

ABCR, the ATP-binding Cassette Transporter Responsible for Stargardt Macular Dystrophy, Is an Efficient Target of All-*trans*-retinal-mediated Photooxidative Damage *in Vitro*

IMPLICATIONS FOR RETINAL DISEASE*

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A large body of experimental and clinical data have documented the damaging effects of light exposure on photoreceptor cells although the identities of the biologically relevant molecular targets of photodamage are still uncertain. Several lines of evidence point to retinoids or retinoid derivatives as chromophores that can mediate light damage. We report here that ABCR, a photoreceptor-specific transporter involved in the recycling of all-*trans*-retinal, is unusually sensitive to photooxidation damage mediated by all-*trans*-retinal *in vitro*. Partial loss of ABCR function is responsible for Stargardt macular dystrophy, which is associated with accumulation of A2E, a diretinoid adduct within the retinal pigment epithelium. Photodamage to ABCR causes it to aggregate in SDS gels and results in the loss of retinal-stimulated ATPase activity. Peripherin/RDS and ROM-1, two structural proteins that colocalize with ABCR at the outer segment disc rim, are also significantly more susceptible to all-*trans*-retinal-mediated photodamage than are the major proteins from the rod outer segment. These observations imply that there may be specific protein targets of photodamage within the outer segment, and they may be especially relevant to assessing the risk of light exposure in those individuals who already have diminished ABCR activity due to mutation in one or both copies of the *ABCR* gene.

The retina is the only part of the central nervous system that is directly exposed to light, and it has long been known that excessive light exposure leads to retinal damage (1). Acute light toxicity, for example that incurred by directly viewing a solar eclipse, has been recognized since antiquity; in Plato's *Phaedo*, Socrates advises that such injuries can be avoided by viewing the eclipse via its reflection in water (2). Indirect exposure to sunlight can also impair visual function if it occurs over an extended period. Among military personnel exposed to bright sunlight and its reflection from water or sand, temporary elevations in the threshold for dark adaptation have been reported in individuals exposed without sunglasses for 3–4 h each day

over a 2-week period (3), and decreased visual acuity and macular pigmentary changes have been reported following several months of exposure (4–8). Of potentially greater significance to public health is the effect that decades of exposure to 'normal' light levels may have on the retina and underlying retinal pigment epithelium (RPE)¹ (9). The clinical relevance of this exposure is suggested by two recent epidemiological studies showing a positive correlation between light exposure and age-related macular degeneration, the most common cause of visual impairment in the elderly (10–12).

An extensive body of work has documented the damaging effects of acute and chronic light exposure in laboratory animals (reviewed in Refs. 13–15). Most of these studies have assessed the appearance of the retina and RPE through the ophthalmoscope and in tissue sections using light or electron microscopy; some studies have also assessed retinal function via the electroretinogram. These studies have employed a variety of animal species and light exposure protocols and, as a result, have produced a number of disparate conclusions regarding the initial cellular targets for light damage, the sensitivity of those targets to different wavelengths and intensities, and the natural history of tissue injury and repair. Despite the lack of a detailed consensus, several broad themes have emerged, and these are summarized below.

Acute light damage involves both the photoreceptors and RPE, but the relative susceptibilities of each cell layer and the subcellular compartments within which damage occurs vary with wavelength (13–15). Experiments in which rodents or monkeys are exposed to intense light over a period of minutes to hours, as well as clinical data from humans exposed to intense lights as an occupational hazard (e.g. arc welders), indicate that light at the blue end of the visible spectrum is ~50-fold more potent in producing acute damage than is light from the midspectral region (2, 16–19). By contrast, chronic exposure to relatively low light levels over a period of weeks or months produces photoreceptor damage with an action spectrum that peaks at ~500 nm and coincides with the absorption spectrum of rhodopsin (20–22), strongly suggesting that visual pigment activation and/or release of the all-*trans*-retinal chromophore plays a role in light damage. A role for retinal or other retinoids in light damage is further supported by the observations that (*a*) photoexcited all-*trans*-retinal can generate singlet oxygen (23, 24) and mediate photooxidative damage of lipids (25, 26), proteins (26, 27), or DNA (28) *in vitro* (reviewed

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¹ The abbreviations used are: RPE, retinal pigment epithelium; OS(s), outer segment(s); ROS(s), rod outer segment(s); RDH, retinal dehydrogenase; ABC, ATP-binding cassette; BSA, bovine serum albumin; mW, milliwatt.

in Ref. 29), (b) dietary vitamin A depletion partially protects the retina from chronic light damage (30, 31), and (c) the retinas of RPE65(−/−) mice, which have little or no rhodopsin secondary to defective production of 11-*cis*-retinal in the RPE, are highly resistant to acute light toxicity (32).

Although the identities of the biologically significant molecular targets of photodamage are not yet known, the mechanisms by which they are damaged have been inferred from experiments in which damage to the retina is abrogated when an antioxidant or free radical scavenger is administered prior to light exposure (33, 34). These experiments indicate that photooxidative mechanisms play a significant role in the generation of light damage. Consistent with this idea, a number of investigators have documented photooxidation of lipids in photoreceptor outer segments (OSs) following irradiation *in vivo* or *in vitro* (25, 35, 36). By contrast, only a few studies have addressed the question of protein targets of light damage within the photoreceptor. These studies have demonstrated oxidation of the rhodopsin sulfhydryl groups following irradiation of rod outer segments (ROSs) *in vitro* (26, 27) and an ~2-fold decline in ROS retinol dehydrogenase (RDH) activity 20 h after exposure of rats to intense visible light (37).

One or more chromophores distinct from those involved in photoreceptor damage are presumed to be responsible for light-mediated RPE damage. In searching for the relevant chromophore(s) a number of investigators have focused on the large quantities of age-dependent pigments, lipofuscin, that accumulate within the RPE (38–41). One major component of human RPE lipofuscin is a diretinal adduct, A2E, that appears to form within the photoreceptor OS from the spontaneous condensation of phosphatidylethanolamine and the all-*trans*-retinal released from photoactivated rhodopsin (42–44). Phagocytosis of OSs by the RPE results in accumulation of A2E within the RPE where it appears to be trapped within phagolysosomes. A2E efficiently absorbs blue light and is phototoxic to RPE cells in culture (45); its progressive accumulation within the RPE suggests that with age the RPE may become increasingly susceptible to phototoxic damage. Thus both photoreceptor and RPE photodamage may be mediated, at least in part, by retinoids or retinoid derivatives.

