Retinal Gene Expression and Visually Evoked Behavior in Diabetic Long Evans Rats

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PURPOSE. Patients with diabetic retinopathy may experience severe vision loss due to macular edema and neovascularization secondary to vascular abnormalities. However, before these abnormalities become apparent, there are functional deficits in contrast sensitivity, color perception, and dark adaptation. The goals of this study are to evaluate early changes (up to 3 months) in retinal gene expression, selected visual cycle proteins, and optokinetic tracking (OKT) in streptozotocin (STZ)-induced diabetic rats.

METHODS. Retinal gene expression in diabetic Long Evans rats was measured by whole genome microarray 7 days, 4 weeks, and 3 months after the onset of hyperglycemia. Select gene and protein changes were probed by polymerase chain reaction (PCR) and immunohistochemistry, respectively, and OKT thresholds were measured using a virtual optokinetics system.

RESULTS. Microarray analysis showed that the most consistently affected molecular and cellular functions were cell-to-cell signaling and interaction, cell death, cellular growth and proliferation, molecular transport, and cellular movement. Further analysis revealed reduced expression of several genes encoding visual cycle proteins including lecithin/retinol acyltransferase (LRAT), retinal pigment epithelium (RPE)-specific protein 65 kDa (RPE65), and RPE retinal G protein–coupled receptor (RGR). These molecular changes occurred simultaneously with a decrease in OKT thresholds by 4 weeks of diabetes. Immunohistochemistry revealed a decrease in RPE65 in the RPE layer of diabetic rats after 3 months of hyperglycemia.

CONCLUSIONS. The data presented here are further evidence that inner retinal cells are affected by hyperglycemia simultaneously with blood retinal barrier breakdown, suggesting that glial and neuronal dysfunction may underlie some of the early visual deficits in persons with diabetes.

Diabetic retinopathy is a major sight-threatening disease in working age people in the developed world, and its severity is clinically classified by vascular changes that can lead to blindness. There is ample evidence, however, that early functional abnormalities are caused by nonvascular effects of abnormally high glucose levels. Diabetic patients without vascular changes in the retina experience abnormal color vision,2 dark adaptation,3 and contrast sensitivity.4 The oscillatory potential (OP) of the electroretinogram (ERG) is anomalous in diabetic patients4–8 and rodents.9–15 which is consistent with altered function of supporting cells in the retina, such as impaired glutamate metabolism14–16 and increased glial fibrillary acidic protein (GFAP) expression in Müller cells.13,15,16,19 Other glial cells, such as astrocytes and microglia, are also affected.17 There is evidence of inflammatory changes in the eyes of diabetic patients20–24 and animals25–27 and cell death of various retinal neurons.28–35

In addition to inner retinal pathology, photoreceptor viability is impacted by hyperglycemia.3,31,34 Before photoreceptor cell death, the visual cycle is altered in diabetic rodents as evidenced by impaired rhodopsin regeneration32 and reduced 11-cis-retinal concentration in the retina.35 The visual cycle is initiated in photoreceptors but also involves other cell types intimately associated with photoreceptors, such as retinal pigment epithelium (RPE) and Müller cells. On light stimulation, 11-cis-retinal is converted to all-trans-retinal in the first step of phototransduction (see Ref. 37 for review), followed by recycling of all-trans-retinal to 11-cis-retinol by the visual cycle which takes place outside the photoreceptor.37,38 It involves several binding proteins as well as enzymes such as lecithin/retinol acyltransferase (LRAT),39 RPE65,40 11-cis-retinol dehydrogenase (RDH5),41 and RPE retinal G protein–coupled receptor (RGR).42 Patients with mutations in LRAT, RPE65, and RGR exhibit severe early-onset retinal dystrophies.37,38 Rod opsin regeneration relies on visual cycle reactions occurring in the RPE, whereas an alternative visual cycle occurring in Müller cells is associated with cone opsin regeneration (see Refs. 43, 44 for reviews).

