Identification of a Novel Lipofuscin Pigment (iisoA2E) in Retina and Its Effects in the Retinal Pigment Epithelial Cells

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Background: Macular degeneration implicates lipofuscin deposition in the retina. Results: Bisretinoid iisoA2E in the retina was characterized; excessive accumulation of iisoA2E was cytotoxic to retinal pigment epithelial cells. Conclusion: Pyridinium iisoA2E is a unique directinal adduct and serves as a fluorescent biomarker of aberrant all-trans-retinal metabolism. Significance: Characterization of iisoA2E gives a more complete understanding of the biosynthesis of retinal bisretinoid lipofuscin.

Lipofuscin accumulation in retinal pigment epithelial (RPE) cells of the eye implicates the etiologies of Stargardt disease and age-related macular degeneration, a leading cause of blindness in the elderly. Here, we have identified a previously unknown RPE lipofuscin component. By one- and two-dimensional NMR techniques and mass spectrometry, we confirmed that this compound is a new type of pyridinium bisretinoid presenting an unusual structure, in which two polyenic side chains are attached to adjacent carbons of a pyridinium ring. This pigment is a light-induced isomer of isoA2E, rather than A2E, referred to as iisoA2E. This pigment is a fluorescent lipofuscin compound with absorbance maxima at ≈430 and 352 nm detected in human, pig, mouse, and bovine eyes. Formation of iisoA2E was found in reaction mixtures of all-trans-retinal and ethanolamine. Excess intracellular accumulation of this adduct in RPE cells in vitro leads to a significant loss of cell viability and caused membrane damage. Phospholipase D-mediated phosphodiester cleavage of the A2PE series generated isoA2E and iisoA2E, in addition to A2E, thus corroborating the presence of isoA2PE and iisoA2PE that may serve as biosynthetic precursors of isoA2E and iisoA2E.

Vision depends on a biochrome that consists of a light-sensitive protein called opsin attached to a chromophore. The visual chromophore for most vertebrate opsins is 11-cis-retinal. After capturing a photon by an opsin pigment, 11-cis-retinal bound to lysine 296 of opsin isomerizes to all-trans-retinal (1, 2). Restoration of light sensitivity to the bleached opsin pigment involves chemical reisomerization of all-trans-retinal back to 11-cis-retinal via an enzymatic process called the visual cycle (3). Most steps of the visual cycle occur within retinal pigment epithelial (RPE) cells. An additional RPE cell function is to phagocytose the distal tips of photoreceptor outer segments, which are shed by photoreceptor cells on a daily basis (4, 5). Based on these functions, RPE cells are extremely critical for the maintenance of photoreceptor viability. Ongoing shedding and phagocytosis of distal photoreceptor outer segments lead to gradual accumulation of fluorescent retinoids, lipids, and protein debris, called lipofuscin, in RPE phagolysosomes (6, 7). Delterious lipofuscin accumulation in the eye is considered to be one of the causative factors responsible for blindness in patients with retinal disorders, particularly age-related macular degeneration (8) and Stargardt disease (9, 10). Throughout the life of an individual, RPE cells of the eye accumulate fluorescent bisretinoids, which constitute the lipofuscin of the cells. These pigments are derived from reactions of all-trans-retinal. The first RPE lipofuscin constituent to be described was A2E (Fig. 1A), an unprecedented pyridinium bisretinoid. Previous evidence revealed that excessive accumulation of A2E in RPE cells in vitro can mediate detergent-like effects on cell membranes (11) as well as lead to the alkalinization of lysosomes (12) and detachment of proapoptotic proteins from mitochondria (13). All of the double bonds along the side arms of A2E assume the trans (E) configuration, whereas isoA2E (Fig. 1B), an isomer of A2E, has one cis (Z) olefin at the C13C14 position and exhibits a long-arm absorbance maximum about 12 nm blue-shifted from that of A2E.

On the other hand, the visual cycle produces a high flux retinoid that can lead to elevated levels of toxic retinoid intermediates, in particular all-trans-retinal, which can cause photore-
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![Image of structures A2E, isoA2E, iisoA2E, and all-trans-retinal]

FIGURE 1. Structures of A2E, isoA2E, iisoA2E, and all-trans-retinal. Shown are UV-visible absorbance (nm) and electronic transition assignments (→). Carbon atoms present in A2E (A), isoA2E (B), iisoA2E (C), and all-trans-retinal (D) are numbered.

dehydrogenase (LDH) assay kit was purchased from Shanghai Biyuntian Biological Science & Technology Co., Ltd. (Shanghai, China). Ethanolamine and HPLC grade trifluoroacetic acid were obtained from Aladdin. HPLC grade acetonitrile and methanol (MeOH) were purchased from Fischer. All other chemical reagents were AR grade.

Animals and Tissues—C57BL/6, BALB/cByJ, and Rpe65rd12 mice were purchased from the Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China) and raised under 12 h on/off cyclic lighting with an in-cage illuminance of 60–90 lux. All procedures were approved by the Institutional Animal Care and Use Committee and complied with guidelines set forth by the Ophthalmology Branch of the Chinese Medical Association. Human donor eyes were received within 12 h post mortem from the Eye Center of the Second Affiliated Hospital of Zhejiang University for Sight Restoration (Hangzhou, ZJ, China). All procedures were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all patients prior to study inclusion. Fresh bovine and pig eyes were obtained from local slaughterhouses.

