Redox Proteomic Identification of Visual Arrestin Dimerization in Photoreceptor Degeneration after Photic Injury

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PURPOSE. Light-induced oxidative stress is an important risk factor for age-related macular degeneration, but the downstream mediators of photoreceptor and retinal pigment epithelium cell death after photic injury are unknown. Given our previous identification of sulfhydryl/disulfide redox status as a factor in photoreceptor survival, we hypothesized that formation of one or more disulfide-linked homo- or hetero-dimeric proteins might signal photoreceptor death after light-induced injury.

METHODS. Two-dimensional (non-reducing/reducing) gel electrophoresis of Wistar rat retinal homogenates after 10 hours of 10,000 lux (4200°K) light in vivo, followed by mass spectrometry identification of differentially oxidized proteins.

RESULTS. The redox proteomic screen identified homodimers of visual arrestin (Arr1; S antigen) after toxic levels of light injury. Immunoblot analysis revealed a light duration-dependent formation of Arr1 homodimers, as well as other Arr1 oligomers. Immunoprecipitation studies revealed that the dimerization of Arr1 due to photic injury was distinct from association with its physiological binding partners, rhodopsin and enolase1. Systemic delivery of tris(2-carboxyethyl)phosphine, a specific disulfide reductant, both decreased Arr1 dimer formation and protected photoreceptors from light-induced degeneration in vivo.

CONCLUSIONS. These findings suggest a novel arrestin-associated pathway by which oxidative stress could result in cell death, and identify disulfide-dependent dimerization as a potential therapeutic target in retinal degeneration. (*Invest Ophthalmol Vis Sci.* 2012;53:3990–3998) DOI:10.1167/iovs.11-9321

Photic (light-induced) injury to photoreceptors and retinal pigment epithelium (RPE) is one of the risk factors for several retinal degenerations, including age-related macular degeneration (AMD), the most prevalent cause of blindness in developed countries.¹ Exposure to intense light in animal models causes retinal injury in otherwise normal animals,²

affecting primarily the rod photoreceptors, responsible for phototransduction, and the RPE, a pigmented tissue with multiple metabolic roles supporting the photoreceptors. Surprisingly, the other retinal neurons and the Müller cell glia are spared. Retinal neuronal cell death after intense light damage is associated with oxidative stress from generation of reactive oxygen species (ROS),^{3,4} and as might be expected, ROS scavengers (dimethylthiourea,⁵ ascorbate,⁶ thioredoxin,⁷ and desferrioxamine⁸) ameliorate the loss of photoreceptors in photic injury models.

The mechanism of RPE death in photic injury is associated with oxidative photocleavage products of fluorescent bisretinoids such as bis-retinaldehyde-phosphatidylethanolamine and other vitamin A derivatives that accumulate with age.^{9–11} For photoreceptors, it is less well understood how ROS transduce photic injury into initiation of a cell death program. There is evidence for lipid peroxidation and subsequent protein modifications in the rod outer segments of light-exposed retinas⁴ and other oxidative processes,¹² but the precise targets for these oxidative modifications within photoreceptors are unknown. Photoreceptors are protected from photic injury when there is an absence of the primary rod phototransduction molecule rhodopsin,¹³ or when its regeneration is blocked,¹⁴ implying that rhodopsin is a necessary component of the photic injury cascade. Yet whether there is a direct link between an oxidative modification or damage and rhodopsin is uncertain.

We approached the question of how photic-oxidative injury could initiate a cell death program in photoreceptors based on our previous observation that sulfhydryl redox status was a factor in photoreceptor survival.¹⁵ Specifically, the survival of acutely dissociated rat retinal cells (which are mostly rod photoreceptors) cultured in antioxidant-free media was remarkably higher when the sulfhydryl/disulfide redox couple was shifted to reduction using varying ratios of dithiothreitol (DTT) and 5,5'-dithiobis-(2-nitrobenzoic acid). We hypothesized that chemical modulation of the redox environment mediated oxidative injury to photoreceptors, and that the oxidative target(s) in photic injury was associated with sulfhydryl oxidative modification. To test this hypothesis, we performed a differential redox proteomic screen for proteins undergoing formation of disulfide-linked homo- or heterodimers in a photic injury model of retinal degeneration. We identified visual arrestin (Arr1) as undergoing a disulfide-dependent dimerization that can be reversed by chemical reduction, the latter resulting in photoreceptor neuroprotection after photic injury.

