Microarray Analysis of Murine Retinal Light Damage Reveals Changes in Iron Regulatory, Complement, and Antioxidant Genes in the Neurosensory Retina and Isolated RPE

Majda Hadziahmetovic,1 Usba Kumar,2 Ying Song,1 Steven Grieco,1 Delu Song,1 Yafeng Li,1 John W. Tobias,3 and Joshua L. Dunaief1

Purpose. The purpose of this study was to investigate light damage–induced transcript changes within neurosensory retina (NSR) and isolated retinal pigment epithelium (RPE). Similar studies have been conducted previously, but were usually limited to the NSR and only a portion of the transcriptome. Herein most of the transcriptome, not just in the NSR but also in isolated RPE, was queried.

Methods. Mice were exposed to 10,000 lux cool white fluorescent light for 18 hours and euthanized 4 hours after photic injury. NSR and isolated RPE were collected, and RNA was isolated. DNA microarray hybridization was conducted as described in the Affymetrix GeneChip Expression Analysis Technical Manual. Microarray analysis was performed using probe intensity data derived from the Mouse Gene 1.0 ST Array. For the genes of interest, confirmation of gene expression was done using quantitative real-time PCR. Immunofluorescence assessed protein levels and localization.

Results. Numerous iron regulatory genes were significantly changed in the light-exposed NSR and RPE. Several of these gene expression changes favored an iron-overloaded state. For example, the transferrin receptor was upregulated in both light-exposed NSR and RPE. Consistent with this, there was stronger transferrin receptor immunoreactivity in the light-exposed retinas. Significant changes in gene expression following light damage were also observed in oxidative stress and complement system genes.

Conclusions. The concept of a photooxidative stress–induced vicious cycle of increased iron uptake leading to further oxidative stress was introduced. (Invest Ophthalmol Vis Sci. 2012;53:5231–5241) DOI:10.1167/iovs.12-10204

Materials and Methods

Animals

Male, 10-week-old Balb/cJ mice (n = 8) were obtained from a commercial laboratory (The Jackson Laboratory, Bar Harbor, ME). All procedures were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania and complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Photic Injury

Mice (n = 4) were exposed to 10,000 lux cool white fluorescent light for 18 hours, as described previously,14,15 with slight modifications. The control group (n = 4) was kept on a regular 12-hour light/dark cycle. Mice were euthanized 4 hours after photic injury ended, and the...
NSR and isolated RPE were collected for mRNA quantification. Four samples per group were analyzed separately.

**Neurosensory Retina and RPE Isolation**

Purified RPE cells were isolated by removing the anterior segment (cornea, iris, and lens) from enucleated mouse eyes after a two-step digestion. The eyes were incubated at 37°C for 40 minutes in 2% w/v dispase in 1X Hanks’ balanced salt solution (HBSS) with Ca²⁺ and Mg²⁺ (HBSS+ (Invitrogen, Carlsbad, CA). After digestion with dispase, slits were made in the cornea with a scalpel blade and the eyes were incubated for additional 10 minutes in 1 mg/mL hyaluronidase in HBSS without Ca²⁺ and Mg²⁺ (HBSS/C₀). After two washes in HBSS/C₀, the anterior segment was removed and the eyecup was placed in HBSS/C₀, where the NSR was removed. The NSR was frozen on dry ice and stored at −80°C until the RNA isolation was performed (RNeasy Mini Kit; Qiagen Inc., Valencia, CA) according to the manufacturer’s protocol. The RPE cells were gently brushed from the eyecup in fresh HBSS/C₀, collected, and pelleted at 1200g for 15 minutes. The supernatant was removed and the cells were stored at −80°C until the RNA isolation was performed (RNeasy Micro Kit; Qiagen Inc.) according to the manufacturer’s protocol. The purity of the NSR and isolated RPE cells was verified by relative quantification of RPE-specific 65-kDa protein (Rpe65) mRNA, specific to the RPE; rhodopsin (Rho), specific to the NSR; and platelet/endothelial cell adhesion molecule 1 (Pecam1), specific to vasculature.

