



Effects of streptozotocin-induced diabetes on taste buds in rat vallate papillae

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Received 20 March 2006; received in revised form 23 October 2006; accepted 25 October 2006

KEYWORDS

Diabetes;
Vallate papilla;
Taste bud;
PGP 9.5;
CGRP;
Immunohistochemistry;
Rat

Summary

Some studies have documented taste changes in patients with diabetes mellitus (DM). In order to understand the relationships between taste disorders caused by DM and the innervation and morphologic changes in the taste buds, we studied the vallate papillae and their taste buds in rats with DM. DM was induced in these rats with streptozotocin (STZ), which causes the death of β cells of the pancreas. The rats were sacrificed and the vallate papillae were dissected for morphometric and quantitative immunohistochemical analyses. The innervations of the vallate papillae and taste buds in diabetic and control rats were detected using immunohistochemistry employing antibodies directed against protein gene product 9.5 (PGP 9.5) and calcitonin gene-related peptide (CGRP). The results showed that PGP 9.5- and CGRP-immunoreactive nerve fibers in the trench wall of diabetic vallate papillae, as well as taste cells in the taste buds, gradually decreased both intragemmally and intergemmally. The morphometry revealed no significant difference in papilla size between the control and diabetic groups, but there were fewer taste buds per papilla (per animal). The quantification of innervation in taste buds of the diabetic rats supported the visual assessment of immunohistochemical labeling, that the innervation of taste cells was significantly reduced in diabetic animals. These findings suggest that taste impairment in diabetic subjects may be caused by neuropathy defects and/or morphological changes in the taste buds.

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Introduction

Taste disturbances are commonly observed in patients affected by various illnesses, or as a side effect of drug therapies. Diabetes mellitus (DM) is a common cause of nervous system disorders that

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manifest in a variety of clinical forms. Sensory deficits in diabetes occur early and are generalized, and the tactile and special senses have been widely studied. A large number of clinical symptoms, including the loss or depression of the tendon reflex, impaired vibration perception and position sense and sensory ataxia are reported (Vinik et al., 1992; Zola and Vinik, 1992). Diabetes also affects taste perceptions. Taste thresholds for hydrochloric acid, sucrose, sodium chloride, and urea are changed in diabetic subjects (Schelling et al., 1965; Ronald et al., 1972; Lawson et al., 1979; Abbasi, 1981; Hardy et al., 1981; Niewoehner et al., 1986; Le Floch et al., 1989; Smith and Gannon, 1991; Lamey et al., 1992; Tepper et al., 1996). Chochinov and Ullyot (1972) demonstrated that the electric-taste threshold in diabetic individuals fell within a wide range of up to 300 μ A, in comparison to the narrow range of 2–30 μ A in the healthy population.

The protein gene product 9.5 (PGP 9.5) is found predominantly in the cytoplasm of neurons and neuroendocrine cells, and is a pan-neuronal marker for nervous tissue (Thompson et al., 1983). Several studies have shown that calcitonin gene-related peptide (CGRP) is a peptide composed of 37 amino acids, whose presence has been demonstrated in the nervous system by recombinant-DNA and molecular biological techniques (Amara et al., 1982; Rosenfeld et al., 1983). Antibodies against PGP 9.5 and CGRP have been used to study the innervation of vallate taste buds in humans, guinea pigs, rats, and mice (Iwanaga et al., 1992; Huang and Lu, 1996a; Wakisaka et al., 1996; Kusakabe et al., 1998; Chou et al., 2001). In these studies, the distribution of PGP 9.5 immunoreactivity was localized both intra- and extragemmally on taste bud cells and nerve fibers. CGRP-immunoreactive nerve fibers are numerous in the subgemmal connective tissue and enter the epithelium to form intragemmal and extragemmal networks. No taste cells immunoreactive for CGRP were reported.

Although neurological complications appear to exacerbate taste deficits (Abbasi, 1981), knowledge of its underlying pathogenesis and its relationship to diabetes is limited. In order to resolve this issue, we studied a streptozotocin (STZ)-induced diabetic rat model. The vallate papillae and their taste buds were surveyed. Morphometric analysis was used to determine possible morphological changes in diabetic vallate taste buds. Variations in the innervation of diabetic vallate taste buds were observed using immunohistochemistry to detect binding of antibodies against PGP 9.5 and CGRP.

