

Review

A2E: A Component of Ocular Lipofuscin[¶]

Laura E. Lamb and John D. Simon*

Department of Chemistry, Duke University, Durham, NC

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ABSTRACT

The presence of lipofuscin in postmitotic cells is considered a hallmark of the aging process. In the retinal pigment epithelium (RPE), lipofuscin is found as micrometer-sized spherical particles and characterized by its yellow autofluorescence when exposed to blue light. This exposure to light is also known to produce reactive oxygen intermediates (ROI), but the particular molecular constituent(s) responsible for this phototoxicity have yet to be completely identified. Resulting mostly from the autophagocytosis of intracellular organelles, the composition of lipofuscin is poorly defined but known to contain protein, lipids and several fluorophores. The subsequent identification of one of the fluorophores in lipofuscin, A2E, generated much interest and resulted in a variety of studies to understand its potential role in the phototoxicity of lipofuscin. Several modes of toxicity have been suggested through which A2E can affect the health of RPE cells. These modes include photoinduced production of ROI, which places additional oxidative stress on RPE cells, the disruption of membrane integrity through its natural role as an amphiphilic detergent and inhibition of key cellular functions. This article presents the current understanding of the photochemistry of A2E and its involvement as a phototoxic agent in RPE cells.

DISCOVERY AND IDENTIFICATION

In a seminal study Eldred and Katz (1) reported that a series of fluorophores were isolated from retinal pigment epithelium (RPE) cellular extracts. The excitation and emission spectra of these molecules revealed the presence of several blue-absorbing chromophores. These unidentified molecules were proposed to be responsible for the blue-light-induced phototoxicity of lip-

ofuscin granules. Purification of RPE extracts led Eldred and Lasky (2) to identify one of these fluorophores. This molecule had a mass of 592.4520 amu and a molecular formula $C_{42}H_{58}NO$. Basic chemical analysis revealed one quaternary nitrogen, one hydroxyl group and 12 double bonds in the structure of the molecule. However, analysis of fragmentation patterns from mass spectrometry (MS) measurements provided ambiguous results. A reaction of all-*trans*-retinal and ethanolamine produced a product with similar chemical and physical characteristics, but in a poor yield of less than 1%. The product of the Schiff base condensation of retinal and ethanolamine was proposed to be *N*-retinylidene-*N*-retinylethanolamine, shown in Scheme 1. However, this proposed structure was quickly shown to be inconsistent with the molecular formula determined (3,4). Thus, refinement of the molecular structure was necessary. Elucidating the exact structure was of great interest because this particular molecule was also purported to "account for a substantial portion of the fluorescence of human RPE lipofuscin" (2). But in fact, as described in a brief review of this work by Wolf (5), no quantitative data was presented, and the thin layer chromatography plates shown indicated the presence of several other unidentified fluorescent components of RPE lipofuscin.

To determine the correct molecular structure of this blue-absorbing chromophore, Sakai *et al.* (6) used MS and nuclear magnetic resonance techniques to establish that the molecule was an unprecedented pyridinium bis-retinoid. The chemical name for the corrected structure is 2-[2,6-dimethyl-8-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1*E*,3*E*,5*E*,7*E*-octatetraenyl]-1-(2-hydroxyethyl)-4-[4-methyl-6-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1*E*,3*E*,5*E*-hexatrienyl]-pyridinium, see Scheme 1. The molecule was coined A2E because the structure is a combination of two all-*trans*-retinal molecules (vitamin A aldehyde) and one ethanolamine molecule (6,7). Note that all the double bonds in the molecule are in the *E* or *trans* configuration.

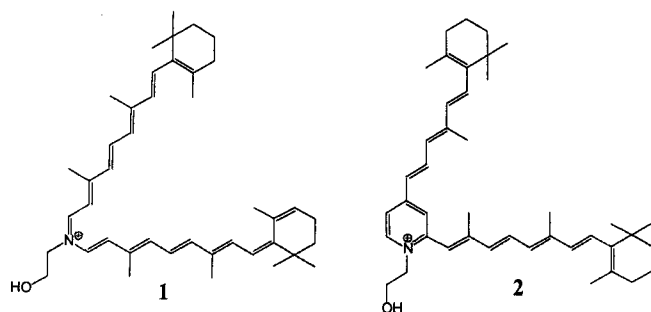
The presence of A2E in albino rat eyes was first confirmed by Reinboth *et al.* (8), who developed a high-pressure liquid chromatography (HPLC) assay capable of detecting A2E concentrations as low as 10 pmol. Similar HPLC studies by Parish *et al.* (9), which aimed at identifying A2E in the extract of human RPE cells, revealed a second isomer of A2E. This isomer, termed iso-A2E, shown in Scheme 2, was also found in small traces in biomimetic synthetic samples and identified as the 13'-*cis* isomer of all-*trans*-A2E. Exposure of a purified sample of all-*trans*-A2E to room light and subsequent analysis revealed that all-*trans*-A2E and 13'-*cis*-A2E achieve a 4:1 photostationary equilibrium. The

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*To whom correspondence should be addressed at: Department of Chemistry, Duke University, Durham, NC 27708-0346, USA. E-mail: jsimon@duke.edu

Abbreviations: Bcl-2, B-cell leukemia/lymphoma 2; DMPO, 5,5'-dimethyl-1-pyrroline-*N*-oxide; DMSO, dimethyl sulfoxide; EPR, electron paramagnetic resonance; FAB, fast atom bombardment; HPLC, high-pressure liquid chromatography; LDH, lactate dehydrogenase; MS, mass spectrometry; PE, phosphatidylethanolamine; POS, photoreceptor outer segments; ROI, reactive oxygen intermediates; ROS, rod outer segments; RPE, retinal pigment epithelium.

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Scheme 1. The first incorrectly proposed structure of *N*-retinylethanolamine (**1**) and the corrected structure of 2-[2,6-dimethyl-8-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1*E*,3*E*,5*E*,7*E*-octatetraenyl]-1-(2-hydroxyethyl)-4-[4-methyl-6-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1*E*,3*E*,5*E*-hexatrienyl]-pyridinium (**2**), coined A2E.

appearance of other unidentified molecules in the equilibrium mixture was noted. After refinement of the HPLC procedure, additional isomers were identified (10). An HPLC-purified sample of all-*trans*-A2E was exposed to room light for 35 min. Subsequent HPLC analysis of the sample showed the presence of seven products, primarily all-*trans*-A2E and 13'-*cis*-A2E. These remaining species had absorbance spectra similar to that of all-*trans*-A2E, and HPLC followed by MS showed that the mass of each of these species was 592.45 amu. These species were also determined to be present in the extract of human RPE cells. Based on light-induced isomerization studies of all-*trans*-retinal to form its 13-*cis*, 11-*cis*, 9-*cis* and 9,13-di-*cis* isomers, it was proposed that these additional A2E species, shown in Scheme 2, represent a range of *cis* isomers formed after the initial synthesis of A2E.

