Differences and Similarities in Development of Corneal Nerve Damage and Peripheral Neuropathy and in Diet-Induced Obesity and Type 2 Diabetic Rats

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PURPOSE. Peripheral neuropathy has been shown to exist in prediabetic and diabetic patients and animal models. However, the development of peripheral neuropathy in prediabetes and posthyperglycemia is likely different. The purpose of this study was to examine the progression of peripheral neuropathy in diet-induced obese rats and high-fat-fed rats treated with a low dose of streptozotocin, a model for type 2 diabetes, using standard endpoints as well as corneal sensitivity and innervation.

METHODS. Diet-induced obese rats and high-fat/low-dose streptozotocin diabetic rats were used to examine standard peripheral neuropathy endpoints and innervation of the cornea and corneal epithelium using corneal and standard confocal microscopy, respectively, and corneal sensitivity using a Cochet-Bonnet esthesiometer at three different time points.

RESULTS. Obese rats and to a greater extent diabetic rats were insulin resistant. Obese and diabetic rats had developed sensory nerve deficits, but only diabetic rats had motor nerve dysfunction as determined by measuring nerve conduction velocity, thermal nociception, and intraepidermal nerve fiber density. In the cornea there was a decrease in corneal nerve fiber length, innervation of the corneal epithelium, and corneal sensitivity in both diet-induced obese and diabetic rats.

CONCLUSIONS. These studies demonstrate that changes in corneal nerve innervation and sensitivity occur in both obese and type 2 diabetic rat models that are consistent with development of peripheral neuropathy. Examination of corneal nerve changes may be valuable endpoints for exploring potential treatments for peripheral neuropathy in both prediabetes with insulin resistance and diabetes.

Keywords: diabetes, diabetic neuropathy, cornea

Diabetic neuropathy is a common complication of diabetes with no known treatment besides normalization of blood glucose.¹ Translation of effective treatments from diabetic animal models to humans has failed in clinical trials.² This is due in part to endpoints in animal studies that were non-predictive or insensitive when applied in human studies and interventions not beginning until after disease was too far advanced.² It has also been reported that peripheral neuropathy complications are present in animal models and patients with impaired insulin resistance.^{3–5} Therefore, an effective approach for treatment of peripheral neuropathy in type 2 diabetes may be to initiate treatment of patients prior to the onset of hyperglycemia. This would require development of markers that are detectable and predictive of peripheral neuropathy. 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 peripheral neuropathy.^{6–11} Loss of corneal nerves in diabetic patients has been shown to correlate with the severity of peripheral neuropathy.¹² Previously we demonstrated in a rat model of type 2 diabetes a decrease in corneal nerve fiber length and

corneal sensitivity, as well as deficits in nerve conduction velocity, thermal hypoalgesia, and decrease in intraepidermal nerve fiber density in the hindpaw.¹³ However, little is known about the changes in corneal nerve structure that occur in an animal model of diet-induced obesity and insulin resistance. In the present study we sought to determine the progression of changes in corneal nerve structure and standard peripheral neuropathy endpoints in a rat model of diet-induced obesity in comparison to a rat model of type 2 diabetes.

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 (approval ACURF

#1202032) and National Institutes of Health guidelines for use of animals were followed. The studies also adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. At 12 weeks of age, rats were separated into three groups. Two of these groups were placed on a high-fat diet (D12451, 45% kcal as fat, 4.7 kcal/g; Research Diets, New Brunswick, NJ). The high-fat diet contained 24 gm% fat, 24 gm% protein, and 41 gm% carbohydrate. The primary source of the increased fat content in the diet was lard. The remaining group was maintained on the control diet (#7001, 3.0 kcal/g; Harlan Teklad), which contained 4.25 gm% fat. Rats were maintained on the high-fat diet for 8 weeks. Afterward, one group of the high-fat-fed rats was treated with streptozotocin (30 mg/kg in 0.9% NaCl, intraperitoneal [IP]). Diabetes was verified 96 hours later by evaluating blood glucose levels with the use of glucose-oxidase reagent strips (Aviva Accu-Chek; Roche Diagnostics, Mannheim, Germany). Rats having blood glucose level of 250 mg/dL (13.8 mM) or greater were considered to be diabetic. All high-fat-fed rats remained on the high-fat diet. Our protocol created two experimental groups of rats—a diet-induced obesity group and a type 2 diabetic group. Our experimental plan also included three time points. The first time point represented rats fed a high-fat diet for 16 weeks with the paired diabetic group within this time point having hyperglycemia for 8 weeks. The second time point represented rats fed a high-fat diet for 26 weeks with the paired diabetic group within this time point having hyperglycemia for 18 weeks. The third time point represented rats fed a high-fat diet for 40 weeks with the paired diabetic group within this time point having hyperglycemia for 32 weeks. Each of the experimental groups within the three different time periods was matched with a control group of rats fed a standard diet throughout the protocol.

