



Review:

Structures and biogenetic analysis of lipofuscin bis-retinoids*

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Abstract: Age-related macular degeneration (AMD) is still an incurable blinding eye disease because of complex pathogenic mechanisms and unusual diseased regions. With the use of chemical biology tools, great progress has been achieved in improving the understanding of AMD pathogenesis. The severity of AMD is, at least in part, linked to the non-degradable lipofuscin bis-retinoids in retinal pigment epithelial (RPE). This material is thought to result from the lifelong accumulation of lysosomal residual bodies containing the end products derived from the daily phagocytosis of rod outer segments by RPE cells. Here, we present previously recognized bis-retinoids with focus on structures and biosynthetic pathways. In addition to a brief discussion on the mutual conversion relationships of bis-retinoids, future perspectives and the medical relevance of such studies on these lipofuscin constituents are also highlighted.

Key words: Age-related macular degeneration (AMD), Lipofuscin, Retinal pigment epithelial (RPE), Bis-retinoids, Structures, Biogenesis, Conversion relationships

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1 Introduction

Time-dependent lipofuscin accumulation in retinal pigment epithelial (RPE) cells has been notoriously deemed as a major risk factor in association with age-related macular degeneration (AMD) (Bressler *et al.*, 1988; Eldred and Katz, 1988; Eldred and Lasky, 1993; Kliffen *et al.*, 1997; Sarks *et al.*, 1999; Delori *et al.*, 2000). AMD, the leading cause of blindness in elderly people, is a degenerative disease of the eye (Bressler *et al.*, 1988; Mata *et al.*, 2000; Telander, 2011), for which there is thus far no remedy. Increasing accumulation of lipofuscin granules in RPE is not a feature of aging RPE alone given that excessive amounts of these fluorophores are also remarkably detected in the RPE of juvenile patients with Stargardt's disease (Eagle *et al.*, 1980; Feeney-Burns

et al., 1984; Rabb *et al.*, 1986; Birnbach *et al.*, 1994; Kennedy *et al.*, 1995) caused by mutations in *Abca4* gene (Delori *et al.*, 1995; Shroyer *et al.*, 1999). RPE lipofuscin is an enzymatically non-degradable heterogeneous mixture of numerous biomolecules (Ng *et al.*, 2008), in which extensive attention has been drawn to the indigestible bis-retinoid constituents that contribute to the etiology of inherited and AMD (Broniec *et al.*, 2005; Sparrow *et al.*, 2010a).

Vitamin A cycling leading to vision occurs in the retina, and is also regarded as the origin of adverse lipofuscin bis-retinoids (Fig. 1) (Borhan *et al.*, 2000; Sparrow *et al.*, 2010d). After capturing a photon, the inherent visual chromophore 11-*cis*-retinal bound to lysine²⁹⁶ of opsin, with a protonated covalent Schiff base bond, isomerizes to all-*trans*-retinal (Rando, 1996; Borhan *et al.*, 2000). A sequence of conformational rearrangements causes the dark-adapted rhodopsin to undergo metarhodopsin II (Meta II) transition (Sakmar, 1997; Gelasco *et al.*, 2000; Burns and Baylor, 2001; Okada *et al.*, 2001; Isin *et al.*, 2006). Given that Meta II comprises all-*trans*-retinal and opsin that bind together

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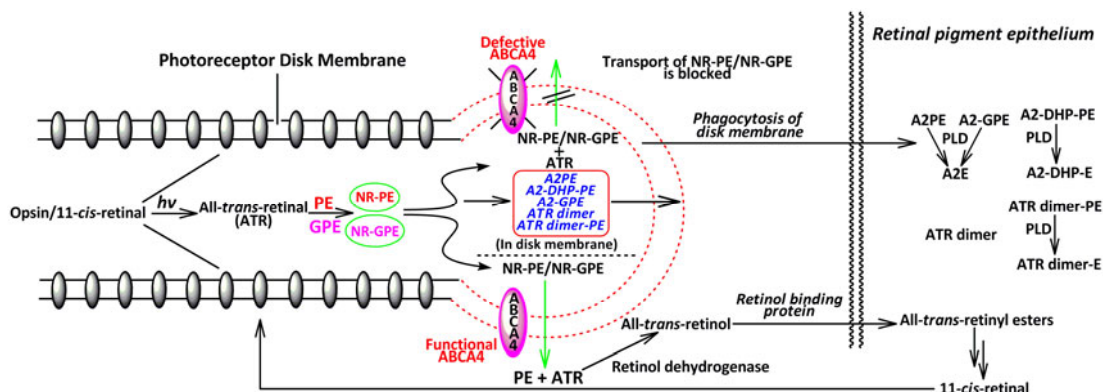


