The Effect of Diabetes on Neuropeptide Content in the Rat Cornea and Iris

Carl F. Marfurt and Stephen F. Echtenkamp

Purpose. To determine the effect of diabetes mellitus on the levels of substance P (SP), calcitonin gene-related peptide (CGRP), and vasoactive intestinal polypeptide (VIP) in the rat cornea and iris.

Methods. Corneas and irides from control and diabetic rats were processed for neuropeptide radioimmunoassay 3 months after induction of diabetes with streptozotocin. Corneas and irides also were processed for SP and CGRP immunohistochemistry and were evaluated qualitatively.

Results. The radioimmunoassay data revealed no significant differences in either the content or concentration of SP, CGRP, and VIP between control and diabetic corneas. In contrast, iridial levels of CGRP and SP were significantly increased by 38% and 256%, respectively, in the diabetic animals. Iridial VIP levels were unchanged in the diabetic versus control groups. Immunohistochemical demonstrations of corneal and iridial SP- and CGRP-immunoreactive fiber plexuses were indistinguishable on the basis of purely qualitative criteria.

Conclusions. The results of this study have demonstrated a target- and peptide-specific effect of short-term diabetes on SP and CGRP expression in ocular nerves of the anterior eye segment. The absence of demonstrable changes in corneal neuropeptide levels argue against the theory that corneal abnormalities seen in clinical diabetes are caused, in part, by deficits in synthesis or axonal transport of "trophic" peptides in corneal sensory nerves. In contrast, elevated iridial SP and CGRP levels may be responsible for reported clinical deficits in pupillary diameter regulation. Invest Ophthalmol Vis Sci. 1995;36:1100-1106.

Diabetes mellitus is associated with a variety of ocular complications. The most serious of these are retinopathy and cataract; however, corneal complications also have been reported in patients with this disease. The corneal epithelium in diabetes exhibits a number of morphologic abnormalities, including cellular enlargement, reduced numbers of hemidesmosomes, basement membrane abnormalities, and ulcerations. Metabolic abnormalities include decreased oxygen consumption and uptake rate, increased permeability to fluorescein, and delayed healing with persistent epithelial defects after vitrectomy.

In the streptozotocin-treated diabetic rat model, corneal nerves demonstrate morphologic abnormalities, including occasional axonal degeneration, changes in the normal periodicity of nerve fiber beading, and ultrastructural irregularities of the Schwann cell basal lamina. Humans with diabetes often exhibit decreased levels of corneal sensitivity, suggesting a partial loss of corneal sensory innervation or functional perturbation of the sensory apparatus. These observations have prompted speculation that corneal abnormalities in diabetes may be related to...
diabetic neuropathy and that they reflect a loss of trophic influences from the trigeminal nerve and its branches.1,6,15

The mechanisms by which trigeminal sensory nerves exert their trophic effects on the cornea are unclear; however, the release of axonally transported neuropeptides has been proposed.18 Two peptides present in abundance within corneal sensory nerve fibers are substance P19 (SP) and calcitonin gene-related peptide (CGRP).20 Substance P and CGRP exert trophic influences on various peripheral tissues and cell types,21-23 and recent evidence suggests that these peptides can modify corneal epithelial mitogenesis,24 cell migration,25 and wound healing.26

Alteration of neuropeptide content from changes in peptide synthesis, axoplasmic transport, and/or release is a common feature of diabetic neuropathy27-32 and has been reported for several ocular tissues. For example, irides of diabetic rats contain increased numbers of vasoactive intestinal polypeptide (VIP)-immunoreactive nerves in the dilator muscle33 and overall approximately three times more SP34 than do irides of age-matched control animals. In the vascular system of the diabetic rat optic nerve sheath, neuropeptide Y content is elevated35 and the numbers of catecholaminergic nerve fibers are decreased.36 In diabetic Chinese hamsters, ocular VIP content is increased 50% over control levels, with most of the increase attributed to increased VIP content of the choroidal nerves.37

Whether similar changes in peptide expression occur in the diabetic cornea has, to our knowledge, never been investigated. This information would shed light on the relationship between diabetic neuropathy and the development of corneal abnormalities in diabetes. In the present study, we have investigated changes in corneal and iridal SP, calcitonin gene-related peptide, and vasoactive intestinal polypeptide content in the diabetic rat cornea and iris by combined radioimmunoassay and immunohistochemical techniques.

