Light Damage in Abca4 and Rpe65rd12 Mice

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PURPOSE. Bisretinoids form in photoreceptor cells and accumulate in retinal pigment epithelium (RPE) as lipofuscin. To examine the role of these fluorophores as mediators of retinal light damage, we studied the propensity for light damage in mutant mice having elevated lipofuscin due to deficiency in the ATP-binding cassette (ABC) transporter Abca4 (Abca4−/− mice) and in mice devoid of lipofuscin owing to absence of Rpe65 (Rpe65rd12).

METHODS. Abca4−/−, Rpe65rd12, and wild-type mice were exposed to 450-nm light to produce a localized lesion in the superior hemisphere of retina. Bisretinoids of RPE lipofuscin were measured by HPLC. In histologic sections, outer nuclear layer (ONL) thickness was measured as an indicator of photoreceptor cell degeneration, and RPE nuclei were counted.

RESULTS. As shown previously, A2E levels were increased in Abca4−/− mice. These mice also sustained light damage–associated ONL thinning that was more pronounced than in age-matched wild-type mice; the ONL thinning was also greater in 5-month versus 2-month-old mice. Numbers of RPE nuclei were reduced in light-stressed mice, with the reduction being greater in the Abca4−/− than wild-type mice. In Rpe65rd12 mice bisretinoid compounds of RPE lipofuscin were not detected chromatographically and light-damage-associated ONL thinning was not observed.

CONCLUSIONS. Abca4−/− mice that accumulate RPE lipofuscin at increased levels were more susceptible to retinal light damage than wild-type mice. This finding, together with results showing that Rpe65rd12 mice did not accumulate lipofuscin and did not sustain retinal light damage, indicates that the bisretinoids of retinal lipofuscin are contributors to retinal light damage.

Keywords: Abca4, bisretinoid, light, lipofuscin, retina, retinal degeneration, retinal pigment epithelium

It is well known that the retina is subject to photochemical damage when exposed to prolonged periods of light of increased intensity.2,3 Light damage to both photoreceptor cells and retinal pigment epithelial (RPE) cells has been observed. However, the mechanisms that lead to the damage and which irradiance levels can be considered safe are not completely understood. Conclusions as to whether photoreceptor cells or RPE are damaged first and which cells are affected more severely have varied. For instance, the loss of photoreceptors cells is greatest when intense light exposure begins during the normal nighttime phase of the diurnal cycle.3,4 The death of both cell types occurs by means of programmed apoptotic processes.5,6 Two action spectra for photochemical damage to the retina have been described: one adheres to the absorption spectrum of rhodopsin and has been recorded by using long exposures (>24 hours) at low irradiances,7,8 while the other exhibits decreasing thresholds at shorter wavelengths and is associated with high irradiances and shorter exposure periods.9,10

Light-induced retinal injury is linked to the availability of the 11-cis-retinal chromophore of visual pigment. This feature is evidenced by the protection against light damage provided by conditions that block or slow the regeneration of 11-cis-retinal. These conditions include null mutations in Rpe6511 and lecithin:retinol acyltransferase12; amino acid variants in Rpe6513,14; systemic delivery of 15-cis-retinoic acid15; and halothane anesthesia that interferes with 11-cis-retinal binding to the opsin molecule.16 On the other hand, an increase in rhodopsin content in rod outer segments increases light damage susceptibility.17 Studies18 also indicate that the superior hemisphere of rat retina may be more sensitive to phototoxicity because of greater concentrations of rhodopsin.

Although light exposure triggers photoreceptor cell apoptosis only after photo-isomerization of visual pigment, phototransduction may not be necessary.6,12,19 Products of bleaching such as all-trans-retinal (λmax ~ 580 nm),6,12 or metarhodopsin II20,21 have been suggested as the damaging agent. An extensive bleach of rhodopsin can potentially release ~3 mM all-trans-retinal,21 yet a single bleaching event does not induce photoreceptor cell death in mice.9 Moreover, all-trans-retinal has an absorbance maximum of 380 nm and only little absorbance above 450 nm;22 at these longer wavelengths retinal photodamage readily occurs.

