Characterization of Peroxy-A2E and Furan-A2E Photooxidation Products and Detection in Human and Mouse Retinal Pigment Epithelial Cell Lipofuscin*

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The nondegradable pigments that accumulate in retinal pigment epithelial (RPE) cells as lipofuscin constituents are considered to be responsible for the loss of RPE cells in recessive Stargardt disease, a blindness macular disorder of juvenile onset. This autofluorescent material may also contribute to the etiology of age-related macular degeneration. The best characterized of these fluorophores is A2E, a compound consisting of two retinoid-derived side arms extending from a pyridinium ring. Evidence indicates that photochemical mechanisms initiated by excitation from the blue region of the spectrum may contribute to the adverse effects of A2E accumulation, with the A2E photooxidation products being damaging intermediates. By studying the oxidation products (oxo-A2E) generated using oxidizing agents that add one or two oxygens at a time, together with structural analysis by heteronuclear single quantum coherence spectroscopy, we demonstrated that the oxygen-containing moieties generated within photooxidized A2E include a 5,8-monofuranoid and a cyclic 5,8-monoperoxide. We have shown that the oxidation sites can be assigned to the shorter arm of A2E, to the longer arm, or to both arms by analyzing changes in the UV-visible spectrum of A2E, and we have observed a preference for oxidation on the shorter arm. By liquid chromatography-mass spectrometry, we have also detected both monofuran-A2E and monoperoxy-A2E in aged human RPE and in eye cups of Abca4/Abcr−/− mice, a model of Stargardt disease. Because the cytotoxicity of endoperoxide moieties is well known, the production of endoperoxide-containing o xo-A2E may account, at least in part, for cellular damage ensuing from A2E photooxidation.

The bisretinoid fluorophores that accumulate in retinal pigment epithelial (RPE) cells as lipofuscin constituents are considered to be responsible for the loss of RPE cells in recessive Stargardt disease (1–3), an early onset form of macular degeneration, and may also be involved in the etiology of age-related macular degeneration (4). The RPE lipofuscin fluorophores isolated thus far include A2E (5–7), iso-A2E (7), less abundant double bond isomers of A2E (8), and all-trans-retinal dimer conjugates (9, 10) (Fig. 1). The most intensely studied of the RPE lipofuscin constituents are A2E and related photoisomers, pigments that, when accumulated by RPE cells in culture, have been shown to bestow a sensitivity to light damage (11–13). Blue light produces the most pronounced effect (11). The augmentation of cell death under conditions that prolong the lifetime of singlet oxygen together with the protection provided by quenchers and scavengers of singlet oxygen has implicated singlet oxygen as having a role in the events leading to the death of the cells (14).

The propensity for A2E to undergo photooxidation was initially revealed by a tendency for fluorescence quenching of intracellular A2E upon blue light illumination (13). HPLC analysis later confirmed this observation, the absorbance of the A2E peak after 430 nm irradiation, exhibiting a corresponding reduction (13). Subsequent analysis by mass spectrometry showed that 430 nm irradiation of A2E, either in an acellular or cellular environment, yielded products that, starting from the M−/m/z 592 peak attributable to A2E, presented as consecutive peaks differing in m/z by 16 (15). Several lines of investigation suggested that singlet oxygen is involved in the photooxidation, with A2E serving as a sensitizer for the generation of singlet oxygen from triplet oxygen (13, 15, 16). For instance, the extent of photooxidation was found to increase in deuterium oxide (D2O), a solvent that prolongs the lifetime of singlet oxygen. Irradiation (430 nm) of A2E in chloroform also produces a luminescence at ~1270 nm, which is typical of the phosphorescence of singlet oxygen. In addition, experiments demonstrated that singlet oxygen generated by an endoperoxide of 1,4-dimethylpyridine could substitute for blue light in mediating A2E oxidation. Singlet oxygen quenchers were also found to be inhibitory. Nonetheless, oxidation by other reactive forms of oxygen may also occur (17, 18).

