Changes in Corneal Innervation and Sensitivity and Acetylcholine-Mediated Vascular Relaxation of the Posterior Ciliary Artery in a Type 2 Diabetic Rat

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PURPOSE. Corneal confocal microscopy is emerging as a clinical tool to evaluate the development and progression of diabetic neuropathy. The purpose of these studies was to characterize the changes in corneal sensitivity and innervation in a rat model of type 2 diabetes in relation to standard peripheral neuropathy endpoints. Assessment of diabetes-induced changes in corneal innervation and sensitivity in animal models will be important for determining the usefulness of corneal markers for preclinical studies to test potential new treatments for diabetic neuropathy.

METHODS. High-fat/low-dose streptozotocin diabetic rats were used to examine diabetes-induced changes in standard diabetic neuropathy endpoints and innervation of the cornea using confocal microscopy, corneal sensitivity using a Cochet-Bonnet esthesiometer, and vascular reactivity of the posterior ciliary artery.

RESULTS. Compared with age-matched control rats, the induction of hyperglycemia in rats fed high-fat diets caused a decrease in nerve conduction velocity, thermal hypoalgesia, and intraepidermal nerve fiber profiles. In the cornea there was a decrease in corneal nerve fiber length and sensitivity. In addition, vascular relaxation in response to acetylcholine was decreased in the posterior ciliary artery.

CONCLUSIONS. These studies suggest that in a type 2 diabetic rat model, changes in corneal nerve innervation and sensitivity occur that are consistent with changes seen in diabetic patients. Corneal sensitivity and innervation may be valuable endpoints for examining the potential treatments of diabetic neuropathy in preclinical studies. (*Invest Ophthalmol Vis Sci*. 2012;53:1182–1187) DOI:10.1167/iovs.11-8806

Diabetic neuropathy is a common complication of diabetes with no known treatment.¹ Translation of effective treatments of diabetic animal models has failed in clinical trials.² This is due in part to endpoints in animal studies that were

insensitive when applied in human studies.² To address this issue, corneal confocal microscopy has emerged as a tool to measure small nerve fiber damage as a surrogate marker for the early detection of diabetic neuropathy.³ Application of this technology has been successful in human studies, but to date few animal studies have been performed.^{3–8} To address this issue, we have compared the effect of diabetes on standard nerve functional endpoints in a rat model of type 2 diabetes with changes in corneal innervation and sensitivity and vascular reactivity in the posterior ciliary artery. The goal of these studies was to determine whether type 2 diabetes causes changes in corneal innervation and sensitivity and to determine how these changes compare with standard peripheral nerve endpoints. These studies are important for verifying corneal confocal microscopy as a marker of diabetic neuropathy in animal models of diabetes that can be used in preclinical studies for evaluating and developing potential treatments.

For these studies we used high-fat-fed/low-dose streptozotocin-treated rats, an animal model for type 2 diabetes.^{9,10} Rats fed a high-fat diet do not become hyperglycemic, presumably because of compensatory hyperinsulinemia.⁹ However, treating high-fat-fed rats with a low dose of streptozotocin damages insulin-producing β -cells so that hyperglycemia develops even though insulin levels are similar or even higher than in chow-fed normoglycemic rats.⁹ The diabetes in these rats is analogous to the development of human type 2 diabetes, when the decline in hyperinsulinemia is not able to compensate for insulin resistance and hyperglycemia occurs.⁹ In our hands this rat models late-stage type 2 diabetes.¹¹

METHODS

Unless stated otherwise all chemicals used in these studies were obtained from Sigma Chemical Co. (St. Louis, MO).

Animals

Male Sprague-Dawley (Harlan Sprague-Dawley, Indianapolis, IN) rats 10 to 11 weeks of age were housed in a certified animal care facility, and food (#7001; Harlan Teklad, Madison, WI) and water were provided ad libitum. All institutional guidelines for the use of animals were followed. The studies adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. At 12 weeks of age, the rats were separated into two groups. One of these groups was placed on a high-fat diet (45% of grams from fat; D12451; Research Diets, New Brunswick, NJ).¹¹ The other group was maintained on the control diet, which contained 4.25% of grams from fat. After 8 weeks, rats on the high-fat diet were treated with streptozotocin (30 mg/kg in 0.9% NaCl, administered intraperitoneally). Rats on the control diet received vehicle. Diabetes was verified 96 hours later by evaluating blood glucose levels with the use of glucose-oxidase reagent strips (Lifescan Inc., Milpitas, CA). Rats with blood glucose levels 250 mg/dL or greater were considered diabetic. Diabetic rats were maintained on the high-fat diet for the duration of the study, which was 12 weeks.

