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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

Table 1
Sequences of specific primers used for RT-qPCR amplification.

Gene	Primer sequences (5'–3')	Size of the PCR products (bp)
ncx1	Forward: GTTGTGTTCGCTTGGGTTGC Reverse: CGTGGGAGTTGACTACTTTC	163
pmca$_{1b}$	Forward: CGCCATCTTCTGCACAATT Reverse: CAGCCATTGTTCTATTGAAAGTTC	109
trpv6	Forward: ATCCGCCGCTATGCAC Reverse: AGTTTTTCTGGTCACTGTTTTTGG	80
cldn 2	Forward: GCTGCTGAGGGTAGAATGA Reverse: GCTCGCTTGATAAGTGTCC	107
Gapdh	Forward: AGTCTACTGGCGTCTTCAC Reverse: TCATATTTCTCGTGGTTCAC	133

ncx1: Na^+/Ca^{2+} exchanger; *pmca$_{1b}$*: plasma membrane Ca^{2+}-ATPase; *trpv6*: transient receptor potential V6; *cldn 2*: claudin 2; *gapdh*: glyceraldehyde-3-phosphate dehydrogenase.

8% (w/v) SDS–polyacrylamide minigels for the other proteins [27]. Gels containing the separated proteins were immersed in the transfer buffer (25 mmol/L Tris–HCl, and 192 mmol/L glycine, 0.05% w/v SDS and 20% v/v methanol) [28]. Nitrocellulose membranes (0.45 μm) were blocked for 1.5 h with 2% w/v nonfat dry milk in 0.5 mol/L Tris-buffered saline solution and incubated overnight at 4 °C with the specific primary antibody at 1:1000 dilution in each case. The antibodies were: anti $PMCA_{1b}$ (human erythrocyte clone 5 F10 A7952 SIGMA Saint Louis, MO, USA), anti-NCX1 monoclonal antibody PPS019 (R&D Systems, Minneapolis, MN, USA), anti-TRPV6 polyclonal antibody (L-15: sc-31445 Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-VDR polyclonal antibody (C-20: sc-1008 Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-cldn 2 monoclonal antibody (Invitrogen, Carlsbad, CA, USA). After three washings, appropriate secondary biotinylated antibodies were incubated at room temperature for 1 h. Then, the blots were washed three times and streptavidin – biotin conjugate (Histostain-SP Broad Spectrum, Invitrogen CA, USA) was added. Detection was performed using 3,3'-diaminobenzidine (DAB) as a chromogen. Monoclonal antibody anti-GAPDH (clone GAPDH-71.1 Sigma–Aldrich, St. Louis, MO, USA), was used to detect GAPDH as a marker to normalize the relative expression of the other proteins. The band intensities were quantified using an Image Capturer EC3 Imaging System, LaunchVisionworksls software in order to obtain the relative expression of proteins.

2.9. Total GSH determination

Total GSH content was also determined in supernatants from intestinal homogenates using the glutathione disulfide reductase-5,5´-dithiobis (2-nitrobenzoate) recycling procedure, as described elsewhere [29]. The data are expressed in nmol/mg of protein.

2.10. Superoxide anion measurement

Mature enterocytes from the duodenal villus tip were isolated as previously described [30]. Cellular viability was assayed by the Trypan blue exclusion technique. Cells were washed twice with Hanks buffer (137 mmol/L NaCl, 5.4 mmol/L KCl, 0.25 mmol/L Na_2HPO_4, 0.44 mmol/L KH_2PO_4, 1.3 mmol/L $CaCl_2$, 1 mmol/L $MgSO_4$, 4.2 mmol/L $NaHCO_3$, 6.24 mmol/L glucose, pH 7.4) and incubated with nitro blue tetrazolium (NBT) (1 mg/mL) at 37 °C for 1 h. The formazan precipitates formed were dissolved in dimethylsulfoxide and quantified by spectrophotometry at 560 nm. OD values are direct indicators of $\dot{O}_2^-$ concentration in the samples [31].

