Lowered Susceptibility of Muscarinic Receptor Involved in Salivary Secretion of Streptozotocin-Induced Diabetic Rats

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ABSTRACT—We investigated the responses of salivary secretion and the susceptibility of the muscarinic receptors in the salivary glands of the streptozotocin (STZ)-induced diabetic rats (STZ rats). Giving water ad libitum, the amount of whole saliva with no stimulation was similar in the STZ and the control rats. Pilocarpine increased salivary secretion in both groups, although the effect in the STZ rats was two to three fold less than in the control rats. If the animals were restricted from taking water for 6 h, salivary secretion was not slightly changed in the STZ rats in spite of a remarkable increase in the control. An obvious decrease in salivary secretion of the STZ rats was negatively correlated with an increase in urination. Furthermore, salivary secretion from the parotid gland was increased in a dose-dependent manner with pilocarpine in the control rats, but not in the STZ rats. In the \(^{3}H\)quinuclidinyl benzilate (QNB) binding studies for muscarinic receptor of the STZ rats, \(B_{\text{max}}\) was decreased in the parotid gland and \(K_{d}\) was increased in the submandibular gland. Competitive inhibition of \(^{3}H\)QNB binding to both glands showed an increase in IC\(_{50}\) of pilocarpine and carbachol. These results suggest that a decrease in salivary secretion of STZ rats is not only induced by a water loss, but also closely associated with the lowered susceptibility of the muscarinic receptors.

Keywords: Salivary secretion, Salivary gland, Muscarinic receptor, Streptozotocin, Diabetic rat

Generally, parasympathetic nerve stimulation induces a considerable flow of saliva that has a low protein content. Sympathetic stimulation results in a relatively low flow of saliva that is rich in protein (1). Dry mouth (xerostomia) is a frequent clinical complaint and is a condition with a marked decrease in the ability of the salivary glands to synthesize, transport and secrete saliva (2 – 4). It is considered that xerostomia occurs by the primary effect due to a consequence of irreversible damage to the salivary glands and by the secondary effect due to the systemic dehydration resulted from polyuria (5). It is commonly known that various chronic complaints such as diabetes mellitus, cirrhosis, chronic renal failure, chronic sialoadenitis and autoimmune diseases induce xerostomia. Alteration of salivary flow and morphological changes in the salivary glands were found in diabetics (6 – 10). Furthermore, numerous investigations have demonstrated the effects of experimental diabetes using streptozotocin (STZ) and alloxan on the structure and function of the salivary glands in the rats (11 – 15) and mice (16, 17). Especially, STZ-induced diabetic rats (STZ rats) has been commonly used in many investigations concerning diabetic mellitus, since it was reported in experimental animals that STZ is diabetogen and induces clinical signs seen in the patients (18, 19). Studies on salivary secretory system using STZ rats showed that a decrease in salivary secretion induced by drugs or electronic stimulation could be associated with histological changes in the salivary glands (20 – 26).

Despite the documentation of both physiological and histological changes, there have been few reported pharmacological studies on saliva secretory responses in experimental diabetic animals. Especially, characteristics of the muscarinic receptor involved in saliva flow by muscarinic receptor stimulation with pilocarpine (17) are not well studied, and there are no reports about salivary secretion on loosing water by polyuria. Therefore, we examined the saliva secretory responses in the various external conditions and the characteristics of muscarinic receptor in the salivary gland membranes in STZ rats.
MATERIALS AND METHODS

Chemicals
STZ, carbachol hydrochloride (carbamylcholine chloride), bovine serum albumin, protease inhibitors were purchased from Sigma Chemical (St. Louis, MO, USA). Pilocarpine hydrochloride and atropine sulfate were purchased from Wako Pure Chemical Industries (Osaka) and Research Biochemical International (Natick, MA, USA), respectively. Insulin (Lente, MC40) was purchased from Novo Industries. \(^{3}H\)Quinuclidinyl benzilate (QNB) were obtained from Du Pont/New England Nuclear (Boston, MA, USA). All other reagents were purchased from analytical grade.