METHODS

Animals

Male Wistar rats were from Harlan Sprague Dawley (Indianapolis, IN) at 6 weeks of age, and reared on 12 hour light/12 hour dark cyclic light

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conditions with in-cage light levels of approximately 25 lux. Light exposure took place between 6 and 14 weeks of age. All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research.

In Vivo Photic Injury

Prior to exposure, rats were dark-adapted for 16 hours by placing them in a cage draped with a heavy black light-tight cloth cage cover in the later part of their normal 12-hour light cycle. Rats were transported and maintained in the dark, and administered injections under a red darkroom safelight to maintain dark adaptation. TCEP in saline (100 μ mol/kg) or saline was given intraperitoneally, or animals left untreated, followed by immediate exposure to 10,000 lux uniform, omnidirectional cool white fluorescent light (Sylvania, Danvers, MA) for 10 hours, within a mechanically ventilated Plexiglas tube with a suspended wire floor and freely available water. Light levels were measured with an Enviro-Meter (Fisher Scientific). After exposure, rats were euthanized immediately for protein studies or returned to the animal facility for 5 days prior to sacrifice for histological studies. Control animals remained unexposed to light without dark adaptation unless otherwise specified.

Two-Dimensional Non-Reducing/Reducing Electrophoresis for Proteomic Analysis of Redox State

Animals used for proteomic studies were sacrificed immediately after light exposure, and retinas harvested into equal volumes of radioimmunoprecipitation buffer containing protease inhibitors and 100 mM iodoacetamide (IAA) to prevent proteolysis and spontaneous disulfide formation, respectively. Approximately 20–40 μ g of retinal protein were separated by their oxidized molecular weights in the first dimension on a 4–20% Tris-glycine minigel. Then gel lanes were excised and incubated in 50 mM dithiothreitol (DTT) for 30 minutes, to reduce disulfide bonds, and rinsed in running buffer. This was immediately followed by a 10 minute incubation with 100 mM IAA again to alkylate the newly reduced disulfides and prevent reformation of the bonds between the proteins within the gel. Each excised gel lane was rinsed with running buffer to eliminate excess iodoacetamide, then rotated 90 degrees and placed into the 2D loading well of a second Tris-glycine minigel, and proteins separated in the second dimension based on their reduced molecular weight. Gels were stained with SYPRO Ruby (Invitrogen), and digitally imaged (Fotodyne, Hartland, WI). Differences in protein oxidation were detected manually for subsequent identification.

Identification of Differentially Disulfide-Dependent Spots by Mass Spectrometry

In each gel there were many spots below the diagonal, representing proteins that normally exist as disulfides within the cell. Any spot that appeared or disappeared with photic injury was picked because its disulfide state presumably reflects the effect of the injury. Only spots below the diagonal that were consistently present in non-reducing/reducing gels of photic injury but not non-photic injured retinas, or vice versa, were chosen for further analysis.

In gel digestion and mass spectrometric analysis of differentially oxidized proteins were performed at the Mass Spectrometry Facility, Biotechnology Center, University of Wisconsin-Madison. Cleavage was with trypsin. Peptide map fingerprint MS/MS analysis was performed on a 4800 Matrix-Assisted Laser Desorption/Ionization-Time of Flight-Time of Flight (MALDI TOF-TOF) mass spectrometer (AB SCIEX, Foster City, CA). Peptide fingerprints were generated scanning a 700–4000 Da mass range using 1000 shots acquired from 20 randomized regions of the sample spot at 4200 intensity of an OptiBeam on-axis Nd:YAG laser with 200 Hz firing rate and 3 to 7 ns pulse width in positive reflectron mode. The fifteen most abundant precursors, excluding trypsin

autolysis peptides and sodium/potassium adducts, were selected for subsequent tandem MS analysis, where 1200 total shots were taken with a 4700 laser intensity and 2 kV collision induced activation (CID) using air. Post-source decay (PSD) fragments from the precursors of interest were isolated by timed-ion selection and reaccelerated into the reflectron to generate the MS/MS spectrum. Raw data was deconvoluted using GPS Explorer (Version 3.6 [build 328]; AB SCIEX) software and submitted for peptide mapping and MS/MS ion search analysis against the Rodentia subset of the NCBI nr database (226,347 rodent protein entries; release date May 12, 2009) with an in-house licensed Mascot search engine (Version 2.1.0; Matrix Science, London, UK). The following variable modifications were considered: carbamidomethyl (C), deamidation (NQ), and oxidation (M). No fixed modifications were required. Enzyme specificity was set for the C-terminal side of KR unless the next residue was P, with one missed cleavage permitted. Peptide and fragment mass tolerances were ± 0.2 Da. Minimum threshold score was 66 ($P < 0.05$).