Glucose Tolerance

Glucose tolerance was determined by injecting rats with a saline solution containing 2 g/kg glucose, IP, after an overnight fast as previously described.³ Rats were briefly anesthetized with isoflurane, and the glucose solution was injected. Immediately prior to the glucose injection and at 15, 30, 45, 60, 120, 180, and 240 minutes, blood samples from the tip of the tail were taken to measure circulating glucose levels using the glucose-oxidase reagent strips.

Thermal Nociceptive Response and Corneal Sensitivity

Thermal nociceptive response in the hindpaw was measured using the Hargreaves method as previously described.^{14,15} Data were reported in seconds. Corneal sensation was measured using a Cochet-Bonnet filament esthesiometer in unanesthetized rats (Luneau Ophtalmologie, Prunay-le-Gillon, France).¹³ 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 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 with Nembutal IP (50 mg/kg, IP; 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.^{14,16} Sensory nerve conduction velocity was

determined using the digital nerve as described by Obrosova et al.¹⁷ Motor and sensory nerve conduction velocity was reported in meters per second.

Corneal Innervation

Subbasal corneal nerves were imaged using the Rostock cornea module of the Heidelberg Retina Tomograph confocal microscope (Heidelberg Engineering, Heidelberg, Germany) as previously described.¹³ Briefly, the anesthetized rat was secured to a platform that allowed adjustment and positioning of the rat in three dimensions. A drop of GenTeal (lubricant eye gel) was applied onto the tip of the lens and advanced slowly forward until the gel contacted the cornea, allowing optical but not physical contact between the objective lens and corneal epithelium. Ten random high-quality images without overlap of the subbasal nerve plexus 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.¹³ 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.¹³ The corneal fiber length for each animal was the mean value obtained from the 10 acquired images and expressed as mm/mm². Based on receiver operating characteristic (ROC) curve analysis, corneal nerve fiber length is the optimal parameter for diagnosing patients with diabetic neuropathy and has the lowest coefficient of variation.^{6,7}

After corneal confocal microscopy, corneas were dissected from the eyes and trimmed around the sclerolimbo region. One cornea was fixed and embedded in paraffin for analysis of innervation of the corneal epithelium (see below); the other cornea was fixed, blocked, and then incubated with neuronal class III *B*-tubulin 1:500 in incubation buffer overnight at 4°C (Covance, Dedham, MA). After washing with incubation buffer, the tissue was incubated with Alexa Fluor 546 goat anti-rabbit IgG 1:2000 in incubation buffer for 2 hours at room temperature (Invitrogen, Eugene, OR). After washing, the cornea was placed epithelium up on a microscope slide. Excess water was carefully aspirated and three radial cuts were made at 120° intervals, nearly to the center of the cornea. The tissue was carefully covered with a cover slip, mounted with ProLong Gold, and sealed with clear nail polish. A 3 × 3 matrix of Z-stack images was collected using a Zeiss LM710 confocal microscope with ZEN Black software (Carl Zeiss Microscopy, Jena, Germany). Corneal nerves located in the subbasal layer were analyzed using simple PCI (Hamamatsu Photonics, Hamamatsu City, Japan), and data were expressed as total nerve fiber length μm/μm².

