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ORIGINAL ARTICLES

Na+-Glucose Cotransporter SGLT1 Protein in Salivary Glands: Potential Involvement in the Diabetes-Induced Decrease in Salivary Flow

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Abstract Oral health complications in diabetes include decreased salivary secretion. The SLC5A1 gene encodes the Na^+-glucose cotransporter SGLT1 protein, which not only transports glucose, but also acts as a water channel. Since SLC5A1 expression is altered in kidneys of diabetic subjects, we hypothesize that it could also be altered in salivary glands, contributing to diabetic dysfunction. The present study shows a diabetes-induced decrease ($p < 0.001$) in salivary secretion, which was accompanied by enhanced ($p < 0.05$) SGLT1 mRNA expression in parotid (50%) and submandibular (30%) glands. Immunohistochemical analysis of parotid gland of diabetic rats revealed that SGLT1 protein expression increased in the luminal membrane of ductal cells, which can stimulate water reabsorption from primary saliva. Furthermore, SGLT1 protein was reduced in myoepithelial cells of the parotid from diabetic animals, and that, by reducing cellular contractile activity, might also be related to reduced salivary flux. Six-day insulin-treated diabetic rats reversed all alterations. In conclusion, diabetes increases SLC5A1 gene expression in salivary glands, increasing the SGLT1 protein content in the luminal membrane of ductal cells, which, by increasing water reabsorption, might explain the diabetes-induced decrease in salivary secretion.

Keywords Submandibular gland · Parotid gland · SGLT1 · SLC5A1 gene · Diabetes mellitus

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Oral health complications associated with diabetes include xerostomia, periodontal diseases, increased incidence and severity of caries, tooth loss, and candidiasis (Vernillo 2001; Cohen et al. 1970; Murrah 1985; Carda et al. 2006; Hintao et al. 2007), and these alterations may be linked to salivary gland dysfunction. In fact, impaired salivary function in diabetic subjects has been described (Lin et al. 2002), and this includes reduced salivary flow rate (Conner et al. 1970; Newrick et al. 1991; Ben-Aryeh et al. 1988) and alterations in the composition of saliva, such as an increase in glucose concentration (Campbell 1965; Forbat et al. 1981; Sharon et al. 1985; Reuterving 1986; Karjalainen et al. 1996; Belazi et al. 1998; Aydin 2007).

SLC5A1 and SLC5A2 genes, which encode the SGLT1 and SGLT2 Na+-glucose cotransporter proteins (Thorens 1996), respectively, are expressed in the apical membrane of epithelial cells of intestine and renal proximal tubule, where they participate in the luminal to the interstitium glucose flux (Wright 2001).

In salivary glands, the SGLT1 protein was described in acinar cells of ovine parotid (Tarpey et al. 1995) and in rat submandibular (Elfeber et al. 2004); however, no clear functional role has been proposed to SGLT1 in these cells. In the duct system, phlorizin-sensitive glucose reabsorption was demonstrated (Takai et al. 1983), suggesting SGLT-mediated glucose transport. An unanswered mechanistic question is what isoform of SGLT is responsible for glucose reabsorption in the luminal membrane of ductal cells.

Additionally, recent reports have demonstrated that the SGLT1 cotransporter plays an important role as a water pump (Loo et al. 2002), and a stoichiometric relationship of transport capacity of 2 Na^+:1 glucose:264 H_2O molecules was shown (Wright et al. 2004).

Considering that changes in SLC5A1 and SLC5A2 gene expression have already been described in kidney of diabetic rats (Vestri et al. 2001; Freitas et al. 2008), we hypothesized that it may also occur in salivary glands. The aim of the present study was to investigate SLC5A1 gene expression, as well as the cellular localization of SGLT1 protein in salivary glands of diabetic rats. We believe these investigations will contribute to the understanding of glucose and water routes to and from saliva, as well as the establishment of the pathophysiological mechanisms of salivary gland dysfunction in diabetes.

