Increased expression of ceruloplasmin in the retina following photic injury

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Purpose: Oxidative stress plays a role in the photic injury model of retinal degeneration and in age-related macular degeneration. Our preliminary microarray analysis of retinal gene expression upon photic injury suggested increased expression of ceruloplasmin, a ferroxidase that could reduce retinal oxidative stress. Patients with aceruloplasminemia have retinal degeneration, indicating that ceruloplasmin is necessary for maintenance of retinal health. The purpose of this study was to determine whether retinal ceruloplasmin is upregulated following photo-oxidation, to localize ceruloplasmin protein, and to determine which ceruloplasmin isoform is present in the retina.

Methods: Balb/c mice were exposed to bright white light for seven hours. TUNEL labeling was used to detect photoreceptor apoptosis. At several intervals after the light injury, retinal ceruloplasmin was studied by quantitative PCR, immunohistochemistry, and western analysis. Expression of the secreted and expression of the membrane-anchored glycosyl phosphatidyl inositol (GPI) linked forms of ceruloplasmin were assesed in rat retina using primers specific for each form. Vitreous ceruloplasmin was detected by immunohistochemistry in Balb/c mouse eyes and by western analysis of aspirated vitreous from post-mortem human eyes.

Results: Retinal ceruloplasmin mRNA was upregulated eight-fold following photic injury. Ceruloplasmin protein was detected throughout normal retinas by immunohistochemistry, with a specific increase in Muller cell labeling following photic injury. Western analysis confirmed an increase in ceruloplasmin protein following photic injury and revealed eight-fold more ceruloplasmin protein in normal retina than in brain. The mRNAs for both the secreted and GPI linked forms of ceruloplasmin were detected by RT-PCR in the retina. Ceruloplasmin protein was detected by western analysis of normal human vitreous and was increased in mouse vitreous following photic injury.

Conclusions: Ceruloplasmin, a retinal ferroxidase, is upregulated at the mRNA and protein levels upon light damage. The increased protein is primarily in Muller cells. Ceruloplasmin is considerably more abundant in retina than in brain. The retina expresses both the GPI-linked and secreted forms of ceruloplasmin, and since vitreous ceruloplasmin increases following photic injury, some of the retinal ceruloplasmin may be secreted into the vitreous. Ceruloplasmin may protect the retina from oxidative stress by decreasing the amount of ferrous iron available to produce reactive oxygen species.

Several studies indicate that oxidative stress is one of the causes of age-related macular degeneration (AMD), the leading cause of irreversible vision loss among people age 60 and older in the United States [1,2]. Retinal oxidative stress can be caused by light exposure, which has been implicated in the pathogenesis of AMD and other retinal degenerations [3]. Photo-oxidative stress is caused by an imbalance between light-induced reactive oxygen species (ROS) and antioxidants. In the retina, absorption of light by photosensitizers results in electron transition to an unstable excited state [4], subsequently generating ROS. Photoactivation of lipofuscin yields singlet oxygen, superoxide anion, hydrogen peroxide, and lipid hydroperoxides [4,5]. These photoinducible ROS have been shown to result in lipid peroxidation, enzyme inactivation [6], and retinal pigment epithelium (RPE) cell death [7]. When photo-oxidative stress is severe, cells may respond to the insult by undergoing apoptosis [8-11].

Photic injury in mice has long been used as a model system to study retinal degeneration [8]. In this model and many others, photoreceptor death occurs through apoptosis, as determined by TUNEL and agarose gel electrophoresis demonstrating apoptosis-specific DNA laddering [9-11]. Oxidative stress has been implicated in photic injury pathogenesis by immunohistochemical detection of biomarkers of oxidative damage [12] and by the use of exogenous antioxidants to protect the rodent retina from photic injury [13,14]. We hypothesized that in the mouse retina, as suggested for AMD [15], light damage not only generates ROS, but also induces overexpression of endogenous antioxidants to minimize the oxidative stress.

Ceruloplasmin is a ferroxidase, converting the hydroxyl-radical producing ferrous (Fe^{2+}) iron to the safer ferric (Fe^{3+}) form [16]. Cultured neural cells from ceruloplasmin knock-out mice are more susceptible to free radical injury, suggesting that ceruloplasmin may combat oxidative stress [17]. Ceruloplasmin is expressed in the human retina [18] and increased retinal ceruloplasmin expression occurs in the rat retina following optic nerve crush, where lipid peroxidation is associated with retinal ganglion cell death [19]. Since photo-oxidative stress is a more direct form of retinal oxidative damage, it seemed likely that retinal ceruloplasmin would be upregulated following photic injury. We now report that ceruloplasmin (Cp) was increased following photo-oxidation of the mouse retina.

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Increased understanding of the antioxidant function of ceruloplasmin in the retina may shed light on the pathogenesis of retinal degenerations caused or exacerbated by oxidative stress.