Since repetitive photon absorption is required to elicit retinal damage, photoproducts formed after sustained activation of rhodopsin could accumulate and act as photosensitizers causing cellular damage.7,23 Potential initiators that fit this description are the lipofuscin fluorophores of retina. The vitamin A aldehyde-derived fluorophores of this lipofuscin form in photoreceptor cells as byproducts of photon absorbance by the 11-cis-retinal chromophore of visual pigment; this origin is consistent with evidence coupling retinal light damage
to activation of visual pigment. Under conditions of dark-rearing, the bisretinoids of lipofuscin probably also form from reactions of 11-cis-retinal. After being transferred to RPE when shed outer segments are phagocytosed, these fluorophores subsequently accumulate as lipofuscin in the lysosomal compartment of RPE. Significantly, native lipofuscin in the RPE exhibits an excitation spectrum with a range of approximately 425 to 580 nm and a peak at approximately 480 nm. Individual bisretinoid fluorophores that have been identified exhibit excitation maxima at 435, 439, 443, 490, and 510 nm. These relatively long excitation wavelengths are enabled by carbon–carbon double bonds that form extended conjugation systems in the bisretinoid molecules. Lipofuscin isolated from RPE cells can serve as a photo-inducible generator of singlet oxygen and superoxide anion and in the presence of light and RPE lipofuscin, lipid undergoes peroxidation. Retinal pigment epithelium cells that contain lipofuscin or one of its constituents, A2E, are subject to mitochondrial and nuclear DNA damage and a loss of viability.

To examine the role of RPE lipofuscin as a mediator of light damage, we studied the susceptibility to light damage of wild-type mice as compared to mice burdened with elevated RPE lipofuscin due to a null mutation in the ATP-binding cassette (ABC) transporter Rpe65Leu450/mice. We also studied mice that do not synthesize lipofuscin owing to deficiency in Rpe65 (Rpe65−/− mice).

**METHODS**

**Mice**

Albino Abca4/Aber null mutant mice and Abca4/Aber wild-type mice (homozygous for Rpe65-Leu450), were generated and genotyped by PCR amplification of tail DNA. For genotyping of the murine Rpe65 variant, digestion of the 545-bp product with Msel restriction enzyme (New England Biolabs, Ipswich, MA) indicated the Leu450 variant if 180- and 305-bp fragments were generated. Individual bisretinoid fluorophores that have been identified exhibit excitation maxima at 435, 439, 443, 490, and 510 nm. These relatively long excitation wavelengths are enabled by carbon–carbon double bonds that form extended conjugation systems in the bisretinoid molecules. Lipofuscin isolated from RPE cells can serve as a photo-inducible generator of singlet oxygen and superoxide anion and in the presence of light and RPE lipofuscin, lipid undergoes peroxidation. Retinal pigment epithelium cells that contain lipofuscin or one of its constituents, A2E, are subject to mitochondrial and nuclear DNA damage and a loss of viability.

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**High-Performance Liquid Chromatography**

A2E was measured by HPLC as we have described previously. Briefly, murine eyecups were homogenized, extracted in chloroform/methanol (2:1) and after drying, the extract was redissolved in 50% methanolic chloroform containing 0.1% trifluoroacetic (TFA) and analyzed by reverse phase HPLC (Alliance System; Waters Corp., Milford, MA) using a C18 column. Gradients of water and acetonitrile with 0.1% TFA were used for the mobile phase. Chromatographic peak areas were determined by using Empower software (Waters Corp.), and molar quantity per eye was calculated by using external standards of synthesized compounds, the structures of which have been confirmed.