Materials and Methods

Experimental Procedures

Male Wistar rats (~280-g body weight) were rendered diabetic (D) by a single intravenous injection (penis vein) of alloxan (40 mg/kg body weight), and nondiabetic control rats (ND) were injected with saline (Vestri et al. 2001). Animals were individually caged and allowed free access to water and standard rodent chow diet (Nuvilab CR-1; Nuvital, Curitiba, Brazil). D and ND animals were sampled 20 days after diabetes induction. Additional 20-day diabetic rats were submitted to 6 days of insulin treatment (NPH insulin, Biohulin N; Biobrás, MG, Brazil, subcutaneously), at a dose of 2 U in the morning (0830 h) and 4 U in the afternoon (1730 h). These insulin-treated diabetic rats (DI) were compared with saline-treated diabetic rats (DS).

Urine was collected for the last 24 h before the experiments, which were conducted in anesthetized rats (sodium pentobarbital, 40 mg/kg body weight). Nonstimulated salivary secretion was measured during 7 min, using four preweighed cotton balls inserted into the oral cavity, two underneath the tongue, and two bilaterally medial to the teeth and oral mucosa. The 7-min volume of secreted saliva was calculated by subtracting the initial from the final weight of the four cotton balls, considering that 1 mg corresponds to 1 µL (Renzi et al. 1993; Cecanho et al. 1999). After that, tail blood was sampled, and salivary glands were carefully excised, weighed, and processed for further analysis. Experimental procedures were approved by the Ethical Committee for Animal Research of the Institute of Biomedical Sciences, of the University of São Paulo (Protocol 22/2005).

Northern Blotting

Total RNA (80 µg), extracted using TRIZOL reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), was electrophoresed in agarose gel and blotted onto a nylon membrane. The membrane was hybridized with a rat SLC5A1 ^{32}P-labeled cDNA probe, striped, and rehybridized with β-actin ^{32}P-labeled cDNA probe. A 2400-bp cDNA of rat SGLT1 and a 400-bp cDNA of human β-actin were generously provided by Dr. M. Kasahara (Teikyo University, Japan). The blots were analyzed by optical densitometry (Image Master 1D; Pharmacia Biotech, Uppsala, Sweden), and the results are expressed as arbitrary units (AU), after normalization by the respective β-actin value.

Western Blotting

Tissue samples were homogenized in buffer (10 mM Tris HCl, 1 mM EDTA, 250 mM sucrose, 5 µg/ml leupeptin, 15 µg/ml aprotinin, pH 7.4) and centrifuged at 3000*g* for 15 min at 4°C. The supernatant was kept and the pellet was resuspended and centrifuged at under the same conditions. Both supernatants were mixed, centrifuged at 7000*g* for 30 min, and the final pellet was resuspended as a plasma membrane enriched fraction (PM). One hundred micrograms of protein was electrophoresed, transferred to nitrocellulose membrane (Hybond-ECL; Amersham Biosciences, Buckinghamshire, UK), and immunodetected using anti-rat SGLT1 antibody (Chemicon International, Temecula, CA, USA) followed by enhanced chemiluminescence (Amersham Biosciences). The blots were quantified by optical densitometry, and the results are expressed as arbitrary units (AU).

Immunohistochemistry

Tissues were fixed in 4 % formaldehyde phosphate buffer (PB), followed by cryoprotection in crescent sucrose solutions (10%, 20%, and 30%) in PB. Seven-micrometer-thick sections were placed on gelatin-coated slides (Sigma Chemical Co., St. Louis, MO, USA) and subjected to immunodetection using anti-rat SGLT1 antibody (1:50; Chemicon International), followed by incubation with anti-rabbit IgG goat antiserum tagged with Cy5 (1:200 l; Molecular Probes, Eugene, OR, USA). Controls for the experiments consisted of the omission of primary antibodies. F-Actin staining was performed with rhodamine phalloidin (1:100; Molecular Probes), and nuclear staining with Sitox Green (1:10,000; Molecular Probes). After washing, the tissue was coverslipped and analyzed in a Nikon PCM2000 confocal microscope (Nikon, Tokyo).

Analytical Procedures

Plasma and urine were assayed for glucose (Glicose Enzimática; ANALISA Diagnostica, Belo Horizonte, MG, Brazil). Plasma was also assayed for insulin (Coat-A-Count; DPC Diagnostic Products, Los Angeles, CA, USA).

