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Fluorescent Pigments of the Retinal Pigment Epithelium and Age-Related Macular Degeneration

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Abstract—The major hydrophobic fluorophore of the retinal pigment epithelium (RPE) is A2E, a pyridinium bis-retinoid derived from all-*trans*-retinal and phosphatidyl-ethanolamine. The accumulation of fluorophores such as A2E is implicated in the pathogenesis of age-related macular degeneration (AMD), a disease associated with the deterioration of central vision and a leading cause of blindness in the elderly. Recent chemical and biological studies have provided insight into the synthesis and biosynthesis of A2E, the spectroscopic properties of this pigment, and the role of A2E and RPE cell death. © 2001 Published by Elsevier Science Ltd.

Age-related macular degeneration (AMD) results in a loss of vision due to deterioration of the central region of the retina (macula). This disease, the leading cause of blindness in persons over the age of 60, manifests itself in two ways—nonneovascular (atrophic, dry)¹ and neovascular (exudative, wet)² forms. Nonneovascular AMD is characterized by the deposition of material (drusen) under the retinal pigment epithelium (RPE), by changes in the distribution of melanin pigment, and by the gradual degeneration of RPE cells. Progression to the neovascular form of AMD occurs with the proliferation of new blood vessels under the RPE and can be accompanied by subretinal hemorrhage, RPE detachment, and the ingrowth of scar tissue. The end result of AMD is the loss of high acuity, central vision due to RPE and photoreceptor cell death.

The cells intimately involved in visual transduction are the rod and cone photoreceptor cells (Fig. 1). Rods and cones are specialized for responding to dim light and color vision, respectively. The outer segments of the photoreceptor cells are replete with light-capturing proteins consisting of rod or cone opsin bound through a

Schiff base linkage to the chromophore 11-*cis*-retinal. It is the isomerization of the retinal chromophore from the 11-*cis* to the all-*trans* form by a photon of light that initiates phototransduction.³ Photoreceptor cells, in turn, are dependent on cooperative interactions with the underlying retinal pigment epithelium (Fig. 1). It is these cells that contain the machinery to regenerate 11-*cis*-retinal from all-*trans*-retinal,⁴ the latter being released from opsin after photoisomerization. RPE cells also phagocytose and degrade outer segment membrane that is discarded daily by the photoreceptor cell to balance the continual production of new disks at the base of the photoreceptor outer

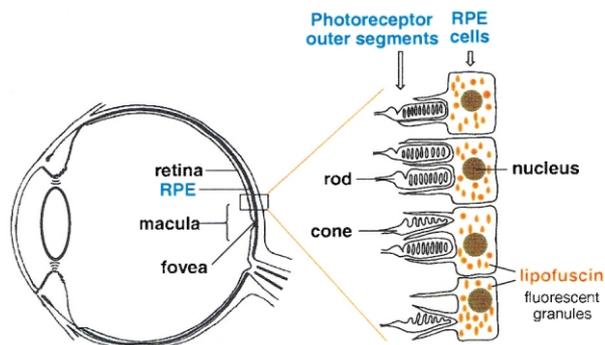


Figure 1. Diagram of the eye. The macula is the central portion of the retina, which contains photoreceptor cells (rods and cones) adjacent to a monolayer of RPE. In this cartoon, the outer segment to RPE ratio is simplified for clarity. RPE, retinal pigment epithelium.

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segments.^{5,6} Since RPE cells are essential to normal visual function and to the health of neural retina, the demise of these cells in some retinal disorders such as AMD is a critical component of the disease process.

RPE Lipofuscin

A major factor contributing to the deterioration and death of RPE cells, particularly in the macula, may be the accumulation of lipofuscin, autofluorescent granules that localize in the RPE lysosomes and are comprised of fluorescent pigments as well as an uncharacterized mixture of lipids and proteins.⁷ The amassing of lipofuscin (Fig. 1) is not a feature of aging RPE alone since excessive amounts of these fluorophores are also present in the RPE in some inherited retinal disorders such as Stargardt's disease, Best's macular dystrophy, and cone-rod dystrophy.^{7–12} The deposition of lipofuscin occurs largely as a consequence of the phagocytotic burden placed on the RPE cell, with the presumption being that the components of the ingested outer segments that cannot be degraded by the cohort of lysosomal enzymes present in RPE accumulate as lipofuscin.^{13–17}

Even at age 20 years, the content of lipofuscin is notable.⁹ By age 80, when it is estimated that approximately 90 million disk membranes will have been internalized,⁶ 20% of the area of an RPE cell in the central retina is occupied by lipofuscin.⁹ Moreover, histological analyses of human donor eyes,^{18–20} in addition to in vivo (non-invasive) fundus spectrophotometry²¹ and confocal laser scanning ophthalmoscopy,^{22,23} have shown that RPE cells overlying the macula, with the exception of RPE in the macula's cone-rich center (fovea), exhibit the most pronounced accumulation of fluorescent material. Lipofuscin levels in RPE cells are also topographically correlated with histopathological indicators of AMD^{8,9} and with the loss of light absorbing photoreceptor cells in aged eyes.⁸ While the accumulation of fluorescent granules in RPE can be simulated by feeding photoreceptor outer segments to RPE,^{24,25} it is not known whether the fluorophores are comparable to those present in native lipofuscin.