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Thermal Nociceptive Response, Tear Secretion, and Corneal Sensitivity

Thermal nociceptive response in the hind paw was measured using the Hargreaves method, as previously described.^{11,12} Data were reported in seconds. Tear secretion was determined using phenol red-impregnated cotton threads (Zone-Quick; Showa Yakuhin Kako Co., Tokyo, Japan).⁸ The threads were placed in the medial canthus for 1 minute, and the length of the wetted section was measured in millimeters. Corneal sensation was measured using a Cochet-Bonnet filament esthesiometer in unanesthetized rats (Luneau Ophthalmologie, Luneau, France).⁸ The testing began with the nylon filament extended to the maximal length (6 cm). The end of the nylon filament was touched to the cornea. If the rat blinked (positive response), the length of the filament was recorded. If the rat did not blink, the nylon filament was shortened by 0.5 cm and the test was repeated until a positive response was recorded. This process was repeated for each eye three times.

Motor and Sensory Nerve Conduction Velocity

On the day of terminal studies, rats were weighed and anesthetized (Nembutal IP, 50 mg/kg; Abbott Laboratories, North Chicago, IL). Motor nerve conduction velocity was determined as previously described using a noninvasive procedure in the sciatic-posterior tibial conducting system.^{11,13} Sensory nerve conduction velocity was determined using the digital nerve, as described by Obrosova et al.¹⁴ Motor and sensory nerve conduction velocity was reported in meters per second.

Corneal Innervation

Corneal nerves were imaged using the cornea module (Rodentstock) of a confocal microscope (Heidelberg Retina Tomograph; Heidelberg Engineering, Heidelberg, Germany). The anesthetized rat was secured to a platform that allows adjustment and positioning of the rat in three dimensions. A drop of lubricant eye gel (GenTeal; Novartis, Basel, Switzerland) was applied onto the tip of the lens and advanced slowly forward until the gel touched the cornea, allowing optical but not physical contact between the objective lens and the corneal epithelium.³ Ten random high-quality images of the subbasal nerve plexus around the perimeter of the central cornea were acquired by finely focusing the objective lens to maximally resolve the nerve layer just under the corneal epithelium. The investigator acquiring these images was masked with respect to identity of the animal condition. For these studies, a single parameter of corneal innervation was quantified. Corneal nerve fiber length defined as the total length of all nerve fibers and branches (in millimeters) present in the acquired images standardized for area of the image (in square millimeters) was determined for each image by tracing the length of each nerve in the image, summing the total length and dividing by the image area.^{3,8} The corneal fiber length for each animal was the mean value obtained from the 10 acquired images and was expressed as millimeters per square millimeter. Based on receiver operating characteristic curve analysis, corneal nerve fiber length is the optimal parameter for diagnosing patients with diabetic neuropathy.⁴

Additional measurements included nonfasting blood glucose and HbA1C levels (Glyco-tek affinity column; Helena Laboratories, Beaumont, TX). Serum was collected for determining free fatty acid, serum triglyceride, and free cholesterol levels using commercial kits from Roche Diagnostics (Mannheim, Germany), Sigma Chemical Co. (St. Louis, MO), and Bio Vision (Mountain View, CA), respectively. Serum thiobarbituric acid reactive substances level were determined as a marker of oxidative stress, as previously described.^{11–13}