The most recent line of evidence suggestive of a role for retinoids in photodamage comes from the study of autosomal recessive Stargardt disease. In Stargardt disease the cycling of retinoids between photoreceptors and RPE, a process referred to as the visual cycle, is defective. Stargardt disease arises from a partial loss of function of ABCR (46–51), an ABC transporter that resides in the internal (disc) membranes of photoreceptor OSs (52–54). Stargardt disease is the most common form of early onset macular degeneration, and it is characterized by a progressive accumulation of large quantities of lipofuscin including A2E within the RPE, delayed dark adaptation, and a progressive loss of central vision (43, 55–61). ABCR gene mutations leading to partial loss of function can also cause recessive cone-rod dystrophy (62), complete loss of ABCR function causes autosomal recessive retinitis pigmentosa (62–64), and heterozygosity for either of two sequence variants in the ABCR gene has been implicated as a risk factor for age-related macular degeneration (65). Purified and reconstituted ABCR exhibits all-*trans*-retinal-stimulated ATP hydrolysis *in vitro* (51, 66, 67), and ABCR knockout mice exhibit an acute light-dependent accumulation of all-*trans*-retinal within the OS and a progressive light-dependent accumulation of A2E in the RPE (43, 68). Based on these data, ABCR appears to function as an all-*trans*-retinal transporter/flippase that delivers all-*trans*-retinal to RDH, the OS enzyme responsible for conversion of released all-*trans*-retinal to all-*trans*-retinol prior to its delivery to the RPE.

In this paper, we report that *in vitro* ABCR is unusually sensitive to photooxidative damage mediated by all-*trans*-retinal. If this type of photodamage also occurs *in vivo* it would diminish the rate of reduction of all-*trans*-retinal in the OS, and the resulting increase in all-*trans*-retinal would be predicted to lead to further phototoxic damage and an accelerated accumulation of A2E. Photodamage of this type may be particularly relevant to understanding pathophysiological mechanisms and assessing the risk of light exposure in those individuals with inherited retinal diseases caused by partial loss of ABCR function.

EXPERIMENTAL PROCEDURES

ROS Preparation—Dark-adapted bovine ROSs were prepared by the method of Papermaster (69). Unless otherwise noted, all ROS preparations were used at a concentration of 1 mg/ml rhodopsin. For depletion of both free and opsin-bound retinal, ROSs were exposed to visible light for 60 min at room temperature in the presence of 10 mM hydroxylamine. An equal volume of BSA buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 4% BSA) was added to the irradiated ROS suspension, and the ROSs were incubated on ice for 20 min and recovered by centrifugation at 15,000 × *g* for 5 min at 4 °C. The pelleted ROSs were resuspended and subjected to a second incubation in BSA buffer, recovered by centrifugation, and stored in aliquots at −80 °C.

SDS-Polyacrylamide Gel Electrophoresis and Immunoblot Analysis of Recombinant ABCR, ROS ABCR, and Other ROS Proteins—Human ABCR was produced in transiently transfected 293 cells and azido-[³²P]ATP labeling was performed with 302 nm irradiation from a Spectrolyne TR-302 transilluminator as described in Ref. 51. For immunoblot assays of light damage, 293 membranes containing ABCR or dark-adapted bovine ROSs in resuspension buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, and 3 mM MgCl₂) were added to a 0.3-ml glass vial or a 1.5-ml microcentrifuge tube, and either all-*trans*-retinal (>95% pure by high pressure liquid chromatography; Sigma) in ethanol or ethanol alone was added to a final ethanol concentration of 1%. In a typical irradiation experiment, each 20 μl of reaction contained 0.5 mg/ml rhodopsin for ROS samples or 1 mg/ml total protein for 293 membrane samples as determined by the Bradford assay (BioRad). For UV damage, samples in glass vials or uncapped microcentrifuge tubes were placed 5 cm from a long-wave UV lamp (peak emission 366 nm; irradiance 2 mW/cm²; model UVL-56, UVP, Inc.) and irradiated from above for the indicated length of time. For exposure to visible light, samples in uncapped microcentrifuge tubes were placed in a room temperature water bath and irradiated at a distance of 2 cm for 2 h from above with a fluorescent light source (F15T8-CW cool white 15 watt lamp; irradiance 1.5 mW/cm²; General Electric) filtered through a 420-nm cut-off filter (GG-420, 1 mm thick, Schott Glass Technologies, Inc.). Light sources were calibrated with a United Detector Technologies model 61 optometer. After irradiation, an equal volume of 2× SDS loading buffer was added, and the samples were immediately loaded onto a 6% (see Figs. 3, 5, upper panel, and 6) or 10% (see Figs. 1, 4, and 5, lower panel) SDS-polyacrylamide gel electrophoresis resolving gel with a 5% stacking gel without sample heating. The gels were transferred to nitrocellulose at 140 volts for 1 h at 4 °C. ABCR was detected by immunoblotting using a rabbit polyclonal antibody against human ABCR (ABCR1156–1258; Ref. 53), peripherin/RDS was detected with monoclonal antibody 2B6, ROM-1 was detected by monoclonal antibody 1D5, the α subunit of the cGMP channel was detected with monoclonal antibody 1D1 (gifts of Dr. R. Molday), and arrestin was detected with rabbit polyclonal antisera (a gift of Dr. T. Shinohara). Following incubation with a horse radish peroxidase-conjugated secondary antibody, the immunoblots were developed using the SuperSignal West Pico chemiluminescent substrate (Pierce).

ATPase Assays of Photodamaged ABCR—ROS ABCR was purified, reconstituted into membranes, and assayed for ATPase as described (51).

ROS GTPase Assays—Each complete GTPase reaction contained 5 μl of ROSs and 100 μl of 1× GTPase buffer (20 mM Hepes, pH 7.5, 50 mM KCl, 5 mM MgCl₂, 100 μM GTP containing 106 cpm [^γ-³²P]GTP). Prior to addition of 1× GTPase buffer, 10 mM all-*trans*-retinal in ethanol was added to an aliquot of dark-adapted ROSs to give a final all-*trans*-retinal concentration of 100 μM. A control aliquot of ROSs received only ethanol. The ROS samples were then exposed to room light for 1 min, an exposure sufficient to bleach >90% of the rhodopsin. Half of each sample was further exposed to 366 nm light as described for ABCR assays for 10 min at room temperature while the other half was kept in the dark. The GTPase reaction was initiated by the addition of 1× GTPase buffer and incubated at 37 °C under dim red light. At 5 min

intervals, 15 μ l of aliquots were removed from each reaction and vigorously mixed with a 200- μ l suspension of activated charcoal; released 32 P was measured in the supernatant after removing the charcoal by centrifugation.

