Early Inner Retinal Astrocyte Dysfunction during Diabetes and Development of Hypoxia, Retinal Stress, and Neuronal Functional Loss

Alice Ly, Peter Yee, Kirstan A. Vessey, Joanna A. Phipps, Andrew I. Jobling, and Erica L. Fletcher

PURPOSE. Neuronal and glial alterations precede the overt vascular change that characterizes diabetic retinopathy. Because retinal astrocytes modulate neuronal and vascular function, this study investigated the time course of astrocyte, Müller cell, and neuronal change during diabetes to determine whether astrocytes may play an early role in diabetic retinopathy.

METHODS. Sprague-Dawley rats were rendered diabetic via streptozotocin and neuronal and glial changes were assessed after 2–10 weeks. Astrocyte change was investigated using connexin-26 immunolabeling, whereas connexin-26 and -43 gene expressions were quantified using real-time PCR. Hypoxia was measured by pimonidazole labeling and the expression of hypoxia-inducible factor-1 alpha (HIF-1α) was quantified using Western blot. Müller cell gliosis was assessed by glial fibrillary acidic protein immunolabeling and retinal function assessed using the electroretinogram.

RESULTS. Astrocyte connexin-26 and -43 gene and protein expression decreased after 4 weeks of diabetes, before significant astrocyte loss. At the same time, the retina became hypoxic, with increased HIF-1α expression and pimonidazole labeling in the ganglion cell layer. This coincided with a decrease in ganglion cell function. After 6 weeks of diabetes, Müller cell gliosis became more evident and there were additional functional deficits in photoreceptor and amacrine cell responses.

CONCLUSIONS. These findings suggest that early changes in astrocytes are coincident with inner retinal hypoxia and ganglion cell functional deficits, whereas Müller cell gliosis and more extensive decreases in neuronal function occur later. Astrocytes may play an early and key role in changes in retinal vasculature and inner retinal dysfunction in diabetes. (Invest Ophthalmol Vis Sci. 2011;52:9316–9326) DOI:10.1167/iovs.11-7879

Diabetic retinopathy is a major complication of diabetes mellitus and is historically characterized by the development of pathologic changes in retinal vasculature, including microaneurysms, acellular capillaries, and neovascularization.1–3 A number of studies have investigated these vascular changes and numerous mechanisms have been proposed, with recent treatment strategies targeting the angiogenic factor, vascular endothelial growth factor (VEGF).4–6 However, despite the undeniable role the retinal vasculature plays in diabetic retinopathy, it is evident that other changes involving the neural retina also occur.7,8 In fact, functional electroretinogram (ERG) testing has indicated that neuronal dysfunction often occurs before any overt vascular change in human diabetics and in animal models of hyperglycemia.9,10 In addition to these functional deficits, other studies have detailed specific retinal neuronal and glial cell change during diabetes.11 Although such data have led to the theory that neuronal/glial changes may drive retinal vascular pathology during diabetes, a causal link between the two has yet to be established.

Numerous studies principally involving animal models of hyperglycemia have investigated the neuronal and glial cell classes that are altered in the retina during diabetes. As far as retinal neurons are concerned, diabetes-induced deficits in rod and cone photoreceptor function have been characterized,10,12,13 as have been changes in downstream neurons such as bipolar, amacrine, and ganglion cells.10,12,14,15 These neuronal functional deficits have been attributed to various changes in key metabolic/support pathways that may ultimately lead to increased diabetic-induced neuronal loss (Phipps JA, et al. IOVS 2004;45:ARVO E-Abstract 3233).11,16 Although there is some variation in the time course of neuronal effects, the data generally show an early (2–4 week postinduction of diabetes) inner retinal dysfunction involving amacrine and ganglion cells followed by later outer retinal effects.

