ORIGINAL ARTICLE

Salivary gland hypofunction in KK-A^y type 2 diabetic mice

Highlights
- Saliva secretion from type 2 diabetic (T2D) mice decreased at the glandular level.
- Membrane proteins critical for fluid secretion are preserved in T2D mice.
- Decreased calcium signaling is the key for diabetes-induced hypofunction of salivary glands.

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Abstract

Background: Hypofunction of different organs in the body is associated with diabetes, including in the oral cavity. Diabetes is often associated with xerostomia, but the underlying mechanism is not well characterized. Thus, the mechanisms underlying diabetes-induced xerostomia were investigated in this study in KK-A^y mice as an experimental model of type 2 diabetes.

Methods: The mechanisms involved in diabetes-induced xerostomia were investigated using the ex vivo glandular perfusion technique, histological analysis, and immunohistochemical and intracellular signaling analyses.

Results: Ex vivo submandibular gland secretions from KK-A^y mice decreased by 30% following stimulation with 0.3 μmol/L carbachol (CCh), a cholinergic agonist. Acinar cell weight was comparable between KK-A^y and control mice, whereas duct cell weight was significantly greater in KK-A^y mice. Concentrations of Na^+ and Cl^- in the secreted saliva decreased significantly in KK-A^y mice, supporting the finding of increased ductal tissue in KK-A^y mice. Immunohistochemistry revealed no significant differences between KK-A^y and control mice in terms of the expression of Cl^- and water channels, Na^+-K^+-2Cl^- cotransporters, and membrane proteins critical for fluid secretion. Cellular signaling analysis revealed that the increase in [Ca^{2+}]_i in response to 0.3 μmol/L CCh was reduced by 30% in KK-A^y mice, although there was no significant difference in the thapsigargin (1.0 μmol/L)-induced increase in store-depleted calcium between KK-A^y and control mice.

Conclusions: These results demonstrate that submandibular fluid secretion is diminished in KK-A^y mice because of a diminished increase in [Ca^{2+}]_i. Duct cell weight increased in KK-A^y mice, possibly leading to increased ion reabsorption and thus decreased Na^+ and Cl^- concentrations in the secreted saliva.

Keywords: cell signaling, saliva, saliva physiology.
component of stimulated saliva in humans and mice. The basic secretory mechanisms are almost identical among these glands. Salivary glands are innervated and controlled by both the sympathetic and parasympathetic nervous systems; in particular, activation of muscarinic cholinoreceptors plays a major role in fluid secretion.

Muscarinic cholinoreceptors are present in the basolateral membrane of acinar cells, secretory end pieces. Following cholinoreceptor activation, there is an increase in \([Ca^{2+}]_i\), mediated via the inositol 1,4,5-trisphosphate (IP3) pathway and calcium-activated calcium influx mechanisms. This increase in \([Ca^{2+}]_i\), then activates anion channels located in the luminal membrane, namely calcium-activated chloride channel transmembrane member 16A (TMEM16A), also known as anoctamin-1, with subsequent anion release into the lumen triggering fluid secretion. Anion accumulation induces cation movement into the lumen, resulting in an osmotic gap between inside the cell and the lumen. Consequently, osmotic pressure induces water movement into the lumen through the water channel aquaporin-5 (AQP5) and tight junctions. Acinar cells are polarized cells; \(Na^+–K^+–2Cl^−\) cotransporters (NKCC1) in the basolateral membrane stimulate the accumulation of \(Cl^−\) (up to 60 mmol/L) in acinar cells. In contrast, duct cells reabsorb large quantities of \(Na^+\) and \(Cl^−\) from the primary saliva produced by acinar cells. The final saliva in the oral cavity then becomes hypotonic relative to plasma.

Reduced salivary gland function is observed in Sjögren’s syndrome, as well as after radiation therapy for head and neck cancerous lesions, where inflammation is observed in acinar cells. Reduced salivary gland function is also associated with metabolic diseases, such as diabetes mellitus. Some of the effects of diabetes on salivary glands include diabetes-induced reductions in sympathetic activity to the salivary gland, a possible role for autonomic neuropathy in the abnormal response of the parotid gland to parasympathetic nerve stimulation in diabetic rats, and reduced parasympathetic vasodilatation in the submandibular gland (SMG) in diabetic rats, which may result in salivary dysfunction. Furthermore, diabetes increases the expression of sodium–glucose cotransporter 1 (SGLT1), which works as a water transporter, in salivary duct cells, which may be one of the causes of diabetes-induced salivary dysfunction.