Vascular Reactivity in Posterior Ciliary Artery

The ophthalmic artery travels along the inferior side of the optic nerve sheath and divides into three branches: central retinal artery, medial posterior ciliary artery, and lateral posterior ciliary artery. We isolated both ciliary arteries for vascular studies using videomicroscopy by

carefully removing the entire eye along with the optic nerve and trimming excess tissue from the desired vessels.¹¹ The isolated vessels were cannulated at both ends with glass micropipettes filled with buffer (4°C) and secured with 10–0 nylon monofilament sutures (Ethicon, Cornelia, GA). The pipettes were then attached to a single pressure reservoir (initially set at 0 mm Hg) under condition of no flow. The organ chamber containing the cannulated vessels was then transferred to the stage of an inverted microscope (CK2; Olympus, Lake Success, NY), as previously described.¹¹ It was connected to a rotary pump (Masterflex; Cole Parmer Instrument, Vernon Hills, IL) that continuously circulated 37°C oxygenated Krebs-Henseleit physiological saline solution (PSS) of the following composition: 118 mmol/L NaCl, 4.7 mmol/L KCl, 2.5 mmol/L CaCl₂, 1.2 mmol/L KH₂PO₄, 1.2 mmol/L MgSO₄, 20 mmol/L NaHCO₃, 0.026 mmol/L Na₂EDTA, and 5.5 mmol/L glucose at 30 mL/min. The pressure within the vessel was then slowly increased to 60 mm Hg. At this pressure, we found that KCl gave the maximal constrictor response. Therefore, all the studies were conducted at 60 mm Hg. Internal vessel diameter (resolution of 2 μ m) was measured by manually adjusting the video micrometer. After a 30-minute equilibration, KCl was added to the bath to test vessel viability. Vessels failing to constrict by at least 30% were discarded. After they were washed with PSS, vessels were incubated for 30 minutes in PSS and then constricted with phenylephrine (10^{–6} mol/L) (Cayman Chemical, Ann Arbor, MI) to 30% to 50% of passive diameter. Afterward, a cumulative concentration-response relationship was evaluated for acetylcholine (10^{–8}–10^{–4} M). At the end of each dose-response curve, papaverine (10^{–5} M) was added to determine maximal vasodilation. Data were presented as percentage of relaxation.

Intraepidermal Nerve Fiber Density in the Hind Paw

Immunoreactive intraepidermal nerve fiber profiles, which are primarily sensory nerves, were visualized using confocal microscopy. Skin samples of the right hind paw were fixed, dehydrated, and embedded in paraffin. Sections (7 μ m) were collected and immunostained with anti-PGP9.5 antibody (rabbit anti-human; AbD Serotec, Morpho Sys US Inc., Raleigh, NC) overnight, followed by treatment with secondary antibody Alexa Fluor 546 goat anti-rabbit (Invitrogen, Eugene, OR). Profiles were counted by two investigators masked to the sample identity. All immunoreactive profiles within the epidermis were counted and normalized to epidermal length.^{11,15}

Statistical Analysis

Results are presented as mean $\pm$ SEM. Comparisons between control and diabetic rats were conducted using unpaired Students *t*-test (Prism; GraphPad, San Diego, CA). Concentration-response curves for acetylcholine were compared using two-way repeated measures analysis of variance with autoregressive covariance structure (Proc Mixed; SAS Institute, Cary, NC).¹¹ *P* < 0.05 was considered significant.

RESULTS

Effect of High-Fat/Streptozotocin Diabetes on Weight, Blood Glucose, and Serum Lipid and Thiobarbituric Acid Reactive Substances

Data in Table 1 demonstrate that control and diabetic rats gained approximately the same amount of weight over the study period. Blood glucose levels were significantly increased in diabetic rats compared with control rats, as were HbA1C levels. Data in Table 2 demonstrate that diabetic rats were hyperlipidemic, with free fatty acid, serum triglyceride, and free cholesterol levels all significantly elevated above control. Serum thiobarbituric acid reactive substances were also significantly increased in diabetic rats.

TABLE 1. Changes in Body Weight, Blood Glucose Level, and Hemoglobin A1C in High-Fat/Streptozotocin Diabetic Rats

Determination	Control Rats (<i>n</i> = 9)	Diabetic Rats (<i>n</i> = 9)
Start weight, g	421 ± 13	415 ± 10
End weight, g	525 ± 18	518 ± 18
Blood glucose, mg/dL	109 ± 8	443 ± 26*
HbA1C, %	8.6 ± 0.3	17.6 ± 0.5*

Data are presented as the mean ± SEM.

* *P* < 0.05 compared with control.

Effect of High-Fat/Streptozotocin Diabetes on Nerve Conduction Velocity, Thermal Nociception, and Intraepidermal Nerve Fiber Density

Data in Figure 1 demonstrate that motor and sensory nerve conduction velocities were significantly decreased in diabetic rats compared with control rats. Diabetic rats were also thermal hypoalgesic, and the number of intraepidermal nerve fiber profiles in the hind paw was significantly decreased (Fig. 2).