Data Analysis

All values weare presented as mean ± SE. Numbers of animals are indicated in the figure legends. Comparisons of means were performed by one-way analysis of variance (ANOVA), followed by Student–Newman–Keuls posttest (GraphPad Prism version 4.00 for Windows; GraphPad Software, San Diego, CA, USA).

Results

Compared with nondiabetic rats (ND; Table 1), 20-day diabetic rats (D) presented a lower body weight ($p < 0.001$), hyperglycemia and glycosuria ($p < 0.001$), and low plasma insulin ($p < 0.001$). After 6 days of insulin treatment, plasma insulin of diabetic rats (DI) was higher ($p < 0.01$) than in nondiabetic rats. The treatment also increased body weight ($p < 0.001$) and decreased the volume of urine and glucose excretion to levels similar to those of nondiabetic rats. Plasma glucose was reduced significantly ($p < 0.001$), although it remained higher ($p < 0.05$) than that observed in nondiabetic rats. Neither the diabetes nor the insulin treatment changed the tissue weight or protein concentration of parotid and submandibular glands (Table 2). However, oral salivary secretion, measured during 7 min, was reduced eightfold ($p < 0.001$) in untreated or saline-treated diabetic rats compared to nondiabetic or insulin-treated diabetic rats (Table 2).

SLC5A1 gene expression is shown in Fig. 1. SGLT1 mRNA was increased in both parotid (58%) and submandibular (22%) glands of 20-day diabetic rats, as well as in parotid of 6-day saline-treated diabetic rats. After 6 days of insulin treatment, the effect of diabetes on SGLT1 mRNA was reversed in both glands. However, the mRNA content also decreased in the submandibular of saline-treated rats. Compared to mRNA, the SGLT1 protein was inversely regulated in both glands of diabetic rats treated or not with insulin, suggesting posttranscriptional regulation of SLC5A1 gene and/or variations in the subcellular localization of the protein.

Figure 2 shows the cellular and subcellular localization of SGLT1 protein in salivary glands. In parotid of nondiabetic rats (Fig. 2, top: A and D), immunofluorescence

Table 1 Body weight, 24-h urine glucose and volume, and plasma glucose and insulin in nondiabetic (ND), untreated diabetic (D), and saline (DS)- or insulin (DI)-treated diabetic rats

	ND	D	DI	DS
Body weight (g)	352 ± 6.9 (21)	271 ± 11.9*** (13)	341 ± 10.8 (10)	246 ± 12.3*** (10)
Urine glucose (mg/24 h)	6.2 ± 1.9 (21)	365 ± 32.5*** (15)	40 ± 15.6 (6)	361 ± 59.7*** (8)
Urine volume (mL)	6.0 ± 0.9 (28)	99 ± 2.2*** (19)	20 ± 1.4 (7)	123 ± 14.4*** (9)
Plasma glucose (mg/dL)	103 ± 2.1 (24)	244 ± 2.3*** (21)	130 ± 25.2$^{\#}$ (9)	237 ± 13*** (12)
Plasma insulin (μU/mL)	51 ± 12.9 (10)	7 ± 1.2** (14)	101 ± 17.3$^{\#\#}$ (9)	13 ± 3.5** (6)

Note: Data are mean ± SE. Number of samples given in parentheses. ** $p < 0.01$ and *** $p < 0.001$ vs. ND and DI; $^{\#}$ $p < 0.05$ and $^{\#\#}$ $p < 0.01$ vs. ND; one-way ANOVA, Student–Newman–Keuls posttest

Table 2 Morphological and functional characteristics of salivary glands of nondiabetic (ND), untreated diabetic (D), and saline (DS)- or insulin (DI)-treated diabetic rats

	ND	D	DI	DS
Tissue weight (mg)				
Parotid	150 ± 5.3 (13)	139 ± 7.9 (11)	159 ± 2.3 (8)	152 ± 7.5 (6)
Submandibular	281 ± 8.9 (12)	273 ± 5.6 (8)	290 ± 18.6 (6)	271 ± 3.9 (9)
Protein recovery (mg/g tissue)				
Parotid	15.7 ± 3.0 (13)	16.6 ± 2.2 (11)	13.7 ± 1.4 (9)	21.1 ± 2.2 (5)
Submandibular	12.8 ± 2.1 (11)	9.4 ± 1.0 (8)	8.4 ± 1.6 (6)	9.5 ± 1.0 (9)
Salivary secretion (μL/7 min)	4.25 ± 0.62 (5)	0.52 ± 0.14*** (5)	4.53 ± 0.17 (5)	0.68 ± 0.15*** (5)

