SGLT1 protein expression in plasma membrane of acinar cells correlates with the sympathetic outflow to salivary glands in diabetic and hypertensive rats


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SALIVARY GLAND FUNCTION IS REGULATED by both the sympathetic and parasympathetic nervous systems, which innervate secretory, myoepithelial, and vascular cells (19). However, the relative contribution to salivary secretion of these autonomic subdivisions, especially in pathological conditions, has not been well established. Salivary glands are target organs for postganglionic sympathetic axons arising from ganglion cells of the superior cervical ganglion (9). Electrical stimulation of sympathetic efferent branch to the salivary glands (14), or application of adrenergic agonists (40), results in a relatively low flow of saliva rich in protein (22). In addition, continuous electrical stimulation, leading to intense vasoconstriction, exacerbates the reduction in saliva secretion (2, 7, 12), a phenomenon that has been erroneously interpreted as a direct effect of the sympathetic activity upon salivary secretion (7).

Paradoxically, sympathectomy also generates decrease in salivary flow (21, 26) by reducing the secretory response to parasympathetic stimulation (17). These findings demonstrate the complexity of sympathetic regulation on salivary flux.

In salivary glands, the sodium glucose cotransporter 1 (SGLT1) participates in salivary dysfunctions through a sympathetic-and protein kinase A (PKA)-mediated pathway. In Wistar-Kyoto (WKY), diabetic WKY (WKY-D), spontaneously hypertensive (SHR), and diabetic SHR (SHR-D) rats, PKA/SGLT1 proteins were analyzed in parotid and submandibular glands, and the sympathetic nerve activity (SNA) to the glands was monitored. Basal SNA was threefold higher in SHR (P < 0.001 vs. WKY), and diabetes decreased this activity (~50%, P < 0.05) in both WKY and SHR. The catalytic subunit of PKA and the plasma membrane SGLT1 content in acinar cells were regulated in parallel to the SNA. Electrical stimulation of the sympathetic branch to salivary glands increased (~30%, P < 0.05) PKA and SGLT1 expression. Immunohistochemical analysis confirmed the observed regulations of SGLT1, revealing its location in basolateral membrane of acinar cells. Taken together, our results show highly coordinated regulation of sympathetic activity upon PKA activity and plasma membrane SGLT1 content in salivary glands. Furthermore, the present findings show that diabetic- and/or hypertensive-induced changes in the sympathetic activity correlate with changes in SGLT1 expression in basolateral membrane of acinar cells, which can participate in the salivary glands dysfunctions reported by patients with these pathologies.

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(WKY-D or SHR-D) by a single intravenous injection (penis vein) of alloxan [40 mg/kg body wt, (33)], and nondiabetic control rats (WKY and SHR) were injected with vehicle. Animals were individually caged and allowed free access to water and standard rodent chow diet (Nuvilab CR-1; Nuvital, Curitiba, Brazil). Animals were studied 1 mo after diabetes induction. Blood and tissue samples were obtained from anesthetized rats (pentobarbital sodium, 40 mg/kg body wt ip) at the end of the experimental period. Twenty-four-hour urine was collected before the experiments.

**Hemodynamic Measurements in Freely Moving Rats**

Arterial pressure was directly measured at the end of the experimental period, as described previously (8). In brief, a catheter was placed in the right femoral artery, tunneled subcutaneously, exteriorized, and fixed on the back of the neck under anesthesia (ketamine-xylazine, 0.7/0.2, vol/vol, 0.4 ml/kg body wt). After a 24-h recovery period, the catheter was connected to the digital recording system (P23Db Transducer, 3400 Recorder; Gould), and the animal was allowed to rest for stabilization of cardiovascular parameters for ~3 min. Basal values of arterial pressure and heart rate were then recorded in conscious and freely moving rats for 30 min.