Tissue Extraction and HPLC Analysis—Murine posterior eyecups (8–12 eyes/sample), human RPE/choroid (1 eye/sample), bovine RPE (1 eye/sample), pig RPE (1 eye/sample), and ARPE-19 cell tissues were analyzed. Tissues were homogenized in a v/v solution of phosphate-buffered saline (PBS) and 50% methanolic chloroform using a 7-ml scale glass tissue grinder. The resulting extract was redissolved in MeOH and centrifuged at 3000 × g for 5 min, the organic layer was placed in a 25-ml round-bottom flask. Residues were collected from three consecutive chloroform extractions. After the removal of combined solvents in a rotary evaporator, the residual material was transferred into a 0.5-ml centrifuge tube with 50% methanolic chloroform and dried under argon gas. The resulting extract was redisolved in MeOH and centrifuged at 7500 × g for 1 min. The supernatant was examined by reverse-phase HPLC using an Alliance System (Waters Corp., Milford, MA) equipped with a 2695 separation module, a 2998 photodiode array detector, and a 2475 multichannel (λ) fluorescence detector. For chromatographic separation, an analytical scale Atlantis® dC18 (3 μm, 4.6 mm × 150 mm) column was used with a gradient mobile phase composed of acetonitrile and water in the presence of 0.1% trifluoroacetic acid: 75–90% acetonitrile (0–30 min), 90–100% acetonitrile (30–40 min), and 100% acetonitrile (40–100 min) with a flow rate of 0.5 ml/min. Photodiode array detection was monitored at 430 nm. HPLC extraction and injection were carried out under dim red light. Integrated peak areas (μV·s) were determined by Empower® version 3 software. Molar quantity per eye was calculated using a calibration curve constructed from known concentrations of synthesized standard and a molar extinction coefficient of 11,500 (iisoA2E in MeOH at 430 nm) and by normalizing to the HPLC injection volume versus sample volume ratio.

Biosynthetic Reaction and HPLC Analysis—A mixture of all-trans-retinal (50 mg) and ethanolamine (4.8 mg) in 3 ml of ethanol (EtOH) was stirred in the presence of acetic acid (5 μl) in a 5-ml round-bottom flask with a sealed ground glass stopper

EXPERIMENTAL PROCEDURES

Materials—All-trans-retinal and dipalmitoyl-L-α-phosphatidylethanolamine (DP-PE) were purchased from Sigma-Aldrich. Phospholipase D (PLD) from Streptomyces chromofuscus was purchased from Merck Millipore. A colorimetric lactate
at room temperature under dim light for 2 days. For HPLC analysis, the reaction mixture was diluted with MeOH and injected into an Atlantis® dC18 reverse-phase column (3 μm, 4.6 × 150 mm). The mobile phase was a gradient of acetonitrile in water with 0.1% trifluoroacetic acid: 75–90% acetonitrile (0–30 min), 90–100% acetonitrile (30–40 min), and 100% acetonitrile (40–100 min) with a flow rate of 0.5 ml/min. The photodiode array detector was set at 430 nm for eluent monitoring.

**NMR Spectroscopy**—One-dimensional (1H and 13C) and two-dimensional (HMBC, HSQC, 1H–1H COSY, and NOESY) NMR spectra were recorded on a BrukerAvance 500-MHz spectrometer in CD3OD. Chemical shifts (δ) in ppm were referenced to the carbon (δc 49.15) and residual proton (δH 3.31) signals of MeOD. Data processing was performed using vendor-supplied software. The NMR DEPT experiment was conducted using a polarization-transfer pulse of 135°.

**Synthesis**—A2E, isoA2E, and iisoA2E were generated from a one-step biosynthetic reaction of all-trans-retinal and ethanolamine. To isolate and prepare these fluorophores, the reaction mixture was concentrated in a rotary evaporator and subjected to reverse-phase HPLC. A preparative scale SunFire™ C18 column (10 μm, 10 mm × 250 mm; Waters Corp.) was utilized with an acetonitrile and water gradient containing 0.1% trifluoroacetic acid (80–100%, 0–30 min; 100% acetonitrile, 30–40 min; flow rate 2 ml/min). For iisoA2E, further purification was performed on an analytical scale Atlantis® dC18 (3 μm, 4.6 mm × 150 mm) with a gradient of acetonitrile in water with 0.1% trifluoroacetic acid: 85–90% acetonitrile (0–10 min), 90–100% acetonitrile (10–40 min), and 100% acetonitrile (40–50 min) with a flow rate of 0.8 ml/min. The photodiode array detector was set at 430 nm for eluent monitoring. The A2PE series (A2PE, isoA2PE, iisoA2PE, etc.) was synthesized as described previously (19) with minor modifications. All-A2E series (A2E, isoA2E, iisoA2E, etc.) was synthesized as described previously (20) with minor modifications. Solutions of HPLC-purified A2E, isoA2E, or iisoA2E in water (200 μM) containing 2% DMSO was irradiated by a 500-W xenon illuminant (20,000 lux) for 10 min. The extent of oxidization was tested by mass spectrometry and reverse-phase HPLC. A Finnigan LCQ Deca XPplus ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with an electrospray ionization (ESI) interface and an ion trap mass analyzer was utilized. A syringe pump was used for direct loop injection of sample solutions with a flow rate of 5 μl/min. Operating parameters in the positive ion mode were as follows: collision gas, ultra-high purity helium; nebulizing gas, high purity nitrogen; ion spray voltage, 4.5 kV; nitrogen sheath gas, 5 arbitrary units; capillary temperature, 275 °C; capillary voltage, 15 V; and tube lens offset voltage, 30 V. The energy for collision-induced dissociation was 35%, and the isolation width of precursor ions was 2.0 Th. For HPLC, an Atlantis® dC18 (3 μm, 4.6 mm × 150 mm) reverse-phase column was used for the stationary phase, and for the mobile phase, a gradient of acetonitrile in water was used with 0.1% trifluoroacetic acid (85–100% acetonitrile (15 min) and 100% acetonitrile (15–30 min) with a flow rate of 0.8 ml/min) monitored at 430 nm with a 30-μl injection volume.