Materials and methods

Animals

This study was approved by the Institutional Animal Use Committee of Taipei Medical University. Adult male Sprague-Dawley rats (weight, 300–350 g) were housed in a temperature-controlled room (22 ± 1 °C) under artificial illumination (lights on from 05:00 to 17:00) and at 55% relative humidity, with free access to food and water.

Measurement of blood glucose levels

Before the start of the experiments, the blood glucose level of each rat was measured using a One Touch II blood glucose meter (Lifescan, Milpitas, CA). The blood of each animal was taken from the tail vein. The blood glucose levels for rats ranged 75–115 mg/dl.

Diabetes induced by STZ

Immediately prior to the injection, STZ (Sigma, St. Louis, MO, USA) was dissolved in 0.01 M citrate buffer (pH 4.5). The rats in the experimental group received a single intraperitoneal (i.p.) injection of STZ at a dose of 75 mg/kg body weight. The rats in the control group received a comparable injection of saline. Forty-eight hours following treatment, the blood glucose levels of all rats was measured, and STZ-treated rats all had concentrations in the range of 320–400 mg/dl which indicated that they were diabetic. Prior to sacrifice, weights and blood glucose levels were measured to confirm the persistence of diabetes.

Tissue preparation

In total, 25 STZ-treated rats survived for 3, 7, 9, 12, and 20 weeks and were divided into groups at various stages after the injection. All of the animals were anesthetized with sodium pentobarbital (30 mg/kg body wt, i.p.) and sacrificed by perfusion through the left ventricle with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The tongue with the vallate papillae was excised and fixed in the same solution at 4 °C for 24 h.

Immunohistochemistry

After cryoprotection with sucrose, 30- μ m tissue sections were prepared using a cryostat. Immunolabelling was performed on free-floating cryostat sections using an indirect immunoperoxidase visualization

technique. Sections were first pre-incubated in a solution containing 10% normal goat serum (NGS) and 0.3% H₂O₂ in 0.1 M phosphate-buffered saline (PBS) for 2 h to block the endogenous peroxidase activity and the nonspecific binding of antibodies. Sections were then incubated with rabbit polyclonal primary antibodies directed against PGP 9.5 or CGRP, both diluted 1:1000 in PBS, for 20 h at 4 °C. Sections were next incubated in biotinylated goat anti-rabbit IgG (diluted 1:100, Vector, USA) and then in the reagents from an ABC kit (Avidin–Biotin Complex, Vector Laboratories, USA), prepared according to manufacturer's instructions, for 1 h at room temperature. Immunolocalization was revealed by incubation with 3,3'-diaminobenzidine (DAB)/H₂O₂ (0.5 mg/ml DAB with 0.003% H₂O₂ in 0.5 M Tris buffer; pH 7.6) for 2–3 min. Sections were mounted on gelatin-coated slides using Permount (Fisher, USA), examined using light microscopy, and photographed.

Morphometric analysis

Tongues with vallate papillae were obtained from both the control rats ($n = 7$) and diabetic rats 9 ($n = 3$) and 12 weeks ($n = 3$) after treatment with STZ; tissues were dehydrated in alcohol, cleared in xylene, and embedded in paraffin wax by routine protocols. 7- μ m-thick coronal sections were cut serially by microtome and stained with hematoxylin and eosin (H&E) for light microscopic observation and morphometric analysis using routine protocols. The number of taste buds in each papilla was counted using the method of Bradley et al. (1980). Briefly, each section was examined using a light microscope, and each longitudinal section of a taste bud was counted. Twenty taste buds from each vallate papilla were then randomly selected, and the number of sections in which each taste bud appeared was counted. From these data, the average number of sections required to cut through a taste bud was calculated. This average was then divided by the total number of taste bud sections for that particular vallate papilla to yield a total taste bud count. Thus, the total number of taste bud longitudinal sections, the average number of longitudinal sections for a single taste bud, and the total number of taste buds per papilla were counted for each vallate papilla. The number of sections from each vallate papilla multiplied by the thickness of each section (7 μ m) was used to calculate the papilla size.