One previous study using gene microarray in the diabetic rat model assessed 5147 genes and expressed sequence tags45 as opposed to current whole genome technology, which probes more than 41,000 rat genes and transcripts. Furthermore the analysis was limited to individual genes grouped into broad functional classes45 rather than specific pathways or biological networks. Others explored changes in a single cell type, specifically Müller cells.46 Finally, Brucklacher et al.47 performed a whole genome microarray with pathway analysis in the retinas of diabetic Sprague-Dawley rats that show inflammatory changes in the retina not detected in other strains.27 Evaluating a rat strain that lacks a potentially overshadowing inflammatory response may reveal more subtle, but equally important, changes in metabolic, signaling, and other pathways. Changes other than inflammation are likely present and partially responsible for functional deficits obvious in changes in ERG,10,46 defective γ-aminobutyric acid signaling,36,47 and abnormal gene expression reported in earlier microarrays.25,45

The data presented here show evidence of abnormal cell metabolism, cellular interaction and proliferation, and decreased expression of retinal genes involved in the visual cycle...
in diabetic Long Evans rats. Furthermore, decreased visual function is observed in these animals. Both changes appear early during the disease before significant cell death in the retina or photoreceptor damage occurs, suggesting that hyperglycemia in the retina affects the vision-supporting function of secondary neurons or glial cells.

**Materials and Methods**

**Animals and Timeline of Experiments**

Male Long Evans rats (Charles River Laboratories, Wilmington, MA), each weighing ~ 250 g, were used in all experiments. The timeline is represented in Figure 1. For microarray experiments, rats were killed 7 days, 4 weeks, and 3 months after the induction of diabetes. Each microarray experiment was performed using one retina from each of three rats as a pooled sample on one array chip (n = 3 rats). Each data point consisted of three independent experiments (n = 3 chips; n = 9 rats per group). For real-time PCR, rats were killed after 1 month (retina and RPE) or 5 months (retina only), and samples from one retina of each of three rats were analyzed separately (retina) or pooled (RPE) for diabetic and control groups. Data were from three independent experiments (n = 9 animals per group). Determination of the spatial frequency threshold was performed on four diabetic and five control animals each in two independent experiments (n = 8 diabetic and n = 10 control rats). Histopathology and immunohistochemistry were performed on four diabetic and three control animals. All data, including microarray, real-time PCR in retina, real-time PCR in RPE, spatial frequency measurements, and histopathology, were collected in individual groups of animals. All animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Induction of Diabetes**

Rats were rendered diabetic by intraperitoneal injection of 65 mg/kg body weight STZ in 0.09 M citrate buffer, pH 4.8 (both Sigma, St Louis, MO). Age-matched control rats were injected with buffer only. Diabetes was confirmed and monitored regularly using a glucose meter (Ascensia; Bayer, Tarrytown, NY). Rats with blood glucose levels above 250 mg/dL after 48 hours were deemed diabetic. Up to 49% of rats remained normoglycemic after STZ treatment and were either injected with a second dose of STZ27,48–50 or used as nonhyperglycemic STZ controls. There was no significant difference in weight or blood glucose levels 6 days after the initial treatment between rats injected once and those receiving a second dose. In addition, real-time PCR and visual acuity measurements were not significantly different for rats injected once compared with those injected twice. Rats injected twice were not used for experiments starting less than 2 weeks after the first STZ injection. Average (± SD) blood glucose levels in diabetic rats were 475 (± 106) and 83 (± 11) for controls over the course of the experiment. The value used to replace glucose levels that exceeded the upper limit of detection was 600 mg/dL. No insulin was supplied at any time.

![Figure 1. Timing of STZ injection and data collection.](image_url)

**Whole Genome Microarray**

One retina from each of three rats was removed and immediately transferred to tissue storage reagent (RNAlater; Ambion, Austin, TX), and the pooled sample was incubated overnight at 4°C. Excess reagent (RNAlater; Ambion) was then removed, and the samples were stored at –70°C. RNA isolation and microarray was performed by Genus Biosystems (Northbrook, IL). Briefly, after RNA isolation (RiboPure; Ambion), RNA concentration was measured by spectrophotometry, and quality was assessed using a bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA was labeled and amplified, and resulting Cy3-cRNA was applied to a one-color rat whole genome array (Agilent Technologies) and scanned on a microarray scanner (G2565; Agilent Technologies).

