Photodegradation of retinal bisretinoids in mouse models and implications for macular degeneration

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Adducts of retinaldehyde (bisretinoids) form nonenzymatically in photoreceptor cells and accumulate in retinal pigment epithelial (RPE) cells as lipofuscin; these fluorophores are implicated in the pathogenesis of inherited and age-related macular degeneration (AMD). Here we demonstrate that bisretinoid photodegradation is ongoing in the eye. High-performance liquid chromatography (HPLC) analysis of eyes of dark-reared and cyclic light-reared wild-type mice, together with comparisons of pigmented versus albino mice, revealed a relationship between intraocular light and reduced levels of the bisretinoids A2E and A2-glycero-phosphoethanolamine (A2-GPE). Analysis of the bisretinoids A2E, A2-GPE, A2-dihydropyridine-phosphatidylethanolamine (A2-DHP-PE), and all-*trans*-retinal dimer-phosphatidylethanolamine (all-*trans*-retinal dimer-PE) also decreases in albino *Abca4*$^{-/-}$ mice reared in cyclic light compared with darkness. In albino *Abca4*$^{-/-}$ mice receiving a diet supplemented with the antioxidant vitamin E, higher levels of RPE bisretinoid were evidenced by HPLC analysis and quantitation of fundus autofluorescence; this effect is consistent with photooxidative processes known to precede bisretinoid degradation. Amelioration of outer nuclear layer thinning indicated that vitamin E treatment protected photoreceptor cells. Conversely, in-cage exposure to short-wavelength light resulted in reduced fundus autofluorescence, decreased HPLC-quantified A2E, outer nuclear layer thinning, and increased methylglyoxal (MG)-adducted protein. MG was also released upon bisretinoid photodegradation in cells. We suggest that the lower levels of these diretinal adducts in cyclic light-reared and albino mice reflect photodegradative loss of bisretinoid. These mechanisms may underlie associations among AMD risk, oxidative mechanisms, and lifetime light exposure.

bisretinoid | visual cycle | retina | retinal pigment epithelium | macular degeneration

The bisretinoid fluorophores of the lipofuscin of retinal pigment epithelial (RPE) cells form nonenzymatically in photoreceptor outer segments as a consequence of indiscriminate reactions of molecules of vitamin A-aldehyde (A2, 2 vitamin A molecules) (1). These bisretinoid pigments are subsequently transferred to RPE during the process of photoreceptor outer segment shedding and phagocytosis. Various bisretinoids of RPE lipofuscin, including A2-GPE (A2-glycero-phosphoethanolamine), A2-DHP-PE (A2-dihydropyridine-phosphatidylethanolamine), all-*trans*-retinal dimer-PE (all-*trans*-retinal dimer phosphatidylethanolamine) and A2E (adduct of A2 and ethanolamine) (1–4) have been isolated and characterized structurally (Fig. S1).

An early-onset form of macular degeneration, recessive Stargardt disease (STGD1), is associated with pronounced deposition of bisretinoid lipofuscin in RPE cells; this accumulation culminates in RPE and photoreceptor cell death. The *ABCA4* gene encodes the ATP-binding cassette transporter (ABCA4) in photoreceptor cells, and mutations in this gene are causal for STGD1 (5). RPE lipofuscin is also elevated in models of dominant Stargardt-like macular degeneration (6) associated with mutations in elongation of very long chain fatty acids-like 4 (*ELOVL4*), but the pathways involved in this perturbation have not been elucidated. Although age-related macular degeneration (AMD) stems from multiple factors (7), there may be a contribution from bisretinoid lipofuscin (1).

The ABCA4 transporter in photoreceptor cells serves in the delivery of retinaldehyde to cytosolic retinol dehydrogenases that reduce vitamin A aldehyde to the nonreactive alcohol form (8). Marked bisretinoid accumulation is replicated not only in *Abca4* null mutant mice (9, 10) but also in mice deficient in retinol dehydrogenase-8 and dehydrogenase-12 (Rdh8; Rdh12) (11–13). In addition, the relationship between RPE lipofuscin accumulation and retinal disease is evidenced in the *Abca4* mouse model by complement dysregulation, carbonyl-deposition, thickening of Bruch's membrane, and a progressive loss of photoreceptor cells (11, 14–17).