Note: Data are mean ± SE. Number of samples is given in parentheses. Total tissue protein recovery was measured in a plasma membrane-enriched fraction, used for SGLT1 protein analysis. *** $p < 0.001$ vs. ND and DI; one-way ANOVA, Student–Newman–Keuls posttest

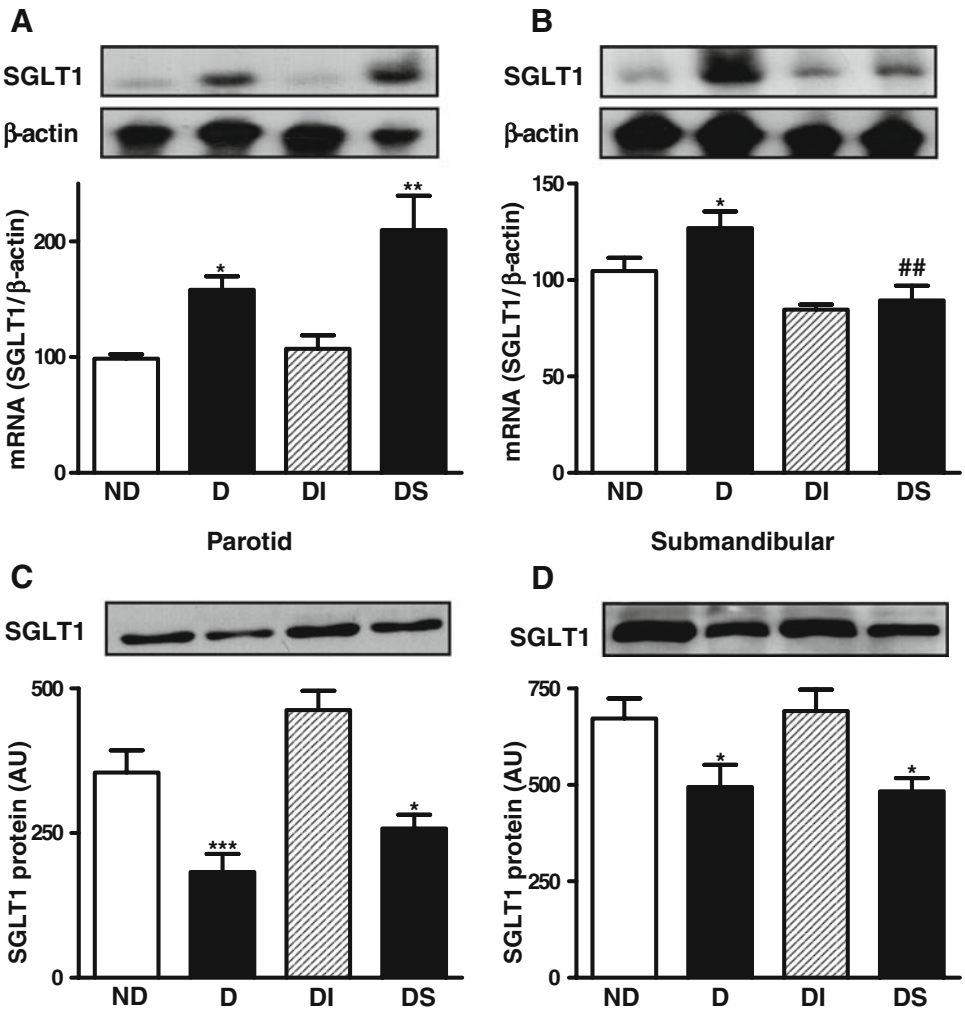

Fig. 1 SLC5A1 gene expression in parotid (**a** and **c**) and submandibular (**b** and **d**) glands of control nondiabetic (ND) and 20-day diabetic rats, before (D) and after treatment with insulin (DI) or saline (DS). A and B: top, representative images of SGLT1 mRNA and β-actin; bottom, SGLT1 mRNA as a ratio of β-actin mRNA content. C and D: top, representative image of SGLT1 protein in plasma membrane fraction; bottom, SGLT1 protein content measured in 100 µg protein. Results, as arbitrary units (AU), are mean ± SE of 4 to 7 rats for mRNA and 5 to 10 rats for protein, analyzed in at least four experiments. $^{*}p < 0.05$, $^{**}p < 0.001$, and $^{***}p < 0.001$ vs. ND; $^{\#\#}p < 0.01$ vs. D; one-way ANOVA, Student–Newman–Keuls post hoc test