METHODS

Rodents used in the experiments presented in this study were handled using methods comparable to those published by the Institute for Laboratory Animal Research. Experiments with mice and rats were approved by the University of Pennsylvania Institutional Animal Care and Use Committee and experiments with post mortem human eyes from anonymous donors were approved by the University of Pennsylvania Institutional Review Board.

Mouse photic injury: For each experiment, ten week old male Balb/c mice (Jackson Laboratories, Bar Harbor, ME), reared in cyclic light and darkness, were divided into groups allowing analysis of gene expression immediately (t0), or at intervals after photic injury. No light control mice were dark adapted for 24 h and then exposed to room light (200 lux) for 7 h before euthanasia. The experimental groups were dark adapted for 24 h and then exposed to 10 klux cool white fluorescent light in a well ventilated, air conditioned room for 7 h from 2 to 9 am, then sacrificed either immediately (t0), 8 h after photic injury ended (t8), or 28 h after photic injury ended (t28). Three retinas from each time point were pooled and used for RNA preparation for quantitative PCR analysis and two more were pooled and used for protein purification. One eye from each time point was used for histology and TUNEL labeling. Three independent photic injury experiments were performed, each with a no light group and a t0 group, two with t8 groups and two with t28 groups.

TUNEL labeling: The enucleated eyes used for TUNEL labeling were immersion fixed in 4% paraformaldehyde for 24 h. Eye cups were generated by removing the anterior segment. Eye cups were cryoprotected with 30% sucrose overnight, then embedded in Tissue-Tek OCT (Sakura Finetek, USA, Torrance, CA). 10 µm frozen sections were cut in the sagittal plane through the optic nerve head. The TUNEL in situ apoptosis detection kit (Roche, Mannheim, Germany) was applied to detect cleaved DNA in the frozen sections using the manufacturer's protocol [20], except that tissue was fixed prior to sectioning [20].

Quantitative PCR (Q-PCR) and Reverse Transcription PCR (RT-PCR): Primers for glyceraldhyde-3-phosphate dehydrogenase (GAPDH) and ceruloplasmin (both secretory form and GPI-linked form) were designed spanning the intron-exon boundaries to amplify the corresponding mRNAs and minimize amplification of potentially contaminating genomic DNA. For GAPDH, the forward primer was 5'-TTC ACC ACC ATG GAG AAG GC-3', and the reverse primer was 5'-GGC ATG GAC TGT GGT CAT GA-3'. For the secretory form of ceruloplasmin, the forward primer was 5'-GTA AAC AAA GAC AAC GAG GAA T-3', and the reverse primer was 5'-TAT TTC ATT CAG CCA GAC TTA G-3'. For the GPI-linked form, the forward primer was 5'-GTA TGT GAT GGC TAT GGG CAA TGA-3', and the reverse primer was 5'-CCT GGA TGG AAC TGG TGA TGG A-3' [21].

Q-PCR was carried out to detect the mRNA levels with mouse retinal cDNA as template, which was synthesized with total retinal RNA, a T7-(dT)24 oligomer primer, and the superscript system (Gibco, BRL Life Technologies, Rockville, MD). The experiment was performed with the SYBR green PCR master mix (ABI, Foster City, CA) using the ABI 7000 fluorescein PCR detection system following the manufacturer's protocol. RT-minus controls (samples containing RNA that was not reverse transcribed), primer-minus, and template-minus controls were included.

RT-PCR was performed to study the ceruloplasmin isoform with rat retina cDNA as the template. Total RNA was isolated was from rat retinas with Trizol (Gibco) Next, cDNA was synthesized as above. PCR was performed with an MJ research PTC-0200 thermocycler.

Immunohistochemistry: Cryostat sections (10 µm) of 4% paraformaldehyde-fixed eyes (prepared as described above for TUNEL labeling) were incubated with anti-ceruloplasmin antibody (DAKO, Carpinteria, CA). Binding of the primary antibody was detected with fluorophore-labeled secondary antibody [20]. Purified human ceruloplasmin protein (Vital Products, Delray Beach, FL) was used to preadsorb the ceruloplasmin and anti-glial fibrillary acidic protein (GFAP) antibodies (DAKO, Carpinteria, CA) to test the specificity of antigen-antibody binding.

