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Time dependent changes in the intestinal Ca^{2+} absorption in rats with type I diabetes mellitus are associated with alterations in the intestinal redox state

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ABSTRACT

The aim was to determine the intestinal Ca^{2+} absorption in type I diabetic rats after different times of STZ induction, as well as the gene and protein expression of molecules involved in both the transcellular and paracellular Ca^{2+} pathways. The redox state and the antioxidant enzymes of the enterocytes were also evaluated in duodenum from either diabetic or insulin-treated diabetic rats as compared to control rats. Male Wistar rats (150–200 g) were divided into two groups: 1) controls and 2) STZ-induced diabetic rats (60 mg/kg b.w.). A group of diabetic rats received insulin for five days. The insulin was adjusted daily to maintain a normal blood glucose level. Five 5 d after STZ injection, there was a reduction in the intestinal Ca^{2+} absorption, which was maintained for 30 d and disappeared at 60 d. Similar changes occurred in the GSH and $\dot{O}_2^-$ levels. The protein expression of molecules involved in the transcellular pathway increased at 5 and 30 d returning to control values at 60 d. Their mRNA levels declined considerably at 60 d. The gene and protein expression of claudin 2 was upregulated at 30 d. Catalase activity increased at 5 and 30 d normalizing at 60 d. To conclude, type I D.m. inhibits the intestinal Ca^{2+} absorption, which is transient leading to a time dependent adaptation and returning the absorptive process to normal values. The inhibition is accompanied by oxidative stress. When insulin is administered, the duodenal redox state returns to control values and the intestinal Ca^{2+} absorption normalizes.

1. Introduction

There is a great body of evidence that human type I Diabetes mellitus (D.m.) is associated with alterations in Ca^{2+} homeostasis resulting in hypercalciuria and reduced bone mass or osteopenia [1]. Hypercalciuria can be rectified by insulin, but only partially if hormone treatment is delayed, an indication that some irreversible changes might occur at early stages of diabetes [2]. The diabetic hypercalciuria in rats involves enhanced glomerular filtration rate with raised urinary output, reduced Ca^{2+} reabsorption, and impaired bone deposition. A reduction of extracellular Ca^{2+}-sensing receptor without alterations in other Ca^{2+} transport molecules were shown throughout the whole kidney section in diabetic rats [1]. However, Western blot analysis has shown that the protein expression of plasma membrane Ca^{2+}-ATPase ($PMCA_{1b}$) and vitamin D receptor (VDR) was significantly decreased in kidneys from streptozotocin (STZ)-treated mice compared to that of controls [3]. With regard to bone, diabetic patients not only show osteopenia [4], but also increased risk of fractures [5,6] and delayed fracture healing [7]. The adverse effects of D.m. on bone have been attributed to insulinopenia, microangiopathy, and alterations in local factors regulating bone remodeling [8]. Serum calcium has been observed to be decreased [9], normal [10] or increased [11] in type I D.m. In other words, Ca^{2+} homeostasis in the type I D.m. is quite controversial and needs to be clarified.

The intestine is another important organ involved not only in the maintenance of Ca^{2+} homeostasis, but also in the proper mineralization of bone preventing osteoporosis and osteoporotic fractures [12]. Earlier studies have shown that diabetic patients have a normal [13], low [14] or high [15] intestinal Ca^{2+} absorption. Hough et al. [11] have found differences in the intestinal Ca^{2+} absorption and in hormonal response in diabetic rats depending on the duration of diabetes (chronic versus short). Since nutritional factors also alter the intestinal Ca^{2+} absorption [16], the variability in the data on the cation transport in diabetic patients or animals might be also due to differences in the diet composition.

Abbreviations: GSH, glutathione; AP, alkaline phosphatase; CAT, catalase; SOD, superoxide dismutase; cldn2, claudin 2; D.m, Diabetes mellitus; $PMCA_{1b}$, Ca^{2+}-ATPase; VDR, vitamin D receptor; STZ, streptozotocin; TRPV6, transient receptor potential cation channel V6; NCX1, Na^+/Ca^{2+}exchanger; cldn 12, claudin 12; RIPA, radio immuno precipitation assay buffer; DAB, 3,3'-diaminobenzidine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

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Calcitriol or $1,25(OH)_2D_3$ is the main stimulus to increase the intestinal Ca^{2+} absorption either in mammals or in birds [17]. As serum levels of $1,25(OH)_2D_3$ have been proved to be decreased in diabetic humans and rats [18], the alteration in the intestinal Ca^{2+} absorption has been associated with vitamin D status [11]. The intestinal Ca^{2+} absorption occurs via paracellular and transcellular pathways. Apparently, calcitriol enhances both routes. The transcellular pathway involves molecules such as the transient receptor potential cation channel V6 (TRPV6), located at the apical side of enterocytes, calbindin D_{9k} in the cytoplasm, and $PMCA_{1b}$ and Na^+/Ca^{2+}-exchanger (NCX1), both proteins at the basolateral membranes [19]. The paracellular via is less known and, apparently, some proteins that form the tight junctions, such as claudin 2 (cldn 2) and claudin 12 (cldn 12), could be involved in Ca^{2+} movements [20]. There is a lack of information on the time dependent changes about the gene and protein expression of these molecules when the D.m. is developing.