In addition to the neuronal dysfunction found during diabetes, changes in the retinal glial cells have also been observed. The retina contains two broad populations of glial cells, the macroglia encompassing Müller cells and astrocytes, and the retinal microglial cells. The role of the retinal macroglia are of particular interest because, in addition to their role in neuronal support,17 Müller cells and astrocytes maintain the integrity of the blood–retinal barrier and regulate blood vessels in response to neuronal activity.18,19 Numerous changes have been reported in Müller cells during diabetes, with the most obvious being gliosis.20,21 In addition to this relatively broad response, changes in Müller cell neurotransmitter processing, ion channel function, as well as altered growth factor and inflammatory gene expression have also been reported in diabetes.22–26 Unlike Müller cells there have been few studies detailing the diabetic changes in retinal astrocytes. Those studies that have investigated retinal astrocytes have detailed very early (4 weeks postinduction of diabetes) cell loss, as evidenced by reduced glial fibrillary acidic protein (GFAP) expression.22–27 Although some alterations in morphology have been observed,20 other studies have found no alteration in certain neurotransmitter transporters.22

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The close proximity of astrocytes to the retinal vasculature and ganglion cell layer, two cell types known to be altered during diabetes, in addition to their role in retinal blood vessel formation, neurovascular coupling, and modulation of pathologic neovascularization, targets them as a critical modulator of early diabetic retinal change. Previous work in the rat brain has shown that astrocyte communication is significantly reduced during diabetes, whereas hyperglycemia decreases the expression of astrocyte gap junction proteins in the retina. Recently, these diabetic alterations in astrocytes have been linked with neuronal dysfunction. Of further interest, retinal astrocytes are known to play a key role in the development of retinopathy of prematurity, another retinal disease involving significant vascular pathology. 33

Given their role in retinal neuronal and blood vessel function, and the finding that they are lost early in diabetic astrocytes may play an early and key role in the development of neuronal dysfunction before overt retinal vessel pathology in diabetic retinopathy. The aim of this study was to characterize the time course of astrocyte, Müller cell, and neuronal change during streptozotocin (STZ)-induced diabetes and to investigate possible mechanisms involved in an astrocyte-mediated effect. Understanding the complex relationship between neuronal and glial function in the retina and how this is altered in diabetes is crucial for explaining the pathology associated with diabetic retinopathy.

METHODS

Animals and Induction of Diabetes

All experimental procedures adhered to the guidelines of the National Health and Medical Research Council of Australia’s Code for the Care and Use of Animals for Scientific Purposes and the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. Six-week-old Sprague-Dawley rats were exposed to a 12-hour light-dark cycle and allowed free access to food and water. After an overnight fast, animals were randomized and received a single interperitoneal (IP) injection of either 50 mg/kg streptozotocin (STZ; Sigma-Aldrich Co., St. Louis, MO) diluted in 0.1 M citrate buffer (pH 4.5; Chem-Supply Ltd., Gillman, Australia) or citrate buffer alone. Serum glucose levels were measured twice weekly for the diabetic animals (Accu-Chek Go; Roche, Mannheim, Germany) and, if required, animals received an injection of insulin (2 to 4 units IP; Ultralatr, Novo Nordisk A/S, Bagsvaerd, Denmark). Control and diabetic animals were taken after 2, 4, 6, and 10 weeks postinjection of citrate buffer or STZ and the various functional and biochemical measures were performed as detailed in the following text.

Retinal Function

Retinal function was assessed by measuring the ERG after 2, 4, and 6 weeks postinjection of citrate buffer or STZ. Control and diabetic animals were deeply anesthetized (60:5 mg/kg ketamine/xylazine; euthanized with sodium pentobarbital (Lethobarb, 120 mg/kg; Merial Australia Ltd, Paramatta, NSW, Australia), and their eyes enucleated. The anterior segments were removed and posterior eyecups either fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 30 minutes and processed for indirect immunofluorescence, or the retinas were removed from the posterior eye cup, snap frozen in liquid nitrogen, and stored at −80°C for total RNA isolation or Western blot analysis.

Four hours before tissue collection, a separate cohort of control and diabetic animals (4 and 6 weeks) were anesthetized and received an intravitreal injection of pimonidazole hydrochloride (1 μL of 400 μM in 0.1 M PB, pH 7.4) to assess retinal hypoxia (see the following text).

