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Naringin prevents the inhibition of intestinal Ca^{2+} absorption induced by a fructose rich diet

V. Rodríguez[1], M. Rivoira[1], S. Guizzardi, N. Tolosa de Talamoni*

Laboratorio "Dr. Cañas", Cátedra de Bioquímica y Biología Molecular, Facultad de Ciencias Médicas, INICSA (CONICET-Universidad Nacional de Córdoba), Córdoba, Argentina

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ABSTRACT

This study tries to elucidate the mechanisms by which fructose rich diets (FRD) inhibit the rat intestinal Ca^{2+} absorption, and determine if any or all underlying alterations are prevented by naringin (NAR). Male rats were divided into: 1) controls, 2) treated with FRD, 3) treated with FRD and NAR. The intestinal Ca^{2+} absorption and proteins of the transcellular and paracellular Ca^{2+} pathways were measured. Oxidative/nitrosative stress and inflammation parameters were evaluated. FRD rats showed inhibition of the intestinal Ca^{2+} absorption and decrease in the protein expression of molecules of both Ca^{2+} pathways, which were blocked by NAR. FRD rats showed an increase in the superoxide anion, a decrease in the glutathione and in the enzymatic activities of the antioxidant system, as well as an increase in the NO content and in the nitrotyrosine content of proteins. They also exhibited an increase in both IL-6 and nuclear NF-κB. All these changes were prevented by NAR. In conclusion, FRD inhibit both pathways of the intestinal Ca^{2+} absorption due to the oxidative/nitrosative stress and inflammation. Since NAR prevents the oxidative/nitrosative stress and inflammation, it might be a drug to avoid alteration in the intestinal Ca^{2+} absorption caused by FRD.

1. Introduction

The intestinal Ca^{2+} absorption is an active process that mainly occurs in the small intestine [1]. To obtain an optimal Ca^{2+} absorption, it is necessary to maintain the proper intestinal redox state [2]. The depletion in the intestinal glutathione (GSH) content is associated with a reduction in the Ca^{2+} transport [3], as a consequence of developing oxidative stress, which leads to apoptosis of epithelial cells [4]. Ca^{2+}enters the organism crossing the enterocytes (transcellular pathway) or the intercellular spaces (paracellular pathway). The transcellular pathway involves Ca^{2+} entry through the brush border membranes (BBM or apical border), a process in which the epithelial Ca^{2+} channels such as TRPV6 and TRPV5 participate; movement from one pole to the other of the cells, which is facilitated by the calbindin D_{9k} (CB D_{9K}) and the exit across the basolateral membranes (BLM), where the plasma membrane Ca^{2+}-ATPase and the Na^{+}/Ca^{2+} exchanger are located to pump Ca^{2+} out against the electrochemical gradient [5,6]. The paracellular Ca^{2+} movement occurs through the tight junctions, and presumably involves proteins such as Cldn-2 and Cldn-12 [7].

There is considerable evidence that fructose rich diet (FRD) causes adverse metabolic perturbations. Many studies have demonstrated that FRD to normal rats induces several features of the metabolic syndrome [8,9]. In addition, it has been found that FRD inhibits intestinal Ca^{2+} absorption and induces vitamin D insufficiency [10,11]. The authors claim that the inhibitory effect of fructose on intestinal Ca^{2+} absorption is specific because the sugar has no affect the transepithelial Pi transport and transapical glucose uptake. The underlying mechanisms of the inhibitory effect of fructose on the intestinal Ca^{2+} absorption are not completely elucidated.

Since FRD is known to cause oxidative stress and inflammation in different tissues [12–14], it is quite possible that the inhibitory effect of FRD on intestinal Ca^{2+} absorption involves exacerbation of ROS and inflammatory cytokines, which could alter the functioning of molecules that participate in the intestinal Ca^{2+} transport. If so, the use of antioxidants could block or avoid, at least in part, the inhibitory action of FRD on intestinal Ca^{2+} transport. Flavonoids are considered very potent natural antioxidants. They are polyphenolic compounds ubiquitously found in plants with positive effects against diverse pathologies such as cancer, neurodegenerative or cardiovascular disease [15]. Among them, naringin (full name, naringenin-7-O-neohesperidin glycoside, NAR) is a flavanone that facilitates the removal of free radicals,

* Corresponding author. Cátedra de Bioquímica y Biología Molecular, Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Pabellón Argentina, 2do. Piso, Ciudad Universitaria, 5000 Córdoba, Argentina.
E-mail addresses: ntolosatalamoni@yahoo.com.ar, ntolosa@biomed.fcm.unc.edu.ar (N. Tolosa de Talamoni).
[1] Have equally contributed.

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Abbreviations

AP	alkaline phosphatase
BMI	body mass index
CAT	catalase
CB D_{9K}	calbindin D_{9k}
CLDN 2	claudin 2
CLDN 12	claudin 12
DAB	3,3′-diaminobenzidine
ECLIA	electro-chemiluminescence
FRD	fructose rich diet
GSH	glutathione
HOMA-IR	homeostasis model assessment
IL-6	interleukin 6
NAR	naringin
NBT	nitro blue tetrazolium
$NO^{\bullet}$	nitric oxide
$^{\bullet}O_2^-$	superoxide anion
$PMCA_{1b}$	Ca^{2+}-ATPase
RIA	radioimmunoassay
RIPA	radio immuno precipitation assay buffer
SOD	superoxide dismutase
TG	triglycerides
TRPV6	transient receptor potential cation channel V6
VDR	vitamin D receptor

oxidative stress and inflammation [16]. It is widely distributed in grapefruit and other citrus as well as in Chinese herbal medicines such as *Drynaria fortunei* [17], and it has interesting biological and pharmacological actions due to its antioxidant, antiapoptotic and anti-inflammatory properties [18].