**Light Exposure**

Mice were anesthetized with 80 mg/kg ketamine and 5 mg/kg xylazine, anesthesia that does not interfere with light-damage models. Pupils of right eyes were dilated with phenylephrine hydrochloride (2.5%; Alcon Laboratories, Fort Worth, TX) and cyclopentolate hydrochloride (0.5%, Alcon Laboratories), and ViscoTears Liquid Gel (2 mg/g; Novartis Pharmaceuticals, North Ryde, NSW, Australia) was applied to the surface of the cornea to maintain hydration and clarity. The cornea was positioned at a constant distance from the light source and to eliminate light scatter at the corneal surface, a thin glass plate (diameter, 7.5 mm; thickness, 0.4 mm) was placed on the cornea; light lost by absorption was <10%. Light intensity was measured with a Newport optical power meter (model 840; Newport Corporation, Irvine, CA). Light (430 ± 20 nm) was delivered to the superior hemisphere of the retina from a mercury arc lamp at an intensity of 50 mW/cm² for 30 minutes. Throughout the period of illumination the operator carefully observed the positioning so as to re-adjust if shifts in the position occurred. Right eyes were light exposed and left eyes served as unexposed controls.

**Histometric Analysis**

Seven days after light exposure, eyes were fixed in 4% paraformaldehyde for 24 hours at room temperature. Hematoxylin-eosin–stained, sagittal, 6-mm paraffin sections were prepared. For measurement of outer nuclear layer (ONL) thickness, three sections through the optic nerve head (ONH) of each eye were imaged digitally (Leica Microsystems, Leica, Buffalo Grove, IL) with an ×10 objective, and ONL thickness was measured at 200-μm intervals from the ONH to a position 2.4 mm superior and inferior to the ONH along the vertical meridian. Outer nuclear layer width in pixels was converted to micrometers (1 pixel = 0.92 μm) and data from the three sections were averaged. For groups of Abca4+/+ and Abca4−/− mice at each age, mean ONL thickness at each position along the vertical meridian was plotted as a function of eccentricity from the ONH. Outer nuclear layer area was calculated as the sum of the ONL thicknesses in superior hemiretina (ONH to 1.0 mm) with multiplication by the measurement interval of 0.2 mm.

To count RPE nuclei, three sections through the ONH were imaged with the ×40 objective. Nuclei were counted within 200-μm intervals beginning at the edge of the ONH and continuing superiorly along the vertical axis. Data from the three sections were averaged to obtain mean values for each measurement interval. Mean nuclei numbers within the 0.2-mm measurement intervals were plotted as a function of distance from ONH to 1.6 mm superiorly. Counts were also summed to obtain total numbers of RPE.

**Statistical Analysis**

Significance was assessed at the 0.05 level (Prism 6; GraphPad Software, Inc., La Jolla, CA) by using statistical tests as indicated.

**RESULTS**

**HPLC Quantitation of A2E**

As formerly reported, deficiency in Abca4 was associated with substantial accumulations of lipofuscin pigments in the RPE (Fig. 1). We quantified A2E, a bisretinoid compound of RPE lipofuscin, by integrating HPLC peaks and normalizing to a calibration curve. A2E levels in the Abca4+/+ mice were 3.8-fold greater than in wild-type mice at 5 months of age. However, the pattern of change in the two groups of mice was different. Specifically, while A2E increased steadily from 2 to 8 months of age in the Abca4+/+ mice, the amounts in Abca4−/− mice rose between 2 and 5 months of age and then leveled off at 8 months of age (Fig. 1).
ONL Thickness