2.11. Catalase and superoxide dismutase activities

All enzymes activities were assayed in supernatants of duodenal homogenates. Catalase (CAT), EC 1.11.1.6, and superoxide dismutase (Mg^{2+}-SOD), EC 1.15.1.1, activities were performed in diluted aliquots from the supernatants of intestinal homogenates (1:5). CAT activity was assayed in 50 mmol/L potassium phosphate buffer pH 7.4 and 0.3 mol/L H_2O_2 [32]. 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 NBT and 40 μmol/L riboflavin [33]. Enzyme activities are expressed in U/mg of protein.

2.12. Statistical analysis

All data are expressed as means ± SE. The results were evaluated by one-way analysis of variance (ANOVA) and the Bonferroni's test as a post hoc test. Differences were considered statistically significant at $p < 0.05$. All the analyses were carried out by using SPSS software (version 17.0) for Windows XP (SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. Characterization of the diabetic rats

Table 2 shows that the weight loss in rats injected with STZ occurred as early as 5 days after the injection. At all times studied, the diabetic rats exhibited lower body weight than the respective control rats. As expected, the body weight increased over time in control animals as well as in diabetic rats, but in less proportion in the latter group. Serum glucose levels were always significantly higher in diabetic rats than those from the control rats. Serum glucose remained unchanged in the control rats, whereas in the diabetic rats the values were gradually increased after the STZ injection. Serum insulin decreased sharply 5 days after STZ injection decreasing even more over time. The percentage of HbA_{1c} increased almost 50 % in diabetic rats as compared to control rats; this difference was kept at all times. Serum Ca values were similar between control and diabetic rats, as well as serum P and serum creatinine at different times (Table 2).

3.2. D.m. inhibits transiently the intestinal Ca^{2+} absorption

A considerable reduction in the intestinal Ca^{2+} absorption was triggered by STZ after 5 days of induction as compared to that of the control rats. The intestinal Ca^{2+} absorption remained low 30 days after STZ injection in the diabetic rats, returning to the control values at 60 days (Table 3).

3.3. D.m. alters the intestinal AP activity

As shown in Fig. 1, the activity of intestinal AP, a protein presumably involved in the intestinal Ca^{2+} absorption, decreased 5 days after STZ injection, continued low after 30 days and, then, increased reaching higher values compared to the control rats one month later.

3.4. D.m. modifies the gene and protein expressions of molecules involved in the transcellular and paracellular pathways of the intestinal Ca^{2+} absorption and the VDR protein expression

The gene expression of proteins involved in the transcellular pathway of the intestinal Ca^{2+} absorption, *pmca$_{1b}$*, *ncx1* and *trpv6*, decreased significantly 60 days after STZ injection. The gene expression of *cldn 2*, a protein presumably involved in the paracellular pathway of the intestinal Ca^{2+} absorption, was more than double in diabetic rats than that of the control rats 30 days after STZ injection and later decreased (Table 4). In contrast, the protein expression of $PMCA_{1b}$, NCX1 and TRPV6 significantly increased either 5 or 30 days after STZ injection returning to the control values at 60 days, except that of the TRPV6. Cldn 2 protein expression highly increased 30 days after STZ induction and normalized 30 days later (Fig. 2). VDR protein expression was enhanced at all

Table 2
General characteristics of STZ induced diabetic rats.

Days after induction	Body weight (g)		Serum glucose (mg/dL)		Serum insulin (ng/mL)		HbA_{1c} (%)		Serum Ca (mg/dL)		Serum P (mg/dL)		Serum creatinine (mg/dL)	
	C	STZ	C	STZ	C	STZ	C	STZ	C	STZ	C	STZ	C	STZ
5	238.01 ± 0.09	196.70 ± 12.20*	142 ± 17	406 ± 13*	1.73 ± 0.21	0.31 ± 0.05*	8.51 ± 0.45	12.58 ± 0.94§	9.93 ± 0.09	9.38 ± 0.20	5.93 ± 0.25	5.73 ± 0.28	0.72 ± 0.25	0.74 ± 0.23
30	255.85 ± 5.50	178.88 ± 8.59*	143 ± 14	450 ± 15*	n.d	0.29 ± 0.11	8.80 ± 0.51	11.91 ± 0.83§	9.52 ± 0.02	9.43 ± 0.22	5.80 ± 0.20	5.82 ± 0.20	0.71 ± 0.31	0.71 ± 0.21
60	369.22 ± 16.73#	221.81 ± 8.31*¤	145 ± 15	496 ± 21*+	n.d	0.18 ± 0.05	8.60 ± 0.29	12.35 ± 1.27*	9.16 ± 0.31	9.46 ± 0.38	5.52 ± 0.25	5.90 ± 0.12	0.71 ± 0.29	0.72 ± 0.15