Immunoblot Analysis

After identification of a candidate protein undergoing dimerization, disulfide formation in the target was verified through immunoblot analysis in non-reducing and reducing conditions. Proteins were prepared as for two-dimensional analysis in the presence of iodoacetamide, separated on a 4–20% Tris-glycine minigel, then transferred overnight to a nitrocellulose membrane. The membrane was blocked in a 5% milk solution in Tris-buffered saline containing 0.05% Tween 20 (TBS-Tween). Membranes were probed with a rabbit polyclonal antibody against Arr1 (H-90; 0.1 μ g/ml; Santa Cruz Biotechnology, Santa Cruz, CA), followed by 3 rinses with TBS-Tween. The membrane was then probed with a peroxidase-conjugated goat anti-rabbit IgG antibody (0.04 μ g/ml; Jackson ImmunoResearch, West Grove, PA), rinsed 5 times with TBS-Tween, and bands detected by enhanced chemiluminescence.

Autoradiography films were scanned in 16-bit grayscale at 1200 dpi on an Expression 1680 scanner with a transparency unit (Epson America, Long Beach, CA), using a red filter to decrease variance that may be associated with polychromatic light¹⁶ and with color correction disabled. Blots were analyzed with standard gel analysis tools in NIH ImageJ software, selecting a region roughly one-third the width of a lane (0.1 inches) in the center of the lane for analysis.¹⁷ Band density was determined as the area under peak for all peaks of interest (arrestin monomer, presumed arrestin-arrestin homodimer, and presumed arrestin-enolase1 heterodimer). Saturation was avoided by requiring the presence of smooth rounded peaks on the densitometry plots for bands at an intensity less than the maximum achieved by saturated marker bands.¹⁷ Band density readings were calculated as the ratio of detected Arr1 dimer to Arr1 monomer, and compared across all experiments.

Immunoprecipitation

Animals were sacrificed immediately following light exposure and retinas harvested into NP-40 lysis buffer with protease inhibitors and 100 mM IAA and homogenized by sonication. Samples were incubated for one hour at 4°C with 100 μ L each of TrueBlot Anti-Mouse IgG bead slurry (eBioscience, San Diego, CA). After incubation, samples were centrifuged and the pellet discarded. The supernatant of each condition was split into two equal volumes, and 2 μ g of mouse anti-rhodopsin antibody (RET-P1; Santa Cruz Biotechnology), mouse anti-enolase1 (Enol2-53; gift of WC Smith, University of Florida, Gainesville, FL), or mouse anti-c-Myc antibody (9E 10; DSHB, University of Iowa, Iowa City, IA) was added to each sample along with 50 μ L of bead slurry. Samples were incubated overnight at 4°C, centrifuged, and the supernatant removed. The pellet was washed with NP-40 lysis buffer, and 50 μ L of 2 $\times$ SDS Tris-Glycine sample loading buffer was added to bead pellets. Samples were vortexed, heated to 100°C for 10 minutes, and centrifuged. Samples were reduced using 50 mM DTT and 25 μ L of

FIGURE 1. Photoc injury induces oxidative changes to visual arrestin. **(A)** Schematic of 2-dimensional non-reducing/reducing polyacrylamide gel electrophoresis. Proteins are prepared in the presence of a sulfhydryl alkylating agent, and separated in non-reducing conditions by molecular weight, allowing protein multimers to run at their combined weight. Lanes from the gel are excised and proteins reduced in situ with DTT, followed by alkylation of the free sulfhydryls to prevent spontaneous re-formation of disulfide linkage. Reduced lanes were then rotated and again separated by weight on a second gel, perpendicular to the first dimension. Cleaved multimers run at the size of their monomeric components, creating spots (circled) below the prominent diagonal of proteins that ran at identical sizes in the first and second dimension. **(B)** Representative diagonal gels of retinal proteins from control rats and those undergoing photoc injury. A protein with a consistently induced disulfide linkage is circled, running at a molecular weight of approximately 50 kDa.

sample was loaded into each lane of a 4–20% Tris-glycine gel. Immunoblots were carried out as described above using antibodies against Arr1, enolase1 (Enol2-53; 1:250), or rhodopsin (RET-P1; 80 ng/mL). Rhodopsin and enolase1 blots were probed using mouse IgG TrueBlot Ultra peroxidase-conjugated reagent (1:1000; eBioscience) to avoid detection of reduced primary antibody fragments.