Rhodopsin Reconstitution—10 mM all-*trans*-retinal in ethanol was added to retinoid-depleted ROSs in 20 mM Hepes, pH 7.5, 50 mM KCl to give a final all-*trans*-retinal concentration of 100 μ M. Control ROSs received only ethanol. Following incubation in the dark or exposure to 366 nm light for 10 min as described for ABCR assays, 30 μ l of aliquots of the ROS samples were added to 400 μ l of 20 mM Hepes, pH 7.5, 50 mM KCl, 5 mM MgCl₂, 10% glycerol, 0.05% BSA, 0.6% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS), and 50 μ M 11-*cis*-retinal, and the mixtures were incubated at room temperature in the dark for 10 min. Preliminary experiments demonstrate that under these conditions reconstitution with 11-*cis*-retinal proceeds with a half-life of less than 1 min. Hydroxylamine was then added to a final concentration of 50 mM, and the incubation continued for an additional 10 min at which point the samples were frozen on dry ice. Photobleaching difference spectra were obtained in a water-jacketed cuvette by bleaching with an intense visible light delivered to the cuvette by a fiber optic cable. All spectra were recorded with a Uvikon 860 spectrophotometer and analyzed using Cricket Graph software.

RDH Assays—Recombinant bovine RDH was prepared by transient transfection of 293 cells with a RDH expression plasmid followed by membrane purification as described in ref. 70. ROS RDH activity was measured starting with retinoid-depleted ROSs. All-*trans*-retinal addition to a final concentration of 100 μ M and irradiation with 366 nm light for 10 min were performed with the 293 and ROS membranes as described above for the ROS GTPase assays. The RDH reaction was initiated by solubilizing the 293 or ROS membranes, which had been stored on dry ice following the pretreatment regimen in 400 μ l of 20 mM Hepes, pH 7.5, 50 mM KCl, 5 mM MgCl₂, 10% glycerol, 0.05% BSA, 0.6% CHAPS, 100 μ M NADPH, and 50 μ M all-*trans*-retinal. The reaction was monitored spectrophotometrically as described in Ref. 70. A control reaction containing 100 μ M NADH in place of NADPH showed barely detectable RDH activity.

RESULTS

Protein Targets of All-*trans*-retinal-mediated Photodamage in Vitro—The investigation reported here began with an experiment to determine whether all-*trans*-retinal affects the affinity of ABCR for ATP. To this end we asked whether all-*trans*-retinal alters the efficiency of photo-cross-linking of azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ to ABCR expressed in 293 cells. As seen in Fig. 1A, all-*trans*-retinal produces a dose-dependent loss in ^{32}P labeling of ABCR at the expected monomer mobility of \sim 250 kDa. By contrast, azido- $[\beta\text{-}^{32}\text{P}]\text{ATP}$ photo-cross-linking to an endogenous protein of \sim 55 kDa is unaffected by addition of all-*trans*-retinal. Although this result might be interpreted as evidence for an inhibition by all-*trans*-retinal of azido-ATP binding to ABCR, immunoblotting with anti-ABCR antibodies revealed instead that exposure of ABCR to UV light (the regimen used for azido-ATP photo-cross-linking) in the presence of all-*trans*-retinal, converts ABCR from a species that migrates as an apparent monomer in SDS gels to one that appears to be aggregated and is largely retained at the origin (Fig. 1B). This all-*trans*-retinal- and UV light-dependent aggregation of ABCR is independent of azido-ATP. Moreover, a comparison of the bulk proteins within 293 membrane preparations by Coomassie Blue staining indicates that aggregation is remarkably selective for ABCR (Fig. 1B).

These observations suggested the possibility that all-*trans*-retinal-dependent photodamage might impair ABCR function in the intact human eye. In humans, the lens protects the retina from UV damage by efficiently absorbing light of $<$ \sim 410 nm (Fig. 2; Ref. 71). Although the peak absorption of all-*trans*-retinal is at 380 nm, the longwave limb of the absorption curve produces appreciable absorption at wavelengths $>$ 410 nm as seen in Fig. 2, suggesting that ambient light might produce a significant level of photoexcitation of all-*trans*-retinal within the human eye. We therefore asked whether all-*trans*-retinal-dependent aggregation of ABCR can be produced by visible

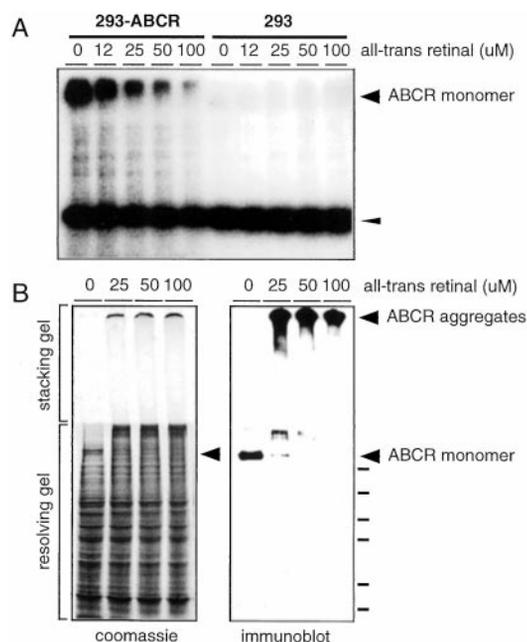


FIG. 1. The loss of azido- $[\beta\text{-}^{32}\text{P}]\text{ATP}$ labeling of ABCR produced by all-*trans*-retinal reflects aggregation of ABCR. A, azido- $[\beta\text{-}^{32}\text{P}]\text{ATP}$ labeling of 293 cell membranes from cells expressing ABCR (left) or from nontransfected cells (right). The samples were exposed to 302 nm light for 10 min at room temperature in the presence of the indicated concentrations of all-*trans*-retinal. A protein of \sim 55 kDa (small arrowhead) is radiolabeled with azido-ATP in 293 membranes and is unaffected by all-*trans*-retinal addition. ABCR (large arrowhead) migrates at \sim 250 kDa. B, Coomassie Blue stain of membrane proteins from transfected 293 cells (left) and the corresponding immunoblot with anti-ABCR antibodies (right) following a 10-min irradiation with 366 nm light in the presence of the indicated concentrations of all-*trans*-retinal. Bars at right indicate from top to bottom the molecular mass standards of 220, 130, 90, 70, 60, 40, and 30 kDa.

light filtered to approximate the filtering effect of a human lens. As seen in Fig. 3, a 2-h exposure to visible light of $>$ 410 nm produces aggregation of ABCR that increases with increasing concentration of all-*trans*-retinal. The loss of monomeric ABCR is more pronounced in 293 membranes than in ROS membranes, a difference that may relate to differences in lipid composition or to the high levels of antioxidants in ROS membranes, as discussed more fully below. In many of the experiments reported below, we have used a brief (10–20 min) exposure to 366 nm light rather than an extended exposure to $>$ 410 nm light because the shorter wavelength light produces efficient photoexcitation of all-*trans*-retinal and aggregation of ABCR and its use minimizes the problem of competing reactions that might occur during an extended incubation time.