Additional measurements were taken, including nonfasting blood glucose and hemoglobin A_{1c} levels (Glyco-tek affinity column; Helena Laboratories, Beaumont, TX). Serum was collected for determining levels of free fatty acid, triglyceride, free cholesterol, and leptin, using commercial kits from Roche Diagnostics, Sigma Chemical Co., Bio Vision (Mountain View, CA), and ALPCO Diagnostics (Salem, NH), respectively.

Intraepidermal Nerve Fiber Density in the Hindpaw and Innervation of the Epithelium of the Cornea

Immunoreactive nerve fiber profiles innervating the skin from the hindpaw and epithelium of the cornea were visualized

TABLE 1. Effect of Duration of High-Fat Diet and High-Fat/Streptozotocin-Induced Diabetes in Rats on Change in Body Weight, Blood Glucose, and Hemoglobin A_{1c}

Determination	Control	High Fat	Diabetic
Start weight, g			
16/8 wk	314 ± 3	313 ± 3	311 ± 2
26/18 wk	311 ± 2	315 ± 1	315 ± 3
40/32 wk	314 ± 2	313 ± 3	312 ± 2
End weight, g			
16/8 wk	454 ± 7*	482 ± 18*	405 ± 17*‡
26/18 wk	491 ± 12*	564 ± 14*†	432 ± 30*‡
40/32 wk	510 ± 17*	677 ± 50*†	411 ± 28*‡
Blood glucose, mg/dL			
16/8 wk	138 ± 7	137 ± 6	454 ± 37‡
26/18 wk	137 ± 5	138 ± 7	411 ± 49‡
40/32 wk	138 ± 9	139 ± 8	487 ± 41‡
Hemoglobin A _{1c} , %			
16/8 wk	6.9 ± 0.5	6.6 ± 0.6	13.6 ± 2.1‡
26/18 wk	7.1 ± 0.2	7.8 ± 0.7	15.1 ± 1.4‡
40/32 wk	6.4 ± 0.5	10.3 ± 0.7†	15.9 ± 1.2‡

For these studies there were two experimental groups, high-fat-induced obesity and type 2 diabetic, and three experimental periods, 16/8, 26/18, and 40/32 weeks (the first value represents the number of weeks the rats were on the high-fat diet and the second value the number of weeks of hyperglycemia for the group of rats treated with a low dose of streptozotocin). Data are presented as the mean ± SEM. * $P < 0.05$ compared to respective start weight; † $P < 0.05$ compared to control; ‡ $P < 0.05$ compared to high fat. The number of experimental animals in each group ranged from 10 to 12.

using standard confocal microscopy as previously described.^{3,13,14} Samples of skin of the right hindpaw and strips of the medial cornea extending across the entire diameter of the cornea were fixed, dehydrated, and embedded in paraffin. Three sections (7 μm for skin and 10 μm for cornea) for each animal 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. Profiles were counted by two individual investigators who were masked to the sample identity. All immunoreactive profiles were counted and normalized to length.^{13,14}

Data Analysis

Results are presented as mean ± SEM. Comparisons between the control, obese, and diabetic rats were conducted using a one-way ANOVA and Bonferroni-Dunn test for multiple comparisons (Prism software; GraphPad, San Diego, CA). Correlation coefficients were determined using Prism software (GraphPad). A P value less than 0.05 was considered significant.

RESULTS

Table 1 provides data for the weight of the three groups of rats at the beginning and end of the study, nonfasted blood glucose, and hemoglobin A_{1c} values for each of the three time points determined. At the beginning of the study, all rats weighed approximately the same. All rats gained a significant amount of weight when compared to their beginning weights. High-fat-fed rats gained significantly more weight than control rats after 26 and 40 weeks on the high-fat diet. Diabetic rats at all three time points weighed significantly less than the high-fat-fed

FIGURE 1. Effect of high-fat diet or high-fat diet and low-dose streptozotocin on glucose tolerance. Glucose clearance was determined as described in the Methods section using rats fed a high-fat diet for 40 weeks treated with or without 30 mg/kg streptozotocin after 8 weeks on the high-fat diet. Data are presented as the mean ± SEM for glucose utilization in mg/dL. The number of rats in each group was from 10 to 12.