Chemical Composition of RPE Lipofuscin

In order to effectively investigate the impact of lipofuscin in RPE cell function, the factors that contribute to fluorescent pigment formation must be understood, the molecular components of lipofuscin must be characterized, and the specific effects of these pigments on RPE cells must be defined. While an early hypothesis attributed the accumulation of fluorescent pigments to the oxidation of RPE lipids, controlled studies of lipid oxidation did not provide material that contained the major fluorophore present in aging RPE cells.²⁶ The most direct approach for identifying the fluorescent constituents of lipofuscin is the isolation of the various components followed by structural elucidation. In this way, the prominent pigments of RPE lipofuscin have been characterized.^{27–30}

Working with the pooled lipofuscin from aged human eyes, Eldred and Katz²⁷ isolated fluorescent compounds by chloroform-methanol extraction and an extensive series of chromatographic separations. While the polar extract contained only a weak blue fluorescence that was not consistent with the orange colored emission of lipofuscin, the hydrophobic extract could be separated into ten fractions, a number of which contained material that did correlate well with the expected orange emission (~ 600 nm). Spectroscopic measurements indicated that the first two of these fractions contained retinol (vitamin A) and retinyl palmitate. The remaining eight fractions, which emitted yellow to orange light upon excitation, appeared to be responsible for the characteristic fluorescence of lipofuscin. Isolation of the most prominent of these compounds from more than 250 eyes of human donors, 40 years of age or older, provided 100 micrograms of the major orange fluorophore.²⁸ Structural elucidation was attempted with this material. The original compound as well as the perhydrogenated fluorophore and its acetate were submitted to FAB tandem mass spectrometry with collisionally activated decomposition analysis. These data, along with NMR and UV analyses, led to the proposal of the zwitterionic structure, *N*-retinylidene-*N*-retinyl-ethanolamine (Fig. 2).²⁸ This structure was thought to be confirmed by the preparation of an identical substance by the reaction of two equivalents of all-*trans*-retinal and one equivalent of ethanolamine. However, several aspects of this structural assignment could not be reconciled: the mass spectrum of the proposed structure would differ by two mass units from the measured value, the hydroxyl group would not be ionized at biological pH, the iminium function would undergo hydrolysis by silica gel during chromatographic separation, and the electronic spectrum of the fluorophore showed none of the fine structure characteristic of the retro retinoid moiety. After retraction of the originally proposed structure,³¹ Eldred brought it to our attention for further elucidation and we initiated a collaborative effort.

The ability to prepare the major fluorophore of RPE lipofuscin by the reaction of retinal and ethanolamine²⁸ provided significant quantities of this compound for

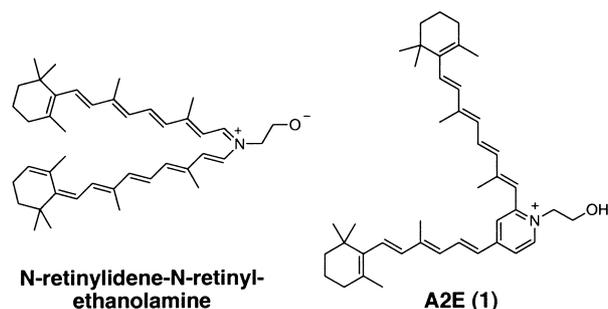


Figure 2. Structure of A2E. The original *N*-retinyl-*N*-retinylidene-ethanolamine structure²⁸ was later revised. Further structural elucidation²⁹ and synthesis³² has confirmed the pyridinium bis-retinoid structure, A2E. The counterion of the pyridinium salt is presumably chloride in vivo and is trifluoroacetate for synthetic samples.

further structural studies. The biomimetic protocol was repeated on a larger scale, reacting one gram of all-*trans*-retinal with ethanolamine in the presence of acetic acid.²⁹ This procedure yielded 5 mg of a compound that was identical to that isolated from eye tissue. The amount was sufficient for detailed NMR and additional spectroscopic studies, and led to the elucidation of an unprecedented pyridinium bis-retinoid structure, a rigid amphiphilic wedge with a pyridinium polar head group and two hydrophobic retinoid tails. This revised structure was given the name A2E (**1**) (Fig. 2) since it is derived from two molecules of vitamin A aldehyde and one molecule of ethanolamine.²⁹ In the case of synthetic A2E, the counterion of the pyridinium moiety is trifluoroacetate, since trifluoroacetic acid is used during A2E purification. However, *in vivo*, the anionic counterion would most likely be chloride.