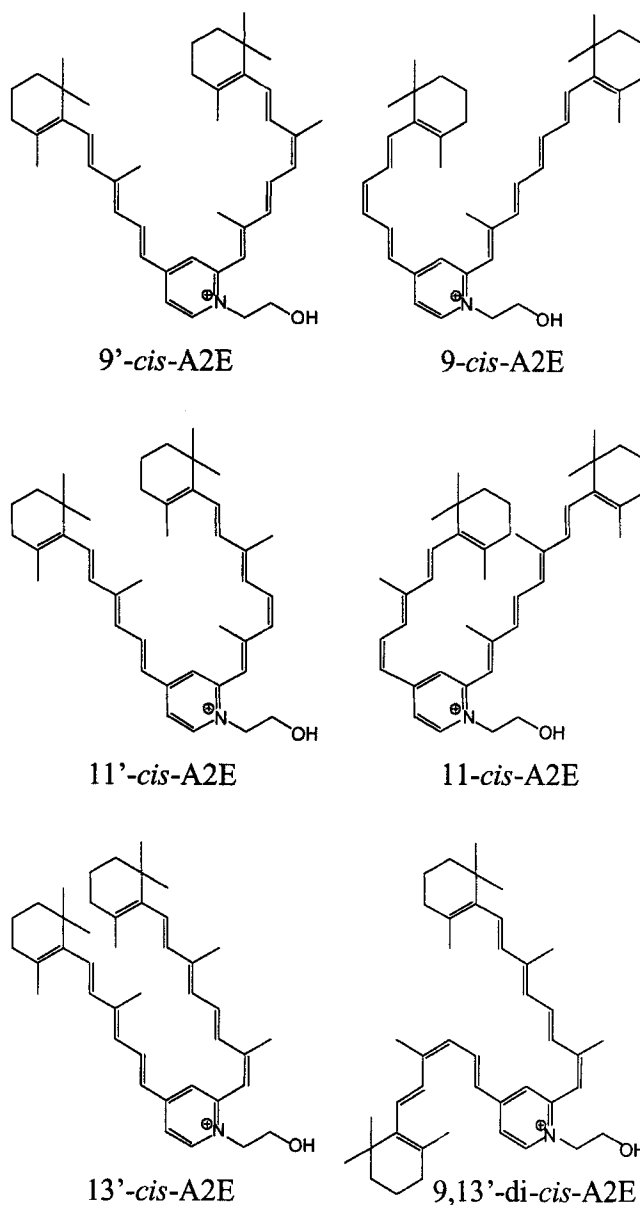
Because of the numerous isomers identified, unless otherwise specified in this review, the abbreviation A2E will refer collectively to all isomers.

SYNTHESIS AND PURIFICATION

Because of the poor yields of A2E using biomimetic methods, Ren *et al.* (11) developed a convergent total synthesis for A2E. This method used a double Wittig olefination of a bis-substituted pyridine moiety with a Wittig reagent comprising a portion of the A2E molecule common to both side chains. This reaction resulted in a mixture of A2E isomers at the 11 and 11' positions. This mixture was refluxed in nitromethane overnight and converted primarily to the all-*trans* isomer. The increased all-*trans* yield indicated that conversion between the A2E isomers could be thermally induced. This method yielded 70% all-*trans*-A2E, as confirmed by traditional analytic techniques.

The preparation of the bis-aldehyde pyridine moiety by Ren *et al.* (11) required several steps before it could be used in the Wittig reaction to prepare A2E. Using aza-6 π -electrocyclization followed by oxidation to form the bis-aldehyde pyridine, Tanaka *et al.* (12,13) condensed the number of steps necessary in the total synthesis of A2E. Refinement of this procedure led to an efficient one-pot synthesis of A2E consistent with its proposed biogenesis (12,13). The mechanism proposed by Sakai *et al.* (14) for the biological synthesis of A2E is discussed in the next section.

Modification of the previous biomimetic method by Parish *et al.* (9) also increased the yield of all-*trans*-A2E, comparable with that achieved using convergent techniques (11–13). The biomimetic



Scheme 2. Structures of possible A2E-derived photoisomers, including the previously identified isomer iso-A2E or 13'-*cis*-A2E.

synthesis of A2E was sensitive not only to the pH but also to the type of acid and solvent used in the reaction. When 2:1:1 equivalents of all-*trans*-retinal, ethanolamine and acetic acid were combined in ethanol and stirred at room temperature in the dark for 2 days, all-*trans*-A2E was formed in greater quantities. After chromatographic purification on a silica gel column, a 49% yield of A2E was achieved (9).

BIOSYNTHESIS, BIOTRANSPORT AND BIOAVAILABILITY

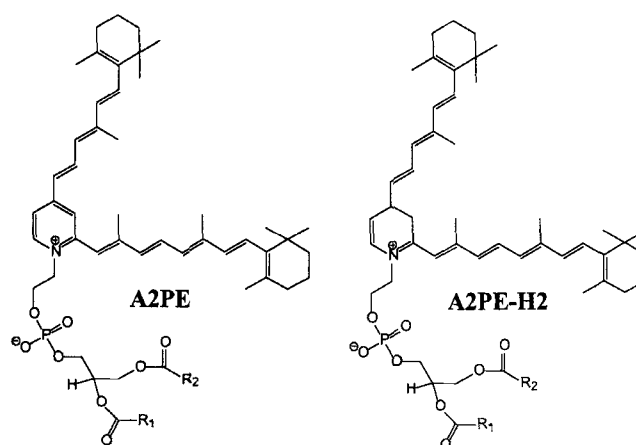
Shortly after the identification of a fluorophore present in lipofuscin, Eldred (7) envisioned the first biosynthetic pathway for its formation. He noted that if all-*trans*-retinal, released from rhodopsin, was not enzymatically reduced, it could reside in the

lipid bilayer of the outer segments disks. Only on disruption of this membrane would all-*trans*-retinal react with phosphatidylethanolamine (PE). Thus, Eldred (7) proposed that the synthesis of A2E likely takes place in the acidic environment of the RPE lysosome after phagocytosis of the outer segment disks containing the all-*trans*-retinal and PE. Once formed A2E would be unable to exit the lysosome and the lysosome would not be capable of degrading A2E, so it must accumulate in the lysosome.