**DNA Microarray Hybridization**

Microarray services were provided by the Penn Molecular Profiling Facility, including quality control tests of the total RNA samples (Agilent 2100 Bioanalyzer; Agilent Technologies, Santa Clara, CA; NanoDrop Spectrophotometer; NanoDrop Products, Wilmington, DE). All protocols were conducted as described in commercially available user guides and technical manuals (NuGEN Ovation User Guide; NuGEN Technologies, Inc., San Carlos, CA; and the Affymetrix GeneChip Expression Analysis Technical Manual; Affymetrix Inc., Santa Clara, CA). Briefly, 10 ng of total RNA was converted to first-strand cDNA using reverse transcriptase primed by poly(T) and random oligomers that incorporated an RNA priming region. Second-strand cDNA synthesis was followed by ribo-SPIA linear amplification of each transcript using an isothermal reaction with RNase, RNA primer, and DNA polymerase (Ovation Pico WTA System; NuGEN Technologies), and the resulting cDNA was fragmented, assessed by quality control testing (Bioanalyzer; Agilent Technologies), and biotinylated by terminal transferase end labeling. Labeled cDNA (3 μl) was added to hybridization cocktails (Affymetrix Inc.), heated at 99°C for 5 minutes, and hybridized for 16 hours at 45°C (to Mouse Gene 1 GeneChips; Affymetrix Inc.). The microarrays were then washed at low (6X SSPE) and high (100 mM MES, 0.1 M NaCl) stringency and stained with streptavidin-phycoerythrin. Fluorescence was amplified by adding biotinylated antistreptavidin and an additional aliquot of streptavidin-phycoerythrin stain. A confocal scanner was used to collect the fluorescence signal after excitation at 570 nm.

**FIGURE 1.** Purity of NSR and isolated RPE. Graphs showing Rpe65 (A) Rho (B) mRNA levels within NSR and RPE and Pecam1 (C) within RPE and RPE/choroid. Isolated RPE has significantly higher expression of Rpe65 relative to NSR (NSR was normalized to 1), whereas NSR has significantly higher expression of Rho (RPE was normalized to 1). Pecam1 mRNA is significantly higher within the RPE/choroid relative to RPE. *P < 0.05.

**FIGURE 2.** Light-induced changes in visual cycle genes. Graphs showing significant reduction in expression of Rho within NSR (A) and Rpe65 within RPE (B) following light damage, measured by qPCR. *P < 0.05.
Data Analysis

Microarray analysis was performed using probe intensity data (.cel files; derived from the Mouse Gene 1.0 ST Array; Affymetrix Inc.). The microarray data were analyzed using commercial software (Partek Genomics Suite, version 6.6; Partek Inc., St. Louis, MO). CEL files (Affymetrix Inc.) were imported into the commercial software suite (Partek Inc.) and background correction, normalization, and summa-

Table 1. List of Differentially Expressed Iron Regulatory Genes in Neurosensory Retina following Light Damage

<table>
<thead>
<tr>
<th>Gene Title, Gene Symbol</th>
<th>Fold Change</th>
<th>Step-up (P Value)</th>
<th>Affymetrix Transcript Cluster ID</th>
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<td>Transferrin, Tf</td>
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Figure 3. PCA of the processed microarray gene expression data. PCA analysis using all genes where NSR is represented in blue and RPE in red, and light exposure and no light exposure as triangles and squares, respectively. Tissue type (NSR and RPE) is the strongest determinant of global gene expression variability and is captured by the first principal component (x-axis). Light exposure is captured by the second principal component (y-axis).
Quantitative Real-Time PCR (qPCR)