staining shows SGLT1 protein in the basolateral membrane of acinar cells and also in the stromal region. Furthermore, high SGLT1 immunoreactivity is observed in myoepithelial cells (Fig. 2, top: G), and no labeling can be seen in striated ducts (Fig. 2, top: H and I). In the parotid of diabetic rats (Fig. 2, top: B and K), SGLT1 protein is also observed in the basolateral membrane of acinar cells, but in a discontinuous pattern and, in some places, at a low intensity. However, diabetes increased SGLT1 in the stromal region and induced additional expression of SGLT1 protein in the luminal membrane of striated duct cells (Fig. 2, top: J and K). Insulin treatment reversed the effects of diabetes in acinar and ductal cells (Fig. 2, top: C and F) but did not change SGLT1 immunoreactivity in the stromal region.

In the submandibular gland of nondiabetic rats (Fig. 2, bottom: A and D), a subtle staining of SGLT1 can be seen in the basolateral membrane of acinar cells. Additionally, immunoreactivity can be seen, at lower intensity, inside the cells of striated and granulous ducts. In the submandibular gland of diabetic rats (Fig. 2, bottom: B and E), SGLT1 staining decreased in the basolateral membrane of acinar cells and increased in the cytoplasm of granulous duct cells, mainly in the apical region. Insulin treatment reversed all changes in SGLT1 protein observed in the submandibular gland of diabetic rats (Fig. 2, bottom: C and F).

SGLT1 protein analysis in parotid and submandibular glands of diabetic rats treated with saline was the same as in diabetic untreated rats (data not shown). Negative controls for the immunoreactivity to SGLT1 are shown for both the parotid (Fig. 2, top: L and M) and the submandibular (Fig. 2, bottom: L and M) glands.

Discussion

Description of SGLT1 expression and function in salivary glands is critical to understanding not only glucose fluxes but also water flow into saliva. Moreover, in diabetic subjects, changes in SGLT1 expression may be paramount to determining salivary dysfunctions such as xerostomia. In the present study we have demonstrated that diabetes