**RPE Cell Culture**—A human adult RPE cell line (ARPE-19; Basic Medical Experimental Center, Fudan University, Shanghai, China) devoid of endogenous iisoA2E was grown in Dulbecco’s modified Eagle’s medium (HyClone, Logan, UT) with 10% heat-inactivated fetal calf serum (HyClone, Logan, UT) in a 6-well (Nunc, Shanghai, China) or 96-well (Laboratory-Tek, Nunc (Roskilde, Denmark)) cell culture plate.

**Treatment and Cellular Uptake of iisoA2E**—iisoA2E was stored as a stock solution in DMSO (10 mM) and kept at −80 °C in the dark. Cells were incubated with serial dilutions of iisoA2E (0.625, 1.25, 2.5, 5, 10, 20, and 40 μM) for 1–3 days to assess cell viability. All cell-based experiments included untreated cells as controls. After incubation with 10 μM iisoA2E for 5 days, ARPE-19 cells were vigorously washed with PBS to ensure that only intracellular iisoA2E remained and then were harvested for HPLC analysis. The cell lysate was extracted with MeOH and chloroform. For compound elution, an Atlantis® dC18 (3 μm, 4.6 × 150 mm) reverse-phase column was used for the stationary phase, and a gradient of acetonitrile in water with 0.1% trifluoroacetic acid was set for the mobile phase: 85–100% acetonitrile, 0.8 ml/min, 15 min; 100% acetonitrile, 0.8–1.2 ml/min, 15–20 min; 100% acetonitrile, 1.2 ml/min, 20–40 min. Photodiode array detection was set at 430 nm.

**Fluorescence Assays**—ARPE-19 cells were incubated with iisoA2E (5, 10, 20, and 40 μM) for 2 days. Afterward, cultures were washed and fixed with 4% paraformaldehyde in PBS for 15 min. Accumulation of iisoA2E in cultured RPE cells was detected and imaged with a Zeiss multiphoton confocal micro-

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scope (LSM780, Carl Zeiss) using an argon-krypton laser (isoA2E, excitation/emission 488/499–631 nm; DAPI, one-photon excitation/emission 405/426–489 nm).

Cell Viability Assay—Cytotoxicity was tested by a 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Roche Applied Science) that measures the ability of viable cells to degrade a yellow tetrazolium salt into purple formazan crystals. Briefly, 20 μl of MTT reagent was added to 200 μl of culture medium in each well. Following a 4-h incubation, 150 μl of DMSO was added. After oscillating for 10 min, solutions were spectrophotometrically measured at 490 nm. Cell viability was expressed as a proportion of control optical density (OD).

Lactate Dehydrogenase Assay—LDH assay was performed by a LDH cytotoxicity assay kit (Biyuntian, Shanghai, China). Cytoplasmic LDH was measured in the media of RPE cultures grown to 80–90% confluence in 96-well plates following a 72-h isoA2E treatment and a specified interval of 24 h (LDH release period) after the addition of fresh serum-free media to isoA2E-containing cells. After the plate was centrifuged at 400 × g for 5 min, cell-free supernatants were collected and assayed in a new 96-well plate at 120 μl/well. Thirty minutes after reaction mixture addition, absorbance was measured at 490 nm. In each experiment, six replicates of each condition were included, and background levels were determined using medium that was not exposed to cells and subtracted from absorbance values obtained for each condition.

PLD-mediated Hydrolysis of the A2PE Series—The A2PE series (550 μg) was dissolved in DMSO (45 μl) and then added to 465 μl of 40 mM MOPS buffer (pH 6.5) containing 100 units/ml PLD and 15 mM CaCl2. The mixture was incubated for 4 h at 37 °C, extracted with 1:1 (v/v) chloroform/MeOH and chloroform, dried under argon, and redissolved in 100 μl of 50% methanolic chloroform. HPLC analysis was performed and monitored at 430 nm. The mobile phase contained a gradient of MeOH in water with 0.1% formic acid (75–90% MeOH (0–30 min), 90–100% MeOH (30–40 min), and 100% MeOH (40–100 min)) with a flow rate of 0.5 ml/min. To simultaneously detect the A2PE series and cleavage products released from its PLD-mediated hydrolysis, an XBridge™ C8 column (5 μm, 4.6 mm × 150 mm) was used with an injection volume of 50 μl and a total running time of 100 min.

Statistical Analysis—Data were analyzed by one-way ANOVA and Newman-Keuls multiple comparison test (Prism 4, GraphPad Software, San Diego, CA).

RESULTS

Detection of Two Unidentified Lipofuscin Pigments in Mouse Eyecups—Chloroform/MeOH extracts of posterior eyecups harvested from C57BL/6 and BALB/cByJ mice were reexamined by reverse-phase HPLC while monitoring eluents at 430 nm. As expected, HPLC profile peaks were readily assigned to A2E, isoA2E, and a previously unrecognized peak (URP) that exhibited absorbance maxima of 445 and 342 nm and a retention time (Rt) of 38.5 min (Fig. 2). In addition to A2E, isoA2E, and URP, an additional unknown peak adjacent to URP was also observed; this minor peak, different from URP, exhibited absorbance maxima at 430 and 352 nm and a Rt of 39.3 min (Fig. 2, A and B). To investigate the origin of these compounds in the visual cycle, the chromatogram generated utilizing eyecup extracts of C57BL/6 (Fig. 2A) and BALB/cByJ (Fig. 2B) mice was compared with that of Rpe65rd12 mice (Fig. 2A, right inset), in which retinal chromophores were not formed in vivo (21). The injectants from the eyecups of Rpe65rd12 mice were distinguished by an absence of bisretinoid components, such as A2E and isoA2E, that were present in C57BL/6 and BALB/cByJ mice. Additionally absent in the case of the Rpe65rd12 mice were URP and the 430/352 nm peak at Rt ~39 min.

