Transducin1, Phototransduction and the Development of Early Diabetic Retinopathy

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PURPOSE. Recent evidence suggests that retinal photoreceptor cells have an important role in the pathogenesis of retinal microvascular lesions in diabetes. We investigated the role of rod cell phototransduction on the pathogenesis of early diabetic retinopathy (DR) using Gnat1−/− mice (which causes permanent inhibition of phototransduction in rod cells without degeneration).

METHODS. Retinal thickness, oxidative stress, expression of inflammatory proteins, electroretinograms (ERG) and optokinetic responses, and capillary permeability and degeneration were evaluated at up to 8 months of diabetes.

RESULTS. The diabetes-induced degeneration of retinal capillaries was significantly inhibited in the Gnat1−/− diabetics. The effect of the Gnat1 deletion on the diabetes-induced increase in permeability showed a nonuniform accumulation of albumin in the neural retina; the defect was inhibited in diabetic Gnat1−/− mice in the inner plexiform layer (IPL), but neither in the outer plexiform (OPL) nor inner nuclear (INL) layers. In Gnat1-deficient animals, the diabetes-induced increase in expression of inflammatory associated proteins (iNOS and ICAM-1, and phosphorylation of IkB) in the retina, and the leukocyte mediated killing of retinal endothelial cells were inhibited, however the diabetes-mediated induction of oxidative stress was not inhibited.

CONCLUSIONS. In conclusion, deletion of transducin1 (and the resulting inhibition of phototransduction in rod cells) inhibits the development of retinal vascular pathology in early DR.

Keywords: retina, photoreceptors, microvasculature, Gnat1, phototransduction

Diabetic retinopathy (DR) is a leading cause of acquired blindness, but its pathogenesis and reason for the relatively unique susceptibility of the retinal vasculature to diabetes-induced injury remains unclear. Recently, several cell types that participate in vision and are unique to the eye (photoreceptor cells and RPE) have been implicated in having key roles in the pathogenesis of DR.1–7 We investigated the possibility that induced injury remains unclear. Recently, several cell types that

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Materials and Methods

All procedures involving animals were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Male C57Bl/6j mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Mice in which the rod transducin α-subunit (Gnat1−/−) was knocked out (on the C57Bl/6 background) were provided originally by Janis Lem (Tufts University, Boston, MA, USA). In the retina, this gene is expressed only in rod photoreceptor cells. Some C57Bl/6j mice were housed in continuous darkness.
for 2 months as a nongenetic control for the effects of eliminating phototransduction on retinal production of superoxide. These chronically dark-adapted animals and their controls that experienced the normal day/night cycle were killed in the morning.

Animals were housed in a ventilated microisolator with free accessibility to food and water. Diabetes was induced at 2 to 3 months of age by intraperitoneal injection of a freshly prepared solution of streptozotocin in citrate buffer (55 mg/kg body weight [bw] for 5 consecutive days). Female mice have been recognized to be resistant to the diabetogenic effects of streptozotocin, so only males were used in this study. Insulin was given as needed (0–0.2 units subcutaneously 0–3 times a week) to inhibit weight loss, while still allowing hyperglycemia. Following streptozotocin, the milieu of the entire body changes rapidly, and the new steady-state takes days to weeks to stabilize; thus, blood glucose concentration was not measured until at least 7 days after the final administration of streptozotocin. Blood glucose was determined with a portable glucose meter, using blood collected from the tail vein under nonfasting conditions. The onset of diabetes was defined as three consecutive measures of blood glucose over 275 mg/dL. Hemoglobin A1c (HbA1c) was measured every 2 to 3 months as reported previously.

Vascular Histopathology

After 8 months of diabetes (D) or in age-matched nondiabetic (N) controls (10 months old), one eye was fixed in formalin. The retina then was isolated, washed, and digested in elastase (acellular) capillaries were quantitated in 6 to 7 field areas of FITC-BSA concentration in neural retina relative to that of inner retina. Leakage of albumin into neural retina was measured as reported previously.