Stepwise and Biomimetic Synthesis of A2E and *iso*-A2E

An independent confirmation of the A2E structure was attained by total stepwise synthesis of **1** through a convergent double Wittig olefination of bis-aldehyde **4** with Wittig reagent **5** (Fig. 3). Phosphonium salt **5** contains the triene moiety common to both side arms of A2E. The final step of the synthesis was the alkylation with iodoethanol at the pyridine nitrogen, providing A2E (**1**).³² To prepare bis-aldehyde **4**, 2-hydroxy-4-methylpyridine (**2**) was oxidized with SeO₂ to give 2-hydroxy-4-formyl-pyridine. The methyl group of **2** was activated due to the coordination of SeO₂ to the pyridine nitrogen with concomitant H-bond formation with the *ortho* hydroxyl group. Reaction of 2-hydroxy-4-formyl-pyridine with Tf₂O/pyridine and subsequent coupling with vinyl stannane in the presence of a catalytic amount of copper(I) iodide according to the procedure of Stille provided vinyl pyridine **3**. Subsequent oxidation with SeO₂ yielded the desired bis-aldehyde **4**.

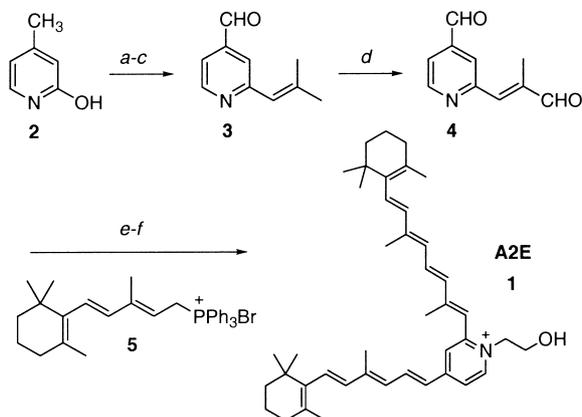


Figure 3. Total synthesis of A2E.³² Reagents and conditions: (a) SeO₂, dioxane (52%); (b) Tf₂O, pyridine, CH₂Cl₂ (77%); (c) Bu₃SnCHC(CH₃)CH₃, Pd(PPh₃)₄, LiCl, CuI, dioxane (78%); (d) SeO₂, dioxane (83%); (e) 2.2 equiv **5**, NaOEt, EtOH (76%); (f) ICH₂CH₂OH, CH₃NO₂ (56%).

An alternative and efficient one-pot procedure for the synthesis of key intermediate bis-aldehyde **4** was executed starting with (*E*)-ethoxycarbonyl-2,4,6-trienal **6** (Fig. 4).³³ Thus treatment of trienal **6** with two equivalents of lithium bis-trimethylsilyl-amide in THF at room temperature cleanly led via a Peterson reaction to the corresponding *N*-TMS-1,2-dihydro derivative, which underwent an aza-6π-electrocyclization of the resulting azatriene. The reaction mixture containing the unstable 1,2-dihydropyridine was oxidized with DDQ, affording the desired ethoxycarbonyl pyridine derivative **7**. This intermediate was converted to bis-aldehyde **4**, the overall yield from trienal **6** being ca. 77%.

The most efficient method for the preparation of A2E is an optimized one-step biomimetic synthesis (Fig. 5), which provides A2E in ca. 50% yield.³⁰ This result was a significant enhancement over the original one-step yield of 0.5%.²⁹ The mechanism of A2E formation is presented in Figure 5. Reaction of all-*trans*-retinal (**8**) and ethanolamine (**9**) leads to Schiff base **10**, which tautomerizes to enamine **11**. After a second molecule of all-*trans*-retinal reacts with **11** to form iminium salt **12**, A2E (**1**) is generated after electrocyclicization (to dihydropyridine **13**) and auto-oxidation. In the improved biomimetic synthesis, a mixture of all-*trans*-retinal (100 mg, 352 μmol) and ethanolamine (9.5 mg, 155 μmol) was stirred in ethanol (3 mL) in the presence of one equivalent of acetic acid (9.3 μL, 155 μmol). Optimization of A2E formation under a variety of conditions indicated that this process is not favored under strongly acidic or basic milieu, consistent with the fact that A2E is generated under physiological conditions. After stirring the reaction for 2 days in the dark at room temperature, the mixture was concentrated and purified by silica gel chromatography (elution with methanol/CH₂Cl₂/TFA) to provide 53.8 mg (76.3 μmol, 49%) of A2E as the TFA salt. This sample contained 5% of an additional component that was similar, but not identical, to A2E as characterized by NMR and UV. The two fractions had identical masses as determined by FAB mass spectrometry, but eluted separately by C18 reverse phase HPLC. Extensive NMR studies (ROESY, COSY) of this material showed that it was a double bond isomer in which one double bond had undergone an *E* to *Z* isomerization. This structure is referred to as *iso*-A2E (**14**). Modeling studies have indicated that *iso*-A2E forms a more streamlined conformation than A2E

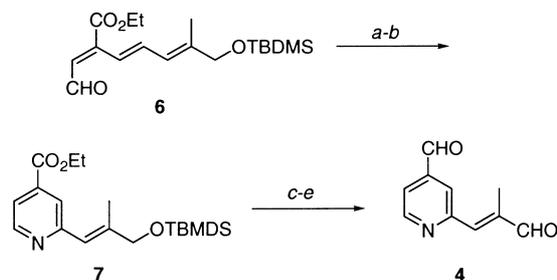


Figure 4. Alternate synthesis of bis-aldehyde **4**.³³ Reagents and conditions: (a) 2 equiv LiN(TMS)₂, THF; (b) 2 equiv DDQ; (c) LiAlH₄, diethyl ether (77%, three steps); (d) TBAF, THF; (e) MnO₂, CH₂Cl₂ (quant, two steps).