Effect of High-Fat/Streptozotocin Diabetes on Corneal Sensitivity and Innervation

Figure 3 provides representative illustrations of intraepidermal nerve fibers and corneal innervation from the hindpaw for control and diabetic rats. For the first time it is shown that in high-fat/streptozotocin diabetic rats, tear secretion was significantly reduced compared with control rats (Table 2). Corneal sensitivity was also significantly decreased in diabetic rats compared with control rats (Fig. 4). In this study a positive blinking response to the 6-cm-long filament (most sensitive) was obtained in 8 of 9 control rats and only 2 of 9 diabetic rats. Data in Figure 4 also demonstrate that the total length of corneal nerves was significantly decreased in diabetic rats compared with control rats.

Effect of High-Fat/Streptozotocin Diabetes on Acetylcholine-Mediated Vascular Relaxation by Posterior Ciliary Arteries

We have previously demonstrated that high-fat/streptozotocin-induced diabetes caused a decrease in acetylcholine-mediated vascular relaxation in epineurial arterioles.¹¹ We also wanted to establish a vascular endpoint that could be used as an independent marker for determining the effect of diabetes on vascular function related to the retina. Using the posterior ciliary artery, we demonstrated that diabetes caused a significant decrease in vascular relaxation in response to acetylcholine compared with control (Fig. 5). Additional characterization of vascular reactivity of the posterior ciliary demonstrated

TABLE 2. Changes in Serum Thiobarbituric Acid Reactive Substance, Triglyceride, Free Fatty Acid, and Cholesterol Levels and Tear Production Length in High-Fat/Streptozotocin Diabetic Rats

Determination	Control Rats (<i>n</i> = 9)	Diabetic Rats (<i>n</i> = 9)
Thiobarbituric acid reactive substance, µg/mL	2.5 ± 1.0	7.9 ± 0.7*
Triglyceride, mg/dL	26 ± 3	64 ± 16*
Free fatty acid, mmol/L	0.08 ± 0.02	0.29 ± 0.06*
Cholesterol, mg/mL	1.7 ± 0.2	5.5 ± 1.9*
Tear production, fiber length, mm	27.4 ± 1.2	20.8 ± 1.3*

Data are presented as the mean ± SEM.

* *P* < 0.05 compared with control.

FIGURE 1. Effect of high-fat/streptozotocin diabetes on motor and sensory nerve conduction velocity. Motor and sensory nerve conduction velocity was examined. Data are presented as the mean ± SEM in meters per second. Each group contained nine control and nine diabetic rats. **P* < 0.05 compared with control rats.

that after the endothelium is removed, the vessel is nonresponsive to acetylcholine but completely relaxes in response to 10^{-4} M sodium nitroprusside (data not shown). This suggests that acetylcholine-mediated relaxation is endothelium dependent. Preincubating posterior ciliary arteries with 10^{-4} M L-arginine methyl ester, an inhibitor of endothelial nitric oxide synthase, reduced maximal acetylcholine (10^{-5} M) vasodilation from $94.4\% \pm 1.9\%$ to $12.5\% \pm 3.2\%$ (*n* = 8), suggesting that acetylcholine-mediated relaxation of posterior ciliary artery is dependent on the production of nitric oxide.¹⁶

DISCUSSION

The important findings originating from this study are that corneal sensation, corneal innervation, and tear secretion were impaired in a rat model of type 2 diabetes. In addition, we found that vascular relaxation of the posterior ciliary artery to acetylcholine was impaired in diabetic rats. Although more studies must be conducted, such as establishing a time course for the development and progression of impaired corneal sensitivity and denervation, the present results suggest that impairment of corneal function and innervation along with vas-

FIGURE 2. Effect of high fat/streptozotocin diabetes on thermal nociception and intraepidermal nerve fiber density. Thermal nociception and intraepidermal nerve fiber density were examined. Data are presented as the mean ± SEM for thermal nociception per second and intraepidermal nerve fiber profiles per millimeter. Each group contained nine control and nine diabetic rats. **P* < 0.05 compared with control rats.