Given the cellular consequences of bisretinoid build-up, efforts have been made to elucidate mechanisms by which these compounds are damaging. Although the bisretinoid fluorophores of lipofuscin do not appear to undergo lysosomal digestion, in in vitro assays, these fluorophores photodegrade. Both whole-lipofuscin mixtures (18, 19) and individual fluorophores such as A2-GPE (3), A2E (20), and all-*trans*-retinal dimer (21) have been shown to initiate photosensitization reactions that generate superoxide anion and singlet oxygen (20–22). Singlet oxygen, as a highly reactive form of oxygen, adds to carbon–carbon double bonds of the bisretinoid molecule, thereby photooxidizing the bisretinoid and creating unstable oxygen-containing moieties that have been detected as oxidized A2E and oxidized all-*trans*-retinal dimer in human and mouse retina (21, 22). It is this oxidation that causes the bisretinoids to photodegrade and release a complex mixture of aldehyde-containing fragments and the

Significance

Visual cycle adducts having bisretinoid structures accumulate in retinal pigment epithelial cells as lipofuscin. These light-sensitive compounds are implicated in disease mechanisms leading to visual impairment in some inherited and age-related forms of macular degeneration. The means by which these diretinal adducts impart chronic damage is not fully understood. By studying mice raised under varying levels of intraocular light and by analyzing mice treated with vitamin E, we provide evidence of chronic bisretinoid photooxidation and degradation in the eye. These processes could shed light on oxidative and light-related mechanisms involved in disease pathogenesis and provide support for therapeutic approaches that target bisretinoids.

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dicarbonyls methylglyoxal (MG) and glyoxal (23) that modify other molecules. Because these carbonyl-modified proteins are present in deposits (drusen) that accumulate on the basal side of RPE cells *in vivo,* and drusen are a major risk factor for AMD progression (24, 25), the photodegradation of bisretinoids likely constitutes a relationship to AMD.

Here we have undertaken to determine whether photodegradation of bisretinoid is ongoing in the eye. We reasoned that photodegradation could be detected, were it to occur, by comparing levels of bisretinoid in mice that were raised in cyclic light as opposed to darkness and by comparing albino versus black and agouti mice.

Results

A2E Accumulation in Albino and Pigmented Wild-Type Mice Housed in Darkness Versus Cyclic Light. Ocular melanin serves as a neutral density filter (0.37 log units) (26). Accordingly, with the absence of melanin in the albino, light entering the eye through the iris and pars plana is substantially increased (27). Thus, pigmented C57BL/6J and albino C57BL/6J^{c2j} mice were housed from birth in either continuous darkness or under cyclic light; except for the mutated tyrosinase gene (c/c), these mice are genetically identical. With analysis by high-performance liquid chromatography (HPLC) and ultraperformance liquid chromatography (UPLC), the bisretinoids A2E, isoA2E, and A2-GPE were present in eyes from both cyclic light and dark-reared mice (Fig. 1 *A* and *B* and Fig. S1). Chromatographic quantitation also revealed that with housing in darkness, bisretinoid amounts were not significantly different in pigmented C57BL/6J versus albino C57BL/6J^{c2j} mice (*P* > 0.05) (Fig. 1*B*). Conversely, in albino C57BL/6J^{c2j} mice maintained under cyclic light conditions, A2E levels were reduced by 45% compared with albino C57BL/6J^{c2j} mice reared in the dark (*P* < 0.05) (Fig. 1*B*). In albino C57BL/6J^{c2j} mice, A2-GPE was not detectable.

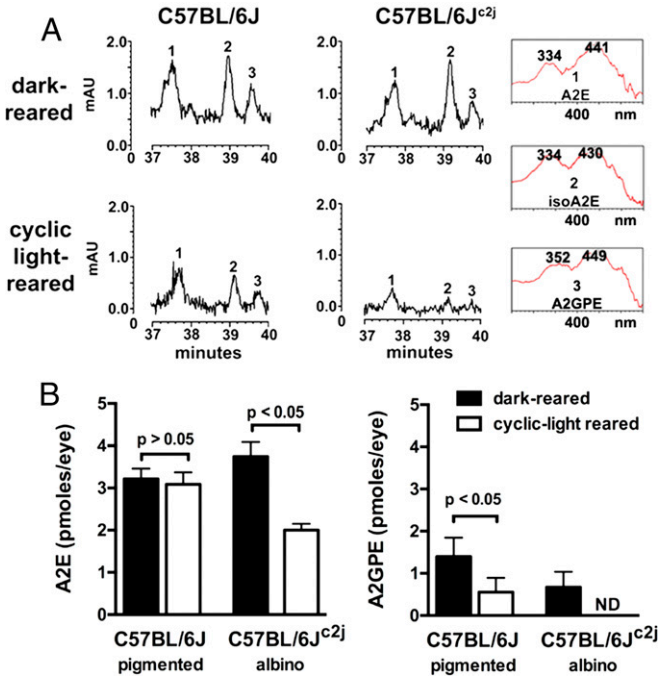

Fig. 1. The RPE bisretinoids A2E and A2-GPE accumulate in both light- and dark-reared mice and are present at reduced levels in light-reared versus dark-reared albino mice. (*A*) Reverse phase chromatographic detection of A2E and A2-GPE in dark-reared and cyclic-light reared pigmented C57BL/6J and albino C57BL/6J^{c2j} mice at age 6 mo. (*Insets*) UV-visible spectra of chromatographic peaks corresponding to A2E (all-*trans*-A2E), iso-A2E (13–14 *cis*), and A2-GPE. (*B*) Chromatographic quantitation. A2E and iso-A2E were quantified by HPLC, and values presented are A2E and iso-A2E summed. A2-GPE was quantified by UPLC. For each sample, six to eight eyes were pooled; means ± SEM of five or seven independent samples are plotted; *P* values determined by one-way ANOVA and Tukey's multiple comparison test.