rats, and after 32 weeks of hyperglycemia, weight of the diabetic rats was also significantly less than in age-matched control rats. Blood glucose levels and hemoglobin A_{1c} were significantly increased in diabetic rats at all three time points compared to age-matched control and high-fat-fed rats. Non-fasting blood glucose was not increased in high-fat-fed rats compared to control rats. However, after 40 weeks of a high-fat diet, hemoglobin A_{1c} values were significantly increased in high-fat-fed rats compared to age-matched control rats. Figure 1 provides data for the glucose clearance rate for control, high-fat-fed, and diabetic rats from the third time point. Glucose utilization is significantly impaired in rats fed a high-fat diet for 40 weeks compared to age-matched control rats; and when these rats were hyperglycemic for 32 weeks of the 40-week period, glucose utilization was impaired to even a greater extent. Glucose utilization was also impaired in high-fat-fed rats and to a greater extent in diabetic rats for the two earlier time points (data not shown).

Table 2 provides data for serum triglyceride, free fatty acid, free cholesterol, and leptin levels of the three groups of rats for each time point in the study. There was a gradual increase with age in triglyceride, free fatty acid, and cholesterol levels in control rats. Serum triglyceride and free fatty acid levels were significantly increased in diabetic rats at all three time points compared to age-matched control and high-fat-fed rats. Serum triglyceride and free fatty acid levels in high-fat-fed rats were not significantly different from those in age-matched control rats. Serum free cholesterol levels were significantly increased in high-fat-fed rats and diabetic rats at all three time points compared to age-matched control rats. Serum free cholesterol levels in diabetic rats were significantly increased compared to those in age-matched high-fat-fed rats at the two later time points. Serum leptin levels were significantly increased in high-fat-fed rats compared to age-matched control and diabetic rats. In diabetic rats, serum leptin levels were significantly decreased compared to those in age-matched control rats at the two later time points.

Figures 2 and 3 provide data for the effect of a high-fat diet and diabetes on motor and sensory nerve conduction velocity of the three groups of rats for each time point in the study. Feeding rats a high-fat diet for 8, 18, or 32 weeks did not affect

TABLE 2. Effect of Duration of High-Fat Diet and High-Fat/Streptozotocin-Induced Diabetes in Rats on Serum Triglycerides, Free Fatty Acids, Free Cholesterol, and Leptin

Determination	Control	High Fat	Diabetic
Triglycerides, mg/dL			
16/8 wk	14 ± 1	20 ± 4	52 ± 16*†
26/18 wk	28 ± 5	26 ± 3	304 ± 78*†
40/32 wk	33 ± 3	34 ± 8	174 ± 53*†
Free fatty acids, mmol/L			
16/8 wk	0.18 ± 0.06	0.26 ± 0.09	0.53 ± 0.13*†
26/18 wk	0.22 ± 0.05	0.23 ± 0.02	0.83 ± 0.08*†
40/32 wk	0.52 ± 0.04	0.68 ± 0.10	1.11 ± 0.12*†
Cholesterol, mg/dL			
16/8 wk	0.9 ± 0.1	1.8 ± 0.2*	2.5 ± 0.7*
26/18 wk	1.2 ± 0.3	2.6 ± 0.2*	12.1 ± 3.3*†
40/32 wk	2.1 ± 0.3	5.1 ± 0.6*	11.1 ± 2.0*†
Leptin, ng/mL			
16/8 wk	1.6 ± 0.2	5.1 ± 0.9*	0.7 ± 0.3†
26/18 wk	2.2 ± 0.2	6.8 ± 1.0*	0.3 ± 0.1*†
40/32 wk	2.8 ± 0.3	12.1 ± 1.0*	0.9 ± 0.3*†

The experimental groups were the same as described for Table 1. Data are presented as the mean ± SEM. * $P < 0.05$ compared to control; † $P < 0.05$ compared to high fat. The number of experimental animals in each group ranged from 10 to 12.

motor nerve conduction velocity compared to that in age-matched control rats (Fig. 2) but did cause a decrease in sensory nerve conduction velocity in rats after 18 or 32 weeks of a high-fat diet (Fig. 3). In diabetic rats, motor nerve conduction velocity was significantly impaired compared to that in age-matched control and high-fat-fed rats at each of the three time points (Fig. 2). Sensory nerve conduction was significantly impaired in diabetic rats compared to age-matched control rats at all three time points (Fig. 3).

