In vivo secretory responses of submandibular glands in streptozotocin-diabetic rats to sympathetic and parasympathetic nerve stimulation

Leigh C. Anderson 1, John R. Garrett 2, Ahmed H. Suleiman 1, Gordon B. Proctor 3, Ka-Ming Chan 1, Robert Hartley 2

1 Department of Oral Biology, University of Washington, 502-22, Seattle, WA 98195, USA
2 Department of Oral Pathology, King’s College School of Medicine and Dentistry, London, UK

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Abstract. Submandibular gland responses to sympathetic and parasympathetic nerve stimulation were studied in streptozotocin-diabetic rats. Morphologically, the acinar cells in control glands were relatively uniform in size and contained electron-lucent granules. The granular ducts were distinguished by the presence of electron-dense granules. With the exception of intracellular lipid droplets and the presence of a few autophagosomes in diabetic glands, no consistent differences in acinar cell structure were observed. In contrast, the diameter of the granular ducts and the granule content of their cells were less in diabetic glands. At 3 weeks sympathetic flow rate, salivary protein concentration, and total protein output were unaffected by diabetes. Sympathetic flow rate was greater at 3 months, and the concentration of protein in the saliva was lower. In 6-month diabetic rats flow rate remained increased, but protein concentration and total protein output were reduced. The decrease in salivary protein concentration at 3 and 6 months was accompanied by a reduction in secretory granule release from acinar and granular duct cells. No consistent differences in flow rate, protein concentration, protein output, or secretory granule release were observed following parasympathetic stimulation. We conclude that the effects of diabetes on nerve-stimulated flow rate and protein release depend on the duration of diabetes and the type of stimulation, and are independent of one another.

Key words: Diabetes – Submandibular gland – Sympathetic innervation – Parasympathetic innervation – Rat (Wistar)

During the past 25 years numerous investigations have demonstrated the effects of experimental diabetes on the structure and function of the major salivary glands in the rat (Li and Lin 1669a, b; Anderson 1983; Takai et al. 1983; Hand and Weiss 1984; High et al. 1985; Anderson and Garrett 1986; Resteverg et al. 1986; Anderson et al. 1989). Liu and Lin (1969a, b) were amongst the first to study the structural changes in the rat submandibular gland following the induction of diabetes. Alloxan diabetes and subsequent insulin treatment were shown to markedly affect the growth and development of the submandibular gland. Significant decreases in gland weight and granular duct diameter were measured, but no discernible histological differences were seen in the acinar portion of the gland were reported. A more recent examination of the effects of streptozotocin-diabetes on the rat submandibular gland (High et al. 1985) confirmed these initial findings. The most striking morphological effect of diabetes, however, is the rapid accumulation of lipid within the acinar cells of the parotid and submandibular glands (Anderson 1983; Anderson and Garrett 1986; Hand and Weiss 1984; Resteverg et al. 1986).

The deposition of lipid is somewhat less in the submandibular than in the parotid gland, and the droplets tend to be smaller and located only in the seromucous acini and intercalated duct cells. In addition, with increasing duration of diabetes the number of acidophilic structures in both the acini and the granular ducts tended to increase (L.C. Anderson and R.K. Gett, unpublished observations).

The morphological observations outlined above lead quite naturally to questions about the effects of diabetes on secretory function in the submandibular gland. In nondiabetic rats, several studies have defined the actions of sympathetic and parasympathetic nerve stimulation in vivo (Abe and Dawes 1978; Garrett et al. 1991) and of pharmacological agonists in vivo and in vitro (Abe and Dawes 1978; Bogart and Picard 1978) on protein and fluid secretion from the submandibular gland. Generally, sympathetic stimulation results in a relatively low flow of saliva that is rich in protein, and is accompanied by an extensive degranulation from both the acinar and granular duct cells. In contrast, parasympathetic nerve stimulation induces a considerable low of saliva that has a low protein content, and is accompanied by little or no degranulation of the secretory cells (Garrett et al.)
1991). Initial reports by Takai et al. (1983) suggested that submandibular gland secretory function in diabetic rats is significantly impaired, but in a preliminary report Anderson and Saidman (1989) were unable to demonstrate any abnormalities in sympathetically stimulated submandibular gland secretory function in diabetic animals. The purpose of the present studies, therefore, was to examine, both morphologically and physiologically, the response of the rat submandibular gland to sympathetic and parasympathetic nerve stimulation after 56 weeks of streptozotocin-induced diabetes.

