Characterization of Native Retinal Fluorophores Involved in Biosynthesis of A2E and Lipofuscin-associated Retinopathies*

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Mutations in the photoreceptor-specific ABCA4 gene are associated with several inherited retinal and macular degenerations. A prominent phenotype of these diseases is the accumulation of cytotoxic lipofuscin fluorophores such as A2E within the retinal pigment epithelium. Another compound, dihydro-N-retinylidene-N-retinylphosphatidylethanolamine (A2PE–H2), also accumulates in retinas of mice and humans harboring ABCA4 mutations and was proposed to be a precursor of A2E. The role of A2PE–H2 in the biogenesis of A2E and its relationship to other retinal fluorophores has not been previously investigated. We report spectral properties and structural relationships of the principal retinal fluorophores that accumulate in retina and retinal pigment epithelium of abca4−/− mice. A long wavelength fluorescence emission intrinsic to abca4−/− retinal explants is shown to emanate from A2PE–H2. All-trans retinal dimer conjugates, which were also identified in the retinal explants, possessed distinct fluorescent and structural properties and, unlike A2PE–H2, did not accumulate in an age-dependent manner. Derivative absorbance and fluorescence spectroscopy revealed that A2PE–H2, A2E, and N-retinylidene-N-retinylphosphatidylethanolamine (A2PE), a known precursor of A2E, share common electronic and resonant structures. Importantly, collision-induced dissociation of A2PE–H2 produced daughter ions that were identical to authentic A2E and its daughter ions. Finally, intravitreal administration of A2PE–H2 to wild-type mice resulted in the formation of A2PE and A2E. These data validate a previously hypothesized biosynthetic pathway for A2E and implicate A2PE–H2 as a precursor in this pathway. Fluorescence properties of A2PE–H2 and other related fluorophores characterized in this report have significance for evaluation of human retinal diseases characterized by aberrant fundus autofluorescence.

Vertebrate photoreceptor cells contain light-sensitive proteins called opsins. Photoreceptor opsins are located in a membranous structure called the photoreceptor outer segment (POS). The visual chromophore for most vertebrate opsins is 11-cis-retinaldehyde. Absorption of a photon by an opsin pigment induces 11-cis to all-trans isomerization of the retinaldehyde chromophore. Restoration of light sensitivity to the bleached opsin pigment involves chemical isomerization of the all-trans-retinaldehyde (atRAL) back to 11-cis-retinaldehyde via an enzymatic process called the visual cycle. Most steps of the visual cycle take place within cells of the RPE, a cell layer adjacent to the photoreceptors. Another function of the RPE is to phagocytose the distal tips of POS membranes. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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4 The abbreviations used are: POS, photoreceptor outer segment; A2E, N-retinylidene-N-retinylethanolamine; A2PE, N-retinylidene-N-retinylphosphatidylethanolamine; A2PE–H2, dihydro-N-retinylidene-N-retinylphosphatidylethanolamine; atRAL, all-trans retinaldehyde; HPLC, high performance liquid chromatography; LC/ESI-MS, liquid chromatography/electrospray ionization mass spectrometry; RPE, retinal pigment epithelium; PE, phosphatidylethanolamine; N-ret-PE, N-retinylidene phosphatidylethanolamine; ATR dimer-PE, all-trans retinal dimer-phosphatidylethanolamine; ATR dimer-Et, all-trans retinal dimer-ethanolamine; STGD1, Stargardt disease; CID, collision-induced dissociation.