Immunocytochemistry

Changes in retinal macroglia (astrocytes and Müller cells) and tissue hypoxia (pimonidazole) were assessed in retinal whole mounts and transverse 12-μm sections cut on a cryostat (Microm 550 cryostat; Menzel-Gläser, Braunschweig, Germany). Immunocytochemistry was performed as described previously. Briefly, nonspecific binding sites were blocked (10% normal goat serum, 1% bovine serum albumin, and 0.5% Triton X-100 in PB) for 1 hour and then tissue samples were incubated with the respective primary antibodies overnight (see the following text; all antibodies diluted in 3% normal goat serum, 1% bovine serum albumin, and 0.5% Triton X-100 in PB). After rinsing, secondary antibodies (see following text) were applied to the sections for 90 minutes, rinsed in PB, and coverslipped. Immunostained tissue was photographed on a confocal microscope (Zeiss LSM 510 Pascal; Carl Zeiss AG, Oberkochen, Germany) using a ×40 oil objective. Offset and gain parameters were held constant for control and diabetic samples.

Retinal Hypoxia. Tissue hypoxia was measured using a commercially available kit (HypoxyProbe; Chemicon International, Temecula, CA) per the manufacturer’s instructions. The mean intensity of pimonidazole immunolabeling was quantified in the inner retina (excluding blood vessels) from the nerve fiber layer (NFL) to the bottom of the inner plexiform layer (IPL), using ImageJ software on a grayscale from black (0) to white (255) (version 1.36b; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). At least 12 photographs were analyzed per retinal eccentricity per retina. Group means were calculated for central and peripheral retina.

Glial Cell Changes. To assess astrocyte change, double-labeling experiments were performed on control and diabetic retinas (2, 4, 6 weeks, n = 5) using antibodies to GFAP (1:5000, mouse anti-bovine; Dako, Carpinteria, CA) and connexin-26 (1:1000, rabbit anti-rat; Zymed Laboratories, South San Francisco, CA), a marker that labels retinal astrocytes but not Müller cells. After incubation with secondary antibodies (1:500, goat anti-mouse Alexa Fluor 594 and goat anti-rabbit Alexa Fluor 488; Molecular Probes), the extent of connexin-26 labeling in transverse retinal sections was assessed and expressed as a percentage of retinal length for central and peripheral retina. In flat-mounted retinas, astrocyte cell bodies and their processes were counted (Image, using the Cell Counter plugin) and group mean values calculated for central and peripheral retinas. Co-labeling experiments were also performed using antibodies to GFAP and connexin-43 (1:500 rabbit intensity flashes, the rod photoreceptor response (PII) was recorded and fitted according to a model described by Hood and Birch, and the amplitude, sensitivity, and latency of response were determined. The rod and cone b-waves (PII) were fitted using an inverted gamma function, providing measures of amplitude and implicit time as described previously. The oscillatory potentials (OP2, OP3, OP4) were extracted by subtracting the fitted rod PII response from the raw waveform and their amplitude and timing determined.
Table 1. Oligonucleotide Primer Sequences for Gene Expression Studies

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Size (bp)</th>
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<tbody>
<tr>
<td>Connexin-26 (Gjb2)</td>
<td>caccagcatggggaaaatct</td>
<td>cacgtgcatacatccagga</td>
<td>235</td>
</tr>
<tr>
<td>Connexin-43 (Gjb1)</td>
<td>tctgctctgggagagct</td>
<td>gttgacccctctgtgcag</td>
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</tr>
<tr>
<td>HPRT</td>
<td>ccagaggactagggtgtaa</td>
<td>ccccaaggtcattcagaag</td>
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<tr>
<td>GAPDH</td>
<td>tgatccggtggtgtcctga</td>
<td>tggctgtgagctcaggag</td>
<td>150</td>
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</table>

Gene-specific primers were designed for real-time qPCR. Primers are shown 5’-3’ and product sizes are included. For generation of the external standards the same primers were used as detailed below; however, a T7-promoter sequence (5’-taatagctgcgtatagg-3’) was included at the 5’ end of the forward primer, whereas a poly-T15 sequence was included at the 5’ end of the reverse primer. Product sizes for the standards were as shown above ± 55 bp.

Müller cell gliosis was estimated in control and diabetic retinas (2, 4, 6, 10 weeks, n = 5) by colabeling transverse retinal sections with antibodies to GFAP (1:10,000, rabbit anti-bovine; Dako) and glial fibrillary acidic protein (GFAP; 1:1000, mouse anti-sheep; Chemicon). After the addition of secondary antibodies (1:500, goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Alexa Fluor 594; Molecular Probes), the proportion of GFAP-positive Müller cell processes that were also colocalized for GFAP immunoactivity were counted in the S1 sublamina of the IPL.