Accumulation of the blood protein, albumin, in the neural retina was measured as reported previously. At 8 months of diabetes, sterile FITC-BSA (50 μg/μL) in phosphate buffered saline (NaCl, 0.138 M; KCl, 0.0027 M; pH 7.4) was injected into the tail veins of mice (100 μL of 0.138 M KCl). After 20 minutes, mice were euthanized, and one retina was harvested. The isolated vasculature was floated out flat on a glass microscope slide, dried overnight, stained with hematoxylin and eosin, and photographed by fluorescence microscopy.

Leakage of Albumin into Neural Retina

Ultrahigh-Resolution Spectral-Domain Optical Coherence Tomography (SD-OCT) Imaging

SD-OCT (Bioptigen, Durham, NC, USA) was used for in vivo imaging of mouse retinas. Mice were anesthetized by intraperitoneal injection of 10 μL/g dilute ketamine/xylazine (16.5/1.65 mg/mL). Pupils were dilated with 1% tropicamide. Five pictures were acquired in the B-scan mode and used to construct each final averaged image. Thickness of the retina and ONL was measured at distances of 150, 300, and 450 μm from the optic nerve.

Superoxide Generation

Superoxide generation and oxidative stress were assessed by two independent methods: (1) lucigenin-induced bioluminescence (LB) and (2) dichlorofluorescein (DCF) staining of unfixed cryosections. For the lucigenin-induced bioluminescence method, freshly isolated retinas were incubated in 200 μL Krebs-Hepes buffer, pH 7.2, with 5 or 25 mM glucose for 5 minutes at 37°C in 5% CO2. Luminescence indicating the presence of superoxide was measured 5 minutes after addition of 0.54 mM (final concentration) lucigenin. Luminescence intensity is reported in arbitrary units per mg protein.

For the DCF method, eyes were collected, immediately embedded in O.C.T. compound, and fresh frozen using liquid nitrogen vapor. Sections (12 μm) were cut, and while still frozen, slides were transferred to ice cold acetone for 10 minutes at −20°C. Slides then were warmed to room temperature for 20 minutes for acetone to evaporate. Next, sections were washed in PBS 3 times 5 minutes. Subsequently (from here on, work is done in dark), sections were submerged in DCF (cat# D6883; Sigma-Aldrich Corp., St. Louis, MO, USA) at 10 μM, and incubated at 37°C for 60 minutes. Sections then were washed 3 times 5 minutes, then cover-slipped using ProLong Gold anti-fade reagent with 4’,6-diamidino-2-phenylendole (DAPI; cat# P36935; Invitrogen, Carlsbad, CA, USA), and photographed by fluorescence microscopy.

Immunoblotting Technique

Retinas were isolated, sonicated, and centrifuged, and the supernatants were used for Western blots. Samples (usually approximately 30 μg) were fractionated by SDS-PAGE, electroblotted onto nitrocellulose membranes, and membranes then blocked in Tris-buffered saline containing 0.2% Tween 20 and 5% nonfat milk. Antibodies for ICAM-1 (1:2000; Proteintech Group, Inc., Chicago, IL, USA) and INOS (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), p-IκB and IκB (1:200 and 1:1000 dilutions, respectively; Santa Cruz Biotechnology) were applied, followed by secondary antibody for 1 hour. After washing, nitrocellulose membranes were visualized for enhanced chemiluminescence. Protein levels were quantified relative to β-actin loading controls (1:5000 dilution, Abcam Inc., Cambridge, MA, USA) in the same samples.

Visual Function

Electroretinography (ERG)

ERGs were measured at 8 months of diabetes (10 months of age). After overnight dark adaptation, mice were anesthetized with ketamine (80 mg/kg) and xylazine (16 mg/kg), the cornea was anesthetized with 1% proparacaine hydrochloride, and the pupils were dilated with 1% tropicamide, 2.5% phenylephrine hydrochloride, and 1% cyclopentolate. Mice were placed on a temperature-regulated heating pad throughout each recording session, which has been described. In brief, responses of the outer retina were recorded with a contact lens electrode...
TABLE. Glycemia in WT and Gnat1−/− Mice Over 8 Months of Study