Detection of isoA2E and URP in Human, Bovine, and Pig RPE—To test for tissue localization of isoA2E and URP, fresh pig eyes and dissected RPE were obtained. In methanolic chloroform extracts of retinal pigment epithelia from a single pig eye (Fig. 2D), isoA2E and URP were readily detected by reverse-phase HPLC. In addition, HPLC analysis of the extract from bovine retinal pigment epithelia also showed URP and isoA2E, eluted at 39.5 min and 39.8 min, respectively (Fig. 2E). To substantiate the consistency of isoA2E in bovine RPE with synthesized standard, the HPLC chromatogram of bovine RPE extract was compared with that of the bovine RPE extract and exogenous isoA2E mixture. As expected, the peak height/area of isoA2E was significantly increased. Quantification by integrating peak areas demonstrated that the percentage of isoA2E relative to URP in the extract/mixture increased by 63.7% (supplemental Fig. S1). More importantly, the human donor eye (age 55, female), dissected as retinal pigment epithelia with attached choroid to maximize the yield of RPE cells, revealed the presence of isoA2E and URP (Fig. 2F).

HPLC Analysis of Biosynthetic Reaction Mixtures—Next we established whether URP and the 430/352 nm compound in mice were generated in a reaction mixture of all-trans-retinal and ethanolamine (Fig. 2C). Besides observing the peaks attributable to A2E and isoA2E, the elution profile revealed a peak with UV-visible absorbance maxima at 430 and 352 nm. ESI mass spectrometry (ESI-MS) analysis of the 430/352 nm pigment in positive ion mode disclosed an m/z peak at 592.5 (Fig. 2C, right inset) consistent with the m/z of A2E and isoA2E. This peak was identical to the 430/352 nm pigment in eyecup extracts of wide-type mice (Fig. 2, A and B) as judged by Rt, ESI-MS, and UV-visible absorbance. By contrast, the peak attributable to URP was not formed in the reaction mixture, as determined by reverse-phase HPLC (Fig. 2C).

Photoisomerization/Photo-oxidation of A2E, isoA2E, and 430/352 nm Pigments—To investigate the light-induced modification of A2E, isoA2E, or the 430/352 nm pigment, solutions of these fluorophores in water (200 μM) containing 0.2% DMSO for solubility purposes were exposed to sunlight, room lamp light, and 430-nm blue light. Exposure of the A2E solution to either sunlight or room lamp light for 35–120 min yielded isoA2E and several minor cis-isomers, all with a molecular ion signal at m/z 592 in the mass spectra (supplemental Fig. S2), consistent with a previous report (20, 22). Moreover, isoA2E was more hydrophobic in comparison with other cis-isomers of A2E, and products behind isoA2E were not monitored in the HPLC chromatograms of the mixture generated from light illumination of A2E. Importantly, subjected the isoA2E solution to sunlight for 35 min yielded a less polar peak absorbing at 430 nm.
and 352 nm (Fig. 3, A and B). Given that Rt (\(~12.5\) min), UV-visible absorbance maxima, and \(m/z\) 592 of this peak were in accordance with that of the 430/352 nm pigment in extracts of mouse eyecups and biomimetic reaction mixtures, we confirmed that this 430/352 nm pigment was produced from light-mediated isomerization of isoA2E. Accordingly, we will refer to this molecule as iisoA2E; this nomenclature is selected to reflect a molecule that can form from facile isomerization of isoA2E. To explore whether light-induced isomerization of iisoA2E to yield isoA2E occurred, a solution of iisoA2E was illuminated with sunlight. As a result, HPLC analysis revealed five isomers of iisoA2E (I–V; Fig. 3, C and D) on the basis of UV-visible absorbance (Fig. 3E) and a molecular ion peak at \(m/z\) 592 by ESI-MS. All of these isomers were eluted in front of iisoA2E, indicative of higher polarity than the parent compound. The chromatographic peaks behind iisoA2E were not observed in the HPLC profiles (Fig. 3D) of the mixture generated from light illumination of iisoA2E, suggesting that irradiation of iisoA2E did not generate products less polar than the parent compound. It was noted that peak V eluted at 9.5 min and showed a weak shoulder at 295 nm in the absorbance spectrum as well as characteristic absorbance maxima at 430 and 339 nm (Fig. 3E, blue), indicating that this product was slightly altered by photo-oxidation (23). According to absorbance maxima and \(R_t\), it was inferred that isoA2E, different from products I–V, was not generated from photoisomerization of iisoA2E. To further corroborate this conclusion, a solution of iisoA2E was illuminated with sunlight for 35 min and injected into HPLC with synthesized isoA2E. As expected from the overlay chromatograms (Fig. 3F), a clear peak (\(R_t = 38.6\) min) was assigned to synthesized isoA2E and a shoulder peak (\(R_t = 38.9\) min) was readily detected that corresponded to product II, confirming that conversion of iisoA2E into isoA2E by light was not feasible. Likewise, these isomerizations were also observed when A2E, isoA2E, and iisoA2E were illuminated for 5 min by monochromatic light (430-nm blue light) (supplemental Fig. S3).