Total RNA Isolation

Total RNA was isolated from diabetic and control retinal tissue samples (2, 4, 6, 10 weeks, n = 9) using a commercial silica-based spin column (RNasey, Qiagen, Valencia, CA). All samples were treated on-column with DNaseI (Qiagen) to eliminate contaminating genomic DNA. Total RNA samples were quantified at 260 nm (Nanodrop 1000; Thermo Scientific, Wilmington, DE) and 500 ng of total RNA was reverse transcribed using a random hexamer-primed commercial reverse transcriptase (RT) (Superscript III, Invitrogen, Carlsbad, CA). Respective negative controls, which lacked the RT enzyme, were included to control for genomic contamination.

Quantitative Real-Time PCR

Gene expressions of connexin-26 and connexin-43 were assessed relative to the housekeeping genes, hypoxanthine guanine phosphoribosyl transferase (HPRT) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; see Table 1 for primer sequences). External standards were used to quantify gene expression. Production of the standards required sequence-specific primers incorporating a T7-promotor sequence at the 5’ end of each forward primer and poly-T15 at the 5’ end of each reverse primer. The amplified gene products were then transcribed into RNA (Megascript T7 High Yield Transcription kit; Ambion Inc., Austin, TX) and dilutions of the RNA standards were combined with yeast tRNA (500 ng; Invitrogen) to reflect the retinal total RNA amount used in the reverse transcription reaction. The RNA standards were reverse transcribed with the retinal RNA standards to standardize efficiency.

Real-Time PCR (Rotor-Gene V3000; Corbett Research, Sydney, Australia) was performed using a commercial reaction mixture (Rotor-Gene SYBR Green PCR kit; Qiagen). Respective four-point standard curves were included in every run and standards and samples were amplified in triplicate. Each primer set yielded only one product of the correct size and negative controls were included in every run. Absolute gene copy number was calculated with reference to the standard curve (Rotor-Gene V6.1 software; Corbett Research) and expressed relative to HPRT and GAPDH.

Western Blot

Diabetic and control retinal tissue samples (2, 4 weeks, each n = 3) were homogenized in radioimmunoprecipitation assay buffer (Sigma) containing protease inhibitors (Roche) and protein concentration measured (BCA; Thermo Fisher Scientific, Rockford, IL). Control and diabetic samples (10 μg) were separated on a 12% acrylamide gel and transferred overnight to nitrocellulose membrane (Hybond; GE Healthcare, Piscataway, NJ). The membrane was probed with antibodies to hypoxia-inducible factor-1 alpha (HIF-1α; 1:500, mouse anti-human; Novus Biologicals, Littleton, CO) and GAPDH (1:5000, rabbit anti-mouse; Sigma), followed by respective fluororescent secondary antibodies (goat anti-mouse 800, goat anti-rabbit 680; Li-Cor Biosciences, Lincoln, NE). Membranes were scanned (Odyssey infrared imager; Li-Cor Biosciences) and pixel intensity of HIF-1α immunolabeling was expressed relative to that of GAPDH.

Results

Table 2. Serum Glucose Levels of Experimental Animals

<table>
<thead>
<tr>
<th>Group</th>
<th>Week 2</th>
<th>Week 4</th>
<th>Week 6</th>
<th>Week 10</th>
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<tr>
<td>Control</td>
<td>5.8 ± 0.2</td>
<td>6.2 ± 0.2</td>
<td>6.4 ± 0.3</td>
<td>Not collected</td>
</tr>
<tr>
<td>Diabetic</td>
<td>28.5 ± 1.5</td>
<td>28.8 ± 2.4</td>
<td>23.6 ± 2.4</td>
<td>29.9 ± 1.7</td>
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Blood glucose was taken weekly and data are expressed as mean blood glucose ± SEM. All blood glucose measurements were taken before the administration of anesthesia.
sion of a second astrocyte gap junction protein, connexin-43, was investigated. Connexin-43 immunoreactivity (green) was found on astrocytes (GFAP, red) in both control and diabetic retinas (Figs. 2A, 2B, respectively). Connexin-26 protein and gene expression were reduced from 4 weeks of diabetes. Data are shown as mean values ± SEM and gene expression data are relative to the housekeeping gene, HPRT. **P < 0.001, *P < 0.01, n > 3 for protein expression, n > 9 for gene expression.