**Microarray Analysis**

Microarray data were analyzed using gene expression software (GeneSpring X; Agilent Technologies). Data were log2 transformed, quantile normalized, and baseline transformed to the median of all samples. Probes with <50% of samples in diabetic or control samples present or marginal were excluded. After calculation of the average of the data from three independent experiments, P value and fold changes were established, and Student’s t-test with a cutoff of <0.05 was used to determine significance. All genes with fold changes >1.1 were included in the analysis.

Gene ontology analysis was performed with gene expression software (GeneSpring X), and biological functions were examined using analysis software (Ingenuity Pathway Analysis; Ingenuity Systems, Redwood City, CA). Functional groups with 10 or fewer genes were excluded.

The data discussed in this article have been deposited in National Center for Biotechnology Information Gene Expression Omnibus and are accessible through GEO Series accession number GSE28831 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc =GSE28831).

**RNA Extraction and Real-Time PCR**

Retinas frozen in stabilization reagent (RNAlater; Qiagen, Valencia, CA) were transferred to 1 ml lysis reagent (Qiazol; Qiagen) and were homogenized (Tissue-Tearor; Biospec, Bartlesville, OK). RPE cells were isolated, directly transferred into lysis reagent, and homogenized by five passes through a 25-gauge needle. RNA extraction was performed identically for both tissues using a purification kit (RNeasy Mini Kit; Qiagen) in accordance with the manufacturer’s instructions. RPE cell mRNA was pooled by successively adding ethanol-precipitated RNA from three eyes over the same column (RNeasy; Qiagen). Retinal RNA quantity was determined by spectrophotometry, and quality was assessed (RNA 6000 Nano Kit; Agilent Technologies, Santa Clara, CA), and then RPE RNA quantity and quality were measured (Nanodrop 2000; Thermo Scientific; Waltham, MA). Reverse transcription of 1 µg RNA using 25 mM MgCl2, 10 mM dNTP mixture, 25 U RNasin, 15 U AMV reverse transcriptase, and reverse transcription buffer (all Promega, Madison, WI) in 20 µL was carried out for 20 minutes at 42°C.
followed by denaturation for 5 minutes at 95°C and 5 minutes at 4°C. Nucleic-acid-free water was added to a final volume of 200 μL.

Real-time PCR was performed on individual retinas using a real-time PCR system (7900HT; Applied Biosystems, Foster City, CA) with commercially available TaqMan probe and primer sets for Acetb (Rn00667869_m1), Gfap (Rn00666605), Lrat (Rn00574091), Opn1m (Rn00585560_m1), Rgr (Rn01754438_m1), and Rpe65 (Rn00585655_m1; all Applied Biosystems). All primers span exon junctions and therefore do not recognize genomic DNA. Gene expression was normalized and converted to a linearized value using the following formula: Unit = 1.8⁷(Ct Actb − Ct gene) × 100,000. Statistical analysis was performed using the Student’s t-test, with significance determined by P < 0.05.

Histopathology and Immunohistochemistry

After 3 months of diabetes, rats were killed and immersion fixed in 10% neutral buffered formalin (NBF) for 24 hours, followed by processing and sectioning using routine histologic techniques. Sections were then stained using standard immunohistochemical procedures. Briefly, sections from pigmented eyes were bleached using 0.25% potassium permanganate for 10 minutes, followed by 0.1% oxalic acid (both Mallinckrodt, Hazelwood, MO) for 10 minutes.

After inhibition of endogenous peroxidase, blocking for endogenous avidin and biotin, and blocking for endogenous protein using serum, sections were incubated with the following polyclonal antibodies: goat anti-RPE65 (ab77381 at 10 μg/mL; Abcam, Cambridge, MA) on the NBF-fixed eye. After overnight incubation at 4°C, sections were reacted with the appropriate biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA) for 30 minutes, followed by horseradish peroxidase-conjugated streptavidin (ABC kit; Vector Laboratories for 30 minutes. Immunoreaction sites were visualized using substrate (NovoRed; Vector Laboratories). Sections were then counterstained with methylene green, washed, dehydrated, and coverslipped.