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ATR dimer-PE (\(\lambda_{\text{max}} = 510\, \text{nm}\))

A2PE (\(\lambda_{\text{max}} = 435\, \text{nm}\))

A2PE-H\(_2\) (\(\lambda_{\text{max}} = 510\, \text{nm}\))

A2E (\(\lambda_{\text{max}} = 435\, \text{nm}\))

**FIGURE 1.** Chemical structures and absorbance maxima of native retinal fluorophores. Shown are the native retinal fluorophores under study in the present investigation. The reported absorbance maxima were determined from UV-visible absorbance spectra acquired during HPLC analysis as described under “Experimental Procedures.” Designations \(R_1\) and \(R_2\) on ATR dimer-PE, A2PE-H\(_2\), and A2PE represent substituent fatty acyl moieties associated with PE (e.g. 16:0, 18:0, 20:4, and 22:6). The chemical structure for ATR dimer-PE has been recently solved (19); however, its relationship to A2PE-H\(_2\) is not known. The proposed chemical structure for A2PE-H\(_2\) is based upon previously determined structures for A2PE and A2E (23, 26). It is theorized that oxidation of the A2PE-H\(_2\) dihydropyridinium ring yields A2PE. Hydrolysis of the A2PE phosphate ester then liberates A2E. These processes are believed to occur in a non-enzymatic fashion within RPE phagolysosomes.

500-nm absorbing species in retina and RPE extracts from abca4\(^{-/-}\) mice. Structural characterization of this compound showed it to be a dimer of aRAl conjugated to phosphatidylethanolamine through a Schiff base linkage (termed ATR dimer-PE) (Fig. 1). Fishkin et al. (19) suggest that ATR dimer-PE is the sole 500-nm absorbing species in abca4\(^{-/-}\) ocular tissues and that A2PE-H\(_2\) may not exist. Similarities in the absorbance spectra of ATR dimer-PE and the compound previously identified as A2PE-H\(_2\), as well as the observation that ATR dimer conjugates rearrange to A2E under acidic conditions in vitro (19), tend to support this contention.

In this report, the relationship of dominant retinal fluorophores that accumulate in abca4\(^{-/-}\) mice and their role in A2E biosynthesis in vivo has been examined. We show that in addition to ATR dimer conjugates, abca4\(^{-/-}\) ocular tissues contain a second 500-nm absorbing species. Significant similarity in the electronic structure and chemical composition of this species with A2PE and A2E has been observed. These features are not shared by ATR dimer conjugates. In addition, this compound serves as a precursor to A2E in vivo. Based on these findings, we suggest that this compound is identical to the previously proposed A2PE-H\(_2\).

**EXPERIMENTAL PROCEDURES**

**Mice**—Retinal tissue explants were dissected from abca4\(^{-/-}\) (B6 × 129 hybrid) mice and age-matched, wild-type (C57BL/6) mice. Mice were maintained at 30–50 lux in a vivarium facility under 12-h cyclic light and were anesthetized by intraperitoneal injection of ketamine (200 mg/kg) plus xylazine (10 mg/kg) before death by cervical dislocation. For the A2PE-H\(_2\) treatment study, A2PE-H\(_2\) (\(~5\) nmol by phosphorus content) was resuspended in a solution containing 0.2 mg/ml dioleoyl phosphatidyl-choline/dioleoyl phosphatidylethanolamine (60:40, v/v) in phosphate-buffered saline, pH 7.2 (final volume ~0.2 ml). The solution was gently sonicated, and 2 \(\mu\)l was administered to anesthetized wild-type (BALB/c) mice by intravitreal injection. Six injections were given over a 12-day period. Mice were aged 1.5 months at study onset and were kept under 12-h cyclic light at 30 lux for 28 days following the final injection.

**Tissue Preparation and Extraction**—Chloroform-soluble compounds were extracted from retinal tissues as described previously (17). The extracts were washed with 4 ml of distilled H\(_2\)O/methanol (1:1, v/v), and the organic phase was then taken to dryness under a stream of N\(_2\). Sample residues were dissolved in 200 \(\mu\)l of 2-propanol for analysis by HPLC. All manipulations were done on ice under dim red light (Kodak Wratten 1A).