To determine whether the 4-week decrease in connexin expression was due to a loss of astrocytes or to alterations in communication, connexin-26 immunoreactivity was colocalized with GFAP, another marker of astrocytes. Connexin-26 colocalized exclusively with GFAP in control and diabetic tissue, confirming its expression by astrocytes (Figs. 3A, 3C). Furthermore, after 6 weeks of diabetes there was a decrease in the tiling of astrocytes across the retina, as indicated by a loss of both GFAP and connexin-26 cell labeling (Figs. 3B, 3D). When this was quantified, there was a significant decrease in astrocyte number in both the central and peripheral retina (central −28%, peripheral −31%, P < 0.001, ANOVA; Fig. 3E). In addition to this cell loss, the remaining astrocytes had fewer processes per cell in the diabetic peripheral retina (−19%, P <
Although there was a mean decrease in process number in the central retina, this was not significant ($P > 0.05$, ANOVA; Fig. 3F). This suggests that by 4 weeks of diabetes there are significant changes in astrocyte communication and by 6 weeks there are fewer astrocytes, in particular in the peripheral retina.

**Inner Retinal Hypoxia Develops Early in Diabetes**

Altered astrocyte gap junction communication and cell loss are known to alter blood vessel function and lead to hypoxia. To determine whether the decrease in astrocyte number and connexin expression observed after 4 weeks of diabetes increased inner retinal hypoxia, the expression of HIF-1$\alpha$ was investigated. HIF-1$\alpha$ is a transcription factor known to be stabilized in hypoxic conditions and is an early marker of hypoxia. Using Western blot, faint HIF-1$\alpha$ staining was observed in the control and 2-week diabetic retinas, but there was no significant difference in the levels of HIF-1$\alpha$ expression. At 4 weeks, the diabetic samples showed increased immunostaining at the reported size (120 kDa; Fig. 4A). When this was quantified relative to GAPDH intensity, there was an increase in HIF-1$\alpha$ protein expression after 4 weeks of diabetes (11%, $P < 0.05$, two-tailed $t$-test; Fig. 4B).

To determine whether the development of hypoxia correlated with the inner retinal astrocyte alterations, vertical sections of control and diabetic retinas were labeled with pimonidazole, a marker of hypoxia. In the control rat retina, very little pimonidazole immunoreactivity was visible at either 4 or 6 weeks (Figs. 5A, 5D, respectively). In the diabetic retinas, increased labeling was observed in the ganglion cell layer (GCL) of the 4-week tissue and this became more evident at 6 weeks of diabetes, with Müller cells also exhibiting label-
ing (Figs. 5B, 5D, respectively). When the extent of pimonidazole labeling was quantified, the 4-week data showed a mean increase in labeling in the central and peripheral retinas, although this was not significantly different from control ($P > 0.05$, ANOVA; Fig 5C). In contrast, at 6 weeks immunoreactivity was significantly higher in the diabetic retinas in both central and peripheral regions (central 84%, $P < 0.05$ and peripheral 84%, $P < 0.01$, ANOVA; Fig. 5F). These data indicate that by 4 weeks of diabetes the retina begins to alter expression levels of factors important in regulating response to hypoxia (HIF-1$\alpha$) and that by 6 weeks of diabetes significant tissue hypoxia can be detected in the inner retina, in particular around the astrocyte/ganglion cell layer in both the central and peripheral retina.