Visually Evoked Behavior

Visual function in Long Evans rats was tested measuring a visually evoked behavior (OptoMotry system; CerebralMechanics, Lethbridge, AB, Canada), as described in Prusky et al. Briefly, a vertical sine wave grating moving at a speed of 12°/s appeared on computer screens at 100% contrast surrounding a rat sitting unrestrained on a platform in the center. Starting spatial frequency was 0.042 c/d, with the widths of the gridlines gradually decreasing for increasing spatial frequency. Reflexive head movements (tracking) were noted by the experimenter, with occasional confirmation from a second examiner. The rat was monitored by video feedback that allowed centering of a cross hair at the rat eyes. This information was used for computerized adjustment of spatial frequency in relation to viewing distance. A staircase method was used by which two correct answers in a row or one wrong answer resulted in a change of spatial frequency that was increased or decreased by 50% until the step size was smaller than the hardware resolution (0.003 c/d). The spatial frequency threshold was determined after seven consistent assessments (four yes, three no) by the experimenter, and testing was performed under photopic conditions (142 cd/m²). The direction of motion was random, and one staircase was performed for each direction. Student’s t-test was used, and P < 0.05 was considered significant. Rats were screened for cataracts after 6 weeks of diabetes following dilation with a handheld ophthalmoscope.

RESULTS

Gene Expression in the Retina of Diabetic Long Evans Rats

Gene expression changes in approximately 40,000 genes and transcripts were measured in the retinas of diabetic rats using a whole genome microarray. Compared with controls, 1781 transcripts were significantly regulated 1 week, 1 month, and 3 months after the induction of diabetes. Ontological analysis showed that of the changed transcripts, 369 were associated with the gene ontology biological processes category. The subcategories most affected when measured by number of genes were cellular process, biological regulation, and metabolic process (Fig. 2).

Time-course analysis revealed that most genes are uniquely upregulated at a single time point (628 transcripts at day 7, 718 transcripts after 4 weeks, and 588 transcripts after 3 months) with little overlap. Fewer than 20 transcripts are shared at 1 week and 4 weeks or at 4 weeks and 3 months, and only two genes are differentially expressed over the whole course of the experiment (Fig. 3).

After 7 days, the five molecular and cellular function pathways most significantly affected by hyperglycemia (P < 0.01) were small molecule biochemistry, cellular movement, cell-to-cell signaling and interaction, cellular assembly and organization, and cell death. Although the number of transcripts significantly changed by diabetes was similar between 1 and 4 weeks, the number of pathways affected was more than doubled from 7 to 18 weeks in the latter group. The five most affected functions were cell-to-cell signaling and interaction, cellular growth and proliferation, free radical scavenging, molecular transport, and cellular movement. After 3 months of diabetes, the number of genes and transcripts differentially expressed was reduced, as was the number of functions affected. The five functions most affected later were cell cycle, cell death, cellular growth and proliferation, cell signaling, and molecular transport. This analysis showed that although there is little overlap in genes differentially expressed, some of the same molecular functions are affected at different times in experimental diabetes.

Genes Significantly Changed at More Than One Time Point

To identify long-term changes in the diabetic retina, focus was shifted to transcripts upregulated or downregulated at more
than one time point. Changes also apparent in rats that received STZ injections but did not become hyperglycemic were excluded from this analysis. Two genes—solute carrier family 46 (folic acid transporter) member 1 (Slc46a1) and RNase, RNase A family 4 (Rnase4)—were upregulated at all three time points. Of 16 genes and transcripts differentially expressed both 7 days and 4 weeks (early) after the induction of diabetes, six encoded known genes (Table 1). Four of the genes were upregulated at both time points, glutathione S-transferase alpha 3 (Gsta3), heat shock 22-kDa protein 8 (Hspb8), inhibitor of DNA binding 3, dominant negative helix-loop-helix protein (Id3), and radical S-adenosyl methionine domain containing 2 (Rsad2). The remaining two genes, gypsy retrotransposon integrase (Gin1) and integrator complex subunit 10 (Ints10), were upregulated early and downregulated at the later time point.