Chromatographic detection of A2E, isoA2E, and photooxidized A2E in albino C57BL/6J^{c2j} and black C57BL/6J mice are presented in Fig. S2. Note that in the albino mice, the sum of the integrated peak areas of A2E and isoA2E (68,049 μV·sec) is lower than in the black mice (89,794 μV·sec), but the peak area of oxidized A2E (Fig. S2, peak 1) is not correspondingly greater in the albino. As shown in Fig. S3, when synthesized samples of A2E are irradiated, the amount of remaining A2E and isoA2E is lower after 100 s irradiation (1,372,034, 179,102 μV·sec) than after 30 s (2,681,180, 357,314 μV·sec). Nevertheless, the quantity of oxidized product is not greater after the longer irradiation because oxidized A2E is lost to photodegradation. Taken together, the latter experiments provide in vivo and in vitro evidence of bisretinoid photooxidation and photodegradation and indicate that greater light exposure does not necessarily increase the amount of oxidized bisretinoid because photodegradation follows the oxidation.

Accumulation of Bisretinoid in Dark- and Light-Reared Albino *Abca4* Null Mutant Mice. Bisretinoid fluorophores form in abundance in the retinae of *Abca4*$^{-/-}$ mice because of impaired handling of all-*trans*-retinal within photoreceptor outer segments (10, 28). Chromatographic detection of the bisretinoids A2E and iso-A2E (2), A2-DHP-PE (4), all-*trans*-retinal dimer-PE (21), and A2-GPE (3) (Fig. 2 and Fig. S1) and quantification by integrating peak areas revealed that all of these fluorophores were present in greater amounts in dark-reared albino mice compared with cyclic-light reared albino *Abca4*$^{-/-}$ mice (Fig. 2*D*). The decline in A2E levels in the albino cyclic light-reared *Abca4*$^{-/-}$ mice was 62% (*P* < 0.05), with decreases in A2-DHP-PE, all-*trans*-retinal dimer-PE, and A2-GPE being similar or greater. Outer nuclear layer (ONL) thinning was also less pronounced in the dark-reared mice (Fig. S4).

Comparing Agouti Versus Albino Mice. Differences were also observed between albino BALB/cJ (albino; Rpe65 Leucine450 variant) and 129/Sv mice having an agouti-coat color (Rpe65 Leucine450 variant) at 4, 6, and 9 mo of age (Fig. 3*A*). At 6 mo of age, A2E, A2-DHP-PE, and A2GPE were from three- to sevenfold higher in the 129/Sv mice (*P* < 0.05). Although A2-DHP-PE and A2-GPE were measurable in 129/Sv mice at all ages, these fluorophores were not always at detectable levels in BALB/cJ mice.

Modulation of A2E Levels by the Rpe65 Leu450Met Variant. The Rpe65 450Met variant expressed by C57BL/6J and C57BL/6J^{c2j} mice slows the isomerization step in the regeneration of 11-*cis* retinal (26, 29); these retarded kinetics are also associated with reduced A2E in albino C57BL/6J^{c2j} versus BALB/cJ (wild-type Rpe65 Leu450) mice (10). Accordingly, A2E levels in 9-mo-old albino C57BL/6J^{c2j} (450Met) mice were ~60% of that in the BALB/cJ (450Leu) mice (Fig. 3*B*). A corresponding difference between the albino BALB/cJ (450Leu) and pigmented C57BL/6J (450Met) mice was not observed, likely because of more pronounced A2E photodegradation in the albino BALB/cJ mice.