Histology

Euthanasia was performed 5 days after photoc injury. Eye orientation was marked with cautery, and the eyes enucleated and fixed in 4% paraformaldehyde overnight at 4°C. Eyes were then rinsed with 100 mM phosphate buffer, and embedded in glycomethacrylate. Eyes were sectioned in the sagittal plane through the optic nerve head at a thickness of 2 microns, stained with toluidine blue, and cover slipped for further analysis.

Measurement of Outer Nuclear Layer Thickness

The retinal section through the optic nerve head that had the least histologic damage was identified for measurement and analysis. Overlapping segments of retina were digitally imaged on an Olympus BX61 microscope using cellSens Dimension software (Olympus America, Center Valley, PA) at 40× magnification, and reassembled into complete montages automatically by the software. The outer margin of the outer nuclear layer (ONL) containing the photoreceptor nuclei was traced from the optic nerve head, and divided into 100 μ m segments. A box bounding the ONL in each 100 μ m segment was drawn, followed by automatic detection of the ONL by the software through a hue saturation value (HSV) threshold, selecting for the darker

blue shades indicative of nuclei and excluding light and purple shades typical of the neighboring RPE, outer plexiform, and photoreceptor outer segments. The detected nuclear area within each 100 μ m segment was measured, and the results compiled across all experimental eyes by condition. Mean ONL nuclear thickness was determined by summing the total area contained in nuclei within the ONL in three consecutive 100 μ m sections, and dividing by 300 μ m.

Statistics

Comparisons of ratios of protein dimer to monomer abundance were with Students' *t*-test on log-transformed values. Comparisons among treatment groups of area measurements across the retina were by multifactorial ANOVA.

RESULTS

Arr1 Dimerization via Sulfhydryl Bond Formation in Photoc Injury

Male Wistar rats were dark adapted for 16 hours prior to exposure to omnidirectional (4200°K) fluorescent light at 10,000 lux for 10 hours. Proteins were then separated by two-dimensional non-reducing/reducing gel electrophoresis (Fig. 1A), stained with SYPRO Ruby, and digitally imaged. Proteins undergoing disulfide oxidation and dimerization as determined by a change in electrophoretic mobility after reduction—proteins running below the diagonal—were compared between unexposed and light-exposed retinal samples (Fig. 1B). One such spot (circled in Fig. 1B) was consistently absent in

FIGURE 2. Identification of the cross-linked protein. The protein of interest from Figure 1B was excised from the gel and identified by MALDI-TOF mass spectrometry as Arr1 (S-antigen). Matched peptide fragments cover 54% of the sequence.

unexposed samples but present in light-exposed samples. The spot was digested and identified by MALDI-TOF-TOF mass spectrometry as S-antigen, or Arr1 (Fig. 2), with a score of 877 (expect = 5.7×10^{-77}). Because of the critical role of Arr1 in phototransduction, it was chosen for further analysis. Enolase1 (α -enolase) was also detected by MALDI-TOF-TOF from the spot, with a score of 103 (expect = 1.1×10^{-5}).

A second spot was inconsistently present in samples from light-exposed but not unexposed retinas, and was identified as collapsin response mediator protein-2 (CRMP-2) by mass spectrometry. However, disulfide formation in CRMP-2 could not be confirmed by immunoblot analysis, and therefore this protein was not studied further.

To confirm the dimerization of Arr1 after photic injury, we studied immunoblots of retinal lysates against Arr1 from unexposed and light-exposed animals, running protein samples in the presence and absence of DTT. Immunoblot analysis confirmed the presence of the Arr1 protein at the expected weight of 48 kDa, as well as a photic injury-induced band at roughly twice the molecular weight of the monomer, and occasionally other oligomers (Fig. 3A). The degree of dimerization of Arr1, as determined by the ratio of the dimer band to the monomer band by densitometry of unsaturated

NCBI BLAST search of **gi|57178** against trypsin digest fragments
Variable modifications: Carbamidomethyl, Deamidation, Oxidation
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **54%**
Matched peptides are shown in **Red and Boxed**