Of 19 transcripts differentially expressed late (4 weeks and 3 months; Table 2), 11 encoded known genes and only one, insulin-like growth factor binding protein 3 (Igfbp3), was significantly upregulated (1.4- and 2.3-fold respectively) at both time points. Four genes were moderately (1.1–1.6 fold) downregulated at both time points, glutathione S-transferase alpha 3 (Gsta3), heat shock 22-kDa protein 8 (Hspb8), inhibitor of DNA binding 3, dominant negative helix-loop-helix protein (Id3), and radical S-adenosyl methionine domain containing 2 (Rsad2). The remaining two genes, gypsy retrotransposon integrase (Gin1) and integrator complex subunit 10 (Ints10), were upregulated early and downregulated at the later time point.

The remaining three genes that were significantly changed (−2.0 to −5.2, Fig. 3A) belong to a highly specialized functional group. Those genes, Lrat, Rgr, and Rpe65 are all part of the visual cycle, which recycles all-trans-retinal from bleached photoreceptors to 11-cis-retinal required for phototransduction.

TABLE 1. Genes Significantly Changed after 7 Days and 1 Month of Diabetes

| Gene Symbol | Entrez Gene Name | Fold Change
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Gin1</td>
<td>Gypsy retrotransposon integrase 1</td>
<td>1.3 1.2</td>
</tr>
<tr>
<td>Gsta3</td>
<td>Glutathione S-transferase alpha 3</td>
<td>1.2 1.2</td>
</tr>
<tr>
<td>Hspb8</td>
<td>Heat shock 22kDa protein 8</td>
<td>1.3 1.4</td>
</tr>
<tr>
<td>Id3</td>
<td>Inhibitor of DNA binding 3</td>
<td>1.3 1.6</td>
</tr>
<tr>
<td>Ints10</td>
<td>Integrator complex subunit 10</td>
<td>1.2 −1.1</td>
</tr>
<tr>
<td>Rsad2</td>
<td>Radical S-adenosyl methionine domain containing 2</td>
<td>1.9 1.6</td>
</tr>
</tbody>
</table>

Genes significantly changed after both 1 week and 1 month of diabetes. Fold change expression of genes in retinas of diabetic mice compared with control that is common to both early time points. Statistical significance was determined using Student’s t-test.
increased at this time point (Fig. 6A), nor was there a change in mRNA levels of retinol-binding proteins. Real-time PCR confirmed significant reductions of \textit{Lrat} and \textit{Rgr} mRNA levels, but not those of \textit{Rpe65} (Fig. 6B).

**RPE65 Protein Localization in the Diabetic and Normal Rat Eye**

To determine the protein levels of differentially expressed genes, immunohistochemistry was performed 3 months after the induction of diabetes. This method did not show any changes in the retina, but RPE65 protein was reduced in the RPE layer in 3 of 4 diabetic rats (Fig. 7). No specific LRAT or RGR staining could be demonstrated in the rat eye using the fixatives available in this study.

**Effect of Diabetes on Visual Function**

There is little evidence of photoreceptor dysfunction by electrophysiological measurements after 4 weeks of diabetes.\textsuperscript{9,11–13} Opsin mRNA levels are unchanged at this point, further suggesting that photoreceptors are not affected until later during the disease. However, a potential defect in vitamin A recycling may lead to decreased visual function before cellular changes. To test this hypothesis, the spatial frequency threshold was measured. Baseline levels were determined 1 week before injection with STZ and were similar to those reported previously in Long Evans rats.\textsuperscript{52} Subsequently, animals were tested after 2, 3, and 4 weeks of diabetes. Although sustained hyperglycemia showed no effect after 2 and 3 weeks, spatial frequency thresholds were significantly reduced 1 month after the induction of diabetes (Fig. 8). To exclude the possibility that cataracts were responsible for the diminished vision, each rat was examined for lens opacity 6 weeks after STZ injection. Although some rats exhibited signs of mild cataract development, there was no significant difference in visual acuity between those rats that did develop cataracts and those that did not (data not shown).

**DISCUSSION**

In recent years, evidence has accumulated that diabetic retinopathy is not simply a microvascular disease, it also affects neurons and glial cells (see Ref. 56 for review). Changes in GFAP protein expression\textsuperscript{13,15,17,19,57} and other changes in astrocytes and Müller cells\textsuperscript{14,16,25} suggest functional impairment of glial cells; retinal neurons including amacrine\textsuperscript{29} and ganglion cells\textsuperscript{28,30,33} as well as photoreceptors\textsuperscript{31} die by apoptosis.