Based upon previous considerations, the aim of this work was to clarify the mechanisms by which FRD causes inhibition of intestinal Ca^{2+} absorption in experimental animals, and determine if any or all underlying alterations are prevented by NAR administration.

2. Material and methods

2.1. Chemicals

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

2.2. Animals and experimental design

After weaning, male Wistar rats (150–200 g) were fed a commercial normal rodent diet (GEPSA mouse-rat, Pilar, Buenos Aires, Argentina) under standard conditions of humidity, temperature (20-25 °C) and light (12 h light/12 h dark). They had access to water and food *ad libitum* (control group). At two-months old, the animals were divided into three groups: a) controls, b) FRD: the same diet plus 10% fructose (Anedra, Research AG S.A., Buenos Aires, Argentina) in the drinking water during 30 days, c) FRD + NAR40: three days after fructose treatment, a group of rats received daily NAR (40 mg/kg b.w.) therapy *via* subcutaneous injection (Fig. 1). After 30 days of treatment and an overnight fast, they were weighed and killed by cervical dislocation. The excised duodena were rinsed with cold 0.15 M NaCl and mucosa or enterocytes were isolated, as described below. In some experiments other NAR doses were utilized (see Results).

The studies were conducted according to the Guide for Care and Use of Laboratory Animals. The protocol was approved by the CICUAL (Res. 07/15, Commission for Care and Use of Laboratory Animals, Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Córdoba, Argentina. All efforts were made to minimize the number of animals used and their suffering.

2.3. Serum measurements

Blood samples from rats were used for serum biochemical determinations. Serum glucose (Glicemia enzimática AA), triglycerides (TG Color, GPO/PAP AA), cholesterol (Colestat enzimático AA), HDL (HDL Cholesterol- Precipitating Reagent), LDL (LDL-Cholesterol, monophase AA), Ca (Ca-Color AA), P (Fosfatemia UV-AA) were determined using kits from Wiener Laboratorios S.A.I.C. (Rosario, Argentina) following the manufacturer's protocol. Serum insulin was measured by radioimmunoassay (RIA) using an anti-rat insulin antibody (Sigma, St. Louis, Missouri, USA); the minimum detectable concentration was 0.04 ng/mL 25(OH)D_3 (Vitamin D total, Roche Diagnostics) was measured by electro-chemiluminescence (ECLIA) immunoassay (Modular Analytics E1701, Roche, Mannheim, Germany) and IL-6 by ELISA (Mouse IL-6 ELISA Set, BD OptEIA, San Diego, CA, USA) according to manufacturer's operating protocol.

The insulin resistance was evaluated by the homeostasis model assessment (HOMA-IR) as follows: HOMA-IR = serum insulin (μIU/mL) × fasting blood glucose (mM)/22.5 [19].

2.4. Intestinal calcium absorption

Animals from different groups were anesthetized with an intramuscular injection of ketamine (50 mg/kg b.w.) and xylazine (10 mg/kg b.w), were laparotomized and a 10 cm segment of duodenum was ligated. One milliliter of 150 mM NaCl, 1 mM $CaCl_2$, plus 1.85×10^5 Bq $^{45}Ca^{2+}$, pH 7.2, was introduced into the lumen of the ligated intestinal segment. After 10 min, 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 [2].

2.5. Duodenal cell isolation

Duodenum epithelial cells were isolated as previously described [21]. Villus tip cells were collected by centrifugation at 500×*g* for 5 min, and then resuspended in an incubation medium (140 mM NaCl, 5 mM KCl, 20 mM HEPES, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 10 mM glucose, pH 7.4). Alkaline phosphatase (AP, EC 3.1.3.1.) activity was performed as a marker enzyme of cell maturation [20]. Cell viability was assessed by the Trypan blue exclusion technique. Only mature cells were used for the different experiments.

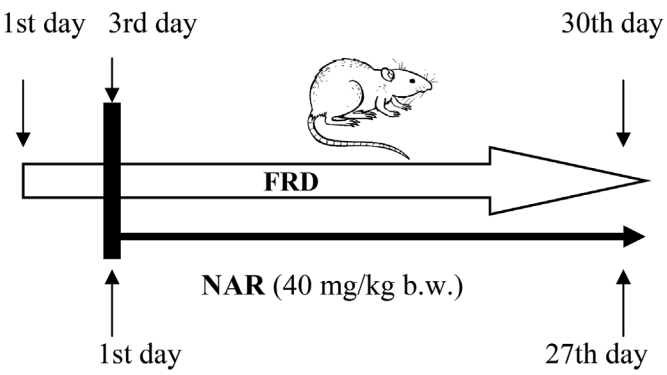

Fig. 1. Schematic diagram of treatment protocol. FRD: fructose-rich diet; NAR: naringin.