Gene expression from the neurosensory retina and RPE samples used for microarray analysis was additionally analyzed using quantitative real-time PCR as previously published. Gene expression assays were obtained (TaqMan; Applied Biosystems, Foster City, CA) and used for PCR analysis. Probes used were rhodopsin (RhO, Mm00520345_m1), (Rpe65, Mm00504133_m1), (Pecam-1, Mm01242584_m1), heme oxygenase (decycling) 1 (Hmox1, Mm00516005_m1), catalase (Cat1, Mm00437992_m1), glutathione peroxidase 1 (Gpx1, Mm00656767_g1), superoxide dismutase 1 (Sod1, 01700393_g1), complement components 3a receptor 1 (C3ar1, Mm00262006_s1), Cd59b antigen (Cd59b, Mm02525679_s1), transferrin receptor (Tfrc, Mm00441941_m1), hephastins (Heph, Mm00515907_m1), ferritin light chain-1 (Fti1, Mm03030414_g1), Eukaryotic 18S rRNA (Hs99999901_s1) served as an internal control. Real-time RTPCR (TaqMan; Applied Biosystems) was performed on a commercial sequence detection system (ABI Prism 7500; Applied Biosystems) using the ΔΔCt method. All reactions were performed in biological quadruplicates (four mice) and technical triplicates (three real-time PCR replicates per mouse).

### Statistical Analysis for qPCR

The mean of four samples and the SE were calculated for each comparison group. The means between the groups were compared using the two-group t test. A value of P < 0.05 was considered to be statistically significant. Correction for multiple comparisons was not performed. Data are reported as means ± SEM. All statistical analysis was performed with a commercial software package (GraphPad Prism version 5; GraphPad Software, San Diego, CA).

### Immunofluorescence

The immunofluorescence was performed as previously published on an additional set of samples. Briefly, mouse globes were fixed in 4% paraformaldehyde for 10 minutes, followed by infiltration in 30% sucrose overnight. Then the eyecups were embedded in optimal cutting temperature compound (Tissue-Tek; Sakura Finetek, Torrance, CA). Immunofluorescence was performed on sections 10 μm thick. Primary antibody was detected using Cy3 fluorophore conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Control sections were treated identically, except that primary antibody was omitted. Sections were analyzed by fluorescence microscopy with identical exposure parameters (Nikon, Tokyo, Japan), with ImagePro Plus version 6.1 software; Media Cybernetics Inc, Bethesda, MD). Quantification of immunoreactivity was performed by measuring the mean pixel intensity within the RPE and neurosensory retina of each photomicrograph.
RESULTS

Purity of the Isolated NSR and RPE

The purity of control NSR and isolated RPE was verified by relative quantification of mRNAs specific for retinal pigment epithelium (Rpe65), photoreceptors (Rho), and abundant in vascular endothelial cells (Pecam1). The Rpe65 mRNA levels were compared between NSR and RPE by qPCR and found to be almost 30,000-fold higher in the isolated RPE cells (Fig. 1A), indicating minimal NSR contamination with RPE cells. Similarly, Rho was 400-fold higher within the NSR relative to RPE (Fig. 1B), additionally verifying purity of the isolated RPE. To assess RPE contamination with choroid, we compared Pecam1 mRNA
levels between isolated RPE and RPE/choroid. The choroid expresses this gene due to the presence of vasculature. Pecam1 mRNA levels were 20-fold higher in RPE/choroid (Fig. 1C) than in isolated RPE, indicating only slight contamination of the isolated RPE cells with choroid.

Photic Injury and Retinal Damage

To confirm that exposure to light caused retinal damage we performed qPCR for Rho and Rpe65. Light exposure caused significant reduction in Rho expression within NSR, suggesting severe photoreceptor damage (Fig. 2A). Analogously in the RPE, Rpe65 was significantly reduced following photic injury (Fig. 2B). Previously we have reported retinal morphology following the light damage.15 Our protocol causes severe retinal degeneration with thinning of the outer nuclear layer and disruption of inner and outer segments by 10 days after light exposure.