### Table 2. Genes Changed after 4 Weeks and 3 Months of Diabetes

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Entrez Gene Name</th>
<th>Fold Change 1 Month</th>
<th>Fold Change 3 Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyp3a62</td>
<td>Cytochrome P450, subfamily 3A, polypeptide 62</td>
<td>2.0</td>
<td>2.4</td>
</tr>
<tr>
<td>Golgi3</td>
<td>Golgi autoantigen, golgin subfamily a, 5</td>
<td>–1.2</td>
<td>–1.1</td>
</tr>
<tr>
<td>Igf4r3</td>
<td>Insulin-like growth factor binding protein 3</td>
<td>1.4</td>
<td>2.3</td>
</tr>
<tr>
<td>Klk7</td>
<td>Kallikrein-related peptidase 7</td>
<td>–3.9</td>
<td>–5.2</td>
</tr>
<tr>
<td>Lalba</td>
<td>Lactalbumin, alpha-</td>
<td>–3.2</td>
<td>–3.6</td>
</tr>
<tr>
<td>Lrat</td>
<td>Lecithin retinol acyltransferase</td>
<td>–2.2</td>
<td>–3.2</td>
</tr>
<tr>
<td>Pop4</td>
<td>Processing of precursor 4, ribonuclease P/ MR subunit</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Rgr</td>
<td>Retinal G protein-coupled receptor</td>
<td>–2.1</td>
<td>–2.0</td>
</tr>
<tr>
<td>Rpe65</td>
<td>Retinal pigment epithelium-specific protein 65 kDa</td>
<td>2.1</td>
<td>2.0</td>
</tr>
<tr>
<td>Skiv2l2</td>
<td>Superkiller viralicidic activity 2-like 2</td>
<td>–1.3</td>
<td>–1.2</td>
</tr>
<tr>
<td>Tmem27</td>
<td>Transmembrane protein 27</td>
<td>–1.6</td>
<td>–1.5</td>
</tr>
</tbody>
</table>

**FIGURE 4.** Expression of visual cycle, \textit{Gfap}, and \textit{Opn1mw} genes in the retina after 1 month of diabetes. Whole genome microarray was performed, and analysis revealed decreased expression of \textit{Lrat}, \textit{Rgr}, and \textit{Rpe65} but not of \textit{Gfap} mRNA in the retinas of diabetic compared with normal rats (A). Real-time PCR using probes show significantly reduced expression of \textit{Rgr} and \textit{Rpe65} but not of \textit{Lrat, Gfap}, or \textit{Opn1mw} mRNA (B). Data are averages of three independent experiments with three rats per group (n = 9). Error bars denote SD. *P < 0.05 compared with control using Student’s t-test.
Whole genome microarray analysis showed global changes in cellular proliferation, movement, signaling, interaction, and cell death but also free radical scavenging and molecular transport. Examination of individual genes revealed reduced expression of visual cycle enzymes before significant photoreceptor damage.31 This occurred simultaneously with decreased visual function in diabetic Long Evans rats.

Changes in the diabetic retina were explored by gene expression analysis after 7 days, 4 weeks, and 3 months of hyperglycemia in Long Evans rats. Grouping changes by gene ontology annotation showed that a large proportion of transcripts was related to cellular process, biological regulation, or metabolic process. Of the 1781 transcripts significantly changed in diabetic rats, 628 were found to be unique after 7 days, 718 after 4 weeks, and 388 after 3 months. Although most genes were differentially regulated between diabetic and normal retina only at a single time point, functionally there was overlap in the molecular and cellular pathways most significantly affected. Two pathways—cellular movement and cell-to-cell signaling and interaction—were significantly changed after both 7 days and 4 weeks, and two—cellular growth and proliferation and molecular transport—were affected after 1 month and 3 months of diabetes. Of note, cell death was affected after 1 week and 3 months, but not after 4 weeks. Previously published microarray gene ontology data in Sprague-Dawley rats showed predominantly inflammatory changes,26 which differed from observations in Long Evans rats.45 This is in agreement with recent data in diabetic rats that showed inflammatory protein expression only in Sprague-Dawley but not Long Evans or Brown Norway retina.27 The current Long Evans study includes analysis of the whole rat genome, as opposed to a limited array of approximately 5000 genes in an earlier study in this rat strain,47 as well as biological function and pathway analysis using a statistical weighted approach not available previously.

