Part I
Methodology
1.1 Introduction

It is well known that the major source of fundus autofluorescence (FAF) is the lipofuscin of retinal pigment epithelial (RPE) cells. Lipofuscin is understood to be material in the lysosomal compartment of nondividing cells that cannot be degraded, and thus it accumulates [16, 74, 89]. For many cell types, lipofuscin originates internally (autophagy), but for the RPE, lipofuscin derives primarily from phagocytosed photoreceptor outer segments. These fluorophores most likely accumulate in RPE cells because the structures of the fluorophores are unusual and not amenable to degradation, rather than because the lysosomal enzymes in these cells are defective. Emerging evidence indicates that the lipofuscin of RPE cells is unique, since much of this material forms as a consequence of the light-capturing function of the retina. An origin from retinoids that leave the visual cycle is consistent with the finding that the accumulation of RPE lipofuscin is most marked in central retina, the area having the greatest concentration of visual pigment. The extensive system of conjugated double bonds within these retinoid-derived fluorophores also explains the long wavelength fluorescence emission of RPE lipofuscin. The excessive accumulation of RPE lipofuscin in autosomal-recessive Stargardt macular degeneration is considered to be the cause of RPE atrophy. This material is also implicated in disease processes underlying dominant Stargardt-like macular degeneration, Best’s vitelliform macular dystrophy, and age-related macular degeneration.

1.2 The Source of RPE Lipofuscin

Evidence that the precursors of RPE lipofuscin originate in photoreceptor outer segments came from work in the Royal College of Surgeons rat showing that in this strain, in which RPE cells fail to phagocytose shed outer segment membrane, RPE lipofuscin is substantially diminished [36]. Lipofuscin was also reduced concomitant with light-induced loss of photoreceptor cells [38]. As with other cell types, the lipo-
fuscin of RPE gathers within membrane-bound organelles of the lysosomal compartment of the cells; because of their ultrastructural appearance, these organelles are referred to as lipofuscin granules [11, 15, 27, 30]. Early theories as to the genesis of RPE lipofuscin focused on the adducts generated following the peroxidation of lipid, particularly those formed by reactions between aldehyde products and biological amines [13]. Products of lipid oxidation have been detected in lipofuscin granules [62], but whether they contribute to the golden-yellow emission of RPE lipofuscin has been a matter of discussion [14, 23, 24].

Current understanding of the molecular composition of RPE lipofuscin originated with experiments documenting that the deposition of lipofuscin fluorophores is dependent on dietary vitamin A [37]. More recently it has been shown that when the 11-cis-retinal and all-trans-retinal chromophores of visual pigment are absent, as in Rpe65−/− mice, RPE lipofuscin, measured as fluorescence intensity, is severely reduced [39]. These findings are consistent with the observation that in patients with early-onset retinal dystrophy associated with mutations in RPE65, RPE lipofuscin is similarly lacking [45].

1.3 Characteristics of Known RPE Lipofuscin Pigments

This evidence—that the RPE lipofuscin that accumulates with age and in some retinal disorders forms largely as a byproduct of light-related vitamin A cycling—is consistent with the finding that a prominent constituent is a di-retinal conjugate A2E (C_{42}H_{58}NO, molecular weight 592), named because it could be synthesized from vitamin A aldehyde (all-trans-retinal) and ethanolamine when combined in a 2:1 ratio [26, 58] (Figs. 1.1 and 1.2). The polar head group of A2E is a pyridine ring carrying a positive charge conferred by a quaternary amine nitrogen; two side arms extend from the ring, a long arm and a short arm. Each arm is derived from a molecule of all-trans-retinal [58]. The structure of A2E is unprecedented.

The polyene structure of the long arm of A2E (Fig. 1.1), including the double bonds within the pyridine and ionone rings, provides the extended conjugation system that allows A2E to absorb at wavelengths in the visible range of the spectrum. Since the absorbance spectrum of A2E has two peaks, it is also clear that the two arms of A2E do not constitute a single continuous conjugation system. Instead, A2E has an absorbance maximum in the visible spectrum of ~440 nm that can be assigned to the long arm and a shorter wavelength absorbance at ~340 nm that is generated within the short arm.