Western analysis: After sacrifice, mice were perfused through the left cardiac ventricle with ice-cold phosphate buffered saline (PBS; pH 7.4) to flush out the ceruloplasmin-containing plasma, and the retinas and cerebral cortex were isolated and immediately frozen at -80 °C. Protein was extracted in a buffer containing 10 mM dibasic potassium phosphate, 150 mM NaCl, 200 mM sucrose, 1% Triton X-100, and one Complete Protease Inhibitor Cocktail tablet (Roche Diagnostics GmbH, Mannheim, Germany). Samples were sonicated at 30% output for 30 pulses using a Branson Model 250 sonifier (VWR International, Westchester, PA). Protein concentration was determined using the BCA kit (Pierce, Rockford, IL). Equal amounts of photic injury retinal protein (1 µg protein/lane) and serial dilutions of "no light" control protein (1 µg, 2 µg, and 4 µg protein/lane) were separated by gel electrophoresis with Nupage 7% Tris-Acetate gels and then transferred to nitrocellulose membranes (Invitrogen Life Technologies, Carlsbad, CA). Western analysis was performed with anti-ceruloplasmin (DAKO, Carpinteria, CA, dilution 1:1000) and anti-GAPDH (Chemicon, Temecula, CA, dilution 1:1000) followed by an alkaline phosphatase-linked secondary antibody. Chemifluorescent bands were detected with ECF (Amersham Pharmacia Biotech, Buckinghamshire, UK) and a Storm Phosphorimager (Molecular Dynamics, Sunnyvale, CA). Band intensities were quantified using Image Quant analysis software (Molecular Dynamics). The intensities of the ceruloplasmin bands were normalized to GAPDH bands within each lane.

For western analysis of human vitreous, both eyes from a 70 year old donor with no significant ocular disease were enucleated 6 h after death and shipped by the National Disease Research Interchange on ice. The anterior segment of each eye was removed by generating a circumferential inci-

sion at the pars plana. The anterior, cortical, and posterior vitreous were then collected by aspiration combined with frequent cutting using Westcott scissors.

RESULTS

Photoreceptors undergo apoptosis following light damage: It has been established that photoreceptors undergo apoptosis given sufficient photic exposure [22]. In order to confirm that our mice had been exposed to light of sufficient intensity and duration to undergo oxidative stress and photoreceptor damage, we used the TUNEL assay to detect photoreceptor apoptosis. TUNEL positive photoreceptor nuclei were present at the t0, t8, and t28 time points (Figure 1). The t8 retinas had the largest number of TUNEL positive photoreceptors (Figure 1C). Only a few TUNEL positive photoreceptors were present in the t0 retinas and none were found in the control group (no light), or in t28 sections in which the terminal deoxytransferase enzyme had been omitted. In t28 retinas only, a few Muller cells near TUNEL positive photoreceptor nuclei had TUNEL label in their cell bodies but not their nuclei (Figure 1D, top left), possibly because they had taken up fragmented chromatin from photoreceptor nuclei.

Ceruloplasmin mRNA increase detected by quantitative PCR (Q-PCR): Microarray analysis of retinal mRNA following photic injury revealed significant increases in ceruloplasmin in three independent experiments (not shown). To confirm the validity of these results, Q-PCR with ceruloplasmin secretory form primers was used to assess ceruloplasmin mRNA levels following photic injury. The housekeeping gene GAPDH was used as an internal control, and its mRNA levels remained similar in photic injury and no light controls (Figure 2). In contrast, in the exponential amplification phase, ceruloplasmin amplification product was present three cycles earlier in the photic injury compared to the no light control retinas, consistent with an eight-fold increase in retinal ceruloplasmin mRNA following photic injury. This eight-fold increase was present at t0 and sustained at t8 and t28. QPCR

results were replicated at least once in independent photic injury experiments for each time point. The Q-PCR analysis was run simultaneously for t0 and t8 samples and utilized the same "no light" control sample. Data from this single control sample are shown on both the t0 and t8 graphs for comparison to results from light damaged retinas. These results were reproducible with independent photic injury experiments, each with its own no light control. Template-minus controls yielded no PCR products, as expected. Ceruloplasmin primers did not yield any PCR products in the RT-minus controls, indicating that the PCR product represented the ceruloplasmin mRNA, not a DNA contaminant. In contrast, the RT-minus controls with GAPDH primers were positive, most likely resulting from the presence of a GAPDH pseudogene [23]. The amount of this contaminant was negligible, less than 0.1% of the cDNA sample, as determined by using the equation $2^{[Ct(RT+)-Ct(RT-)]}$ [24]. In the equation, Ct is the threshold cycle, the point at which the fluorescence exceeds a threshold limit that is 10 times the standard deviation of the baseline.

Increased ceruloplasmin protein in Muller cells following photic injury: Following photic injury, ceruloplasmin label was increased throughout the retina, with strongest label in Muller cells. Comparing retinas that had either no photic injury or photic injury followed by several intervals before sacrifice (Figure 1), the label was notably increased in Muller cells at t0, t8, and t28. No label was present at t28 when the primary antibody was omitted (Figure 1E) or when antibody was pre-adsorbed with ceruloplasmin protein (not shown). Double labeling with TUNEL confirmed that the light exposure had induced photoreceptor apoptosis in these retinas.