Sakai *et al.* (14) expanded this hypothesis, suggesting the first biosynthetic mechanism for the formation of A2E in the RPE lysosome from all-*trans*-retinal and ethanolamine. In brief, the acidic environment of the RPE lysosome protonates the ethanolamine, which forms a Schiff base with one retinal molecule. This molecule undergoes tautomerization to form an intermediate enamine. This enamine reacts with a second retinal molecule yielding an iminium cation. This cation undergoes aza-6 π -electrocyclization and oxidation to form the final product A2E. Subsequent to the proposal of this mechanism, Parish *et al.* (9) suggested that the formation of A2E occurs not in the lysosome but in the photoreceptor outer segments (POS). It was suggested that all-*trans*-retinal, released from activated rhodopsin, could react with PE, present in high amount in the disk membranes of the outer segments. However, most of these proposals concerning the biological formation of A2E relied on little experimental data and a fair amount of conjecture.

To determine if these proposed mechanisms were occurring *in vivo*, Liu *et al.* (15) investigated the biosynthesis of A2E. First, *in vitro* experiments showed that retinal reacted with a variety of PE to produce A2PE, a proposed intermediate in the biosynthetic pathway, shown in Scheme 3. Additional experiments showed that retinal reacted with isolated bovine rod outer segments (ROS) to produce A2PE. To confirm the biological presence of A2PE, retinas from blind Royal College of Surgeons mice were extracted and subjected to HPLC analysis. A2PE was detected, supporting the proposed mechanism that A2E formation begins in the POS. This mechanism was further supported by the absence of A2PE in the retina of normal rats. Analysis of the extract of the RPE cells from both blind and normal control rats showed no detection of A2PE. Incubation of synthetic A2PE with phospholipase D generated A2E through enzymatic hydrolysis. Thus, this study concluded that A2PE, formed in the POS, is a biosynthetic precursor of A2E. Further work by Mata *et al.* (16) identified a precursor to A2PE in the biosynthesis of A2E, A2PE-H2, shown in Scheme 3. Located exclusively in the POS, oxidation of isolated and purified A2PE-H2 in the presence of acid yielded A2PE. Further overnight incubation and hydrolysis of the phosphate ester yielded A2E.

Additional biosynthetic studies by Ben-Shabat *et al.* (10) showed that A2PE was formed in the isolated bovine retinas after light exposures to release endogenous all-*trans*-retinal. This formation was accelerated in dark-adapted rats exposed to 6 h bright light as compared with that formed by rats maintained on a cyclic light schedule. The hydrolysis of A2PE was examined in greater detail. A2PE was incubated under acidic conditions similar to that of the lysosome for 24 h, after which no detectable levels of A2E were present. After 1 week of incubation only 0.01% of the original A2PE had been converted to A2E. Thus, based on previous results, it seems likely that the conversion of A2PE to A2E is enzyme mediated in the POS. After phagocytosis of the shed outer segments by the RPE, the A2E ingested remains in the lysosome.



Scheme 3. Structures of A2PE and A2PE-H2, biosynthetic precursors of A2E. R₁ and R₂ represent a variety of biological lipids.

Recent work by Fishkin *et al.* (17) identified a new fluorophore with emission properties similar to A2E. When purified bovine ROS were exposed to a 10-fold molar excess of all-*trans*-retinal, the generation of a fluorescent protein was observed. This protein was a rhodopsin adduct containing retinal moieties. It was determined that all-*trans*-retinal reacted with the lysines of rhodopsin in a manner similar to its reactions with ethanolamine to form a bis-substituted pyridinium moiety. This species was termed A2Rh and contained three such moieties at different lysines. It is interesting to note that when the ROS were exposed to only a three-fold excess of all-*trans*-retinal, the major product formed was A2PE, the precursor of A2E. A2Rh was formed only after this three-fold excess was exceeded. The authors suggested that the formation of A2PE is mediated by lipid binding to rhodopsin. This suggestion is consistent with the observation that A2PE is produced in greater yield by reacting all-*trans*-retinal with ROS than with pure PE molecules.

Quantification of the bioavailability of A2E in the RPE remains difficult to determine. Two different groups have measured the concentration of A2E *in vivo* (18,19). Sparrow *et al.* (18) isolated the RPE from a variety of donors aged 58–70 years. After quantifying the numbers of cells isolated, the RPE cells were extracted and HPLC analysis was performed. This revealed a range of A2E concentrations in the various donors from 34 to 134 ng/10⁵ cells with an average of 86 ng/10⁵ cells. The concentration did not appear to depend on donor age (18). In a similar experiment, Davies *et al.* (19) isolated and pooled lipofuscin granules from the RPE of a variety of donors aged 60–70 years. The number of granules was determined, and the granules were extracted. The extract was then subjected to HPLC analysis and the concentration of A2E quantified. In each lipofuscin granule 7.8 × 10⁻²⁰ mol of A2E was present. These workers report that on average a cultured RPE cell ingests approximately 300 granules; thus, each RPE cell contains 2.34 × 10⁻¹⁷ mol of A2E. This corresponds to an A2E concentration of 13 ng/10⁵ cells (19). This value is lower than any of the values determined by Sparrow *et al.* (18). This is possibly due to an underestimation of the number of granules present per cell by Boulton or the extraction of A2E present elsewhere in the cell in addition to the lipofuscin granules by Sparrow. In either case the concentrations of A2E used in any cellular experiment should span the range of values detected to mimic *in vivo* concentrations.

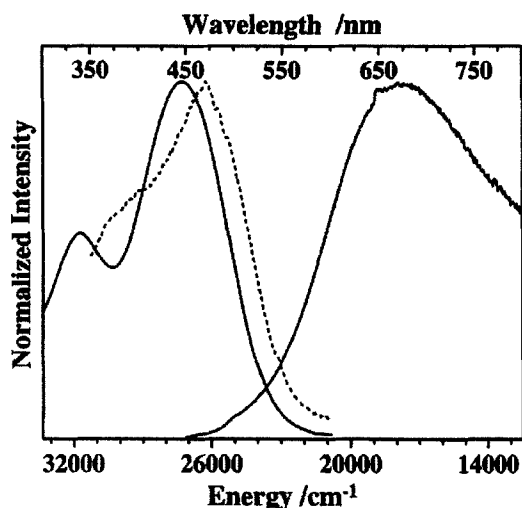


Figure 1. The absorption (—), emission excited at 434 nm (---) and excitation spectra monitored at 650 nm (···) of A2E in room-temperature methanol solution. The intensity shown is as a function of energy. Reprinted with permission from Lamb *et al.* (20).

PHOTOCHEMICAL AND PHOTOPHYSICAL STUDIES

The blue absorption of A2E generated the initial interest in its characterization. The absorbance, excitation and emission spectra of A2E are shown in Fig. 1 (20). The shift between the emission and excitation spectra indicates that the initial absorption of blue light populates the S_2 state. When corrected for instrumental response, the peak of the A2E emission is at 600 nm, within the range of the emission observed from lipofuscin granules. The emission quantum yield of A2E in ethanol was determined to be 0.01 (20).