Quantification of taste bud innervation

Measurements of taste bud innervation were obtained from both control rats and rats after each

stage of STZ treatment (two rats in each group) in which axons and cells had been visualized by immunohistochemistry for binding of primary antibodies against PGP 9.5 or CGRP. Three 30- μ m cryostat sections from the approximate center of each vallate papilla with a periodicity of two were used. Twenty profiles of the taste bud from each section that passed longitudinally through the basal lamina and extended to the free surface of the trench were then randomly selected. The selected sections were viewed using a 20 \times objective and a Nikon Eclipse E600 microscope. The computerized image analysis system Image-Pro Plus 5.1 for Windows was used to quantify the immunoreactive cells and nerve fibers in the taste bud.

Statistical analyses

Data are expressed as mean \pm SD. One-way analysis of variance (ANOVA) was used to analyze the significance of differences between the groups, with a p value of <0.05 considered significant.

Results

STZ-induced diabetes in rats

As indicated in Table 1, the mean blood glucose levels for each group before the start of the experiments ranged from 90.2 to 96.0 mg/dl. Within 2 days after injection of STZ, the blood glucose levels of the diabetic-induced rats rose to the range of 512 and 591 mg/dl and no individual rat's blood glucose level dropped below 500 mg/dl before the time of sacrifice. The mean body weight for all groups before the day of STZ injection was in the range 317.2–326.6 g. The control rats continued to grow normally, ultimately weighing over 500 gm. In contrast, STZ-induced diabetic rats gained little additional body weight during the experiment. While the blood glucose level was significantly ($p < 0.0001$) increased in each group of diabetic rats compared with control rats, the body weight was decreased. After injection of STZ, the rats displayed typical signs of diabetes, including hyperphagia and polyuria.

Immunohistochemistry

The results of immunohistochemistry to visualize binding of the antibody against PGP 9.5 is illustrated in Fig. 1. The PGP 9.5-immunoreactive nerve fibers in the basal part of the papilla ascended in the lamina propria to the basal part

Table 1. Body weight and blood glucose levels in control and STZ-induced diabetic rats at each stage in the experiment

Group	<i>n</i>	Start BW (g)	End BW (g)	Start BS (mg/dl)	End BS (mg/dl)
Control	10	324.3 ± 13.6	567.3 ± 33.3	90.2 ± 13.6	89.0 ± 10.4
3W	5	331.8 ± 15.8	371.4 ± 19.3*	96.0 ± 14.9	527.4 ± 12.6*
7W	5	326.6 ± 9.2	352.8 ± 19.8*	91.4 ± 13.5	557.8 ± 23.8*
9W	5	317.2 ± 17.1	332.0 ± 18.3*	93.8 ± 15.6	575.4 ± 15.6*
12W	5	324.2 ± 14.4	347.4 ± 12.3*	91.6 ± 14.2	568.6 ± 24.1*
20W	5	322.0 ± 12.4	333.6 ± 14.5*	94.8 ± 12.6	578.0 ± 16.1*

BW = body weight; BS = blood glucose levels. W = number of weeks. All data are expressed as mean ± SD; **P* < 0.0001 versus control group at each stage.