Ceruloplasmin protein is increased in the light damaged retina and is abundant even in the normal retina: Western analysis was used to assess ceruloplasmin protein levels following photic injury (Figure 3A). After sacrifice but before enucleation, mice were perfused with ice cold saline to eliminate plasma ceruloplasmin. Western analysis of retinal protein with an anti-ceruloplasmin antibody revealed a single
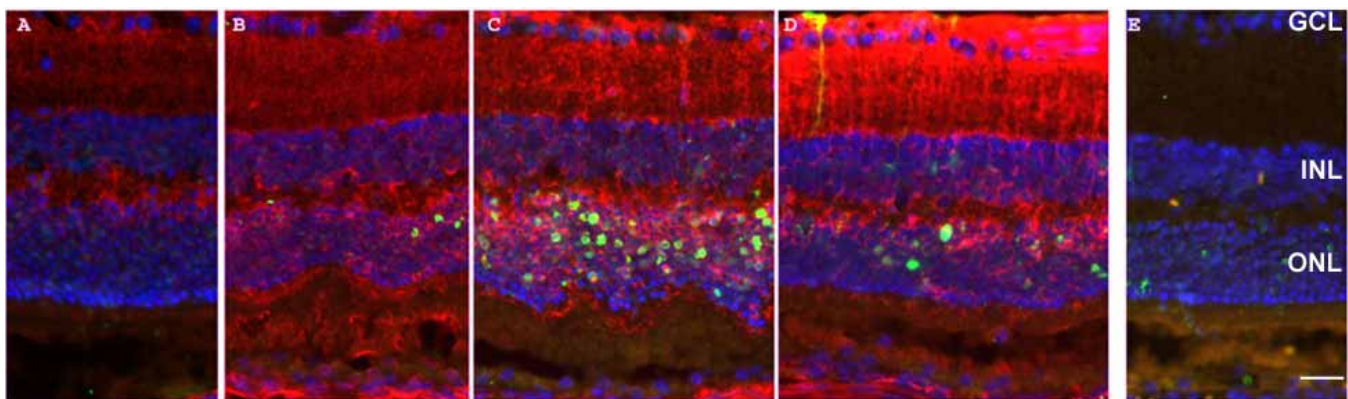

Figure 1. Photic injury induces TUNEL positive photoreceptors and elevated ceruloplasmin. Fluorescence photomicrographs of TUNEL (green) and anti-ceruloplasmin (red) labeled retinas at various times following photic injury. **A**: No light control. **B**: Immediately after photic injury (t0). **C**: Eight hours after photic injury (t8). **D**: Twenty eight hours after photic injury (t28). **E**: No primary ceruloplasmin antibody. Nuclei are labeled with DAPI (blue). Ganglion cell layer (GCL), inner nuclear layer (INL), outer nuclear layer (ONL). Scale bar 50 µm.

band, and the signals in lanes t0, t8, and t28 were higher than that in lane NL (no light, 1µg). Normalized to GAPDH levels, the t0 (immediately after 7 h of light exposure) ceruloplasmin was 3.2X greater than NL, t8 was 1.8X greater and t28 was 1.8X greater. Serial dilutions of total protein from the NL retina (1, 2, and 4 µg) were loaded on the same gel to ensure that detection was in the linear range.

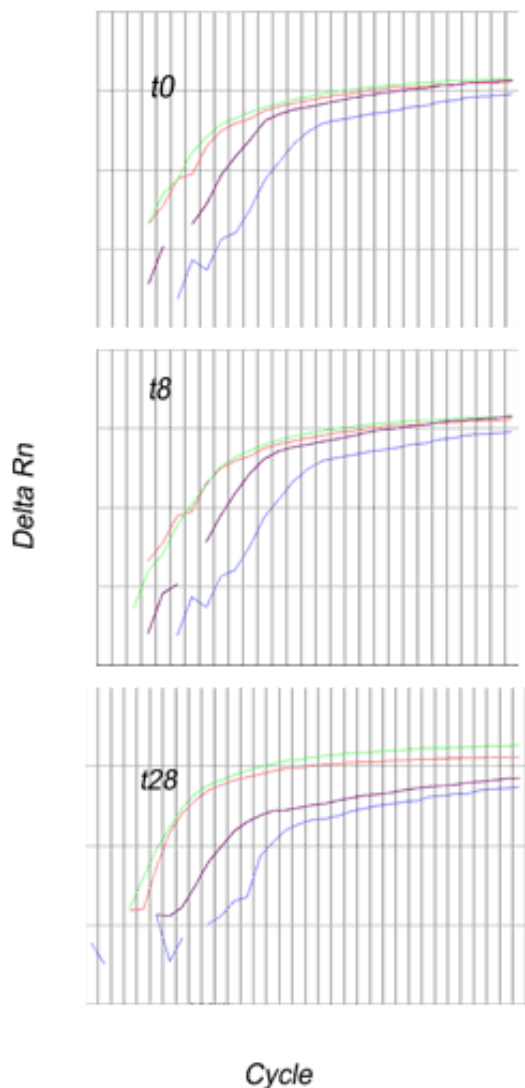In addition to the retina, ceruloplasmin is also expressed in brain, liver, lung, testis, kidney and spleen [25]. Western analysis was used to compare the abundance of retinal ceruloplasmin to brain ceruloplasmin (Figure 3B). Serial dilutions revealed that retinal ceruloplasmin was eight-fold more abundant than brain ceruloplasmin. Densitometry revealed similar ceruloplasmin signals for 1 µg of retinal protein and 8 µg of brain protein; 2 µg of retinal protein and 16 µg of brain protein were also similar. The retinal ceruloplasmin band, approximately 130 kDa, was the same size as the fastest-migrating of the three brain bands. The middle brain band migrated at approximately 142 kDa and the upper at 150 kDa.