Initial studies by Cubeddu *et al.* (21) measured the fluorescence lifetime of A2E in solution to be multiexponential, best fit by three time constants. The fluorescence lifetime of RPE cells loaded with A2E was found to be similar (21). However, an explanation for this multiexponential behavior was not offered. Later work by Lamb *et al.* (20) showed that the fluorescence lifetime of A2E was monoexponential with a time constant of approximately 12 ps. The previously observed nonexponentiality was partially attributed to trace amounts of fluorescent impurities in the samples (20). In addition, this lifetime was not previously observed because of the time resolution constraints of the instrument setup (21).

Photoacoustic studies observed that the primary relaxation event in A2E is nonradiative (20). When compared with a standard, A2E showed almost quantitative release of heat after excitation. This release was not affected by temperature and occurred on the subnanosecond timescale. Given the precision of the technique, it was estimated that the maximum quantum yield for intersystem crossing was at most 0.03. To explore the relaxation dynamics on a faster timescale, femtosecond transient absorption studies were performed. These experiments confirmed the S_1 lifetime of 12 ps and furthermore observed that the $S_1 \leftarrow S_2$ relaxation occurs in approximately 1 ps.

Photosensitization studies by Cantrell *et al.* (22) revealed that the excited triplet state of A2E has a lifetime of 30 μ s and a λ_{max} of 550 nm. This triplet was efficiently quenched by molecular oxygen with a second-order rate constant of $1 \times 10^9 \text{ dm}^3/\text{mol s}$. Direct

excitation of A2E at 355 nm produced a weak transient with a λ_{max} of 450 nm, which displayed an exponential decay with a rate constant of $1 \times 10^5 \text{ s}^{-1}$. Thus, the quantum yield for its formation is less than 0.01, consistent with the previously suggested low intersystem crossing efficiency (20). This study also found little evidence for the formation of A2E radicals after electron transfer from 1-nitronaphthalene. They suggested that the formal positive charge of A2E might affect its oxidation to a radical cation (22).

The photodynamics of A2E are dependent on the surrounding environment as reported by Ragauskaite *et al.* (23). When solubilized by Triton X-100 micelles as compared with methanol solution, the A2E emission maximum shifts to 615 nm from 510 nm. A blueshift in the emission maximum was also observed in a series of solvents with decreasing dielectric constants. In addition to shifts in the emission maxima, the quantum yield for fluorescence is also affected, ranging from 0.011 in Triton X-100 micelles to 0.002 in acetonitrile. Despite the changes observed in the steady-state emission of A2E, the absorption spectrum is similar in all solvents. Blue-light excitation of A2E in several solvents produced no observable long-lived transients or any measurable singlet oxygen production. However, when solubilized in Triton X-100 micelles, a weak transient was observed with a λ_{max} of 430 nm, which decayed exponentially with a rate constant of $7 \times 10^4 \text{ s}^{-1}$. Similar to the transient observed by Cantrell *et al.* (22), this transient species is also quenched by molecular oxygen in addition to several reducing agents (23).

AEROBIC REACTIVITY

The action spectrum for the photoconsumption of oxygen by A2E is shown in Fig. 2 (24). The normalized absorbance spectrum of A2E is superimposed over the same wavelength region. These data establish that the photoconsumption of oxygen is proportional to the absorption cross section of A2E. Identical experiments were performed on lipofuscin granules, lipofuscin extract and the material remaining after the extraction of lipofuscin. The action spectra for the photoconsumption of oxygen by all three samples are similar to but distinct from that of A2E. These results suggest that A2E is not responsible for the aerobic photoreactivity of lipofuscin. To provide a more quantitative comparison, oxygen uptake experiments were performed on absorbance-matched samples of A2E and the extract of lipofuscin. The rate of photoconsumption of oxygen at 434 nm for the lipofuscin extract was determined to be $9.38 \times 10^{-8} \text{ Ms}^{-1}$, whereas the corresponding A2E sample has a rate of $2.28 \times 10^{-8} \text{ Ms}^{-1}$. However, at this wavelength A2E cannot account for all the lipofuscin extract absorbance. In fact, from HPLC analysis of the extract, A2E likely is only responsible for 25% of the absorbance of the extract at 434 nm. Combining these results, A2E can account for approximately 6% of the photoconsumption of the lipofuscin extract. Thus, it is unlikely that A2E is the only molecule in lipofuscin contributing to its photoreactivity. However, a greater understanding of the reactions between A2E and oxygen was necessary.

To explore reactive oxygen intermediates (ROI) photoproduction by A2E, a standard cholesterol peroxidation assay was used (25). A2E and cholesterol were incorporated into lipid micelles and irradiated with blue light. Any ROI photogenerated by A2E reacts with the cholesterol to produce stable oxidized products. Using HPLC and electrochemical detection, the formation of these

products can be examined. This assay observed the production of several standard cholesterol peroxidation products, including the 5 α , 6 α , 6 β and 7 α / β , as shown in Fig. 3 (25). The 6 α , 6 β and 7 α / β products can be formed by reaction mechanisms involving several ROI, whereas the formation of the 5 α product can only come about by the reaction of cholesterol with singlet oxygen. These experiments establish the quantum yield for the photogeneration of singlet oxygen by A2E as approximately 1% depending on solvent conditions but do not explore the specific generation of other ROI. Related cholesterol assay experiments confirm these results (26).

To look for other ROI, standard detection methods for the production of superoxide have been used (26). Superoxide is known to react with several spin traps to produce a long-lived, stable species. These species can then be detected using electron paramagnetic resonance (EPR) techniques. The photoproduction of superoxide by A2E was measured using the spin trap 5,5'-dimethyl-1-pyrroline-*N*-oxide (DMPO) in dimethyl sulfoxide (DMSO). The EPR spectra produced displayed the splitting pattern typical for a DMPO spin adduct with superoxide as shown through comparison with a simulated spectra. The kinetics of this process were monitored over time and the yield for the formation of superoxide by A2E was found to be significantly less than that of lipofuscin extracts.

Complementary to the cholesterol peroxidation assay, the detection of emission directly from singlet oxygen is unambiguous confirmation of its production. Comparing the $^3\Sigma_g^- \leftarrow ^1\Delta_g$ emission of singlet oxygen produced from a standard to that of A2E determined the quantum yield for the production of singlet oxygen by A2E was at most 0.02 (20). Similar experiments have also measured singlet oxygen production by A2E with quantum yields ranging from 0.004 to 0.0008 depending on solvent and irradiation conditions (25,27). These yields are consistent with the low efficiency for intersystem crossing upon blue-light excitation of A2E (20). In addition to producing a small amount of singlet oxygen, A2E also has the ability to quench singlet oxygen. The effect of A2E on the lifetime of singlet oxygen was determined as a function of A2E concentration. A2E was shown to quench singlet oxygen with a rate constant of $10^8 \text{ M}^{-1} \text{ s}^{-1}$. This rate constant is similar to those for the endogenous antioxidants vitamin E and vitamin C (27). Thus, it is debatable whether A2E is capable of producing singlet oxygen in sufficient quantities to induce oxidative stress in RPE cells. The efficient quenching observed suggests that it is unlikely that any singlet oxygen produced diffuses away from the A2E before reacting with the A2E molecule itself.