The emission spectrum of in vivo FAF exhibits strong similarities with that of cell-associated A2E and with the emission spectra of native lipofuscin present in RPE isolated from human eyes (F. Delori and J.R. Sparrow, unpublished observations) and isolated lipofuscin granules [11]. All have emission maxima at 590–620 nm and exhibit a characteristic red shift with increasing excitation wavelengths (Fig. 1.3).
Conversely, the in vivo excitation spectra are broader and peak at longer wavelengths (470–500) than that of A2E (448 nm) and native lipofuscin (460–475 nm).

The biosynthesis of A2E begins in photoreceptor outer segments with condensation reactions between all-trans-retinal and phosphatidylethanolamine (Fig. 1.2). All-trans-retinal that participates in A2E biosynthesis is generated by photoisomerization of 11-cis retinal. N-retinylidene-PE (NRPE), the product of the Schiff-base reaction between a single all-trans-retinal and phosphatidylethanolamine [42, 77, 84], is likely the substrate for ABCA4 (ABCR), the photoreceptor-specific ATP-binding cassette transporter [4, 50, 51, 53, 76–79] that is the protein product of the gene responsible for recessive Stargardt disease, a majority of cases of autosomal recessive cone-rod dystrophy, and a form of autosomal recessive retinitis pigmentosa (RP19) [3, 63]. A2E formation continues with the reaction of NRPE and a second all-trans-retinal and then proceeds through a multistep pathway that includes the generation of a phosphatidyl-dihydropyridinium (dihydro-A2PE) compound. The latter intermediate is unstable and undergoes oxidative aromatization [6, 42, 54] to form A2PE, the phosphatidyl-pyridinium bisretinoid that is the immediate precursor to A2E [6, 42, 54]. Because the intermediates that form before dihydro-A2PE are likely capable of reverse-synthesis, auto-oxidation of dihydro-A2PE may be the last step at which it is possible to intervene in the synthesis of A2E [71]. A2PE has a stable aromatic ring and is the fluorescent pigment detected in photoreceptor outer segments [6, 42]. Since the shedding and phagocytosis of outer segment membrane leads to the complete replacement of the photoreceptor outer segment approximately every 10 days [90], A2PE is not continuously amassed by the photoreceptor cell. In Royal College of Surgeons rats, a strain in which RPE cells fail to phagocytose shed outer segment membrane, A2PE is responsible, at least in part, for a golden-yellow autofluorescence in outer-segment degenerating debris [25, 36, 42]. A2PE may also account in part for the lipofuscin-like fluorescence detectable in photoreceptor cells in recessive Stargardt disease and in some forms of retinitis pigmentosa [10, 12, 80]. Although cleavage of A2PE to generate A2E has been suggested to occur by acid hydrolysis within RPE lysosomes [6], it is just as likely that the generation of A2E from A2PE is enzyme mediated, and phospholipase D has been implicated in this process [42].