Vitamin E-Treated Mice. Evidence for the involvement of photooxidative processes in determining A2E levels at a given age was also obtained in experiments in which albino *Abca4*$^{-/-}$ mice received a diet supplemented with vitamin E (α-tocopherol), a potent lipid-soluble antioxidant. In studies using a singlet oxygen generator, we have previously shown that vitamin E can protect against A2E-oxidation by quenching singlet oxygen (30). In the current study, plasma levels of vitamin E measured at 8 mo of age were 6.7 ± 0.1 μg/mL (mean ± SEM) in control mice and 11.7 ± 0.5 μg/mL in

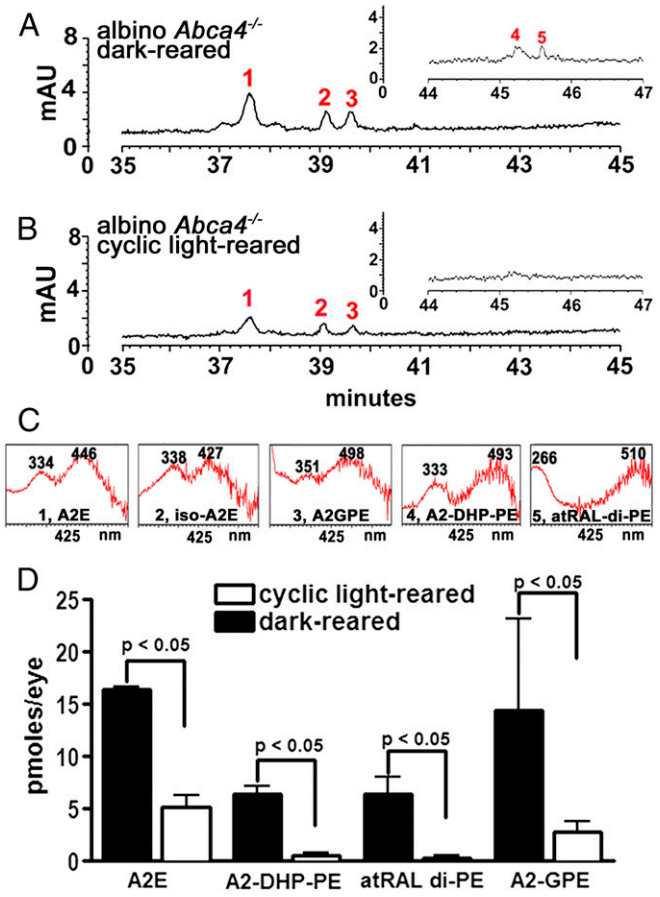

Fig. 2. Comparison of bisretinoid levels in cyclic light-reared and dark-reared albino *Abca4*$^{-/-}$ mice. (*A* and *B*) Representative reverse-phase chromatograms with 430-nm monitoring to detect A2E (1) and iso-A2E (13–14 *cis*) (2), A2-GPE (3), A2-DHP-PE (4), and all-*trans*-retinal dimer-PE (atRAL dimer-PE) (5) in dark-reared (*A*) and cyclic light-reared (*B*) albino *Abca4*$^{-/-}$ mice (8 mo). (*Insets*) Chromatograms expanded between retention times 44–47 min. (*C*) UV-visible absorbance spectra of the indicated compounds. (*D*) Quantitation of bisretinoid compounds in albino *Abca4*$^{-/-}$ mice (6 mo) that were cyclic-light or dark-reared; picomoles of A2E and iso-A2E were summed. A2E, A2-DHP-PE, atRAL di-PE were quantified by HPLC, and A2-GPE was quantified by UPLC. Mean ± SEM of two to five samples, four to six eyes per sample; *P* values determined by one-way ANOVA and Sidak's multiple comparison test.

vitamin E-treated mice. Mice housed in cyclic light and treated with vitamin E from 1 to 6 mo of age exhibited levels of the bisretinoids A2E, A2-DHP-PE, and all-*trans*-retinal dimer-PE that were greater than in control mice (Fig. 4 *A* and *B*). For example, A2E levels in the vitamin E-treated mice were 54% higher ($P < 0.05$). Similarly, noninvasive in vivo measurement of RPE lipofuscin by quantitative fundus autofluorescence (qAF) analysis (16), revealed higher levels of fundus autofluorescence in mice treated with vitamin E from age 1–8 mo ($P < 0.05$) (Fig. 4*C*). Importantly, the thinning of ONL that is indicative of reduced photoreceptor cell viability in albino *Abca4*$^{-/-}$ mice (15, 16, 31) was less pronounced in the vitamin E-treated mice (Fig. 4*D*). In vitamin E-treated albino *Abca4*$^{-/-}$ mice, ONL area in superior and inferior hemiretina was 10.0×10^4 μm^2 and 10.1×10^4 μm^2, respectively, compared with 7.9×10^4 and 7.7×10^4 μm^2, respectively ($P < 0.05$, two-tailed *t* test) in the control *Abca4*$^{-/-}$ mice.

The higher levels of bisretinoid and qAF in the vitamin E-treated mice are consistent with a mechanism involving a reduction in photooxidation-associated photodegradation of bisretinoid in the presence of vitamin E. Support for the latter mechanism was also forthcoming in noncellular experiments in which we observed a concentration-dependent protection of A2E when irradiation (430 nm) was carried out in the presence of vitamin E (Fig. 4*E*).