Consistent with previous work, our light damage model yielded a lesion in superior central retina; the lesion size was \(~800\) \(\mu\)m along the vertical meridian. The measurement of ONL is an established approach to assessing photoreceptor cell integrity. Thus, to detect the light-induced lesion, we measured and plotted ONL thicknesses as a function of distance superior and inferior to the optic nerve head in the vertical plane (Fig. 2). In wild-type (\(Abca4^{++}\)) mice at 2 months of age, no ONL thinning was observed in superior hemiretina of light-stressed (right) eyes after 1 week (Fig. 2A). Conversely, ONL width was reduced in superior retina of light-exposed eyes of \(Abca4^{-/-}\) null mutant mice, with the differences at the 0.4- and 0.6-mm positions being statistically significant (\(P < 0.05\); one-way ANOVA and Sidak’s multiple comparison test, Fig. 2B). For mice at 5 months of age, 450-nm light induced a superior lesion in both the \(Abca4^{++}\) (0.6 mm, right versus left; \(P > 0.05\)) and \(Abca4^{-/-}\) (0.2–0.8 mm, right versus left; \(P < 0.05\), one-way ANOVA and Sidak’s multiple comparison test) hemiretina, although visual inspection revealed that ONL thinning was more pronounced in the \(Abca4^{-/-}\) null mutant versus wild-type mice (Figs. 2C, 2D, respectively). The lesions in the 8-month-old \(Abca4^{-/-}\) and \(Abca4^{++}\) superior hemiretinae (Figs. 2E, 2F) were also distinct. However, although the difference between exposed and unexposed retinas in \(Abca4^{-/-}\) mice was significant (0.2–0.6 mm, right versus left; \(P < 0.05\), one-way ANOVA and Sidak’s multiple comparison test), the plots of ONL thickness revealed that the lesions in the \(Abca4^{-/-}\) mice were less pronounced at 8 months of age than at age 5 months. In this regard it was also apparent that even in the absence of experimental light damage (Fig. 2F, left eyes), ONL width was reduced in the 8-month-old \(Abca4^{-/-}\) mice; this observation has been previously reported.\(^{47,48}\) Thus, in the case of the 8-month-old \(Abca4^{-/-}\) mice, experimental light exposure was delivered on a background of genetically induced photoreceptor cell degeneration.

For further quantitation, we also calculated ONL area by summing ONL thicknesses in superior hemiretina (0.2–1.0 mm) and multiplying by the measurement interval of 0.2 mm (Fig. 3A). For the \(Abca4^{++}\) mice exposed to 430-nm light, ONL area was not reduced at 2 months of age. However, at 5 and 8 months of age, ONL area was decreased by 9% (\(P < 0.05\)) and 15% (\(P < 0.05\)), respectively, when compared to the control (left eyes, paired t-test). In the \(Abca4^{-/-}\) mice, ONL area in light-exposed eyes (right) was decreased by 19% even at 2 months of age (\(P > 0.05\), right versus left, paired t-test), and at 5 months of age, the reduction reached 47% (\(P < 0.05\), left versus right eyes, paired t-test). The reduction in ONL area associated with light stress was also significantly greater in the \(Abca4^{-/-}\) versus \(Abca4^{++}\) mice at 2 and 5 months of age when compared to wild type (\(P < 0.05\), unpaired t-test). In addition, an effect of age was observed, with the light-induced ONL thinning in \(Abca4^{-/-}\) mice being greater at 5 months than at 2 months and thinning in \(Abca4^{++}\) mice being greater at 8 months than at 2 months (\(P < 0.05\), one-way ANOVA and Tukey’s multiple comparison test). Shown in Figure 3B are representative images of hematoyxin-eosin-stained superior retinae from \(Abca4^{++}\) and \(Abca4^{-/-}\) mice (age 5 months). In the \(Abca4^{-/-}\) mice, ONL thickness was severely reduced as compared to wild type.

Light Damage and the \(Rpe65^{rd12}\) Mouse

The \(Rpe65^{rd12}\) mouse is characterized by loss of function of Rpe65 due to a naturally occurring severe truncation of the protein together with nonsense-mediated mRNA degradation.\(^{49}\) It has previously been reported that Rpe65 null mutation prevents an accumulation of the RPE fluorescence attributable to lipofuscin.\(^{50}\) Consistent with the latter observation, we found that bisretinoids of lipofuscin were not detected in \(Rpe65^{rd12}\) mice (Fig. 5). The bisretinoids examined chromatographically (Fig. 5A) included A2E, A2E isomers, A2-retinaldehyde, A2-RAL dimer–PE (all-trans-retinal dimer phoshatidylethanolamine), and atRAL dimer–PE (all-trans-retinal dimer phoshatidylethanolamine). Rpe65 deficiency is also reported to confer a resistance to light damage.\(^{11,51}\) Thus, we tested our light damage model under conditions of a null mutation in Rpe65 by submitting \(Rpe65^{rd12}\) mice (5 months of age) to 430 ± 20 nm light (50 mW/cm² for 30 minutes). There was no difference in ONL thickness in light-stressed (right) compared to non-light-stressed (left) eyes of \(Rpe65^{rd12}\) mice (\(P > 0.05\), 0.2–2.0 mm, right versus left eyes; \(P < 0.05\), one-way ANOVA and Sidak’s multiple comparison test). Thinning was, however, observed owing to the Rpe65 mutation. Specifically, when non-light-stressed \(Rpe65^{rd12}\) mice (age 5 months) were compared to C57BL/6J mice (age 6 months), there was a noticeable decrease in ONL thickness (Fig. 5B). This change was consistent with previous reports indicating that mice homozygous for the \(Rpe65^{rd12}\) mutation undergo thinning of ONL after 5 months of age, with a 50% reduction in rows of ONL nuclei being measurable in the \(Rpe65^{rd12}\) mutants by 7 months of age.\(^{49,51}\)