Fig. 2 Correlation of the ABCA4 transporter with lipofuscin bis-retinoid biosynthesis

All-*trans*-retinal (ATR) comes from the isomerization of 11-*cis*-retinal that is released from light-activated rhodopsin in the retina. Incubation of ATR with phosphatidylethanolamine (PE)/glycerophosphoethanolamine (GPE) would give rise to *N*-retinylidene-phosphatidylethanolamine (NR-PE)/*N*-retinylidene-glycerophosphoethanolamine (NR-GPE) that can be delivered to the cytoplasmic side of disk membranes by ABCA4. When the activity of this transporter attenuates or becomes extinct, NR-PE and NR-GPE have no choice but to reside in the rod outer segments, and react with a second molecule of ATR to form various bis-retinoids including *N*-retinylidene-*N*-retinyl-phosphatidylethanolamine (A2PE), *N*-retinylidene-*N*-retinyl-dihydropyridine-phosphatidylethanolamine (A2-DHP-PE), *N*-retinylidene-*N*-retinyl-glycerophosphoethanolamine (A2-GPE), ATR dimer, and ATR dimer-PE via a multi-step cascade. Rod outer segments are routinely discarded on a daily basis, and subsequently are phagocytosed by RPE cells, resulting in the transfer of these di-retinal pigments to RPE where PLD, a lysosomal acid enzyme, can cleave A2PE, A2-GPE, A2-DHP-PE, and ATR dimer-PE to produce *N*-retinylidene-*N*-retinyl-ethanolamine (A2E), *N*-retinylidene-*N*-retinyl-dihydropyridine-ethanolamine (A2-DHP-E), and ATR dimer-E, respectively. However, ATR dimer remains intact due to the inability to be further digested by the lysosomal enzymes

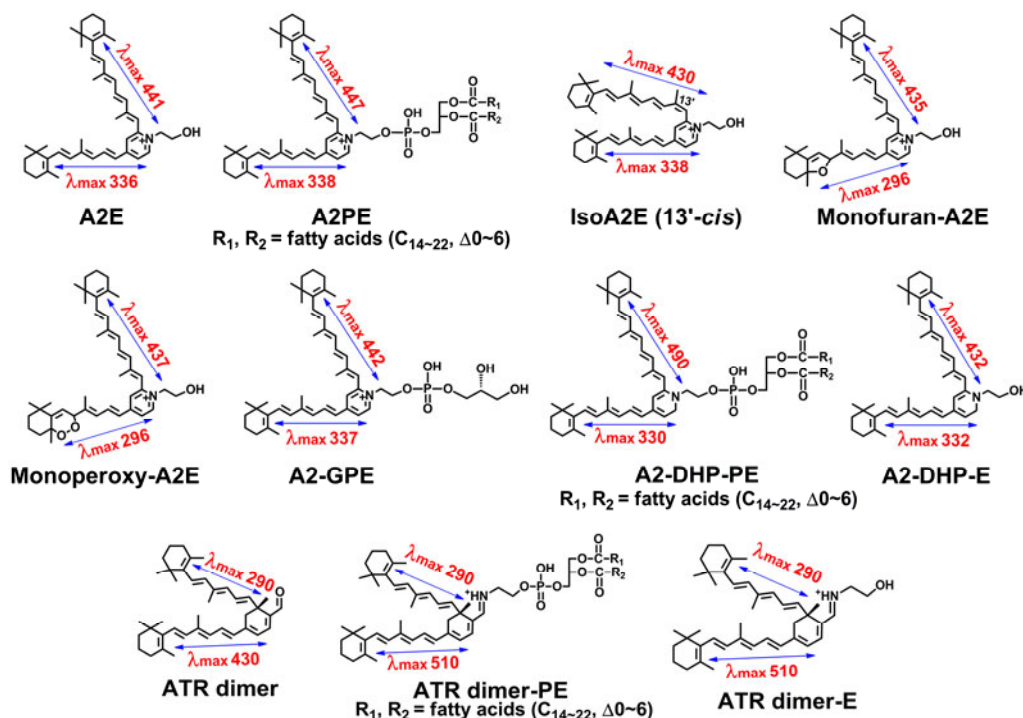


Fig. 3 Eleven bis-retinoids associated with formation of retinal pigment epithelial (RPE) lipofuscin