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1 MAACVKTNKS HVIFKKVSRD KSVTIYLGKR DYIDHVSQVE
41 PVDGVVLVDP ELVK GK KVYV TLTCFRYQ EDIDVIGLTF
81 RRDLYFSRVQ VYPPVGAMSA PTQLQLSLR K KLGDNTYPFL
121 LTFPDYLPSC VMLQPAPQDV GKSCGVDFEV KAFATDITDA
161 EEDKIPK KSS VRLIRKQVH APPEMGQPC AEASWQFMS
201 DKPLHLVSLS SK EIYFHGEP IPVTVTVNN TEK VVKKIK V
241 SVEQIANVVL YSSDYVVKPV ASEETOEK VQ PNSTLT TLV
281 LVPLIANNRE R RGIALDGKI KHEDTNLASS TIKEGIDRT
321 VMGILVSYHI K VKLTVSGFL GELTSSEVAT EVFRLMHPQ
361 PEDPAKESVQ DENLVFEFA RQNLKDTGEN TEGK KDEDA
401 QDE
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blots, was significantly increased by intense light exposure (from 1.0% [95% CI 0.5, 2.1] to 7.2% [95% CI 4.4, 11.9]; $P = 0.0002$; $n = 17$). The presence of DTT eliminated the higher molecular weight bands, indicating that its presence was due to disulfide-linked dimer, and not a non-covalent association with another binding partner (Fig. 3A). The degree of dimerization appeared to increase with duration of photic exposure (Fig. 3B).

Arr1 associates with light-activated phosphorylated rhodopsin, thereby inactivating the latter in phototransduction. It was possible that the higher molecular weight Arr1 moiety identified by immunoblot analysis was an arrestin-rhodopsin heterodimer. To test this possibility, we performed two sets of experiments. First, immunoblots of retinal lysates from normal and light-exposed animals for both Arr1 and rhodopsin showed that the dimer band is not detected by a rhodopsin antibody (based on molecular weight of band) (Fig. 4A). The relative density of rhodopsin between the two conditions is also the opposite of what is seen with induction of the dimeric species, suggesting that what is observed is not due to an Arr1-rhodopsin complex. Second, co-immunoprecipitation of the proteins bound to rhodopsin shows that Arr1 binds to rhodopsin, but that the association does not show

FIGURE 3. Photic injury causes dimerization of visual arrestin. (A) Immunoblot analysis demonstrates induction of an Arr1 dimer in retinas exposed to 10 hours of photic injury (PI). This band could be eliminated by treating samples with a reducing agent (DTT, 50 mM) prior to electrophoresis. (B) Dimerization of Arr1 is associated with greater duration of photic injury. Levels of dimer formation showed a general upward trend correlating with duration of photic injury. Dark-adapted rats were exposed to photic injury for 0-10 hours, sacrificed, and the retinas electrophoresed and immunoblotted with antibody to Arr1.

FIGURE 4. Photic injury does not induce dimerization of visual arrestin with its physiological partners rhodopsin and enolase1. **(A)** The detected species is not an Arr1-rhodopsin heterodimer based on immunoblot analysis. Retinal lysates from control and light-exposed animals were blotted in non-reducing conditions and probed for Arr1 and rhodopsin. Rhodopsin was not detected at a molecular weight consistent with the presumed Arr1 dimer, nor was the distribution of rhodopsin immunoreactivity consistent with the observed pattern in the Arr1 blot. **(B)** The detected species is not an Arr1-rhodopsin heterodimer based on immunoprecipitation. Retinal proteins from control and light-exposed animals were immunoprecipitated for rhodopsin or c-Myc, reduced, and then probed for Arr1 or rhodopsin. Although the two proteins co-immunoprecipitated, their association did not increase with photic injury. Immunoprecipitation against c-Myc confirmed that observed bands were not due to denatured IgG present in the samples or non-specific precipitation of the proteins of interest. **(C)** The detected species is not an Arr1-enolase1 heterodimer based on immunoblot analysis. Retinal lysates from control and exposed animals were blotted in non-reducing conditions for both Arr1 and enolase1. Enolase1 was not detected at a molecular weight consistent with the presumed Arr1 dimer. **(D)** The detected species is not an Arr1-enolase1 heterodimer based on immunoprecipitation. Retinal proteins from control and exposed animals were immunoprecipitated for enolase1 or c-Myc, reduced, and then probed against both Arr1 and enolase1. Although the two proteins co-immunoprecipitated, their association did not increase (and in fact decreased) with photic injury. Immunoprecipitation against c-Myc confirmed that observed bands were not due to denatured IgG present in the samples or non-specific precipitation of the proteins of interest. **(E)** The Arr1 homodimer has a mass distinct from the Arr1-enolase1 heterodimer. Retinal lysates from control and exposed animals were blotted in non-reducing conditions, probed for Arr1 and enolase1, and films intentionally overexposed. Overexposure reveals a weak band on the enolase1 blot ("A-E"), consistent in size with an arrestin-enolase homodimer, which was distinct from the presumed Arr1 homodimer ("A-A").

the same light dependency as formation of Arr1 dimers (Fig. 4B), confirming that Arr1 dimerization was not confused with rhodopsin-Arr1 association. Co-immunoprecipitation against c-Myc was used to demonstrate that detected bands were not due to non-specific binding of rod photoreceptor proteins or denatured antibody from the immunoprecipitation process.