Ceruloplasmin protein is present throughout normal retina: Western analysis revealed significant quantities of ceruloplasmin in the normal retina (Figure 3B). To localize ceruloplasmin in a normal retina, immunohistochemistry employed higher antibody concentration and less stringent blocking than the experiment shown in Figure 1. For some sections, the antibody was pre-adsorbed with ceruloplasmin protein to test the specificity of antigen-antibody binding (Figure 4). In the normal, uninjured retina, ceruloplasmin label was present throughout the retina (Figure 4B). When the ceruloplasmin antibody was pre-adsorbed with purified human ceruloplasmin protein, the label was completely eliminated (Figure 4C),
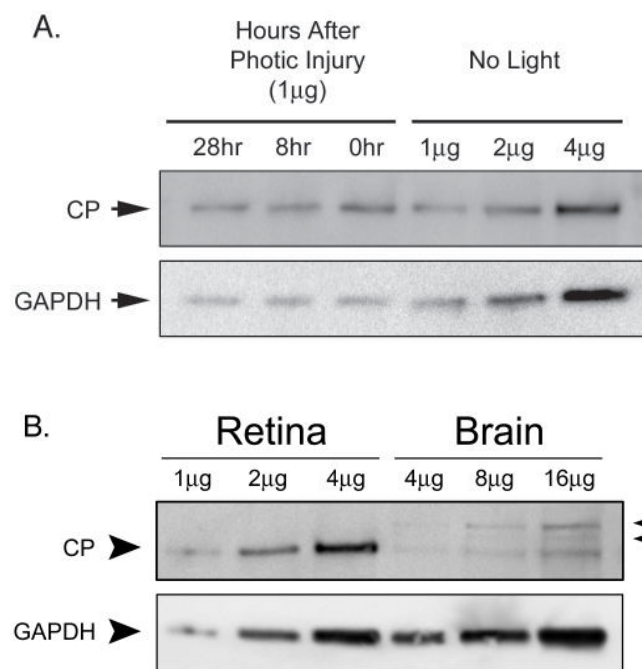

Figure 2. Ceruloplasmin mRNA levels. Ceruloplasmin mRNA levels increases eight-folds following photic injury. Quantitaitve PCR for ceruloplasmin and GAPDH was performed with retinal cDNA from the no light control, the light exposed t0 (top panel), t8 (middle panel), and t28 (bottom panel) experimental groups. Fluorescence emission is measured continuously during the PCR amplification and delta Rn (increase in fluorescence emission substracted from the background fluorescence signal) is plotted against cycle number. Each space between each horizontal lines represents a 2.8-fold difference in fluorescence intensity, while each vertical line indicates one PCR cycle. Red lines: GAPDH from no light retinas. Green lines: GAPDH from photic injury retinas. Blue lines: ceruloplasmin from no light retinas. Purple lines: Ceruloplasmin from light damaged retinas.

Figure 3. Ceruloplasmin protein levels. **A**: Western analysis showing increased Cp protein in retinas following photic injury. The top half of the filter was exposed to anti-Cp and the bottom half to anti-GAPDH antibody. The band intensities at 0, 8, and 28 h after the termination of 7 h light exposure were compared to serial dilutions of total retinal protein as indicated from mice not exposed to light. **B**: Western analysis demonstrating higher levels of Cp protein in retina than in brain. Serial dilutions as indicated of total protein from retina and brain were compared. The top half of the filter was exposed to anti-Cp and the bottom half to anti-GAPDH antibody. Arrowheads on the right indicate two bands detected in the brain but not the retinal samples.

providing evidence that the ceruloplasmin label was specific. To test whether the pre-adsorption was specific, ceruloplasmin protein was mixed with an unrelated antibody, the glial fibrillary acidic protein (anti-GFAP) antibody. Anti-GFAP labels Muller cells in photic injury sections (Figure 4D). When anti-GFAP antibody was pre-adsorbed with ceruloplasmin protein there was no reduction in Muller-specific label (Figure 4E). This suggests that ceruloplasmin protein specifically blocks the binding of anti-ceruloplasmin antibody to ceruloplasmin antigen in the retina but does not non-specifically block the interaction between anti-GFAP and retinal GFAP. Together, these data suggest that the label in Figure 4B more likely represents ceruloplasmin in the normal retina than non-specific antibody adherence to the tissue.