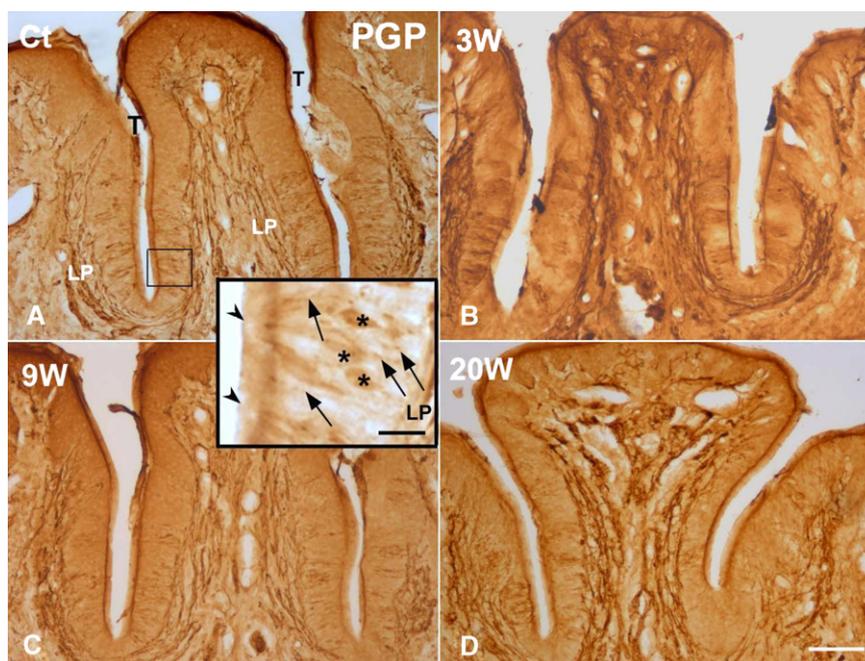


Figure 1. PGP 9.5 immunoreactivity in vallate papillae from control rats (A) and diabetic rats 3 (B), 9 (C), and 20 weeks (D) after the STZ injection. Inset. High magnification photomicrograph from rectangle in the control rats (A) shows the organization of the immunoreactivity of PGP 9.5 is localized in oval-shaped cells (*) in the vallate taste buds and nerve fibers (arrow) penetrating from the subgemmal nerve plexus in the lamina propria (LP) into the taste buds as intragemmal nerve fibers and between the taste buds as extragemmal nerves fibers. The PGP 9.5-immunoreactive nerve fibers and cells decreased in the diabetic groups from 3 to 20 weeks. T, vallate trench and arrowhead, taste pore. Scale bars 100 μm and 25 μm for inset.

of the apical epithelium and the epithelium of the trench walls, and formed dense plexuses in the lamina propria of the papillae. Many thin PGP 9.5-immunoreactive nerve fibers penetrated into the epithelium both intragemmally (into the taste bud) and intergemmally (between the taste buds) from the nerve plexuses in the lamina propria and then ramified perpendicularly to the epithelial surface. Oval PGP 9.5-immunoreactive cells were observed within the lightly labeled barrel-shaped taste buds, which were located in both the medial and lateral trench walls. The diabetic groups at 3, 7, 9, 12, and

20 weeks after STZ treatment contained fewer PGP-9.5 immunoreactive nerve fibers, both in the lamina propria and trench wall, compared to control rats. The number of PGP-9.5 immunoreactive cells in the taste buds also gradually decreased. PGP-9.5 immunoreactivity decreased systematically with the time period after the STZ injection.

The results of immunohistochemistry to visualize binding of the antibody against CGRP is illustrated in Fig. 2. A similar pattern of CGRP and PGP 9.5 immunoreactivity was seen; no CGRP-immunoreactive taste cells were seen in taste buds. The nerve

fibers formed dense plexuses in the underlying subgemmal connective tissue; some of these fine and varicose fibers penetrated the apical and trench wall epithelia of papillae. The immunoreactivity distribution pattern of CGRP in the diabetic groups, at 3, 7, 9, 12, and 20 weeks after the STZ injection was similar to that of the control group, but the fibers penetrating the trench wall epithelium were attenuated. The CGRP-immunoreactive nerve fibers in the lamina propria of the diabetic groups showed less intense labeling than in control rats.