Smith et al. recently demonstrated the interaction of enolase1 and arrestin in dark-adapted bovine and *Xenopus* photoreceptors.¹⁸ Given that enolase1 was also present in the photic injury-induced spot below the diagonal (Fig. 1B), it was possible that the presumed Arr1 homodimer actually represented a light-induced Arr1-enolase1 heterodimer. To explore

this possibility, we studied the interaction of Arr1 and enolase1 in light-exposed and control retinas by immunoblot analysis and immunoprecipitation, in order to determine whether Arr1 binding to enolase1 was responsible for the observed dimer band. Retinal lysates probed for enolase1 did not demonstrate an induced band at the size of the detected arrestin dimer (Fig. 4C), and retinal protein extracts immunoprecipitated for enolase1 and probed for Arr1 did not show increased levels of arrestin with light exposure (Fig. 4D). Co-immunoprecipitation with antibodies against c-Myc demonstrated specificity of the immunoprecipitation analysis.

To further study whether an Arr1-enolase association could be confused with Arr1 dimerization in photic injury, we deliberately overexposed blots probed against Arr1 and enolase1 (Fig. 4E). Blots probed for enolase1 contained a faint high molecular weight band that ran at a higher mass than the observed Arr1 homodimer, and was consistent in mass with an Arr1-enolase1 heterodimer. Quantitative densitometry of this presumed Arr1-enolase1 heterodimer on arrestin blots failed to demonstrate a significant increase with photic injury (ratio photic injury to control = 1.7 ± 0.5 ; $P = 0.25$; $n = 12$), which was very different from what was seen with the Arr1 homodimer band (ratio photic injury to control = 5.8 ± 1.9 ; $P = 0.03$; $n = 17$). In retrospect, the Arr1-enolase band was also visible in non-reduced retinal immunoblots probed for Arr1 (Fig. 3A), and dark-adaptation increased its intensity, consistent with the finding of Smith et al.¹⁸ of an Arr1-enolase1 association in darkness. A low level of Arr1-enolase1 association in light-exposed animals, coupled with their similar monomeric weights, could be responsible for the identification of enolase1 by mass spectrometry in the excised Arr1-dimer spot in non-reducing/reducing two-dimension gel electrophoresis.

Reduction of Arr1 Dimers and Photoreceptor Degeneration after Photic Injury In Vivo

To determine if there was a relation between photic injury-induced neural death and the formation of Arr1 dimers, we tested whether pharmacological reduction of disulfides was neuroprotective of photoreceptors. Rats were treated with tris(2-carboxyethyl)phosphine (TCEP; 100 $\mu\text{mol/kg}$) or saline control by intraperitoneal injections immediately prior to 10 hours light exposure. Retinas were studied histologically and in non-reducing gel electrophoresis. The outer nuclear layer showed significant thinning when examined histologically 5 days post-injury, and this effect was significantly reduced in animals treated with TCEP, particularly in the inferior retina ($P = 0.005$; $n = 9, 5$, and 7) (Figs. 5A, 5B). Immunoblot analysis of retinal extracts harvested immediately after light exposure demonstrated that TCEP significantly diminished Arr1 dimer formation in exposed animals compared to saline (10.1% [95% CI 3.8, 21.1] vs. 3.5% [95% CI 1.1, 10.8]; $P = 0.037$; $n = 6$) (Fig. 5C). Together, these results are consistent with chemical reduction of one or more protein disulfides ameliorating cell death after photic injury, and suggest the possibility that dimerization of Arr1 or other targets could play a critical role in photic degeneration.