Structures, UV-visible absorbance (nm), and electronic transition assignments (\leftrightarrow) are shown. All of these fluorescent di-retinal pigments have dual conjugation systems located in two side-arms, conferring light-induced absorbance in both the ultraviolet and visible regions

Up to the present time, at least eleven bis-retinoid pigments (Fig. 3) present in human RPE and mouse eyecups have been characterized structurally and chromatographically. Given significant implications of lipofuscin bis-retinoids in AMD, this review will mainly focus on the advances in both structure and biogenetic analysis of these inadvertent fluorophores.

2 Bis-retinoids with a pyridinium ring

To date, the identified pyridinium bis-retinoids with a positive charge on the nitrogen atom residing in the six-membered heterocyclic ring include A2E, isoA2E (13'-*cis*), A2PE, oxidized derivatives of A2E and A2-GPE. A2E, an unprecedented pyridinium bis-retinoid was the most studied ocular age pigment, and was structurally characterized by Sakai *et al.* (1996). Ren *et al.* (1997) completed its first total synthesis.

One year later, Parish *et al.* (1998) optimized *in vitro* A2E synthesis, which led to the one-step biomimetic preparation of this pigment in 49% yield. Given that A2E has two hydrophobic side-arms derived from all-*trans*-retinal, together with a hydrophilic pyridinium head group, this molecule is conferred with an unusual amphiphilic property (Sparrow *et al.*, 2003a). Evidence from several fronts (Poincelot *et al.*, 1969; Anderson and Maude, 1970; Mata *et al.*, 2000; Sparrow *et al.*, 2010a) denotes that the biosynthetic route of A2E (Fig. 4) begins in the photoreceptor outer segment membrane as a condensation reaction between PE and a single molecule of all-*trans*-retinal that generates a Schiff base conjugate NR-PE. NR-PE goes through a [1,6]-proton tautomerization to yield an *N*-retinylidene-phosphatidylethanolamine. It is postulated that subsequent condensation of the latter with a second molecule of all-*trans*-retinal and 6 π -*aza*-electrocyclization will produce an imperative