Morphometric and quantitative analyses

Morphometric analysis by light microscopy, illustrated in Fig. 3, showed no significant difference in papilla size (Fig. 3A) between the control ($971 \pm 81.6 \mu\text{m}$) and 9 and 12 weeks diabetic (924 ± 80.7 , and $1024 \pm 81.1 \mu\text{m}$) rats. In the control group, the number of the taste buds per papillae was 655 ± 39.52 , which was significantly higher than that of the 9 and 12 weeks diabetic groups at 610 ± 8.06 and 597 ± 31.98 ($p < 0.05$; Fig. 3B). There were significantly ($p < 0.01$) fewer PGP-9.5-immunoreactive cells, PGP-9.5- and CGRP-immunoreactive

nerve fibers per taste bud profile (Fig. 3C) in the diabetic groups. The control rats exhibited 35.6 PGP-9.5-immunoreactive cells per taste bud profile (35.6 cells/taste bud profile), and after the STZ injection, the number of PGP-9.5-immunoreactive cells per taste bud profile decreased very obviously from 3 weeks (24.6 cells/taste bud profile) to 7 weeks (21.0 cells/taste bud profile) and 9 weeks (14.9 cells/taste bud profile), then remained stable to the end of the experimental period (15.9 cells/taste bud profile at 12 weeks and 15.4 cells/taste bud profile at 20 weeks). Similar degrees of the PGP-9.5- and CGRP-immunoreactive nerve fibers in STZ-induced diabetic groups were seen in the vallate taste bud profiles ($p < 0.01$).

Discussion

Adult taste buds are sustained by the on-going trophic influence of axons that transport putative trophic agents, and they are thought to be a classic example of neurotrophically dependent receptor cells (Barry and Frank, 1992). The subgemmal fibers can certainly be activated by partially processed signals at the level of the basal cells or modulation

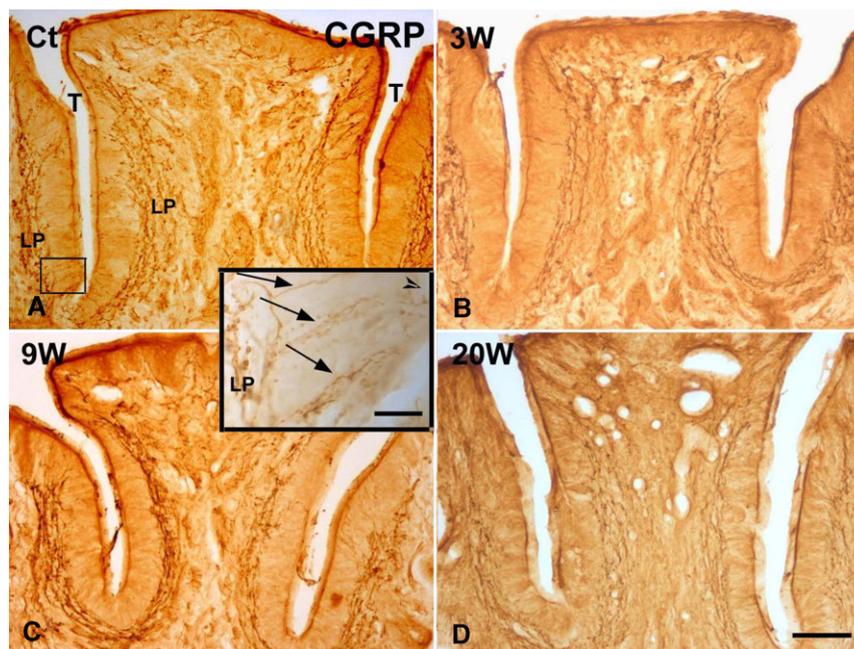


Figure 2. CGRP immunoreactivity in vallate papillae from control (A) and diabetic rats 3 (B), 9 (C), and 20 weeks (D) after the STZ injection. Inset: high magnification of the area indicated by the rectangle in the control group (A) shows typical immunoreactivity of CGRP. CGRP-immunoreactivity was only located in the nerve fibers (arrow). The fine and varicose CGRP-immunoreactive nerve fibers were observed in the taste bud (intragemmal nerve fibers) and between the taste buds and were derived from the subgemmal nerve plexus in the underlying lamina propria (LP). The immunoreactivity distribution pattern of CGRP in the diabetic groups (B, C, and D) was similar to that of the control group, but the fibers penetrating into the trench wall epithelium were attenuated. T, vallate trench and arrowhead, taste pore. Scale bars 100 μm and 25 μm for inset.