FIGURE 3. Representative illustration of the effect of high-fat/streptozotocin diabetes on intraepidermal nerve fiber profiles and corneal innervation. *Top, white arrows:* intraepidermal nerve fibers from the hind paw of a control rat (*left*) and a diabetic rat (*right*). *Bottom, black arrows:* corneal nerve fibers of a control rat (*left*) and a diabetic rat (*right*). In the diabetic rats, fewer nerve fibers are found in the skin of the hind paw or cornea.

cular reactivity of the posterior artery may be valuable endpoints for preclinical studies for treatments of diabetic neuropathy.

Therapies for diabetic neuropathy found to be successful in diabetic animal models have not been successful when applied in clinical trials.^{2,17,18} Many possible reasons have been presented to explain the lack of success in translating successful results from animal studies to humans.^{2,17–20} However, from these failures has come the development of new endpoints identifying early small nerve fiber damage in the skin or cornea that can accurately quantify the severity of diabetic neuropathy and progression. These endpoints could lead to improved clinical trials and discovery of treatments for diabetic neuropathy.^{4–7,21,22} Even though these new developments provide hope for future clinical trials, it must be remembered that

FIGURE 4. Effect of high-fat/streptozotocin diabetes on corneal sensitivity and innervation. Corneal sensation was determined with a Cochet-Bonnet esthesiometer and corneal innervation using a confocal microscope. Data are presented as the mean $\pm$ SEM for corneal sensitivity in centimeters and corneal innervation as total nerve fiber length in millimeters per square millimeter. Each group contained nine control and nine diabetic rats. * $P < 0.05$ compared with control rats.

FIGURE 5. Effect of high-fat/streptozotocin diabetes on vascular relaxation by acetylcholine in posterior ciliary arteries. Pressurized vessels (60 mm Hg and 180- to 250- μ m luminal diameter) were constricted with phenylephrine (30%–50%), and incremental doses of acetylcholine were added to the bathing solution while recording steady state vessel diameter. Data are presented as the mean of percentage relaxation $\pm$ SEM. Each group contained nine control and nine diabetic rats. * $P < 0.05$ compared with control.

diabetic neuropathy is a complicated disorder with multifactorial mechanisms.²³ It is unlikely that a monotherapeutic approach will be successful in treating diabetic neuropathy. Testing multitherapeutics for diabetic neuropathy in humans will be difficult and expensive. Therefore, it seems that animal studies will be needed to advance our understanding and to test the efficacy of multiple drug approaches. However, future animal studies must be performed using translational endpoints that will increase the likelihood of success in clinical trials. For this reason, we have initiated studies to compare the effect of diabetes on corneal sensitivity and innervation to standard diabetic neuropathy endpoints.

For these studies, we used the high-fat-fed/low-dose streptozotocin-treated rat as an animal model for type 2 diabetes.^{9,10} Rats fed a high-fat diet do not become hyperglycemic, presumably because of compensatory hyperinsulinemia.^{9,11,15} However, treating high-fat-fed rats with a low dose of streptozotocin damages insulin-producing β -cells so that hyperglycemia develops even though insulin levels are similar to or even higher than those in chow-fed normoglycemic rats.^{9,11} The diabetes in these rats is analogous to the development of human type 2 diabetes when the decline in hyperinsulinemia is not able to compensate for insulin resistance and hyperglycemia occurs.⁹ In our hands, this rat models late-stage type 2 diabetes.¹¹ This diabetic model has been used for pharmacologic drug screening, which will become important as we begin to conduct studies examining potential treatments for diabetic neuropathy using confocal microscopy of corneal nerves as a surrogate marker for diabetic neuropathy.^{9,10} For instance, Reed et al.⁹ demonstrated that treating these rats with metformin and troglitazone, common treatments for patients with type 2 diabetes, reduced hyperglycemia and insulin resistance. We were the first to characterize vascular and neural complications in this model and have demonstrated that treating these diabetic rats with a vasoactive peptide improves vascular and neural function.^{11,24} We could have chosen other rat models of type 2 diabetes for this study. The Zucker diabetic fatty (ZDF) rat has been widely used by many investigators, including us.^{25,26} For these studies, we decided not to use this model because of concerns that deletion of the leptin receptor could have unknown effects on vascular function and nerve struc-

ture. In addition, the lipid abnormalities in the ZDF rat are much more severe than is seen in most patients with type 2 diabetes. Another common type 2 diabetic rat model is the Goto-Kakizaki (GK) rat. The GK rat is a nonobese model of type 2 diabetes with modest hyperglycemia and few to no changes in serum lipid values. Most patients with type 2 diabetes have moderate abnormal lipid values, and similar serum lipid changes are observed in high-fat-fed/low-dose streptozotocin diabetic rats.¹¹