**Monitoring of Sympathetic Nerve Activity to Salivary Glands**

**Surgical procedures.** In a second group of animals, the sympathetic nerve activity (SNA) to salivary glands was evaluated. On the day of the experiment, the rats were anesthetized with halothane (5% in O₂ inspired air), followed by urethane (750 mg/kg) administered via penial vein. Rectal temperature was measured by a thermometer and maintained between 36.5 and 37°C by using a heating blanket. A saline-filled polyethylene catheter (PE-10 connected to PE-50; Bioscop Australia, Huntingdale, Victoria, Australia) was inserted into the left femoral artery and vein. The arterial catheter was used for pressure recording and heart rate recordings. The venous catheter was used for injection of hexamethonium to block the sympathetic ganglion activity and determining the background noise of SNA to salivary gland. After a middle incision on the ventral surface of the neck, a catheter (PE-250) was inserted into the trachea to facilitate respiratory movements. During the experimental procedure, the animals were allowed to breathe spontaneously. Afterward, the left common carotid, the vagus nerve, the left superior cervical ganglion, the external carotid, and the postganglionic sympathetic branch, which project to the salivary glands along with the arterial supply (9), were carefully identified and dissected by using a stereomicroscope. A thin bipolar platinum electrode was placed around a branch of postganglionic fibers at C1 level for subsequent nerve activity monitoring.

**Data acquisition and analysis.** The raw SNA signal to the salivary gland was amplified (10 K of gain, AN502 Differential Amplifier; Tektronix, Beaverton, OR) and filtered (band pass filter 0.1–3.0 kHz). Further processing was performed using a data acquisition system assembled on a microcomputer equipped with an analog-to-digital converter (CODAS, 10-kHz sampling rate; Dataq Instruments).

Resting values of SNA, arterial blood pressure, and heart rate were recorded for 30 min. SNA values were expressed as a percentage of the maximal (100%) and minimal (0%) nerve discharge. The maximal SNA was induced by hypoxia-hypercapnia stimulus by blocking the tracheal catheter with a clip for 20 s, enough time to saturate the nerve discharge, which was totally reversible after catheter releasing. The minimal SNA was determined at the end of the experiment before the animal euthanasia by an intravenous bolus injection of hexamethonium (30 mg/kg body wt), a ganglion blocker that allows us to determine the basal SNA. Every animal underwent at least three hypoxia-hypercapnia stimuli every 30 min for comparison of consistent responses in the SNA. Rats were euthanized by an overdose of intravenous injection of urethane. The postmortem signal of nerve discharge was measured and used to subtract the background noise of the raw sympathetic nerve activity to achieve the real sympathetic outflow.

SNA data were analyzed offline in Spike 2 software (CED; Cambridge Electronic Design, Cambridge, UK) by measuring basal percentage of change and discharge rate among all groups studied.

**Stimulation of Sympathetic Efferent Branch to Salivary Glands**

To demonstrate that the efferent output of previously monitored SNA was projecting directly to the salivary glands and to demonstrate the effects of sympathetic outflow upon the PKA activity and plasma membrane SGLT1 protein content, WKY rats were anesthetized (pentobarbital sodium, 40 mg/kg body wt ip), and the left postganglionic nerve was stimulated at the same point in which the sympathetic nerve branch was previously recorded. A thin bipolar platinum electrode was placed around a branch of postganglionic fibers and stimulated at 50 Hz, 5 V, in bursts of 1 s every 10 s (21) for 2 h, using the PowerLab/430 stimulator (AD Instruments). After the stimulation period, the right (basal, nonstimulated) and left (stimulated) salivary glands were gently removed for protein analysis.

Another set of WKY rats was injected intravenously with saline or the nonselective β-adrenoceptor blocker propanolol (2 mg/kg, used to block the cyclic AMP-PKA pathway) 20 min before nerve stimulation began (18). The sympathetic branch of postganglionic fibers to salivary glands was stimulated for 30 min or 2 h at the same point, as described previously. After the stimulation period, the right (basal, nonstimulated) and left (stimulated) salivary glands were gently removed for immunohistochemical SGLT1 analysis.