Figures 4 and 5 provide data for the effect of a high-fat diet and diabetes on intraepidermal nerve fiber density and thermal nociception of the hindpaw of the three groups of rats for each time point in the study. Data in Figure 4 demonstrate that

FIGURE 2. Effect of high-fat diet or high-fat diet and low-dose streptozotocin on motor nerve conduction velocity. Motor nerve conduction velocity was examined as described in the Methods section. The groups examined and the number of rats in each group were the same as shown in Table 1. Data are presented as the mean ± SEM for motor nerve conduction velocity in m/s. * $P < 0.05$ compared to control rats; † $P < 0.05$ compared to high-fat-fed rats. **FIGURE 3.** Effect of high-fat diet or high-fat diet and low-dose streptozotocin on sensory nerve conduction velocity. Sensory nerve conduction velocity was examined as described in the Methods section. The groups examined and the number of rats in each group were the same as shown in Table 1. Data are presented as the mean ± SEM for sensory nerve conduction velocity in m/s. * $P < 0.05$ compared to control rats.

intraepidermal nerve fiber profiles were significantly decreased to a similar extent in rats fed a high-fat diet or induced with diabetes compared to age-matched control rats at each of the three time points. Thermal nociception latency was significantly increased in the two later time points for both the high-fat-fed rats and diabetic rats compared to age-matched control rats (Fig. 5).

Corneal sensitivity, as measured using a Cochet-Bonnet filament esthesiometer in unanesthetized rats, was found to be significantly impaired in high-fat-fed rats and diabetic rats compared to age-matched control rats at all three time points (Fig. 6). At the latest time point, corneal sensitivity was more significantly impaired in diabetic rats compared to age-matched high-fat-fed rats.

Data in Figure 7 demonstrate that corneal nerve fiber length was significantly decreased in rats fed a high-fat diet for 26 and 40 weeks compared to age-matched control rats. In diabetic

FIGURE 4. Effect of high-fat diet or high-fat diet and low-dose streptozotocin on intraepidermal nerve fiber density. Intraepidermal nerve fiber density was examined as described in the Methods section. The groups examined and the number of rats in each group were the same as shown in Table 1. Data are presented as the mean ± SEM for intraepidermal nerve fiber as profiles/mm. * $P < 0.05$ compared to control rats.

FIGURE 5. Effect of high-fat diet or high-fat diet and low-dose streptozotocin on thermal nociception. Thermal nociception was examined as described in the Methods section. The groups examined and the number of rats in each group were the same as shown in Table 1. Data are presented as the mean $\pm$ SEM for thermal nociception in seconds. * $P < 0.05$ compared to control rats.

rats, corneal fiber length was significantly decreased at all three time points (Fig. 7). We also examined innervation of the corneal epithelium (Fig. 8). There was a decrease in innervation of the corneal epithelium with age. At the time of analysis, the rats in the three groups of this study were 28, 38, and 52 weeks of age. There was a trend for a decrease in innervation of the corneal epithelium at 38 weeks of age and a significant decrease in rats at 52 weeks of age compared to 28 weeks of age. Innervation of the corneal epithelium was significantly decreased to a similar extent in rats fed a high-fat diet or induced with diabetes compared to age-matched control rats at each of the three time points.

At the latest time point we evaluated corneal nerve fiber density more extensively by examining corneal fiber length in whole corneas stained with class III B-tubulin. Figures 9A, 9B, and 9C provide a visual demonstration that initial loss of nerves in the subbasal region of the cornea occurs near the inferior whorl. In high-fat-fed rats and type 2 diabetic rats there was an

FIGURE 6. Effect of high-fat diet or high-fat diet and low-dose streptozotocin on corneal sensitivity. Corneal sensitivity was examined as described in the Methods section. The groups examined and the number of rats in each group were the same as shown in Table 1. Data are presented as the mean $\pm$ SEM for corneal sensitivity in centimeters. * $P < 0.05$ compared to control rats; + $P < 0.05$ compared to high-fat-fed rats.