Materials and methods

**Animals**

Male Wistar rats initially weighing 200-250 g were used throughout three studies. Diabetes was induced with a single intravenous injection of streptozotocin (65 mg/kg i.v.). A group of animals served as controls. All animals were maintained on a 12-h light-dark cycle, and allowed free access to food and water. Nonfasting serum glucose levels were determined, and only animals having glucose values greater than 15 mmol/L were considered diabetic.

**Stimulation of submaxillary secretion**

Three weeks and 3 and 6 months after the induction of diabetes, the animals were fasted overnight. Anaesthesia was induced with pentobarbital (35 mg/kg i.p.) followed by dihydorurate (37 mg/kg i.v.) via the femoral vein. The trachea was cannulated with a polythene tube and the right submandibular duct cannulated with a fine glass cannula. Body temperature was recorded by means of a rectal thermometer and maintained between 37°C and 38°C.

One group of rats (r) each time point received stimulation of the right sympathetic nerve trunk, which had been sectioned in the neck. The peripheral stump was placed on bipolar electrodes for stimulation at 50 Hz in bursts of 1 s every 10 s at 4 V with a pulse width of 0.1 ms (Anderson et al., 1986). The other group of animals received parasympathetic nerve stimulation of 5 Hz continuously at 4 V, 2 ms by carefully reflecting the lingual nerve onto the submandibular duct and placing both on a bipolar electrode.

Salivary flow was recorded both by observing and recording the number and rate of drops falling from the cannula, and by collection of the saliva into preweighed vials, cooled by ice, which were then reweighed after stimulation (d g of saliva was taken to be equivalent to 1 ml). Protein content of each sample was assessed by absorbance at 215 nm (Amesnevil 1974) using a human albumin globulin standard.

**Tissue fixation**

Following each experiment, the right (stimulated) and left (control) submandibular gland lobes were removed and weighed. For stereological measurements segments of tissue were always taken from the middle of the gland. Each wedge of tissue was immersed in a glutaraldehyde-perchloric acid mixture (Karnovsky, 1965), transected into small cubes, and fixed for 2-4 h at room temperature. The tissue was then rinsed overnight at 0°C-4°C in 0.15 M cacodylate buffer (pH 7.2) containing 7.5% sucrose. The tissue was then postfixed in 1% OsO , for 1 h, dehydrated through graded alcohol and embedded in Epon. Thick sections (2-3 μm) were stained with toluidine blue for light-microscopic assessment. Thin sections were stained with uranyl acetate and lead citrate for electron microscopy.

**Morphometry**

Intraductal volume densities were determined using thick sections of submandibular ducts and volume density of secretory granules within the granular ducts. The minimal sample size for visual analysis was determined by finding the cumulative mean of a large number of consecutive sections but smaller samples were independently assessed using a Kontron Videoplan. A summation average permeation point beyond which the graph started at 100% was determined, and the curve within ±3% was used. The minimal sample size was 20 A 1-mm index profile.

To determine intraductal volumes in animal cells, blocks of tissue from each gland were shown rankby rank. Thin sections were cut, and a total of four grids with four or five sections on each were stained. Twenty micrographs of individual acinar cells with a visible nucleus and that bordered a stained lumina were used to determine the volume density of secretory granules within these cells. Paper prints, final magnification × 5000, were used for morphometric analysis.

**Statistical analysis**

Differences between means were analysed using Student's t-test, and differences between groups were analysed using ANOVA for statistical significance using Student's t-test.

**Results**

**Body weight and serum glucose**

The effects of diabetes on body weight and serum glucose levels are shown in Table 1. The body weight of diabetic animals was always significantly less than in control animals. Mean serum glucose levels in diabetic rats were approximately 5.6 mM whereas glucose levels in rats ranged from 15 mM to 35 mM.