Yin et al.⁸ has recently reported corneal complications in streptozotocin-induced type 1 diabetic rats. Eight weeks after the induction of diabetes in young Sprague-Dawley rats, tear secretion was reduced by approximately 50%, and corneal sensitivity, determined using an esthesiometer, was mildly but significantly decreased; in addition, corneal nerve fibers were thinner and had fewer branches, and the overall fiber length was reduced by approximately 75%.⁸ These results of corneal nerve structure and function in type 1 diabetic rats after 8 weeks of hyperglycemia are similar to our results obtained in type 2 diabetic rats after 12 weeks of hyperglycemia. In addition, the diabetes-induced changes in nerve conduction velocity, thermal sensitivity, and intraepidermal nerve fiber density in high-fat-fed/low-dose streptozotocin diabetic rats after 12 weeks of hyperglycemia were similar to changes we have reported in streptozotocin-treated type 1 diabetic rats.²⁷ Together these results indicate that corneal sensitivity and innervation are impaired early after the induction of hyperglycemia in type 1 and type 2 diabetic rats. These changes are consistent with changes in standard neural endpoints observed in peripheral diabetic neuropathy.

Determination of corneal nerve fiber length by corneal confocal microscopy, which is considered a better test for diagnosing diabetic neuropathy than nerve fiber density and nerve fiber branching, has been found to be reliable and repeatable.^{4,28} It accurately detects corneal small nerve fiber damage, which is directly related to the degree of severity of neuropathy and intraepidermal nerve fiber density found on skin biopsy.^{3,4,6,29} However, compared with intraepidermal nerve fiber density measurements, corneal confocal microscopy is capable of quantifying small nerve fiber damage rapidly and noninvasively and detects changes at earlier stages of nerve damage.³ It has also been shown that corneal confocal microscopy is useful in longitudinal studies to assess progression and improvement in human diabetic neuropathy.⁵ Innervation of the Bowman's layer of the rat is approximately 50% less than that of the human comparing nerve fiber length based on area.⁴ Nonetheless, we were able to detect approximately a 50% loss of corneal nerve fibers after 12 weeks of hyperglycemia in high-fat-fed, low-dose streptozotocin diabetic rats. A similar decrease in corneal nerve fiber length is observed in diabetic patients determined to have mild diabetic neuropathy.⁴ Moreover, the decrease in corneal nerve fiber length in diabetic rats was associated with a decrease in corneal nerve sensitivity, as determined by a Cochet-Bonnet filament esthesiometer and measuring tear secretion. In diabetic patients, noncontact corneal esthesiometry has been shown to be a good predictor of diabetic neuropathy.²² With the same instrumentation used for diabetic patients, we were able to detect the damage or loss of small corneal nerve fibers in a rat model of type 2 diabetes after 12 weeks of hyperglycemia. Future studies will examine the progression of corneal nerve fiber damage in this rat model and potential treatments for diabetic neuropathy using corneal nerve fiber assessment by confocal microscopy as a surrogate marker.

We have examined the effect of diabetes on vascular relaxation of the posterior ciliary artery to provide a vascular endpoint for future studies. Even though the cornea is a nonvascular structure, capillary vessels, which are derived from the

anterior ciliary artery and which anastomose with the posterior ciliary artery, surround the cornea.³⁰ The posterior ciliary artery is the main source of blood to the optic nerve head and also supplies the choroid, retinal pigment epithelium, outer segment of the retina, and medial and lateral segments of the ciliary body and iris.³⁰ Posterior ciliary artery circulation is the most important aspect of ocular and optic nerve head circulation, and disturbances to it can result in varying degrees of visual loss.³⁰ Our studies demonstrated that diabetes caused a decrease in acetylcholine-mediated vascular relaxation of the posterior ciliary artery.