Animals
Male Wistar strain rats weighing 200 – 250 g were purchased from Japan SLC, Inc. (Hamamatsu). They were housed in an air-conditioned room (temperature: 23 ± 2°C, humidity: 55 ± 5%, and lighting 6:00 AM – 6:00 PM) and maintained on commercial laboratory chow and water. All animals received humane care in accordance with the Guideline for the Treatment of Experimental Animals approved by Tokyo Dental College.

Induction of diabetes
Rats were starved overnight and administered with a single intraperitoneal injection of STZ at 60 mg/kg in a volume of 0.2 ml per 100 g body weight. STZ was dissolved in physiological saline. Control rats were given an equal volume of physiological saline alone. Two and five days after administration with STZ, the urinary glucose levels of rats were inspected by test strips. Two weeks after administrating STZ, the rats showing a positive reaction to the glucosuria test were used in the experiments. To evaluate the effect of insulin on the functional recovery in the salivary gland of diabetic rats, insulin (4 units/day) was subcutaneously injected daily to the back of rats on the 7th until the 13th day after the STZ administration.

Saliva collection
Collection of whole saliva by absorption method: The whole saliva of a rat was measured accordingly to the method of Yamanaka et al. (27). Briefly, three pellets of paper weighing 30 mg and a container (polyethylene microtube of 1 ml) were prepared for each measurement. Immediately after wiping remaining saliva in the oral cavity with a paper ball, the whole saliva in the oral cavity was collected by absorbing it into three pellets for 30 s at each time point. Each pellet was exchanged every 10 s. After the pellet was taken out, it was immediately transferred into the container in order to prevent moisture loss. Then the container including three pellets was weighed on an electric balance in a room with controlled temperature and humidity at 25°C and 60% to 70%, respectively. The volume of the secreted saliva was obtained from the difference of the weight between before and after the measurement. Mean whole saliva volume in untreated rats was 22.1 ± 2.7 μl (mean ± S.E.M.), being the same as the reported data (27). Pilocarpine was used as the saliva secretory stimulator and dissolved in physiological saline. Whole saliva was measured at 0, 30 and 60 min after administrating pilocarpine (2 mg/kg, i.p.).

Collection of saliva from parotid gland by cannulation method: The rat was anesthetized with pentobarbital (50 mg/kg, i.p.), secured in a supine position, and a tracheal tube was placed for the support of respiration. Rat parotid saliva was collected as described in the previous report (28). Briefly, the tapered end of each capillary cannula (PE-50; Clay-Adams, Becton Dickinson, MD, USA) was inserted into the parotid duct and the other end was put in a 0.5-ml microtubes to collect the secreted saliva. The microtubes were exchanged to another every 10 min for 2 h after administrating pilocarpine (2 to 8 mg/kg, i.p.).

Urine collection
In the experiment of collecting urine, the rats were housed in the urine collecting cage for 6 h. The excreted urine was collected into a mess-cylinder attached to the cage. They were restricted from taking water throughout this experiment.

Membrane preparation
Membranes were prepared as described previously (29). Briefly, the parotid and submandibular glands were quickly removed from rats and dissected free of fat and connective tissues under ether anesthesia. Both glands were homogenized in 10 vol ice-cold 0.32 M sucrose containing protease inhibitors (aprotinin, 15 μg/ml; antipain, 5 μg/ml; pepstatin A, 5 μg/ml; and leupeptine, 5 μg/ml) using a Brinkmann Polytron at a speed setting of 5 for 1 min. The homogenate was centrifuged at 1,000 × g for 10 min. Then the supernatant was centrifuged at 20,000 × g for 20 min. The resulting pellet was resuspended in 10 vol ice-cold 50 mM Tris/HCl buffer (pH 7.4) and then recentrifuged at 48,000 × g for 20 min. The same step was repeated two times. The final pellet was resuspended in ice-cold same buffer and used as the membrane source for \(^{3}H\)QNB binding assays (below). All preparative steps were performed at 4°C. Protein concentration was determined by the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA).