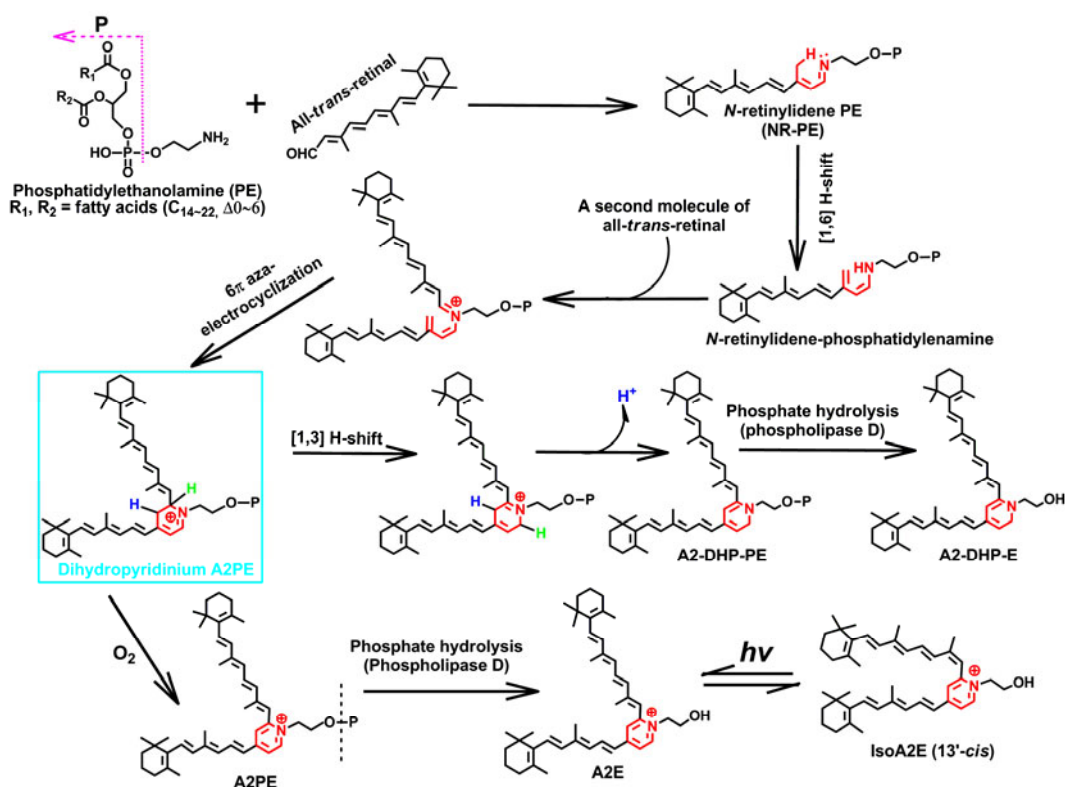


Fig. 4 Biosynthetic pathways of *N*-retinylidene-*N*-retinyl-ethanolamine (A2E) and *N*-retinylidene-*N*-retinyl-dihydropyridine-phosphatidylethanolamine (A2-DHP-PE)

These two bis-retinoids form from a proposed uniform intermediate, dihydropyridinium *N*-retinylidene-*N*-retinyl-phosphatidylethanolamine (A2PE). The latter is enclosed by a rectangle and is not detectable in the eye and biomimetic chemical reactions, probably resulting from the facile auto-oxidation of a unique dihydropyridinium ring, from which one or double particular hydrogen is eliminated with the electronic rearrangements. Hydrogens in blue and green represent the active atoms involved in the shifting and eliminating processes in the biosynthesis of these two heterocyclic fluorophores (Note: for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

phosphatidyl-dihydropyridinium bis-retinoid intermediate (dihydropyridinium A2PE) (Fig. 4) (Kim *et al.*, 2007; Sparrow *et al.*, 2012). Automatic oxidation of dihydropyridinium A2PE with loss of two hydrogens ($-2H$) yields A2PE, the immediate precursor of A2E and isoA2E (13'-*cis*). It is interesting to observe that A2E and isoA2E (13'-*cis*) would reach photo-equilibrium at a ratio of 4:1 when exposed to room light (Parish *et al.*, 1998). The (430 \pm 20) nm blue light irradiation of A2E-laden ARPE-19 cells in a dark room generated two detectable types of oxidized A2E: monofuran-A2E and monoperoxy-A2E, and they also were found in aged human RPE and in eyecups of *Abca4*^{-/-} mice, an animal model of Stargardt's disease (Jang *et al.*, 2005).

Most recently, Yamamoto *et al.* (2011) mentioned that Sparrow and his colleagues illustrated a fresh bis-retinoid of retina, A2-GPE, to be more abundant than A2E in the eyecups of *Abca4*^{-/-} mice and to form from incubation of two molecules of all-*trans*-retinal and GPE. Due to the similarity in structure between PE and GPE, the biosynthesis of A2-GPE (Fig. 5), slightly different from that of A2E, starts with a condensation reaction between GPE and all-*trans*-retinal, which

creates a Schiff base conjugate NR-GPE. Subsequently, a sequence of chemical processes including [1,6]-proton tautomerization, condensation reaction with a second molecule of all-*trans*-retinal and 6 π -*aza*-electrocyclization, yields dihydropyridinium A2-GPE, the proposed intermediate in the biosynthetic pathway. With loss of two hydrogens ($-2H$) arising from the automatic oxidation, dihydropyridinium A2-GPE is converted into A2-GPE containing a pyridinium ring. An in vitro enzyme assay manifested that phospholipase D (PLD) activity can cleave A2-GPE to release A2E, thereby reflecting that A2-GPE, like A2PE, may also serve as a precursor of A2E (Sparrow *et al.*, 2008). Like NR-PE acting as a key intermediate to form A2PE, NR-GPE also plays an important role in the biosynthetic pathway of A2-GPE. To corroborate A2-GPE biogenesis, it will be highly urgent to detect NR-GPE in mouse eyecups and human RPE. It is also worth noting that dihydropyridinium A2PE enclosed with a rounded rectangle (Fig. 4) and dihydropyridinium A2-GPE (Fig. 5) were not detectable in eyes and biomimetic chemical reactions on account of the transient auto-oxidation behavior.