Given that A2E has nine double bonds besides the pyridinium ring, together with the observation that nine m/z peaks (592 + m/16) culminating in the m/z 736 peak (592 + 9/16) (Fig. 2) (15, 18, 19) appear following 430 nm irradiation of A2E, we previously suggested that A2E undergoes oxidation at all nine double bonds to form an unprecedented nonaoxirane structure. Because of the likelihood that numerous stereoisomers of the nonaoxirane species were present, together with its instability and the small amount of compound that was available, structural studies were not performed. As part of our effort to elucidate mechanisms involved in A2E oxidation, we also oxidized A2E with meta-chloroperbenzoic acid (MCPBA) (15). This approach led to the appearance of an m/z 624 peak (parent peak), to which was assigned a 7,8,7′,8′-bisoxo structure based on the nonaoxirane and mass spectrometry and 1H NMR data (15). More recently, it has been reported that photooxidation products of A2E can include a mono-furanoid oxide, a bis-
furanoid oxide (Fig. 3B), and a mono-furanoid oxide with a second oxygen attached to the cyclohexenyl ring (20). However, this conclusion was based on the fragmentation patterns generated by collision-induced dissociation and tandem mass spectrometry, an approach that cannot discriminate between a 5,8-furanoid and a 7,8-epoxide. Nor can the use of $^1$H NMR spectra distinguish a furanoid from an epoxide moiety because the chemical shift of the 7,8-protons in $^1$H NMR does not reveal the resonance of the 7,8-carbon. Here we provide definitive evidence from HSQC-NMR spectroscopy that the complex mixture of oxidized species resulting from A2E photooxidation includes a 5,8-monofuran-A2E. Further investigation has also uncovered a bisoxygenated photo-product that has been structurally characterized as 5,8-monoperoxy-A2E. More importantly, through an analysis of chromatographic properties and UV-visible spectra together with mass spectrometry to provide molecular weight information, we have also detected a mono-furan-A2E and monoperoxy-A2E in aged human RPE and in the eye-cups of mice with null mutations in $\text{Abca4/Abcr}$, the gene responsible for recessive Stargardt disease.

**FIGURE 1.** Structures of the RPE lipofuscin fluorophores A2E, isoA2E, and all-trans-retinal dimer-PE conjugate. PE, phosphatidylethanolamine.

**FIGURE 2.** Photoisomerization and photooxidation of A2E. Under the influence of light, A2E and iso-A2E undergo photoequilibrium to generate an ~4:1 mixture (A2E/iso-A2E). ESI mass spectrum of A2E (m/z 592) after irradiation at 430 nm is shown. The addition of oxygens is evidenced by a series of peaks that differ in m/z by 16 with the peak at m/z 736, indicating the formation of nonaoxo-A2E, a compound that is likely a complex mixture of stereoisomers.
Photooxidation Products of A2E

EXPERIMENTAL PROCEDURES

Reagents—MCPBA, ethanamine, and trifluoroacetic acid were purchased from Aldrich; HEPES was obtained from Sigma; acetonitrile was purchased from Fisher; and Dulbecco’s phosphate-buffered saline was from Invitrogen. All of the other chemicals were from Sigma. A2E was synthesized as described previously (7).

MCPBA Oxidation of A2E—To a solution of A2E (20.0 mg) in methanol (1.0 ml) was added MCPBA (33.0 mg, 2 eq), and the mixture was stirred for 12 h at room temperature in the dark. After concentration in vacuo, the reaction mixture was subjected to HPLC analysis. A2E mono- and bisfuranoid (Fig. 4) were eluted at 31 and 21 min, respectively, using a YMC C18 column (22 mm x 250 mm) under the following conditions: solvent A, CH3CN, 0.1% trifluoroacetic acid; solvent B, H2O, 0.1% trifluoroacetic acid; gradient mode (A/B), 0 min, 80:20; 20 min, 90:10; 50 min, 100:0; flow rate 4.0 ml/min. UV-visible spectra were monitored at 430 nm.

Endoperoxide of 1,4-Dimethylnapthalene and A2E Oxidation—1,4-Dimethylnapthalene endoperoxide was synthesized as described previously (21). Subsequently, endoperoxide of 1,4-dimethylnapthalene (48.0 mg, 10 eq of A2E) was added to a solution of A2E (15.0 mg) in CD3OD (1.0 ml), and the mixture was stirred overnight at room temperature in the dark. After removal of solvent, the oxidation products of A2E were isolated and purified by HPLC using a YMC C18 column (10 x 250 mm) with the following solvent system: solvent A, CH3CN, 0.1% trifluoroacetic acid; solvent B, H2O, 0.1% trifluoroacetic acid; gradient mode (A/B), 0 min, 75:25; 40 min, 100:0; flow rate 2.5 ml/min. A2E mono- and bisperoxide (Fig. 5) were eluted at 21 and 12.5 min, respectively.