We next asked whether other ROS proteins are also subject to light- and all-*trans*-retinal-dependent aggregation. As seen in Fig. 4, there is little or no aggregation of two integral membrane proteins, the α subunit of the cGMP gated channel and rhodopsin, or of two peripheral membrane proteins, arrestin and rhodopsin kinase. By contrast, peripherin/RDS and ROM-1, homologous integral membrane proteins that, like ABCR, are localized to the OS disc rim, aggregate upon exposure of ROSs to all-*trans*-retinal and 366 nm light as does ABCR. At present, the chemical basis for protein aggregation is unknown. The resistance of the aggregates to incubation in 2% SDS and 5% β -mercaptoethanol at room temperature suggests that aggregation involves covalent cross-links distinct from disulfide bonds.

As noted in the introduction, it has long been known that photoexcitation of all-*trans*-retinal leads to the production of singlet oxygen (23, 24). To determine whether ABCR and pe-

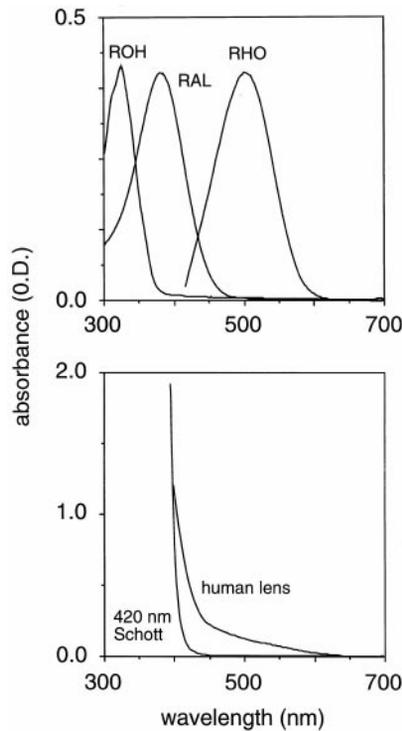


FIG. 2. Absorption spectra of retinoids and filters. Upper panel, all-*trans*-retinol (ROH, absorption maximum 325 nm), all-*trans*-retinal (RAL, absorption maximum 380 nm), rhodopsin (RHO, absorption maximum 500 nm). Lower panel, the human lens (71) and a shortwave cutoff filter (1 mm thick GG-420; Schott Glass Technologies, Inc.) used to approximate the human lens for irradiation experiments with visible light. Both the lens and the GG-420 filter exhibit an absorbance >1 at wavelengths <410 nm (OD).

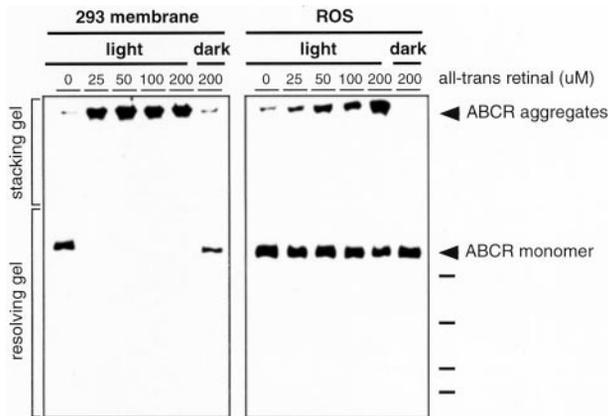


FIG. 3. Visible light and all-*trans*-retinal induce dose-dependent aggregation of both expressed ABCR and ROS ABCR *in vitro*. Samples containing the indicated concentrations of all-*trans*-retinal were irradiated for 2 h with a fluorescent light filtered through a GG-420 shortwave cutoff filter. For a given all-*trans*-retinal concentration, aggregation of ABCR is greater in 293 membranes than in ROSs. Bars at right indicate from top to bottom the molecular mass standards of 220, 130, 90, and 70 kDa.

ripherin/RDS aggregation is oxygen-dependent, increasing concentrations of all-*trans*-retinal were added to ROSs in room air, 100% oxygen, or argon and exposed to 366 nm light (Fig. 5). In this experiment, efficient aggregation of both proteins was observed in room air and in 100% oxygen but not in argon, strongly suggesting that all-*trans*-retinal-dependent photo-damage involves the production of oxygen radicals.

The experiments described above involve the addition of exogenous all-*trans*-retinal to ROS or 293 membranes. In the experiments with ROS membranes, the rhodopsin concentra-

tion in the reaction mixture is $12.5 \mu\text{M}$, and therefore $25 \mu\text{M}$ exogenous all-*trans*-retinal, the lowest concentration tested, corresponds to twice the concentration of rhodopsin-bound retinal. *In vivo*, free all-*trans*-retinal is released from the photo-activated visual pigment and is subsequently reduced within the OS to all-*trans*-retinol by RDH in a NADPH-dependent reaction. Conversion of all-*trans*-retinal to all-*trans*-retinol would be predicted to dramatically reduce photooxidation by >410 nm light because the absorption spectrum of all-*trans*-retinol is blue-shifted by ~ 55 nm relative to that of all-*trans*-retinal (Fig. 2). In addition, all-*trans*-retinol has been reported to have modest antioxidant activity (72). To determine whether light-dependent aggregation of ABCR is promoted by endogenous all-*trans*-retinal and whether conversion of all-*trans*-retinal to all-*trans*-retinol protects ABCR from aggregation, dark-adapted ROSs were exposed to >410 nm or 366 nm light in the presence or absence of NADPH without exogenous all-*trans*-retinal (Fig. 6, left). This experiment reveals appreciable light-dependent aggregation of ABCR that is largely abrogated by the inclusion of NADPH during the period of irradiation. The protective action of NADPH presumably reflects enzymatic conversion of endogenous all-*trans*-retinal to all-*trans*-retinol because no protection is seen upon addition of NADH or NADP (Fig. 6, right), neither of which can support reduction by photoreceptor RDH (70, 73).

Functional Assays of *in Vitro* Photodamage—The aggregation of ABCR observed in SDS gels presumably reflects a significant structural alteration and suggests a corresponding functional impairment. *In vitro*, ABCR exhibits ATPase activity, which is stimulated by all-*trans*-retinal, a coupling that is presumed to reflect the vectorial transport of all-*trans*-retinal driven by ATP hydrolysis. At present, no *in vitro* transport assay exists for ABCR. In an initial experiment to assess the functional effects of photodamage, we immunoaffinity purified ABCR, reconstituted it into lipid membranes, and asked whether ATPase activity was affected by exposure to 366 nm light in the presence of $20 \mu\text{M}$ all-*trans*-retinal. As seen in Fig. 7A, irradiation of ABCR in the presence of $20 \mu\text{M}$ all-*trans*-retinal led to an $\sim 60\%$ loss of ATPase activity relative to a control reaction in which all-*trans*-retinal was irradiated prior to the addition of ABCR. As shown below, most of this loss of ATPase activity is referable to that part of the total ATPase activity that is stimulated by all-*trans*-retinal.