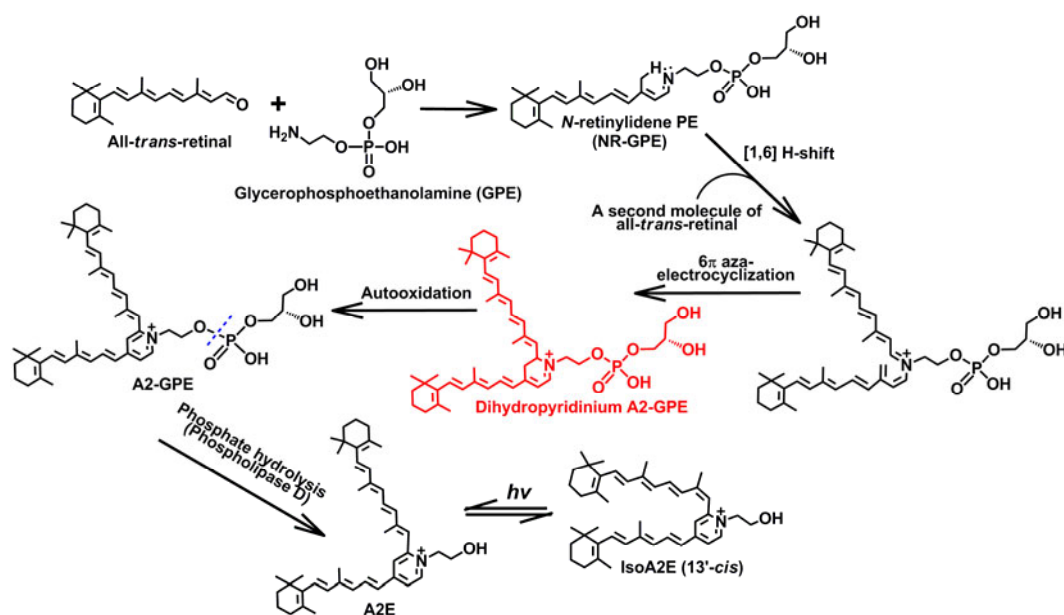


Fig. 5 Biosynthetic pathway of *N*-retinylidene-*N*-retinyl-glycerophosphoethanolamine (A2-GPE)

N-retinylidene-glycerophosphoethanolamine (NR-GPE) and dihydropyridinium A2-GPE, like *N*-retinylidene-phosphatidylethanolamine (NR-PE) and dihydropyridinium *N*-retinylidene-*N*-retinyl-phosphatidylethanolamine (A2PE) in the *N*-retinylidene-*N*-retinyl-ethanolamine (A2E) biosynthetic route, ought to play important roles in the biogenesis of A2-GPE. Due to the susceptibility of the dihydropyridinium ring to auto-oxidation, it is concluded that dihydropyridinium A2-GPE, like dihydropyridinium A2PE, is not found in the eye and biomimetic chemical reactions, from which NR-GPE is, however, possible to be trapped by high-performance liquid chromatography (HPLC)

3 Bis-retinoids with a dihydropyridine ring

In addition to pyridinium bis-retinoids, Wu *et al.* (2009) identified two novel but correlated di-retinal pigments (A2-DHP-PE and A2-DHP-E) that were distinguished by containing a dihydropyridine ring rather than a pyridinium ring. As shown in Fig. 4, A2-DHP-PE biogenesis is closely associated with that of A2E. Following the generation of an estimated intermediate (dihydropyridinium A2PE), there is a bifurcation in the pathway. As mentioned above, one way gives rise to A2E and its isomers (Liu *et al.*, 2000; Mata *et al.*, 2000; Sparrow *et al.*, 2010a). Conversely, another path to form A2-DHP-PE is involved with [1,3]-proton tautomerization and elimination of a hydrogen (Wu *et al.*, 2009; Sparrow *et al.*, 2010a). PLD-mediated hydrolysis of A2-DHP-PE yields A2-DHP-E, revealing that A2-DHP-PE may act as the precursor of A2-DHP-E (Wu *et al.*, 2009).