NMR—1H NMR and heteronuclear singlet-quantum coherence spectroscopy (HSQC) spectra of 5,8,5'-8',bisfuran-A2E were recorded at 500 MHz using a Bruker DMX-500 spectrometer. 1H NMR and HSQC spectra of 5,8,5'-8',bisperoxide-A2E were recorded at 400 MHz (Bruker DRX-400). All data were recorded in CD3OD, and TMS was used as internal standard.

NMR of Bisfuran—A2E: 1H NMR (500 MHz, CD3OD, 25 °C, TMS): δ = 1.14 (s, 6H), 1.41 (s, 3H), 1.42 (s, 3H), 1.77 (s, 3H), 1.91 (s, 3H), 2.07 (s, 3H), 3.87 (s, 2H), 4.50 (s, 2H), 5.15 (s, 1H, H-8), 5.19 (s, 1H, H-8'), 5.22 (s, 1H, H-7), 5.26 (s, 1H, H-7'), 6.26 (d, J = 11.3 Hz, 1H), 6.43 (d, J = 11.2 Hz, 1H), 6.56 (d, J = 15.0 Hz, 1H), 6.66 (s, 1H), 6.73 (d, J = 15.3 Hz, 1H), 6.93 (dd, J = 11.3, 15.0 Hz, 1H), 7.81 (dd, J = 11.2, 15.3 Hz, 1H), 7.86 (s, 1H, H-1), 7.91 (d, J = 7.0 Hz, 1H), 8.53 (d, J = 7.0 Hz, 1H). HSQC (500 MHz, CD3OD): 86.7 (C-8), 86.8 (C-8').

NMR of Bisperoxy—A2E: 1H NMR (400 MHz, CD3OD, 25 °C, TMS): δ = 1.16 (s, 6H), 1.18 (s, 6H), 1.28 (s, 3H), 1.59 (s, 3H), 1.60 (s, 3H), 2.08 (s, 3H), 2.11 (s, 3H), 3.90 (t, J = 4.8 Hz, 2H), 4.54 (t, J = 4.8 Hz, 2H), 4.71 (d, J = 4.0 Hz, 1H, H-8), 4.77 (d, J = 4.0 Hz, 1H, H-8'), 5.63 (d, J = 4.0 Hz, 1H, H-7), 5.67 (d, J = 4.0 Hz, 1H, H-7'), 6.23 (d, J = 11.2 Hz, 1H), 6.41 (d, J = 11.2 Hz, 1H), 6.58 (d, J = 15.2 Hz, 1H), 6.71 (s, 1H), 6.77 (d, J = 15.5 Hz, 1H), 6.98 (dd, J = 11.2, 15.5 Hz, 1H), 7.85 (dd, J = 11.2, 15.5 Hz, 1H), 7.91 (d, J = 2.0 Hz, 1H), 7.96 (dd, J = 2.0 Hz, 1H), 8.57 (d, J = 6.6 Hz, 1H). HSQC (400 MHz, CD3OD): 82.9 (C-8), 83.6 (C-8').

RESULTS

To generate a partially oxidized A2E (oxo-A2E) species, we began by using MCPBA as the oxidizing agent. Reaction of A2E with MCPBA (2 eq) in the dark followed by HPLC analysis revealed a bisoxo product with 1H NMR and mass spectral profiles that were identical to those reported previously for MCPBA-oxidized A2E (15). We surmised that the oxygen-containing moiety could exhibit one of three possible structures: 5,6-epoxide, 5,8-furanoid, or 7,8-epoxide (Fig. 3). The 5,6-epoxide and 5,8-furanoid were considered to be candidates because MCPBA oxidation of β-carotene with the same ring moiety yields a 5,6-epoxide; moreover, the 5,6-epoxide readily rearranges to the 5,8-furanoid structure even under mild acidic conditions (20, 24). However, the 5,6-epoxide was eliminated as a possibility because NMR revealed that the 7,8,8'-proton signals at 5.15–5.26 ppm are shifted upfield (see the