MATERIALS AND METHODS

Twenty-four female, young adult Sprague-Dawley rats, each weighing 233 g on average, were used in this study. All animals were obtained and cared for in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Twelve of the animals were injected intraperitoneally with streptozotocin, a pancreatic beta cell toxin,28 to produce a rat model of insulin-dependent diabetes mellitus.29 The remaining 12 animals were injected with vehicle only and served as age-matched controls. Before the injections, all animals were weighed, and their blood glucose levels were determined by assay of tail capillary blood using glucose oxidase-impregnated test strips and an Ames Blood Glucometer II (Miles; Elkhart, IN). Streptozotocin solutions (65 mg/kg body weight in 0.01 M citrate buffer, pH 4.5) were prepared fresh for each animal immediately before use. Seventy-two hours later, induction of diabetes in the streptozotocin-injected animals was confirmed by assay of non-fasting serum glucose levels.

Diabetic and control animals were maintained for 3 months under identical conditions, and they ingested food and water ad libitum. Three of the diabetic rats died unexpectedly 1 to 3 weeks before the conclusion of the experiment; thus, data were obtained from 9 diabetic and 12 control animals. Body weights and serum glucose levels were Redetermined for all surviving animals immediately before sacrifice. Each animal was killed by lethal injection of sodium pentobarbital and exsanguination. Before enucleation, the corneas were examined macroscopically for evidence of corneal opacities, ulcers, neovascularization, or other corneal abnormalities.

Radioimmunoassay

Corneas and irides from six diabetic and six control animals were prepared for neuropeptide radioimmunoassay (RIA). Immediately after death, the eyes were enucleated and transferred to a petri dish filled with ice-cold saline. Under a dissecting microscope, the whole cornea was cut from each eye and was trimmed to exclude the corneoscleral limbus. The whole iris was peeled from its site of attachment and carefully trimmed to remove all vestiges of the adherent ciliary processes. The tissues were then blotted for 1 to 2 seconds on a filter paper disk, weighed, and immediately frozen at −90°C. The frozen specimens were extracted for neuropeptides by boiling in 1 M acetic acid for 10 minutes and homogenization with a Brinkman Polytron (Brinkman Instruments, Westbury, NY). The boiled homogenized mixtures were sedimented in a refrigerated centrifuge, and the supernatant was lyophilized and stored at −90°C until RIA was performed. Standard competitive binding RIAs for rat CGRP, SP, and VIP were performed using specific antisera and reagents obtained from Peninsula Laboratories (Belmont, CA).

Radioimmunoassay Data Treatment

Peptide levels were calculated in femtomoles per milligram of wet tissue. Group data were expressed as means ± standard error of the mean. Analysis of variance, in conjunction with a modified t-statistic, was used to make comparisons between treatment groups.40 Values of \( P < 0.05 \) were considered statistically significant.
Immunohistochemistry

Corneas and irides from three diabetic animals and six control animals were processed for SP and CGRP immunohistochemistry. The tissues were immersion-fixed for 2 to 3 hours in ice-cold 4% paraformaldehyde-0.2% picric acid in 0.1 M phosphate buffer. Corneas were infiltrated with OCT compound (Miles), frozen flat on chucks, and serially sectioned at 30 μm from anterior to posterior in a cryostat. Conventional immunohistochemical procedures were applied to free-floating corneal sections and iridal whole mounts by incubating for 2 hours at 37°C in 1:500 to 1:2000 dilutions of primary antibody (anti-SP or anti-CGRP; Amersham, Arlington Heights, IL) and using a Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA). Material from diabetic and control rats were incubated simultaneously in adjacent tissue culture wells under identical conditions to minimize immunohistochemical variability. Standard tests for specificity of the immunocytochemical reactions were performed on randomly selected control sections from each group by incubating the tissues in normal rabbit serum lacking primary antibody or by reacting the tissue according to the ABC method with one of the steps omitted.