**Müller Cell Gliosis Occurs Later in Diabetes**

Müller cell gliosis is a hallmark of retinal disease, and has been characterized in the diabetic retina. To determine the time course of Müller cell gliosis in relation to the early astrocyte change and hypoxia detailed earlier, the expression of the intermediate filament, GFAP, was assessed in vertical sections of retina after 2, 4, 6, and 10 weeks of diabetes. Müller cells of 4- and 6-week control retinas showed no labeling with GFAP, with expression restricted to astrocytes (Figs. 6A, 6C, respectively). In the case of the diabetic retinas, some Müller cell gliosis was evident after 4 weeks, although this became more evident by 6 weeks of diabetes (Figs. 6B, 6D, respectively). Müller cell gliosis was quantified by assessing the percentage of GS-immunoreactive Müller cell processes that expressed GFAP in the sublayer 1 of the IPL. At 6 weeks of diabetes the peripheral retinas showed extensive gliosis with 58% of Müller cells positive for GFAP (6 weeks, $P < 0.001$, ANOVA; Fig. 6F). Despite this increase in gliosis in the periphery at 6 weeks, Müller cell gliosis became signif-

*Figure 4.* Altered HIF-1$\alpha$ expression in the retina during early diabetes. Protein extracts from control and diabetic retinas at 2 and 4 weeks were separated and probed with antibodies to HIF1-$\alpha$ and GAPDH (A). The immunoreactivity was scanned and pixel intensity quantified relative to GAPDH (B). Whereas there is no change in protein expression after 2 weeks of diabetes, there is an increased expression of HIF-1$\alpha$ in diabetic retinas from 4 weeks of diabetes. Data are presented as mean ± SEM. *$P < 0.05$, $n = 3$.

*Figure 5.* Inner retinal hypoxia during early diabetes. Control (A, D) and diabetic (B, E) retinas were assessed for pimonidazole immunolabeling after 4 weeks (A, B) and 6 weeks (D, E) of diabetes. The extent of immunoreactivity was quantified in both central and peripheral retinas after 4 weeks (C) and 6 weeks (F) of diabetes. Although there is diffuse labeling with pimonidazole in the inner retinal layers at 4 weeks, extensive labeling can be detected in ganglion and Müller cells in both central and peripheral retinas at 6 weeks of diabetes. Data are presented as mean ± SEM. **$P < 0.01$, *$P < 0.05$, $n = 3$ for 4 week data, $n = 5$ for 6 week data. Scale bar: 50 $\mu$m. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.
icant in the central retinas only at 10 weeks of diabetes (73% of Müller cells positive for GFAP, $P < 0.001$, ANOVA; Fig 6E). These Müller cell changes occur at a later time point in diabetic progression than those astrocyte and hypoxia-related changes observed earlier in the study, indicating that the inner layers of retina may be more susceptible and first affected in diabetes-induced pathology.

**Inner Retinal Function Is Affected Early in Diabetes, whereas an Outer Retinal Deficit Occurs Later**

To determine whether the inner retinal changes observed in diabetes at 4 weeks (astrocytes, hypoxia) and the later Müller cell alterations (6–10 weeks) could be correlated with specific functional change, retinal function was assessed using the ERG. The rod and cone ERG and the scotopic threshold response (STR) were collected from control and diabetic rats at 4 and 6 weeks and representative waveforms from control and diabetic rats are shown after 6 weeks of diabetes (Figs. 7A–C). Individual components of the waveforms were extracted to assess outer and inner retinal function, including rod PIII (rod photoreceptor response), rod PII (rod-post photoreceptor response, primarily depolarizing bipolar cells), OP3 and nSTR (amacrine cell responses), pSTR (ganglion cell response), and cone PII (cone-post photoreceptor response, primarily depolarizing bipolar cells). Changes in these components in diabetes were expressed as a percentage of the average response of control animals at 4 (Fig. 8A) and 6 weeks of diabetes (Fig. 8B). At 4 weeks of diabetes there was a significant decrease in the pSTR amplitude in diabetes relative to control ($-34\%$, $P < 0.05$, two-tailed $t$-test; shaded bar, Fig. 8A) and the PIII and PII responses ($-28\%$ and $-38\%$, respectively, $P < 0.05$, ANOVA, Fig. 8A). There was no change in any of the other ERG components assessed at 4 weeks of diabetes. After 6 weeks of diabetes there were multiple neuronal effects, with the rod PIII, rod PII, OP3, and pSTR amplitudes all significantly decreased relative to controls (PIII, $-25\%$; PII, $-31\%$; OP3, $-56\%$; pSTR, $-55\%$; $P < 0.05$, two-tailed $t$-test; shaded bars, Fig. 8B). In contrast, there was no alteration in the nSTR or cone PII responses ($P > 0.05$).