Global Perspective of Microarray Results

In response to light damage, in the NSR 595 genes were upregulated and 218 were downregulated. In contrast, in the RPE more genes (611) were downregulated than upregulated (569). PCA reveals close correspondence in global gene expression levels in the no light group, and a wide separation from the light-exposed group (Fig. 3). Among the four light-exposed mice, there was moderate separation, most likely reflecting biological variability in light damage severity and/or response to light. The full set of microarray data has been deposited in the National Center for Biotechnology Information Gene Expression Omnibus repository under accession number GSE37773. Because iron dysregulation, oxidative stress, and complement have been implicated in light damage and human retinal degenerations, the following sections will describe light-induced changes in genes from these categories. Although genes assigned to these categories as a group did not show statistically significant changes by the Fisher exact test, a number of genes in each category were significantly altered.

Light Damage and Iron Homeostasis

Microarray analysis showed that photic injury causes significant changes in several retinal iron regulatory genes, seemingly favoring increased iron uptake (Tables 1 and 2). Since microarray is not as quantitative as qPCR, we confirmed gene expression changes by qPCR. In addition, since qPCR is more sensitive than microarray, we used qPCR to test some iron regulatory genes that did not show expression changes in the microarray analysis. We confirmed by qPCR significant expression changes in the no light group, and a wide separation from the light-exposed group (Fig. 3). Among the four light-exposed mice, there was moderate separation, most likely reflecting biological variability in light damage severity and/or response to light. The full set of microarray data has been deposited in the National Center for Biotechnology Information Gene Expression Omnibus repository under accession number GSE37773. Because iron dysregulation, oxidative stress, and complement have been implicated in light damage and human retinal degenerations, the following sections will describe light-induced changes in genes from these categories. Although genes assigned to these categories as a group did not show statistically significant changes by the Fisher exact test, a number of genes in each category were significantly altered.

**Table 3.** List of Differentially Expressed Oxidative Stress Genes in Neurosensory Retina following Light Damage

<table>
<thead>
<tr>
<th>Gene Title, Gene Symbol</th>
<th>Fold Change</th>
<th>Step-up (P Value)</th>
<th>Affymetrix Transcript Cluster ID</th>
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<tr>
<td>Heme oxygenase (decycling) 1, Hmox1</td>
<td>6.71</td>
<td>0.002</td>
<td>10572897</td>
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<tr>
<td>Growth factor receptor bound protein 2-associated protein 1, Gab1</td>
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<td>0.006</td>
<td>10579925</td>
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<tr>
<td>Uveal autoantigen with coiled-coil domains and ankyrin repeats, Uaca</td>
<td>1.51</td>
<td>0.01</td>
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<tr>
<td>Apolipoprotein E, Apoe</td>
<td>-1.75</td>
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<td>10560624</td>
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upregulation of transferrin receptor 1 mRNA within both NSR and isolated RPE (Figs. 4A, 4B), as well as ferritin light chain upregulation in both tissues (Figs. 4C, 4D). Transferrin receptor (TIR1) mediates cellular iron uptake, whereas ferritin is the major iron storage protein. Additionally, expression of the ferroxidase hephaestin went significantly down in both tissues following light damage (Figs. 4E, 4F), whereas its homolog ceruloplasmin’s expression was significantly upregulated in NSR only (Figs. 4G, 4H). To localize TIR1, retinas from mice following light damage were compared with untreated controls using immunofluorescence with an anti-TIR1 antibody (Fig. 5A). TIR1 immunoreactivity was stronger throughout the whole retina following the photic injury relative to untreated controls. This effect was the most pronounced within the RPE, where relative pixel density showed a significant difference (Fig. 5B). TIR1 also colocalized with the marker for endothelial cells PECAM-1 (Fig. 5C).