Methods

Materials and animals

Unless stated otherwise, reagents were purchased from Sigma Japan (Tokyo, Japan). Diabetic KK-A\(^v\) and control C57BL/6J mice, 4–8 weeks of age, were purchased from CLEA Japan (Tokyo, Japan). Mice were maintained under a 12-h light/dark cycle and were fed ad libitum. All experiments were approved by the Animal Committee of Kyushu Dental University (No. 14-001 and No. 15-011).

Blood glucose measurements

Venous blood was drawn from the tail vein of KK-A\(^v\) and control mice aged 4, 6 and 8 weeks. Blood glucose levels were measured using the glucose dehydrogenase flavin adenine dinucleotide method and a FreeStyle Freedom Lite glucose monitor (Abbott Japan, Chiba, Japan).

Ex vivo perfusion of mouse SMG

The details of the surgical procedures used in the present study have been described previously. Briefly, mice were anesthetized with chloral hydrate (400 mg/kg, i.p.), after which the SMGs were removed, along with the common carotid artery and duct. The carotid artery was immediately cannulated and perfused at a rate of 1 mL/min with physiological saline solution (PSS; composition [in mmol/L]: NaCl 120; KCl 4.3; NaHCO_3 25; MgCl_2 1.0; CaCl_2 1.0; glucose 5; HEPES 10) equilibrated with 95% O_2 and 5% CO_2. Saliva secreted in response to the muscarinic agonist carbachol (CCh; 0.3 μmol/L) was collected over a 10-min stimulation period into a glass capillary tube. The collected saliva was stored in 500-μL tubes at −80°C until analysis. Salivary Na\(^+\) and Cl\(^−\) concentrations were determined with electrodes using the Dri-Chem 7000 system (Fuji Film Medical, Tokyo, Japan).

Histological analysis

After SMGs had been dissected from mice, they were fixed in 4% paraformaldehyde phosphate buffer solution for 24 h before being embedded in paraffin. Sections were deparaffinized before hematoxylin–eosin (HE) staining and were subsequently examined under a digital microscope (VHX-5000; Keyence, Osaka, Japan). The area of acinar and duct cells in the SMG was calculated from HE-stained images using the automatic area measurement function of the digital microscope.
Detection of NKCC1, AQP5, and TMEM16A in mouse SMGs

Mouse SMGs were processed for immunohistochemistry as described previously. Prepared sections were incubated overnight with an anti-peptide antibody against TMEM16A (Abcam, Tokyo, Japan), AQP5 (EMD Millpore, Darmstadt, Germany), or NKCC1 (Santa Cruz Biotechnology, Santa Cruz, TX, USA) and immunostaining was developed as described previously.

Measurement of $[Ca^{2+}]_i$

In salivary acinar cells, $[Ca^{2+}]_i$ is an important intracellular signal for water secretion. Submandibular glands were surgically isolated from mice that had been anesthetized with chloral hydrate (400 mg/kg, i.p.). Glands were minced using fine scissors and then digested with 520 U/mL collagenase L (Nitta Gelatin, Osaka, Japan) for 15 min at 37°C. The digested tissue was washed three times with Eagle’s minimum essential medium (MEM; Life Technologies, Carlsbad, CA, USA) and dispersed in 5 mL MEM; the cells were then incubated for 10 min at 37°C with 2 μmol/L Fura-2-acetoxymethyl ester (Fura-2AM). The $[Ca^{2+}]_i$, in Fura-2AM-loaded acinar cells was measured in the presence of external $Ca^{2+}$, using the aforementioned PSS, after the addition of 0.3 μmol/L CCh or 1 μmol/L thapsigargin. To investigate $Ca^{2+}$ mobilization in the absence external $Ca^{2+}$, the PSS was replaced with calcium-free solution, in which the $CaCl_2$ in the PSS was replaced with NaCl. Fluorescence was detected under a microscope equipped with a fluorescence analysis system (Aquacosmos; Hamamatsu Photonics, Hamamatsu, Japan) at an excitation wavelength of 340 or 380 nm and an emission wavelength of 510 nm. The $[Ca^{2+}]_i$ is expressed as a ratio of fluorescence at 340/380 nm. The increase in $[Ca^{2+}]_i$ was calculated from the integral value of $[Ca^{2+}]_i$ for the CCh or thapsigargin stimulation periods as the area under the curve.

Statistical analysis

Data are presented as the mean ± SEM. The significance of differences between control and KK-A^y^ mice was evaluated using unpaired Student’s t-test. P < 0.05 was considered significant. All experiments were performed using at least three different mice for each condition, “n” referring to the number of experiments performed.