DISCUSSION

Here we reported that a mutant mouse line (\(Abca4^{-/-}\)) that accumulates RPE lipofuscin at amplified levels is more susceptible to short-wavelength light damage than wild-type mice. A2E, one of the components of lipofuscin, was 3.6-fold more abundant at 5 months of age in \(Abca4^{-/-}\) mice than wild-type mice. In 5-month-old light-stressed \(Abca4^{-/-}\) mice, ONL...
area (0.2–1.0 mm from ONH), a measure of photoreceptor cell viability, was reduced by 47% relative to control fellow eyes, while the light-induced reduction in the wild-type eyes was only 10% at this age. Moreover, A2E amounts increased with age (2 vs. 5 months) in the \textit{Abca4}/\textit{C0}/\textit{C0} and wild-type (2, 5, and 8 months) mice, as did the severity of light damage. We observed that light-induced damage was more pronounced in central retina than the periphery. In this regard it is significant that the level of A2E, just one of the bisretinoids of lipofuscin, is also higher centrally in the mouse. These findings are consistent with a role for RPE lipofuscin in mediating light damage. A functional 11-cis-retinal chromophore is necessary to elicit...
retinal light damage, but a direct effect of the chromophore cannot explain these results, since rhodopsin and 11-cis-retinal levels are not different in Abca4/C0/C0 versus wild-type mice.20 Whereas Abca4 deficiency increases bisretinoid formation and light damage, we showed that an absence of Rpe65 activity abrogates bisretinoid formation (Fig. 5) and, as has been reported previously,11 the mice are correspondingly refractory to light damage (Fig. 5). Rpe65 activity is rate limiting in the visual cycle and the failure to generate 11-cis-retinal owing to Rpe65 deficiency is the cause of photoreceptor cell degener-

**Figure 3.** ONL thinning in light-stressed Abca4+/+ and Abca4−/− mice. (A) ONL areas in superior hemiretinae of right (light-stressed) and left (nonstressed control) eyes 1 week after light irradiation. ONL area was calculated by summing thickness measurements (0.2–1.0 mm from ONH) and multiplying by 0.2 mm. Values are means ± SEM of 5 to 11 mice. *P < 0.05, right eyes as compared to fellow left eyes (paired t-test); **P < 0.05, right eyes of Abca4−/− mice as compared to age-matched right eyes of Abca4+/+ mice (unpaired t-test, P < 0.05); bracket indicates P < 0.05, in Abca4+/+ mice, age 2 vs. 8 months, and in Abca4−/− mice, 2 vs. 5 months of age (ANOVA and Tukey’s multiple comparison test). (B) Representative light micrographs of superior hemiretina of right eyes of 5-month-old Abca4+/+ (upper) and Abca4−/− (lower) mice 1 week after short-wavelength light exposure. Scale bar: 50 μm. GC, ganglion cell layer; INL, inner nuclear layer.