4 Bis-retinoids with a cyclohexadiene ring

In comparison with the nitrogen-containing heterocyclic bis-retinoids, all-*trans*-retinal dimer series, including ATR dimer, ATR dimer-PE, and ATR dimer-E (Fishkin *et al.*, 2005), are absolutely

distinctive, given that their chemical structures contain a simpler cyclohexadiene ring in which no nitrogen atom resides. Cyclohexa-1,3-diene-carbaldehydes, incredibly similar to ATR dimer, could be released from a base-catalyzed condensation reaction between α,β -unsaturated aldehydes and a β -methyl substituent. Thus, the proposed pathway for ATR dimer biogenesis (Fig. 6) is rooted in the reported mechanism relevant to this type of transformation (Thomas and Guntzdubini, 1976; Duhamel *et al.*, 1991). Like A2PE and A2-DHP-PE, ATR dimer is supposed to form in the photoreceptor cell outer segments (Fig. 2). All bis-retinoids appear to form from the same tautomer (*N*-retinylidene-phosphatidylethanolamine), probably arising from a [1,6] H-shift of NR-PE/NR-GPE (Liu *et al.*, 2000; Mata *et al.*, 2000; Wu *et al.*, 2009; Sparrow *et al.*, 2010a). Via a Michael-type addition reaction (Michael, 1887; Mather *et al.*, 2006), the C₂₀ of NR-PE/NR-GPE would tie with C₁₃ of a second molecule of all-*trans*-retinal. After a Mannich reaction to close the ring (Mannich and Krösche, 1912), the elimination of amine group in PE/GPE yields ATR dimer, along with an electronic rearrangement in the six-membered ring (Fishkin *et al.*, 2005). Apart from the nucleophilicity of C₂₀ residing in *N*-retinylidene-phosphatidylethanolamine (Fig. 6), this carbon also possesses the ability to participate in the [1,4] conjugate

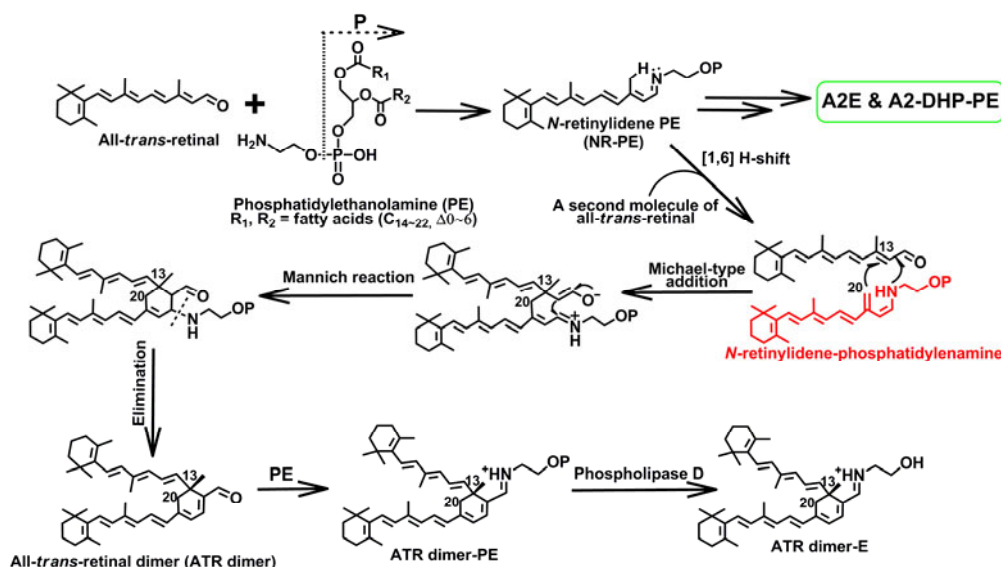


Fig. 6 Biosynthetic pathways of all-*trans*-retinal (ATR) dimer, ATR dimer-E, and ATR dimer-PE