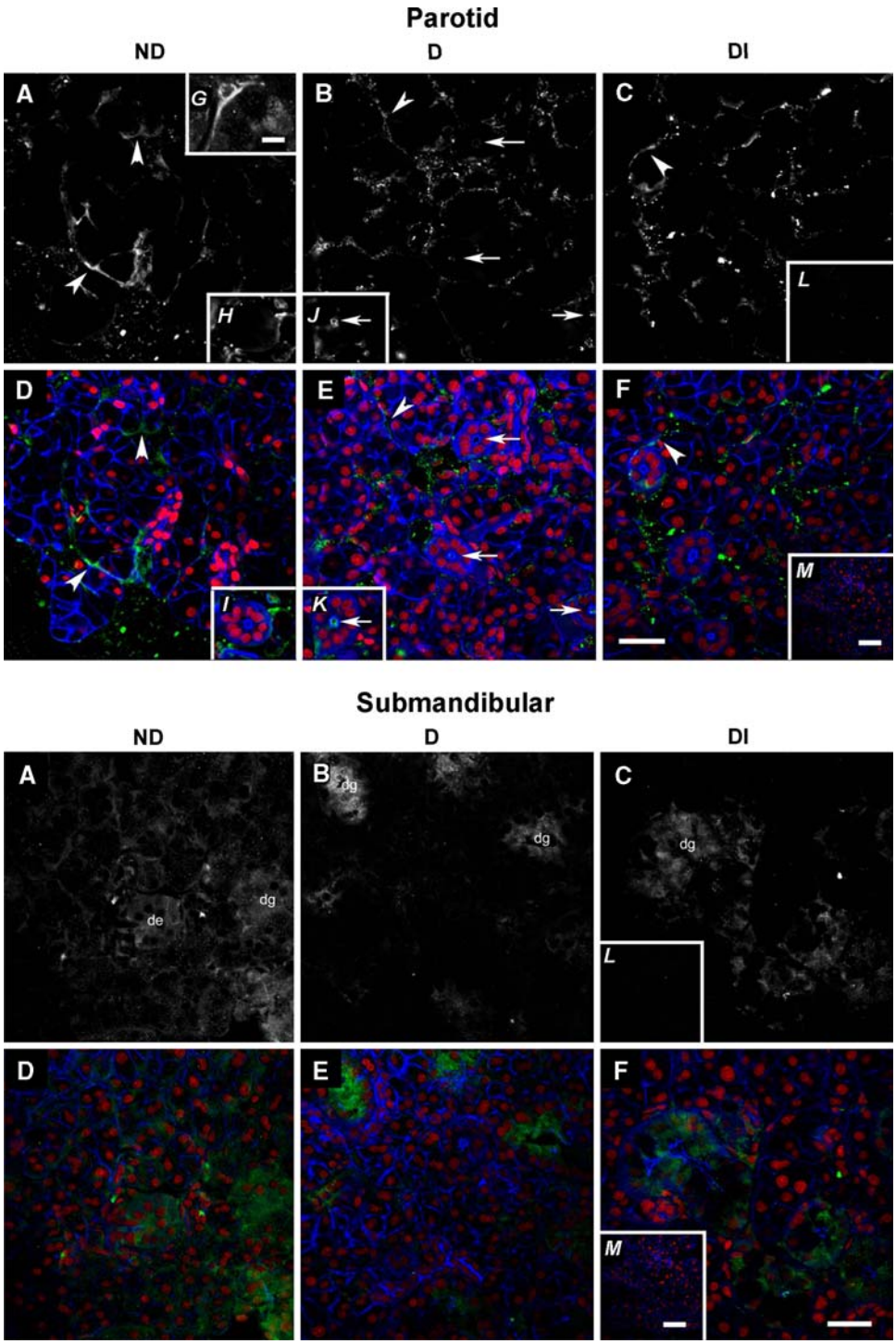

Fig. 2 Immunolocalization of SGLT1 protein in parotid (*top*) and submandibular (*bottom*) glands of nondiabetic (ND; *left-hand columns*), diabetic (D; *middle columns*) and insulin-treated diabetic (DI; *right-hand columns*) rats. Upper panels (**a**, **b**, and **c**) show SGLT1 immunoreactivity in white, and lower panels (**d**, **e**, and **f**) show the same photomicrographs, revealing SGLT1 (*green*), F-actin (*blue*), and nuclear marker (*red*). SGLT1 protein is indicated by arrowheads in the basolateral membrane of acinar cells and by arrows in the luminal membrane of ductal cells. In the parotid, details show a myoepithelial cell (**g**), a striated duct without luminal marking (**h** and **i**), and SGLT1 immunoreactivity in the luminal membrane of striated ducts cells (**j** and **k**). In the submandibular, a nondiabetic rat shows subtle staining in the basolateral membrane of acinar cells and uniformly distributed staining in striated duct (sd) and granulous duct (gd) cells; a diabetic rat reveals an increased amount of SGLT1 in granulous ducts, which is reverted by insulin treatment. Bars represent 45 μm in insets and 30 μm in other photomicrographs. Negative controls for reaction to SGLT1 are shown in **l** and **m**

induces significant changes in SGLT1 mRNA expression and in the cellular distribution of the SGLT1 protein, which may play an important role in the pathophysiology of salivary glands.

Diabetic animals treated or not with insulin showed the same metabolic-hormonal profile described in previous studies, in which SLC5A2 gene (SGLT2 protein) expression in renal epithelium increased in diabetes and was restored to nondiabetic levels with insulin treatment (Vestri et al. 2001; Freitas et al. 2008). SGLT1 mRNA was also reported to be increased in kidney of diabetic rats, decreasing with insulin treatment (Vestri et al. 2001). These regulations are the same as that currently described in salivary glands. However, SGLT1 mRNA in the submandibular gland of saline-treated diabetic rats was regulated the same way as in insulin-treated rats. This may

be related to the unexpected local tissue concentration of glucose and/or insulin, since autocrine effects of insulin have been proposed (Kerr et al. 1995).