**HPLC**—Phospholipid extracts and synthetic retinal fluorophores were analyzed/purified by normal phase HPLC on a silica column (Zorbax Rx-Sil 5 \(\mu\)m, 250 × 4.6 mm, Agilent, Palo Alto, CA) using an Agilent model 1100 liquid chromatograph equipped with photodiode array and fluorescence detectors (Agilent Technologies, Wilmington, DE). The mobile phase (hexane/2-propanol/ethanol/25 mM potassium phosphate/acetic acid, 485:376:100:37:0.275, v/v) was pumped through the system at 1 ml/min. Column and solvent temperatures were maintained at 40 °C.

**Liquid Chromatography/Electrospray Ionization Mass Spectrometry (LC/ESI-MS)**—Samples were analyzed by reverse-phase chromatography on a C18 column (Zorbax 300 SB-C18; 5 \(\mu\)m, 250 × 0.5 mm, Agilent, Palo Alto, CA) using an Agilent 1100 series capillary liquid chromatograph equipped with a photodiode array detector. The column was equilibrated with 25% methanol/75% chloroform/methanol (2:1, v/v). A linear gradient to 100% chloroform/methanol was initiated 5 min after sample injection. Total run time was 25 min. Flow rate was 10 \(\mu\)l/min, and column temperature was maintained at 40 °C. The LC was coupled to an LCQ Deca XP ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA). MS conditions were as follows: electrospray ionization = 5.0 kV; N\(_2\) gas flow = 20 units; capillary voltage = 15 V; tube lens offset = 46 V; capillary temperature = 225 °C. Helium colli-
Biosynthesis of Lipofuscin Fluorophores

Excitation energy was varied between 35 and 45% to optimize fragmentation of the desired ions.

Synthesis and Analysis of A2E, A2PE, and ATR Dimer Conjugates—A2E and A2PE were synthesized as described previously (37). ATR dimer and ATR dimer-ethanolamine conjugate (ATR dimer-Et) were synthesized and purified according to published methods (19). A2E and A2PE were purified using normal phase HPLC as described above. ATR dimer-Et was purified on the same system except that the mobile phase was replaced with a gradient of 10% dioxane in n-hexane (16). Quantitation of the purified compounds and comparison of ATR dimer-Et to native A2PE-H2 were achieved by UV-visible absorbance spectroscopy using a Shimadzu UV-2401 spectrophotometer (Shimadzu/Cole Scientific, Moorpark, CA). Successful synthesis of unprotonated ATR dimer-Et was confirmed by mass spectroscopy and by demonstration of a bathochromic shift following treatment with acetic acid (final [HAc] = 0.1 M in methanol).

Fluorescence Spectroscopy of Retinal Explants—Fluorescence emission spectra from RPE-choroid and retina explants were acquired using a Tecan Safire II fluorescence microplate reader (Tecan US, Research Triangle Park, NC). Briefly, dissected tissue samples were washed in ice-cold phosphate-buffered saline (pH 7.2) and placed anterior side up in a modified 384-well microplate. Emission spectra were measured following excitation at 420 nm using top read mode. The z axis of the optical unit was optimally calibrated for each sample to account for differences in tissue depth and thickness.

Fluorescence Spectroscopy of Synthetic and Native Fluorophores—Fluorescence spectroscopy of purified, synthetic, and native fluorophores was performed using a Fluorolog FL3-22 spectrofluorometer (Jobin Yvon, Edison, NJ). Emission and excitation spectra for ATR dimer-Et (in methanol) were acquired with excitation and emission wavelengths of 363 and 550 nm, respectively. Bandpass was set at 5 nm. Excitation spectral intensities of unprotonated and protonated ATR dimer-Et were normalized to better compare spectral shape. Native A2E, A2PE, and A2PE-H2 were extracted from the eyecups (RPE-choroid + retina) of 14 abca4−/− mice aged 12–24 months. A2E, A2PE, and A2PE-H2 were purified from the sample extracts, as described above, taken to dryness under argon, and resuspended in hexane. Emission and excitation spectra were acquired for these samples using excitation and emission wavelengths of 420 and 590 nm, respectively. Bandpass was set at 10 nm.