In summary, the failure of clinical trials of diabetic neuropathy has been due in part to the lack of sensitive diagnostic tests to detect early changes. In past studies, electrophysiological testing or nerve biopsy have been used, but changes in these endpoints generally involve large nerve fibers, are inefficient, and occur later in the disease course.³¹ Recently, it has been shown that skin biopsy can reveal early changes in small nerve fiber damage, but the disadvantage of this procedure is that it is invasive.³¹ The cornea is the most densely innervated structure of the human body; it contains A δ and unmyelinated C fibers that are derived from the ophthalmic division of the trigeminal nerve.³² Imaging of the subbasal nerve plexus of the cornea using confocal microscopy has emerged as a means to noninvasively assess the development and progression of diabetic neuropathy and perhaps treatments for it.^{3-6,22,32-34} Our studies and those by Yin et al.⁸ demonstrate that early nerve damage in the cornea can also be detected in rats with type 1 or type 2 diabetes. The nerve changes in these diabetic rats appear similar to those that occur in human diabetic patients, indicating that these measurements are potentially translational. Therefore, measuring functional and structural changes in corneal nerves in diabetic rats may be useful in future studies seeking effective treatments for diabetic neuropathy.

References

1. Tesfaye S, Selvarajah D. The Eurodiab study: what has this taught us about diabetic peripheral neuropathy? *Curr Diab Rep.* 2009;9:432-434.
2. Yorek MA. The potential role of angiotensin converting enzyme and vasopeptidase inhibitors in the treatment of diabetic neuropathy. *Curr Drug Targets.* 2008;9:77-84.
3. Quattrini C, Tavakoli M, Jeziorska M, et al. Surrogate markers of small fiber damage in human diabetic neuropathy. *Diabetes.* 2007;56:2148-2154.
4. Tavakoli M, Quattrini C, Abbott C, et al. Corneal confocal microscopy: a novel noninvasive test to diagnose and stratify the severity of human diabetic neuropathy. *Diabetes Care.* 2010;33:1792-1797.
5. Tavakoli M, Kallinikos P, Iqbal A, et al. Corneal confocal microscopy detects improvement in corneal nerve morphology with an improvement in risk factors for diabetic neuropathy. *Diabetic Med.* 2011;28:1261-1267.
6. Malik RA, Kallinikos P, Abbott CA, et al. Corneal confocal microscopy: a non-invasive surrogate of nerve fibre damage and repair in diabetic patients. *Diabetologia.* 2003;46:683-688.
7. Tavakoli M, Hossain P, Malik RA. Clinical applications of corneal confocal microscopy. *Clin Ophthalmol.* 2008;2:435-445.
8. Yin J, Huang J, Chen C, Gao N, Wang F, Yu F-SX. Corneal complications in streptozotocin-induced type 1 diabetic rats. *Invest Ophthalmol Vis Sci.* 2011;52:6589-6596.
9. Reed MJ, Meszaros K, Entes LJ, et al. A new rat model of type 2 diabetes: the fat-fed, streptozotocin-treated rat. *Metabolism.* 2000;49:1390-1394.
10. Srinivasan K, Viswanad B, Asrat L, Kaul CL, Ramarao P. Combination of high-fat diet-fed and low-dose streptozotocin-treated rat: a model for type 2 diabetes and pharmacological screening. *Pharmacol Res.* 2005;52:313-320.
11. Davidson EP, Coppey LJ, Holmes A, Dake B, Yorek MA. Effect of treatment of high fat fed/low dose streptozotocin-diabetic rats

