The Effects of Diabetes and High-Fat Diet on Polymodal Nociceptor and Cold Thermoreceptor Nerve Terminal Endings in the Corneal Epithelium

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PURPOSE. There is a substantial body of evidence indicating that corneal sensory innervation is affected by pathology in a range of diseases. However, there are no published studies that have directly assessed whether the nerve fiber density of the different subpopulations of corneal sensory neurons are differentially affected. The present study explored the possibility that the intraepithelial nerve fiber density of corneal polymodal nociceptors and cold thermoreceptors are differentially affected in mice fed with a high-fat high cholesterol (HFHC; 21% fat, 2% cholesterol) diet and in those that also have diabetes.

METHODS. The mice were fed the HFHC diet for the duration of the experiment (up to 40 weeks). Mice in the diabetes group had hyperglycaemia induced with streptozotocin after 15 weeks on the HFHC diet. Age-matched control animals were fed a standard diet. All corneal nerve fibers were labeled with a pan neuronal antibody (antiprotein gene product 9.5), and polymodal nociceptors and cold thermoreceptors were labeled with antibodies directed against transient receptor potential cation channel, subfamily V, member 1 and transient receptor potential cation channel subfamily M member 8, respectively.

RESULTS. The mice fed a HFHC diet and those that in addition have hyperglycemia have similar reductions in corneal nerve fiber density consistent with small fiber neuropathy. Importantly, both treatments more markedly affected the intraepithelial axons of cold thermoreceptors than those of polymodal nociceptors.

CONCLUSIONS. The results provide evidence that distinct subpopulations of corneal sensory neurons can be differentially affected by pathology.

Keywords: diabetes, TRPM8, corneal innervation, neuropathy

Diabetic neuropathy is a term used to describe a range of effects that diabetes has on the peripheral nervous system. In both type 1 and type 2 diabetes, the most common type of diabetic neuropathy is distal symmetric neuropathy. This is a sensory polyneuropathy that is characterized at its earliest stages by changes in sensitivity to thermal stimuli and the generation of abnormal sensations (dysesthesia and neuropathic pain) due to the effects on small diameter (C and Aβ fiber) sensory axons.1–2 These changes typically manifest in the skin of the feet and are accompanied by decreases in intraepithelial nerve fiber density (IENFD) in foot skin. Using in vivo confocal microscopy (IVCM), reductions in the density of sub-basal corneal nerve fibers have also been demonstrated in patients with diabetes who have clinical signs and symptoms of small fiber neuropathy.3–5 Furthermore, there are reductions in corneal sensitivity to mechanical, chemical, and thermal stimuli in patients with type 1 and type 2 diabetes.6–8 Importantly, reduced thermal sensitivity in foot skin and reductions in IENFD in foot skin and sub-basal corneal nerve fiber density (CNFD) have also been reported in patients with prediabetes that have impaired glucose tolerance but have not yet developed type 2 diabetes.9,10 Consistent with these findings, mice fed a high-fat diet to induce obesity and impaired glucose tolerance and those that also had diabetes induced have similar reductions in IENFD and thermal sensitivity in hindpaw skin.11 Although these studies suggest an association between insulin resistance and small fiber sensory neuropathy; there are reports in patients with prediabetes and type 2 diabetes that obesity and hyperlipidemia are independent risk factors for small fiber neuropathy.12–14 Sensory deficits have also been reported to occur prior to the appearance of impaired glucose tolerance in mice fed a high-fat diet.15