It is thought that ATR dimer series, *N*-retinylidene-*N*-retinyl-ethanolamine (A2E) and *N*-retinylidene-*N*-retinyl-dihydropyridine-phosphatidylethanolamine (A2-DHP-PE), form from the same intermediate *N*-retinylidene-phosphatidylethanolamine (see structure in red). Also, it is interesting to know that ATR dimer-E (λ_{\max} 290, 510 nm) and saturated ATR dimer-PE (λ_{\max} 290, 510 nm) exhibited a visible absorption maximum of about 80 nm red shifted from that of ATR dimer (λ_{\max} 290, 430 nm) because of a protonated Schiff base linkage formed in the position of aldehyde moiety (Note: for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

addition. These analyses connote that ATR dimer ought to form in the rod outer segments rather than in RPE. Incubation of ATR dimer with PE produces ATR dimer-PE with creation of a protonated Schiff base linkage (Fishkin *et al.*, 2005). ATR dimer-E is released from the phosphate hydrolysis of ATR dimer-PE by PLD (Fishkin *et al.*, 2005). In addition to A2-DHP-PE, examination of the 7-d reaction mixture between all-*trans*-retinal and 1,2-diacyl-*sn*-glycero-3-phosphatidylethanolamine from egg yolk (egg-PE) by high-performance liquid chromatography (HPLC) also exhibited the formations of ATR dimer and ATR dimer-PE (Wu *et al.*, 2009). Evidence gleaned from HPLC chromatograms of mouse eyecup extracts showed that ATR dimer-PE is more readily detectable than ATR dimer in the *Abca4*^{-/-} mice. These findings, together with evidence that an incubation of ATR dimer with PE produces ATR dimer-PE, corroborate that ATR dimer is obliged to form firstly and functions as a precursor of ATR dimer-PE (Figs. 1 and 6).

5 Structural features and properties

A common feature in structures of these bis-retinoids is two side-arms consisting of alternating single and double bonds that begin from an aromatic head group and extend into terminal β -ionone rings. The systems of conjugated double bonds in these bischromophores confer their own absorbance maxima in the visible region. It is noted that all of the bis-retinoids listed in Fig. 3 possess fluorescent properties and are the source of fundus autofluorescence (Sparrow *et al.*, 2010b; 2010c). Moreover, it seems that these bis-retinoids form by the random condensation reactions of two molecules of all-*trans*-retinal, and are highly susceptible to photooxidation (Ben-Shabat *et al.*, 2002; Sparrow *et al.*, 2002; Dillon *et al.*, 2004; Fishkin *et al.*, 2005; Jang *et al.*, 2005; Wang *et al.*, 2006; Kim *et al.*, 2008; Wu *et al.*, 2010). RPE cells, different from other types of cells, are constantly exposed to sunlight strongest at 400–700 nm. Given that the human eye is taking full advantage of the solar energy distribution, we envision that light-mediated actions on the malignant bis-retinoids accumulated in RPE with age, will of necessity influence RPE cells to some extent. Indeed, events initiated by the blue-light illumination of some

bis-retinoids allow for the release of epoxides and cleavage products that have been verified to damage RPE cells (Sparrow *et al.*, 2000; 2002; 2003b; Sparrow and Cai, 2001).

6 Relations between ABCA4 transporter and bis-retinoid formation

ABCA4 (formerly known as ATP-binding cassette transporter, retina-specific (ABCR)) is a member of the ABCA subfamily of adenosine triphosphate (ATP) binding cassette (ABC) transporters expressed in vertebrate rod and cone cells, and is localized to the outer segment disk edges. Allikmets *et al.* (1997) first cloned and characterized *Abca4* gene. Mutations in this gene have been verified to account for all cases of early- and late-onset autosomal recessive Stargardt macular degeneration and the major part of autosomal recessive cone-rod dystrophies (Gerber *et al.*, 1998; Maugeri *et al.*, 2000; Briggs *et al.*, 2001; Ducroq *et al.*, 2002) as well as some exceptional cases of retinitis pigmentosa (Martinez-Mir *et al.*, 1998; Rozet *et al.*, 1999; Fukui *et al.*, 2002). These hereditarily degenerative diseases would cause severe vision loss in affected individuals with age and reflect a continuum of disease phenotype associated with severity of gene mutations. As a critical intermediate to form bis-retinoids, NR-PE is the putative ligand translocated from the lumen to the cytoplasmic side of the disk membrane by the ABCA4 acting as a flippase (Fig. 2) (Sun *et al.*, 1999; Weng *et al.*, 1999). According to a recent finding of A2-GPE by the Sparrow group (Yamamoto *et al.*, 2011), we here propose its biosynthetic pathway (Fig. 5), in which a newly estimated intermediate named NR-GPE is considered to form. Due to the abundance of A2-GPE in the eyecups of ABCA4 null mutant mice, ABCA4 is also considered to be the carrier of NR-GPE. Following the hydrolysis of NR-PE and NR-GPE, all-*trans*-retinal is released and reduced to all-*trans*-retinol. The retinol binding proteins carry vitamin A to RPE where it is isomerized and re-oxidized to 11-*cis*-retinal. This chromophore is subsequently reincorporated into opsin, yielding ground state rhodopsin (Fig. 2). When ABCA4 activity is reduced or absent, NR-PE and NR-GPE abundantly accumulate in the disk membrane, and trigger further reaction with an additional molecule of