Radioligand binding assays
The parotid and submandibular gland membranes (0.2 mg) were suspended in 0.5 ml of 50 mM Tris/HCl buffer (pH 7.4) with \(^{3}H\)QNB in the presence or absence
of unlabeled drugs. After incubation for 90 min at 37°C, bound [3H]QNB was separated from free [3H]QNB by rapid filtration under vacuum through a Whatman GF/B glass fiber filter using a Brandel M-24S filtering manifold (Brandel Instruments, Gaithersburg, MD, USA). The filter was then washed twice with 5-ml aliquots of the same ice-cold buffer. The filter was dried and placed in a vial containing 6 ml of Sintisol (Dojin, Kumamoto), and the radioactivity was determined by a liquid scintillation spectrometer (Packard, Meriden, CT, USA). Non-specific binding was defined as the amount of [3H]QNB bound in the presence of 10 µM unlabeled atropine. The specific binding was defined as the total binding minus the non-specific binding. The dissociation constant (Kd) and the maximal binding capacity (Bmax) for [3H]QNB binding to the salivary gland membranes were obtained from the results of saturation experiments by Scatchard plot analysis. In competitive inhibitory experiments, the 50% inhibition concentration (IC50) value was determined by using at least eight concentrations of the inhibitor. The Kd, Bmax, and IC50 values were calculated by the Kell program (Biosoft, Feruson, MO, USA). All assays were performed in triplicate.

Histological examination

In the histological experiments, the parotid and submandibular glands of normal and diabetic rats anesthetized with pentobarbital at 50 mg/kg, i.p. were removed and fixed with 10% formalin solution. Then the tissues were embedded in paraffin for making sections. Both gland sections (5-µm-thick) were stained with hematoxylin/eosin and observed by light microscope. To detect lipid droplets, both tissues were also stained with Oil red-O and examined microscopically.

Statistical analyses

The data are expressed as the means ± S.E.M. Equal variance analysis was performed between the control and STZ group. Significant differences between both groups were determined by Student’s t-test and the Aspin-Welch t-test in the case of equal and unequal variance, respectively. In the experiment on the functional recovery by insulin, the significant differences were analyzed by ANOVA and t-tests described above. A P value of less than 0.05 was considered to be statistically significant.

RESULTS

Blood glucose levels in STZ rats

The blood glucose level in the animals receiving STZ at 60 mg/kg was 560 ± 8.5 mg/dl (means ± S.E.M., n = 5). This was approximately a fourfold increase compared with the level of 141 ± 13.4 mg/dl (n = 5) in the untreated animals.

Saliva secretory response in STZ rats given freely or on restricted water

To examine the effect of diabetes-induced polyuria on the saliva secretory responses, we measured whole saliva secreted in the oral cavity of rats that were given water freely or on restricted water for 6 h (Table 1). If given water freely, the amount of whole saliva with no stimulation was not different between the STZ and control rats. On stimulation with pilocarpine (2 mg/kg, i.p.), salivary secretion was increased in both groups, although the effect in the STZ rats was two- to three-fold less than in the control rats. When the animals were restricted from taking water, the amount of non-stimulated whole saliva in the STZ rats demonstrated a two third decrease compared with the control rats. Upon stimulation with pilocarpine, salivary secretion was slightly changed in the STZ rats. In contrast, in the control rats, it remarkably increased by 1.7 to 2.3 times.

Recovery effects of insulin and water restriction on whole saliva secretion

Figure 1 shows recovery effects on secretory function of whole saliva when the STZ rats were treated with insulin. Insulin treatment was effective for the recovery from the reduced whole saliva secretion in STZ rats and restored up to the level of control rats. In STZ rats given little water, the amount of the collected whole saliva was decreased, but the excreted urine volume was increased. The excreted urine volume for the 6-h non-drinking period was 3.0 ± 1.6 ml (n = 5) in control rats, whereas it was 7.0 ± 2.6 ml (n = 31) in STZ rats. The water loss of the whole body due to urination was equivalent to 0.9% and 2.4% in the control rat.