Moreover, the propensity of iisoA2E to undergo photo-oxidation, as do A2E and isoA2E, was investigated. Samples in water with 0.2% DMSO were irradiated by 500-W xenon light (20,000 lux). In the case of A2E and isoA2E, subsequent ESI-MS analysis (Fig. 4) demonstrated the formation of several higher molecular mass adducts differing by 16 mass units; the structure of the highest \(m/z\) species at 736 was that of a polyoxygenated species corresponding to the addition of nine oxygen atoms to the polyenic side arms (Fig. 4, D and E). Conversely, subjecting the mixture generated from iisoA2E light illumination to ESI-MS resulted in the release of five peaks that differ in \(m/z\) by 16. The \(m/z\) 672 species indicated that only five oxygen atoms were inserted into the carbon-carbon double bonds
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FIGURE 3. Biomimetic photoisomerization of isoA2E and iisoA2E. A–D, representative HPLC profiles (monitored at 430 nm) were obtained before and after 35-min exposure to sunlight of isoA2E (A and B) and iisoA2E (C and D) in water with 2% DMSO. Right insets (B and D), UV-visible absorbance spectra of isoA2E and iisoA2E. Right insets (A and C), UV-visible absorbance spectra of isoA2E and iisoA2E. Right inset (B), UV-visible absorbance spectra of iisoA2E generated from sunlight-mediated isomerization of isoA2E. Red asterisks, cis-isomers of A2E; E, UV-visible absorbance spectra of isoA2E and its isomers (I–VI). F, an overlay of chromatograms generated from illumination of isoA2E by sunlight for 35 min (black) and when injectant was the mixture from 35-min sunlight illumination of isoA2E and synthesized isoA2E (blue). abs, absorbance; mAU, milliabsorbance units.

residing in the side chains of isoA2E (Fig. 4F). Relative abundances of A2E and isoA2E as starting compounds in the mass spectra were significantly decreased compared with that of their photo-oxidized species, whereas the relative abundance of iisoA2E as the parent molecule was still far more greater than that of its photo-oxidative products. With chromatographic monitoring at 430 nm, peak heights of A2E, isoA2E, and iisoA2E were considerably diminished when these compounds were irradiated for 10 min with xenon light (Fig. 4, G–L), and quantitation by integrating peak areas reflected that levels of A2E, isoA2E, and iisoA2E were reduced by 99.65, 99.95, and 99.8%, respectively. These data support the conclusion that photo-oxidation of iisoA2E is much less efficient than that of A2E and isoA2E under identical experimental conditions.

Identification of iisoA2E—iisoA2E, a reddish solid, presented a molecular formula of C_{42}H_{34}NO as determined by high resolution ESI-MS at m/z 592.4497 [M]^+ (theoretically, 592.4513) requiring 15 degrees of unsaturation. As with A2E (supplemental Fig. S4) and isoA2E (supplemental Fig. S5), it was not possible to obtain an interpretable 1H NMR spectrum of this compound when isolated from eyes because each extract contained trace amounts of iisoA2E. Biomimetic synthesis of iisoA2E and subsequent two-step HPLC preparation allowed us to obtain sufficient purified samples and enabled NMR studies (supplemental Figs. S6–S12) to identify its full structure (Figs. 1C and 5). The structure of iisoA2E was observed to be very special as that of A2E and isoA2E, which extended into the meta-carbon (Fig. 1). The 125-MHz 13C NMR spectrum in CH3OD with the aid of spectroscopic techniques, including DEPT135°, HSQC, and HMBC, resolved 42 carbon signals, including 9 methyls, 8 methylenes, 11 quaternary carbons, and 14 methines. All proton and carbon chemical shifts present in iisoA2E 1H and 13C NMR spectra were carefully assigned (Fig. 5; also see numbering in Fig. 1).

1H-1H COSY allowed for complete identification of methyl and olefinic proton resonances. HMBC correlations of H-15 to C14 (δ, 122.63), N-CH2 (δ, 61.78), C15 (δ, 142.24) and C13 (δ, 153.80), and H-14 to C13 (δ, 153.80), C15 (δ, 146.03), and C16 (δ, 153.60) indicated attachment of the long side chain at C16 rather than C15. This was further corroborated by the absence of a cross-peak between C12-H and C16-H in the NOESY spectrum, which was featured in isoA2E but not A2E (20, 24). Coupling constants of vicinal olefinic protons C7-H/C8-H, C11-H/C12-H, C7’-H/C8’-H, and C11’-H/C12’-H were 16.0, 15.1, 16.1, and 15.0, respectively; these values, similar to that of A2E (supplemental Fig. S4) and isoA2E (supplemental Fig. S5), were large, thereby confirming (E)-configurations for the double bonds. NOESY correlations of C8-H/C10-H, C9-CH2/C7-H, C9-CH2/C11-H, C8’-H/C10’-H, C9’-CH2/C7’-H, and C9’-CH2/C11’-H revealed (E)-stereochemistry for C9C10 and C9’C10’ double bonds. The C13C14 double bond was identified as being in z-geometry based on NOESY correlation of C13-H/C14-H and absorbance maxima of the long side chain (λ_{max} 430 nm), consistent with that of isoA2E.