Data Analyses

Substance P- and CGRP-immunoreactive fiber populations were critically examined under an Olympus (Lake Success, NY) BH-2 light microscope. Corneal and iridal tissues were evaluated with reference to several qualitative criteria, including numbers of labeled nerve fibers per corneal section or iridal whole mount (innervation density), individual fiber staining intensities, and fiber morphology and distribution patterns. The specimens were evaluated independently by two different observers, and the slides were coded by a technician so as not to reveal the source of the specimens under examination.

RESULTS

The 3-month period after streptozotocin treatment resulted in a significant 15% weight loss in the diabetic animals and a significant 7% weight gain in the control animals. Mean fasting blood glucose levels before treatment were 78 mg/dl for both groups. After 3 months, the vehicle-treated animals' blood glucose levels had not changed significantly, whereas the blood glucose levels in the streptozotocin-treated animals averaged 458 mg/dl, indicating a severely insulin-depleted state.

At time of sacrifice, no obvious corneal or iridal abnormalities were detected with the dissecting microscope. The weights of the corneas and irides were not significantly different between the diabetic and control groups, in spite of the differences in body weights between these animals.

Figure 1 shows the results of RIA for CGRP, SP, and VIP in the corneas and irides of control versus streptozotocin-treated rats. The data are expressed as femtomoles per milligram (fmol/mg) of wet weight concentrations. No significant differences in corneal neuropeptide levels between diabetic and control groups were seen with reference to any of the peptides examined. In contrast, significant differences (P < 0.05) in iridal substance P and CGRP levels between diabetic and control groups were observed and are indicated by asterisks.

Figure 1. Neuropeptide levels ± SEM in corneas and irides from diabetic (black bars) and control (white bars) rats. Levels of calcitonin gene-related peptide (CGRP), substance P (SP), and vasoactive intestinal polypeptide (VIP) are expressed as femtomole per milligram (fmol/mg) of wet weight concentrations. No significant differences in corneal neuropeptide levels between diabetic and control groups were seen with reference to any of the peptides examined. In contrast, significant differences (P < 0.05) in iridal substance P and CGRP levels between diabetic and control groups were observed and are indicated by asterisks.
FIGURE 2. Immunohistochemical demonstration of CGRP- and SP-immunoreactive nerves in control (A, C, E) and diabetic (D, B, F) rat eyes. Three months after induction of diabetes, the numbers, morphologies and staining intensities of corneal (A, B) and iridial (C–F) CGRP- and SP-immunoreactive fibers in control and diabetic animals were qualitatively indistinguishable.
the irides of the streptozotocin-treated rats showed significantly elevated concentrations of CGRP (38% increase) and even more pronounced elevations of SP (256% increase) (Fig. 1). In contrast, iridial VIP levels were not significantly altered in the diabetic state. In contrast to the RIA data, qualitative assessment of control and diabetic irides processed immunohistochemically for CGRP (Figs. 2c, 2d) or SP (Figs. 2e, 2f) revealed no obvious differences in peptidergic fiber density or staining intensity.

DISCUSSION

Diabetic neuropathy is a symmetric polyneuropathy characterized by distal-to-proximal progression, varying degrees of axonal atrophy and cytoskeletal abnormalities, and impaired regenerative capabilities. In the streptozotocin diabetic rat model, axonal structural abnormalities and conduction velocity deficits occur as early as 2 to 4 weeks after induction of the disease. Alterations in axonal neuropeptide content have been reported in the gastrointestinal tract, skin, seminal vesicle, penis, and eye of experimental animals beginning 4 to 8 weeks after induction of diabetes; however, the described changes do not follow predictable patterns, and levels of particular peptides have been reported to increase, decrease, or remain unchanged depending on the target tissue innervated. Whether or not similar changes in neuropeptide content occur in peripheral nerves of humans with diabetes is unknown.