Another constituent of RPE lipofuscin is the fluorophore isoA2E (Figs. 1.1 and 1.2) that forms by photoisomerization of A2E [54]. While the double bonds along the side arms of A2E are all in the trans (E) position, the double bond at the C13–14 position of isoA2E assumes the cis (Z) configuration [54]. Other less abundant cis-isomers—Z-olefins at the C9/9’–10/10’ and C11/11’–12/12’ positions—are also detectable as additional components of the lipofuscin isolated from aging human RPE [6]. For isoA2E and the other photoisomers, absorbance spectra are slightly blue-shifted relative to A2E (Fig. 1.3). A2E and its isomers have been detected in isolated human RPE [54], wherein their levels have also been shown to increase with age (Jang and Sparrow, unpublished observation). These pigments have also been demonstrated within eyecups harvested from mice, with levels being increased several fold in the Abcr null mutant mouse, a model of recessive Stargardt macular degeneration [40, 47–49, 84].
At least two compounds in RPE lipofuscin have ~510 nm absorbance and form by pathways distinct from that of A2E [28, 29]. Studies indicate that the pigment all-trans-retinal dimer, phosphatidylethanolamine (atRAL dimer-PE) (Fig. 1.1), is generated after two molecules of all-trans-retinal condense to form an aldehyde-bearing dimer (atRAL dimer) (Fig. 1.1). By means of its aldehyde group, atRAL dimer then forms a conjugate with phosphatidylethanolamine, thus forming atRAL dimer-PE [28, 29] (Fig. 1.1). A second ~510 nm absorbing species, all-trans-retinal dimer-E (atRAL dimer-E) (Fig. 1.1), can be subsequently generated by phosphate cleavage of atRAL dimer-PE. The pigments atRAL dimer-PE and atRAL dimer-E are composed of two polyene arms—seven double-bond conjugations on the long arm and four on the short—extending from a cyclohexadiene ring that is linked by Schiff base to phosphatidylethanolamine or ethanolamine, respectively. These pigments exhibit an absorbance maximum in the visible spectrum that is red-shifted relative to A2E (A2E/isoA2E, λ<sub>max</sub> ~340, 440 nm; atRAL dimer-PE, λ<sub>max</sub> ~290, 510 nm). The relatively long wavelength absorbance of atRAL dimer-PE and atRAL dimer-E is attributable to protonation of the Schiff base linkage in these compounds. The fluorescence emission of these pigments peaks at approximately 600 nm and is relatively weak in intensity (Fig. 1.3). It is also significant that when deprotonated, these pigments undergo hydrolysis to revert to atRAL dimer. Unprotonated/unconjugated atRAL dimer is detected in mice and human eyes along with conjugated/protonated atRAL dimer-PE and atRAL dimer-E, indicating that in the acidic environment in which these pigments are housed (lysosomal compartment), an equilibrium exists between the deprotonated/unconjugated and protonated/conjugated states. The absorbance

![Fig. 1.1 Structures of retinal pigment epithelial (RPE) lipofuscin pigments A2E, isoA2E, all-trans-retinal dimer-ethanolamine (atRALdi-E), all-trans-retinal dimer-phosphatidylethanolamine (atRALdi-PE), and all-trans-retinal dimer (atRALdi) with corresponding ultraviolet-visible absorbance spectra](image-url)
spectrum of unconjugated atRAL dimer exhibits maxima at ~290 and 432 nm, and its fluorescence emission profile (emission maximum 580 nm with 430 nm excitation) is slightly different from that of A2E (Fig. 1.3). The pigments atRAL dimer-PE and atRAL dimer-E are present at elevated levels in the lipofuscin-filled RPE of Abcr null mutant mice (S. Kim and J.R. Sparrow, unpublished).

Additional components of RPE lipofuscin are generated by photooxidation. In the case of A2E, photooxidation was originally suspected because of the fluorescence bleaching that accompanies irradiation. Subsequent analysis by mass spectrometry revealed that following blue light irradiation of A2E, the profile not only consisted of the M+ 592 peak attributable to A2E but also included a series of peaks, the sizes of which increased by increments of mass 16, indicating the addition of oxygens at carbon–carbon double bonds [5, 70].

The mixture of oxygen-containing moieties within photooxidized A2E includes cyclic peroxides (peroxy-A2E), furanoid oxides (furano-A2E), and probably epoxides [5, 21, 35]. We have also shown that oxidation occurs more readily on the short arm of A2E. Intracellular A2E has been shown to undergo photooxidation upon blue-

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**Fig. 1.2** Biosynthesis pathway of A2E and the photoisomer isoA2E from all-trans-retinal and phosphatidylethanolamine. The immediate precursor A2PE forms in outer segments; phosphate hydrolysis of A2PE to generate A2E and isoA2E probably occurs in retinal pigment epithelial lysosomes via an enzyme-mediated mechanism.
light exposure [70], and we have detected monofuran-A2E and monoperoxy-A2E in RPE from human eyes and in eyecups from mice with null mutations in Abcr−/− [35], the gene responsible for recessive Stargardt disease. Since the cytotoxicity of oxygen-containing groups such as endoperoxides is well known [46, 81], these moieties may account, at least in part, for cellular damage ensuing from A2E accumulation [73]. The addition of oxygens at olefins of A2E is associated with blue shifts in absorbance. For instance, when one furan or peroxo moiety is positioned on the long arm of A2E, the long wavelength absorbance changes to ~402 nm. Although monoperoxy-A2E exhibits a fluorescence emission that is more intense than A2E, with further oxidation, autofluorescence diminishes.