To identify effects outside a narrow time window, attention was focused on genes that were changed on more than one time point. Only two genes were found to be differentially expressed at all three time points, Rnase4 which has no defined function and Slc46a1, a high-affinity folate transporter.58 Slc46a1 transporter deficiency causes malabsorption of folate across intestine and into the brain, resulting in several neurologic symptoms including seizures, delayed development, and generalized electroencephalogram slowing.59 This transporter is widely expressed in the neurosensory retina, including Müller and ganglion cells as well as inner segments of photoreceptors.60 Increased transcription of this receptor could be a protective mechanism in damaged retinal neurons.

Four of the six genes differentially expressed after both 1 and 4 weeks of diabetes function to protect cells from various stresses. Id3 has been shown to be upregulated by high glucose and regulates gene transcription.61 Rsad2 inhibits viral replication,62 probably affecting gene transcription, and is upregulated by lipopolysaccharide.63 Gin1 has no described function, whereas Ints10 encodes a component of the spliceosome that is involved in pre-mRNA processing.64 The protein product of Hspb8 is expressed in a number of tissues including the brain and eye65 and is inducible by stress.66 It acts as a chaperone66 by inhibiting translation and clearing aggregated proteins67 present in diabetes.68 Finally, the protein encoded by Gsta3 protects cells from oxidative stress,69 a proposed contributor to retinal damage during diabetes.70 Upregulation of

**Figure 6.** Expression of visual cycle genes and Gfap after 3 months of diabetes. Analysis of a whole genome array shows continued decreases in Lrat, Rgr, and Rpe65 but not in Gfap mRNA expression in the retinas of diabetic rats (A). Reduced Lrat and Rgr expression is confirmed by real-time PCR, but Rpe65 or Gfap are not significantly lower in diabetic rats (B). Data are the average of three independent experiments with three rats per group (n = 9). Error bars denote SD. *P < 0.05 compared with control using Student's t-test.
mRNA may compensate for its decreased activity in the rat retina observed after 6 weeks of diabetes.71

Three of the 11 genes significantly changed after 4 weeks and 3 months of diabetes are not obviously linked to hyperglycemia. Tmem27 encodes an amino acid transporter,72 and Skiv2l2 encodes a predicted DEAD-box RNA helicase.73 Cyp3A62 metabolizes drugs as well as endogenous chemicals.74

Protein products of several late-phase genes, including Golga3, Lalba, Igfbp3, Pop4, and Klk7, are related to known pathology in the diabetic retina. Golga3,75 Lalba,76 and Igfbp377 control apoptosis. Pop4, which encodes a subunit of mitochondrial Rnases,78 is downregulated, which may be an early sign of the mitochondrial dysfunction observed in diabetic retinas.79 Downregulation of Klk7, a fibronectin-specific serine protease,80 may contribute to the increase in fibronectin in diabetic retinas.81

Of interest, significant decreases in retinal expression of Rgr, Rpe65, and Lrat were detected by whole genome microarray after 4 weeks and 3 months. The protein products of these three genes are involved in the visual cycle. No significant effect on other members of the visual cycle could be detected.

Real-time PCR confirmed changes in Rgr and Rpe65 after 1 month and in Lrat and Rgr expression after 3 months. Failure to detect significant changes by qPCR may be attributed to relatively low mRNA copy number of genes encoding visual cycle enzymes in the neural retina. This becomes obvious when compared to opsin expression levels. Detection of low expression levels is not surprising as rodent retina is rod rich, and it is well established that the rod visual cycle takes place almost exclusively in the RPE (see Refs. 57, 43 for reviews), but regeneration of cone opsin can take place in the isolated neural retina.43,82–84 Earlier results were derived from cone-rich species, such as chicken82,84 and frog,85 but recent evidence has been presented that an alternative cone visual cycle also exists in rodent as well as human retinas.86 However, despite Lrat mRNA detection in the retina in this model, LRAT protein expression has been demonstrated only in RPE, not retina.86 Coupled with the low gene expression levels encoding the three visual cycle enzymes, this raised the possibility that changes detected in the retina were due to contamination with RPE tissue. To exclude this explanation of the findings, mRNA of Rgr, Rpe65, and Lrat were measured in RPE of diabetic and control rats. There was no difference in expression levels in RPE from diabetic rats compared with control tissues, demonstrating that RPE is not the source of these gene changes. Although contamination with RPE tissue cannot be completely excluded, the data suggest that low expression levels are more likely due to the scarcity of cones and the associated cone visual cycle in the rod-rich rat retina. Further studies are needed to explore the effect of hyperglycemia on the cone visual cycle.