- with Ilepatril on vascular and neural complications. *Euro J Pharmacol.* 2011;688:497-506.
12. Oltman CL, Davidson EP, Coppey LJ, Kleinschmidt TL, Lund DD, Yorek MA. Attenuation of vascular/neural dysfunction in Zucker rats treated with Enalapril or Rosuvastatin. *Obesity.* 2008;16:82-89.
 13. Coppey LJ, Davidson EP, Dunlap J, Lund DD, Yorek MA. Slowing of motor nerve conduction velocity in streptozotocin-induced diabetic rats is preceded by impaired vasodilation in arterioles that provide circulation to the sciatic nerve. *Int J Exp Diabetes Res.* 2000;1:131-143.
 14. Obrosova IG, Li F, Abatan OI, et al. Role of poly(ADP-ribose) polymerase activation in diabetic neuropathy. *Diabetes.* 2004;53:711-720.
 15. Davidson EP, Coppey LJ, Calcutt NA, Oltman CL, Yorek MA. Diet induced obesity in Sprague-Dawley rats: effect on microvascular and neural function. *Diabetes Metabolism Res Rev.* 2010;26:306-318.
 16. Terata K, Coppey LJ, Davidson EP, Dunlap JA, Gutterman DD, Yorek MA. Acetylcholine-induced arteriolar dilation is reduced in streptozotocin-induced diabetic rats with motor nerve dysfunction. *Br J Pharmacol.* 1999;128:837-843.
 17. Calcutt NA. Future treatments for diabetic neuropathy: clues from experimental neuropathy. *Curr Diabetes Rep.* 2002;2:482-488.
 18. Calcutt NA, Cooper ME, Kern TS, Schmidt AM. Therapies for hyperglycemia-induced diabetic complications: from animal models to clinical trials. *Nature Rev.* 2009;8:417-429.
 19. Gabbay KH. Aldose reductase inhibition in the treatment of diabetic neuropathy: where are we in 2004? *Curr Diabetes Rep.* 2004;4:405-408.
 20. Mojaddidi M, Quattrini C, Tavakoli M, Malik RA. Recent developments in the assessment of efficacy in clinical trials of diabetic neuropathy. *Curr Diabetes Rep.* 2005;5:417-422.
 21. Tesfaye S, Boulton AJM, Dyck PJ, et al. Diabetic neuropathies: update on definitions, diagnostic criteria, estimation of severity, and treatments. *Diabetes Care.* 2010;33:2285-2293.
 22. Pritchard N, Edwards K, Shahidi AM, et al. Corneal markers of diabetic neuropathy. *Lab Sci.* 2011;9:17-28.
 23. Brownlee M. The pathobiology of diabetic complications, a unifying mechanism. *Diabetes.* 2005;54:1615-1625.
 24. Davidson EP, Coppey LJ, Holmes A, Yorek MA. Effect of inhibition of angiotensin converting enzyme and/or neutral endopeptidase on vascular and neural complications in high fat fed/low dose streptozotocin-diabetic rats. *Euro J Pharmacol.* 2012;677:180-187.
 25. Oltman CL, Davidson EP, Coppey LJ, et al. Vascular and neural dysfunction in Zucker diabetic fatty rats: a difficult condition to reverse. *Diabetes Obes Metab.* 2008;10:64-74.
 26. Oltman CL, Davidson EP, Coppey LJ, et al. Treatment of Zucker diabetic fatty rats with AVE7688 improves vascular and neural dysfunction. *Diabetes Obes Metab.* 2009;11:223-233.
 27. Oltman CL, Davidson EP, Coppey LJ, et al. Role of the effect of inhibition of neutral endopeptidase on vascular and neural complications in streptozotocin-induced diabetic rats. *Euro J Pharmacol.* 2011;650:556-562.
 28. Efron N, Edwards K, Roper N, et al. Repeatability of measuring corneal subbasal nerve fiber length in individuals with type 2 diabetes. *Eye Contact Lens.* 2010;36:245-248.
 29. Kallinikos P, Berhanu M, O'Donnell C, et al. Corneal nerve tortuosity in diabetic patients with neuropathy. *Invest Ophthalmol Vis Sci.* 2004;45:418-422.
 30. Hayreb SS. Posterior ciliary artery circulation in health and disease. *Invest Ophthalmol Vis Sci.* 2004;45:749-757.
 31. Midena E, Brugin E, Ghirlando A, Sommariva M, Avogaro A. Corneal diabetic neuropathy: a confocal microscopy study. *J Refractive Surg.* 2006;22:S1047-S1052.
 32. Hossain P, Sachdev A, Malik RA. Early detection of diabetic peripheral neuropathy with corneal confocal microscopy. *Lancet.* 2005;366:1340-1343.
 33. Efron N. The Glenn A. Fry award lecture 2010: Ophthalmic markers of diabetic neuropathy. *Optometry Vis Sci.* 2011;88:661-683.
 34. Pritchard N, Edwards K, Vagenas D, et al. Corneal sensitivity as an ophthalmic marker of diabetic neuropathy. *Optometry Vis Sci.* 2010;87:1003-1008.