Values are expressed as means ± S.E from 8 rats for each experimental condition. C: control; STZ: streptozotocin induced diabetic rats. #p < 0.05 vs control of 5 and 30 days, *p < 0.001 vs the corresponding control, §p < 0.01 vs 30 days after STZ injection, +p < 0.05 vs 5 days after STZ injection, ¤p < 0.01 vs the corresponding control. n.d.: not determined.

Table 3
Intestinal calcium absorption in control and diabetic rats (STZ).

Days after induction	Control	STZ
	nmol $^{45}Ca^{2+}$/mL plasma	
5	3.03 ± 0.13	1.22 ± 0.06*
30	3.07 ± 0.25	2.39 ± 0.11#
60	3.05 ± 0.25	3.09 ± 0.33

Values are expressed as means ± S.E from 10 rats for each experimental condition. STZ: streptozotocin induced diabetic rats. *p < 0.001 vs corresponding control and 30 and 60 days after STZ injection, #p < 0.01 vs corresponding control.

different times after STZ injection, but in less proportion 60 days after the induction (Fig. 2).

3.5. Diabetes triggers oxidative stress in the rat duodenum

Fig. 3 shows that STZ caused GSH depletion in mucosa from the rat duodenum either 5 or 30 days after injection, being the values normalized 30 days later. The mucosal levels of $\dot{O}_2^-$ were highly increased 5 days after STZ injection, and progressively decreased reaching the normalization after 60 days. Similarly, duodenal CAT activity was enhanced 5 and 30 days after STZ injection and returned to control values at 60 days. Duodenal SOD activity did not change after STZ injection at any time.

3.6. Insulin restores the intestinal Ca^{2+} absorption and the intestinal redox state of the diabetic rats, but not the levels of serum vitamin D metabolites

The inhibition of the intestinal Ca^{2+} absorption caused 30 days after STZ injection to rats was blunted by using a single daily injection of insulin for 5 days. The same hormonal treatment normalized GSH content, CAT activity and $\dot{O}_2^-$ levels (Fig. 4). The serum levels of $25OHD_3$ and $1,25(OH)_2D_3$ were decreased by STZ injection at different times, and remained low after insulin treatment for 5 days (Table 5).

Discussion

The present study demonstrates that the intestinal Ca^{2+} absorption decreases by insulin deficit in rats injected with STZ. The effect is relatively rapid and transient leading to a time dependent adaptation, which makes that the absorptive process returns to normal values. The inhibitory effect on the intestinal Ca^{2+} absorption is accompanied by redox changes that cause oxidative stress, which may alter the

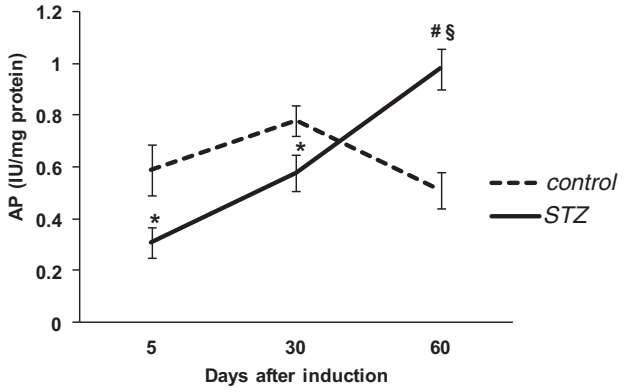

Fig. 1. Intestinal alkaline phophatase activity from duodenal sac of control and STZ induced diabetic rats. Alkaline phosphatase was measured in water homogenates (1:10) of intestinal mucosa using p-nitrophenyl phosphate as a substrate in 0.5 mol/L diethanolamine buffer pH 9.8. Values represent means ± SE from nine rats for each experimental condition. *p < 0.05 vs the corresponding control, #p < 0.001 vs the corresponding control, §p < 0.05 vs 5 and 30 days after STZ injection.