In a second experiment, irradiation of ABCR was carried out in the presence of a variable concentration of all-*trans*-retinal to determine whether there was a dose-dependent loss of basal or retinal-stimulated ATPase activity (Fig. 7B). Control reactions in which various concentrations of all-*trans*-retinal were irradiated prior to the addition of ABCR show no loss of ATPase with increasing concentration of irradiated all-*trans*-retinal (open squares); indeed, this set of samples shows an increase in ATPase activity with increasing concentrations of preirradiated all-*trans*-retinal indicating that irradiated all-*trans*-retinal still retains its ability to stimulate the ABCR ATPase activity, consistent with the previously observed stimulatory activity of various retinal isomers (66). By contrast, an otherwise identical set of samples in which ABCR and variable concentrations of all-*trans*-retinal were irradiated together show no stimulation of ATPase activity with increasing concentrations of irradiated all-*trans*-retinal (open circles). Addition of fresh all-*trans*-retinal to a final concentration of $30 \mu\text{M}$ produces an ~ 2 -fold increase in ATPase activity over the basal level (filled squares), and this increase is inhibited in a dose-dependent manner by prior irradiation of ABCR in the presence of all-*trans*-retinal (filled circles) with nearly complete inhibition at $30 \mu\text{M}$ all-*trans*-retinal. This experiment reveals

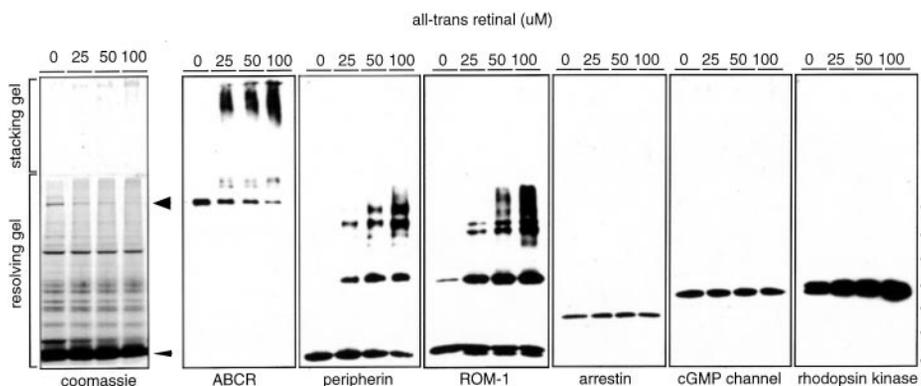


FIG. 4. UV light and all-*trans*-retinal induce dose-dependent aggregation of a subset of ROS membrane proteins *in vitro*. Irradiation was with a 366-nm light for 10 min. Both the stacking and resolving gels are shown. *Left*, Coomassie Blue stain of ROS proteins. *Large arrowhead* indicates the position of monomeric ABCR; *small arrowhead* indicates opsin. *Right*, immunoblotting results for six ROS proteins. ABCR, peripherin/RDS, and ROM-1 show all-*trans*-retinal-dependent aggregation; arrestin, the α subunit of the cGMP-gated channel, rhodopsin kinase, and the major Coomassie Blue-stained proteins do not. *Bars at right* indicate from top to bottom the molecular mass standards of 220, 130, 90, 70, 60, 40, and 30 kDa.

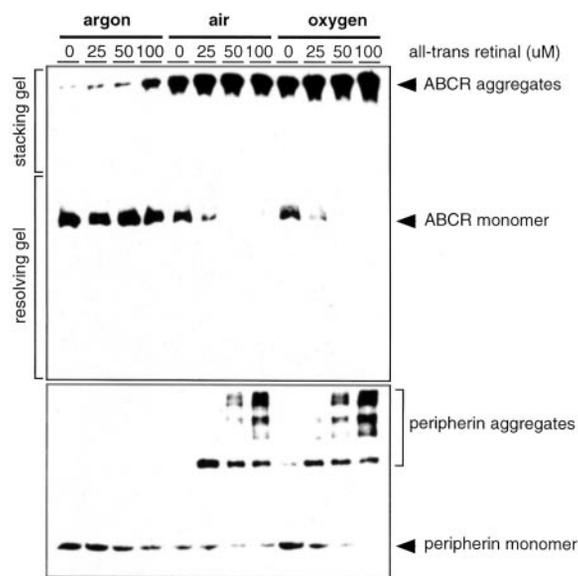


FIG. 5. UV light and all-*trans*-retinal promote aggregation of ABCR and peripherin/RDS *in vitro* in an oxygen-dependent reaction. Samples of ROSs containing the indicated concentrations of all-*trans*-retinal were sealed in glass vials, and the atmosphere within the vial was either maintained as room air, exchanged for argon, or exchanged for oxygen prior to irradiation for 10 min with 366 nm UV light. The *upper* immunoblot probed with anti-ABCR antibodies shows both stacking and resolving gels; the *lower* immunoblot probed with anti-peripherin/RDS antibodies shows only the resolving gel.

that all-*trans*-retinal-dependent photodamage has little effect on the basal ATPase activity of ABCR (compare the data points represented by *open circles* and *open squares*). As site-directed mutagenesis shows that most of the basal ATPase activity appears to be derived from the first nucleotide binding domain (51), the selective loss of retinal-stimulated ATPase may reflect photodamage to the transmembrane transport domain and/or to the second nucleotide binding domain with little or no damage to the first nucleotide binding domain.

To extend the functional analysis of ABCR described above to other ROS proteins, we examined the effect of irradiation in the presence or absence of exogenous all-*trans*-retinal on the regeneration of opsin within ROS membranes, the activity of ROS GTPase, and the activity of ROS and recombinant RDH. For the first three assays we used ROSs that had been largely depleted of endogenous retinoids by extensive photobleaching in the presence of hydroxylamine followed by washing with BSA. As seen in Fig. 8, exposure of retinoid-depleted ROSs to 366 nm light in the presence of 100 μ M all-*trans*-retinal had no

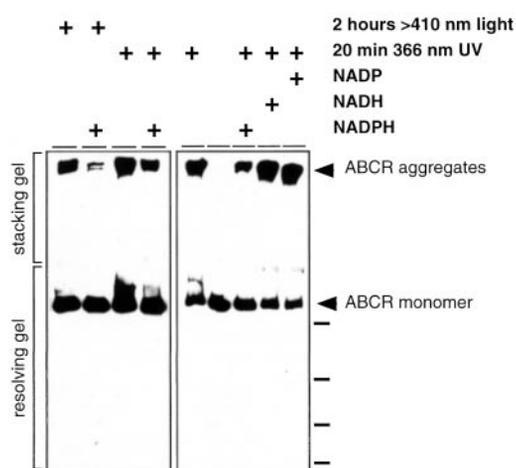


FIG. 6. NADPH but not NADH protects ROS ABCR from UV and visible light damage *in vitro* in the absence of exogenous all-*trans*-retinal. Wavelengths and times of irradiation are indicated. NADP, NADH, or NADPH was added to a final concentration of 1 mM to the indicated tubes. Of the three compounds only NADPH confers protection, presumably reflecting the reduction of released all-*trans*-retinal by ROS RDH. *Bars at right* indicate the molecular mass standards as described for Fig. 3.

effect on the subsequent regenerability of opsin with fresh all-*trans*-retinal. By contrast, ROS GTPase activity was reduced \sim 40%, and ROS RDH activity was reduced \sim 30% relative to unirradiated controls. Similar treatment of recombinant RDH in 293 cell membranes produced an \sim 60% loss in activity consistent with a protective effect of ROS membranes relative to 293 membranes as noted above for ABCR photodamage. The decrease in ROS GTPase and ROS RDH activities in samples exposed to UV light without added all-*trans*-retinal relative to the samples that were not exposed to UV light may reflect photodamage mediated by residual endogenous all-*trans*-retinal. The modest decreases in ROS RDH and GTPase activities following light exposure in the presence of 100 μ M all-*trans*-retinal indicates that these proteins are relatively resistant to photodamage.