Diabetes induced an increase in SGLT1 mRNA content but a decrease in SGLT1 protein expression. This discrepancy between mRNA and protein regulation might be explained by posttranscriptional regulation of the SLC5A1 gene, a feature that has been observed for other glucose transporter genes such as SLC2A4 (Klip et al. 1994; Seraphim et al. 2007).

SGLT1 protein content was measured in a plasma membrane-enriched subcellular fraction, where the transporter was expected to be inserted. Thus, an additional explanation for mRNA and protein discrepancies might involve impaired cellular trafficking of the transporter, in a way such that the increased cellular protein content (similar to the mRNA content) could not be detected in the plasma membrane fraction. Immunohistochemistry pointed out this explanation, revealing that glands from diabetic rats increased SGLT1 protein detection in the stromal region of the parotid and in cytoplasm of ductal cells of the submandibular, proteins that were not included in the Western blotting analysis. Additionally, reduced SGLT1 staining was observed in the basolateral membrane of acinar cells in both the parotid and the submandibular glands of diabetic rats. These findings may explain why in diabetes, despite the increased SLC5A1 gene expression, the plasma membrane SGLT1 protein analyzed in the Western blotting experiment was decreased.

The expression of SGLT1 has been described especially in the luminal membrane of polarized epithelial cells (Hediger et al. 1995), thus the presently observed SGLT1 protein in the stromal region was unexpected. Regarding that, stromal SGLT1 might be related to diabetes-induced inflammation, leading to macrophagic cellular digestion (Hand and Weiss 1984) or autophagic/apoptotic cellular destruction (Hand and Weiss 1984; Robinson et al. 1997), all resulting in fragments of SGLT1 protein with a preserved immunoreactive epitope.

One important question concerns the functional repercussion of the changes in SLC5A1 gene expression and SGLT1 protein localization observed in glands of diabetic rats. This is very complex since no functional role has been proposed for SGLT1 in acinar cells, and the glucose flux from blood to primary saliva has not been clearly described yet. It has been proposed that from the interstitium to the acinar lumen, glucose can flow through paracellular and transcellular routes (Takai et al. 1983; Mangos et al. 1973). Considering the important paracellular glucose transport in acinous glands (Takai et al. 1983), we can suppose that the diabetes-induced reduction in SGLT1 protein in acinar cells may be not important for the glucose concentration in primary saliva.

Functional expression of SGLT1 in the luminal membrane of ductal cells is reported in the present study for the first time, and it can explain the previously reported phlorizin-sensitive glucose reabsorption in the duct (Takai et al. 1983). However, the participation of other SGLT isoforms cannot be excluded. Furthermore, increased SGLT1 protein expression was observed in the luminal membrane of ductal cells from the parotid of diabetic rats, and that may increase not only glucose but also water reabsorption. Considering SGLT1's high capacity of water transport, the increase in this protein in the luminal membrane of ductal cells may explain the decreased salivary flow rate observed in diabetic rats. In addition, this reduction in salivary volume may contribute to the increased salivary glucose concentration, despite its reabsorption. These data point out, for the first time, the role of SGLT1 as a water pump in salivary glands. Finally, the changes observed in the parotid were not detected in the submandibular, suggesting tissue-specific diabetes-induced regulation.

SGLT1 protein was also detected in myoepithelial cells, where it was reduced in diabetic animals, and that might also be involved in the reduced salivary flux. The lower the SGLT1 protein content, the lower the cellular glucose disposal (Wright et al. 2004), decreasing the main metabolic substrate for contractile activity. Decreased myoepithelial contraction might be involved in the reduction of salivary flux by permitting increased dilatation of the acinous and/or by reducing ductal flux propulsion.

In summary, our data reveal the presence of SGLT1 protein in ductal and myoepithelial cells of salivary glands. In ducts, as a water pump, SGLT1 could regulate salivary flux. Furthermore, the results point out that diabetes decreases nonstimulated salivary flux, which was accompanied by enhanced SLC5A1 gene expression and increased SGLT1 protein in ductal cells, despite its decrease in acinar and myoepithelial cells. These alterations can be reversed by insulin therapy and may explain the reduced salivary flow of diabetic subjects.

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