Table 4
Gene expression of *pmca$_{1b}$*, *ncx1*, *trpv6* and *cldn 2*, in control and STZ induced diabetic rats.

Days after induction	*pmca$_{1b}$*		*ncx1*		*trpv6*		*cldn 2*	
	Control	STZ	Control	STZ	Control	STZ	Control	STZ
5	1.00 ± 0.18	0.70 ± 0.08	1.00 ± 0.15	1.22 ± 0.08$^{\#}$	1.00 ± 0.17	0.79 ± 0.17	1.00 ± 0.11	0.87 ± 0.07
30	1.00 ± 0.20	0.87 ± 0.15	1.00 ± 0.20	0.82 ± 0.08	1.00 ± 0.18	0.86 ± 0.07	1.00 ± 0.01	2.53 ± 0.50$^{*\#}$
60	1.00 ± 0.27	0.35 ± 0.07$^{*\#}$	1.00 ± 0.09	0.66 ± 0.07*	1.00 ± 0.07	0.66 ± 0.09*	1.00 ± 0.01	0.64 ± 0.10*

Real time quantitative PCR analysis of rat duodenae mRNA expression levels. Gene expression levels represent the relative mRNA expression compared with the GAPDH mRNA (arbitrary units). Values represent means ± SE from 6 rats for each experimental condition. STZ: streptozotocin induced diabetic rats. $^{*}p < 0.05$ vs the corresponding control, $^{\#}p < 0.001$ vs STZ from the other induction times.

duodenum permeability. When insulin is administered, the redox state of the intestine returns to control values and the intestinal Ca^{2+} absorption is normalized, independently of vitamin D status.

The adaptability of the intestine to absorb more or less Ca^{2+} according to the physio-pathological conditions has been known for many years [34,35]. However, the mechanisms of adaptation of intestinal Ca^{2+} absorption in diabetic patients and animals are not quite clear. In this work, we have followed the intestinal Ca^{2+} absorption for two months after a single STZ injection in rats as compared to control animals. As expected, 5 days after STZ injection the rats show alterations