DISCUSSION

The principal conclusions of this study are that irradiation of ABCR in the presence of all-*trans*-retinal and oxygen *in vitro* leads to its rapid modification and functional inactivation. ABCR, together with peripherin and ROM-1, are significantly more susceptible to all-*trans*-retinal-mediated photodamage than are the major membrane and membrane-associated pro-

FIG. 7. UV light and all-trans-retinal produce a dose-dependent loss in the ATPase activity of purified and reconstituted ABCR. The schematic diagram at the bottom shows (from left to right) the order of addition of components and of irradiation for each of the sample sets. Irradiation was at 366 nm for 10 min in all cases. *A*, all-trans-retinal at 20 μM was irradiated either in the presence or absence of reconstituted ABCR, and then ABCR ATPase was monitored in the presence of fresh all-trans-retinal added to the indicated final concentrations. Note that for panel *A* 100% ATPase activity refers to the activity level in the presence of 20 μM preirradiated all-trans-retinal. *B*, the indicated concentrations of all-trans-retinal were irradiated either in the presence or absence of ABCR, and then ABCR ATPase was monitored in the presence or absence of 30 μM fresh all-trans-retinal. The final concentrations of lipid and ABCR were identical in all samples. Note that for panel *B* 100% ATPase activity refers to the basal activity level, i.e. the activity in the absence of added all-trans-retinal.

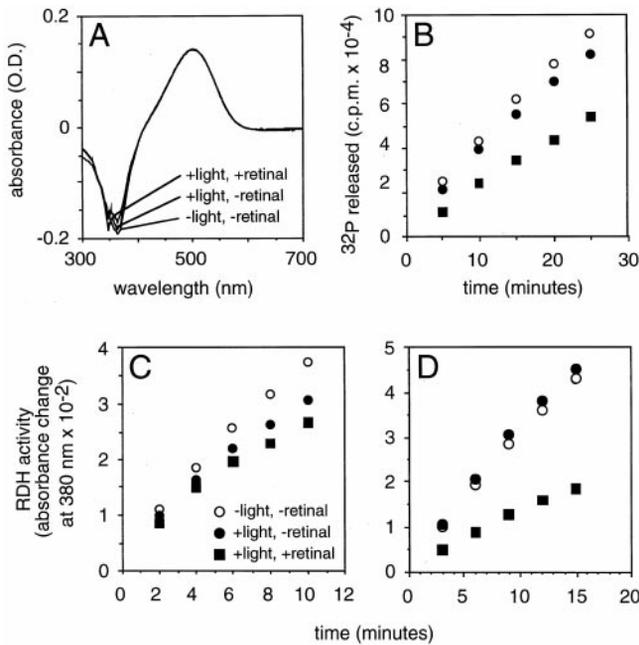
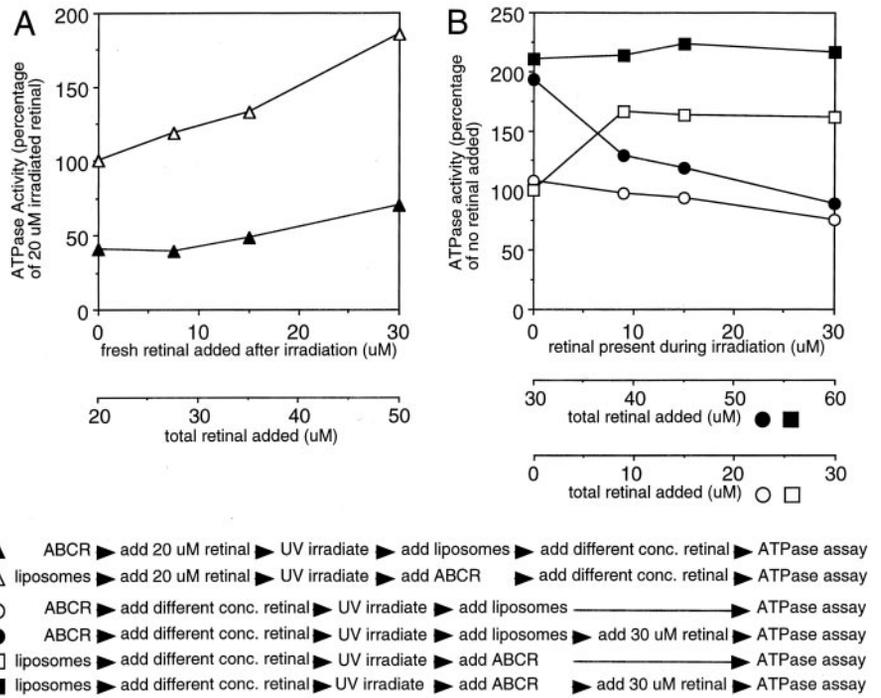


FIG. 8. Effect of all-trans-retinal and UV light exposure *in vitro* on reconstitution of bovine opsin with 11-cis-retinal (A), ROS GTPase activity (B), ROS RDH activity (C), and recombinant RDH activity in transfected 293 membranes (D). Samples were exposed to the following pretreatments: a 10-min incubation in the dark (-light, -retinal; open circles in B-D), irradiation with 366 nm light for 10 min (+light, -retinal; filled circles in B-D), irradiation with 366 nm light for 10 min in the presence of 100 μM all-trans-retinal (+light, +retinal; filled squares in B-D). ROSs were largely depleted of endogenous retinoid prior to UV irradiation as described under "Experimental Procedures."

teins from transfected 293 cells or from ROSs. ROS RDH and GTPase activities show moderate susceptibility to all-trans-retinal-mediated photodamage, but the regenerability of opsin is unaffected under the same conditions. All-trans-retinal-mediated photodamage is produced efficiently by longwave UV light, but it is also produced by light that has been filtered to approximate the wavelength distribution impinging on the hu-

man retina. ABCR photodamage occurs more readily in membranes from 293 cells than from ROSs consistent with the previously described high concentration of antioxidants in ROSs (72, 74). Following release of endogenous all-trans-retinal from rhodopsin in photobleached ROSs, ABCR photodamage can be significantly abrogated by addition of NADPH to the preparation presumably by promoting the reduction of all-trans-retinal to all-trans-retinol (Fig. 9). Finally, ABCR photodamage selectively eliminates retinal-stimulated but not basal ATPase activity, suggestive of damage to the transmembrane transport domain. These data suggest the interesting possibility that the susceptibility of ABCR to all-trans-retinal-mediated photodamage is related to the presumed function of ABCR as a retinal transporter.