Reduction of Bisretinoid and Release of Dicarbonyl by Exposure to Blue Light. To further test for photodegradation of bisretinoid in vivo, we exposed albino C57BL/6J^{c2j} (8 mo) and BALB/cJ (9 mo) mice to 430 nm light, in-cage, for 2 h each day for 7 d. For the remaining hours, the mice were housed in cyclic light. qAF disclosed a reduction of 18% ($P < 0.05$) in the exposed BALB/cJ mice (Fig. 5*A*), whereas HPLC quantitation of A2E revealed a 24% ($P < 0.05$) and 17% reduction ($P > 0.05$) in C57BL/6J^{c2j} and BALB/cJ mice, respectively (Fig. 5*B*), compared with unexposed age- and strain-matched mice. In agouti *Abca4*$^{-/-}$ mice exposed to the same lighting conditions, we detected MG-derived hydroimidazolone (MG-H1), an adduct of MG with arginine, in posterior eyecups by indirect competitive ELISA (Fig. S5*A*). MG is released as a photodegradation product of bisretinoid (Fig. S5*B*). The photodegradation of bisretinoid in the BALB/cJ mice was also accompanied by a reduction in ONL thickness (Fig. 5*C*). ONL area in the exposed mice was 8.2×10^4 μm^2 (inferior) and 8.4×10^4 μm^2 (superior), as opposed to 10.4×10^4 μm^2 (inferior) and 9.9×10^4 μm^2 (superior) in control mice ($P < 0.05$, two-tailed *t* test). Conversely, when younger (age 2 mo) albino mice having less pronounced bisretinoid accumulation, were exposed to 430 nm light under the same conditions, no difference in ONL thickness was observed compared with in unexposed control mice (Fig. S6).

Comparison of A2E and 11-Cis-Retinal in Light-Reared Albino and Pigmented Mice. An additional issue relevant to these experiments is that to adjust for increased photon catch, albino mice have reduced rhodopsin content (photostasis) (32). Thus, we measured 11-*cis*-retinal and A2E in the same eye samples obtained from C57BL/6J and C57BL/6J^{c2j} mice. In the albino mice, 11-*cis*-retinal was 14% and 23% lower, at 6 and 9 mo, respectively, whereas the decrease in A2E in the albino was greater (6 mo: 55% lower; 9 mo: 59% lower) (Fig. S7).

A2E Synthesis from *Cis* and *Trans* Isomers of Retinaldehyde. Because the retinaldehyde double-bond isomers contributing to bisretinoid formation in the dark (Fig. 1) are uncertain, we examined *trans*- and *cis*-isomers of retinaldehyde for their ability to form the all-*trans*- and *cis*-isomers of A2E (2) when reacted with ethanolamine. A2E and isoA2E were both generated when the starting material was all-*trans*-, 13-*cis*-, or 11-*cis*-retinal (Fig. S8 *A*–*C*). 11-*cis*-A2E was also generated when 11-*cis*-retinal reacted with ethanolamine. A2E and isoA2E did not form when 9-*cis*-retinal was the precursor (Fig. S8*D*); of the two products (4, 5) that were generated, one was likely 9-*cis*-A2E. Total conversion from 11-*cis*-retinal and 9-*cis*-retinal to bisretinoid was lower than conversion from all-*trans*-retinal ($P < 0.05$) (Fig. S8 *C* and *D*, *Right*).

Discussion

Levels of RPE bisretinoid that are measured appear to be the result of the opposing activities of synthesis and photodegradation (20–22). By assessing bisretinoid levels in albino versus pigmented mice, and in mice housed in darkness compared with cyclic light, we observed that the levels of the lipofuscin fluorophores in cyclic light-reared albino mice were less than in cyclic light-reared pigmented mice and in mice reared in darkness. With dark-rearing, the presence or absence of ocular melanin had no effect on the levels of RPE bisretinoid lipofuscin. These findings, together with our previous work (20–23), are indicative of light-driven mechanisms involving bisretinoid photosensitization and oxidation followed by photodegradative consumption of bisretinoid. It is possible that bisretinoid formation under cyclic light is more pronounced than is apparent here; for instance, photodegradative loss could mask a greater propensity for lipofuscin formation under cyclic light. On the practical side, investigators quantifying bisretinoids in mouse