DISCUSSION

We employed a two-dimensional redox proteomic screen of proteins from light-exposed and control albino rat retinas to identify molecules undergoing disulfide-linked oligomerization as a result of photic injury. This screen yielded a single molecule, visual arrestin (Arr1) as consistently undergoing dimerization after photic injury. Disulfide bond formation was

confirmed by one-dimensional immunoblot analysis for Arr1 under non-reducing and reducing conditions. Dimer formation increased with duration of light intensity, in agreement with an inverse relationship between length of light exposure and photic damage. The dimer did not form stable complexes with rhodopsin or enolase1, physiological targets for Arr1 during phototransduction and dark adaptation, respectively. Finally, the disulfide reducing agent TCEP protected against thinning of the outer nuclear layer in a model of photic injury, and at the same time decreased levels of Arr1 dimerization.

Arr1 is a highly expressed protein in rod photoreceptors, with a primary role of binding light-activated phosphorylated rhodopsin (P•Rh*) and thereby inactivating phototransduction. Insufficient Arr1 results in shorter, disorganized outer segments, longer photoresponse from flash stimulation,¹⁹ and increased susceptibility to photic injury²⁰ in a mouse Arr1 knockout model. Bovine Arr1 is known to self-associate into dimeric and tetrameric forms for storage.²¹ These oligomeric structures are fully functional in binding microtubules and can be formed even by Arr1 molecules lacking cysteine residues, indicating that physiological oligomerization of Arr1 is not disulfide-mediated. Only the monomeric species are able to bind P•Rh*. The three cysteines contained in Arr1 are located near sites involved in P•Rh* binding,^{22,23} but are otherwise not normally exposed.^{22,24} However, it is likely that the interaction of Arr1 and P•Rh* results in exposure of those cysteines, based on studies with a peptide containing P•Rh*-like sequence.²⁵ This result could explain how continuous Rh activation would increase the probability of Arr1 disulfide-mediated dimerization. Conversely, disulfide formation via any of these cysteines could interfere with the interaction of Arr1 and P•Rh*.

It is tempting to speculate that Arr1 dimerization resulting from toxic levels of light exposure could be involved in the pathophysiology of photoreceptor loss in photic injury. However, our data do not prove that Arr1 dimerization is necessary for photoreceptor death, and there are several other explanations for the observation that Arr1 dimerizes in photic injury.

First, the formation of Arr1 disulfide-linked dimers in photic injury and the neuroprotection with the disulfide reducing agent TCEP from photic injury could simply be a coincidence. If that were the case, then the ability of TCEP to decrease photoreceptor cell death would likely be through the chemical reduction of a protein other than Arr1. Although we did not detect other retinal proteins undergoing disulfide formation after photic injury and that were also reduced by TCEP, low abundance proteins could easily be missed in our relatively sensitive redox proteomic screen. Given that cysteine residues are not critical for either P•Rh* binding or physiological (storage) oligomerization, then Arr1 dimerization (as a result of oxidative stress associated with toxic levels of light exposure) would likely interfere with Arr1 function, but not necessarily induce photoreceptor death.

Second, Arr1 dimers could be a necessary component for the mechanism by which light induces photoreceptor degeneration, for example, by transducing a light-dependent signaling pathway for cell death. Against this possibility is the observation that Arr1^{-/-} mice are not resistant, and in fact are more susceptible, to light-induced photoreceptor degeneration.²⁶ They also have a cone dystrophy independent of light exposure.²⁷ These findings imply that Arr1 dimerization is not necessary for photic injury in these mice.

Third, Arr1 dimerization could be sufficient but not necessary for photic injury, that is, Arr1 dimers could be toxic. This would not explain the resistance to photic injury in RPE65^{-/-} mice, where the failure to make 11-*cis*-retinal leads to decreased rhodopsin.¹³ An alternative explanation would be if

FIGURE 5. Reduction reduces photic injury in vivo. (A) Light exposure induces significant thinning of the ONL in Wistar rats. This thinning is significantly diminished by intraperitoneal administration of TCEP prior to light exposure. Representative sections shown of the region 1.8 to 2.0 mm inferior to the ONH. Scale bar shown represents 25 μm . (B) Cumulative ONL nuclear area (*left*) and ONL nuclear thickness (*right*) as a function of distance from the optic nerve head. Cumulative distance is plotted at 0.1 mm intervals, while mean ONL nuclear thickness is plotted at 0.3 mm intervals. The *thin dark lines* represent the mean area or thickness, and the *shaded bands* represent the corresponding SEM for each point. (C) Immunoblot analysis and 2D non-reducing/reducing gel electrophoresis confirm the visual Arr1 dimer as a target for reduction in vivo.

Arr1 dimer-dependent toxicity was also dependent on rhodopsin.