Corneal sensory neurons can be subdivided into one of the following three functional types by their response to different stimuli: cold thermoreceptors that are stimulated by cooling and by increases in tear osmolarity; polymodal nociceptors that are activated by mechanical, chemical, and thermal stimuli; and mechano-nociceptors that are activated only by mechanical stimuli.16–18 Although animal studies show that a high-fat diet with or without diabetes can affect CNFD,11,19 it is not known if these treatments differentially affect the different subpopulations of corneal sensory neurons. In animals, we have shown that nerve fibers of polymodal and cold thermoreceptors in the corneal epithelium can be distinguished by their molecular
phenotype and nerve terminal morphology. The axons of corneal polymodal nociceptors express transient receptor potential cation channel, subfamily V, member 1 (TRPV1), but not transient receptor potential cation channel subfamily M member 8 (TRPM8), and after leaving sub-basal plexus either terminate without further branching (simple endings) or branch in the squamous cell layer into a small number of terminating branches that run parallel to the corneal surface (ramifying endings). In contrast, the axons of cold thermoreceptors express TRPM8, but not TRPV1, and form a cluster of highly branched fibers that have endings in both the wing and squamous cell layers and possess many large en-passant and terminal boutons (complex endings). In the present study, we explored the possibility that the intraepithelial nerve fiber density of corneal polymodal nociceptors and cold thermoreceptors are differentially affected in mice fed a high-fat diet and in those that also had diabetes induced with streptozotocin.

**MATERIALS AND METHODS**

A total of 36 male C57Bl/6 mice were used in this study. All experiments conformed to the Australian National Health and Medical Research Council code of practice for the use of animals in research and were approved by the Austin Health Animal Ethics Committee. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Experimental Groups**

All animals were initially maintained on a normal diet (Barastock Walter and Elizri Hall Institute mice cubes; Ridley AgriProducts, Melbourne, Victoria, Australia) containing 9% (by weight) fat derived primarily from canola oil. At 6 to 8 weeks of age, they were divided into three treatment groups (n = 12/group). The first treatment group (HFHC) received only the HFHC diet for the duration of the experiment. The third treatment group (HFHC) received only the HFHC diet for the duration of the experiment. The second treatment group (HFHCþDb) was fed a high-fat, high-cholesterol (HFHC) diet (21% fat, 2% cholesterol, 10% fructose; Catalogue No. SF11-109; Specialty Feeds, Glen Forrest, WA, Australia) for the duration of the experiment. The fat in this diet was derived primarily from clarified butter (ghee) and supplies 40% of total energy intake, which is at the high end of the range for western diets (26%–46% of total energy intake). After 15 weeks on this diet, the HFHCþDb mice were injected intraocularly with streptozotocin (65 mg/kg) on 2 consecutive days. In mice, this treatment with streptozotocin causes destruction of pancreatic β cells and induces diabetes (hyperglycemia) without causing absolute insulin deficiency requiring insulin treatment. The second treatment group (HFHCþDb) was injected with streptozotocin (65 mg/kg) on 2 consecutive days. In mice, this treatment with streptozotocin causes destruction of pancreatic β cells and induces diabetes (hyperglycemia) without causing absolute insulin deficiency requiring insulin treatment. The third treatment group (control) was composed of age-matched control animals that were maintained on the normal diet for the duration of the experiment. Each treatment group was further subdivided into two experimental groups that were terminated at 25 to 30 weeks (timepoint 1; n = 6) or 35 to 40 weeks (timepoint 2; n = 6) after starting the HFHC diet. For the HFHCþDb group, timepoints 1 and 2 occurred 10 to 15 weeks and 20 to 25 weeks after the induction of diabetes with streptozotocin, respectively. At each timepoint, the mice were fasted for 10 to 12 hours, then deeply anesthetized with an intraperitoneal injection of pentobarbitone sodium (120 mg/kg) and had blood collected by cardiac puncture for the measurement of blood glucose levels. The measurement of blood glucose was made with a GM7 Micro-Stat blood glucose analyser (Analox Instruments, Stourbridge, UK). After the blood was withdrawn, each animal was killed by decapitation and eyes dissected and processed to reveal immunolabeling as described next.

**Tissue Preparation and Immunolabeling**

The eyes were dissected and immediately placed into cold (4°C) Zamboni’s fixative (2% formaldehyde and 15% saturated picric acid) for 30 minutes. The anterior segment (cornea and 1 mm of sclera) was dissected away from the rest of the eye, and the lens and iris were carefully removed to allow access of fixative to both sides of the cornea. Tissue was then returned to the Zamboni’s fixative for another 30 minutes. Each cornea was washed with 0.1 M phosphate buffered saline and divided into four quadrants to allow the use of different antibodies on the tissue from a single cornea. Each quadrant was processed free floating using the protocol we have previously published to reveal immunolabeling through the full thickness of the cornea. Antibodies directed against TRPV1, TRPM8, and protein gene product 9.5 (PGP9.5) were used to identify polymodal nociceptors, cold thermoreceptors, and all nerve terminal endings, respectively. The source and concentration of the primary and secondary antibodies used are given in the Table. The specificity and characterization of the primary antibodies used have been detailed in our previous publications.