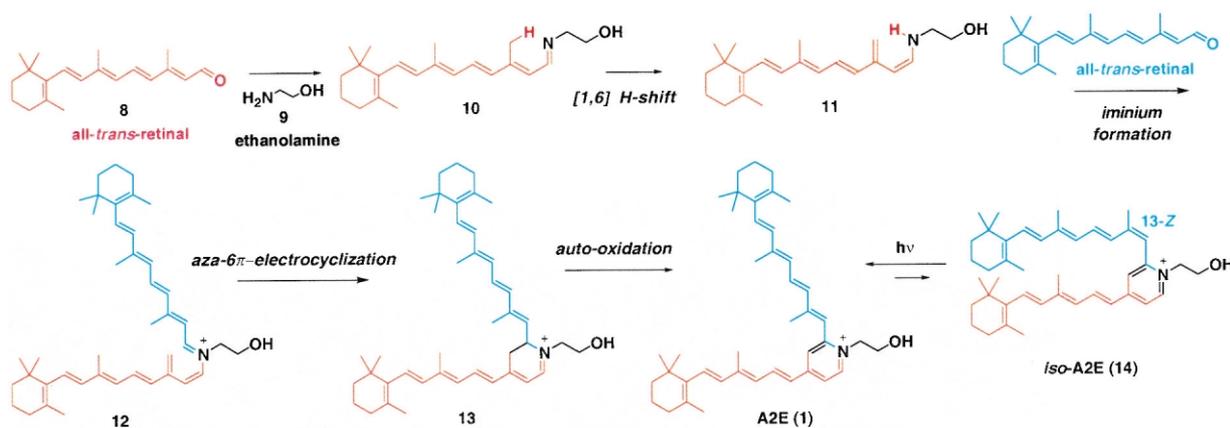


Figure 5. Biomimetic synthesis of A2E from all-*trans*-retinal and ethanolamine. Two molecules of retinal and one molecule of ethanolamine react to provide A2E. A2E equilibrates with *iso*-A2E, the 13-*Z* isomer, in the presence of light, providing a 4:1 A2E/*iso*-A2E mixture.

and hence may penetrate cell membranes even more readily.

A2E: Spectroscopy, Photochemistry, and Analysis

A detailed description of the physical properties of A2E is necessary both to understand its reactivity, photochemistry, and spectroscopy as well as to contribute to our understanding of A2E in a cellular environment. Measurement of the absorbance, emission, and excitation spectra of A2E provided data that was comparable to that observed with the pigment isolated from lipofuscin.²⁷ The absorbance spectrum (Fig. 6) of synthetic A2E in methanol has two peaks with λ_{\max} 439 nm ($\epsilon = 36,900$) and 336 nm ($\epsilon = 25,600$).³⁰ The excitation spectrum, monitored at 600 nm emission, is similar in shape with the maximum at 418 nm.³⁴ Measurements of the emission spectra of A2E in *n*-butyl chloride, methanol and aqueous buffer have shown that the emission maximum of A2E is dependent on the nature of the solvent. In the more hydrophobic solvent, *n*-butyl chloride, the maximum is blue-shifted (585 nm) in compar-

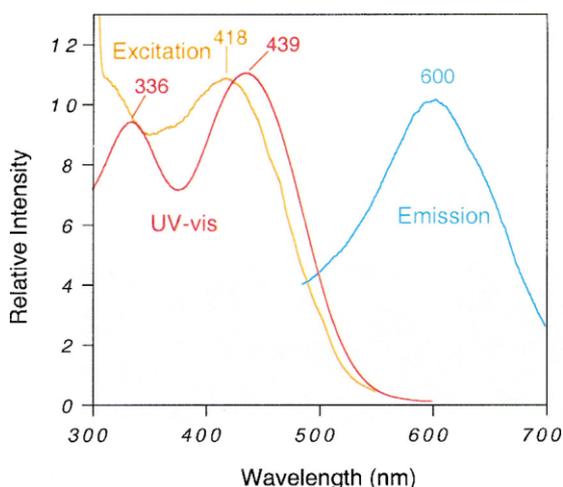


Figure 6. Absorbance (UV-vis), emission, and excitation spectra of synthetic A2E in methanol. The excitation spectrum was measured while detecting at 600 nm emission and the emission was measured with an excitation wavelength of 400 nm.