FIGURE 3. Candidates for the structure of the oxygen-containing moiety generated by MCPBA oxidation of A2E. In each case, the moiety on only one retinoid arm of oxo-A2E is represented. A, 5,6-epoxide; B, 5,8-furanoid; C, 7,8-epoxide.
NMR data under ”Experimental Procedures”) compared with those of A2E at 6.18 to 6.53 ppm (6). Because fragment ions generated using collision-induced dissociation tandem mass spectral analysis for structural determination of o xo-A2E can form by rearrangement during ionization, we sought confirmatory evidence of a furanoid ring by NMR analysis. Accordingly, by using HSQC-NMR spectroscopy to reveal correlations between carbon atoms and directly attached protons (hydrogen), the structure was shown to be that of a 5,8-furanoid (Fig. 4). Specifically, HSQC analysis revealed that the two 87 ppm sp² carbons are coupled to 5.15 (8-H)/5.19 (8'-H) ppm protons, whereas the two 117 ppm sp² carbons are coupled to 5.22 (7-H)/5.26 (7'-H) ppm protons. In addition, because both of the two UV-visible absorbance maxima of A2E were blue-shifted (see below) in this bisfuranoid-A2E, it was established that the bisfuranoid-A2E is 5,8,5'-bisfuranoid-A2E (Fig. 4). We were also able to identify the monofuranoid-A2E as 5,8-monofuranoid-A2E.

Because A2E photooxidation is likely to occur, at least in part, via a singlet oxygen-mediated pathway (13, 15), A2E was also reacted with 1,4-dimethyl napthalene endoperoxide, an aromatic compound that decomposes to singlet oxygen and 1,4-dimethyl napthalene with a convenient half-life of 5 h at 25 °C. Analysis of the product by LC-MS revealed the presence of a peak that exhibited an UV-visible absorption spectrum suggestive of a bisfuranoid-A2E but a molecular mass (m/z 656) that was consistent with the addition of 4 oxygens. NMR studies, including ¹H and HSQC NMR, showed upfield-shifted protons at the 7, 8, and 7', 8' positions, which together with the existence of sp² carbons evidenced by HSQC data (115.2 (C-7)/116.7 (C-7')) confirmed this tetraoxo-A2E as 5,8,5',8'-bisperoxy-A2E (Fig. 5). The presence of the 1,2-dioxin moieties in this o xo-A2E species was corroborated by a previous investigation that demonstrated the production of a cyclic 5,8-peroxide upon photolysis of A1E, a nonbiological single side arm counterpart to A2E (25).

We found that oxidation-associated changes in the UV-visible spectrum of A2E served as a means to determine the oxidation site (Fig. 6). This was possible because the absorption peaks of A2E at 337 nm (band S) and 439 nm (band L) could be assigned to the shorter and longer chains that extend from the pyridinium ring, respectively. Thus, whether the hypsochromic shift occurred in either band S or band L revealed the side arm on which the loss of conjugation had occurred. For instance, comparison of the UV-visible spectra of the mono-furanoid and mono-peroxide with that of A2E revealed that only band S was blue-shifted. In contrast, both bands were observed to undergo a hypochromic shift in the bisfuranoid and bisperoxide. Both furanoid and peroxide formation resulted in the loss of two successive conjugation systems, hence the absorption maximum (either band S or L) of the affected side arm was shifted toward the blue region by ~40 nm.

HPLC analysis of products generated by photooxidation and chemical oxidation also revealed the presence of 335 and 400 nm absorbance peaks that corresponded to blue shifts in only the L band. This observation indicated oxidation exclusively on the longer arm. However, the minuscule amount of these compounds precluded further structural studies. Oxidation apparently occurs more readily on the shorter arm of A2E. Of the two polynenes extending ortho and para to the pyridinium nitrogen, electron delocalization is favored along the former ortho arm, which has one extra conjugation π bond; this leads to relatively lower density of electrons in each of the sp² carbons, thus making it less nucleophilic and less susceptible to the MCPBA attack.

In order to examine the formation of the oxidative products of A2E in a cellular environment, ARPE-19 cells were allowed to incorporate A2E, and the A2E-laden ARPE cells were irradiated at 430 nm. Chloroform/methanol extracts of the cells were analyzed by LC-MS. As shown in Fig. 7, two eluting components that were more polar than A2E and with m/z of 624 and 608 were resolved. The UV-visible spectra of these peaks had similar profiles; specifically, absorbance maxima occurred at 296 nm (band S) and ~435 nm (band L). Because only band S exhibited a hypsochromic shift, relative to A2E, it was apparent that in both cases two successive conjugate systems on the shorter arm of A2E were lost by oxidation. On the basis of the presence of two peaks, which by LC-MS exhibited molecular sizes that corresponded to a mono-oxo (m/z 608) and bisfuranoid-A2E (m/z 624), together with our prior NMR analysis that established the structures of these products, it was evident that in this cellular system the mono-furanoid and mono-peroxide formed.