The main goal of the present study was to test the hypothesis that corneal abnormalities seen clinically in diabetes may be due in part to deficiencies in expression, transport, and/or release of "trophic" substances (specifically, SP and CGRP) from corneal sensory nerve terminals. The findings of the present study using short-term diabetic rats do not support this hypothesis; however, one cannot rule out totally the possibility that subtle alterations in SP and CGRP synthesis, transport, or storage may, over several years, contribute to the long-term impairment in corneal morphology and metabolism seen in clinical diabetes. Alternatively, the pathogenesis of corneal abnormalities in clinical diabetes may involve deficiencies in other trophic factors or it may be largely nonneurogenic in origin.

In contrast, iridial sensory nerves are, under the conditions examined in this study, clearly susceptible to diabetes-induced changes in peptide expression. The dramatic (approximately 250%) increase in SP in the 3-month-old streptozotocin-diabetic rat iris shown here extends earlier observations by Tomlinson et al in long-term survival (11 months) animals that had been given long-acting insulin injections to reduce morbidity. Thus, this study demonstrates that the upregulation of SP expression that occurs within iridial nerves in diabetes develops relatively quickly. In addition, we have shown that the pronounced increase in iridial SP is accompanied by a smaller increase in iridial CGRP levels. This is of interest because the vast majority of iridial sensory nerves that contain SP also contain CGRP, and it is probable that the two peptides colocalize within the same synaptic vesicles. One way to interpret this data is that diabetes induces selective biochemical changes in iridial sensory nerves that result in pronounced "upregulation" of certain neuropeptides, with lesser effects on others. Thus, we have shown that the increases in SP and CGRP expression seen here are target specific (that is, they occur in the iris but not in the cornea) as well as peptide specific (the increase in SP is more pronounced than CGRP).

The increases in iridial peptide levels in the diabetic rat are especially notable in light of the fact that peripheral nerves in short-term diabetes exhibit several defects of axonal transport, including diminished transport of SP and CGRP. Moreover, SP levels in the trigeminal and dorsal root ganglia of diabetic animals are substantially decreased, suggesting possible decreased synthesis of this peptide by the primary afferent perikarya. Thus, the mechanism by which iridial levels of substance P and CGRP are increased in the face of decreased neuronal synthesis and diminished axonal transport remains to be determined, although it indirectly suggests a defective peptide release mechanism.

Interestingly, the increases in iridial SP and CGRP seen here by RIA were not obvious in the iridial whole mounts processed for immunohistochemistry. The reason for this apparent discrepancy is unclear; however, it may be that changes in SP or CGRP peptide contents are more readily detected by quantitative (RIA) than qualitative (immunohistochemical) methods or that the two methods recognize slightly different forms, or pools, of the same peptide.

The pronounced increases in iridial SP content and concentration that we have demonstrated in the diabetic animals of the present study may be of functional significance. Substance P is a potent miotic in humans and other mammalian species, and it is tempting to speculate that elevated tissue levels of this peptide (especially in the presence of an existing iridial autonomic neuropathy) is responsible for the small pupil size seen in many patients with clinical diabetes.

In summary, the results of this study have shown that induction of diabetes in the rat results in a target-specific and peptide-specific alteration of neuropeptide content in the anterior eye segment. Within 3
Neuropeptides in the Diabetic Cornea and Iris

months of disease onset, significant increases in SP and CGRP content occur in the iris. In contrast, iridial VIP levels, and corneal SP, CGRP, and VIP levels remain unchanged. The data suggest that alterations in corneal neuropeptide expression may underlie deficits in pupillary diameter regulation, but not corneal abnormalities, seen in clinical diabetes.

Key Words
substance P, calcitonin gene-related peptide, vasoactive intestinal polypeptide, diabetic neuropathy

Acknowledgments
The authors thank Mr. Lawrence Ellis for excellent technical assistance and Dr. Richard Peterson for advice in injecting rats with streptozotocin.

References
31. Willars GB, Calcutt NA, Compton AM, Tomlinson DR, Keen P. Substance P levels in peripheral nerve, skin, atrial myocardium and gastrointestinal tract of rats.