ison with the peaks observed in methanol (600 nm) and PBS (610 nm).³⁵ The emission maxima of intracellular A2E (565–570 nm) corresponds most closely to that observed in *n*-BuCl, indicating that A2E most likely exists in a hydrophobic environment. These spectra are significant in terms of the efforts to monitor lipofuscin fluorescence by fundus spectrophotometry^{21,36,37} and confocal laser scanning ophthalmoscopy.^{22,23}

When illuminated with room light in methanol, A2E and *iso*-A2E undergo photoequilibration leading to a 4:1 A2E/*iso*-A2E mixture (Fig. 5).³⁰ This isomerization was also observed with monochromatic blue light (430 nm), indicating that visible light that reaches the retina is capable of accomplishing the same transformation. It is interesting to note that EPR studies on lipofuscin crude extracts and synthetic pigment mixtures indicate that irradiation leads to the formation of radical species,^{38–40} which may be related to the A2E/*iso*-A2E photoconversion.

While several protocols for HPLC detection of A2E have been described,^{40,41} an optimized procedure³⁰ with excellent sensitivity employed C18 reversed-phase HPLC (Fig. 7) to identify as little as 5 ng of A2E and *iso*-A2E in aged human eyes. The quantity of A2E detected in RPE-choroid isolates from individual human eyes, as determined from integrated peak intensities, was found to be in the 200–800 ng range. The sensitivity of the HPLC procedure allowed the analysis to be performed on only 5% of the sample from a single human eye. When performed under dim light to prevent photochemical degradation of the fluorophores, tissue isolation and extraction with chloroform and methanol provided a crude fluorophore mixture. This extract, when characterized directly by HPLC (Fig. 7), not only identified A2E and *iso*-A2E as the major hydrophobic components,³⁰ but also indicated that the mixture was substantially simpler than the complex combination of fluorophores originally observed by Eldred.²⁷ Since Eldred's purification was more laborious, it may be speculated that some fluorophore degradation may have led to the formation of additional components that were not observed with a more abbreviated protocol. It is likely that one of Eldred's components corresponded to

iso-A2E. We have also observed what we believe to be additional A2E double bond isomers in small quantities (Fig. 7), and these too may have been isolated in some amount without characterization by Eldred. Additional peaks in the HPLC profile were identified as all-*trans*-retinol (vitamin A) and 13-*cis*-retinol (Fig. 7).³⁰ However, no all-*trans*-retinal was observed; this is not surprising since free retinal is reduced to retinol by retinol dehydrogenase in photoreceptor outer segments. Retinal that escapes reduction is available for further reaction, with A2E being one of the products.⁴² In contrast to adult eyes, the extracts of choroid/RPE isolated from two fetal eyes (18 and 20 weeks of gestation) did not contain A2E.³⁰

Biosynthesis of A2E

Recent evidence indicates that the biogenesis of A2E is initiated when all-*trans*-retinal (**8**), which is released from photoactivated rod and cone visual pigment, reacts with the membrane phospholipid phosphatidylethanolamine (PE, **15**) to form a PE-all-*trans*-retinal Schiff base conjugate (*N*-retinylidene-phosphatidylethanolamine; NRPE) **16** (Fig. 8).^{42–45} This adduct undergoes a [1,6]-proton tautomerization generating the phosphatidyl analogue of enamine **11**, which reacts with a second molecule of all-*trans*-retinal (cf. Fig. 5).³⁰ After aza-6 π -electrocyclization and auto-oxidation, the fluorescent phosphatidyl-pyridinium bis-retinoid A2-PE (**17**) is formed. Finally, hydrolysis of the phosphate ester of A2-PE yields A2E (**1**).^{30,42} This biogenic scheme is summarized in Figure 8.

Mass spectrometry was utilized to confirm the formation of A2-PE and to identify this pigment in complex mixtures. In unpurified reaction samples prepared from synthetic dipalmitoyl-PE, NRPE and A2-PE were identified by electrospray ionization (ESI) mass spectrometry. Protonated molecular ions of NRPE and A2-PE provided peaks at *m/z* 958.6 and 1222.9, respectively

(Fig. 9A). Collision-induced dissociation analysis (FAB-CID MS/MS) revealed the product ions *m/z* 551.4 and 408.2 for the synthetic NRPE (Fig. 9B) while only one product ion was observed from A2-PE at *m/z* 672.8, representing the phosphoryl-A2E fragment (Fig. 9C).⁴² FAB mass spectrometric analysis has clearly indicated that A2-PE was generated when retinal was incubated with ROS membranes. In the absence of retinal, no peaks corresponding to A2-PE were observed.⁴²