Both the secreted and membrane-anchored GPI linked forms of ceruloplasmin are expressed in the retina: Two forms of ceruloplasmin have been described. The secreted form is the predominant form expressed by the liver, resulting in significant plasma ceruloplasmin levels. The GPI-linked form is the predominant form in the brain [21]. The mRNA encoding the GPI linked form is produced by alternative splicing of the final exon, and, relative to the secreted form, replaces the 5' C-terminal amino acids with 30 amino acids containing a GPI linkage signal at the protein's C-terminus. To determine which form is expressed in the retina, RT-PCR using previously described primers [21] that differentiate the two forms of rat ceruloplasmin was performed. Primers specific for either the GPI-linked form or the secreted form each resulted in amplification of a fragment of the predicted size (Figure 5). Omission of reverse transcriptase as a negative control did not result in detectable bands, indicating that the original template was RNA and not contaminating DNA.

Murine vitreous ceruloplasmin protein increases following photic injury and human vitreous contains abundant ceru- *loplasmin:* We next tested the possibility that some of the increased ceruloplasmin produced in the retina after photic injury might be secreted into the vitreous. Three observations prompted investigation of this hypothesis. First, the secreted form of ceruloplasmin is produced in the retina. Second, Muller cells, which express ceruloplasmin, can secrete proteins into the vitreous [26]. Third, ceruloplasmin is present in at least one intraocular compartement, in the the aqueous humor [27]. We used ceruloplasmin immunohistochemistry on sections from whole eyes following photic injury (rather than on eye cups, as described above). This technique allowed detection of ceruloplasmin in the vitreous of t28 eyes. The label was absent when primary antibody was omitted or when ceruloplasmin antibody was pre-adsorbed with ceruloplasmin protein (not shown), consistent with specific vitreous label. Minimal label was present when t28 sections were labeled with an unrelated antibody or in ceruloplasmin-labeled control no light retinas consistent with an increase in vitreous ceruloplasmin following photic injury. To determine whether ceruloplasmin is present in vitreous and retina from a normal human eye, the anterior, cortical and posterior vitreous as well as retina from a post mortem eye were tested using western analysis with an anti-ceruloplasmin antibody. Bands corresponding to ceruloplasmin were readily detected in each sample (Figure 6), suggesting that ceruloplasmin is present in the normal human vitreous.

DISCUSSION

We demonstrate increased retinal levels of ceruloplasmin mRNA and protein following photic injury. An increase in ceruloplasmin mRNA was detected with Q-PCR and an increase in protein, especially in Muller cells, was ascertained by immunohistochemistry and western analysis. In the normal retina, ceruloplasmin protein levels were significantly
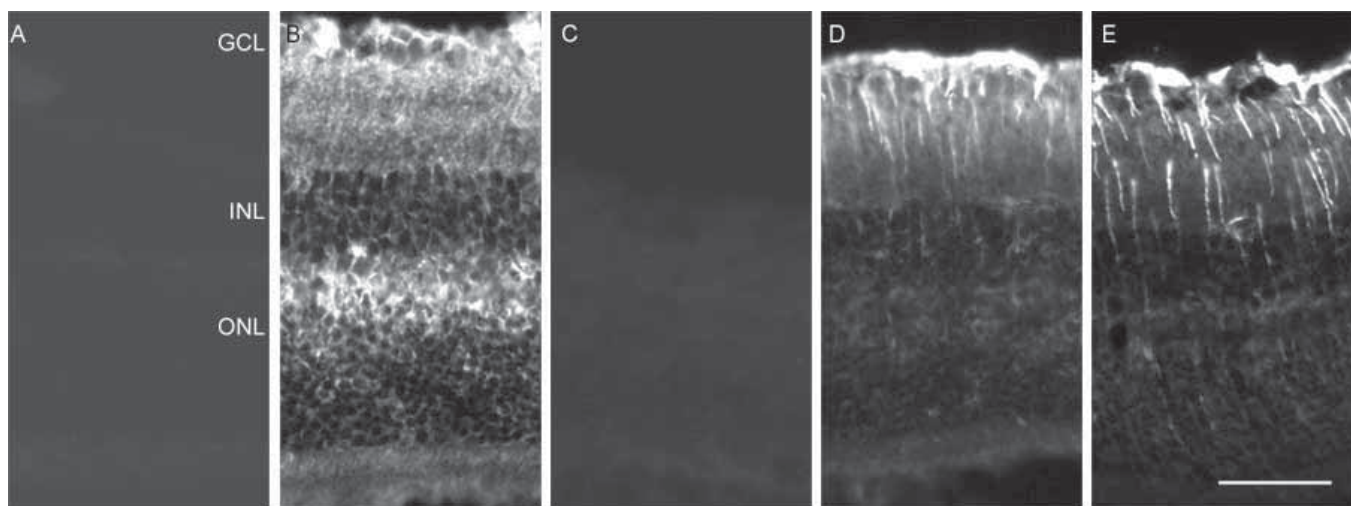