RESULTS

Fluorescence Properties of abca4−/− RPE and Retina Explants—Biogenesis of A2E is thought to begin within the retina. Therefore, it is reasonable to expect that precursor fluorophores might be present in this tissue. We have explored this possibility by examining fluorescence properties of explants prepared from abca4−/− retina and RPE/eyecups. The emission spectrum acquired for RPE/eyecups in this study (Fig. 2A, dashed trace) is comparable with the spectra of A2E-laden human RPE cells (20) and human lipofuscin granules (21). In contrast, the fluorescence spectrum from retina explants demonstrated lower intensity relative to RPE/eyecups and an emission maximum of ~625 nm (Fig. 2A, solid trace). The intensity of this emission increased in an age-dependent manner (Fig. 2B) and was not detected in age-matched wild-type mice.

To identify and characterize the fluorescent compound(s) in abca4−/− retinas, chloroform-soluble extracts were prepared from the retina explants. These extracts were analyzed by HPLC with online UV-spectral and fluorescence detection. Two major peaks with similar absorbance maxima (λmax ~ 510 nm) and spectra were observed (Fig. 3A, compare left and center insets). However, only the peak eluting at ~26 min demonstrated fluorescence emission when excited at 440 nm (Fig. 3A, solid trace). Importantly, the acquired emission spectrum (Fig. 3A, right inset) was very similar to the emission spectrum observed during epifluorescence analysis of retina explants. Like the fluorophore detected in retina explants, the fluorescence intensity associated with this peak increased in an age-dependent manner (Fig. 3B). An age-dependent accumulation was not observed for the early eluting peak.

Characterization of Dominant Fluorophores in abca4−/− Retinas—It is likely that one of the two compounds detected in abca4−/− retinal extracts is the ATR dimer-PE species that was recently identified in...
**Biochemical Relationships of Native Retinal Fluorophores**—We next explored the relationship of A2PE-H₂ to A2E and a known A2E precursor, A2PE (23). In this analysis, the native fluorophores were purified from eyecups of aged abca4⁻/⁻ mice and analyzed by second derivative absorbance and fluorescence spectroscopy. Chromatography of eyecup extracts with fluorescence detection demonstrates that these are abundant fluorophores in abca4⁻/⁻ eyecups (Fig. 5A, solid trace). The absorbance spectrum for each fluorophore is shown in the figure inset. Excitation and emission spectra of the purified compounds (measured in hexane) are provided in Fig. 5B. It is noteworthy that although A2E, A2PE, and A2PE-H₂ demonstrate distinct absorbance spectra, their excitation spectra are quite similar. A structural homology among these fluorophores becomes readily evident upon examination of the second derivative absorbance and excitation spectra. Second derivative transformation of the absorbance spectra of Fig. 5A (inset) reveals two distinct bands at ~330 and ~420 nm that are present in each of the fluorophores (Fig. 5, C–E, indicated by arrows). Similarly, transformation of the excitation spectra of Fig. 5B exposes two common resonant features at ~420 and ~465 nm in each fluorophore (Fig. 5, F–H, indicated

*abca4⁻/⁻* retinas (19). Because the fluorescence intensity of the peak eluting at ~26 min increased in an age-dependent manner and was not present in wild-type retinas, it is likely associated with the *ABCA4* mutation. We sought to determine whether this compound might be an ATR dimer conjugate. To address this issue, we purified large amounts of the ~26-min peak (designated 26mp) from *abca4⁻/⁻* retinal extracts and determined its relationship to a synthetic ATR dimer conjugate using absorbance and fluorescence spectroscopy.

ATR dimer-Et, rather than ATR dimer-PE, was used for comparison based upon increased product yield after synthesis, its validation in a previous study (19), and the fact that PE does not contribute to absorbance or fluorescence in the examined wavelength range (250–450 nm). We confirmed that the synthesized ATR dimer-Et possessed the expected molecular mass (m/z = 594.48, Fig. 4A) and a Schiff base that was susceptible to protonation. Thus, in the presence of 0.1 N HAc, an immediate and significant bathochromic shift in the absorbance spectra (from 420 to 510 nm) was observed (Fig. 4B). This same behavior has been described for ATR dimer-PE (19). In contrast, neither bathochromic nor hypsochromic shifts were observed for 26mp under either acidic or basic conditions (data not shown). These data indicate a qualitative difference in the bond configuration and/or chemical composition between ATR dimer-Et and 26mp.