**Harvesting of Salivary Glands**

Rats were anesthetized (pentobarbital sodium, 40 mg/kg body wt ip), blood was collected from the tail vein, and parotid and submandibular glands were carefully excised, weighed, and processed for further analysis.

**Western Blotting for SGLT1 and PKA proteins**

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 3,000 x g for 15 min at 4°C. The supernatant was kept, and the pellet was resuspended and centrifuged under the same conditions. Both supernatants were mixed and centrifuged at 7,000 x g for 30 min, and the final pellet was suspended as a plasma membrane-enriched fraction. 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) followed by enhanced chemiluminescence (Amersham Biosciences). After that, the membranes were stripped and reprobed with anti-β-actin antibody (Sigma-Aldrich, St. Louis, MO). The blots of single band were quantified by optical densitometry and the results expressed as arbitrary units in accordance with a previous study (33) after normalization by the respective β-actin value. For PKA analysis, samples (100 μg of protein) were collected from the first supernatant fraction, which represents total protein of salivary glands, electrophoresed, and probed using anti-rat catalytic subunit of PKA (Abcam, Cambridge, MA).

**Immunohistochemistry**

Tissues were fixed in 4% formaldehyde phosphate buffer (PB), followed by cryoprotection in cryosection sucrone solutions (10, 20, and 30%) in PB. Seven-micrometer-thick sections were placed on gelatin-coated slides (Sigma Chemical, St. Louis, MO) and subjected to immunodetection using anti-rat SGLT1 antibody (1:50; Chemicon International), followed by incubation with goat antiserum against rabbit IgG tagged to Cy5 (1:200; Molecular Probes, Eugene, OR). Controls for the experiments consisted of the omission of primary

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antibodies. F-actin staining was performed with rhodamin-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.

Analytical Procedures

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

Data Analysis

All values are presented as means ± SE. The number of animals used in each experiment is shown in the figure legends. Comparisons of the means were performed by one-way analysis of variance, followed by Student-Newman-Keuls posttest (GraphPad Prism version 4.00 for Windows; GraphPad Software, San Diego, CA).

RESULTS

Body weight, volume of urine, glycosuria, and glycemia were similar (Table 1) in WKY and SHR. As expected, diabetes reduced (P < 0.001) body weight and increased glycemia, urinary volume, and glucose excretion in both WKY and SHR diabetic animals (P < 0.01 to P < 0.001). The plasma insulin in SHR was higher (P < 0.05) than in WKY rats. Diabetes decreased plasma insulin in WKY (P < 0.05) and SHR (P < 0.001) compared with their respective controls. These data demonstrated the severity of the diabetic state achieved as well as the similarity of the diabetic state in both WKY and SHR.

Diabetes Does Not Alter Hemodynamic Parameters in Hypertensive Rats

Hemodynamic parameters were assessed in awakened, freely moving animals (Fig. 1). In WKY rats, diabetes did not alter diastolic, systolic, or mean arterial pressure, although the heart rate was decreased by 15% (P < 0.05). As expected, the blood pressure data increased by ~30% (P < 0.001) in SHR and the heart rate by 19% (P < 0.01). The induction of diabetes in SHR did not affect these parameters.

Diabetes Reduces SNA in Both Normotensive and Hypertensive Rats

To assess the sympathetic output to the salivary glands in the normal and diabetic state in normotensive and hypertensive animals, we recorded nerve activity from the postganglionic sympathetic fibers arising from the superior cervical ganglion in SHR and WKY animals. Representative recordings of the SNA to the salivary gland obtained during basal and hexamethonium-treated conditions in all groups are shown in Fig. 2, A–D. Decreased basal and discharge rate of SNA in both WKY-D (~60%, P < 0.001) and SHR-D (~40%, P < 0.05)
was observed when compared with the respective nondiabetic controls. In addition, we observed in Fig. 2, E and F, that SNA is higher in SHR when compared with WKY, demonstrating enhanced sympathetic activity in this strain. Furthermore, although diabetes reduced the SNA to a significant degree in SHR-D group, it remained higher than in WKY-D rats.