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Final bw, g</th>
<th>Blood Glucose, mg/dL</th>
<th>HbA1c, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>N mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>42 ± 7</td>
<td>156 ± 18</td>
<td>3.1 ± 0.1</td>
</tr>
<tr>
<td>Gnat1−/−</td>
<td>41 ± 8</td>
<td>156 ± 10</td>
<td>3.1 ± 0.1</td>
</tr>
<tr>
<td>D mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>28 ± 3</td>
<td>489 ± 48</td>
<td>9.7 ± 0.9</td>
</tr>
<tr>
<td>Gnat1−/−</td>
<td>29 ± 4</td>
<td>514 ± 53</td>
<td>10.1 ± 0.4</td>
</tr>
</tbody>
</table>

* Glucose levels and HbA1c were measured as described in the Methods section.

referenced to a needle electrode placed in the cheek in response to strobe-flash stimuli presented in the dark or superimposed on a steady 20 candelas (cd)/m² rod-desensitizing adapting field. The amplitude of the a-wave was measured 6 ms after flash onset from the pre-stimulus baseline. The amplitude of the b-wave was measured from the a-wave amplitude at 6 ms to the peak of the b-wave.

Leukocyte-Mediated Cytotoxicity Toward Endothelial Cells

Transformed retinal endothelial cells were grown in control medium (Dulbecco’s modified Eagle medium [DMEM] with 5 mM glucose) containing 10% serum. The serum concentration was reduced to 2% just before cells were placed either in low glucose or high glucose (30 mM). Media was changed every other day for 3 days. When cells reached 80% confluency level (~300,000 cells), freshly isolated leukocytes from blood (100,000 cells) were added and incubated for 6 additional hours, after which cells were collected and washed with PBS. Cells were stained with an antibody against CD144 (1:50 dilution; BD Biosciences Pharmingen, San Diego, CA, USA) to identify endothelial cells, and the viability of the endothelial cells was identified by flow cytometry based on 7-AAD staining. Endothelial cell death was expressed as the percentage of endothelial cells that stained positively with dye. Approximately 10,000 cells were counted in each sample. Experiments were repeated twice with similar results each time.

Leukostasis

At 2 to 3 months of diabetes, blood was removed from the vasculature of anesthetized animals (100 mg/mL ketaset; 100 mg/mL xylazine = 5:1) by complete perfusion with PBS via a heart catheter. Animals then were perfused with fluorescein-coupled Concanavalin A lectin (20 µg/mL in PBS; Vector Laboratories, Burlingame, CA, USA) as described previously. Flat-mounted retinas were imaged via fluorescence microscopy, and the number of leukocytes adherent to the vascular wall was counted.

Statistical Analyses

Data are expressed as mean ± SD. All statistical analyses were performed with ANOVA followed by Fischer’s test. P < 0.05 was considered statistically significant.

RESULTS

There was no significant difference with respect to bw or glycemia between N members of the strains studied. Glycemia was elevated in all animals assigned to D groups, and the severity of diabetes was not different among the D groups. HbA1c and blood glucose were measured multiple times over the entire duration of the 8-month experiment, and these values were significantly greater than normal in all D groups (P < 0.01). Data are summarized in the Table.

Effect of Diabetes and Deletion of Gnat1 on Photoreceptor Survival

OCT analysis indicated that diabetes of 8 months duration did not cause a significant increase or decrease in retinal thickness in wildtype (WT) animals (213 ± 5 µm compared to 212 ± 5 for N and D groups 450 µm from the optic nerve, respectively). Gnat1−/− mice that had been diabetic for a comparable duration showed only a slight (but significant, P < 0.05) reduction in thickness (196 ± 6 µm) compared to age-matched WT mice. The thickness of the ONL was not significantly different from normal in D WT and in Gnat1−/− mice (Fig. 1).