**Image Acquisition and Data Analysis**

In this study, CNFD was assessed through the full thickness of the corneal epithelium, and this includes both the sub-basal nerve fibers in the basal epithelium and the nerve terminal axons that terminate in the wing and squamous cell layers. To allow for direct comparisons of CNFD between animal treatment groups, image acquisition and data analysis were performed precisely the same ways for tissue labeled with each of the different antibodies. Nerve fibers and terminals in the corneal epithelium were imaged using a Zeiss Axioskop-
pe.Z1 fluorescence microscope (Carl Zeiss, Oberkocken, Germany) with Zen imaging software (version 5.5, Carl Zeiss). Z-stack images were collected through the entire thickness of the corneal epithelium. Each corneal quadrant was divided into peripheral and central zones (Fig. 1). The peripheral zone of each quadrant was defined as the outermost part of the cornea captured with a rectangular field placed with its outer edge adjacent to the limbus. The central zone was defined as the part of the cornea closest to the center of the cornea and captured with a rectangular field placed with its inside edge at the apex of each quadrant. The rectangular field imaged for each zone was of the same size (895 × 670 μm). After completion of image collection for each of the two zones, maximum intensity projected images of the Z-stacks were produced using Zen imaging software.

The quadrants immunolabeled with the PGP9.5 antibody were imaged using a 10× objective because the labeling was of sufficient intensity to permit efficient imaging at this magnification. Quadrants immunolabeled with TRPM8 and TRPV1 antibodies were imaged using a 20× objective because the labeling was not as intense and required higher magnification to capture properly. Importantly, to maintain the same area for calculation of nerve fiber density across all experiments, four images per zone were collected using the 20× objective in each corneal quadrant immunolabeled with TRPM8 and TRPV1. All images that were collected in this way were...

**FIGURE 1.** A schematic of the areas sampled for analysis of CNFD in each corneal quadrant.

**FIGURE 2.** The upper panels show the percent of body weight gain from the time the HFHC diet was initiated for the control, HFHC, and HFHC+Db groups terminated at 25 to 30 weeks (A) and 35 to 40 weeks (B). These data are presented as mean and SE and pairwise statistical comparisons were made with Tukey’s range tests. The lower panels show the blood glucose levels for the control, HFHC, and HFHC+Db groups terminated at 25 to 30 weeks (C) and 35 to 40 weeks (D). These data are presented as median and interquartile range, and pairwise statistical comparisons were made with Dunn’s tests. *P < 0.05, **P < 0.01.
stitched together using the Mosaic plugin of Image J software (National Institutes of Health, Bethesda, MD, USA). Binary, thresholded images were then generated using Metamorph image analysis software (Metamorph offline 64 bit, version 7.7.0; Molecular Devices, Inc.). This involved enhancing the resolution of the image using the unsharp mask filter and the Neurite Outgrowth module to threshold the images. This module works on 16-bit fluorescent images and requires the approximate width of the nerve fibers and their intensity above the local background (threshold) to be defined prior to processing the image. The width of the nerve fibers was estimated for each image. To determine a consistent threshold value, the difference between the nerve terminal’s pixel intensity and the local background was calculated. A mean of the differences was determined for five clearly labeled nerve terminals in the image. The threshold was set at 70% of this value. The area fraction of the nerve terminals, relative to the total area captured in the thresholded images, was determined using Image J software and used as a representation of CNFD.