### 1.4 Adverse Effects of RPE Lipofuscin

In autosomal-recessive Stargardt disease caused by ABCA4/ABCR gene mutations [2, 64, 65, 88], RPE lipofuscin has a composition similar to age-associated lipofuscin; the accumulation of this material is also accelerated [19, 22, 43, 44, 82] and is considered the cause of RPE atrophy [56]. Since RPE lipofuscin is amassed with age and is of highest concentration underlying central retina [20], it is also implicated in atrophic age-related macular degeneration. Additionally, in-vivo monitoring of RPE lipofuscin as FAF in patients with age-related macular degeneration has revealed areas of intense FAF that correspond to sites of reduced scotopic sensitivity [59, 60] and are

![Emission spectra of A2E, isoA2E, all-trans-retinal dimer-ethanolamine (atRALdi-E), all-trans-retinal dimer-phosphatidylethanolamine (atRALdi-PE), and all-trans-retinal dimer (atRALdi) with excitation at 430 or 500 nm, as indicated](image)
prone to atrophy [31, 33]. Because mutations in ABCA4/ABCR lead to increased RPE lipofuscin, it is also of interest that heterozygous mutations in the gene have been associated with increased susceptibility to age-related macular degeneration [2, 9]. Poorly understood is the relationship between RPE lipofuscin and vitelliform macular dystrophies (e.g., Best disease, adult-onset vitelliform dystrophy) caused by mutations in VMD2, the gene encoding bestrophin-1 (Best1).

Work in in-vitro models suggests mechanisms by which lipofuscin constituents may damage the RPE cell [18, 61, 67, 68, 75]. Thus, as an amphiphilic molecule, not only can A2E mediate detergent-like effects on cell membranes [18, 67, 72, 75], but its accumulation can lead to the alkalinization of lysosomes [32], possibly by interfering with the ATPase-dependent proton pump located in the lysosomal membrane [7]. A2E appears not to directly inhibit the activities of lysosomal enzymes [8]. A2E has also been shown to confer a susceptibility to photo-induced apoptosis [61, 68, 69], with sensitivity to blue light being directly dependent on the A2E content of the cells; green light (540 nm) is considerably less damaging. The photochemical events triggering apoptosis when A2E-laden RPE are exposed to blue light probably involve the generation of reactive forms of oxygen and photooxidative products of A2E (discussed above). The amount of A2E that undergoes photooxidation and cleavage in a lifetime may be significant: we observed that the amount of formed A2-PE, the immediate precursor of A2E, is many times greater than the amounts of A2E that accumulate in RPE cells [41]. One explanation for this finding is that a portion of the A2E that forms is normally lost. Because it is known that levels of A2E in RPE do not diminish under dark conditions [48], it is likely that the light-dependent conditions involve photooxidative processes. Decreased FAF is observed in areas of photoreceptor cell degeneration [83]; perhaps this observation can be accounted for by halted deposition (due to the absence of photoreceptor cells) together with depletion due to photooxidation.