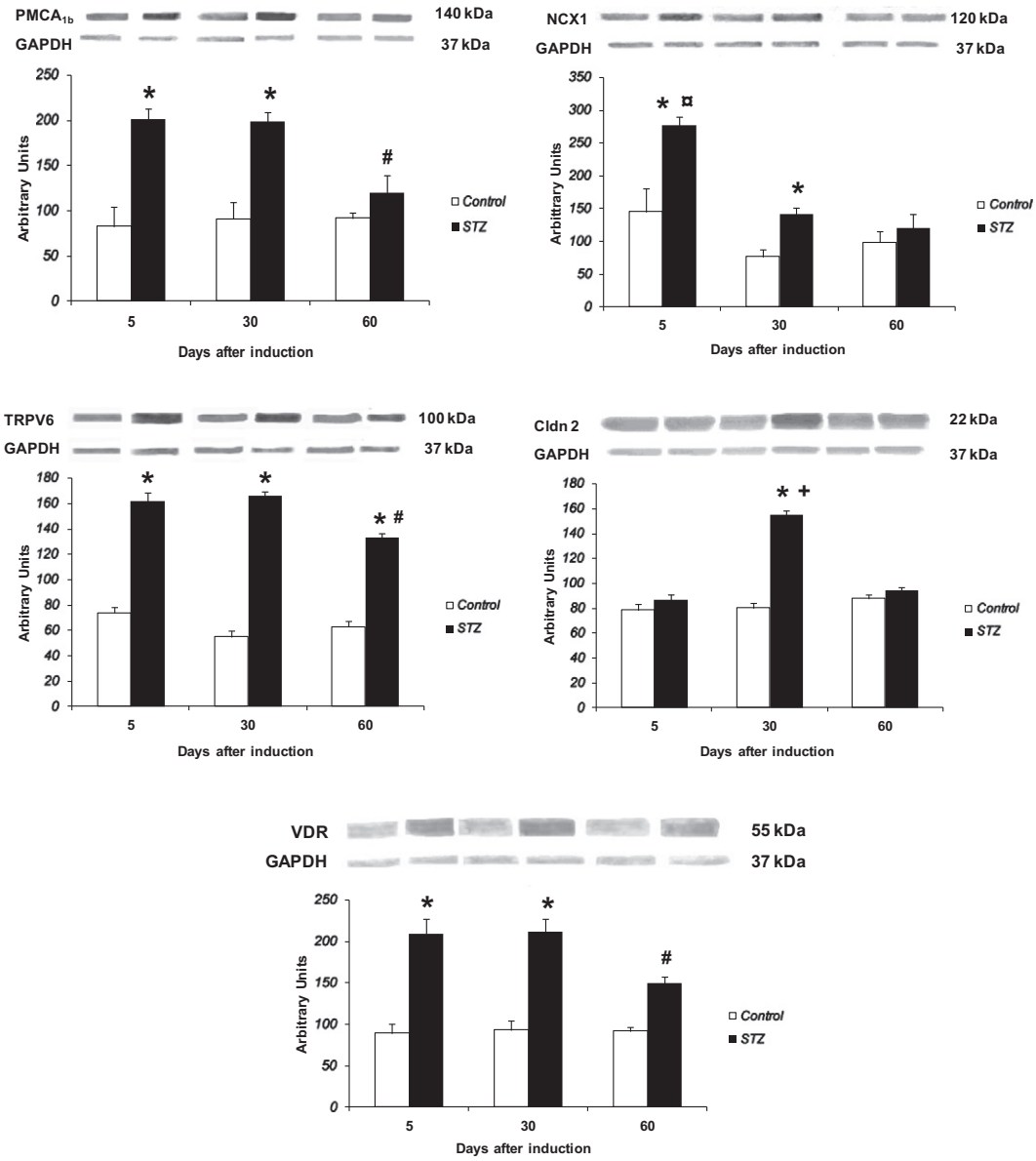

Fig. 2. Protein expression of $PMCA_{1b}$, NCX1, TRPV6, claudin 2 and VDR was analyzed by Western blot in pool of mucosa from two rat duodenae for each experimental condition. One hundred micrograms of duodenal mucosa homogenate suspension was separated by SDS-PAGE and immunoblotted with the corresponding antibodies followed by detection with DAB as a chromogen. Three independent experiments were accomplished. Values are expressed as means ± S.E. $^{*}p < 0.05$ vs control at the same time; $^{\alpha}p < 0.001$ vs 30 and 60 days after STZ injection, $^{\#}p < 0.001$ vs 5 and 30 days after STZ injection, $^{+}p < 0.001$ vs 5 and 60 days after STZ injection.