GraphPad Prism 5.0 (Graphpad Software, San Diego, CA, USA) was used for statistical testing. Statistical comparisons of the percent of body weight gain between the groups were made with 1-way analysis of variance. Because of unequal variance between the groups, the fasting blood glucose levels and the measures of CNFD were compared with the Kruskal-Wallis test (KW). Post hoc pairwise comparisons were made using the Tukey’s range tests (percent body weight gain) or Dunn’s tests (fasting blood glucose levels and CNFD) if a significant difference was indicated between the groups. To test for changes in CNFD between the two timepoints for the control (normal diet) group, the CNFD generated from the PGP9.5 immunolabeled cornea quadrants at each timepoint were compared using the Mann-Whitney U test. P values < 0.05 were considered to indicate significant differences.

RESULTS

Body Weight

The percent body weight gain from the time the HFHC diet was initiated differed between the treatment groups at both timepoints of 25 to 30 weeks and 35 to 40 weeks (1-way ANOVA, 25–30 weeks F[2] = 23.54, P < 0.001; 1-way ANOVA, 35–40 weeks F[2] = 28.4, P < 0.001). The body weight gain for the HFHC group was greater than in the control group (Figs. 2A, 2B). The body weight of the HFHC + Db group had increased by 64 ± 4% (n = 12) at the time of streptozotocin treatment (at 15 weeks). However, at termination their net percent body weight gain was 20% to 25% and was lower than in either the control or HFHC groups (Figs. 2A, 2B).

Blood Glucose Levels

In comparison with animals in the control and HFHC groups, those in the HFHC + Db group had elevated fasting blood glucose levels (KW, 25–30 weeks H[2] = 9.71, P = 0.008; KW, 35–40 weeks H[2] = 10.79, P = 0.005; Figs. 2C, 2D). The fasting blood glucose levels in HFHC animals did not differ from those in the control animals (Figs. 2C, 2D).

Corneal Nerve Fiber Density

PGP9.5 immunolabeling was used to identify all nerve fibers in the corneal epithelium (Fig. 3). In the peripheral cornea, there were no differences in PGP9.5 CNFD between any of the experimental groups and at either of the timepoints tested (KW, 25–30 weeks H[2] = 2.117, P = 0.366; Fig. 3D; KW, 35–40 weeks H[2] = 4.784, P = 0.088; Fig. 3E). In contrast, PGP9.5 CNFD in the central cornea was decreased in the HFHC + Db and the HFHC groups, relative to the control group, at 35 to 40 weeks.

FIGURE 3. An example of PGP9.5 immunolabeling in the peripheral cornea of control (A, A’), HFHC (B, B’), and HFHC + Db (C, C’) mice at 35 to 40 weeks. Left side panels (A–C) show Z-series projections of fluorescent PGP9.5 immunolabeling through the full thickness of the corneal epithelium. Right side panels (A’–C’) are thresholded representations of the field of view immediately to the left. Scale bars: 50 μm. PGP9.5 CNFD is shown in the peripheral (D, E) and central (F, G) corneas at 25 to 30 weeks (D, F) and 35 to 40 weeks (E, G). CNFD is expressed as an area fraction (%) and presented as the median and interquartile range. Pairwise statistical comparisons were made with Dunn’s tests. *P < 0.05, **P < 0.01.
were no differences in TRPM8 CNFD between the HFHC+Db and the HFHC treatment groups at either timepoint (Figs. 5D-G).

Polymodal Nociceptor Nerve Fiber Density

TRPV1 immunolabeling was used to identify the intraepithelial nerve fibers of polymodal nociceptors (Fig. 6). As previously reported, the TRPV1 immunolabeled nerve terminals projecting from the sub-basal plexus had simple or ramifying morphology.21,22 There were no differences in TRPV1 CNFD between the HFHC+Db and HFHC treatment groups, relative to the control group, in either the peripheral (KW, 25–30 weeks H[2] = 1.636, P = 0.459; Fig. 6D; KW, 35–40 weeks H[2] = 2.561, P = 0.290; Fig. 6E) or central cornea (KW, 25–30 weeks H[2] = 4.713, P = 0.092; Fig. 6F; 35–40 weeks H[2] = 4.262, P = 0.118; Fig. 6G). This suggests that the density of corneal polymodal nociceptor nerve fibers was not greatly affected by either of the two treatments.