1.5 Modulators of RPE Lipofuscin Formation

All-trans-retinal that avoids reduction to all-trans-retinol by all-trans-retinol dehydrogenase is available to undergo the random inadvertent reactions that lead to formation of the all-trans-retinal-derived fluorophores of RPE lipofuscin. Correspondingly, conditions that increase the availability of all-trans-retinal enhance the opportunity for these fluorophores to form. Not surprisingly, therefore, since the generation of all-trans-retinal in photoreceptor outer segments is light-dependent, light is also a determinant of the rate of A2E formation. Thus, in-vivo experiments have shown that the A2E precursor A2PE in photoreceptor outer segments is augmented by exposing rats to bright light [6]. Moreover, dark rearing of ABCR+/− mice inhibits the deposition of A2E [48]. Since A2E levels are not diminished if mice are raised in cyclic light and then transferred to darkness, it is also clear that once A2E is formed, it is not eliminated from the RPE [48]. Another well-known factor that modulates
A2E formation is the activity of ABCA4 (ABCR), the photoreceptor-specific ATP-binding cassette transporter [50, 53, 76, 79] that is thought to aid in the movement of all-trans-retinal to the cytosolic side of the disc membrane [1, 34, 76, 79, 84], where it is accessible to retinol dehydrogenase, the enzyme responsible for its reduction to all-trans-retinol [57]. As a consequence of the loss of ABCR protein activity in Abercr-/- mice, the levels of A2E in RPE cells are several fold greater than those in normal mice [40, 47, 84]. In Abercr+/- mice, accumulation of A2E is approximately 40% of that in the null mutant mouse [49].

Because the source of all-trans-retinal for A2E formation is the photoisomerization of 11-cis-retinal, another determinant of A2E accumulation is the kinetics of 11-cis-retinal regeneration. Evidence for this factor has come from studies of an amino acid variant in murine Rpe65, the visual cycle protein that may have a rate-determining role in the visual cycle [87]. Specifically, in albino and pigmented mice in which the amino acid residue at position 450 of RPE 65 is methionine (C57BL/6J-c2J; Abercr/- Met/Met; Abercr+ Met/Met) instead of leucine (BALB/cByJ; Abercr/- Leu/Leu; Abercr+ Leu/Leu), recovery of the electroretinographic response following a photobleach and rhodopsin regeneration are retarded [17, 52, 85, 86]; the content of A2E in the RPE is diminished by a similar magnitude [40].

Therapeutic approaches aimed at retarding the visual cycle also serve to reduce A2E accumulation. For instance, the acne medication isotretinoin (13-cis-retinoic acid, Accutane; Roche Laboratories, Nutley, NJ), which was shown to reduce visual sensitivity under darkened conditions by retarding 11-cis-retinal regeneration [66], reduces A2E deposition in the RPE of ABCR-/- mice [55]. Nonetheless, 13-cis-retinoic acid has severe side effects, including teratogenicity [71] and is thus not appropriate for long-term therapy. Nonretinoid isoprenoid compounds that compete with retinyl esters for binding to RPE65, thereby interfering with visual cycle kinetics [47], also serve to mediate substantial reductions in A2E accumulation when administered chronically to Abercr-/- mice. Indeed, RPE65 appears to be an excellent therapeutic target, with A2E levels in eyecups of Abercr-/- mice treated with the RPE65 antagonists being as much as 85% lower than in vehicle-treated mice [47]. In another form of intervention, A2E levels in Abercr-/- mice have also been reduced by daily administration of the retinoic acid analog N-(4-hydroxyphenyl)retinamide (HPR) for 1 month [56]. HPR acts by competing for binding sites on retinol-binding protein, thus reducing serum retinol levels. Consequently, retinol uptake by the eye is reduced, visual cycle retinoids are decreased, and the associated decrease in all-trans-retinal leads to retarded A2E formation.

1.6 Summary

RPE cells are unusual in that they are exposed to visible light while at the same time housing photoreactive molecules that accumulate as lipofuscin. There are many in-
indications that an excessive accumulation of RPE lipofuscin can lead to cellular dys-
function and contribute to retinal aging and degeneration [74]. Insight into the
composition, biogenesis, and photoreactivity of RPE lipofuscin has improved our
understanding of the extent to which the accumulation of these pigments renders
the macula prone to insult. Novel therapeutic approaches are being developed to min-
imize lipofuscin accumulation in order to reduce the progression of retinal condi-
tions such as recessive Stargardt disease and age-related macular degeneration.

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