Figure 4. Ceruloplasmin IHC label in mouse retina. Immunohistochemistry with ceruloplasmin antibody demonstrates label throughout a normal retina, with increased label following photic injury. Fluorescence photomicrographs of mouse retinas labeled with no primary antibody (**A**), anti-ceruloplasmin (**B,C**), or anti-GFAP (**D,E**). **A-C** were normal retinas. **D** and **E** were 28 h after the end of photic injury (t28). Primary antibody in **C** and **E** was pre-adsorbed with ceruloplasmin protein before it was applied to the section.

higher than in the brain. There are two isoforms of ceruloplasmin, the secreted form and the GPI-linked membrane anchored form, and the retina produces both forms. Since the mouse vitreous contains increased ceruloplasmin following photic injury, ceruloplasmin may be secreted into the vitreous by retinal cells.

Q-PCR was performed with retinas obtained from three individual photic injury experiments. The consistency of the eight-fold increase in retinal ceruloplasmin mRNA following photic injury, further validated by microarray analysis detecting an eight-fold increase in three independent photic injury experiments (data not shown) warranted further characterization of retinal ceruloplasmin. Q-PCR experiments are limited to the study of gene expression at the mRNA level and mRNA levels do not necessary correlate with protein levels [28,29]. Thus, we used western analysis and immunohistochemistry to study ceruloplasmin expression at the protein level. Although there is increased ceruloplasmin mRNA and protein, the increase in protein (approximately two-fold) is lower than the increase in mRNA (eight-fold). This discrepancy could be explained by secretion of a portion of the ceruloplasmin made in the retina into the vitreous. This hypothesis is supported by our finding of abundant ceruloplasmin in human vitreous and increased ceruloplasmin in mouse vitreous following photic injury. Muller cell secretion of other proteins into the vitreous has been demonstrated [26]. A second explanation for only a two-fold protein increase is the abundant ceruloplasmin protein in normal retinas (Figure 4B); the new ceruloplasmin protein produced acutely following photic injury would represent a smaller percentage of total retinal ceruloplasmin pro-

tein if the baseline level of retinal ceruloplasmin is high and the protein is stable. Alternatively, if ceruloplasmin protein is less stable under photic injury conditions, protein degradation could partially offset the increased ceruloplasmin protein production.

Increased expression of ceruloplasmin could help protect the retina from oxidative stress. Ceruloplasmin is a copper binding glycoprotein found mainly in plasma but also present in several other tissues including retina and brain [30]. Ceruloplasmin may reduce the amount of reactive oxygen species in the retina. By oxidizing iron, ceruloplasmin converts the hydroxyl-radical producing ferrous form of iron to the safer ferric form [16]. Ferrous iron can generate highly reactive hydroxyl and superoxide free radicals in the presence of hydrogen peroxide or molecular oxygen. Recent work with ceruloplasmin (Cp) knockout mice showed that $Cp^{-/-}$ neural cells have increased susceptibility to oxidative stress [17].

Not only does ceruloplasmin oxidize iron to a less toxic form, but it also facilitates iron binding to transferrin, the major extracellular iron transporter, which can only bind ferric iron. Ceruloplasmin may facilitate iron loading onto transferrin in the retina, as transferrin is made by RPE and photoreceptors and bound by photoreceptors and other retinal cells [31,32]. It may also facilitate iron loading onto transferrin in the vitreous, which contains transferrin [33] and ceruloplasmin, although we can not rule out a post mortem artifact resulting in vitreous ceruloplasmin; studies with fresh vitreous will be needed to confirm this.

Ceruloplasmin is also essential for regulating the efficiency of iron export from specific cell types: Cp knockout mice have a defect in iron export from the reticuloendothelial system [34], and our preliminary studies with these mice (unpublished data) and studies by others [17] suggest that ceruloplasmin plays a critical role in iron transport in the retina. Evidence that iron plays a role in photic injury comes from the demonstration that administration of the iron chelator deferoxamine ameliorates photic injury [35].