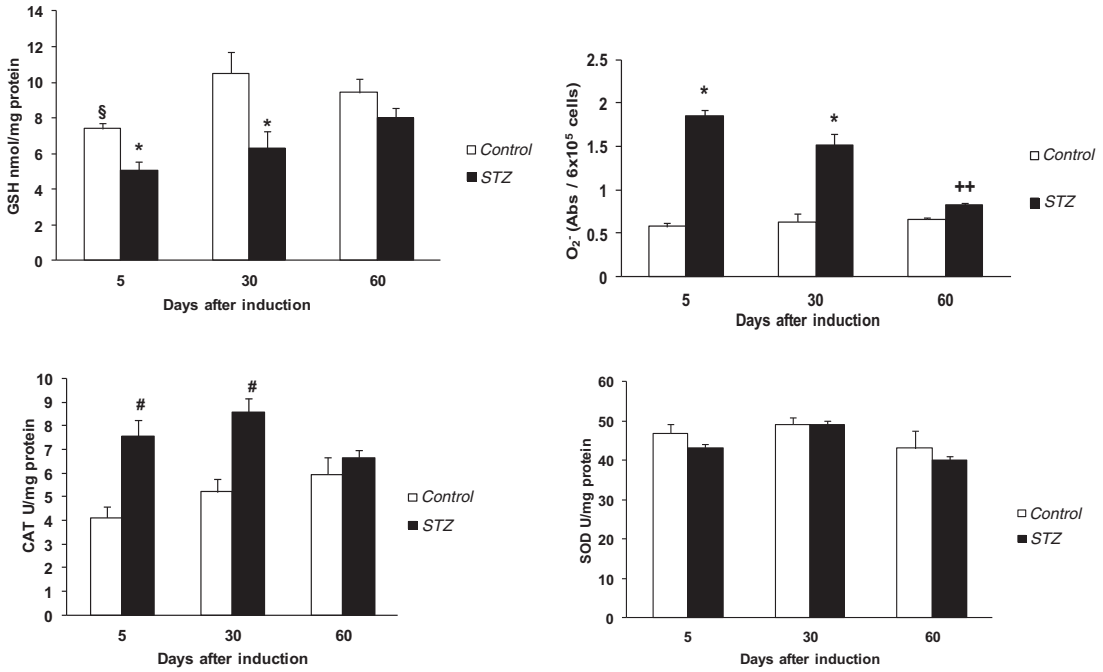

Fig. 3. Intestinal total GSH content, superoxide anion, SOD and CAT activities from control and STZ induced diabetic rats. Values represent means ± SE from nine rats for each experimental condition. $^{\S}p < 0.05$ vs control of 30 and 60 days, $^{*}p < 0.01$ vs control at the same time, $^{++}p < 0.001$ vs 5 and 30 days after STZ injection, $^{\#}p < 0.001$ vs control at the same time.

in body weight and in biochemical parameters, all of them compatible with type I D.m. [36]. At this time, the intestinal Ca^{2+} absorption is highly inhibited; it remains low at 30 days and returns to control values at 60 days. The activity of intestinal AP, enzyme that has been proposed to be involved in the intestinal Ca^{2+} absorption, exhibits similar changes 5 and 30 days after STZ injection, but it increased over the control values 30 days later. The intestinal AP plays multiple roles in maintenance of intestinal homeostasis [37]. It is quite possible that the late upregulation

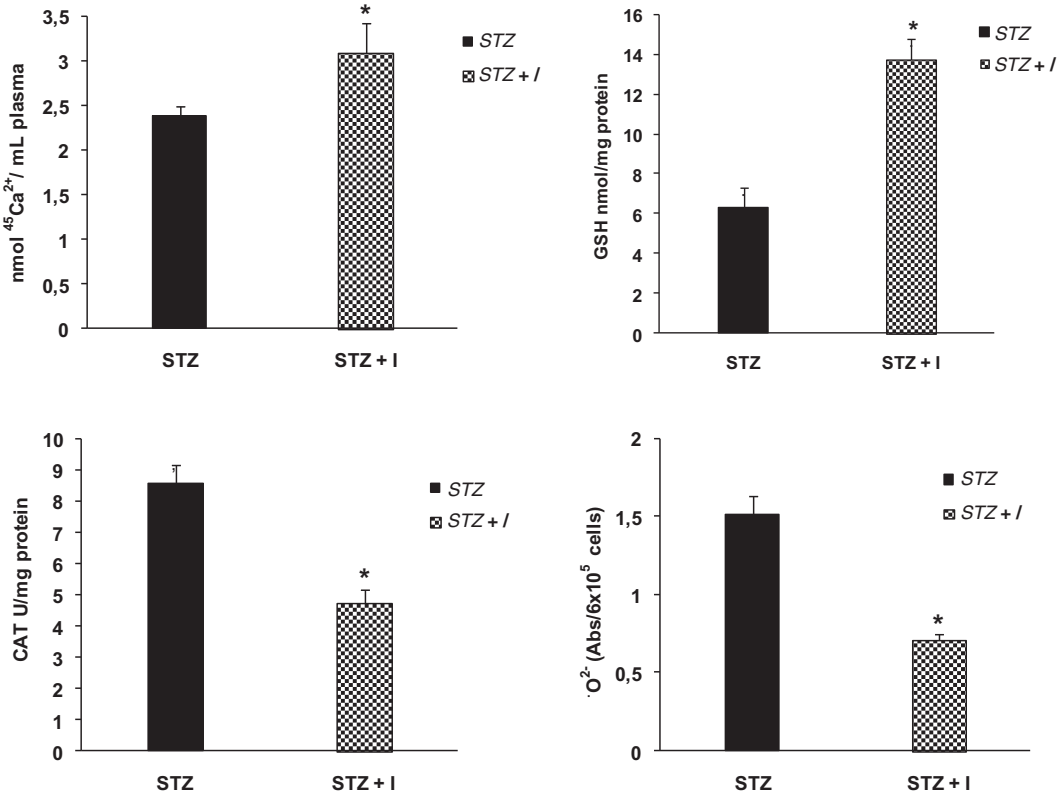

Fig. 4. Intestinal calcium absorption, intestinal total GSH content, CAT activities and superoxide anion from STZ induced diabetic rats and STZ treated with insulin. Values represent means ± SE from nine rats for each experimental condition. $^{*}p < 0.01$ vs 30 days after STZ injection.