**Light and electron microscopy**

All control glands showed normal features as described previously (Garrett et al., 1971). Adenocrine sections of control glands showed normal

<table>
<thead>
<tr>
<th>Group</th>
<th>Time</th>
<th>Body wt</th>
<th>Gland weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6 weeks</td>
<td>233.2 ± 7.6</td>
<td>199 ± 8</td>
</tr>
<tr>
<td>Diabetes</td>
<td>6 weeks</td>
<td>232 ± 7.7</td>
<td>195 ± 8</td>
</tr>
<tr>
<td>Control</td>
<td>3 months</td>
<td>280 ± 10</td>
<td>231 ± 10</td>
</tr>
<tr>
<td>Diabetes</td>
<td>3 months</td>
<td>250 ± 12</td>
<td>230 ± 12</td>
</tr>
<tr>
<td>Control</td>
<td>6 months</td>
<td>250 ± 12</td>
<td>230 ± 12</td>
</tr>
<tr>
<td>Diabetes</td>
<td>6 months</td>
<td>235 ± 20</td>
<td>195 ± 25</td>
</tr>
</tbody>
</table>

* Mean ± S.E.M.; n equals the number of animals
* P < 0.001; diabetes vs control

All control glands showed normal features as described previously (Garrett et al., 1971). Adenocrine sections of control glands showed normal...
Fig. 1A, B. Electron micrographs of axini from unstimulated submandibular glands. A. Lumens, × 4300. Bar: 5 μm. A: Normal control submandibular gland showing acinar cells containing numerous secretory granules. B: Submandibular gland from diabetic rats with diabetes of short duration not showing similar appearance of normal secretory granules and acinar cells as in control gland. There is an increase in lipid (*) in the basal region of the cell.
Fig. 2A, B. Electron micrographs of granular ducts from unstimu-
lated submandibular glands. L. Lumen. x 4000. Bar: 5 mm. A Nor-
mal control submandibular gland showing granular ductal cells
filled with secretory granules of varying size and electron density.
B Submandibular gland from diabetic (6 weeks duration) rat
showing granular duct cells of smaller size that contain fewer gran-
ules than in the control gland.
Table 2. The effects of streptozotocin diabetes on the proportional volumes of secretory granules (V<sub>S</sub>) within acinar and granular duct cells

<table>
<thead>
<tr>
<th>Group</th>
<th>Time</th>
<th>% Volume density</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>V&lt;sub&gt;S&lt;/sub&gt; (Acinar cells)</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>3 weeks</td>
</tr>
<tr>
<td></td>
<td>Diabetic</td>
<td>58.3</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>3 months</td>
</tr>
<tr>
<td>Diabetic</td>
<td>48.4 ± 1*</td>
<td>39.4 ± 1</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>6 months</td>
</tr>
<tr>
<td>Diabetic</td>
<td>50.2 ± 2</td>
<td>36.3 ± 2</td>
</tr>
</tbody>
</table>

* Mean ± S.E.M.; n equals the number of animals

P < 0.05; diabetes vs control

P < 0.01; diabetes vs control

Histological features with a regular distribution of parenchyma, acini, and granular ducts within a connective tissue stroma. At the electron-microscopic level (Fig. 1A), the acinar cells were relatively uniform in size, and contained electron-lucent secretory granules of varying size that filled most of the cytoplasmic volume. Nuclei, endoplasmic reticulum, and Golgi apparatus were all located in the basal one-third of the cell. The granular ducts were easily distinguished from the striated ducts by the presence of numerous electron-dense secretory granules of variable size (Fig. 2A).