DISCUSSION

The present study shows that mice fed a high-fat diet and those that also have diabetes have altered corneal nerve fiber density consistent with small fiber neuropathy. Importantly, both treatments more markedly affected the intraepithelial axons of cold thermoreceptors than those of polymodal nociceptors, providing clear evidence that these subpopulations of corneal sensory neurons can be differentially affected by pathology.

Corneal Cold Thermoreceptors Are More Susceptible to Small Fiber Neuropathy Than Polymodal Nociceptors

Our findings of reduced TRPM8 expressing CNFD provides the first direct demonstration of small fiber neuropathy preferentially affecting cold thermoreceptors in the cornea. There is only one study that has assessed corneal thermal sensitivity in patients with diabetes.8 This study reported a reduction in sensitivity to both heating and cooling as well as to acidic and mechanical stimuli in patients with type 1 and type 2 diabetes.8 This suggests that all subpopulations of corneal sensory neurons are affected by diabetes. However, the average time since diagnosis of diabetes for the patients in this study was 15 years, and they all had evidence of diabetic retinopathy. Therefore, the effects reported for these patients are the consequence of prolonged diabetes and are not directly comparable with the findings of the current study. In clinical studies, cold detection thresholds are reduced in the foot skin of patients with type 2 diabetes and preclinical small fiber neuropathy, and this change had a much greater sensitivity for detecting early small fiber neuropathy than did the change in axon reflex-induced flare produced by activation of peptidergic polymodal nociceptors.25 This finding suggests that cutaneous cold thermoreceptors may be preferentially affected at an early stage of small nerve fiber neuropathy in patients with type 2 diabetes.

A number of studies have shown reduced IENFD and altered heat and/or cold sensitivity in the foot skin of patients with type 1 or type 2 diabetes.4,5,26 Notably, Quattrini et al.4 reported that the reduction in cold sensitivity was correlated with the reduction in both IENFD and the sub-basal nerve fiber density assessed by IVCM. Thus, the evidence presented here suggests that diabetes produces dysfunction of cold thermoreceptors in both the skin and cornea.

The observation that the TRPM8 expressing nerves in the central cornea were more affected than those in the periphery...
is consistent with a length-dependent “die-back” neuropathy because the longest axons projecting within the cornea were most affected. In the future, it will be important to establish a more complete timecourse of changes in TRPM8 expressing CNFD and if the changes are observed first in the most superficial layers of the corneal epithelium.

There is one report that TRPV1 nerve fiber density is reduced in the skin of patients with diabetes.27 Although this study suggests that polymodal nociceptors are affected by diabetic neuropathy, no information was provided regarding whether the patients had type 1 or type 2 diabetes or for how long they had diabetes. This makes comparisons with the findings of the present study impossible.

We were unable to assess effects on the mechano-nociceptor neurons because currently there are no tools available to discriminate their nerve fibers in the corneal epithelium. Therefore, it is possible that a reduction in the intraepithelial axon density of mechano-nociceptors contributes to the overall reduction in nerve fibers detected with PGP9.5 labeling at 35 to 40 weeks.

Hyperglycemia Is Not the Primary Cause of Early Changes in CNFD

Our finding that there is no difference in CNFD between the treatment groups suggests that the primary cause of nerve fiber loss is metabolic stress induced by the high-fat diet. Two previous studies have assessed changes in sub-basal nerve fiber density using IVCM in rodents receiving a high-fat diet or a high-fat diet together with streptozotocin-induced diabetes, and both reported similar reductions in both treatment groups.11,19 Yorek et al.11 used a diet containing 55% fat (compared to 21% in the present study), and it is possible that this results in an earlier onset of neuropathy. In rats fed a diet containing 24% fat and treated with streptozotocin after 8 weeks, reductions in sub-basal nerve fiber density were observed at 26, but not at 16, weeks on the high-fat diet.19 This timecourse is more similar to that observed in the present study.