Table 5
Serum calcidiol ($25(OH)D_3$) and calcitriol ($1,25(OH)_2D_3$) levels from control, STZ induced diabetic rats and STZ + I.

Days after induction	$25(OH)D_3$ (ng/mL)			$1,25(OH)_2D_3$ (pg/mL)		
	Control	STZ	STZ + I	Control	STZ	STZ + I
5	7.64 ± 0.49	3.17 ± 0.09*	3.22 ± 0.10*	n.d.	n.d.	n.d.
30	10.89 ± 1.16	4.25 ± 0.82*	3.13 ± 0.06*	115.00 ± 3.51	35.83 ± 7.92#	36.33 ± 0.88#
60	9.94 ± 0.74	4.53 ± 0.77*	3.45 ± 0.20*	98.33 ± 0.66	25.33 ± 7.12#	30.20 ± 0.40#

Values are expressed as means ± S.E from 4 rats for each experimental condition. STZ: streptozotocin induced diabetic rats, STZ + I: diabetic rats treated with insulin. *$p < 0.01$ vs the corresponding control, #$p < 0.001$ vs the corresponding control. n.d.: not determined.

of AP activity, which was also observed by other groups [38], could be an anti-inflammatory response in order to protect the intestine from the inflammatory cell infiltration, as suggested [37]. The protein expression of $PMCA_{1b}$, NCX1 and TRPV6, all proteins presumably involved in the Ca^{2+} transcellular pathway, is increased either 5 or 30 days after STZ injection returning to control values at 60 days, except that of the TRPV6. The data are indicative of an adaptive mechanism to improve the intestinal Ca^{2+} absorption for avoiding Ca^{2+} loss, which has been detected 60 days after STZ injection. The TRPV6 protein expression goes down at 60 days, but it is still significantly higher than that of the control group, which could mean that the Ca^{2+}entrance step needs more time to be normalized than the Ca^{2+} exit step from enterocytes. Lee et al. [39] have also found a significant increase in the abundance of renal calbindin D_{28k} and TRPV5 in diabetic rats for the 2-week duration compared to control group. Both proteins belong to the transcellular pathway of Ca^{2+} reabsorption in rat kidney and the increment of them has been proposed as a compensatory mechanism for the enhancement of renal Ca^{2+} excretion. It has been also reported similar compensatory upregulations of sodium and water transporters in uncontrolled D.m. in rats [40,41].

The gene expression of $PMCA_{1b}$, NCX1 and TRPV6 is not modified 5 or 30 days after STZ injection. However, the mRNA levels codifying for these proteins decline considerably 30 days later, when the intestinal Ca^{2+} absorption returns to normal values. Our data suggest that the transient inhibition of intestinal Ca^{2+} absorption caused by diabetes induction would trigger a compensatory mechanism as judged by an increase in the protein expression of molecules involved in the intestinal transcellular Ca^{2+} movement. This response could downregulate the gene expression of those proteins to avoid an overproduction of the molecules responsible for taking the cation from one pole to the other pole within the cell. In other words, the transcription of these genes is not altered by the insulin deficit at early stages of diabetes, but the protein synthesis seems to be stimulated in order to rapidly compensate the inhibition of the intestinal Ca^{2+} absorption.

Although the molecular basis for paracellular Ca^{2+} absorption in the intestine is not well known [42], tight junction proteins cldn 2 and cldn12 have been proposed to form the paracellular pores for cations [20]. Our data on gene and protein expressions of cldn 2 show no changes at 5 days after STZ injection. However, a sharp rise in the protein expression of cldn 2 occurs 30 days after STZ injection, which is also accompanied by increased gene expression. It is quite possible that the enhanced protein expression of molecules involved in the transcellular pathway is not enough for restoring the intestinal Ca^{2+} absorption and, consequently, the paracellular pathway is induced, a possible interplay of both mechanisms that has been previously suggested [19]. In other words, the insulin deficit promotes the transcellular and the paracellular pathways of the intestinal Ca^{2+} absorption in order to normalize the cation absorption.