All of the experiments described here were performed *in vitro*. It will therefore be important to determine the extent to which this type of photodamage occurs in the living eye and in which species and under which conditions it occurs. In the paragraphs that follow, we relate the present observations to the cell biology and physiology of photoreceptors and to the pathophysiology of degenerative retinal disease under the working hypothesis that photodamage of the type described here can occur in the living human eye.

ABCR, All-trans-retinal, and the Gradient of Recovery of Photosensitivity along the OS—Photoreceptor OS renewal occurs at a constant rate throughout life. New ROS constituents are synthesized within the photoreceptor cell body and as new discs are added at the base of the ROS older discs are progressively displaced to more distal locations within the ROS until they are engulfed and degraded by the RPE. Because discs move along the ROS without exchanging their integral membrane protein components, these proteins age as a cohort. In mammals, 10% of the ROS length is turned over per day, and therefore the membrane proteins at the distal end of each ROS are uniformly 10 days old (Fig. 9B).

It has long been assumed that ROS turnover exists as a mechanism for eliminating proteins or lipids that are impaired because of accumulated damage (9). Evidence that discs exhibit a progressive functional impairment as they age comes from suction electrode recordings in which different regions along

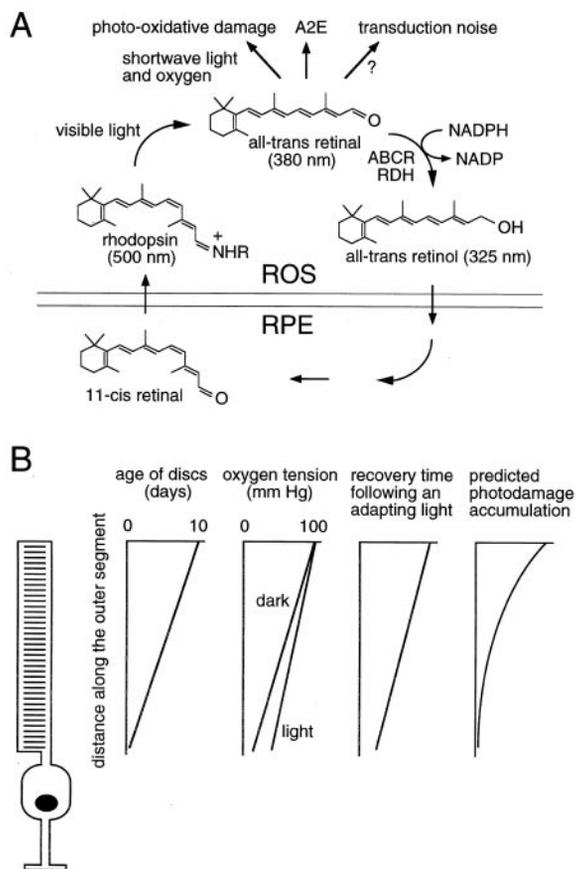


FIG. 9. Schematic diagrams showing the visual cycle and the characteristics that are known or are predicted to change along the length of the ROS. *A*, the principal transformations of the visual cycle that occur within the ROS. Three aberrant fates for released all-*trans*-retinal are shown at the top of the diagram. The absorption maxima of the relevant retinoids are indicated. The surface membranes of the ROS and RPE are indicated by horizontal lines. *B*, a rod cell (*left*) and summary plots (*right*) indicating, as a function of distance along the outer segment, the age of the discs (for mammals), oxygen tension (data from cats and monkeys; Refs. 88, 89), recovery time following an adapting stimulus (data from toads; Ref. 75), and the predicted accumulation of photodamage as described under "Discussion."

the OS of a toad rod are exposed to an adapting light. After the period of adaptation, the time course of local recovery of sensitivity is measured with a series of dim test lights (75). In dark-adapted rods, test flashes elicit responses of nearly identical amplitude independent of position along the OS, indicating little or no effect of disc age on the efficiency of the phototransduction cascade. However, following a stimulus estimated to bleach several percentage of the rhodopsin within the irradiated OS region, there is a delay in recovery of sensitivity that increases with increasing distance from the inner segment (Fig. 9*B*). Additional experiments in which an adapting stimulus delivered to the proximal OS was found to have no effect on the recovery kinetics of the distal OS suggest that the delay in recovery of the distal OS does not reflect limiting amounts of a diffusible substance from the inner segment but rather is an intrinsic property of the distal OS.

A number of studies have pointed to opsin, released all-*trans*-retinal, and/or other as yet unidentified products of photobleaching as potential sources of transduction noise, which limit photosensitivity during recovery from an adapting stimulus (Fig. 9*A*). For example, binding of all-*trans*-retinal to opsin *in vitro* leads to a low level of activation (76–78), and *in vivo* the initial rise and subsequent decline in all-*trans*-retinal following intense illumination parallels the loss and recovery of visual sensitivity (79–84). These studies suggest that reduction of

all-*trans*-retinal to all-*trans*-retinol by the combined action of ABCR and RDH plays a role in recovery of photosensitivity by lowering the concentration of free all-*trans*-retinal. In keeping with this idea, Stargardt disease patients and ABCR knockout mice exhibit a pronounced delay in dark adaptation (58, 68). With respect to the delay in the recovery of photosensitivity in the distal OS described in the preceding paragraph, a decrement in ABCR activity secondary to accumulated photodamage would be predicted to slow the rate of reduction of all-*trans*-retinal and could therefore contribute to the observed delay in recovery of sensitivity.

Following a strong bleaching light, direct measurements of the rate of reduction of all-*trans*-retinal along the length of the OS, made by observing the fluorescence of all-*trans*-retinol, show a spatially uniform accumulation of all-*trans*-retinol during the ensuing 10–20 min (85). However, extrapolation of these data to physiological bleaching levels is difficult because the experimental requirement for extensive photobleaching releases all-*trans*-retinal in quantities far above saturation for RDH, and this may mask local differences in the efficiency of ABCR-mediated transport. Whether reduction of all-*trans*-retinal occurs more slowly in the distal OS following an adapting light in the physiological intensity range remains an open question.