A comparison of protonated ATR dimer-Et and 26mp by absorption spectroscopy revealed subtle spectral differences (Fig. 4C). These differences became more obvious after second derivative transformation. This technique improves the signal-to-noise ratio of absorbing bands and is useful in extracting qualitative information from overlapping or incompletely resolved peaks (22). Examination of the second derivative spectral shape and trough locations provides information regarding the electronic structure of a compound. Second derivative spectra for ATR dimer-Et and 26mp spectra showed significant variations in the 250–450 nm region despite the common ~500-nm absorption peaks (Fig. 4D). These variations indicate differences in the chemical structures of ATR dimer-Et and 26mp.

Fluorescence properties of ATR dimer-Et were also distinct from those of 26mp. Although the excitation spectrum of ATR dimer-Et was not significantly affected by protonation (excitation maximum ~363 nm, Fig. 4E), the emission spectrum was dramatically altered. Unprotonated ATR dimer-Et demonstrated a very broad emission (500–570 nm) with 363-nm excitation. Protonation of the ATR dimer-Et Schiff base narrowed the emission, resulting in a spectrum with a well defined maximum at 450 nm (Fig. 4F). Excitation of either protonated or unprotonated ATR dimer-Et at 400–440 nm produced no detectable emission, a result consistent with the excitation spectra of this compound (Fig. 4E). In contrast, 26mp showed pronounced emission intensity with excitation in this wavelength range (Fig. 3A, right inset). These data demonstrate that 26mp is not an ATR dimer conjugate. The fact that only two ~500-nm absorbing species, which possess similar UV-visible spectra, have been identified in *abca4⁻/⁻* retinas (i.e., ATR dimer conjugates and A2PE-H₂) leads us to deduce that the early eluting peak in Fig. 3A is likely an ATR dimer conjugate. Indeed, the absence of fluorescence emission from this peak with 440-nm excitation (Fig. 3A) and an observed hypsochromic shift (from 510 to 425 nm) at alkaline pH (data not shown) are consistent with the behavior of an ATR dimer conjugate (19). Clearly, the long wavelength fluorescence emission observed in *abca4⁻/⁻* retinas cannot be attributed to ATR dimer conjugates. We can therefore conclude that the compound referred to here as 26mp is, in fact, A2PE-H₂.
by arrows). These data demonstrate a significant structural relationship among A2PE-H₂, A2PE, and A2E.

**Analysis of A2PE-H₂ by Mass Spectrometry**—Similarities in the electronic and resonant structures of A2PE-H₂, A2PE, and A2E prompted an analysis of the A2PE-H₂ chemical composition. In this study, A2PE-H₂ was first purified using normal phase HPLC as described above (Fig. 5A). The purified A2PE-H₂ sample was then analyzed by reverse-phase LC/ESI-MS. Like the normal phase system, the reverse-phase system provided baseline separation of the three fluorophores (data not shown). The retention time and absorbance spectrum of the injected A2PE-H₂ sample was used to confirm its identity and purity (Fig. 6A and inset). A portion of the eluted A2PE-H₂ peak (from 7.3 to 8.2 min) was delivered to the mass spectrometer in real time.

In the first scan event (Full ms), a major ion at 1274.85 m/z was detected (Fig. 6B). Collision-induced dissociation (CID) of this ion in the second scan event (ms2) produced a prominent daughter ion at 672.44 m/z that is consistent with a phosphoryl A2E fragment (Fig. 6C and inset). In addition, a neutral loss of 602.54 from the 1274.85 m/z parent ion was also detected (not shown). This loss is consistent with the mass of dehydrated dioleoylglycerol. Based on these data, it appears that the 1274.85 m/z parent ion may be dioleoyl A2PE. To better elucidate the composition of the 672.44 m/z ion, a second CID was performed in the third scan event (ms3). The daughter ions from this fragmentation (Fig. 6D) included an ion with an m/z identical to that of A2E (592.50) and an ion that is consistent with the m/z of dehydrated A2E (574.46).