The observation that A2E is generated when A2-PE is incubated with phospholipase D⁴² confirms that A2-PE is the precursor to A2E. Several lines of evidence indicate that A2-PE can form within the photoreceptor outer segment membrane before phagocytosis by the RPE cell. Thus, the absence of all-*trans*-retinal in RPE cell extracts³⁰ and the observation that A2-PE formation from all-*trans*-retinal and PE is optimal at neutral pH⁴² are incompatible with the formation of A2E in the acidic environment of RPE cell lysosomes.⁴⁶ In addition, the abundant lipofuscin that is observed in RPE cells of antioxidant-deficient rats⁴⁷ and in the eyes of patients with Stargardt's disease and retinitis pigmentosa^{20,48,49} coexists with deposits of autofluorescent pigment in photoreceptor cells. Furthermore, mass spectrometric analysis⁴² confirmed that A2-PE is the orange-colored fluorophore^{17,28,50,51} present in degenerating photoreceptor outer segment debris of RCS rats, an animal model of retinal degeneration. Significant accumulations of A2-PE have also been observed in the photoreceptor outer segments of knockout mice lacking the *abcr* gene,⁴⁴ which encodes a putative all-*trans*-retinal transporter.^{52,53} And lastly the formation of [¹⁴C₂]-A2-PE has been measured in excised whole retinas following [¹⁴C₂]-ethanolamine incorporation and illumination of the retinas to release endogenous all-*trans*-retinal (unpublished observation). The observation that [¹⁴C₂]-A2-PE was not formed when retinas were incubated in the dark (unpublished observation), together with the finding that A2-PE and A2E formation in *abcr* null

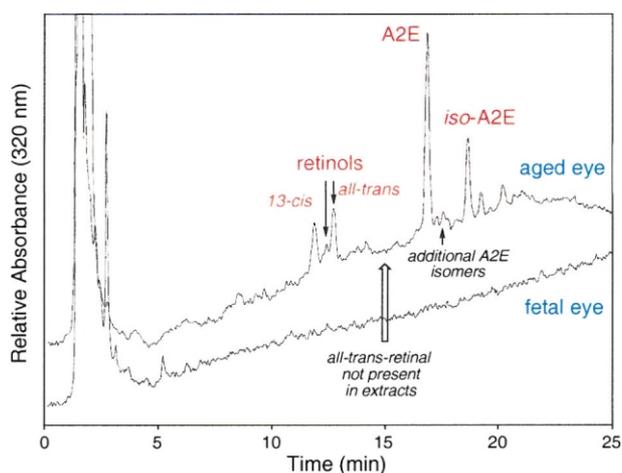


Figure 7. HPLC of eye extracts. The chromatograms are the analysis of an aged (80-year-old) human eye and fetal eye tissue by C18 reverse-phase HPLC, as described in Parish et al.³⁰ Only 5% of the aged eye (upper trace) was injected, while analysis of the fetal eye (lower trace) indicated no A2E or *iso*-A2E.

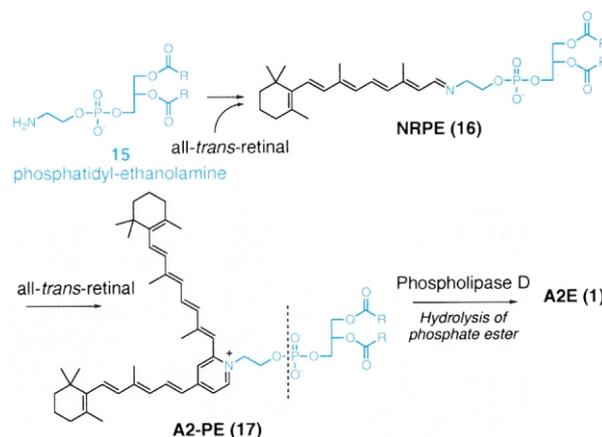


Figure 8. Biosynthesis of A2-PE and A2E in the retina. all-*trans*-Retinal, which is released from opsin after photoisomerization of ground state 11-*cis*-retinal, reacts with phosphatidylethanolamine in the disk membrane to produce the *N*-retinyl-phosphatidylethanolamine Schiff base (NRPE). If additional all-*trans*-retinal is present in the photoreceptor outer segment, NRPE will react further to generate A2-PE. Hydrolysis of the phosphate ester, potentially by phospholipase D in the RPE, yields A2E.

mutant mice are dependent on light exposure⁴⁴ also confirms that the light-dependent release of all-*trans*-retinal is a prerequisite of A2-PE formation.

The cleavage of A2-PE by phospholipase D to generate A2E indicates that, as opposed to being acid-mediated, the *in vivo* hydrolysis of A2-PE may occur enzymatically. Additional support for this notion is provided by the fact that A2E is not generated when A2-PE is incubated in an acidic environment that mimics the lysosome (pH 5.0) (unpublished observations). Nevertheless, the site of hydrolysis of A2-PE has not been clarified.