As previously shown [43,44], the diabetic condition alters the vitamin D metabolism, as judged by the low levels of serum calcidiol and calcitriol. This could partly explain the inhibition in the intestinal Ca^{2+} absorption that occurs as early as 5 days after STZ injection, but not the compensatory stimulation of molecules of both transcellular and paracellular pathways. Therefore, other molecules could be responsible for the compensatory mechanisms. In addition, insulin replacement therapy for 5 consecutive days does not improve the impaired vitamin D metabolism. Maybe a longer insulin therapy or other factors are necessary to normalize serum calcitriol levels.

The occurrence of oxidative stress in the small intestine has been demonstrated in diabetic rats 6 weeks after STZ injection [24]. Our study shows that oxidative stress is triggering in the duodenum at early stages of STZ-induced diabetic rats as revealed by GSH depletion, increased $\dot{O}_2^-$ and increase in the CAT activity after 5 days of induction. These alterations are also very noticeable at 30 days, but disappear 30 days later. There is a temporal correlation between changes in the intestinal Ca^{2+} absorption and the oxidative stress. The recovery of the normal redox status of the enterocytes occurs at the same time that the intestinal Ca^{2+} absorption returns to control values. When consecutive insulin injections for 5 days are administered to rats 30 days after STZ injection, the intestinal Ca^{2+} absorption is normalized as well as the intestinal GSH content, the $\dot{O}_2^-$ levels and the CAT activity. Therefore, the lack of insulin promotes intestinal oxidative stress, which in turn causes inhibition of intestinal Ca^{2+} absorption. In our laboratory we have well documented that GSH depleting drugs such as menadione, deoxycholate and BSO inhibit the intestinal Ca^{2+} absorption through oxidative stress and apoptosis mediated by both the extrinsic and intrinsic pathways [21–23]. In diabetic animals there are compensatory mechanisms trying to normalize the redox state and, consequently, the intestinal Ca^{2+} absorption. It is interesting to note that these mechanisms occur independently of the levels of plasma vitamin D metabolites. However, we have shown that the intestinal VDR protein expression is increased by the STZ injection at all times studied. This upregulation of VDR has been suggested as a unique compensatory response of intestine in untreated D.m. animals, which has been associated with the intestinal hyperplasia [45]. In addition, a higher affinity of calcitriol to intestinal VDR has been also demonstrated in type I D.m [46].

Many studies indicate that oxidative stress plays a crucial role in the pathogenesis of late diabetic complications [47,48]. It has been proved that hyperplasia and hypertrophy of intestinal epithelial cells and decrease in fluidity of the brush border membranes occur in the small intestine of diabetic rats 6 weeks after STZ injection [49], and a reduction in the density of myenteric neurons of the duodenum was observed 120 days after STZ administration [50]. In the jejunum of diabetic rats 15 days after STZ injection, increase in the length of villi, congestion, goblet cell hyperplasia and infiltration of inflammatory cells have been found [38]. Our study shows that oxidative stress in the duodenum of diabetic rats occurs at early stages, but if an appropriate compensatory response occurs from the antioxidant network, the redox imbalance can be overcome and the intestinal Ca^{2+} absorption returns to normal values. We do not discard that transitory alterations in the intestinal absorption of other ions or nutrients could be also occurred.

To conclude, oxidative stress in the rat intestine occurs at early stages of developing type I D.m., leading to inhibition of the intestinal Ca^{2+} absorption. Time-dependent adaptive mechanisms trigger an increment of protein expression of molecules involved in both the transcellular and the paracellular pathways, normalizing the intestinal Ca^{2+} absorption as well as the duodenal redox state. Insulin injection

also restores the duodenal redox state and the intestinal Ca^{2+} absorption without improving the serum levels of vitamin D metabolites. The characterization of the intestinal Ca^{2+} absorption in diabetic animals is an important issue that might help to understand the alterations in the Ca^{2+} homeostasis and the bone disease associated with the type I D.m. in humans.

Conflict of Interest

No conflicts of interest, financial or otherwise, are declared by the authors.

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