We also detected o xo-A2E in chloroform/methanol extracts of RPE cells isolated from human donor eyes and in extracts of posterior eye cups of Abcat4/Abcr<sup>−/−</sup> mice. The latter mice are considered to be a model of Stargardt macular degeneration. Again we used LC-MS for this analysis. It is our experience that the use of MS/MS fragmentation analysis with collision-induced dissociation is not a reliable approach to the assignment of o xo-A2E structure because the fragmentation patterns vary, probably because the oxygen-containing fragment ions can undergo complex intramolecular rearrangements. As shown in Fig. 8, examination of the human and mouse samples by LC-MS revealed the presence of reversed phase HPLC eluates with extracted ion signals indicative of m/z 592, 608, and 624. The corresponding compounds

figure 4. HSQC study of bisfuran-A2E isolated following MCPBA oxidation of A2E. Numbers represent chemical shifts of the protons. Numbers in parentheses represent chemical shifts of carbons directly connected to protons.

figure 5. Determining the structure of the tetraoxo-A2E isolated following endoperoxide oxidation of A2E. A. HSQC study reveals the structure to be that of 5,8,5',8'-bisperoxy-A2E. Numbers represent chemical shifts of the protons. Numbers in parentheses represent chemical shifts of carbons directly connected to protons. B, the mono-retinoid A1E also undergoes oxidation to form peroxo-A1E.
Photooxidation Products of A2E

could be identified as A2E ($m/z$ 592), monofuran-A2E ($m/z$ 608), and monoperoxy-A2E ($m/z$ 624) because they had the same retention time and UV-visible absorbance spectra as the species detected in A2E-laden ARPE-19 cells following 430 nm irradiation (Fig. 7).

To determine whether these partially oxidized forms of A2E could serve as intermediates on a pathway to further oxidation of A2E, we incubated A2E with MCPBA and isolated the product 5,8-monofuran-A2E by HPLC. The monofuranoid in phosphate-buffered saline with 0.5% Me$_2$SO was subsequently submitted to blue light irradiation (430 nm), and unirradiated and irradiated samples were analyzed by MS. As shown in Fig. 9, the 5,8-monofuran-A2E ($m/z$ 608) underwent further oxidation to generate higher molecular species representing the incorporation of additional oxygens (i.e., $m/z$ 640, 672, and 720 peaks corresponding respectively to addition of 2, 4, and 7 oxygens). Most interestingly, however, if irradiation of 5,8-monofuran-A2E was carried out with methanol as solvent, no additional oxidation occurred.

We also proceeded to revisit the issue of solvent effects on A2E photooxidation. To this end, we performed HPLC analysis of unirradiated samples of A2E and samples in which A2E was irradiated in an environment of Me$_2$SO/H$_2$O and samples irradiated in Me$_2$SO/D$_2$O (Fig. 10). Consistent with the ability of deuterium solvent to extend the lifetime of singlet oxygen (26), irradiation in D$_2$O resulted in more pronounced...

FIGURE 6. Correlation between hypsochromic shifts in the UV-visible spectra and A2E oxidation. The UV-visible spectrum of A2E exhibits two major absorbance bands at 337 and 438 nm. These bands can be assigned to the shorter (S) and long (L) arms of A2E, respectively. Oxidation of a carbon-carbon double bond is accompanied by a hypsochromic shift. A hypsochromic shift in band S corresponds with oxidation on the short arm; a blue-shift in band L reflects oxidation on the long arm; hypsochromic shifts in both peaks indicates oxidation on both side arms.

FIGURE 7. Generation of monoperoxy-A2E and monofuran-A2E upon irradiation of intracellular A2E. A2E-laden ARPE-19 cells were irradiated at 430 nm and subjected to LC-MS analysis. HPLC was obtained by monitoring at 430 nm. UV-visible absorbance spectra were recording by a photodiode array detector. Assignment of mass to the chromatographic peaks was performed by coupled electrospray ionization analyses. mAU, milli-absorbance units.