To understand the photoinduced oxidation of A2E, several irradiation experiments were undertaken (28). In the presence of oxygen, blue-light irradiation of A2E, solubilized with 0.1% DMSO in phosphate-buffered saline, generated several oxidized species. HPLC purification combined with fast atom bombardment (FAB)-MS showed the formation of epoxides at 7,8 or 7',8' double bonds. Additional FAB-MS showed that all the nine double bonds in A2E were subject to epoxide formation. It was suggested that the formation of the epoxides was due to the reactivity of singlet oxygen with double bonds. Irradiation experiments in H₂O, D₂O, CH₃OH and CD₃OD were consistent with this proposal; the consumption of A2E as determined by HPLC analysis was greater in the deuterated solvents, which prolonged the singlet oxygen lifetime. The rate constant for quenching singlet oxygen was estimated to be $3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$.

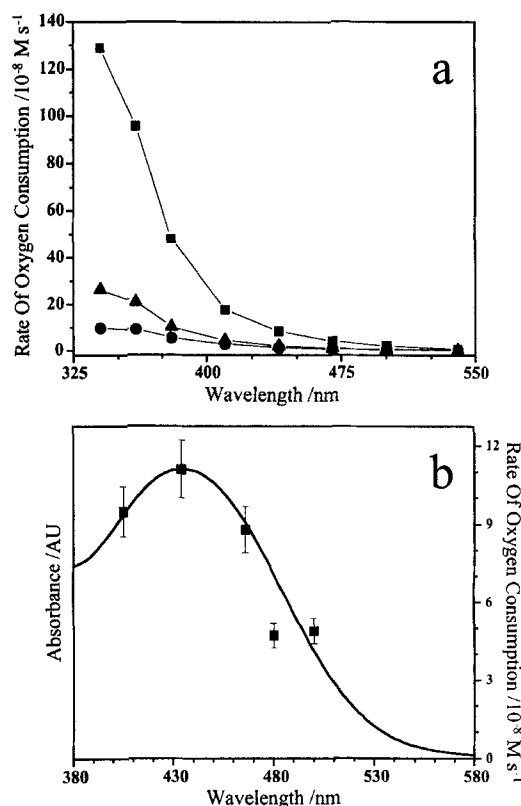


Figure 2. a: The action spectra for the uptake of oxygen by phosphate-buffered saline solutions of lipofuscin granules (■), LF extract solubilized in POPC liposomes (●) and the insoluble material remaining after the extraction process (▲). The three spectra have similar shapes. The similarity in the shape of the action spectrum of the LF extract to that of the LF granule argues that the photoactive molecule(s) in the granule are present in the LF extract. b: The action spectrum for the uptake of oxygen in a phosphate-buffered saline solution of A2E solubilized in POPC (L- α -phosphatidylcholine, β -oleoyl- γ -palmitoyl) liposomes (■) follows the shape of the absorption spectrum of A2E (—). This action spectrum, however, differs significantly from the LF action spectra shown in Fig. 1. The final concentration of A2E in the sample was 115 μM . Reprinted with permission from Pawlak *et al.* (24).

NONPHOTOINDUCED INTERACTIONS

Toxicity studies performed by Cubeddu *et al.* (21) showed that RPE cell growth was inhibited after 3 days of exposure to media containing A2E at a concentration greater than 1 μM . This response was found to be dose dependent. However, the mechanism through which cell growth was diminished was not determined. Holz *et al.* (29) demonstrated that when cultured RPE cells are exposed to A2E for 4 weeks, A2E accumulates almost exclusively within the lysosomal compartment. Of note, to facilitate the internalization of A2E into the cell, A2E was coupled to low-density lipoprotein particles. However, a small portion of A2E did appear to be associated with the cellular membrane.

Eldred (7) first noted the inhibition of lysosomal function in cultured fibroblasts when exposed to A2E. The mode of action was proposed to be similar to other quaternary amines shown to inhibit lysosomal functions, possibly involving complexation of A2E to specific enzymes. The effects of specific loading of A2E in the lysosome were examined by Schutt *et al.* (30) using acridine orange staining. This revealed that the accumulation of A2E in the lysosome causes an elevation of the lysosomal pH. When RPE

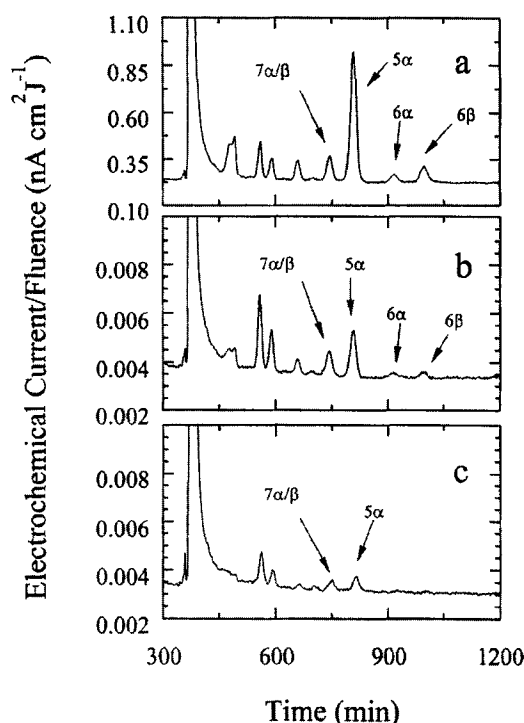


Figure 3. Cholesterol hydroperoxide formation from equilibrium mixtures of A2E and iso-A2E irradiated at 420 nm in 80% cyclohexane- d_{12} -20% acetone- d_6 , 0.2% ethanol. Panel a: Solvent containing 0.53 μM TPP (tetraphenylporphine) and 0.29 J/cm^2 fluence. Panel b: Initial A2E concentration, 7.4 μM ; fluence, 20 J/cm^2 . Panel c: Solvent alone; fluence, 20 J/cm^2 . Note the difference in the ordinate scale between Panel a and Panels b and c. Time is measured from the sample injection. The large peak at the left of each panel is caused by oxygen dissolved in the injected sample. Reprinted with permission from Kanofsky *et al.* (25).

cells are loaded with A2E, dose-dependent, selective inhibition of lysosomal function was observed by Holz *et al.* (31). Bermann *et al.* (33) examined whether A2E directly inhibits the function of lysosomal hydrolases by measuring the activity of a variety of such enzymes in the presence of A2E (32). At concentrations significantly higher than those predicted *in vivo*, A2E showed no ability to directly inhibit the function of lysosomal hydrolases, including proteases, lipases, glycosidases, nucleases, sulfatases and phosphatases. The results suggest that direct inhibition of lysosomal function by A2E may not explain the role of A2E in a variety of retinal disease; however, A2E may affect these disorders through other secondary pathways.