The importance of ceruloplasmin in vivo is most clearly illustrated by studies of aceruloplasminemia, a hereditary deficiency of ceruloplasmin [36-40]. Patients with aceruloplasminemia in the fourth or fifth decade of life exhibit symptoms and signs of retinal degeneration from iron
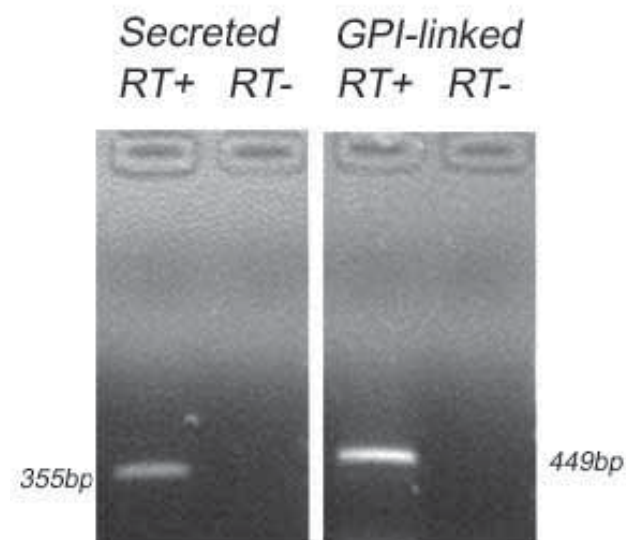
Figure 5. Secreted and GPI-linked forms of ceruloplasmin. RT-PCR shows that both the secreted and GPI-linked forms of ceruloplasmin are present in the retina. PT/PCR performed with rat retina RNA and primers specific for secreted ceruloplasmin (lanes 1,2) or GPI-linked ceruloplasmin (lanes 3,4) resulted in DNA fragments detected with ethidium bromide following agarose gel electrophoresis. Lanes 2 and 4 are RT-minus controls.
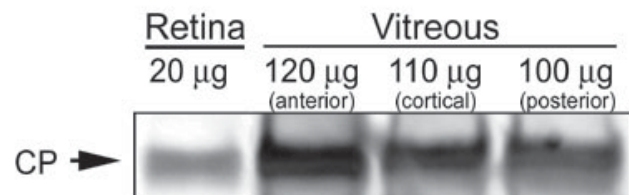
Figure 6. Ceruloplasmin protein in human vitreous and retina. Western analysis detects ceruloplasmin in post mortem human vitreous and retina. The indicated quantities of total vitreous or retinal protein were loaded on the gel as follows: retina (lane 1), anterior vitreous (lane 2), cortical vitreous (lane 3), and posterior vitreous (lane 4).

overload in the retina. RPE atrophy in the mid-peripheral fundus and blocked fluorescence in the macula, possibly caused by iron overload has been described [41]. Aceruloplasminemic patients also accumulate iron in their basal ganglia and exhibit dementia, dystonia and dysarthria.

Tissue in situ hybridization indicated that ceruloplasmin is mainly expressed in the astrocytes in the ganglion cell layer and in the Muller cells in the inner nuclear layer [19,25]. Consistent with these data, we observed by IHC increased labeling of Muller cells in the photic injury group suggesting increased synthesis of ceruloplasmin in Muller cells. Since ceruloplasmin protein is distributed diffusely in the normal mouse retina (Figure 4B), Muller cells, like hepatocytes, may secrete ceruloplasmin. Brain astrocytes predominantly synthesize a membrane-bound glycosylphosphatidylinositol (GPI)-anchored form of ceruloplasmin. RT-PCR analysis indicates that both the secreted and GPI-linked forms of ceruloplasmin are produced by the retina (Figure 5).

Western analysis with anti-ceruloplasmin antibody detected one band in the retina, but in brain, three bands were present. The two additional, higher molecular weight forms in the brain could represent cross reacting proteins, such as the ceruloplasmin homologue hephaestin [42,43], which should migrate in this region. Because of the significant size differences, they were not likely alternatively spliced and/or post-translationally modified forms of ceruloplasmin. The membrane-bound glycosylphosphatidylinositol (GPI)-anchored form of ceruloplasmin made by brain astrocytes results from alternative splicing leading to production of a C-terminus with a GPI linkage signal sequence [21]. However, this form of ceruloplasmin was unlikely to be resolved from the secreted form, only a few KDa smaller. In addition, the retina also has the GPI-linked form, which, if significantly different in size from the secreted from, should have been visible as a second band in the lane loaded with retinal protein.

Consistent with the previous report of higher levels of ceruloplasmin mRNA in retina than in brain [19], we find that ceruloplasmin protein levels are higher in normal retina than in brain (Figure 3B). Since the retina is exposed to large amounts of ROS produced because of its exposure to focused light, it may require higher levels of antioxidants to combat oxidative stress.

In summary, we show that ceruloplasmin levels increase following photic injury. Since the Age-Related Eye Disease Study (AREDS) indicates that oxidative stress is one of the causes of vision loss in AMD, further study of the potential antioxidative function of ceruloplasmin may suggest new preventative measures or treatments for AMD or other retinal diseases involving oxidative stress. The ferroxidase and iron transport functions of ceruloplasmin are particularly relevant to AMD since AMD maculas have increased iron relative to normal maculas (unpublished data). Our future studies will probe the anti-oxidative function of ceruloplasmin in retina in ceruloplasmin knock-out and transgenic mice, and in post mortem human retinas.

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