Results

Body weight, gland weight, and blood glucose concentrations

Body weight increased in both KK-A^y^ and control mice with age (Fig. 1a). Body weight was comparable between 4-week-old KK-A^y^ and control mice, but was significantly greater in 6- and 8-week-old KK-A^y^ than control mice (P < 0.001). The weight of SMGs was significantly lower in 4-week-old KK-A^y^ than control mice (P < 0.05), but at 6 and 8 weeks of age was significantly greater in KK-A^y^ mice (P < 0.001; Fig. 1b). Blood glucose concentrations in KK-A^y^ and control mice are shown in Fig. 1c. Blood glucose concentrations were stable in control mice at all ages, but increased with age in KK-A^y^ mice. Although blood glucose concentrations were comparable between KK-A^y^ and control mice at 4 weeks of age, they were significantly higher in KK-A^y^ mice at 6 and 8 weeks of age (Fig. 1c).

Salivary secretion by ex vivo mouse SMG

Salivary flow rates in KK-A^y^ and control mice are shown in Fig. 2a,b. Perfusion of SMGs with the muscarinic agonist CCh (0.3 μmol/L) induced stable salivary flow in both KK-A^y^ and control mice. The total volume of saliva secreted by SMGs from KK-A^y^ and control mice during the 10-min stimulation period is shown in Fig. 2c. At all ages examined, SMGs from KK-A^y^ mice secreted significantly less saliva than SMGs from control mice, with the difference between KK-A^y^ and control mice increasing with age.

In 6- and 8-week-old KK-A^y^ mice, the volume of saliva secreted during the 10-min stimulation period normalized against gland weight was significantly lower than in control mice (Fig. 2f). Similarly, concentrations of both Na^+^ and Cl^−^ in the saliva were significantly lower in 6- and 8-week-old KK-A^y^ than control mice (Fig. 2g,h).

Acinar and duct cells in mouse SMG

To investigate the mechanism underlying the reduced salivary function in KK-A^y^ mice, morphological analyses were conducted. As indicated in HE-stained images of SMGs from KK-A^y^ and control mice (Fig. 3a,b), there was a decrease in the area of acinar cells in KK-A^y^ compared with control mice, whereas the duct cell area was greater in KK-A^y^ mice. As indicated in Fig. 1b, the weight of SMGs from KK-A^y^ mice was greater than that of SMGs from control mice. Therefore, we calculated acinar and duct cell weight on the basis of gland weight (Fig. 1b) and acinar and duct cell area (Fig. 3c). Acinar cell weight was comparable...
between KK-Aʸ and control mice (Fig. 3d), whereas duct cell weight was significantly greater in KK-Aʸ mice (Fig. 3e), indicating that decreased salivary secretion in KK-Aʸ mice was not caused by a reduction in acinar cells.

To confirm the secretary function of acinar cells in KK-Aʸ and control mice, salivary flow rate (Fig. 3f,g) and the total volume of saliva secreted during the 10-min stimulation period (Fig. 3h) were normalized against acinar cell weight. As indicated in Fig. 3h, the total volume of saliva secreted, normalized against acinar cell weight, was comparable between KK-Aʸ and control mice at 4 weeks of age, but significantly lower in KK-Aʸ than control mice at 6 and 8 weeks of age.

Expression of TMEM16A, AQP5, and NKCC1 in SMGs from KK-Aʸ and control mice

To investigate difference in acinar cell function between KK-Aʸ and control mice, immunohistochemistry was used to detect three major membrane proteins in acinar cells, namely TMEM16A, AQP5, and NKCC1. In control mice, TMEM16A was expressed at the acinar apical membrane and intercalated duct cells (Fig. 4a), whereas in KK-Aʸ mice it was expressed at only the acinar apical membrane (Fig. 4b). In contrast, AQP5 was expressed at the apical membrane of the acinar cells in both control (Fig. 4c) and KK-Aʸ mice (Fig. 4d) and NKCC1 was highly expressed at the basolateral membrane of acinar cells and faintly expressed in duct cells (Fig. 4e,f).