The effect of A2E on the ability of cultured RPE cells to phagocytose POS was examined by Finnemann *et al.* (34). Incubation of RPE cells with 100 μM A2E for 6 h did not inhibit the phagocytosis of fluorescently labeled POS. This suggests that A2E loading of the lysosomal compartment in RPE cells does not disrupt the initial steps of POS phagocytosis. However, the rate at which RPE cells degraded the POS was slowed by the presence of A2E. However, antibody staining of POS proteins revealed that the presence of A2E did not inhibit the degradation of the POS protein opsin, which comprises 80% of the total POS protein content. This is consistent with the results of Bermann *et al.* (33) described above, which examined many of the lysosomal enzymes involved in protein degradation. Further studies revealed that the presence of A2E affected the ability of the RPE cells to degrade the POS lipids (34). Specifically, the degradation of phosphatidylcholine,

the most abundant lipid in the POS, was decreased perhaps through inhibition of phospholipase A₂ or phospholipase C. It was suggested that this slowing in phosphatidylcholine degradation would over time result in the build up of undigested lipids in RPE cells.

Preliminary studies by Suter *et al.* (35) demonstrated that A2E could localize in the mitochondria of murine cerebellar granule cells, which have high mitochondrial content. This accumulation decreased mitochondrial activity and subsequently released cytochrome *c* and apoptosis-inducing factor, two proapoptotic proteins. This initiation of apoptosis did not appear to involve the caspases, specifically caspase-3, because caspase inhibitors were unable to modulate the onset of apoptosis. Additional experiments on mitochondria, isolated from rat livers, showed that all-*trans*-A2E decreased oxygen consumption, whereas 13'-*cis*-A2E showed little effect. The addition of cytochrome *c* or cardiolipin reversed this decrease in oxygen consumption, suggesting that A2E aids in the detachment of cytochrome *c* from the mitochondria. Cardiolipin assists in the binding of cytochrome *c* to cytochrome *c* oxidase; thus, A2E may interfere with binding, interrupting the electron flow of respiration. A2E was also shown to decrease oxygen consumption of isolated cytochrome *c* oxidase in the presence of cytochrome *c*, supporting this conclusion.

Extensive exploration of the interactions of A2E with cytochrome *c* oxidase in the presence of cytochrome *c* was performed by Shaban *et al.* (36). Coprecipitation with cytochrome *c* oxidase demonstrated that A2E binds tightly to the protein, but non-covalently because it can be detached. It is presumed that the binding involves electrostatic and hydrophobic interactions. A2E only exhibits toxic effects when bound to cytochrome *c* oxidase. The binding of A2E to cytochrome *c* oxidase ultimately results in the production of ROI, adding additional oxidative stress to RPE cells, and in the formation of the proapoptotic complexes involving cytochrome *c*. Investigation of this mechanism in human RPE cells was carried out by Shaban *et al.* (37). At concentrations of A2E as low as 5 μM , the formation of ROI and nitrogen species was observed by the conversion of 2,7-dichlorofluorescein to dichlorofluorescein. However, when RPE cells were exposed to 10 μM A2E, the presence of phosphatidylglycerol modulated the effect, decreasing cell death. Negatively charged phosphatidylglycerol is a precursor to cardiolipin and is proposed to function similar to cardiolipin. The antioxidant coenzyme Q was also found to mediate the response of RPE cells to A2E. For a detailed discussion of this work see the review by Shaban and Richter (38).

The inherent positive charge of the quaternary nitrogen and hydrophobic retinoid side chains in A2E suggest that it may behave as a surfactant (2,7). Sparrow *et al.* (18) examined the amphiphilic nature of A2E in RPE cells with an assay quantifying the release of the cytoplasmic enzyme lactate dehydrogenase (LDH) into the culture medium. Cells were incubated with 50 or 100 μM A2E for 2 h, after which the media was replaced. After two additional hours, the amount of LDH released into the fresh media was determined. Compared with control experiments, which were not exposed to A2E, the levels of LDH present were elevated. Increasing the A2E incubation time to 6 h caused the levels of released LDH to remain higher for up to 3 days after the initial exposure but returned to levels similar to unexposed or control cells after 7 days. The amount of LDH released was also dependent on the concentration of A2E incubated with the cells. In cells challenged with 10 μM of A2E no detectable traces of LDH release were observed.

Similar experiments by Schutt *et al.* (39,40) explored the effect of A2E on the integrity of cellular organelle membranes. Intact lysosomes, mitochondria and microsomes were isolated from RPE cells. The intact organelles were incubated in solutions of A2E at a variety of concentrations and compared with controls incubated with detergent Triton X-100. The activities of organelle specific enzymes were assayed after exposure to A2E to assess the latency of the membranes. Increases in the enzyme activity indicated a loss of membrane integrity. These experiments determined that A2E affected the integrity of the organelle membranes at low, biologically relevant concentrations, as shown in Fig. 4 (39). Leakage was observed at 1 μM A2E for the mitochondria and 2 μM A2E for the lysosomes, whereas 5 μM A2E was necessary for the microsomes. Additional experiments assessed the integrity of the cellular membrane, observing no leakage at concentrations of 100 μM A2E. Other synthetic retinoids and detergents have been shown to have similar effects on membrane integrity and initiate apoptosis through lysosomal leakage. Thus, apoptosis may be initiated by the degeneration of organelle membranes by A2E.

The effect of A2E on model membranes was examined by De and Sakmar (41). The critical concentration at which A2E undergoes aggregation was determined to be 300 μM in water as measured by fluorescence anisotropy. The anisotropy of A2E in model membranes was also found to be dependent on the lipid composition of the vesicles. This demonstrates that the concentration of A2E necessary for the onset of membrane solubilization varies depending on the type of vesicle. Increases in the concentration of A2E caused the lipid vesicles to transition to a micelle structure. Additional studies demonstrated that A2E induced membrane leakage, consistent with the previously described studies by Sparrow *et al.* (18) and Schutt *et al.* (39,40).