Because the ERG is a serial waveform, a reduction in the photoreceptor response may result in at least a similar magnitude loss of the b-wave, OPs, and pSTR. At the 6-week time
whether astrocytes may play an early role in the subsequent neuronal and glial cell change during diabetes to determine alterations, in addition to its clinical presentation of overt diabetic retinopathy is characterized by neuronal and glial cell dysfunction at 6 weeks of diabetes.

DISCUSSION

Diabetic retinopathy is characterized by neuronal and glial cell alterations, in addition to its clinical presentation of overt vascular pathology. This study investigated the time course of the neuronal and glial cell change during diabetes to determine whether astrocytes may play an early role in the subsequent outer retinal dysfunction at 6 weeks of diabetes.

Astrocyte Change, Hypoxia, and Altered Ganglion Cell Function in Early Diabetes

Retinal astrocytes had reduced connexin-26 labeling in the peripheral retina from 4 weeks of diabetes, whereas retinal connexin-26 and -43 gene expression profiles were also decreased at this early time point. These data support a previous report of decreased connexin-43 in the diabetic mouse retina and other studies carried out in different tissues showing reduced connexin expression in diabetes. Because connexins are integral to gap junction formation and cell-cell communication, the decrease in retinal connexin expression early in diabetes may reflect a reduction in gap junction formation and a subsequent alteration in astrocyte communication. A similar disruption of gap junctions has been reported in the retinal vasculature during diabetes. Furthermore, reduced astrocyte communication has been detected in brain slices from diabetic mice. Although both connexin-26 and -43 gene data show decreases at 4 weeks of diabetes, connexin-43 gene expression was also upregulated at 2 weeks, whereas connexin-26 was unaffected. This increase in connexin-43 expression was surprising since most reports describe a hyperglycemic-induced downregulation of connexin-43 expression. Whether this increase is indicative of early dysregulation in response to high glucose or a transient STZ-related effect is unclear and requires further investigation.

Although the decrease in connexin expression may reflect altered gap junction expression, it may also be due to the reduction in peripheral astrocyte number, which was detected from 6 weeks of diabetes. This loss of astrocytes is supported by previous studies in the rat retina after 4 weeks of STZ-induced diabetes, as well as in other tissues such as the cerebellum and hypothalamus. Despite this, one study has reported increased retinal astrocytes in the GFAP-GFP diabetic mouse. Although the current data do not allow the mechanism of this cell loss to be determined, in vitro studies have shown that hyperglycemia results in reduced astrocyte viability possibly via altered gap junction signaling. However, due to the importance of astrocytes in blood vessel growth and regulation, the loss of astrocytes early in diabetes would likely result in associated blood vessel change. Furthermore, the loss of the astrocyte template has been suggested to lead to blood vessel pathology in diseases such as retinopathy of prematurity (ROP). Although further work is required to determine whether the diabetes-induced astrocyte loss directly affects retinal blood vessels, current data do provide some evidence of al-

Figure 7. Altered retinal function during diabetes. Representative ERG waveforms derived from rod mediated pathways (A), cone mediated pathways (B), and the positive scotopic threshold response (pSTR; C) of control and diabetic retinas after 6 weeks of diabetes. Both the rod and cone-mediated waveforms and pSTR are reduced in amplitude at this stage of diabetes.