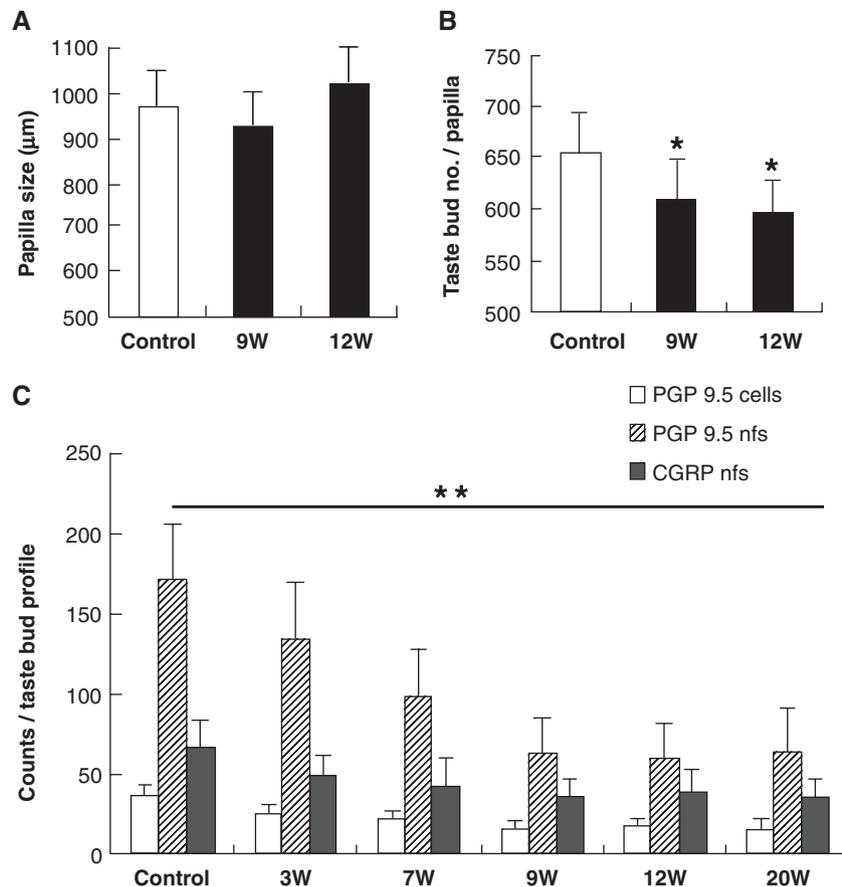


Figure 3. Porhometric measurements of the vallate papilla size (A), taste bud number (B), and quantitative analysis of PGP-9.5-immunoreactive cell and PGP-9.5- and CGRP-immunoreactive nerve fiber number per taste bud profile (C) in control and diabetic rats. * $p < 0.05$, ** $p < 0.01$ vs. the control.

of these signals by the antidromic release of neuropeptides at this level (Roper, 1989). Thus in adult subjects, taste buds degenerate when taste axons are removed, and regenerate when taste axons return (Kennedy, 1972; Oakley et al., 1993; Huang and Lu, 1996b). Chou et al. (2001a,b) demonstrated that nerve fibers invade the trench wall epithelium of vallate papillae earlier than taste cell and taste bud formation is apparent. As a result, we found that the number of PGP 9.5-immunoreactive taste cells and taste buds in diabetic rats decreased with diminished PGP 9.5- and CGRP-immunoreactive nerve fibers. Possible physiological roles which have been proposed for CGRP include either a neurotrophic effect on taste buds or a regulatory role on gustatory neurotransmission (Montavon and Lindstrand, 1991a, b; Huang and Lu, 1996a). The distributions of PGP 9.5 and CGRP immunoreactivity in the rat vallate taste buds in our study were consistent with those described in former studies (Iwanaga et al., 1992; Huang and Lu, 1996a; Wakisaka et al., 1996; Kusakabe et al.,