Cell-based Assays of iisoA2E—It was first confirmed that ARPE-19 cells did not contain endogenous iisoA2E by reverse-phase HPLC (supplemental Fig. S13). After incubation of ARPE-19 cells with iisoA2E, cellular uptake was verified by analysis of intracellular granules. In a representative HPLC chromatogram generated from the extract of RPE cells receiving 10 μM iisoA2E, a clear peak attributable to iisoA2E was observed that was not present in the extract of cells unexposed to iisoA2E. In addition to iisoA2E, we did not detect additional products in iisoA2E-containing cells, indicating that further
FIGURE 4. Light-induced oxidization of A2E, isoA2E, and iisoA2E. A–C, ESI-MS spectra of A2E, isoA2E, and iisoA2E before irradiation by 500-W xenon light. D–F, ESI-MS spectral analysis of A2E, isoA2E, and iisoA2E after 10-min irradiation with xenon light. The addition of oxygen atoms is evidenced by a series of molecular ion peaks that differ in m/z by 16. The peak at m/z 736 in D and E indicates the formation of nonaoxo-A2E/isoA2E, a compound that is probably a complex mixture of stereoisomers. G–L, A2E, isoA2E, and iisoA2E (200 μM in water with 2% DMSO) were irradiated for 10 min by a 500-W xenon light (20000 lux). Non-irradiated and irradiated samples were subsequently analyzed by reverse-phase HPLC. As highlighted in red, the percentage of irradiated sample relative to non-irradiated samples based on chromatographic peak areas (mAU) negatively correlated with the extent of photo-oxidation. mAU, milliabsorbance units.
degradation of this pigment did not occur. Online HPLC fluorescence detection demonstrated that iisoA2E exhibited clearly fluorescent signals with excitation/emission of 430/600 nm (Fig. 6A). Based on the autofluorescent nature of this molecule, cells with an accumulation of iisoA2E manifested autofluorescence viewed by laser-scanning fluorescence microscopy. Confocal imaging in the horizontal plane at depths within the cells (Fig. 6, B–F) confirmed that exogenously delivered iisoA2E was internalized by ARPE-19 cells in culture. Autofluorescent intensity of internalized iisoA2E granules was augmented, increasing in concentration from 5 to 40 μM (Fig. 6, C–F).

Next, the health of ARPE-19 cells with accumulated iisoA2E was examined. 1–3 days after introducing iisoA2E to cultures, cell viability was tested by MTT assay. Concomitant loss of cell viability was observed at 0.625 μM (1 day). A significant increase in LDH degradation of this pigment did not occur. Online HPLC fluorescence detection demonstrated that iisoA2E exhibited clearly fluorescent signals with excitation/emission of 430/600 nm (Fig. 6A). Based on the autofluorescent nature of this molecule, cells with an accumulation of iisoA2E manifested autofluorescence viewed by laser-scanning fluorescence microscopy. Confocal imaging in the horizontal plane at depths within the cells (Fig. 6, B–F) confirmed that exogenously delivered iisoA2E was internalized by ARPE-19 cells in culture. Autofluorescent intensity of internalized iisoA2E granules was augmented, increasing in concentration from 5 to 40 μM (Fig. 6, C–F).

Next, the health of ARPE-19 cells with accumulated iisoA2E was examined. 1–3 days after introducing iisoA2E to cultures, cell viability was tested by MTT assay. Concomitant loss of cell viability was not observed with iisoA2E concentrations ranging from 2.5 to 40 μM (Fig. 6G). However, when the concentration of iisoA2E increased to 40 μM, a significant loss of cell viability was detected. After 2 days, loss of cell viability was not detected at 0.625 μM but was significantly observed at higher concentrations (1.25–40 μM). After 3 days, a significant loss of cell viability was observed at 0.625 μM. However, at 0.625 μM and 1.25 μM, loss of cell viability was not significantly associated with incubation time, whereas cell viability by MTT assay clearly decreased with time at concentrations starting from 2.5 μM. To test the ability of iisoA2E amassed in RPE cells to perturb membrane integrity, evidence of membrane damage was assayed by investigating release of cytoplasmic LDH into the culture medium. It was found that RPE cells exposed to 0.625, 1.25, 2.5, 5, 10, and 20 μM iisoA2E did not exhibit elevated LDH levels after 72 h of treatment, followed by 24-h incubation in fresh medium (Fig. 7). A significant increase in LDH levels was observed when cultures accumulated iisoA2E from the 40 μM concentration.

**Enzymatic Cleavage of the A2PE Series Releases A2E, isoA2E, and iisoA2E—A2PE was a compound mixture with phosphatidic acid moieties that vary in fatty acids in vivo (25). DP-PE is**
a phospholipid (C_{37}H_{74}NO_{8}P) component of the cell membrane. To exclude the formation of A2PE with variable fatty acid compositions, all-trans-retinal was reacted with DP-PE to yield an A2PE species (dipalmitic A2PE), which contained a dipalmitic acid. Interestingly, HPLC analysis of the reaction mixture-derived A2PE sample showed a cluster of chromatographic peaks rather than a single peak attributable to dipalmitic A2PE, suggesting that this material may be a compound mixture (an A2PE series) in which A2PE, isoA2PE, and iisoA2PE were included (Fig. 8A). Further evidence identifying the analyte of interest as a mixture of A2PE and its isomers was also provided by ESI-MS in positive ion mode (Fig. 8A, right inset). The mass spectrum only exhibited prominent ion peaks at m/z 1223.3 and 1245.5, corresponding to dipalmitic A2PE (C_{77}H_{124}NNaO_{8}P, calculated as 1222.914) and the Na⁺ adduct of the molecular ions of dipalmitic A2PE (C_{77}H_{125}NO_{8}P, calculated as 1244.896). In experiments aimed at determining whether isoA2PE and iisoA2PE are formed in the reaction of all-trans-retinal with DP-PE and act as the substrates for PLD, we incubated the A2PE series with PLD and observed a reduction in all chromatographic peaks corresponding to the A2PE series together with the appearance of four more hydrophilic peaks in the HPLC profile at 21.97, 22.74, 24.13, and 24.83 min, respectively (Fig. 8B). On the basis of absorbance spectra and by co-injection with synthetic A2E, isoA2E, and iisoA2E, the first three peaks were readily identified as A2E, isoA2E, and iisoA2E, respectively (Fig. 8B, left inset). These observations corroborate the fact that besides A2PE, isoA2PE and iisoA2PE are generated in vivo as the immediate precursors of isoA2E and iisoA2E, with isoA2E and iisoA2E being released from isoA2PE and iisoA2PE by enzymatic hydrolysis (Fig. 8, C–E).