Light-microscopic observations readily demonstrated the reduction in the relative volume of granular ducts in diabetic glands, from 38% ± 1% in controls to 21.5 ± 2% at 3 weeks, 22.5 ± 2% at 3 months, and 20% ± 2% at 6 months (P < 0.01). The secretory granule content (% intracellular volume density) of these structures was also less in diabetic glands than in controls (Table 2). As a result of the decreases in granular tubule volume density, there was a corresponding increase in acinar cell volume density from 48% ± 1% in controls to 55% ± 2%, 64% ± 3%, and 62% ± 2% in 3-week, 3- and 6-month diabetic rats (P < 0.025). No observable differences in individual acinar cell area or granular content were seen, but there was a marked accumulation of lipid in the basal regions of many acinar cells (Fig. 3B). In addition, there was an increase in the rough endoplasmic reticulum, a variable coalescence of macular granules, and the appearance of a variable number of phagocytosed structures. Nevertheless, most acinar cells were relatively normal in appearance, and there were few pathological changes (lipid, phagosome accumulation, and coalescence of macular granules) that might suggest an ongoing process of cellular degeneration and death. The cells of the persisting granular ducts also appeared relatively normal (Fig. 2B), with the exception of the readily apparent decrease in size and secretory granule volume density.

Table 3. The effects of sympathetic nerve stimulation on secretion from diabetic and control rat submandibular glands

<table>
<thead>
<tr>
<th>Group</th>
<th>Time</th>
<th>Saliva volume (μl/g tissue/min)</th>
<th>Protein conc. (mg/ml)</th>
<th>Protein output (μg/g tissue/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>3 weeks</td>
<td>17.0 ± 2.0*</td>
<td>46.3 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>Diabetic</td>
<td>20.7 ± 2.4</td>
<td>44.9 ± 4.0</td>
<td>797 ± 37</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>3 months</td>
<td>13.3 ± 1.2</td>
<td>59.3 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>Diabetic</td>
<td>33.9 ± 7.6*</td>
<td>23.4 ± 4.0</td>
<td>722 ± 105</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>6 months</td>
<td>16.2 ± 9.3</td>
<td>93.5 ± 10.0</td>
</tr>
<tr>
<td>Diabetic</td>
<td>35.4 ± 3.5*</td>
<td>20.7 ± 2.0*</td>
<td>701 ± 127</td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± S.E.M.; n equals the number of animals

P < 0.05; diabetes vs control

P < 0.02; diabetes vs control

P < 0.001; diabetes vs control

P < 0.05; control vs control (3 weeks)

In control animals, submandibular salivary flow rates evoked by sympathetic stimulation remained fairly constant over time (Table 3). Protein concentration (mg/ml), however, increased significantly from 3 weeks to 3 months, and then again from 3 to 6 months (P < 0.01). Although protein concentration increased at both 3 and 6 months, the protein output (μg/g tissue/min) was only significantly greater at 6 months (P < 0.001), compared with 3-week control rats. In 3-week diabetic animals, sympathetic flow rate, salivary protein concentration, and total protein output were similar to those observed in control rats. Three months after the induction of diabetes, sympathetic flow rate was significantly greater (P < 0.05) and salivary protein concentration was lower (P < 0.0025) than in age-matched controls. Total protein output was still unaffected. In 6-month diabetic animals, sympathetic flow was still increased, protein concentration was reduced, and total protein output was similar to that at 3 weeks and 3 months. Because of the significant increase in protein concentration in 6-month control salivars, however, total protein output from diabetic glands was significantly lower than that from control glands (P < 0.001).

In contrast to the results obtained during sympathetic stimulation, no consistent differences in salivary flow rate, protein concentration, or total protein output were observed during parasympathetic stimulation (Table 4).
rate was evident at both 3 and 6 months. As with the morphological evidence, therefore, the physiological data are clearly not consistent with the previously reported fibrous degeneration of the submandibular gland (Cutler et al. 1979).

Nevertheless, a serendipitous finding by one of the authors may shed some light on the matter. We were extremely cautious about the housing of animals, in particular, about not placing mice and rats in the same housing unit. During a preliminary investigation using aloxan-diabetic animals, Anderson (unpublished data) found that all diabetic, but not control, rats housed with mice (a relatively common practice at the time) developed a marked fibrotic degeneration of the submandibular glands. Unfortunately, details about the housing and care of animals used in the earlier studies (Cutler et al. 1979; Takai et al. 1983) are not available.