PHOTOINDUCED TOXICITY

The appearance of intracellular yellowish brown granules in cultured RPE cells after exposure to A2E was indicative of successful uptake, as shown by Sparrow *et al.* (18). The amount of these granules formed was dependent on the concentration of the A2E exposure, the higher the concentration the more the number of granules observed. These granules were fluorescent and not present in control RPE cells not exposed to A2E. It was also determined using HPLC that the cell line used has no detectable native A2E. The spectrum of the A2E fluorescence in the cells was determined; the emission maximum is 565 nm. Several control samples of A2E in a variety of solvents showed that the shape of the emission spectra is dependent on the solvent environment. The more hydrophobic the solvent, the more the emission maximum is blueshifted. As previously discussed, *in vivo* A2E concentrations range from 34 to 134 ng/10⁵ cells. This study also estimated that only 5% to 10% of the A2E exposed to the cultured cells was internalized; thus, concentrations of at least 10–25 μM A2E were necessary to achieve internal loading of concentrations comparable with those observed in native samples.

Cellular studies reported by Sparrow *et al.* (18) show that the spatial distribution of the A2E loading was similar to the typical punctuate perinuclear arrangement of lysosomes. Cultured cells were exposed to concentrations of A2E and two organelle specific dyes: LysoTracker Red DND and Mitotracker Red CM-H2XRos. LysoTracker Red DND is a membrane-permeable acidophilic fluorophore that localizes in lysosomes, whereas Mitotracker Red

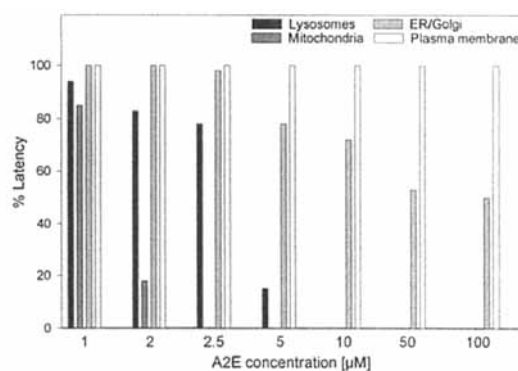


Figure 4. Latency of purified lysosomes was compared with partially purified mitochondria (taken from the 20%:5% metrizamide interface of the second ultracentrifugation step), ER–Golgi membranes (in the postnuclear supernatant) and the plasma membrane (in intact cells, as described in Materials and Methods). Comparisons of different latencies were assessed after 30 min incubation time. Results are the means of three determinations (SD was always less than 5%). Reprinted with permission from Schutt *et al.* (39).

CM-H2XRos localizes on the mitochondria. Confocal microscopy revealed that A2E colocalized with the LysoTracker Red DND. Thus, after the internalization of A2E by the RPE cells, A2E selectively accumulates in the lysosome. Additional flow cytometry experiments by Schutt *et al.* (42) demonstrated that the uptake of A2E by the lysosomes is dose dependent and showed more than an 80-fold increase in fluorescence as compared with controls. This study also observed that the pH of the lysosome was affected by the internalization of A2E, which colocalized in the lysosome with acridine orange. The fluorescence of acridine orange is dependent on its pH; under acidic condition it emits red, whereas it emits green in neutral or basic conditions. RPE cells stained only with acridine orange emitted red, whereas those with A2E uptake fluoresced mainly green, indicating a increase in the pH of the lysosome. However, as discussed above, this does not appear to affect the function of the lysosomal hydrolases (33).

In a study by Sparrow *et al.* (43), confluent-cultured RPE cells were exposed to A2E at a variety of concentrations. Using HPLC analysis, the amount of A2E present in individual cells ranged from 64 ± 6 ng/10⁵ cells for exposure to 100 μM A2E concentration down to less than 5 ng/10⁵ cells for exposure to an A2E concentration of 10 μM . The A2E internalized by the cells was present for at least 7 days after the initial challenge. Control cells not exposed to A2E showed no detectable autofluorescence in the same spectral region.

To examine the photoinduced toxicity of A2E, 1 week after loading with A2E, the RPE cells were exposed to 480 ± 20 nm light (43). A fluorescence assay to determine nuclear viability was used to measure the effect of A2E and blue light on RPE health. The number of nonviable cells after loading with A2E and exposure to blue light scaled with the concentration of the A2E loaded and the length of blue light illumination. A summary of the experiments performed is shown in Fig. 5 (43). Note that after 60 s of 75 mW/mm² blue-light exposure, cells loaded through exposure to 100 μM A2E showed a 75% loss of viability 18 h after irradiation. This is an underestimation of cell death because by this time some cells had detached from the substrate. Cells exposed to the same light condition but not loaded with A2E showed no loss

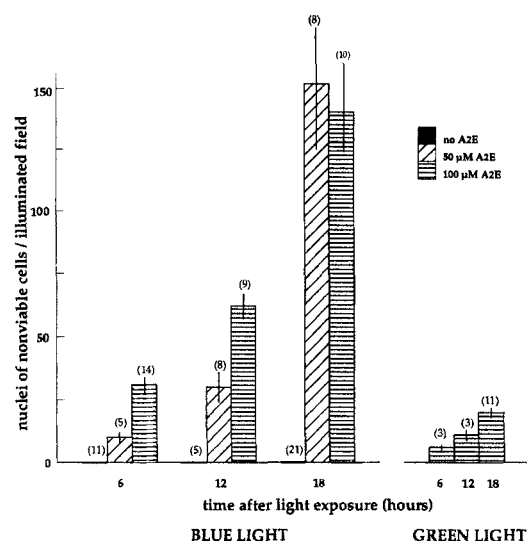


Figure 5. Quantization of nonviable cells after the illumination of A2E-containing ARPE-19 cells in culture. ARPE-19 cells accumulated A2E from 50 and 100 μ M concentrations in media. Control cells (without A2E) did not accumulate A2E. Fields of cells (0.5 mm diameter) were exposed to 480 nm (blue) or 545 nm (green) light for 60 s. After 6, 12 and 18 h the nuclei of nonviable cells were fluorescently labeled. Values are means $\pm$ standard error of mean. The numbers in parentheses indicate the total number of replicates pooled from one to four experiments. The values for untreated cultures are less than 0.5. Note that the value for 18 h at 100 μ M is underestimated because in some experiments cells had begun to detach from the culture dish. Reprinted with permission from Sparrow *et al.* (43).

of cellular viability. Additional control experiments used 545 ± 15 nm green light to illuminate the confluent cells. Green light was less efficient at inducing cell death, consistent with the decreased absorbance cross section of A2E, even though the light intensity used, 210 mW/mm², was almost three times the radiant energy of the blue light. Using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay, Schutt *et al.* (42) monitored decreases in cell viability when RPE cells fed A2E were exposed to broadband blue light, 390–550 nm at 2.8 mW/cm². After 5 days, viability was decreased by as much as 70% when compared with control cells. A variety of microscopic indicators of cell damage was noted, including membrane blebbing, rounded refractile bodies, cytoplasmic vacuolization, condensation and detachment from the substrate. These results are consistent with those observed by Sparrow *et al.* (43).