Analysis of [Ca²⁺]i signaling

In the present study, [Ca²⁺]i signaling in response to 0.3 μmol/L CCh in the presence of extracellular Ca²⁺ was investigated. In these experiments, CCh induced an increase in [Ca²⁺]i in SMGs from both KK-Aʸ and control mice (Fig. 5a). The increase in [Ca²⁺]i, was calculated as the area under the curve during CCh stimulation. Acinar cells from KK-Aʸ mice exhibited a significantly smaller increase in [Ca²⁺]i than acinar cells from control mice, indicating that reduced [Ca²⁺]i may be responsible for the salivary dysfunction in KK-Aʸ mice. The increase in [Ca²⁺]i was made up of two stages: (i) Ca²⁺ release from the endoplasmic reticulum (ER); and (ii) store-operated Ca²⁺ entry (SOCE). Which of these stages was impaired in KK-Aʸ mice was determined by performing experiments in the absence of extracellular Ca²⁺ or in the presence of 1.0 μmol/L thapsigargin, respectively. In the absence of extracellular Ca²⁺, the increase in [Ca²⁺]i, in acinar cells was lower in both the KK-Aʸ and control groups (Fig. 5b). In the presence of thapsigargin, which releases Ca²⁺ by inhibiting the Ca²⁺-ATPase in the ER, the thapsigargin-induced increase in [Ca²⁺]i, in acinar cells was comparable between the KK-Aʸ and control groups, suggesting that SOCE was not diminished by diabetes (Fig. 5c).

Discussion

In the present study, we used KK-Aʸ mice to analyze the mechanism underlying diabetes-induced
xerostomia. The KK-A^Y mouse, a model of type 2 diabetes (T2D), was established by Nishimura. To create KK-A^Y mice, the Ay mutation at the agouti locus was introduced into the KK strain, which was developed by Kondo et al. Although KK mice are also a model of T2D, KK-A^Y mice develop diabetes more stably because of the introduction of the Ay mutation. In the present study, we were able to...
Figure 3  Gland weight and cell composition of submandibular glands (SMGs) from 8-week-old control and KK-A^Y^ mice. (a,b) Representative hematoxylin and eosin (HE)-stained images of SMGs from control (a) and KK-A^Y^ (b) mice. Bars, 200 μm. (c) Area of acinar and duct cells calculated from HE-stained images. The area of duct cells was significantly greater in KK-A^Y^ than control mice (n = 9 and 8, respectively). (d,e) Weight of acinar (d) and duct (e) cells per, calculated as gland weight (mg) × acinar cell area (%). Although acinar cell weight in SMGs was comparable between the KK-A^Y^ and control groups, duct cell weight was significantly greater in KK-A^Y^ than control mice. (f,g) Salivary flow rate normalized by acinar cell weight in control (f) and KK-A^Y^ (g) mice following stimulation with 0.3 μmol/L carbachol (CCh). (h) Total amount of saliva secreted over a 10-min period of CCh stimulation normalized by acinar cell weight. Normalized for acinar cell weight, the volume of saliva secreted was significantly lower in KK-A^Y^ than control mice at 6 and 8 weeks of age. Where appropriate, data are given as the mean ± SEM. **P < 0.01, ***P < 0.001.
confirm that KK-A^y mice developed diabetes symptoms of obesity and hyperglycemia because their body weight and blood glucose concentrations were higher than in control (C57BL/6J) mice. Because in our experiments in vivo stimulation (i.e. intraperitoneal injection) with a muscarinic agonist kills KK-A^y mice, in the present study we used an ex vivo perfusion technique. We speculate that the death of KK-A^y mice following intraperitoneal injection of muscarinic agonists is associated with coronary artery disease (CAD), because T2D increases the risk of CAD. It has been reported muscarinic agonists induce coronary artery spasm in patients who have vascular endothelial cell abnormalities.26,27

According to previous reports, diabetic patients have reduced salivary secretion.28,29 As expected, in the present study the salivary secretion of KK-A^y mice was significantly decreased. Therefore, we hypothesized that organic changes in the salivary glands are the cause of reduced salivary secretion, and tested this using histological analyses. There was no significant difference in acinar cell weight between KK-A^y and control mice. Furthermore, we examined the expression of three important membrane proteins for salivation in KK-A^y and control mice and found no obvious differences between the two groups except for the disappearance of TMEM16A expression in intercalated ducts in KK-A^y mice. It has been reported that TMEM16A is expressed...
hand panels show the time course of the [Ca\textsuperscript{2+}]i increase, as the ratio (a) of extracellular Ca\textsuperscript{2+}. (c) However, there was no signif