1998; Chou et al., 2001). However, the densities of PGP 9.5 and CGRP immunoreactivity in the taste buds of diabetic rats were gradually attenuated; this phenomenon being obvious as the experimental period advanced after STZ treatment to 9 weeks, and was then sustained at the same level through to the end of the experimental period (20 weeks). Although peripheral neuropathy is a serious complication of diabetes compared with other peripheral nerves, few studies on the innervations of diabetic taste buds have been performed. Abbasi (1981) compared taste reactions in diabetics with clinically established neuropathy and those who showed no neuropathy, and found that those with neuropathy showed increased taste thresholds to all taste modalities. Moreover, a steady increase in the threshold of taste acuity was demonstrated with the evolution and progression of diabetic neuropathy. These results support the possibility that taste impairment in diabetes is another expression of diabetic neuropathy. The quantitative analysis of the different neuropeptide-containing

nerve fibers including substance P in the tongue of the diabetic rat was demonstrated by Batbayar et al. (2004), who showed that the number of total immunoreactive nerve fibers decreased after 1 week of STZ treatment. Christianson et al. (2003) reported that 7 weeks after STZ administration, diabetic mice showed an obvious decrease in PGP 9.5- and CGRP-immunoreactive cutaneous axons from the flank region of the hind limb. Insulin-dependent diabetics affected by diabetes for more than 10 years exhibited significantly reduced epidermal CGRP- and PGP 9.5-immunoreactivity as reported by Properzi et al. (1993). In the present study, we provide morphological evidence consistent with neuropathy contributing towards taste impairment in diabetic subjects.

A decrease in the number of taste buds has been reported after interruption of innervation to the taste buds (Kennedy, 1972; Oakley et al., 1993; Huang and Lu, 1996b) and resulting from zinc deficiency (Chou et al., 2001). In the study reported here, STZ-treated rats showed fewer taste buds than the controls ($p < 0.05$). However, there was no difference in papilla size. It was proposed by Kennedy (1972) and Oakley et al. (1993) that the lost taste buds are replaced by lingual epithelium due to proliferation of epithelium to fill the space left by the taste buds. Zn (II) is known to be an essential trace element involved in the physiology of insulin (Arquillq et al., 1978; Klinlaw et al., 1983; Levine et al., 1983; Faure et al., 1992). In both animals and humans, it has been mentioned that taste disturbance is one symptom of zinc deficiency (Henkin et al., 1971; Catalanotto and Nanda, 1977; Kobayashi and Tomita, 1987; Kondo et al., 1987; Gibson et al., 1989). The main effects of zinc deficiency in rat vallate taste buds were demonstrated by Chou et al. (2001a), who reported decreases in the number and profile size of taste buds and fine structural changes in taste bud cells. It was considered that neurotrophins BDNF and NGF in the developing vallate papillae might act as local tropic factors for the embryonic growth of the fibers, inducing differentiation of the taste buds (Chou et al., 2001b). Christianson et al. (2003) reported that NGF-responsive cutaneous axons are affected in diabetic mice. These observations, taken together with those reported here, suggest that neurotrophic depletion also plays a role in events resulting in reduced taste perception in diabetics.

While all of the primary taste sensations may be blunted in diabetic patients (Schelling et al., 1965; Ronald et al., 1972; Lawson et al., 1979; Abbasi, 1981; Hardy et al., 1981; Niewoehner et al., 1986; Le Floch et al., 1989; Smith and Gannon, 1991;

Lamey et al., 1992; Tepper et al., 1996), the effect is not usually severe and is generally tolerated without complaint. Patients with diabetes who have a high threshold for taste sensation may consume larger quantities of food in order to obtain the same level of gustatory satisfaction, which consequently exacerbates the hyperglycemia. Retrospective and prospective studies have suggested a relationship between hyperglycemia and the development and severity of diabetic neuropathy. We believe that understanding the cause of taste disorders has an important role to play in protecting a patient's quality of life.

In conclusion, results from the present study show that taste cells and nerves were altered in the diabetic model; and the number of taste buds were decreased. We propose that such results transposed to human diabetes would indicate that insulin affects taste acuity through its neuropathy.

Acknowledgments

This work was supported by Grant nos. TMU-90-Y05-A109 and TMU-91-Y05-A121 from Taipei Medical University. The technical assistance of Ms. S.M. Lai is greatly appreciated.

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