Lipofuscin and N-Retinylidene-N-Retinylolethanalamine (A2E) Accumulate in Retinal Pigment Epithelium in Absence of Light Exposure

**THEIR ORIGIN IS 11-cis-RETINAL**

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**Background:** Toxic components of lipofuscin in the retina are proposed to arise from all-trans-retinal, a by-product of light detection.

**Results:** Lipofuscin precursors form from 11-cis-retinal; lipofuscin accumulation is independent of light exposure.

**Conclusion:** 11-cis-Retinal is the primary source of lipofuscin components.

**Significance:** 11-cis-Retinal may play a major role in the pathogenesis of macular degenerations.

The age-dependent accumulation of lipofuscin in the retinal pigment epithelium (RPE) has been associated with the development of retinal diseases, particularly age-related macular degeneration and Stargardt disease. A major component of lipofuscin is the bis-retinoid N-retinylidene-N-retinylolethanalamine (A2E). The current model for the formation of A2E requires photoactivation of rhodopsin and subsequent release of all-trans-retinal. To understand the role of light exposure in the accumulation of lipofuscin and A2E, we analyzed RPEs and isolated rod photoreceptors from mice of different ages and strains, reared either in darkness or cyclic light. Lipofuscin levels were determined by fluorescence imaging, whereas A2E levels were quantified by HPLC and UV-visible absorption spectroscopy. The identity of A2E was confirmed by tandem mass spectrometry. Lipofuscin and A2E levels in the RPE increased with age and more so in the Stargardt model Abca4−/− than in the wild type strains 129/sv and C57Bl/6. For each strain, the levels of lipofuscin precursor fluorophores in dark-adapted rods and the levels and rates of increase of RPE lipofuscin and A2E were not different between dark-reared and cyclic light-reared animals. Both 11-cis- and all-trans-retinal generated lipofuscin-like fluorophores when added to metabolically compromised rod outer segments; however, it was only 11-cis-retinal that generated such fluorophores when added to metabolically intact rods. The results suggest that lipofuscin originates from the free 11-cis-retinal that is continuously supplied to the rod for rhodopsin regeneration and outer segment renewal. The physiological role of Abca4 may include the translocation of 11-cis-retinal complexes across the disk membrane.

Lipofuscin, a fluorescent pigment, has been shown to accumulate with age in granules in postmitotic cells (1, 2), including the retinal pigment epithelium (RPE)³ of the eye (3–6). Lipofuscin is a complex mixture of partially digested lipid and protein components, with recent analysis showing that protein contributes ~2% by weight (7). The best characterized components are bis-retinoids (8), with A2E, the product of the condensation of two molecules of all-trans-retinal with ethanolamine, being one of the most abundant (9, 10). As lipofuscin and A2E have been shown to be cytotoxic in vitro (9, 11–13), it has been suggested that their accumulation contributes to retinal degenerative diseases such as age-related macular degeneration (14, 15) and Stargardt disease (16, 17). Stargardt disease has been associated with mutations in the multidrug resistance family transport protein ABCA4 (18, 19). Mice that lack Abca4 exhibit a large increase in RPE lipofuscin and bis-retinoid, including A2E, accumulation (17, 20, 21).

Previous studies have shown that the accumulation of lipofuscin has two prerequisites as follows: 1) the generation of 11-cis-retinal, as Rpe65−/− animals have strongly reduced levels of lipofuscin (22); and 2) the phagocytosis of photoreceptor outer segments by the RPE (23, 24). The mechanism of A2E and lipofuscin accumulation in the RPE is not well understood, but it is likely that components generated from retinal in the outer segment are introduced to the RPE through phagocytosis of photoreceptors. According to current models (1, 8, 22), A2E formation begins with the photoactivation of rhodopsin, the initial step in the detection of light by photoreceptor cells. Rhop...
dopsin photoactivation involves the isomerization of its 11-cis retinyl chromophore to all-trans (25). Subsequently, all-trans-retinal is released into the photoreceptor disk membrane. Once released, all-trans-retinal can covalently bind to the amine group of phosphatidylethanolamine (PE), forming N-retinylidene-PE (NRPE). Then a second molecule of retinal binds to NRPE, and one 14-methyl group forms an aromatic ring at the 15C position of the other retinyl chain (26, 27) generating N-retinylidene-N-retinylphosphatidylethanolamine (A2PE). A2PE can be digested by phospholipase D (27), cleaving the phospholipid portion of PE and producing A2E.

In the context of this model, ABCA4 is proposed to function as a transporter of N-retinylidene-PE that has been formed from the all-trans-retinal released by photoactivated rhodopsin (17). The transporter is proposed to flip N-retinylidene-PE from the intradiscal to the cytosolic side of the disk (17, 28, 29), making all-trans-retinal available for reduction to all-trans-retinol by retinol dehydrogenase. As such, deficiencies in ABCA4 function would lead to an accumulation in N-retinylidene-PE, driving the formation of the precursors of A2E and lipofuscin after photoactivation of rhodopsin.