All-trans-retinal and the Gradient of Photodamage along the OS—In both rats and monkeys, acute exposure to broad spectrum visible light produces a disorganization of photoreceptor OSs that is most prominent in the distal region of the OS where the discs are oldest (34, 86, 87). Assuming that photooxidative damage via all-*trans*-retinal represents one of the mechanisms of this damage, we suggest that this spatial pattern could reflect the following several factors either singly or in combination: (a) an intrinsic vulnerability of older discs perhaps related to the cumulative effect of previous photodamage; (b) the proximity to the choroidal blood supply, which produces an oxygen concentration gradient such that the distal OS is at a pO_2 of ~100 mm of mercury (equivalent to arterial blood), whereas the proximal OS is at a pO_2 of ~10 mm of mercury in the dark and ~30 mm of mercury in the light as a result of its proximity to the mitochondria-rich inner segment, the principal region of oxygen consumption within the retina (Fig. 9*B*, Refs. 88 and 89); (c) the proximity to the RPE, which in the mammalian retina, produces more rapid rhodopsin regeneration in the distal OS (90) presumably because of more efficient access to RPE stores of 11-*cis*-retinal; and (d) the greater distance from the inner segment, which could limit access to NADPH or substrates coupled to the local production of NADPH by ROS glucose-6-phosphate dehydrogenase (91, 92) thereby limiting the rate of reduction of all-*trans*-retinal to all-*trans*-retinol. We speculate that the morphological disorganization of OS discs following light damage might reflect all-*trans*-retinal-mediated photooxidative damage to peripherin/RDS and/or ROM-1, which together are proposed to form part of the scaffold that maintains discs morphology (93) and which, as shown here, are both highly susceptible to all-*trans*-retinal-mediated photodamage.

The Visual Cycle, All-trans-retinal-mediated Photodamage, and A2E—All-*trans*-retinoids released by photobleaching of rhodopsin flow from the OS to the RPE to be reisoimerized and then returned to the OS (Fig. 9*A*). Reduction of all-*trans*-retinal to all-*trans*-retinol appears to be the rate-limiting step in the visual cycle as measured in the living rodent eye (81, 84). As noted in the introduction, a small fraction of all-*trans*-retinal, together with OS phosphatidylethanolamine, reacts to form A2E. This reaction requires the condensation of two all-*trans*-retinal molecules, and therefore the rate of A2E production

should be proportional to the square of the concentration of free all-*trans*-retinal. Because a brief high intensity light stimulus will lead to a high peak concentration of free all-*trans*-retinal, such a stimulus will lead to the formation of more A2E than would the same number of photons delivered as a prolonged low intensity stimulus. Moreover, those stimuli that are strong enough to release quantities of all-*trans*-retinal that saturate the ABCR/RDH pathway will lead to the accumulation of free all-*trans*-retinal for an extended period within the OS.

Complete loss of ABCR gene function in mice leads to an accumulation of all-*trans*-retinal within the OS following a light stimulus, and complete or partial loss of ABCR gene function in mice or humans leads to an increase in the accumulation of A2E in the RPE (43, 68). Once formed, A2E is extremely stable as judged by the observation that placing ABCR^(-/-) mice in the dark does not detectably lower the level of A2E that has already accumulated within the RPE (43). If this result can be extrapolated to humans and to time scales of decades rather than months, it would suggest that small decrements in ABCR function may be of clinical significance because small increases in A2E production would be cumulative. By contrast, decrements in the function of phototransduction or structural proteins would not be expected to produce a cumulative effect. Based on the logic of the preceding paragraph, it is reasonable to suppose that inherited ABCR defects also lead to an increase in all-*trans*-retinal-mediated photooxidation products within the OS, which, like A2E, could accumulate within the RPE. Presumably photodamage to ABCR would lead to the same constellation of effects observed with inherited ABCR defects.

Photodamage and OS Degradation within the RPE—Each human RPE cell contacts ~50 rod OSs, and therefore each RPE cell engulfs and degrades the equivalent of five OSs/day (94), a level of constitutive phagocytic activity greater than that of any other cell in the human body. As noted above, one obvious risk in such a system is that relatively small quantities of nondegradable compounds produced within the OS may, over time, accumulate to appreciable levels within the RPE, a problem compounded by the minimal turnover of RPE cells *in vivo* (95). The problem of nondegradable compounds may be especially acute with photooxidation products, which are likely to encompass a large variety of molecular structures, at least some of which may not be substrates for lysosomal enzymes, and which therefore may accumulate in lysosomes/phagosomes. By comparison, keratinocytes, the principal class of light-exposed cells in the skin, are shed externally so that there is no requirement for lysosomal processing of photooxidation products. These considerations emphasize the advantages of minimizing photooxidative damage within the OS especially in species that are long-lived.

Protective Mechanisms within the OS—The significance of photooxidation for the retina is underscored by the long-standing observation that photoreceptor OSs are rich in endogenous antioxidants, including vitamin E, glutathione, and taurine (71, 73). The greater susceptibility of ABCR to photooxidative damage when irradiated in 293 *versus* ROS membranes (Fig. 3) demonstrates the functional protection afforded by these ROS anti-oxidants. A second protective strategy within the OS is the reduction of all-*trans*-retinal to all-*trans*-retinol (Fig. 9A). In addition to decreasing any effects that all-*trans*-retinal may have in generating postbleaching noise, reduction of all-*trans*-retinal would be expected to decrease photodamage because the all-*trans*-retinol absorption spectrum is blue shifted ~55 nm relative to that of all-*trans*-retinal (Fig. 2). In humans, the ~410-nm shortwave cutoff provided by the lens effectively eliminates light absorption by all-*trans*-retinol but admits light

that can be absorbed by the longwave tail of the all-*trans*-retinal absorption spectrum (Fig. 2). Moreover, *in vivo*, the protective effect of reduction of all-*trans*-retinal may be even greater than the comparison between these two absorption spectra might indicate because all-*trans*-retinal in the OS efficiently forms Schiff bases with phosphatidylethanolamine. As the pK_a of these Schiff bases is ~6 (96), 5–10% of them will be protonated at physiological pH, and these will absorb maximally at 440 nm. Finally, sequestration of free retinal by intracellular retinoid-binding proteins would be expected to protect other outer segment constituents from photodamage.

Implications for Retinal Disease Susceptibility—If accumulation of A2E and/or OS-derived photooxidation products represent a risk factor for retinal disease then by implication light exposure *per se* should also constitute a risk factor both because light promotes the release of all-*trans*-retinal from bleached visual pigment and because light absorption by released all-*trans*-retinal promotes photooxidation (Fig. 9A). The efficiency with which all-*trans*-retinal produces ABCR photodamage *in vitro* suggests an additional risk of light exposure: partial inactivation of ABCR with a resulting decrease in the rate of reduction of all-*trans*-retinal. The observation that partial loss of ABCR function by gene mutation produces progressive retinal disease is consistent with the hypothesis that any environmental processes that lead to a decrease in ABCR function may increase the risk or accelerate the development of retinal disease. An environmental effect of this type may be most relevant to individuals with partial loss of ABCR activity due to gene mutation(s) because a decrease in ABCR activity would presumably have a greater adverse impact in this context than it would in the context of normal ABCR function.

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