Fourth, Arr1 dimerization could induce photoreceptor death by reducing the amount of monomer available to arrest rhodopsin phototransduction. This would be consistent with the need for rhodopsin in photic injury¹³ and the light-dependent photoreceptor degeneration in Arr1-deficient animals¹⁹ or animals with reductions in levels of Arr1.²⁸ However, the dimer comprised only approximately 7–10% of the total Arr1 on immunoblot analysis, implying that a similarly small reduction in Arr1 monomer would have to significantly affect rhodopsin signaling. Even though the Arr1-rhodopsin ratio is

exquisitely controlled,²⁹ it is difficult to envision a biological system where a 7–10% change in concentration of a protein caused such a radical effect on cell survival over a short term.

Fifth, the Arr1 dimer could interfere with the physiological translocation of Arr1 monomer from the inner segments to outer segments, and indirectly induce photoreceptor death by inadequately arresting rhodopsin-dependent phototransduction. Equivalently, the dimer formation could be in the outer segments, where there are low levels of reduced glutathione, and the problem could be the translocation of Arr1 back to the inner segments. This abnormal translocation would result from the size-dependence of soluble protein distribution within the

photoreceptor, as has been recently demonstrated.³⁰ This hypothesis would also be consistent with a requirement for rhodopsin in photic degeneration.¹³ It is possible that arrestin dimers could also prevent migration of transducin from the outer segments, leading to a greater photoreponse. The relatively small amount of arrestin dimer could theoretically have a greater effect on monomer distribution than monomer amount, that is, act nonlinearly. And if dimerization were to block any of the regions required for selective binding of P•Rh*, it could serve to exacerbate the oxidative process by sequestering functional Arr1 monomers into an inactive dimeric state, creating a cycle of amplified levels of ROS.

Interpretation of these data has several caveats. First, the model of photic injury that we used, that is, albino rats and 10 hours of white light, is one of several models for photic injury, and is not necessarily predictive of other models, for example, with non-albino animals or chronic light exposure. This is of particular importance when comparing our results to studies of photic injury in Arr1-deficient mice, RPE65-deficient mice, and other genetic models, which are typically on a C57BL/6 background. Second, we only saw robust neuroprotection with disulfide reduction in the inferior retina. We presume that the relative lack of neuroprotection in the superior retina relates to the much greater severity of injury there, but we cannot rule out that there is a mechanistic difference between the nature of the pathophysiological response to injury in the two hemispheres. Third, we observed low but non-zero levels of Arr1 homodimers and other oligomers in retinas not exposed to photic injury. Although the levels were small (1.0% of the monomer concentration), we do not know why Arr1 dimers would exist at low abundance in normal retinas. We also cannot exclude that this finding is artifact from the isolation procedure.

Chemical Reduction of Disulfide Bonds and Photoreceptor Neuroprotection

TCEP is a potent disulfide reductant, which our previous studies have shown to be an effective neuroprotectant against axonal injury in retinal ganglion cells. TCEP increases ganglion cell survival after axotomy in both in vitro¹⁵ and in vivo³¹ models. Protection from light-induced photoreceptor degeneration has been achieved using scavengers of ROS^{5,8,32,33} and upregulation or delivery of gene products that create a reducing environment in the retina.^{7,34,35} However, the mechanism of action of TCEP is different, in that it reduces disulfide bonds.³¹ The most likely explanation is that sulfhydryl oxidation of one or more proteins is downstream of generation of one or more reactive oxygen species. Consequently, reduction of these “toxic” dimers is a potential therapeutic modality that would be downstream of scavenging of reactive oxygen species.

The ability of TCEP to chemically reduce protein dimers such as Arr1 and provide neuroprotection when administered close to the onset of injury was significant, despite its low membrane permeability.³⁶ Glutathione is virtually absent in the outer retina and photoreceptor layers, and it has been hypothesized that photoreceptors renew their outer segments as a way to cope with constitutive levels of oxidative damage in the presence of normal light in lieu of producing high levels of antioxidants and reductants.³⁷ Therefore, even a small concentration of TCEP achieved within photoreceptors might be effective given the lack of glutathione in the outer segments and consequent low levels of reduction potential.

Given the findings of the present study, it is possible that prevention or reversal of signaling protein dimerization with disulfide reducing agents could be a novel pathway for treatment not only of photic injury, but also diseases associated with oxidative damage to photoreceptors, such as dry age-

related macular degeneration. Further study is needed to determine if such dimers are formed in models more closely resembling macular degeneration, and if those dimers play a causal role in light-induced and other retinal degenerations.

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