Table 1. Volume of whole saliva in STZ rats under normal and non-drinking conditions

<table>
<thead>
<tr>
<th>Group</th>
<th>No stimulation</th>
<th>After pilocarpine stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 min</td>
<td>60 min</td>
</tr>
<tr>
<td>Freely given water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>60.0 ± 4.0</td>
<td>111.8 ± 2.7</td>
</tr>
<tr>
<td>STZ</td>
<td>53.6 ± 4.6</td>
<td>70.1 ± 10.2</td>
</tr>
<tr>
<td>Restricted from water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>56.2 ± 5.6</td>
<td>132.0 ± 20.6</td>
</tr>
<tr>
<td>STZ</td>
<td>36.8 ± 4.1</td>
<td>39.3 ± 14.9</td>
</tr>
</tbody>
</table>

Whole saliva of both control and STZ rats was collected for 30 s in the absorbent paper pellets with or without pilocarpine (2 mg/kg, i.p.) stimulation. The same measurement was carried out also after forbidding rats to take water for 6 h. The data are means ± S.E.M. of 5 rats per group. \( P<0.05 \), significant difference between rats with and without pilocarpine stimulation; \( P<0.05 \), significant difference between the control and STZ rats.
and STZ rats, respectively. Plots of the secretory saliva and the excreted urine significantly ($P<0.01$) showed a negative co-relationship with a coefficient index of 0.6 (Fig. 2).

**Salivary secretion from parotid gland of STZ rats**

We measured the amount of saliva secreted from the parotid gland by means of cannulation, in order to examine time-dependent salivary secretion (Fig. 3). Parotid saliva of control rats reached a maximum level within the first 10 min after stimulation of pilocarpine and then gradually decreased. A similar salivary secretory response was also observed in STZ rats. However, parotid saliva in STZ rats was decreased about 70%, compared with control rats at each measurement time. When pilocarpine was administered to the STZ and control rats, saliva secretion was obtained from the control rat in a dose-dependent manner, but not from the STZ rats (Fig. 4).
Xerostomia in Diabetic Rats

To examine the characteristics of the muscarinic receptors in STZ rats, we measured [\(^3\)H]QNB binding to the salivary gland membranes. Scatchard plot analysis of the saturation curve in the control rats yielded a K\(_d\) of 37.7 ± 3.3 pM and a B\(_{max}\) of 134.2 ± 5.9 fmol/mg protein in the parotid gland, while in the submandibular gland, the K\(_d\) was 37.2 ± 1.8 pM and the B\(_{max}\) was 105.8 ± 12.0 fmol/mg protein. In STZ rats, the obtained kinetic parameters demonstrated the following: i) a single component of non-interacting binding sites to both parotid and submandibular gland membranes, ii) a significant decrease of 20% (P<0.05) in B\(_{max}\) and no change in K\(_d\) in the parotid gland membranes (Fig. 5), and iii) a significant increase of 20% (P<0.05) in K\(_d\) and no change in B\(_{max}\) in the submandibular gland membranes (Fig. 6). Furthermore, the results of competitive inhibition of [\(^3\)H]QNB binding to both gland membranes showed an increase in IC\(_{50}\) values of pilocarpine and carbachol in STZ rats (Table 2). Especially, increase of IC\(_{50}\) values was significantly found against pilocarpine in the parotid membranes (P<0.05) and carbachol in the submandibular membranes (P<0.01).

Histological observation of salivary glands in STZ rats

In STZ rats, many acinar cells in the parotid glands showed slightly degenerative changes such as vacuolation and atrophy. By Oil red-O stain, the vacuolation proved to be lipid droplets (Fig. 7B). The submandibular glands in STZ rats showed minimal degenerative changes with vacuolation in acinar cells, but which were Oil red-O negative (Fig. 7D).

**DISCUSSION**

In this study, we examined the saliva secretory response to muscarinic receptor stimulation and the characteristics of muscarinic receptor and histological change in the salivary glands of STZ rats. Even though diabetic conditions have a marked effect on the functions of the salivary gland, there are no reports on the saliva secretory response under non-stimulated conditions. Therefore, we examined the secretory response of whole saliva under no stimulation in STZ rats (Table 1). There was no obvious difference between the control and STZ rats when the animals were freely given water. However, water-restriction induced a remarkable decrease in salivary secretion of the STZ rats compared...
Fig. 7. Light micrographs of the parotid and submandibular glands from control and STZ rats. Left side is by H.E. stain and right side is by Oil red-O stain. A: Parotid gland from control rats (normal), B: Parotid gland from STZ rats (slight fatty degeneration), C: Submandibular gland from control rats (normal), D: Submandibular gland from STZ rats (minimal degenerative change with vacuolation). Scale bar: 100 μm.
with the control. On the other hand, pilocarpine-stimulated whole saliva secretion was remarkably decreased in STZ rats, compared to the control rats, as well as those reported by other investigators (15, 23, 24). The volume of saliva elicited by the parotid gland was also decreased after stimulation of pilocarpine and its level was almost 30% of the control level (Fig. 3). No satisfactory dose-response correlation between pilocarpine dose and parotid saliva volume was found in STZ rats, and a remarkable decrease in salivary secretion was observed at high dose of pilocarpine (Fig. 4). These results strongly suggest that STZ rats have lowered susceptibility for muscarinic receptor stimulation in vivo.