A2E in a Cellular Environment

When A2E is delivered to RPE cells in culture, it accumulates intracellularly (Fig. 10) and demonstrates an affinity for acidic organelles,³⁵ a behavior which replicates the lysosomal compartmentalization of A2E *in vivo*. The mechanism by which A2E is amassed within lysosomes is not understood, nor is the means known by which A2E causes an elevation in lysosomal pH.²³ In general, lysosomotropic tertiary amines become locked inside lysosomes as a result of being protonated within the acidic environment of these organelles.^{54,55} The

neutral, unprotonated form of the amine can cross the hydrophobic phospholipid membrane, but cannot traverse back across the membrane after protonation. Accumulation of lysosomotropic tertiary amines leads to an increase in lysosomal pH. However, in the case of A2E, amine protonation cannot be the explanation for lysosomal compartmentalization and decreased acidity since A2E is a quaternary pyridinium salt that clearly cannot be protonated and deprotonated. An alternative explanation for an A2E-associated change in lysosomal pH is that it occurs as a result of detergent mediated perturbations of the membrane-bound adenosine triphosphatase that actively pumps protons into the lysosome.^{56–58} Indeed, the ability of A2E to exert detergent-like activity on membranes, leading to a loss of membrane integrity, has not only been demonstrated,³⁵ but it is also consistent with the amphiphilic nature of A2E.

While it is clear that there are levels of A2E that are tolerated by RPE cells, critical intracellular concentrations can be reached, above which A2E is damaging to cells.³⁵ Thus, not only can A2E mediate detergent-like effects on cell membranes,³⁵ but when accumulated by cultured RPE cells, A2E also bestows a sensitivity to blue light damage^{34,59} that is proportional to the A2E content of the cells and that is not manifested by cells devoid of A2E.³⁴ While the blue region of the spectrum has a marked ability to induce the death of A2E-containing RPE, longer wavelength green light is considerably less effective.³⁴ This wavelength dependence is consistent with the absorbance and excitation spectra of A2E. Based on several criteria, the loss of A2E-loaded RPE that are exposed to blue light occurs by means of apoptotic cell death,³⁴ and involves the activation of cysteine-dependent proteases (caspases) to execute the cell death program.⁶⁰

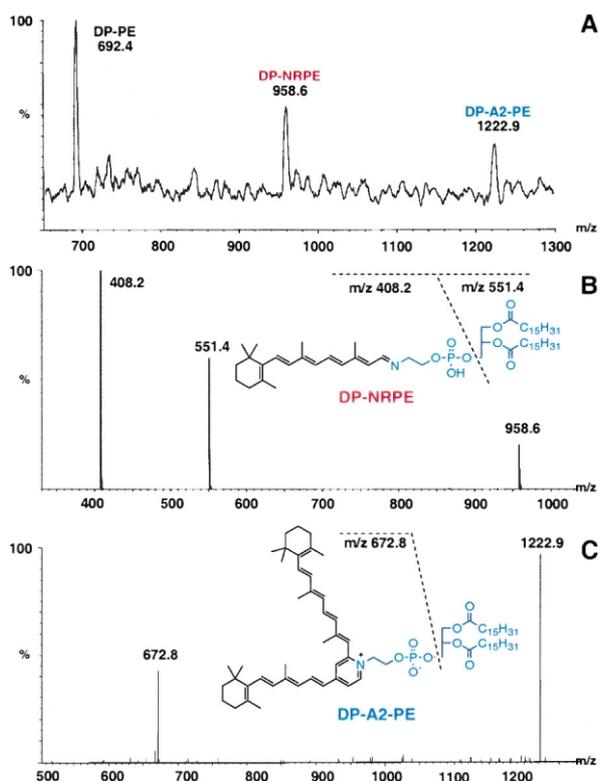


Figure 9. Mass spectrometric analysis of NRPE and A2-PE. (A) Dipalmitoyl-PE (DP-PE) was reacted with all-*trans*-retinal to provide a mixture of PE, NRPE and A2-PE, which was analyzed by electrospray ionization MS. Peaks corresponding to the masses of DP-PE (starting material), dipalmitoyl-NRPE (DP-NRPE), and dipalmitoyl-A2-PE (DP-A2-PE) were observed. (B) Collision-induced dissociation analysis of DP-NRPE provided peaks that correspond to the fragmentation shown in the figure. (C) Collision-induced dissociation analysis of DP-A2-PE provided one fragment peak, which corresponded to phosphoryl-A2E.

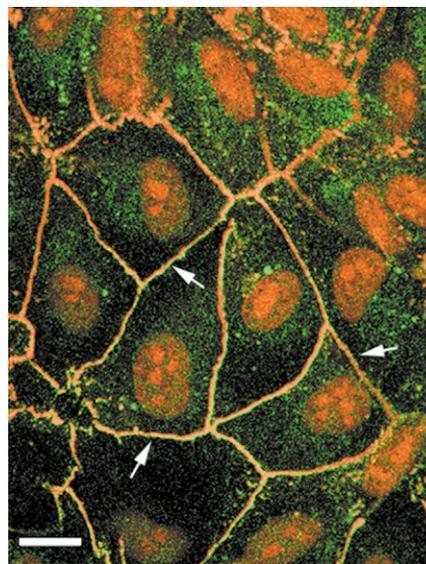


Figure 10. Fluorescence scanning confocal microscopic imaging of RPE cells that have accumulated A2E in culture. Internalized A2E presents as autofluorescent granules having a perinuclear distribution. Nuclei (red) are stained with propidium iodide. Cell borders are outlined by immunolabeling with an antibody to ZO-1, a tight junction-associated protein (arrows). In this image, A2E appears green because of the use of FITC appropriate filters. Scale bar, 20 μ M.