The intactness of intestinal redox state is essential to have an optimal intestinal Ca^{2+} absorption. We have demonstrated that normal levels of intracellular glutathione (GSH) are important to maintain a proper intestinal Ca^{2+} absorption [21–23]. Although oxidative stress in the small intestine during diabetes has been reported in STZ-induced diabetic rats [24], there is no information about a possible relationship between the intestinal Ca^{2+} absorption and the oxidative damage and the antioxidant status of the intestine.

The aim of this study was to determine the intestinal Ca^{2+} absorption in STZ-induced diabetic rats (type I D.m.) after a short and long lasting period of induction, as well as the gene and protein expression of molecules involved in both the transcellular and paracelluar pathways of Ca^{2+} transport. In addition, the redox state and the antioxidant enzymatic system of the enterocytes were evaluated in the duodenum from either diabetic or insulin-treated diabetic rats as compared to control rats.

2. Materials and methods

2.1. Chemicals

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

2.2. Animals

Eight-week-old male Wistar rats (150–200 g) were maintained at 20–25 °C on a 12 h light–12 h dark cycle, with access to water and food ad libitum. Calcium content in the diet was 1 %, and vitamin D content was about 1000 IU/kg of diet (GEPSA mouse-rat, Pilar, Buenos Aires, Argentina). The animals were divided into two groups: 1) control rats, and 2) STZ-induced diabetic rats. Rats from the second group received a single intraperitoneal injection of STZ (60 mg/kg b.w. dissolved in 0.1 mol/L citrate, pH 4.5 solution), whereas the control rats were injected with vehicle alone. After 3 days of STZ injection, the blood glucose levels were measured by using a glucometer (AccuCheck; Roche, Germany). The animals were considered diabetic when their blood glucose values exceeded 250 mg/dL and glucose was detected in urine (Multistix, Siemens Medical Solutions Diagnostics, Malvern, USA). STZ-treated rats were sacrificed by cervical dislocation at 5, 30 or 60 days after induction. All experimental protocols followed the Guide for the Care and Use of Laboratory Animals from the Medicine School of the Universidad Nacional de Córdoba, Córdoba, Argentina. All efforts were made to minimize the number of animals used and their suffering.

2.3. Insulin-treated diabetic rats

Thirty days after STZ injection, a group of rats received daily insulin therapy (Insulina Glargina, Lantus, Sanofi-Aventis, Uruguay S.A.) via subcutaneous injection for five days until they were sacrificed. The insulin dosage was adjusted daily to maintain blood glucose level in the range of 100–200 mg/dL.

2.4. Serum biochemical determinations

Blood samples from rats were used for biochemical measurements. Serum glucose (Glicemia enzimática AA), Ca (Ca-Color AA), P (Fosfatemia UV-AA), and creatinine (Creatinina-enzimática AA) were determined using kits from Wiener Laboratorios S.A.I.C. (Rosario, Argentina), HbA_{1c} and insulin were determined by Glycohemoglobin Reagent (Teco Diagnostics, Anaheim, CA, USA) and Rat insulin ELISA (Millipore, Billerica, MA, USA) respectively, according to manufacturer's operating protocol. $1,25(OH)_2D_3$ was determined by RIA (DiaSorin, Saluggia, Italy) and $25(OH)D_3$ by ECLIA immunoassay (Modular Analytics E1701, Roche, Mannheim, Germany).

2.5. Intestinal Ca^{2+} absorption

Rats were anesthetized with an intramuscular injection of ketamine (50 mg/kg b.w.) and xylazine (10 mg/kg b.w.). One 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. After ten minutes, 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 [21].

2.6. Alkaline phosphatase activity assay

Alkaline phosphatase (AP), E.C. 3.1.3.1., was measured in water homogenates (1:10) of intestinal mucosa using p-nitrophenyl phosphate as substrate in 0.5 mol/L diethanolamine buffer pH 9.8. This was performed by following an adaptation of Walter and Schütt method [25]. Enzyme activities are expressed in IU/mg of protein.

2.7. RNA isolation and analysis of $pmca_{1b}$, ncx1, trpv6 and cldn 2 gene expression by qRT-PCR

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. Quantitative RT-PCR amplification was performed in a thermocycler (Quantitative PCR thermocycler Stratagene Mx 3000P, Agilent Technologies, Inc., Santa Clara, CA, USA). Amplification mixture (total volume: 25 μL) contained 0.5 μg RNA, 0.3 μmol/L each primer, 0.4 μL of the diluted reference dye, 1.0 μL of reverse transcriptase (RT)/RNase block enzyme mixture and 12.5 μL of 2× Brilliant IISYBR 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 [26]. The amount of copy numbers of mRNA from each gene was normalized relative to that of GAPDH. The primers sequences of the studied genes are listed in Table 1.

2.8. Western blot analysis

Immunoblotting analysis of $PMCA_{1b}$, NCX1, TRPV6, VDR and cldn 2 was performed in pools of mucosa from two rat duodenae each. Suspensions were done in RIPA (radio immuno precipitation assay buffer) lysis buffer (1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate in PBS, containing 1 PMSF and 1 mmol/L 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 cldn 2 and VDR and in