**DISCUSSION**

A growing body of clinical and laboratory evidence demonstrates that anomalous lipofuscin accumulation is implicated in the pathogenesis of RPE cell degeneration in patients with age-related macular degeneration, recessive Stargardt disease, and other forms of macular disease (26–28). Here, we have isolated and structurally characterized iisoA2E, a new type of pyridinium bisretinoid of RPE lipofuscin. It was found that the C15 proton of A2E and isoA2E is substituted with a long side chain, whereas the long hydrophobic retinal arm of iisoA2E is attached to C16 rather than C15 (Fig. 1). In the case of iisoA2E, the extension of two polyenic side arms into the adjacent carbon atoms of the pyridinium ring narrows the distance between long and short side chains and probably strengthens steric hindrance of active carbon-carbon double bonds, thereby alleviating the extent of oxidation arising from light (29, 30). Thus, we presume that the ability of iisoA2E to resist photo-oxidation may be partly attributed to its unusual structure (Fig. 1C).

Evidence indicates that two side arms of A2E have nine carbon-carbon double bonds that are strongly susceptible to light. Both long and short polyenic side chains beside the pyridinium...
ring in A2E undergo a series of chemical processes, including cis-isomerization, photo-oxidation, and photo-cleavage during irradiation with blue light to produce cis-isomers, epoxides, furanoid oxides, cyclic peroxides, and cleavage products (23, 31–34). Several lines of investigation have established that there is a mutual conversion between A2E and isoA2E when each pigment is illuminated with light (20, 35), which is in accord with our findings by HPLC (Fig. 3, A and B). Thus, light-induced isomers of isoA2E are very similar, but not identical, to that of A2E. It is especially interesting that isoA2E undergoes light-mediated isomerization to yield iisoA2E. Irradiation of iisoA2E with light yields several isomers in which A2E and isoA2E do not exist, indicating that the unprecedented structure of iisoA2E (Fig. 1C) does not allow it to be converted into either A2E or isoA2E. The compounds attributable to peaks I–V in the HPLC chromatograms (Fig. 3D), different from light-induced isomers of A2E and isoA2E, represent cis/trans-isomers of isoA2E at carbon-carbon double bonds. In addition to isoA2E, a Z-isomer of A2E at the C13C14 double bond, several additional cis double bond isomers of A2E are clearly formed in human RPE (22), whereas the structures of the minor cis-isomers of A2E have so far not been corroborated due to the inability to obtain sufficient samples for NMR studies (22). It has been confirmed that the C13C14 double bond of all-trans-retinal is most prone to cis-isomerization, yielding 13-cis-retinal, this chromophore is considered to coexist with all-trans-retinal in vivo at low levels. All-trans-retinal reacts with PE to generate NRPE. After NRPE undergoes a [1,6]-proton tautomerization, the intermediate reacts with 13-cis-retinal to form a 13-cis-iminium salt, the latter undergoes a hydrogen shift, C13C14 double bond cleavage, and 6-aza-electrocyclization to generate an intermediate bearing a three-membered ring that readily opens. Following opening of the three-membered ring at the C14C15 single bond and shift of C13-H to C15, dihydropyridinium iisoA2PE is generated. Facile auto-oxidation of this intermediate with the loss of two hydrogen atoms gives rise to isoA2E, which is cleaved by hydrolysis of the phospholipid to release isoA2E. A2E and isoA2E can be interconverted by light. isoA2E undergoes C13C14 double bond cleavage and a shift of 6-H to C13 to produce an intermediate with a three-membered ring. Photochemical ring opening and intramolecular hydrogen shift occur in this intermediate, leading to formation of iisoA2E. PLD, phospholipase D.

**Figure 9. Proposed iisoA2E formation cascade.** A, the proposed biosynthetic pathway by which iisoA2E, A2E, isoA2E, and A2-DHP-PE form in the retina. All-trans-retinal released from activated rhodopsin after photoisomerization of ground state 11-cis-retinal reacts with phosphatidylethanolamine (PE) in the disk membrane to produce the NRPE Schiff base that undergoes a [1,6]-proton tautomerization to generate PAE. After reaction with a second molecule of all-trans-retinal and 6-aza-electrocyclization, dihydropyridinium A2PE is generated. This intermediate readily undergoes a 1,3-H shift and hydrogen atom elimination to give A2-DHP-PE or can eliminate two hydrogens to form A2PE. Hydrolysis of the A2PE phosphate ester yields A2E. Because the C13C14 double bond of all-trans-retinal is most prone to cis-isomerization, yielding 13-cis-retinal, this chromophore is considered to coexist with all-trans-retinal in vivo at low levels. All-trans-retinal reacts with PE to generate NRPE. After NRPE undergoes a [1,6]-proton tautomerization to PAE, the intermediate reacts with 13-cis-retinal (not all-trans-retinal). Following 6-aza-electrocyclization, dihydropyridinium isoA2PE forms and undergoes facile auto-oxidation to isoA2PE. Estar hydrolysis removes phosphatidic acid from isoA2PE to produce isoA2E. As an alternative, after PAE reacts with 13-cis-retinal to form a 13-cis-iminium salt, the latter undergoes a hydrogen shift, C13C14 double bond cleavage, and 6-aza-electrocyclization to generate an intermediate bearing a three-membered ring that readily opens. Following opening of the three-membered ring at the C14C15 single bond and shift of C13-H to C15, dihydropyridinium iisoA2PE is generated. Facile auto-oxidation of this intermediate with the loss of two hydrogen atoms gives rise to iisoA2E, which is cleaved by hydrolysis of the phospholipid to release iisoA2E. A2E and isoA2E can be interconverted by light. B, proposed mechanism by which isoA2E is converted into iisoA2E by light. isoA2E undergoes C13C14 double bond cleavage and a shift of 6-H to C13 to produce an intermediate with a three-membered ring. Photochemical ring opening and intramolecular hydrogen shift occur in this intermediate, leading to formation of iisoA2E. PLD, phospholipase D.
undergoes a [1,6]-proton tautomerization generating a phosphatidyl anolog of enamine (PAE). Following reaction with a second molecule of all-trans-retinal, an all-trans-iminium salt is suggested to form. Now with the isolation of iisoA2E, a compound that we suggest could form from a reaction of PAE with one molecule of 13-cis-retinal, rather than all-trans-retinal, with proton transfer/elimination, single/double bond cleavage, and minimal electronic reorganization (Fig. 9). The condensation reaction of PAE and 13-cis-retinal generates a 13-cis-iminium salt, which would go through a 6-H to C13 shift, cleavage and minimal electronic reorganization (Fig. 9). The condensation reaction of PAE and 13-cis-retinal generates a 13-cis-iminium salt, which would go through a 6-H to C13 shift, cleavage of the C13C14 double bond, and 6π azelalectrocytization, thereby leading to formation of an intermediate bearing a three-membered ring. After opening of the three-membered ring (42, 43) at the C14C15 single bond and shift of C13-H to C15, dihydropyridinium iisoA2PE forms, characteristic of the attachment of the long side arm to C16. Subsequent auto-oxidation eliminates two hydrogen atoms from this intermediate to give iisoA2PE, which hydrolyzes to release iisoA2E. As an alternative, 13-cis-iminium salt undergoes 6π azelalectrocytization to yield dihydropyridinium iisoA2PE. After auto-oxidation and a two-hydrogen loss, iisoA2PE is generated and subsequently produces iisoA2E by hydrolysis of the iisoA2PE phosphodiester bond.