**Light Damage and Oxidative Stress**

Microarray analysis performed on NSR and isolated RPE samples following light damage showed, as expected, marked elevation of several genes related to oxidative stress (Tables 3 and 4). To better quantify obtained results we performed qPCR for some of these genes. Within NSR light damage caused significant increases in heme oxygenase-1, catalase, glutathione peroxidase, and superoxide dismutase 1 expression (Figs. 6A, 6C, 6E, 6G). RPE samples showed significant elevation of heme oxygenase-1 and glutathione peroxidase following the insult (Figs. 6B, 6F), whereas catalase and superoxide dismutase 1 showed no change (Figs. 6D, 6H).

**Light Damage and Complement**

Following light damage, microarray analysis also showed significant elevation of genes related to the complement system (Tables 5 and 6). Additional confirmation of these results by qPCR showed that following light damage C3 and C3ra1 were significantly upregulated within NSR (Figs. 7A, 7C). C3 was also significantly upregulated within the isolated RPE (Fig. 7B), whereas within the isolated RPE protectin (Cd59b) a complement regulatory protein that inhibits the membrane attack complex (MAC) was significantly downregulated (Fig. 7F). This change was not observed within NSR.

<table>
<thead>
<tr>
<th>Gene Title, Gene Symbol</th>
<th>Fold Change</th>
<th>Step-up (P Value)</th>
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<td>Serine (or cysteine) peptidase inhibitor, clade G member 1, Serping1</td>
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<td>Complement component factor i, Cfi</td>
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<td>Complement component 5, C5</td>
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Differential Expression of Genes Related to Ophthalmic Diseases following Light Damage

We compared our microarray data to the database of genes and diseases currently being tested (eyeGene, National Ophthalmic Disease Genotyping Network through the National Eye Institute [http://www.nei.nih.gov/resources/eyegene/tableforgenes.asp]). Following light damage, expression of 12 genes known to be related to six ophthalmic diseases were altered within the NSR (Table 7), whereas expression of 6 genes related to six ophthalmic diseases was changed within the isolated RPE (Table 8). Several of the genes, including complement factor 3, ApoE, and TLR3 have been implicated in age-related macular degeneration (AMD), and two have been associated with Stargardt’s disease: ELOVL4 and ABCA4.

**DISCUSSION**

Light-induced retinal degeneration has been studied in experimental animals for several decades. In the retina, absorption of light results in generation of reactive oxygen species (ROS). ROS have been shown to result in retinal cell damage and death. RPE cells and photoreceptors are particularly susceptible to oxidative damage due to high oxygen tension, large numbers of mitochondria, and abundant polyunsaturated fatty acids in photoreceptor membranes. When phototoxic stress is severe, cells may respond to the insult by undergoing apoptosis. Additionally, iron overload can cause retinal degeneration and has been implicated in pathogenesis of AMD. In our previous work we have shown that light damage caused elevation of retinal ceruloplasmin levels, in particular within the Müller cells. This elevation of the ferroxidase ceruloplasmin may protect the retina against oxidative stress by decreasing the amount of ferrous iron available to produce ROS. In this study we use an updated, whole transcriptome microarray to further elucidate the role of light damage on alteration of gene expression within the retina, including genes that are responsible for tight regulation of retinal iron levels. One novelty of this study is that by using isolated RPE cells we can better understand the response of this cell type to light-induced retinal damage. Our results suggest that light-induced upregulation of transferrin receptor
FIGURE 6. Light-induced changes in oxidative stress–related genes measured by qPCR. Graphs showing significant upregulation in Hmox1, Cas-1, Gpx, and Sod1 mRNA levels in NSR following LD (A, C, E, G). The right column shows oxidative stress–related genes in RPE following LD, with only Hmox1 and Gpx being significantly upregulated (B, D, F, H). *P < 0.05.
could lead to a vicious cycle of iron uptake and oxidative stress.