The 592.50 m/z ion was isolated for CID in the fourth scan event (ms4) to confirm its identity. Daughter ions generated in the fourth scan event (Fig. 6E) were then compared with daughter ions generated from CID of authentic A2E (Fig. 6F). The fragmentation patterns in these two scans were nearly identical and contained the same ions at similar relative abundances. It is clear from this analysis that the 592.50 m/z ion generated from fragmentation of A2PE-H₂ was indeed A2E and, therefore, the 1274.85 m/z parent ion is likely A2PE. The facile conversion of A2PE-H₂ to A2PE observed here has been previously noted (18) and is consistent with the oxidative lability of this compound.

**The Relationship between A2PE-H₂ and A2E in Vivo**—Data gathered from the various in vitro analyses have revealed significant similarities between A2PE-H₂ and A2E. However, a direct precursor-product relationship has not been established in vivo. We have explored this issue by examining the fate of A2PE-H₂ following intravitreal administration into wild-type mice.

Purified A2PE-H₂ was suspended in a lipid emulsion and injected into the vitreous cavity of one eye. The contralateral eye was untreated. A separate group of age and strain-matched wild-type mice received intravitreal injections of the lipid suspension alone. At the end of the treatment period, an analysis of the A2PE-H₂-lipid suspension was performed to ensure that it had not degraded during the treatment period. The analysis revealed a single peak that was identified as A2PE-H₂, thereby confirming the integrity of the injected sample (Fig. 7A and inset). Extracts prepared from eyes that received a single injection of the A2PE-H₂-lipid suspension (1 day after injection) showed three peaks that corresponded to the retention times of A2E, A2PE, and A2PE-H₂ (~17, 20, and 27 min, respectively, Fig. 7B). Spectral analysis of the ~27-min peak confirmed that it was A2PE-H₂. At the conclusion of the study period (28 days after injection), the A2PE-H₂ peak was not detectable, and the peaks at ~17 and ~20 min increased ~2-fold (Fig. 7B, compare red and black traces). Spectra acquired for the peaks identified in Fig. 7B (at 17.4 and 20.8 min) were compared with spectra of authentic A2E and A2PE (Fig. 7, D and E, respectively). The spectral overlays confirmed that the identified peaks were A2E and A2PE. Extracts prepared from either uninjected (contralateral) eyes or eyes injected with the lipid suspension alone did not contain these peaks (Fig. 7C). These data clearly establish A2PE-H₂ as a precursor fluorophore involved in the biogenesis of A2PE and A2E in vivo.
FIGURE 6. Analysis of A2PE-H2 by mass spectroscopy. A2PE-H2 was purified from abca<sup>−/−</sup> eyecups and analyzed by LC/ESI-MS. Ions of interest were isolated and fragmented by CID. Chromatographic analysis (absorbance at 440 nm) of A2PE-H2 is shown in A. Absorbance spectra taken at the apex of the major peak (7.8 min) verified that the eluted peak was A2PE-H2 (A, inset); mAU, milliabsorbance units. A portion of the A2PE-H2 peak, indicated in A, was delivered to the mass spectrometer. The major ion detected in the full scan (Full ms) analysis (1274.85 m/z, B) was subject to CID in the second scan event (ms2), resulting in a 672.44 m/z daughter ion (C). The chemical structure for a compound of this mass (phosphoryl A2E fragment) is provided in the inset of C. Relative Fragmentation of the 672.44 m/z ion in the third scan event (ms3) produced daughter ions that were consistent with the m/z of A2E (D). In the final scan event (ms4), the 592.50 m/z ion detected in the third scan event was fragmented. Comparison of the fragmentation pattern generated in the fourth scan event (E) to the pattern produced from fragmentation of authentic A2E (F) reveals nearly identical daughter ion spectra.