Müller cells are essential in cone opsin regeneration and convert all-trans-retinol to 11-cis-retinol through isomerase activity,82 and also contain retinoid-binding proteins, which shuttle retinoids between cells.77,86 In addition, RGR protein is present in RPE and Müller cells,12,90 whereas RPE65 mRNA and protein are expressed in cone photoreceptors,90 explaining the presence of RNA encoding these visual cycle proteins in our RPE-free preparation of neural retina. Conversely, LRAT protein has not been detected in the retina,90 and inhibiting LRAT activity in the retina does not block the cone visual cycle,91 suggesting that its role in this alternative pathway is limited at most.

Because of their location it is likely that Müller cells are exposed to high glucose levels, which may induce apoptosis.70 In diabetes, this cell type develops hyperplasia13 and dysfunctional glutamate homeostasis14 early, and their dysfunction may affect the cone visual cycle directly. It could also be a signal of more widespread inner retinal damage. Upregulation of Gjap mRNA was not observed in whole retina after 4 or 12 weeks, though others have found protein15,16,17–19 and mRNA25 to be increased in Müller cells. Gene ontology shows that genes involved in metabolism are disproportionately affected by diabetes, suggesting that impaired glial cell function may initiate a cascade leading to outer retinal damage and, therefore, decreased visual function.

Immunohistochemistry showed reduced RPE65 protein expression in the RPE layer after 3 months of diabetes. This indicates that the rod visual cycle at this time point may also be impaired by hyperglycemia, which is a novel finding deserving further exploration. Visual cycle protein levels in the retina
could not be quantified, though there does appear to be light staining of RPE65 in ganglion cells. RPE function is affected by diabetes, and downregulation of RPE65 protein could explain why mice with diabetes have a deficit in regenerating rhodopsin and reduced 11-cis-retinal in the retina 4 weeks after onset.

To evaluate a possible effect of the observed changes on visual function, a visually evoked behavior test was used to compare photopic OKT thresholds between diabetic and normal rats. Reports of ERG deficits do not necessarily correlate with changes in OKT thresholds. No difference in OKT thresholds between normal and diabetic animals was found after 2 or 3 weeks, but after 4 weeks of diabetes there was significant reduction in the OKT threshold in diabetic rats indicating cone dysfunction. Systemic neuropathy may influence the outcome of this test, but the effect is most likely minimal because it is a reflex-like behavior that does not involve the visual cortex but is driven by low-frequency visual pathways. High glucose may also affect cornea, vitreous, or lens and, therefore, acuity in rats. Though we cannot rule out this possibility, measurements in diabetic rats that developed slight lens opacity did not differ significantly from those without. This is the first report of a deficit in visually evoked behavior in a rodent model of diabetes.

ERG data in STZ-treated rodents show abnormal OP early, strongly suggesting that dysfunction starts in the secondary neurons of the inner retina and that photoreceptor damage occurs later. There is evidence of impaired dark adaptation in diabetic patients, which could be attributed to disturbed opsin regeneration. There is also abnormal color vision indicating that cones are affected. However, similar to the rodent model, the most consistent finding is an abnormal OP reflecting inner retinal dysfunction.

Treatment for diabetic retinopathy centers on reduced vascular leakage caused by blood retinal barrier breakdown (edema) or neovascularization. The present study supports the hypothesis that there is functional impairment in cells of the inner retina in rodent models in conjunction with blood retinal barrier breakdown in the absence of edema. This finding could have implications for the treatment of early pathology and suggests that visual function in diabetic rats may be an appropriate model for the screening of neuroprotective agents.

References


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