$[^3H]QNB$ binding experiments in STZ rats revealed that: i) a decrease in binding site of muscarinic receptor in the parotid gland membranes (Fig. 5), ii) a decrease in affinity of muscarinic receptor in the submandibular gland membranes (Fig. 6), and iii) a decrease in drug susceptibility for muscarinic receptor agonists (Table 2). As for parotid gland membrane experiments, the protein concentration of prepared membranes in STZ rats was less than those of the control. This suggests that the number of binding sites of muscarinic receptors may be further reduced in STZ rats. Thus, qualitative and quantitative changes of muscarinic receptors in diabetic animals were found in salivary glands as well as heart and lung (30, 31). It is considered that changes in characteristics of muscarinic receptor in the salivary gland membranes contribute to the inhibitory response of salivary secretion by muscarinic agonists in STZ rats.

Numerous investigators have demonstrated that histological changes in the salivary glands are observed in the experimental diabetic rats (21 – 26). In this study, we used rats at 2 weeks after a single administration of STZ (60 mg /kg, i.p.), as the relatively initial diabetic conditions. As shown in Fig. 7, STZ rats showed slight fatty degenerative changes in parotid glands of the STZ-rat, otherwise minimal with vacuolation in the submandibular glands. Our results obtained under the relatively initial diabetic conditions were consistent with the previous reports observed under advanced diabetic conditions (13, 15, 32). The findings such as a lipid accumulation, a decrease in amylase release, and an increase in acetylcholinesterase activity are found in the salivary glands, especially parotid glands of the initial diabetic rats (13, 22, 23, 33). Differences of characteristics between parotid and submandibular glands in muscarinic receptor binding assay may be involved in the differences in the histological lesion between both salivary glands. From these results, it is considered that perhaps degenerative changes in acinar cells in the salivary glands may be correlated to change in the characteristics of muscarinic receptors in the salivary gland membranes and a decrease in the salivary flow rate in STZ rats.

It has been pointed out that xerostomia in diabetics is induced by the secondary effect due to systemic dehydration resulting from polyuria (5). In this study, we examined the effects of non-drinking on the salivary secretion in STZ rats (Table 1). Six-hour non-drinking condition caused a water loss of 0.9% and 2.4% of body weight in the control and STZ rats, respectively. This indicates that possible systemic dehydration due to polyuria occurs in STZ rats by the present condition. Saliva secretory response after non-drinking conditions for 6 h was very different with that of normal conditions. In STZ rats, pilocarpine-unstimulated and -stimulated whole saliva volumes were remarkably decreased, compared to control rats. Furthermore, there was a negative correlation between the whole saliva and urine volume when the rats were restricted from taking water (Fig. 2). These findings suggest that the inhibitory response of salivary secretion in STZ rats are enhanced by losing water resulting from polyuria.

In addition, we found that insulin treatment had a recovery effect on retarded secretion of the salivary glands in STZ rats after non drinking (Fig. 1). Some investigations clarified that the insulin administration to STZ rat was very effective for recovering secretory dysfunction (12, 15, 21, 32), histological degeneration (12, 21), and fatty accumulation (34) in the salivary gland. Our findings also supported their results and indicated that a decrease of salivary secretion was induced by diabetic mellitus, but not a direct action of STZ.

These results indicate that decrease in salivary secretion in STZ rats is not only induced by a loss of water due to the accelerated urination, but also closely associated with the lowered susceptibility of the muscarinic receptors.

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