FIGURE 8. LC-MS analysis of extracts of isolated human RPE (A) and eye cups of Abca4/Abcr$^{-/-}$ mice (B). Extracted ion chromatograms acquired in ESI mode with selection for mass to charge ($m/z$) ratios 592, 608, and 624 and recorded as a function of retention time in a reversed phase HPLC column.
photooxidation, the latter reflected in the greater diminution in the A2E peak relative to the sample irradiated in H$_2$O. Specifically, quantitation of the peak area revealed that with irradiation in Me$_2$SO/H$_2$O A2E was decreased by 75%, whereas in Me$_2$SO/D$_2$O under the same conditions of irradiation the reduction was 90%. In these experiments, only the singlet oxygen that escaped to the medium would be affected by the deuterated solvent.

**DISCUSSION**

Based on the foregoing results, we propose that A2E oxidation occurs by means of multiple independent mechanisms. For instance, the addition of one atom of oxygen can occur at the 5,6 position in the cyclohexenyl ring to form an epoxide followed by, at least in some cases, a rearrangement to a 5,8-furanoid product. The initial epoxidation occurs at the 5,6 double bond as it is the most electron-rich carbon-carbon

![FIGURE 9. Photooxidation of the oxo-A2E product generated by chemical oxidation with MCPBA. A, fast atom bombardment mass spectra of the $m/z$ 608 product (5,8-monofuran-A2E) generated from MCPBA oxidation of A2E. B, irradiation (430 nm) of the $m/z$ 608 product resulted in photooxidation and generation of oxo-A2E species ($m/z$ 640, 672) with additional oxygens.](image-url)
of reactive intermediates and products can form, including aldehydes, epoxides, and epoxyketones (28). In this way the endoperoxide moiety of oxo-A2E can be expected to promote reactivity (29). Indeed, the instability of A2E-endoperoxide may account for the observation that upon irradiation of A2E in cells, wherein the environment is conducive to reactivity, the peak at m/z 624 (monoperoxide) exhibits reduced intensity relative to the peak at m/z 608 (monofuranoid) (13). It is also important to note that the cytotoxicity of metabolites and synthetic endoperoxide-containing compounds is well known (29, 30). The involvement of singlet oxygen in the formation of the peroxy-A2E photooxidation product is corroborated by the observation, in the present work, that the photochemical consumption of A2E was greater in D2O than in H2O. A similar effect of deuterium solvent was reported previously (15). The deuterium effect observed in the present work is also consistent with our previous observation that the singlet oxygen quencher 1,2,2,6,6-pentamethyl-4-piperidinol can protect against the photooxidation of A2E (13). It was also found that azide, histidine, and 1,4-diazabicyclo[2.2.2]octane all of which are efficient quenchers/scavengers of singlet oxygen, protected A2E-containing RPE from irradiation-induced cell death, whereas in deuterium-based media cell death was potentiated (13). These observations contrast with the report that photooxidation-associated loss of A2E occurred twice as fast in H2O than in D2O and that the rate of loss of A2E was also increased in the presence of azide, a singlet oxygen quencher (18).

We have shown previously that the monoretinoid A1E, a synthetic compound designed to model A2E except that it has a single side arm instead of two, undergoes photooxidation by mechanisms that are oxygen- and solvent-dependent. For instance, irradiation of A1E (425 nm) in methanol solution resulted in pericyclization to form pyridinium terpenoids, presumably secondary to photoisomerization of the C-7–C-8 double bond and ring closure. The quantum yield for this cyclization reaction was very low because of efficient photoisomerization of the 7-cis-isomer back to the all-trans-isomer of A1E. It is possible that irradiation-induced pericyclization of A2E could also occur, but because we have not observed this product, if present it must be very minor. Irradiation of A1E in air-saturated carbon tetrachloride or deuterated chloroform also generated a cyclic 5,8-peroxide, the same product as that observed in the current experiments with A2E. The yield of cyclic 5,8-peroxide formation on A1E was increased in environments that extended the singlet oxygen lifetime. Most interestingly, an increase in the concentration of A1E also increased the yield. We did not test the effect of A2E concentration on the yield of cyclic 5,8-peroxide in the present experiments, but we expect there to be a similar effect. Most interestingly, A1E photooxidation always ceases after the addition of two oxygens, whereas A2E oxidation can proceed until nine oxygens are added. These observations suggest that polyoxygenation of A2E is enabled by the presence of two closely spaced side arms.