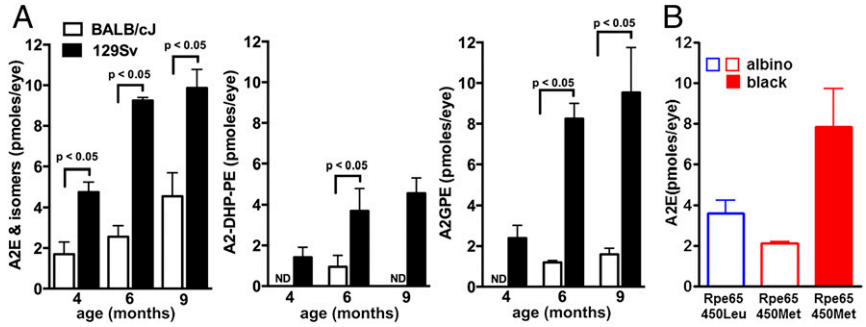

Fig. 3. Modulation of bisretinoids levels in albino and pigmented mice under cyclic-light rearing. (*A*) Quantitation of A2E, A2-DHP-PE, and A2GPE in albino BALB/cJ and agouti 129/Sv wild-type mice at 4, 6, and 9 mo of age. Mice express the wild-type leucine-450 variant in Rpe65. A2E and A2-DHP-PE were quantified by HPLC, and A2E values consist of A2E and iso-A2E summed. A2-GPE was quantified by UPLC. For each sample, six eyes were pooled; means ± SEM of two to six independent samples are plotted; *P* values determined by one-way ANOVA and Sidak's multiple comparison test. ND, not detected. (*B*) A2E levels in cyclic light-reared albino BALB/cJ (Rpe65 leucine-450), albino C57BL/6J^{c2j} (Rpe65 methionine-450), and pigmented C57BL/6J (Rpe65 methionine-450) mice age 9 mo. Values are means ± SEM of eight (BALB/cJ), five (C57BL/6J), and four (C57BL/6J^{c2j}) independent samples per group; six eyes were pooled per sample.

models should anticipate measurement variability caused, at least partially, by variations in ambient light intensities experienced by mice in the vivarium.

Here we also observed that in mice treated with the antioxidant vitamin E, photodegradation of bisretinoid was suppressed, resulting in increased bisretinoid and sparing of photoreceptor cells. Vitamin E intercepts bisretinoid oxidation by scavenging reactive oxygen species generated by bisretinoid photosensitization (23). These results show that bisretinoid photodegradation is damaging and that bisretinoid levels can be modulated by mechanisms not involving a change in synthesis.

Photodegradation products of bisretinoids such as A2E constitute a mixture of aldehyde-bearing fragments, together with the dicarbonyls MG and glyoxal (GO) (23), that ravage cellular and extracellular molecules. Quantitation of MG-adducts by ELISA revealed that in albino light-reared *Abca4*$^{-/-}$ mice accumulating bisretinoid at elevated levels, MG-adducts were elevated threefold compared with wild-type mice (17). Using immunocytochemical approaches that detected MG-carbonyl-modified proteins, labeling in the location of Bruch's membrane was more pronounced in *Abca4*$^{-/-}$ versus *Abca4*$^{+/+}$ mice and in older versus younger mice. The photodegradation products released from bisretinoids cross-link protein and suppress matrix metalloproteinase activity (17). Proteins modified by these reactive species are also constituents of drusen (24, 25), the sub-RPE deposits associated with AMD (33). A limitation of this