all-*trans*-retinal to generate A2PE, A2-DHP-PE, A2-GPE, ATR dimer, and ATR dimer-PE (Fig. 2). Daily phagocytosis of the shed outer segment membrane by RPE cells would take these bis-retinoid pigments to RPE lysosomal bodies where the PLD, an acidic hydrolase in the lysosomes of RPE cells, serves as a blade to cleave to A2PE, A2-GPE, A2-DHP-PE and ATR dimer-PE to give A2E, A2-DHP-E, and ATR dimer-E, respectively (Fig. 2).

7 Medical relevance and future perspective

With regard to treatment for AMD, some attempts at mediating the retinoid cycling rate have been made by utilizing the exogenous small-molecule inhibitors of 11cRDH (Radu *et al.*, 2003), RPE65 (Maiti *et al.*, 2006; Golczak *et al.*, 2008), retinol binding protein and transthyretin (Radu *et al.*, 2005). Indeed, these compounds slow lipofuscin accumulation in RPE cells of the eye. Thus, it will be a promising strategy to screen a large body of low-molecular-weight antagonists limiting the visual cycle with an eventual aim of developing clinical medicines in favor of alleviating AMD. In contrast, no remedies can reverse malicious accumulation of lipofuscin bis-retinoids refractory to lysosomal enzyme degradation, once it has already occurred. In view of this fact, it would be beneficial if there are some exogenous enzymes that could be delivered to RPE cells and directly degrade these aging pigments. A promising finding by Wu *et al.* (2011) attested to the ability of horseradish peroxidase (HRP) to degrade A2E loaded in RPE cells.

To contribute towards a better understanding of biosynthetic pathways of toxic bis-retinoids, a mutual conversion relationship among the bis-retinoids is expected to be clarified. As illustrated in Fig. 7, a future objective will be to establish whether either A2-DHP-PE or A2-DHP-E can be converted into A2E and to investigate whether A2-DHP-PE can undergo further transformation to form A2PE under certain conditions. On the other hand, continuing research and advances in this field should be directed towards characterizing those unrecognized compositions of RPE lipofuscin, understanding the properties of each lipofuscin constituent, and clarifying in vivo mechanisms by which they form and the adverse

effects of these pigments on retina. Characterization of bis-retinoid constituents in RPE lipofuscin is essential for providing insights into the burden placed on RPE cells by the life-long deposition of these vitamin A aldehyde-derived compounds. An understanding of bis-retinoid biogenesis is fundamental to scientific efforts aimed at blocking the formation of these aggressive pigments.

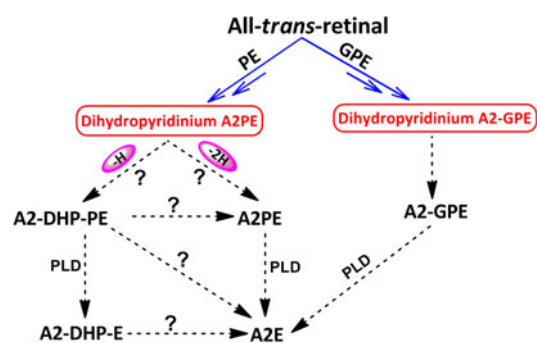


Fig. 7 Diagram of mutual conversion relationships among some bis-retinoids

The symbols (?) represent the potential conversion relationships amongst dihydropyridinium *N*-retinylidene-*N*-retinyl-phosphatidylethanolamine (A2PE), *N*-retinylidene-*N*-retinyl-dihydropyridine-phosphatidylethanolamine (A2-DHP-PE), A2PE, *N*-retinylidene-*N*-retinyl-dihydropyridine-ethanolamine (A2-DHP-E), and *N*-retinylidene-*N*-retinyl-ethanolamine (A2E), for which more evidence is needed

Compliance with ethics guidelines

Ya-lin WU, Jie LI, and Ke YAO declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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