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling assays revealed that 3 h of blue-light exposure used by Sparrow *et al.* (43) was sufficient to cause DNA fragmentation in such A2E-loaded cells. Such a DNA fragmentation can be indicative of the onset of apoptosis. This response was dose dependent and not observed in cells loaded through exposure to 10 μ M of A2E. Further analysis of phosphatidylserine on the cell surface using Annexin V staining confirmed apoptosis as the photoinduced mode of cellular death. Therefore, it can be concluded under the conditions used in this study that A2E photoinduced apoptosis in cultured RPE cells.

The steps involved in the photoinduced onset of apoptosis are not clear, but the initiation of apoptosis is well regulated by the caspases (44). The caspase class of proteases is responsible not only for the initiation of apoptosis but also for its execution. However, it is also possible to trigger apoptosis while the actions of

the caspases are blocked. Apoptosis can also be initiated from the mitochondria by the release of cytochrome *c*, which leads to the activation of the caspases. The B-cell leukemia/lymphoma 2 (Bcl-2) family of proteins regulates this release of cytochrome *c*. Thus, the roles of caspase-3 and Bcl-2 in the photoinduced onset of apoptosis in A2E-loaded RPE cells were examined.

The activation of caspase-3 was observed in A2E-loaded cells exposed to blue light (44). A conjugate caspase-3 was used, which fluoresces red only after it is activated. The hallmark red fluorescence was observed as early as 5 h after blue-light illumination of A2E-loaded cells. This fluorescence was colocalized with the presence of A2E. Treatment of the cells with a known caspase-3 inhibitor modulated the fluorescence signal. The viability of the cells after activation of caspase-3 was examined and found to increase in the presence of the inhibitor.

Altering the expression of Bcl-2 in RPE cells could affect the photoinduction of apoptosis (44). RPE cells were transfected, and several of the resulting clones showed increased Bcl-2 expression as compared with the parent nontransfected RPE cell line. Two of the stably transfected cell lines were subjected to A2E phototoxicity studies. After exposure to blue light a reduction of the toxicity was observed. The altered cell lines displayed a 50–60% reduction in cell death after illumination when compared with control transfected and nontransfected cell lines. The ratio between pro- and antiapoptotic molecules has been shown to be of importance in resisting the onset of apoptosis. Thus, increasing the expression of Bcl-2, an antiapoptotic molecule, demonstrates an increased resistance to apoptosis. However, the exact mechanism for the photoinduced onset of apoptosis by A2E remains unclear.

The role of oxidation in the onset of photoinduced apoptosis was examined by Sparrow *et al.* (45). Cultured RPE cells were loaded with A2E and subjected to blue-light irradiation under a variety of conditions, including the presence and absence of molecular oxygen. The effect of several suppressors and enhancers of oxidative damage on cellular viability was explored. When cells were irradiated in media depleted of its endogenous dissolved oxygen, cell viability was greatly affected. A significant decrease in cell death was achieved through the depletion of oxygen. However, removal of oxygen from cells that were not irradiated showed no effect on their overall health as compared with cells maintained in non-oxygen-depleted media. Conversely, when the A2E-loaded cells were illuminated while exposed to D₂O-based buffer salts, a 66% increase in cell death was observed. These results were proposed to be consistent with the lengthened lifetime of singlet oxygen in D₂O. When known quenchers of singlet oxygen were added to the cells, suppression of cell death was observed, suggesting that the induction of apoptosis involves singlet oxygen. When the susceptibility of cells to hydrogen peroxide-induced oxidative damage was increased through the inhibition of a key antioxidant enzyme, no decrease in cell viability was observed. Similar results were obtained with a strong scavenger of hydroxyl radical, which was also capable of scavenging superoxide anion radical and hydrogen peroxide. These results support the hypothesis that singlet oxygen is responsible for the modulation of apoptosis, as induced by the blue-light irradiation of A2E.

Interactions of A2E in aqueous media and blue light were explored by Sparrow *et al.* (45) when intracellular A2E also displayed fluorescence quenching. HPLC analysis revealed loss of A2E after illumination, a response that was dependent on the amount of dissolved oxygen present in the media. If the oxygen

was depleted, the damage to A2E decreased. MS of the irradiated A2E revealed the formation of higher mass species separated by 16 mass units. Increased irradiation yielded more intense higher mass peaks. When A2E in RPE cells was irradiated and then extracted, the formation of some of the same higher mass species was observed. However, these products were not present in A2E-loaded cells, which were not irradiated. The determination of these structures as A2E-epoxides (28) was discussed in Aerobic Reactivity.

The specific damage to RPE cells initiated by blue-light irradiation of A2E was examined in detail by Sparrow *et al.* (46). This study determined that DNA was oxidatively damaged and its bases modified. After elucidating the structure of the A2E-epoxides, Sparrow *et al.* (47) examined the toxicity of this class of molecules on cultured RPE cells. Alkaline comet assays were performed to examine the effect of A2E-epoxides on DNA single-strand breaks and alkali-labile sites. Incubation of the cells with a variety of A2E-epoxides showed significant damage to cellular DNA as compared with exposure of A2E or other controls. This damage was observed whether or not the cells were permeabilized and independent of the presence of singlet oxygen. Such a singlet oxygen produced by A2E upon blue-light irradiation would likely be quenched before reacting to induce damage in DNA. Specific monoclonal antibody staining for the oxidation of guanine to its 8-oxo form determined that A2E-epoxides damage DNA in a manner similar to other epoxide moieties. The damage to cellular DNA was modulated by natural antioxidants such as vitamins E and C. However, to induce DNA damage, A2E-epoxides must first be produced *in vivo*. The formation of these species is dependent on the production of singlet oxygen and subsequent reaction with A2E to produce the epoxides. The production of singlet oxygen by A2E is photoinduced by blue light. Several experiments revealed that vitamin E is particularly efficient at quenching singlet oxygen before it is able to react with A2E to form an epoxide. Thus, the damage is mediated by prevention of A2E-epoxide formation.

FUTURE DIRECTIONS

The photochemistry of A2E is now well characterized. The quantum yields for the production of ROI are low, significantly less than those of its precursor molecule, all-*trans*-retinal. Most of the energy absorbed by A2E is converted into heat or dissipated through the isomerization of its many double bonds. The efficiency of its isomerization remains to be measured. Despite its limited aerobic reactivity, A2E appears capable of inducing cellular apoptosis in cultured RPE cells, mostly through the formation of A2E-epoxides. Further exploration to understand the phototoxic mechanisms and signaling pathways of such epoxides is important. The combined *in vitro* and *in vivo* data to date indicate that the reactivity of A2E accounts for only a small portion of the observed reactivity of lipofuscin granules. Several of the blue-absorbing chromophores isolated by Eldred and Katz (1) remain unidentified and may be responsible for the additional phototoxicity of lipofuscin. The identification of these molecules and the characterization of their photochemistry are important to fully understand the role of lipofuscin in the photoinduced death of RPE cells.

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