We have demonstrated that PLD-mediated cleavage of the A2PE series generated A2E, iisoA2E, and iisoA2E, thereby confirming the presence of iisoA2PE and iisoA2PE in the DP-PE/all-trans-retinal reaction mixture. The data also manifest that iisoA2PE may serve as the precursor of iisoA2E, together with iisoA2E as a precursor of iisoA2E in the biosynthetic pathway, and corroborate, at least in part, our proposed biogenic scheme (Fig. 9A). For light-mediated mechanisms by which iisoA2E is converted into iisoA2E (Fig. 9B), we proposed that iisoA2E undergoes a 6-H to C13 shift and cleavage of the C13C14 double bond to generate a tautomer that contains a three-membered ring. As is known, three-membered rings readily open and participate in nucleophilic addition reactions (42, 43); ring opening of the three-membered ring at the C14C15 single bond and shift of C13-H to C15 yield iisoA2E.

Next, we tested the stability of iisoA2E in polar solvents (see supplemental Methods and Results and Fig. S14). By HPLC, we elucidated that iisoA2E was safe in DMSO and MeOH, whereas EtOH and chloroform alter this pigment. Interestingly, iisoA2E altered by either EtOH or chloroform could be recovered by MeOH within 30 s (supplemental Fig. S15). Because biomimetic synthesis of iisoA2E ran in EtOH, we believe that a part of iisoA2E previously formed in the condensation reaction was probably altered. Indeed, the iisoA2E synthetic yield increased by 9.5% after using MeOH to recover altered iisoA2E. This study also provides insight into the proper usage of iisoA2E and facilitates the establishment of a correct process for its measure in the eyes.

We also detected an additional unrecognized RPE lipofuscin component, URP. As a neighbor of iisoA2E in the HPLC chromatograms (Fig. 2), URP appears more abundant than iisoA2E in human RPE and eyecups of C57BL/6 and BALB/cByJ mice. It should be noted that a peak attributable to URP was not detected in all mouse eyes and bovine RPEs. We have corroborated that URP is not a light-induced isomer of iisoA2E based on absorbance spectra and HPLC Rt values. Like other bisretinoid compounds, URP has two absorbance maxima (A\text{max} 342 and 443 nm) in its UV-visible spectrum, whereas the 342 nm absorbance intensity appears very weak. At this time, the structure of URP is not likely to be identified given that in vitro synthesis of this compound is still not established to obtain sufficient samples for NMR studies. In addition, we HPLC-quantified the levels of iisoA2E in each type of eye: C57BL/6 mice (0.52 pmol/eye, average content, four samples, 8 eyecups/sample); BALB/cByJ mice (0.33 pmol/eye, average content, four samples, 8 eyecups/sample); pig (20.72 pmol/eye, average content, two samples, 1 RPE/sample); bovine (35.44 pmol/eye, average content, two samples, 1 RPE/sample); female human (45.25 pmol/eye, age 55, one sample, 1 RPE/sample); and male human (80.82 pmol/eye, age 49, one sample, 1 RPE/sample). With cell-based assays, we have confirmed that excessive accumulation of iisoA2E in RPE cells precipitates cell death (Fig. 6) and causes membrane damage (Fig. 7). However, the levels at which iisoA2E is presented in these experiments are much higher than what is expected in vivo. Based on the obvious discrepancies, iisoA2E may not play an important role in RPE cell damage in vivo, but photo-oxidation of this adduct is likely to cause increased cytotoxic activity (44–46). As a vitamin A aldehyde-derived compound, iisoA2E serves as a fluorescent biomarker of aberrant all-trans-retinal metabolism (47), and its characterization will give a more complete understanding of the biosynthesis of this class of compounds in the retina. Careful identification of RPE lipofuscin components is fundamentally important because this knowledge increases awareness of the total burden placed on RPE cells by the deposition of this material. Improved understanding of the biosynthetic pathways of RPE lipofuscin pigments could open avenues toward additional therapies based on limiting the formation of these adverse compounds.

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