PKA and SGLT1 Expression is Correlated to SNA

The PKA catalytic subunit content in salivary glands was highly correlated to the degree of sympathetic activity, whereby higher levels of PKA catalytic subunit expression were observed in animals with high sympathetic outflow (Fig. 3, A and B). Moreover, SGLT1 protein expression mirrored PKA catalytic subunit expression (Fig. 3, B and C). In parotid and submandibular glands of WKY, diabetes significantly decreased PKA (30 and 31%; respectively, P < 0.01) and SGLT1 proteins (58 and 47%, respectively, P < 0.05). In parotid and submandibular glands of SHR, increased PKA protein content (86 and 38%, respectively, P < 0.01) and SGLT1 (164 and 68%; respectively, P < 0.001) were observed. In the SHR groups, although diabetes decreased PKA expression, a significant reduction of SGLT1 was observed only in parotid gland. These proteins remained significantly higher than that observed in nondiabetic WKY. The correlations between the mean values of sympathetic outflow (Table 2) and PKA or SGLT1 (arbitrary units) were confirmed statistically in both glands by linear Pearson correlation analysis (P values from 0.0280 to 0.0031, r² values from 0.9355 to 0.9939). Furthermore, the PKA and SGLT1 data were also highly correlated in both parotid (P = 0.023, r² = 0.9537) and submandibular (P = 0.0015, r² = 0.9971) glands.

To further investigate the effects of sympathetic activation on the expression levels of PKA and SGLT1, we electrically stimulated the sympathetic nerves supplying parotid and submandibular glands. The PKA and SGLT1 contents in salivary glands of WKY, after sympathetic nerve stimulation, are shown in Fig. 4. As expected, the PKA activity of sympathetically stimulated glands was increased (P < 0.001) in both the parotid (32%) and submandibular (29%) glands. In parallel to the increase in the catalytic subunit of PKA, plasma membrane SGLT1 protein content also increased in both glands after the stimulus (31 and 21% in parotid and submandibular, respectively, P < 0.05).

SNA-Induced SGLT1 Translocation to the Basolateral Membrane of Acinar Cells

To confirm the sympathetic activity role in SGLT1 protein translocation from cytoplasm to the basolateral membrane, we
performed immunohistochemical analysis of nonstimulated and sympathetically stimulated parotid glands of WKY rats subjected or not to previous blockade with propranolol (Fig. 5). As soon as after 30 min of stimulus, the SGLT1 protein content clearly increased in basolateral membrane and decreased in intracellular membrane (Fig. 5, A, B, E, and F). In basal (nonstimulated) parotid gland of propranolol-treated rats (Fig. 5, C and G), a high intracellular spread staining is observed, with low condensed reactivity in membrane, revealing that the β-blockade reduced the basal standard translocation to basolateral membrane. In glands of rats that received propranolol and were sympathetically stimulated (Fig. 5, D and H), the SGLT1 remained diffuse in intracellular membrane, and only a little amount of condensed immunoreactivity in basolateral membrane is observed compared with glands of rats not injected with propranol. Similar results were observed in glands from rats previously treated or not with propranolol but subjected to 2 h of sympathetic stimulus (Fig. 5, bottom).