FIGURE 7. Effect of high-fat diet or high-fat diet and low-dose streptozotocin on innervation of subbasal layer of the cornea. Innervation of the subbasal layer of the cornea was determined by using corneal confocal microscopy as described in the Methods section. The groups examined and the number of rats in each group were the same as shown in Table 1. Data are presented as the mean $\pm$ SEM for innervation of the cornea in mm/mm². * $P < 0.05$ compared to control rats.

approximately 50% decrease in corneal nerve fiber length in the region of the whorl (Table 3). As one moved away from the central region of the cornea, nerve loss was decreased; and near the periphery there was no difference in corneal nerve fiber length between control, high-fat-fed, and diabetic rats (Figs. 9D-F; Table 3). Table 3 also provides tabular data for corneal nerve fiber length (Fig. 7) and innervation of the corneal epithelium (Fig. 8) for the last time point of this study (40/32 weeks). Correlations were also determined for this same group of animals (Table 4). This analysis demonstrates that a significant correlation exists for intraepidermal nerve fiber density of the skin of the footpad and corneal nerve fiber length as determined by corneal confocal microscopy and corneal nerve fiber density of the corneal epithelium. There is also a significant correlation for corneal nerve fiber length as

FIGURE 8. Effect of high-fat diet or high-fat diet and low-dose streptozotocin on innervation of the corneal epithelium. Innervation of the corneal epithelium was determined using standard confocal microscopy as described in the Methods section. The groups examined and the number of rats in each group were the same as shown in Table 1. Data are presented as the mean $\pm$ SEM for innervation of the corneal epithelium in profiles/mm. * $P < 0.05$ compared to the 16/8-week control rats; + $P < 0.05$ compared to age-matched control rats.

FIGURE 9. Effect of high-fat diet or high-fat diet and low-dose streptozotocin on innervation of subbasal layer using isolated intact cornea. Innervation of the subbasal layer of cornea was determined by examining expression of corneal nerves following isolation of the intact cornea (whole mount) and staining with anti-tubulin as described in the Methods section. A representative image of the inferior whorl region of the cornea from a control rat (A), high-fat-fed rat (B), and diabetic rat (C) and periphery of the cornea from a control rat (D), high-fat-fed rat (E), and diabetic rat (F) is shown.

determined by corneal confocal microscopy and corneal nerve fiber density of the corneal epithelium.

DISCUSSION

Corneal confocal microscopy, a noninvasive in vivo imaging procedure that can be performed repeatedly, provides an assessment of subbasal corneal sensory nerve structure and is able to detect nerve damage in diabetic patients.^{6,9,18,19} It has been proposed that imaging of diabetes-induced changes of these nerves and measurement of corneal sensitivity may be useful surrogate markers for diabetic neuropathy.^{7,9,20,21} We have previously demonstrated that changes in corneal nerve innervation and sensitivity occur in both type 1 and type 2 diabetic rats and that these changes are consistent with

diabetic patients.^{13,22} We have also shown that innervation of corneal epithelium was decreased prior to a detectable decrease of subbasal corneal nerves.²² In this study we examined the impact of duration of a high-fat diet compared to type 2 diabetes in rats on endpoints for peripheral diabetic neuropathy, as well as innervation of subbasal region of the cornea and corneal epithelium. Several laboratories have now demonstrated that innervation of the cornea is impaired in diabetic patients^{9,23–26} and animal models.^{11,13,22,27} However, less is known of the impact of prediabetes as documented by impaired glucose tolerance on innervation of the cornea.