Visual Function

Spatial frequency threshold is a psychophysical measure that assesses the function of retinal and central visual pathways in cone cells. At 5 months of age, N mice from the Gnat1−/− mice showed spatial frequency thresholds that were equivalent to those observed in WT N mice (Fig. 2a). The fact that this model did not have a major effect on vision is not surprising, since the optokinetic technique measures predominantly cone function, and not the rod cells. Diabetes for 3 months’ duration (5 months old) caused a significant decrease in spatial frequency threshold in WT and Gnat1-deficient mice (Fig. 2a), and this decrease was maintained also at 8 months of diabetes (0.397 ± 0.003 c/deg; 0.371 ± 0.008, and 0.350 ± 0.006 for WT N and D, Gnat1−/− D groups, respectively; all P < 0.001 compared to WT N).

We next examined ERGs as a confirmatory test (Fig. 2b). WT mice diabetic for 8 months (10 months of age) exhibited reductions in a- and b-wave amplitudes as well as the light-adapted response. N Gnat1-deficient mice exhibited small dark-adapted responses, and low a- and b-wave. Light-adapted responses were of normal amplitude in the mutants, and the results seemed not affected by diabetes.

Retinal Vascular Histopathology

N WT C57Bl/6j mice had few degenerate capillaries at 10 months of age (Fig. 3). Gnat1-deficient N mice showed a significant increase in retinal capillary degeneration compared to N WT C57Bl/6j mice (Fig. 3b; P < 0.0001).
As expected, diabetes 8 months in duration (age 10 months) significantly increased the number of degenerated capillaries in WT C57Bl/6J mice compared to their N C57Bl/6J controls (Fig. 3). Since even the N Gnat1-deficient mice showed an increase in retinal capillary degeneration, we evaluated the diabetes-induced increase in capillary degeneration as the number of degenerate capillaries per unit retinal area (Fig. 3b), and as a ratio compared to the number of degenerate capillaries in nondiabetic members of the same mutant strain (Fig. 3c). Gnat1-deficient D mice showed fewer degenerated retinal capillaries compared to WT D controls, and showed no diabetes-induced increase compared to their N Gnat1-deficient controls. Thus, the D mice lacking phototransduction in their rod cells were protected from the diabetes-induced capillary degeneration, and this protection occurred without any reduction in glycemia.

Diabetes of 8 months' duration significantly increased the amount of leakage of FITC-BSA into each multiple layer of the neural retina containing a vascular plexus (IPL, IN, OPL) in WT animals (Fig. 4). The diabetes-induced increase in permeability was inhibited in D Gnat1−/−/C0/C0 mice in the IPL, but there was no significant inhibition of the diabetes-induced increase in albumin leakage in the OPL or INL layers at this time point.

**Retinal Oxidative Stress**

Diabetes of 3 months' duration (5 months of age) appreciably increased the generation of superoxide or reactive oxygen species in the photoreceptors of WT D mice (Fig. 5). Our studies as well as others have reported previously that this increase is present in vivo and maintained for at least an additional 6 months of diabetes,18 demonstrating that this oxidative stress is not an artifact due to ex vivo processing or a
transient abnormality. Generation of reactive oxygen species also was evaluated by measuring DCF staining in freshly isolated retinal slices. Under these conditions, no DCF fluorescence was observed in slices obtained from N control mice (Fig. 5b), while it was consistently detected in photoreceptor cells of D WT mice. The DCF fluorescence was detectable predominantly in the rod outer segments. We found that the deletion of transducin1 (and, thus, phototransduction) in \( \text{Gnat}^1 \)/\( \text{C0} \)/\( \text{C0} \) mice did not inhibit that retinal oxidative stress in diabetes, and in fact, induced it in photoreceptor cells even in N mice.

To further investigate the effects of eliminating phototransduction on retinal superoxide generation, we maintained other N and D WT animals in total darkness for 2 months to mimic the absence of phototransduction that would occur in rods of the \( \text{Gnat}^1 \)-deficient D mice. Compared to N mice under a standard day/night cycle, superoxide generation in the retinas of D mice with the same diurnal light cycle was 316% ± 155% above that in the N mice, whereas superoxide generation by retinas from the dark-maintained N and D animals was 259% ± 137% and 389% ± 244% above that in WT N mice maintained under a diurnal cycle, respectively (n = 3–8 per group).