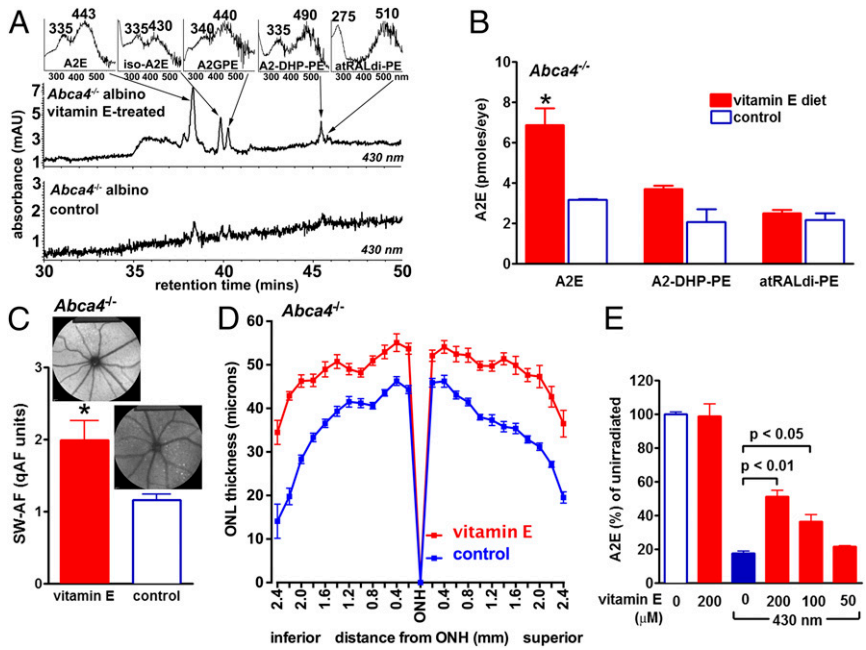

Fig. 4. Vitamin E protects against bisretinoid consumption associated with photooxidation/photodegradation. (*A*) Chromatographic detection of the bisretinoids A2E, iso-A2E (13–14 *cis*), A2-GPE, A2-DHP-PE, and atRAL di-PE in vitamin E-treated versus untreated albino *Abca4*$^{-/-}$ mice raised in cyclic light. Age, 6 mo. (*A*, *Inset*) UV-visible spectra of chromatographic peaks corresponding to indicated compounds. (*B*) Quantitation of A2E, A2-DHP-PE, and atRAL di-PE. For each sample, six eyes were pooled; means ± SEM of two independent samples are plotted. A2E values consist of A2E and iso-A2E summed. Levels of A2E in vitamin E-treated and untreated *Abca4*$^{-/-}$ mice (age, 6 mo) are statistically different (ANOVA and Sidak's multiple comparison test, $P < 0.05$). (*C*) Quantitative fundus autofluorescence in vitamin E-treated and untreated *Abca4*$^{-/-}$ mice (age, 8 mo). Values are means ± SEM of 8 mice; *$P < 0.05$, two-tailed *t* test. (*D*) Outer nuclear layer thickness measured in vitamin E-treated and untreated albino *Abca4*$^{-/-}$ mice (age, 8 mo) and plotted as distance from optic nerve head (ONH). (*E*) Vitamin E protects against the consumption of 430 nm-exposed A2E in an in vitro assay. Values are means ± SEM of two experiments; *P* values based on ANOVA and Newman-Keul multiple comparison test.

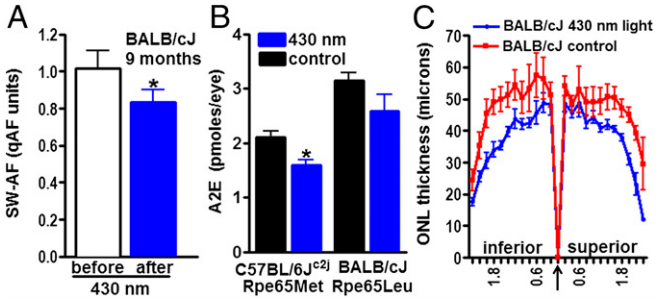

Fig. 5. Exposure of mice to short-wavelength light. Mice were illuminated with 430 nm (1 mW) light for 2 h/day on 7 consecutive days. (*A*) Quantitative fundus autofluorescence was used to measure RPE lipofuscin noninvasively in BALB/cJ mice (9 mo) before and after exposure to the light paradigm; $*P < 0.05$, two-tailed *t* test. (*B*) HPLC quantitation of A2E in eyes of exposed and control (nonexposed) C57BL/6J^{c2j} mice (8 mo; Rpe65 450Met) and BALB/cJ mice (9 mo; Rpe65 Leu450). For each sample, six eyes were pooled; means ± SEM of five (control C57BL/6J^{c2j}) or two independent samples are plotted. $*P < 0.05$, two-tailed *t* test. (*C*) Outer nuclear layer thicknesses in exposed and control (nonexposed) BALB/cJ mice (9 mo) are plotted as distance from optic nerve head (arrow).

work is that, because of practical considerations, the study did not include measurement of MG-adducts in dark-reared versus light-reared mice.

We have previously shown that *Abca4*$^{-/-}$ mice burdened with increased bisretinoid are more susceptible to retinal light damage than are wild-type mice (31). Older mice carrying greater levels of bisretinoid are also more susceptible than younger mice, and light damage was not observed in Rpe65^{rd12} mice that carry little or no RPE bisretinoid. These findings are consistent with reports in the literature indicating that light damage can occur independent of phototransduction (34) and that photooxidative mechanisms are involved (35, 36). Depending on the conditions, light damage can also originate within photoreceptor cells or RPE, although the greatest damage is reported for RPE (36).

In the present study, we demonstrated by in vitro synthesis assays that the bisretinoid A2E can form when 11-*cis*-retinal, all-*trans*-retinal, and 9-*cis*-retinal are used as starting material, although steric hindrance introduced by the 9-*cis*-retinaldehyde configuration reduces the synthetic efficiency of the latter. In disease marked by 11-*cis*-retinal deficiency, 9-*cis*-retinal can form iso-rhodopsin (37) and generate small light-evoked rod responses (38). The propensity for 9-*cis*-retinal to form *cis*-A2E likely explains the detection of some RPE autofluorescence in *Rpe65*$^{-/-}$ mice (39).