Figure 5  Increases in [Ca\textsuperscript{2+}]i in Fura-2-acetoxymethyl ester (Fura-2AM)-loaded submandibular gland acinar cells from 8-week-old KK-A\textsuperscript{y} and control mice in response to either (a,b) the muscarinic agonist carbachol (CCh; 0.3 \mu M) in the presence (a) or absence (b) of extracellular Ca\textsuperscript{2+}, or (c) 1 \mu M thapsigargin, a calcium pump inhibitor, again in the presence or absence of extracellular Ca\textsuperscript{2+}. The left-hand panels show the time course of the [Ca\textsuperscript{2+}]i increase, as the ratio of fluorescence at 340/380 nm, whereas the area under the curve for the period of CCh or thapsigargin stimulation is shown in the graphs on the right. (a,b) The increase in [Ca\textsuperscript{2+}]i was significantly lower in response to CCh in the KK-A\textsuperscript{y} than control group, regardless of the presence (n = 8 and 9, respectively) or absence (n = 5 and 6, respectively) of extracellular Ca\textsuperscript{2+}. (c) However, there was no significant difference in the increase in [Ca\textsuperscript{2+}]i between the KK-A\textsuperscript{y} and control groups in response to thapsigargin (n = 5 and 6, respectively). Data are the mean ± SEM. *P < 0.05, **P < 0.01.

determine why TMEM16A disappeared from duct cells in KK-A\textsuperscript{y} mice. Controlled temporal and spatial regulation of [Ca\textsuperscript{2+}]i is necessary for fluid secretion.\textsuperscript{21} In the present study changes in [Ca\textsuperscript{2+}]i were measured in response to the muscarinic agonist CCh (0.3 \mu M/L) under different conditions (i.e. in the presence and absence of external Ca\textsuperscript{2+}; Fig. 5a–c). Regardless of the presence or absence of extracellular Ca\textsuperscript{2+}, both the initial response of [Ca\textsuperscript{2+}]i and the sustained phase were significantly decreased in KK-A\textsuperscript{y} mice (Fig. 5a,b), suggesting that the muscarinic response is decreased in KK-A\textsuperscript{y} mice, presumably due to the release of Ca\textsuperscript{2+} stores, such as from the ER, because this is the one of the most apparent effects of diabetes.\textsuperscript{30} However, SOCE may not be affected by diabetes stress in KK-A\textsuperscript{y} mice because thapsigargin, a store pump inhibitor, had no effect on [Ca\textsuperscript{2+}]i (Fig. 5c). Conversely, several studies have demonstrated that the increase in [Ca\textsuperscript{2+}]i is associated with AQP5 translocation into the acinar luminal membrane, and impaired AQP5 translocation is induced by diabetes.\textsuperscript{31,32} Thus, the diminished increase in [Ca\textsuperscript{2+}]i in KK-A\textsuperscript{y} mice found in the present study may affect AQP5 translocation, which, in turn, may result in salivary dysfunction.

The relationship between diabetes and salivary glands is often discussed with regard to oxidative stress.\textsuperscript{33–35} Secretory glands are affected in diabetes, but, with regard to fluid secretion, it remains contentious as to whether diabetes directly reduces salivary flow.\textsuperscript{36} In the present study we demonstrated that diabetes mellitus significantly reduced muscarinic agonist-stimulated fluid secretion by approximately 30%, even though the acinar cell weight was identical between KK-A\textsuperscript{y} and control mice. One possible explanation for the reduced flow in KK-A\textsuperscript{y} mice is the increase in the ductal cell population. Water-permeable ductal cells are thought to be present in some tissues.\textsuperscript{37} Although we did not find AQP5 water channels in duct cells in mouse SMGs, SGLT1, which is located at the luminal membrane in salivary duct cells, may work as a water pump\textsuperscript{38} and induce water reabsorption by duct cells. Furthermore, we speculate that the increase in the duct cell population reduced Na\textsuperscript{+} and Cl\textsuperscript{−} concentrations in the secreted saliva because of epithelial sodium channels and/or cystic fibrosis transmembrane conductance regulator Cl\textsuperscript{−} channels located in the apical membranes of duct cells.\textsuperscript{39} Despite some limitations, this animal study improves our understanding of the effect of T2D in the oral environment.

Conclusion

The results of the present study demonstrate that submandibular salivation decreases in KK-A\textsuperscript{y} mice due to
reductions in Ca\(^{2+}\) release from the ER, potentially caused by hyperglycemia-induced ER stress. Duct cell weight increases in KK-A\(^{y}\) mice, possibly leading to an increase in ion reabsorption and decreased concentrations of Na\(^{+}\) and Cl\(^{-}\) in the saliva. Therefore, ion compositions may become useful diagnostic tools in the prediction of diabetes. Well-controlled human studies are necessary in the future to improve our understanding of the effect of T2D on saliva secretion.

Acknowledgement

This work was supported by a grant from Japan Society for the Promotion of Science (JSPS) KAKENHI (No. 16H05527 and 25463011).

Disclosure

None declared.

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