**Markers of Inflammation**

Since increased expression of proinflammatory proteins and cytokines has been implicated in the pathogenesis of the retinopathy,5,18,27 we measured the effect of diabetes and transducin1 deficiency on expression of iNOS, ICAM-1, and other proinflammatory proteins in the retina. Diabetes of 2 to 3 months in duration resulted in a significant increase in expression of retinal iNOS, ICAM-1, and in the ratio of p-IκB/total IκB in WT animals (expression of iNOS and ICAM-1 is regulated by NF-κB, and IκB is an important regulator of NF-κB activation; Fig. 6). The expected diabetes-induced increase was significantly inhibited for all of these proinflammatory proteins in retinas from D \( \text{Gnat}^1 \)-deficient mice (Figs. 6a–c).

Leukocytes are intimately involved in inflammatory processes, and we previously implicated leukocytes as contribut-
According to the development of DR, Consistent with prior studies, we found that diabetes significantly increased the leukocyte-mediated cytotoxicity against retinal endothelial cells in WT D mice, and increased also leukostasis within retinal capillaries (Fig. 7). Even though transducin1 is expressed only in photoreceptor cells, the data indicate that deletion of the protein (and, thus, phototransduction) from rod cells significantly inhibited or tended to inhibit leukocyte-mediated killing of retinal endothelial cells and leukostasis, respectively.

**Figure 6.** Effect of diabetes and deletion of Gnat1 on retinal expression of proinflammatory proteins in mice. (a-c) summarize expression of iNOS, ICAM, and the ratio of phosphorylated IkBα/total IkBα at 4 months of age (2 months of diabetes). (d) Representative Immunoblots of inflammatory proteins quantified, retinal homogenates, and expressed relative to actin in the same sample (n = 4–5 in all groups).

**Figure 7.** In WT animals, (a) leukocyte-mediated cytotoxicity toward retinal endothelial cells and (b) leukostasis are significantly increased by diabetes. In contrast, the leukocyte-mediated cytotoxicity is significantly inhibited in Gnat1-deficient mice (Gnat1−/−), and leukostasis tends to be inhibited (but these results did not achieve statistical significance in this limited sample). Total duration of diabetes was 12 weeks. Horizontal lines above the figure indicate significant differences (n = 4 per group for the cytotoxicity assay, and n = 3–5 per group for leukostasis).
DISCUSSION

Compared to other tissues, the retinal vasculature has been recognized to be uniquely sensitive to adverse effects of hyperglycemia, but the cause of that susceptibility remains unclear. One unique function of the retina is phototransduction, which occurs at the photoreceptors and converts light energy into an electrical signal that can be transmitted to the brain. In diabetes, photoreceptors are well known to be particularly susceptible, and a number of studies have demonstrated that photoreceptor function is affected by diabetes.

While photoreceptor function is affected by diabetes, the role of phototransduction itself has been less well characterized. For example, it is not clear whether phototransduction contributes to photoreceptor degeneration caused by diabetes.

Our study demonstrated that the deletion of Gnat1, a component of the vertebrate phototransduction pathway, protected mice from diabetes-induced retinal vasculopathy. The mechanism underlying this protection is not yet clear, but it is possible that phototransduction may contribute to retinal microvascular leakage in diabetes.

In conclusion, phototransduction is a key component of the vertebrate phototransduction pathway and may contribute to retinal microvascular leakage in diabetes.

REFERENCES

defect. Thus, diabetes does impair aspects of vision, but we have no data that the cause of this defect includes cones. Moreover, the present studies provide no evidence that cone cells contribute to diabetes-induced retinal vascular injury.

Our findings suggested that light-induced phototransduction in rod cells and the molecular processes regulated by it contributing to the diabetes-induced degeneration of the retinal vasculature. Whether pharmacologic inhibition of phototransduction is a meaningful therapeutic target to inhibit retinopathy will depend on whether a partial reduction in phototransduction that does not compromise normal daytime vision can inhibit the microangiopathy. As an alternate approach, however, pharmacologic slowing of visual cycle activity (which is activated by phototransduction) by retinylamine already has been shown to significantly inhibit the development of the vascular lesions of the retinopathy.1

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