Our findings in dark-reared mice and the data of Boyer et al. support the notion that bisretinoid can form from 11-*cis*-retinal in vivo (40). The relative contributions of 11-*cis*- and all-*trans*-retinal to bisretinoid formation under lighted conditions are not known, but in both cases, only a small portion of retinoid escapes the visual cycle to form bisretinoid. With respect to bisretinoid formation in *Abca4*$^{-/-}$ mice, it is significant to note that ABCA4 can transport both 11-*cis*-retinal and all-*trans*-retinal as a reversible Schiff base with PE (40).

The phagocytosis of shed photoreceptor cell disk membrane continues in an entrained fashion in rats raised in total darkness, although the numbers of phagosomes may or may not be be altered (41). Nevertheless, bisretinoid formation in darkness or in cyclic light is unlikely to depend on phagocytosis, as the bisretinoid constituents form in photoreceptor cells before disk shedding and phagocytosis, and even in the absence of RPE phagocytosis (42–45). We can exclude reduced numbers of photoreceptor cells as an explanation for the decrease in lipofuscin, as in 12-mo-old C57BL/6J^{c2j} mice, thinning of ONL is not observed (46). In addition, photostasis (32) cannot account for bisretinoid differences in albino versus pigmented mice.

The use of the albino mouse in these studies presumably allowed us to observe photodegradative consumption of A2E during a relatively short period, whereas the dense ocular melanin of black mice likely enabled the differences between C57BL/6J and C57BL/6J^{c2j} mice to emerge. Nevertheless, photooxidation and photodegradation of RPE bisretinoids can be observed in humans and nonhuman primates. For instance, photobleaching of RPE lipofuscin likely explains aberrations in fundus autofluorescence images (488 nm excitation) observed after retinal translocation in humans (47), and the diminished RPE fluorescence observed after in vivo fluorescence imaging by adaptive optics scanning laser ophthalmoscopy in nonhuman primates (36). At higher light levels, but still within approved safety standards, this photobleaching is accompanied by permanent RPE cell damage, indicating that bisretinoid photodegradation is not benign.

Although epidemiological evidence of an association between sunlight exposure and AMD has been inconsistent over the years (48–50), recent studies have provided supported for such a link (51–53). The adverse consequences of lipofuscin bisretinoid photodegradation could be a pathway that explains this relationship. Similarly, the ability of the antioxidant vitamins E and C and minerals to reduce AMD progression (54) may be understood, at least in part, from the perspective of antioxidant suppression of lipofuscin photooxidation/degradation.

Because the photodegradative processes examined here could over time be responsible for cumulative damage to retina and Bruch's membrane, the present findings have implications with respect to development of therapeutics that address bisretinoid lipofuscin deposition in recessive Stargardt disease and AMD (55–60) and justify efforts to protect the retina of STGD1 patients by reducing light exposure through the use of a black contact lens (61).

Materials and Methods

Mouse Models. BALB/cJ (albino, Rpe65 Leu450), C57BL/6J (black, Rpe65 450Met variant), C57BL/6J^{c2j} (B6(Cg)-Try < c-2J>/J, albino, Rpe65 450Met variant), and 129S1/SvImJ (agouti, Rpe65 Leu450) mice were purchased or bred and housed (see details in *SI Materials and Methods*). In some experiments, mice were exposed in-cage to 430 (± 30 nm) light from a halogen source (1 mW; measured with Newport Optical Power Meter Model 840) for 2 h each day for 7 d before euthanasia. Animal protocols were approved by the Institutional Animal Care and Use Committee of Columbia University and complied with guidelines set forth by the Association for Research in Vision and Ophthalmology (ARVO) Animal Statement for the Use of Animals in Ophthalmic and Vision Research.

Quantitative Fundus Autofluorescence. Images were acquired and analyzed as previously described (16).

Vitamin E Treatment. Albino *Abca4*$^{-/-}$ (Rpe65 450Leu) mice were treated from age 1 mo to 6 or 8 mo with Adjusted Vitamin E Diet (960) (Harlan Teklad), which included 960 mg (IU)/kg vitamin E (as dl-alpha tocopheryl acetate) or a standard diet (vitamin E, 99IU/kg; control). Plasma vitamin E levels were measured (62).

Quantitative HPLC and UPLC. Mouse eyecups (5–12 eyes per sample, as indicated) were homogenized, extracted in chloroform/methanol, concentrated, and analyzed by HPLC and UPLC, as previously described (3, 4) (see details in *SI Materials and Methods*).

In Vitro Photooxidation Assay. Synthesized A2E (2) (100 μM) with vitamin E (50–200 μM) in PBS and was irradiated (430 ± 30 nm; 30 s), extracted, and quantified.

Measurement of Outer Nuclear Layer Thickness. Three histological sections through the optic nerve head were analyzed (31). ONL area was calculated as the sum of the ONL thicknesses in superior and inferior retina (0.2–2.0 mm) multiplied by the measurement interval of 200 microns.

Statistical Analyses. Prism 6 (GraphPad Software) was used.

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