As expected, light damage caused significant changes in expression of several genes related to oxidative stress (Tables 3 and 4; Fig. 3). One of these genes, upregulated within both NSR and isolated RPE, is \textit{Hmox1}. Under oxidative stress conditions, \textit{Hmox1} can have a dual role: it may protect cells by inducing the catabolism of prooxidant heme and hemoproteins to the antioxidants, biliverdin and bilirubin, but \textit{Hmox1} also catalyzes production of ferrous iron and carbon monoxide that may exacerbate cellular injury and oxidative stress by generating free radicals.\textsuperscript{20–22} Broad upregulation of the genes associated with oxidative stress within the NSR, but not isolated RPE, suggests that this modification might be an attempt of the neural retina to protect itself from further damage. In contrast, following the light damage RPE cells did not upregulate catalase. This could make more hydrogen peroxide available to react with the iron taken up by the

**Table 6.** List of Differentially Expressed Complement System Genes in Retinal Pigment Epithelium following Light Damage

<table>
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<tr>
<th>Gene Title, Gene Symbol</th>
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upregulated transferrin receptor, in effect producing more of the highly reactive hydroxyl radical. Another seemingly maladaptive behavior of RPE was downregulation of Cdy5b, a complement regulatory protein that inhibits the MAC. Reduction in genes that regulate or inhibit the complement system might be detrimental to the RPE. Similarly, reduction in one of the major membrane-associated complement regulatory proteins, CD46, has been reported in AMD. Decreased expression of a complement regulatory gene combined with increased expression of C3 and the receptor for the C3 cleavage product C3a suggest that complement expression within the neural retina and RPE may contribute to the retinal degeneration induced by bright light. A previous light damage study found C3 and C3αr upregulation in macrophages and microglia in the neural retina. Further, knockout of complement alternative pathway activator factor D diminishes retinal light damage.

Maintaining iron homeostasis is critical for retinal health because excess iron may be toxic to the retina. Elevation of retinal iron levels is a normal part of aging, but also has been observed in retinal degenerations such as AMD and aceruloplasminemia. In this study we investigated whether light-induced oxidative stress within the NSR and isolated RPE is associated with changes in expression of iron homeostasis-related genes. We expected that following light damage, the retina would downregulate Tfrc to protect itself from further oxidative damage, but both NSR and RPE significantly downregulated Tfrc (Fig. 6). Tfrc mRNA stability is regulated by labile iron levels, with increased labile iron resulting in decreased Tfrc mRNA.

Despite the fact that upon the light damage both NSR and RPE significantly upregulated Tfrc, only NSR counteracted this by significant upregulation of ceruloplasmin, a multicopper ferroxidase that facilitates iron export from the cell. Following the light damage, Cp homolog hephaestin was significantly downregulated in both tissues. These results suggest that Cp and Heph are regulated differently, perhaps to facilitate some distinct roles of each protein. Further, both Hfe and Bmp6 were downregulated. Since these are most likely inhibitors of...
retinal iron import.\textsuperscript{9,32} Their downregulation is predicted to result in increased retinal iron import.

Additionally, \textit{Elovl4} and \textit{Abcr} genes were significantly downregulated within the NSR and RPE following light damage. These genes are associated with inherited macular degenerative diseases encode proteins that function in the processing of lipids in photoreceptor cells and were thought to be photoreceptor specific.\textsuperscript{33,34} Interestingly, we observed their expression within the RPE, as well as significant alterations following the photic injury. In no light damage, samples of \textit{Elovl4} had a 6-fold higher expression within the NSR relative to RPE; \textit{Abcr} was only 1.6-fold higher, suggesting its abundance within the RPE. Since the NSR relative to RPE showed 400-fold higher expression of \textit{Rho} mRNA (Fig. 1B), contamination of RPE with photoreceptor mRNA is unlikely to explain the presence of these mRNAs in our isolated RPE cells. Thus, these genes may have important functions in the RPE.

Our data suggest that following photic injury, the retina alters expression of iron regulatory genes that favor increased iron uptake. Increased labile iron may cause additional oxidative stress in a vicious cycle of cellular self-destruction.

References