FIGURE 7. Intravitreal administration of A2PE-H2. A lipid emulsion containing purified A2PE-H2 was injected into the vitreous cavity of wild-type mice. Injections were given every other day over a 12-day period. Immediately following the final injection, an analysis of the A2PE-H2 sample was performed to ensure that it had not degraded during the treatment period. Chromatographic analysis (absorbance at 440 nm) showed a single peak (at ~27 min) with the appropriate UV-visible absorbance (abs.) spectrum of A2PE-H2 (A and inset). At the times indicated, eyecup extracts were prepared and analyzed by HPLC (absorbance at 440 nm); mAU, milliabsorbance units. Extracts prepared from eyes that had received A2PE-H2 were analyzed at 1 and 28 days after injection (B). At 1 day after injection, three peaks corresponding to the retention times of A2E (~17 min), A2PE (~20 min), and A2PE-H2 (~27 min) were detected. Analysis at 28 days after injection revealed increased levels of the presumptive A2E and A2PE peaks and no detectable A2PE-H2. An overlay of the UV-visible absorbance spectra from the peaks identified as A2E and A2PE (17.4 and 20.8 min, respectively, in B) to authentic A2E (D) and A2PE (E) confirmed their identity. No fluorophores were detected in uninjected (contralateral) eyes (C) or in age-/strain-matched control mice that received injections of the lipid emulsion vehicle alone (not shown).
DISCUSSION

The ocular fundi of individuals afflicted with degenerative macular diseases such as STGD1 and age-related macular degeneration show dramatic, age-dependent accumulation of lipofuscin fluorophores (24–28). These fluorophores emit light in the broad range of 500–750 nm (27, 29, 30, 31). It has been theorized that this fluorescence is due to lipofuscin material contained within the RPE (29, 31). Ocular tissues from abca4+/− mice also accumulate lipofuscin fluorophores (32). In this animal model of STGD1, the fluorescence emission has been attributed to A2E, which accumulates within the RPE. Importantly, A2E is known to be the major long wavelength emitting fluorophore in both rodent and human lipofuscin granules (4–6, 33). In addition to mediating photo-oxidative damage (8, 34), A2E compromises an inherent cellular viability and may compromise the RPE support role on photoreceptors (1). Continued accumulation of this undigested debris reduces the capacity of the RPE for proper digestion and disposal of phagocytosed retinal debris (7, 35). Accumulation of this undigested debris reduces the capacity of the RPE for proper digestion and disposal of phagocytosed retinal debris, and may compromise the health of retinal tissues. In the present report, the A2E biosynthetic pathway comes from the observed biosynthesis of A2E and A2E from exogenous A2PE-H2 in vivo. The fact that this conversion took place within ocular tissues of wild-type mice indicates that A2E can be generated from A2PE-H2 under normal physiological conditions. It is noteworthy that the immediate precursor of A2E, A2PE, was generated during the treatment period. Thus, as the various in vitro analyses described above indicate, A2PE-H2, A2E, and A2E are related fluorophores involved in an unusual non-enzymatic biosynthetic pathway. A growing body of clinical evidence has implicated aberrant fundus autofluorescence and lipofuscin accumulation in the pathogenesis of retinal degeneration in patients with STGD1 and atrophic age-related macular degeneration. Studies have shown that the presence and accumulation of lipofuscin fluorophores precedes RPE and retinal cell death (24–26). Thus, it is conceivable that retinal fluorophores may directly compromise the health of retinal tissues. In the present report, the abca4 null mutant mouse, which excessively accumulates retinal fluorophores and lipofuscin in an age-dependent manner, has been used as a model system to characterize the native fluorophores and validate a previously proposed biosynthetic pathway. The data reveal that A2PE-H2 is a precursor in the A2E biosynthetic pathway and support the hypothesis that A2PE-H2 is an important mediator of lipofuscin-associated retinopathies.

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