Small fiber neuropathy has been reported in humans with prediabetes who have impaired glucose tolerance but have not yet developed type 2 diabetes,9,10,28 and patients with prediabetes who have been assessed by IVCM have been reported to have reduced sub-basal nerve fiber density.4 In the present study, glucose tolerance was not assessed, but in a previous study, C57Bl/6 mice fed the same HFHC diet for 33 weeks did not have glucose intolerance or changes in their blood insulin levels.29 However, they did have histological signs of hepatic steatosis and elevated blood levels of alanine transferase indicative of compromised liver function. In this previous study, there was 15% to 30% loss of enteric neurons in the myenteric plexus of the ileum, cecum, and colon, with the nitric oxide synthase–expressing neurons being most affected. These findings indicate that the lipotoxic effect of the HFHC diet, in the absence of impaired glucose tolerance, is a cause of neuropathy. This conclusion is supported by the present study because the induction of hyperglycemia did not increase the effects produced by the HFHC diet on the corneal innervation. Indeed, there is growing evidence that obesity and hyperlipidemia are independent risk factors for small fiber neuropathy in humans with prediabetes and type 2 diabetes.30

**FIGURE 5.** An example of TRPM8 immunolabeling in the peripheral cornea of control (A, A’), HFHC (B, B’), and HFHC+Db (C, C’) mice at 35 to 40 weeks. Left side panels (A–C) show Z-series projections of fluorescent PGP9.5 immunolabeling through the full thickness of the corneal epithelium. Right side panels (A’–C’) are thresholded representations of the field of view immediately to the left. Scale bars: 50 μm. TRPM8 CNFD is shown in the peripheral (D, E) and central (F, G) corneas at 25 to 30 weeks (D, F) and 35 to 40 weeks (E, G). CNFD is expressed as an area fraction (%) and presented as the median and interquartile range. Pairwise statistical comparisons were made with Dunn’s tests. *P < 0.05, **P < 0.01.
Implications for Management of Ocular Surface Pathology

There is growing interest in understanding how ocular surface pathology interacts with nerves that innervate the ocular surface. Changes in corneal sensitivity and/or sub-basal nerve fiber density are observed in a range of diseases. Our findings indicate that the nerve fibers of cold thermoreceptors are more affected by the metabolic stress induced by a HFHC diet than polymodal nociceptors. Corneal cold thermoreceptors can sense changes in temperature associated with tear film evaporation, and they are also stimulated by increases in tear fluid osmolarity. Furthermore, selective activation of cold thermoreceptors stimulates tear formation and deletion of the cold-sensor protein TRPM8 in mice selectively reduces basal tear formation. For these reasons, it is believed that cold thermoreceptors form the afferent arm of the reflex arc that mediates homeostatic regulation of the tear film and that disruptions of their function are likely to contribute to the etiology of dry eye disease. In support of this suggestion, dry eye disease patients have reduced sensitivity to cold stimuli applied to the cornea. The incidence of dry eye disease is increased in patients with type 2 diabetes, and there is evidence that this is associated with distal symmetric polyneuropathy. For these reasons, it is believed that cold thermoreceptors form the afferent arm of the reflex arc that mediates homeostatic regulation of the tear film and that disruptions of their function are likely to contribute to the etiology of dry eye disease. In support of this suggestion, dry eye disease patients have reduced sensitivity to cold stimuli applied to the cornea. The incidence of dry eye disease is increased in patients with type 2 diabetes, and there is evidence that this is associated with distal symmetric polyneuropathy. Furthermore, a meta-analysis has revealed that patients with type 2 diabetes and dry eye symptoms have impaired tear functions and reduced corneal sensitivity to mechanical stimuli indicative of neuropathy. There is also a recent meta-analysis that has identified hyperlipidemia as a risk factor for dry eye disease. We therefore suggest that the decrease in the density of cold thermoreceptor endings we have reported in the present study could form the basis of the dysfunction that leads to the increased incidence of dry eye disease in people with hyperlipidemia and/or type 2 diabetes. Understanding the mechanisms that produce this selective change in a specific subpopulation of corneal sensory will be important to resolve as this knowledge could lead to new targets for therapies to manage dry eye disease or potentially other ocular surface pathologies.

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