There is some evidence that NaDOC may act as a carcinogen not only in human colon, but also in other segments of the gastrointestinal (GI) tract (Burnat et al., 2010; Bernstein et al., 2011).

A variety of mechanisms are believed to contribute to the deleterious effects of NaDOC on the GI tract. NaDOC was found to perturb membrane structures by alteration of membrane microdomains (Jean-Louis et al., 2006). Activation of protein kinase C and stimulation of prostaglandin E2 production are also involved in the procarcinogenic effects of NaDOC in the colon. Miyaki et al. (2009) have recently found that both chenodeoxycholate and NaDOC appear to increase the release of prostaglandin E2 from colon cancer cells by downregulating catabolism in addition to stimulating synthesis. NaDOC was also found to decrease the transepithelial electrical resistance in the Caco-2 cell line through ROS generation and other signaling mechanisms, which indicates that the tight junctions constitute another target of the bile salt on the intestinal tissue (Araki et al., 2005). NaDOC induces apoptosis in the same cell line as well as in other human colon cancer cell lines (Wachs et al., 2005). In normal colonocytes, a low dose of NaDOC induces cellular proliferation through increased expression of cyclin A and a high dose induces decreased expression of cyclin E and CDK2 (homeobox transcription factor) causing suppression of cell proliferation (Ha and

Abbreviations: BAs, bile acids; BBM, brush border membrane; BLM, basolateral membrane; CAT, catalase; CB, calbindin D_{28k}; DAB, 3,3′-diaminobenzidine; ESR, electron spin resonance; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GI, gastrointestinal; GPx, glutathione peroxidase; GSH, glutathione; GSSG, oxidized glutathione; NaDOC, sodium deoxycholate; NBT, nitro blue tetrazolium; NCX1, Na^{+}/Ca^{2+} exchanger; NFkB, nuclear factor kappa B; O_2^-, superoxide anion; $PMCA_{1b}$, plasma membrane Ca^{2+}-ATPase; QT, quercetin; SOD, superoxide dismutase.

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Park, 2010). NaDOC and other unconjugated bile salts induce breakdown of the junctional integrity in jejunal mucosa leading to exfoliation of the epithelium (Oumi and Yamamoto, 2000). It has also been demonstrated that NaDOC inhibits intestinal transport such as the sodium-coupled glucose transport (Guiraldes et al., 1975), and reverses absorption of Na^+, Cl^- and H_2O to secretion in the distal colon (Mauricio et al., 2000).

The intestinal Ca^{2+} absorption is an important process to control calcium homeostasis. Ca^{2+} ions are absorbed mainly in the small intestine, which is responsible for about 90% of overall Ca^{2+} absorption (Wasserman, 2004). The intestinal Ca^{2+} absorption occurs by two mechanisms: paracellular (passive) and transcellular (active) pathways. The last one comprises 3 steps: 1) Ca^{2+} entrance to the brush border membrane (BBM) through the epithelial Ca^{2+} channels TRPV6 and TRPV5, 2) cation movement from the BBM to the basolateral membrane (BLM), which is mediated by binding to the calcium binding protein calbindin D_{28k} (CB) in avian or calbindin D_{9k} in mammals, and 3) Ca^{2+} exit through the BLM via the plasma membrane Ca^{2+}-ATPase ($PMCA_{1b}$) and/or the Na^+/Ca^{2+} exchanger (NCX1) (Pérez et al., 2008). In our laboratory we have demonstrated that some of these molecules are sensitive to the oxidative stress leading, consequently, to the inhibition of intestinal Ca^{2+} absorption (Tolosa de Talamoni et al., 1996; Marchionatti et al., 2003, 2008). Although it is well known that hydrophobic BAs produce cellular stress, there is very little information about the effect of BAs on intestinal Ca^{2+} absorption and much less about the role of NaDOC, which is present in millimolar concentration in the fecal water of the human intestine (Mauricio et al., 2000).

Based upon previous considerations, our hypothesis was that high concentrations of NaDOC in the intestine could alter the intestinal Ca^{2+} absorption by triggering oxidative stress that might cause apoptosis of intestinal epithelial cells resulting in a decrease of the capacity to transport Ca^{2+}. Thus, the aim of this study was to know the molecular mechanisms triggered by a single high concentration of NaDOC in chick duodenal loop, the site where the most potent active Ca^{2+} absorptive mechanism has been demonstrated.