Diabetes and Hypertension Alter the Subcellular Distribution of SGLT1

We also performed immunofluorescence studies to analyze the subcellular localization of SGLT1 protein in acinar cells of diabetic and/or hypertensive rats (Fig. 6). In parotid gland of WKY rat, immunofluorescence staining shows SGLT1 protein mainly in basolateral membrane, although it can be seen in intracellular membrane (Fig. 6, A and E). In diabetic WKY rat (Fig. 6, B and F), the SGLT1 protein is also present in basolateral membrane of acinar cells, but a strong and diffuse intracellular staining can be observed, indicating reduced trans-
location. In parotid gland of SHR rats (Fig. 6, C and G), SGLT1 immunoreactivity is highly increased in basolateral membranes, which is accompanied by weak intracellular staining. In SHR, diabetes decreased the membrane staining intensity, increasing the diffuse intracellular immunoreactivity (Fig. 6, D and H). Similar results of subcellular distribution and content of SGLT1 protein were also observed in submandibular glands (Fig. 6, bottom).

DISCUSSION

The present study investigated the effects of diabetes or hypertension and their association with sympathetic outflow, PKA, and Na⁺-glucose cotransporter SGLT1 protein expression in salivary glands. Importantly, the sympathetic activity to salivary glands has never been directly measured in diabetic and hypertensive animals despite its critical role in regulating salivary composition and flux, which is linked to the development and/or maintenance of the oral diseases that affect diabetic and hypertensive subjects.

In the present study, we are showing for the first time in diabetic hypertensive and normotensive rats an in vivo approach for measuring the sympathetic outflow projecting directly to the salivary glands. The results show evidence of increased sympathetic nerve activity in SHR and SHR-D compared with WKY and WKY-D rats. Furthermore, in both strains, diabetes reduced the sympathetic nerve activity. Morphological damage to the sympathetic innervation of salivary glands in diabetic rats includes fiber degeneration (5, 16) and decreased number and size of fibers (41) as well as focal dilatations of axons and accumulation of subcellular organelles. Additionally, reduced noradrenaline content and release in sympathetic neurons of salivary glands has been described in diabetic rats (40). Taken together, these data suggest that diabetes induces autonomic neuropathy that alters sympathetic innervation of the salivary glands. However, by showing decreased sympathetic nerve activity to the salivary glands of diabetic rats, the present study strongly supports previous observations of morphological damage to the sympathetic innervation to salivary glands. Nevertheless, increased sympathetic activity to the salivary glands was also shown in hypertensive rats for the first time.

Bearing in mind the classic effects of autonomic outflow to salivary glands, the diabetes-induced decrease in the sympathetic activity to this territory might explain the previously
described reduction in salivary protein content (3, 4, 25, 28) and the proposed increase in baseline blood perfusion of salivary glands of diabetic rats (1). On the other hand, the high sympathetic activity detected in SHR might induce converse modulations in salivary composition and blood flow. In fact, gradual increase of sympathetic stimulus was reported to be accompanied by parallel increase in salivary protein content in normal rats (6), but these parameters have never been investigated in diabetic nor in hypertensive rats.

Taking into account that the \(\beta\)-adrenergic receptor is the most abundant adrenergic receptor in the acinar cells, which in turn are the most abundant cells in the salivary glands, it should be expected that the increased or decreased sympathetic activity measured in experimental conditions would be accompanied by parallel changes in salivary protein content in normal rats (6), but these parameters have never been investigated in diabetic nor in hypertensive rats.

The \(\beta\)-adrenergic receptor is the most abundant adrenergic receptor in the acinar cells, which in turn are the most abundant cells in the salivary glands, it should be expected that the increased or decreased sympathetic activity measured in experimental conditions would be accompanied by parallel changes in the PKA activity. To our knowledge, this is the first report investigating the catalytic subunit of PKA in the parotid and submandibular glands, and the observed regulations correlate with the measured sympathetic activity. In normal rats, we confirmed that the sympathetic stimulation is accompanied by increased PKA activity and established the importance of the \(\beta\)-adrenergic system in the glands.