**Figure 4.** Effect of light stress on RPE cells in superior retina of Abca4+/+ and Abca4−/− mice. (A–F) Numbers of RPE cells (mean ± SEM) counted within 0.2-mm intervals and plotted as function of distance from ONH in superior hemiretina at 2, 5, and 8 months of age in Abca4+/+ (A, C, E) and Abca4−/− mice (B, D, F). (G) Total numbers of RPE cells from ONH to 1.0 mm along vertical axis in superior hemisphere of light-stressed (right) and control non–light-stressed eyes of Abca4+/+ and Abca4−/− mice at 2, 5, and 8 months of age (mean ± SEM; means are based on three values). *P < 0.05 as compared to fellow control eye; paired t-test; **P < 0.05 light-stressed Abca4+/+ versus Abca4−/− mice; unpaired t-test.
Various chromophores in RPE have been suggested to mediate short-wavelength light damage, including riboflavin, the mitochondrial enzyme cytochrome c oxidase, and melanin. However, riboflavin and cytochrome c oxidase are not expected to increase with a null mutation in Abca4 or with age, and these molecules would not be modulated by Rpe65 knockout. Ocular melanin can be excluded as a damaging agent, since pigmented and albino animals are equally susceptible to retinal light damage. In fact, ocular melanin decreases retinal irradiance, thereby lengthening the time to retinal damage. Another model, the RdbS/rdb12/Abca4−/−, A2E levels were also many-fold greater than in wild type, and photoreceptor cell degeneration 7 days after a 30-minute white light exposure (10,000 lux) was more pronounced than in wild-type retinas. In this case, however, the damaging agent was assumed to be all-trans-retinal.

While most studies of light damage address photoreceptor cell loss, the site of origin of light damage in primates may be photoreceptor outer segments and/or RPE cells; indeed, some investigators have reported the greatest damage in RPE cells. In the work presented here, we observed light-induced loss of both RPE and photoreceptor cells. The light stress–induced reduction of RPE numbers was greater with the Abca4−/− null mutation than in wild type; however, for reasons unknown to us, we were unable to detect an age-related increase in light stress–induced RPE loss in the wild-type and null mutant mice. we cannot draw conclusions as to whether damage was initiated in photoreceptor cells and RPE independently or whether for instance, the primary injury resided in photoreceptor outer segments with transfer to the RPE when these cells phagocytose packets of distal outer segment membrane. Accordingly, bisretinoids are present in both
Photo-oxidative mechanisms are clearly involved in retinal light damage, since antioxidants can protect against photic damage, and increased oxygen availability lowers the threshold.\(^{61,67-69}\) Inhibition of NADPH oxidase activity also suppresses rod photoreceptor light-induced degeneration.\(^{70}\) A role for antioxidants in protection against light damage is significant since bisretinoids are well known to be capable of short-wavelength light-induced singlet oxygen generation, with the latter reacting back at carbon–carbon double bonds and thereby photo-oxidizing the bisretinoid.\(^{71}\) Interestingly, TEM-POL-H, a compound having antioxidant properties, has been shown both to inhibit A2E photo-oxidation elicited by short-wavelength light exposure\(^{22}\) and to protect against light-induced damage to both photoreceptor cells and RPE.\(^{73,74}\) Of additional note, the increase in retinal proteins modified by the reactive aldehyde 4-hydroxynonenal, a product of polyunsaturated fatty acid oxidation, is a feature of retinal light damage in rats\(^{49}\) and of cultured RPE cells that have accumulated A2E and are irradiated with short-wavelength light.\(^{75}\)

The photo-oxidation and photodegradation of bisretinoid injury cells\(^{36,58}\) and can be visualized as photobleaching, a light-induced decline in fluorescence.\(^{76}\) Evidence that lipofuscin photobleaching is linked to RPE cell death in vivo has been provided by observations made during imaging of the RPE monolayer by fluorescence adaptive optics scanning light ophthalmoscopy in nonhuman primates. At irradiance levels below American National Standards Institute safety standards\(^{18,66}\) it is reported that photoreceptor cell damage is initiated distally in the outer segment and subsequently progresses to include the entire length of the outer segment.\(^{18,66}\) While this observation may be explained by apical–basal differences in the lipid composition of the outer segment membrane,\(^{2}\) another factor could be that retinally situated more aged discs contain higher levels of the bisretinoid compounds that become the lipofuscin of RPE.

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References