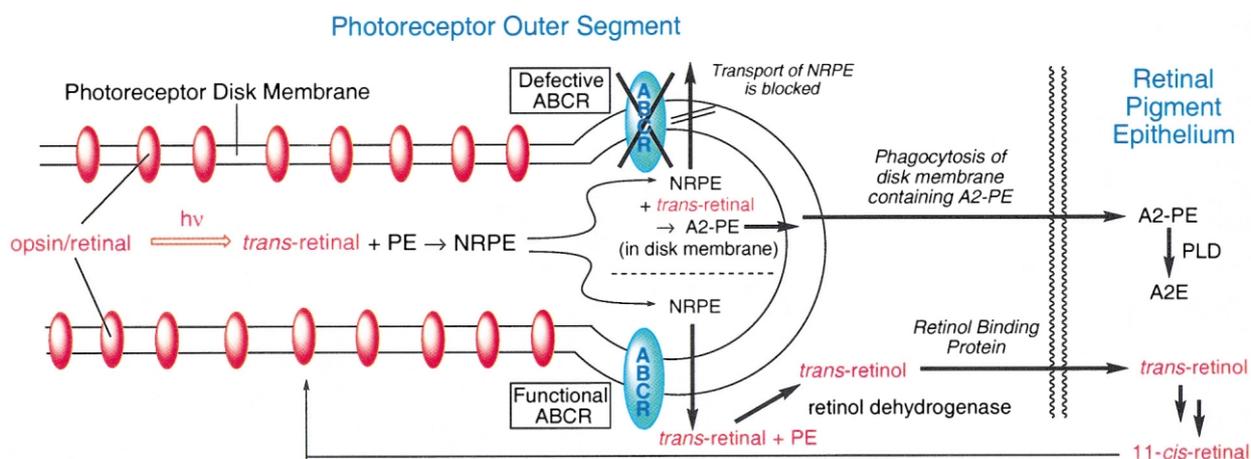


Figure 11. Model of the relationship between the ABCR transporter and A2E biogenesis. The putative ligand exported by the ABCR transporter is NRPE, the Schiff base of all-*trans*-retinal with PE. It is proposed that NRPE is transported to the cytoplasmic face of the outer segment disk where all-*trans*-retinal is reduced to all-*trans*-retinol. A retinol binding protein then carries retinol to the RPE where it is isomerized and oxidized back to 11-*cis*-retinal. The chromophore can then be reincorporated into opsin to form the ground state pigment, which is then ready to receive a photon of light. When ABCR activity is reduced or absent, NRPE accumulates in the disk membrane. Reaction with an additional molecule of all-*trans*-retinal generates A2-PE. Phagocytosis of shed outer segment membrane and phosphate hydrolysis of A2-PE, perhaps by phospholipase D (PLD), leads to the deposition of A2E in the RPE cell. When accumulated to critical concentrations, A2E can lead to RPE cell death. This schematic was derived by compiling existing models and published data.^{30,34,35,42,44,52,53,64,65} ABCR, ATP-binding cassette transporter in the retina; PE, phosphatidyl-ethanolamine; NRPE, retinal-PE Schiff base.

Clinical Significance of A2E Accumulation

Studies demonstrating that A2E, when present in sufficient intracellular concentrations, can be damaging to RPE cells^{23,34,35,59} are particularly relevant to the non-neovascular, atrophic form of AMD that accounts for up to 21% of the blindness caused by AMD¹ and that is characterized by a massive accumulation of lipofuscin preceding RPE cell death.²³ While multiple factors contribute to the onset of AMD, nonexudative AMD has been linked, in some cases, to mutations in the gene encoding for ABCR, the photoreceptor-specific ATP-binding cassette transporter.^{61–63} Interestingly, the substrate transported by ABCR in cone and rod outer segments⁶⁴ is suggested to be NRPE (16), the Schiff base adduct formed in the first step of A2E biogenesis (Fig. 10).^{52,53,65} It has been proposed that the function of ABCR is to transport all-*trans*-retinal across the outer segment disk membrane thereby aiding its reduction to all-*trans*-retinol by retinol dehydrogenase (Fig. 11).^{52,53,65} Moreover, it is clear that reduced ABCR activity results in NRPE accumulation and, with the availability of free all-*trans*-retinal, production of A2-PE in the disk membrane (Fig. 11). Subsequent phagocytosis of A2-PE-containing disk membranes together with A2-PE hydrolysis leads to the deposition of A2E in the RPE. Support for this model is provided by analyses demonstrating that A2-PE and A2E levels are significantly accentuated in photoreceptor outer segments⁴⁴ and RPE cells,⁵³ respectively, when ABCR function is eliminated in mice. Moreover, the means by which *abcr* mutations predispose the RPE to cell death in nonneovascular AMD and some other forms of retinal disease^{62,66,67} is understandable when considered within the framework of A2E biogenesis and the harmful effects of its accumulation in the cell.

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