Figure 8. Early inner retinal and later outer retinal dysfunction in diabetes. The amplitude of the rod PII (a-wave), rod PII (b-wave), OP3, pSTR, nSTR, and cone PII (cone b-wave) were measured after 4 (A) and 6 (B) weeks. Data are expressed relative to age-matched control animals. After 4 weeks only the pSTR is decreased in amplitude, whereas after 6 weeks diabetes, all components except the nSTR and cone PII are decreased relative to controls (shaded bars, \( P < 0.05 \)). In addition, the decreases in OP3 and pSTR were found to be significantly greater than that produced by the photoreceptor response. Data are presented as mean ± SEM relative to control data and shaded bars identify statistically significant differences (\( t \)-test, \( P < 0.05 \)). * \( P < 0.05 \), \( n = 10 \).
tered retinal vasculature. After 4 weeks of diabetes, when astrocyte change was occurring, increased retinal HIF-1α expression and increased tissue hypoxia (pimonidazole labeling) in the ganglion cell layer were also observed. Such early increases in retinal hypoxia have been attributed to rapid (2–4 weeks) decreases in retinal blood flow and capillary density after diabetes.54,55 These hypoxic changes are consistent with other studies that show increased pimonidazole labeling in the mouse retina after 5 months of diabetes,56 decreased oxygen profiles in the diabetic cat retina57 and HIF-1α increases from 1 to 12 weeks of diabetes.58,59 Because whole retinas were taken for the HIF-1α analysis, it is unclear what cell type(s) are producing this transcription factor, although previous reports have suggested HIF-1α is produced by retinal neurons rather than glia.53 Furthermore, Poulaki et al.60 identified increased HIF-1α expression in the ganglion cell layer of diabetic rats. A ganglion cell source of the increased HIF-1α correlates not only with the location of the pimonidazole labeling, but also with the pSTR decrease which is known to reflect ganglion cell function.61 This early decrease in ganglion cell function was the only neuronal deficit found at 4 weeks and is consistent with previous reports showing later deficits.62 This early neuronal dysfunction may result from cell loss, since previous work has shown increased ganglion cell apoptosis as early as 4 weeks of diabetes.63,64

Müller Cell Gliosis and Outer Retinal Functional Change in Later Stages of Diabetes

In addition to the earlier changes in the inner retina, changes in Müller cell gliosis and more widespread neuronal deficits were observed at later time points in the progression of diabetes. Müller cell gliosis was observed from 6 weeks of diabetes, as indicated by an increase in expression of the intermediate filament GFAP, a known marker of gliosis. The gliosis was initially observed in the periphery, with central regions involved as of 10 weeks of diabetes. These observations are in line with numerous other studies that have shown Müller cell gliosis in animal models of diabetes and humans with diabetic retinopathy.20,26,65 Although gliosis is generally reported relatively later in animal models of diabetes, there is evidence of earlier Müller cell changes,20 with enhanced glutamate uptake as early as 1 week after induction of diabetes.22 Despite this, it is unclear whether other Müller cell alterations occur early since most other documented changes have been assessed later (>6 weeks) in diabetes.25,66

After 6 weeks of diabetes more extensive functional deficits were also observed that included a reduction in the rod photoreceptor response (a-wave), rod b-wave (PII), and OP3 response. Similar decreases in photoreceptor and b-wave responses have been reported previously, whereas changes in the OPs have been consistently reported in the literature.10–13,67 Changes in neurotransmitter processing and cell viability have been proposed to result in these decreases in neuronal function during diabetes (Phipps JA, et al. IOVS 2004;45:E-Abstract 3233).11,16,68 In addition to general neuronal dysfunction, our data also showed that the amplitudes of pSTR and OP3 were reduced to a greater extent than that expected due to the loss of rod a-wave. This suggests a continuation of the ganglion cell deficit identified at 4 weeks and the subsequent development of an amacrine cell dysfunction, highlighting continual inner retinal dysfunction. Previous studies have shown that the inner retina is particularly vulnerable to the effects of diabetes.10,14,69

Increased Sensitivity of the Peripheral Retina to Diabetes

An intriguing finding from this study was that changes in glia, and the development of hypoxia were more pronounced in the peripheral retina compared with more central regions, suggesting a vulnerability in the peripheral retina to diabetes. Interestingly, ROP, which also presents with extensive vascular pathologic changes, also shows an increased vulnerability in peripheral retina, with loss of astrocytes, neuronal dysfunction, and vascular pathology showing a peripheral bias.70 This peripheral sensitivity may result from underlying morphologic differences such as reduced astrocyte tiling and decreased peripheral vasculature,20,71 although more work is required to confirm this effect and identify the mechanisms involved in this apparent preferential development of pathology.

Conclusions

The results of this study indicate that astrocyte change occurs early in the progression of diabetes and is accompanied by inner retinal hypoxia and ganglion cell dysfunction. Müller cell gliosis and more extensive changes in neuronal function occur after the astrocyte alterations. Because astrocytes are known to modulate neuronal and vascular function, these alterations in astrocytes early in diabetes may be a significant factor in the development of tissue hypoxia and ganglion cell dysfunction.

References

Early Astrocyte Change in the Retina during Diabetes