2. Material and methods

2.1. Animals

One-day-old Cobb Harding chicks (*Gallus gallus domesticus*) were obtained from Indacor S.A. (Rio Ceballos, Córdoba, Argentina) and were fed a commercial normal avian diet (Cargill, S.A.C.I., Pilar, Córdoba, Argentina). At 4 weeks of age, they were divided into two groups: a) normal chicks (controls), and b) normal chicks treated with NaDOC (Sigma-Aldrich, St. Louis, MO, USA) in the intestinal lumen at different concentrations and times, as indicated in the Results section. Chicks were laparotomized under anesthesia and a 10 cm segment of duodenum was ligated as previously described (Tolosa de Talamoni et al., 1996). One milliliter of vehicle, phosphate buffer saline (PBS), or NaDOC was introduced with a syringe in the distal portion of the sac and the loop was reintroduced in the abdomen for the time of experiment. 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. They were killed by cervical dislocation and the excised duodenae were rinsed with cold 0.15 mol/L NaCl. Samples were obtained for: 1) histological TUNEL assay, 2) isolation of enterocytes, 3) purification of mitochondria, 4) enzymatic activity measurements and immunoblotting, and 5) gene and protein expression determinations.

2.2. Intestinal Ca^{2+} absorption

After injecting NaDOC or vehicle in the duodenal lumen for a period of time (15, 30 or 60 min) and rinsing the intestinal segment with saline solution, 1 mL of 150 mmol/L NaCl, 1 mmol/L $CaCl_2$, containing 1.85×10^5 Bq $^{45}Ca^{2+}$, pH 7.2, was introduced into the lumen of the ligated intestinal segment for 30 min. Then, 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 (Tolosa de Talamoni et al., 1996). Similar experiments were done by injecting NaDOC plus 50 μmol/L quercetin (QT) or QT alone. QT was a gift from Dr. A. Pacciaroni, Departamento de Química Orgánica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Córdoba, Argentina.

2.3. Spectrophotometric procedures

All the enzymes activities were assayed in supernatants of duodenal mucosa homogenates dissolved with 50 mmol/L phosphate buffer pH 7.5 and 0.1 mmol/L EDTA. 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 supernatants of duodenal mucosa homogenates (1:5). Mg^{2+}-SOD activity was determined in 1 μmol/L EDTA, 50 mmol/L potassium phosphate buffer, pH 7.8, 13 mmol/L methionine, 75 μmol/L nitro blue tetrazolium (NBT) and 40 μmol/L riboflavin (Beauchamp and Fridovich, 1973). CAT activity was assayed in 50 mmol/L potassium phosphate buffer pH 7.4 and 0.3 mol/L H_2O_2 (Aebi, 1974). GPx activity was determined in 50 mmol/L potassium phosphate buffer pH 7.8, 1 mmol/L EDTA, 1 mmol/L NaN_3, 1 mmol/L glutathione (GSH) and 1 U GSH reductase (Cheng et al., 1999). Total GSH content was also assayed in supernatants from duodenal mucosa homogenates performed in 5% w/v 5-sulfosalicylic acid (1:5) (Anderson, 1985).

2.4. Determination of free hydroxyl radicals

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.

A spin trapping technique was used in order to identify hydroxyl groups from villus tip cells previously exposed to 10 mmol/L NaDOC for 30 min (Chamulitrat, 1999). The incubation was accomplished at 37 °C under gentle shaking. The spin trap 5,5-dimethyl-1-pyrroline-1-oxide was employed and the electron spin resonance (ESR) spectra were measured at room temperature by an ESR spectrometer system Bruker ECS 106, cavity ER 4102 ST, Germany.

2.5. Changes in mitochondrial membrane permeability (swelling)

Mitochondria isolation: mitochondria were isolated from intestinal mucosa of both groups of animals by differential centrifugation, as previously reported (Tolosa de Talamoni et al., 1985).

Isolated intestinal mitochondria (3 mg protein) were incubated in 3 mL of respiratory buffer (0.1 mol/L NaCl, 10 mmol/L MOPS, 1 mmol/L glutamate, 1 mmol/L 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 mitochondrial absorbance were measured for 5 min and the optical density was followed for 5 more min, after addition of increasing concentrations of NaDOC (0.1–1.0 mmol/L) (Rodrigues et al., 1998).

2.6. TUNEL assay

Intestines of chicks were fixed in 4% paraformaldehyde in 10 mM 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 (TUNEL) procedure 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