In contrast to its effects on flow rate, diabetes (6 months) led to a reduction in salivary protein concentration, total protein output, and secretary granule release during sympathetic stimulation. These data suggest that in the rat submandibular gland the effects of diabetes on fluid secretion and protein release are independent of one another. The reduction in protein output from diabetic submandibular glands at 6 months very likely results from the interaction of several different effects on submandibular structure and function. First, the submandibular gland contains two different secretory compartments, the acinar cells and the granular ducts (Theunis and Sweeley 1965). Neither acinar cell size nor the relative volume density of secretory granules within the acinar cells appear to be affected in diabetic rats. In contrast, histological observations demonstrated a reduction in the granular duct compartment (see also Liu and Lin 1969a; b; High et al. 1985; Reutervig et al. 1985). The smaller diameter and lower number of granular ducts in the diabetic submandibular gland is probably related to the hypoglycemic effect of diabetes (Murray et al. 1983) since both the development and maintenance of the granular ducts are androgen dependent (Shaffer and Holm 1980). Further, the decrease in granular ducts may reflect a significant reduction in the relative volume density of secretory granules within the granular duct cells, which reflects the decreased protein content of diabetic submandibular glands noted previously (Jaffa et al. 1984a, b). This may also be related to the hypoglycemic effects of diabetes, as the levels of several proteases found in the granular ducts are androgen dependent (Rieklinen and Niemi 1968). Finally, morphometric evaluation of stimulated (Anderson et al. 1983) isolated glands within the same animal suggests that, as in the parotid gland (Anderson et al. 1990), the degree of degranulation from both the acinar and granular duct cells after sympathetic stimulation was significantly lower in the diabetic animals than in controls. Thus, the reduction in total protein output after sympathetic stimulation appears to be related to (1) a reduction in the size and number of granular ducts, (2) a reduction in the number of secretory granules contained in the granular duct cells, and (3) a decrease in secretory granule release from both the ductal and acinar cells. This final conclusion is particularly interesting with respect to secretory physiology. Unlike the response of the parotid gland to sympathetic stimulation, submandibular gland fibrosis does not appear to be a β-adrenergically mediated event. Whilst secretion of granules from the acini is a β-adrenergic response, that from the granular ducts is predominantly an α-adrenergic response (Abb and Dawes 1978). However, the intracellular signals generated by α- and β-adrenergic stimulation differ significantly. β-Adrenergic agonists exert their intracellular effects through a cAMP-dependent protein kinase, whereas α-adrenergic stimulation leads to the generation of a variety of putative intracellular messengers, including diacylglycerol and IP3, that result in a rise in intracellular calcium and the activation of Ca2+ -dependent protein kinases (Quissell et al. 1992). Diabetes, therefore, may affect the coxystox of secretory proteins at a point prior to receptor binding and initial second messenger production. Alternatively, sympathetic nerve function could in some way be impaired, leading to a decrease in receptor activation. Since flow rates during both parasympathetic and sympathetic stimulation was not impaired at any time studied, it seems reasonable to infer that nerve impulse formation and the release of conventional neurotransmitters (acetylcholine and noradrenaline) remained relatively normal throughout all the stimulation frequencies used. Nevertheless, it is possible that the effects of diabetes on protein secretion could be related to as yet undefined changes either in adenergic receptor densities, or in nonadrenergic, noncholinergic (peptidergic) transmitter content or release from sympathetic nerve terminals.

Finally, although parasympathetic nerves serve as the main route for flow in the salivary glands, it is well known that both adrenergic and other agents can affect the flow rate. Indeed, recent reports have suggested that adrenergic nerves are important in controlling salivary flow; for example, direct stimulation of the buccal sympathetic nerves can increase salivary flow. However, the role of adrenergic nerves in the control of salivary flow is less clear. It is possible that the stimulation of adrenergic nerves could result in an increase in the release of neurotransmitters such as norepinephrine, which could then stimulate the parasympathetic nerves, leading to an increase in salivary flow. However, this possibility has not been directly tested.