Photoreceptor cell degeneration in recessive Stargardt macular degeneration likely occurs as a result of the RPE cell dysfunctioning and death that is precipitated by the accumulation of lipofuscin pigments (31–33). Although RPE lipofuscin consists of a mixture of fluorophores, our understanding of the adverse effects of specific lipofuscin fluorophores has come largely from studies of A2E and its photoisomers. For instance, the photooxidative processes initiated in cultured RPE cells through blue light-induced sensitization of A2E can lead to DNA base lesions, modifications of protein, and changes in protein expression (14, 34). Several areas of investigation suggest that the photooxidation products of A2E may be damaging agents that are biologically significant. For instance, cellular damage is realized in the presence of oxo-A2E even under conditions that eliminate singlet oxygen and other reactive forms of an oxygen radical (27). However, the biological relevance of these experimental conditions is not clear.

The conjugated double bonds at the 5,6 and 7,8 carbons of A2E are excellent substrates for [4 + 2] cycloaddition of singlet oxygen. Thus the formation of a cyclic peroxy following the photooxidation of intracellular A2E is likely to be a major product. Because of the weakness of the O–O bond, the major pathway from the endoperoxide is homolytic O–O bond cleavage to form an unstable diradical from which a number of reactive intermediates and products can form, including aldehydes, epoxides, and epoxyketones (28). In this way the endoperoxide moiety of oxo-A2E can be expected to promote reactivity (29).
of oxygen as the damaging intermediate (16). Additionally, fragmentation of photooxidized A2E with diffusion of the products may be the explanation for the observation that cellular damage can be observed at sites other than the lysosomal compartment in which A2E is housed, sites that are too distant for the damage to be accounted for by short-lived singlet oxygen and other cytotoxic forms of oxygen generated by A2E photosensitization (35). A species corresponding to mono-oxo-A2E was detected previously in human retinal lipofuscin (36), whereas in the present work, monofuran-A2E and monoperoxy-A2E were detected in extracts of both human RPE and in eucups of Abca4−/− mice. Also suggestive of the presence of blue-shifted photooxidation products are spectral studies of fundus autofluorescence, demonstrating that in patients with Stargardt disease the fluorescence emission is shifted toward lower wavelengths over hyperfluorescent lipofuscin-rich sites corresponding to ophthalmoscopically visible yellow flecks (37). Flecks are prognostically significant because they are a sign of a more severe disease (38). Although the spectral shift may indicate a difference in the relative levels of individual fluorophores in these areas, it is also conceivable that the blue shift reflects an increased content of oxo-A2E. In this regard, it may also be significant that fundus spectrophotometric measurements obtained in human subjects using 550 nm excitation indicate that RPE lipofuscin fluorescence declines after age 70 (39). Although there is potentially more than one explanation for the declining fluorescence at these excitation wavelengths (40), perhaps the spectral shifts associated with the photooxidation of A2E are a contributing factor.

REFERENCES

1. Lois, N., Halfdyk, A. S., Bird, A. C., Holder, G. E., and Fitzke, F. W. (2004) Am. J. Ophthalmol. 138, 55–63.
2. Shroyer, N. F., Lewis, R. A., Allikmets, R., Singh, N., Dean, M., Leppert, M., and Lupski, J. R. (1999) Vision Res. 39, 2537–2544.
3. Weng, J., Mata, N. L., Azarian, S. M., Tzekov, R. T., Birch, D. G., and Travis, G. H. (1999) Cell 98, 13–23.
4. Sparrow, J. R., and Boulton, M. (2005) Exp. Eye Res. 80, 595–606.
5. Eldred, G. E. (1993) Nature 364, 396.
6. Sakai, N., Decatur, J., Nakanishi, K., and Eldred, G. E. (1996) J. Am. Chem. Soc. 118, 1529–1560.
7. Parish, C. A., Hashimoto, M., Nakanishi, K., Dillon, J., and Sparrow, J. R. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 14609–14613.
8. Ben-Shabat, S., Parish, C. A., Vollmer, H. R., Itagaki, Y., Fishkin, N., Nakanishi, K., and Sparrow, J. R. (2002) J. Biol. Chem. 277, 7183–7190.
9. Fishkin, N., Sparrow, J. R., Allikmets, R., and Nakanishi, K. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 7091–7096.
10. Fishkin, N., Pescitelli, G., Sparrow, J. R., Nakanishi, K., and Berova, N. (2004) Chirality 16, 637–641.
11. Sparrow, J. R., Nakanishi, K., and Parish, C. A. (2000) Investig. Ophthalmol. Vis. Sci. 41, 1981–1989.