The main findings were that corneal sensitivity and corneal innervation of the subbasal layer and epithelium were decreased to a similar extent in rats with diet-induced obesity accompanied by impaired glucose utilization as in rats that were fed the same high-fat diet but also received a low-dose

TABLE 3. Effect of High-Fat Diet and High-Fat/Streptozotocin-Induced Diabetes in Rats on Innervation of Subbasal Layer of the Cornea by Corneal Confocal Microscopy, Innervation of the Corneal Epithelium, and Innervation of the Inferior Whorl and Periphery of the Cornea In Vitro by Standard Confocal Microscopy

Determination	Control	High Fat	Diabetic
Corneal nerve fiber length, mm/mm ²	4.8 ± 0.3	3.3 ± 0.2*	2.8 ± 0.2*
Corneal epithelial nerve fibers, profiles/mm	9.5 ± 0.9	7.4 ± 0.5*	7.1 ± 0.6*
Corneal nerve fiber length, μm/μm ²			
Inferior whorl	0.080 ± 0.008	0.042 ± 0.010*	0.042 ± 0.003*
Periphery	0.119 ± 0.007	0.107 ± 0.014	0.124 ± 0.008

The experimental groups were fed a high-fat diet for 40 weeks with or without hyperglycemia for 32 weeks. Data are presented as the mean ± SEM. * *P* < 0.05 compared to control. The number of experimental animals in each group ranged from 8 to 10.

TABLE 4. Correlations for Intraepidermal Nerve Fiber, Corneal Nerve Fiber Length, and Corneal Epithelial Nerve Fiber Density

	Intraepidermal Nerve Fiber Density	Corneal Nerve Fiber Length	Corneal Epithelial Nerve Fiber Density
Intraepidermal nerve fiber density	1.00	0.47*	0.59*
Corneal nerve fiber length	0.47*	1.00	0.46*
Corneal epithelial nerve fiber density	0.59*	0.46*	1.00

The experimental groups were fed a high-fat diet for 40 weeks with or without hyperglycemia for 32 weeks. * $P < 0.05$.

treatment of streptozotocin to induce hyperglycemia. A second outcome was that initial loss of corneal nerves in the subbasal layer occurred predominantly in the region of the inferior whorl. If this finding is translational to human diabetes, quantification of the inferior whorl could improve the diagnostic ability of corneal confocal microscopy for diabetic neuropathy. The third finding was a decrease in innervation of the corneal epithelium with age, although at each time point studied, diet-induced obesity or type 2 diabetes exacerbated the loss of nerves in the corneal epithelium compared to that in age-matched controls. The fourth finding was significant correlations for intraepidermal nerve fiber density, corneal nerve fiber length as determined by corneal confocal microscopy, and corneal nerve fiber density of the corneal epithelium.

In previous studies we demonstrated that high-fat-fed rats were insulin resistant and developed a sensory neuropathy as documented by a slowing of sensory nerve conduction velocity, decrease in intraepidermal nerve fibers in the footpad of the hindpaw, and thermal hypoalgesia.¹⁴ Patients diagnosed as prediabetic with insulin resistance have been found to have a sensory neuropathy.^{4,5} In this study we examined for the first time the effect of duration of a high-fat diet in the absence or presence of hyperglycemia on endpoints relating to peripheral neuropathy. High-fat-fed rats had impaired glucose utilization as early as 16 weeks after initiation of the high-fat diet, and this was exacerbated by hyperglycemia for 8 weeks (data not shown). There was little difference in the severity of impaired glucose utilization when the duration of the high-fat diet and hyperglycemia was extended to 40 and 32 weeks, respectively. Interestingly, we found that rats fed a high-fat diet for 40 weeks did have an elevated hemoglobin A_{1c} value, but fasting and nonfasting blood glucose levels were not different from those in controls. As previously reported, motor nerve conduction was not impaired in rats fed a high-fat diet; and this did not change when the duration of the diet was extended to 40 weeks.¹⁴ However, with the onset of hyperglycemia, motor nerve conduction was impaired, and extending the duration of the hyperglycemia period from 8 to 32 weeks did not exacerbate slowing of motor nerve conduction velocity. Feeding rats a high-fat diet for 26 weeks caused a decrease in sensory nerve conduction velocity that was further impaired after 40 weeks. When high-fat-fed rats were made diabetic, sensory nerve conduction velocity was impaired as early as 8 weeks after the onset of hyperglycemia; and extending the duration of hyperglycemia from 8 to 32 weeks trended to further reduce sensory nerve conduction velocity. Decrease in intraepidermal nerve fibers in the skin of the hindpaw was present in rats after 16 weeks of the high-fat diet, and this was not further affected by hyperglycemia. Moreover, prolonging the duration of the high-fat diet or hyperglycemia did not further reduce the innervation of the skin. Likewise, thermal hypoalgesia was detected in high-fat-fed rats after 26 weeks of the high-fat diet and was not further impacted by hyperglycemia or duration.