We have previously reported decreased SGLT1 protein content in the parotid and submandibular glands of diabetic rats, which could be reversed by insulin treatment (33). In the present study, we confirmed the effect of diabetes and extended these findings by exploring the potential mechanism by which sympathetic activity, via PKA, could be regulating the SGLT1 content in plasma membrane. In SGLT1-transfected oocytes, it has been reported that the activation of the cAMP-PKA pathway enhances the SGLT1 protein content in plasma membrane (23). Here, we are showing a strictly coordinated regulation of sympathetic activity, catalytic subunit of PKA, and SGLT1 protein in plasma membrane of diabetic normotensive and hypertensive rats. These results strongly suggest that the sympathetic effects upon SGLT1 in salivary glands are the same as those demonstrated in transfected oocytes. Additionally, it is important to highlight that our current findings reflect the

![Image of immunolocalization of SGLT1 protein in acinar cells of parotid glands from WKY rats that received physiological saline (A, B, E, and F) or propranolol (C, D, G, and H) and were subjected to 30 min (top) or 2 h (bottom) of sympathetic stimulation. A–D: SGLT1 (green), F-actin (red), and nuclear marker (blue). E–H: only SGLT1 in green color. Scale bar, 30 \(\mu\)m. I and J: negative controls for SGLT1 immunoreactivity are shown (top). Arrows and arrowheads indicate membrane condensed and intracellular spread SGLT1 protein, respectively. Images are representative of 4 animals in each group.](http://ajpendo.physiology.org/)
SGLT1 regulation of acinar cells, where the role of SGLT1 has not yet been clearly defined. That may be different from the potential role of SGLT1 in luminal membrane of ductal cells or plasma membrane of myoepithelial cells, where the SGLT1 has also been observed (33).

The glucose flux from blood to primary saliva has not yet been clearly described. Considering the great paracellular glucose transport in acinous (39), we envisage that diabetes-induced reduction in SGLT1 protein in acinar cells does not play an important role for glucose concentration in primary saliva.
saliva. This can be reinforced by the observation that the microinjection of phlorizin, a SGLT1 inhibitor, reaching the basolateral membrane of acinar cells, did not alter the glucose concentration (39). Most likely, the main role of SGLT1 in basolateral membrane of acinar cells is to provide glucose for the cellular metabolism. This way, SGLT1 regulation could importantly participate in sympathetic effects such as altering salivary protein concentration by modulating acinar cell metabolism.

The imunohistochemistry analysis of SGLT1 protein in the plasma membrane of acinar cells in both the parotid and submandibular glands of diabetic, hypertensive, or normotensive rats revealed the same changes as those observed by Western blotting analysis in a subcellular fraction enriched in plasma membrane protein. The staining intensity surrounding the cell, which represents the SGLT1 translocation into the plasma membrane, clearly correlated with the local sympathetic activity of the animals. Furthermore, in response to sympathetic stimulus, with or without propranolol blockade, we confirmed the β-adrenergic involvement in SGLT1 subcellular distribution. Thus, we propose a sympathetic-induced pathway leading to SGLT1 translocation into the plasma membrane of acinar cells in salivary glands (Fig. 7). Our results indicate that diabetes suppresses whereas hypertension activates this pathway.

In summary, we show that diabetes induces significant reductions in sympathetic activity to salivary glands in both normotensive and hypertensive rats, with the latter group showing a high sympathetic input to the salivary glands. The degree of sympathetic input to the salivary glands is highly correlated with the regulation of catalytic subunit of PKA and SGLT1 protein in plasma membrane of acinar cells of salivary glands in diabetic, SHR, and SHR-D compared with nondiabetic WKY rats. Additionally, in salivary glands of WKY rats, electrical stimulation of sympathetic nerves supplying salivary glands was followed by increased PKA activity and SGLT1 content in plasma membrane. This study demonstrates that SGLT1 protein expression into the basolateral membrane of acinar cells correlates with the local sympathetic activity in salivary glands. Furthermore, the present findings show that diabetic- and/or hypertensive-induced changes in the sympathetic activity correlate with changes in SGLT1 expression in basolateral membrane of acinar cells, and that can play a role in the salivary gland dysfunctions reported by patients with these pathologies.

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DISCLOSURES

The authors have nothing to disclose.
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