Decrease in corneal nerve fiber length in patients with diabetic neuropathy has been shown to be associated with

severity of diabetic neuropathy, with the greater loss of nerve fibers occurring in patients with more severe disease.¹² There is also evidence that progression of peripheral neuropathy is linked to duration of disease.^{8,28,29} In this study there was some evidence of progression of subbasal cornea nerve fiber loss and decreased corneal sensitivity with severity of disease. There was a significant decrease in corneal nerve length at the early time point in diabetic rats but not for rats fed a high-fat diet. However, by the second time point, there was a significant decrease in subbasal cornea nerve fiber length in rats fed a high-fat diet and in diabetic rats. There was a trend for greater decrease of cornea nerve fiber length in diabetic rats, but this was not statistically different from the value in high-fat-fed rats at the two later two time points. The same could be said for corneal sensitivity. At the latest time point, impairment of corneal sensitivity was greater in diabetic rats compared to high-fat-fed rats. There was a significant decrease in innervation of the corneal epithelium at the earliest time point in both high-fat-fed rats and diabetic rats. This agrees with our previous finding in type 1 diabetic rats that a decrease in innervation of the corneal epithelium is an early event in diabetes that occurs prior to detection of corneal nerve fiber loss in the subbasal layer.²² We also observed that decrease in innervation of the corneal epithelium is age dependent. At 52 weeks of age there were significantly fewer nerves in the corneal epithelium compared to the number in rats at 28 weeks of age. Dvorscak and Marfurt,³⁰ using Fischer-344 rats age 6 to 24 months, reported an age-related decrease in rat corneal epithelial nerve density. They also reported that corneal subbasal nerve density increased as a function of age. A similar trend was observed in our studies at 52 weeks of age. Even with the gradual decline in innervation of the corneal epithelium with age, diet-induced obesity and type 2 diabetes caused a greater loss of nerves at each time point studied. The loss of nerve terminal density seen in the cornea epithelium may contribute to the pathogenesis of dry eye disease that is associated with increasing age and diabetes.^{30,31}

Comparisons of nerve fiber length acquired by corneal confocal microscopy and by immunohistochemical staining of isolated corneas has revealed that only a fraction of the nerves present in the cornea are visualized with use of in vivo corneal confocal microscopy. This is not only true for smaller distal nerve endings penetrating the epithelium that may be below the resolution of current in vivo confocal imaging devices, but also true for nerves in the subepithelial plexus comprising the whorl configuration. The reason for this is unknown, as it is not well understood what physical properties of corneal nerves allow them to be visualized in some cases and not in others using in vivo corneal confocal microscopy—or whether this is specific to a certain class of corneal nerves. However, it appears that analysis of corneal nerves both in vivo and in vitro shows a decrease in corneal nerves caused by prediabetes or diabetes. Effort should be made to further enhance visualization of corneal nerves in vivo, as this may improve upon our ability to detect a decrease at an earlier stage of neuropathy, when treatment may be more beneficial, and may also help to detect smaller changes over time in patients.

In summary, these studies have demonstrated that decreased innervation of the cornea and cornea sensitivity occur in rats fed a high-fat diet, an animal model for prediabetes and insulin resistance, to a degree similar to that seen high-fat-fed rats treated with a low dose of streptozotocin, an animal model for type 2 diabetes.^{3,13} We also found that the earliest decrease of subbasal corneal nerve fiber length was detected through imaging the region of the whorl. This would make sense if the phenomenon of nerve axonopathy explains the loss of peripheral nerve fibers.³² The termination of many corneal nerve fibers occurs in the whorl region and corneal epithelium. We have already demonstrated that early loss of corneal nerve fibers can be detected in the corneal epithelium.²² At this time there is no way to assess those fibers in vivo; however, these studies suggest that early loss of subbasal corneal nerve fibers can be detected by imaging the region of the whorl, which is possible in patients through the creation of a montage from multiple images of the subbasal nerve plexus.³³

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