The proposed models for lipofuscin and A2E accumulation in normal and Abca4-deficient retinas posit free all-trans-retinal as a necessary precursor, the generation of which requires light. We have therefore measured the levels of lipofuscin fluorescence and A2E in mice of different ages, reared either in dark or in cyclic light. We found no significant differences of lipofuscin or A2E levels between animals reared in the dark and those reared in cyclic light. This observation held for wild type as well as Abca4−/− animals, although levels of both lipofuscin and A2E were much higher in the Abca4-deficient mice. Conversely, lipofuscin levels were considerably lower, and A2E was undetectable in Rpe65−/− animals. These results demonstrate that light activation of the visual pigment is not necessary for the formation of lipofuscin or A2E, suggesting that other forms of retinal may be involved. Pursuing the potential identity of these forms of retinal, we have detected lipofuscin-like fluorophores in the outer segments of dark-adapted isolated rod photoreceptors, including those from dark-reared animals. Addition of 11-cis-retinal to intact wild type dark-adapted rods led to formation of lipofuscin-like fluorophores.

The results suggest that 11-cis-retinal is a major source for the formation of lipofuscin. They further suggest that N-11-cis-retinylidene-PE (11-cis-NRPE) may be a physiologically important substrate for ABCA4. The results are consistent with ABCA4 working in the same direction as other ABCA transporters (30), translocating the substrate away from the cytoplasm, i.e. from the cytosolic to the intradiscal leaflet of the disk membrane.

**EXPERIMENTAL PROCEDURES**

*Animals*—Wild type 129/sv and C57Bl/6, as well as Abca4−/− and Rpe65−/−, transgenic mice originated from established colonies at the Medical University of South Carolina. 129/sv and C57Bl/6 mice were originally obtained from Harlan Laboratories (Indianapolis, IN); to establish the colonies, breeding pairs of Abca4−/− and Rpe65−/− animals were generous gifts of Drs. G. H. Travis and T. M. Redmond, respectively. The background strain of the Abca4−/− animals was 129/sv. Animals of all strains were reared in cyclic light with a 12-h light cycle (06:00–18:00); 129/sv and Abca4−/− animals were also born and reared in the dark, in ventilated cabinets, exposed to dim red light only when checking on their health and for cage changes. Animal ages were 1–12 months. All animal procedures were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee of the Medical University of South Carolina and were consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. For experiments, animals were dark-adapted overnight and sacrificed under dim red light.

**Dissection of RPE and Retina**—Eyes were enucleated, cleaned of fat and muscle, and then hemisected at the level of the ora serrata under a mammalian physiological solution (in mmol/liter: 130 NaCl, 5 KCl, 0.5 MgCl2, 2 CaCl2, 25 hemisodium-HEPES, 5 glucose, pH 7.40). The procedure was carried out under infrared light. The dark-adapted state of the animals and the dissection under dim red light allowed the separation of the retina from the RPE with minimal cross-contamination (supplemental Fig. 1).

**Flat Mounting for RPE Imaging**—The lens, vitreous, and retina were carefully removed, and four shallow incisions were then made in the eyecup, which was flattened on a glass slide with the RPE facing upward. The eyecup was then gently covered with a glass coverslip.

**Color Photography**—Color photographs were taken on a Zeiss Axioskop 2 microscope (Carl Zeiss, Thornwood, NY) using a ×63 oil immersion objective (NA = 1.4) with a Nikon D200 (Nikon, Inc., Melville, NY) digital camera. Fluorescence was excited with 450–490 nm light, and the emission was collected >510 nm.

**RPE Fluorescence Measurement**—Eyecups on slides were imaged on an SP2 Leica laser scanning confocal microscope (Leica Microsystems, Inc., Buffalo Grove, IL) using a ×10 lens (NA = 0.3) with 488 nm excitation, with the pinhole fully open, and emission collected from 565 to 725 nm. Fluorescence intensity was measured over a 3.2 × 3.2-mm square centered at the optic nerve, covering >80% of the total eyecup area. Using the same size area allowed for fluorescence comparison across eyes of different sizes and did not include the eyecup areas around the rim that were damaged during dissection. These areas typically had low levels of lipofuscin fluorescence, and their exclusion does not affect the conclusions of this study. Eyecup fluorescence was corrected for background by subtracting the fluorescence intensity of an area of equal size adjacent to the eyecup that did not contain any tissue.

Fluorescence levels varied significantly across eyes from animals of different strains or ages, necessitating the use of different detector settings. Thus, to allow comparisons across eyes, the total fluorescence for each eyecup was converted to “bead units” (BU) using InSpecOrange (540/560) microscope image calibration kit beads (Invitrogen). Lipofuscin fluorescence is reported as “bead units per megapixel” (BU/MP). The area of one megapixel is equal to 2.44 mm2. For each age, three animals providing six eyecups were used.
Lipofuscin granule spectra were obtained from the same eyes with a ×63 oil immersion lens (NA = 1.4) using 488 nm excitation and measuring the emission across the range from 515 to 739 nm (bandwidth 14 nm). The pinhole aperture was reduced to ~1 Airy to eliminate as much as possible the inclusion of nongranule tissue fluorescence. Spectra were corrected for background by subtracting the fluorescence intensities of a spectrum of a tissue area from the same field that did not contain granules.

**A2E Quantification**—After enucleation, hemisection, and removal of the lens, vitreous, and retina, the RPE-choroids were collected by gently scraping them off the sclera in mammalian physiological buffer solution. For each experiment, two to six eyecups were used; higher numbers of eyecups were needed in cases of younger animals, which contain lower A2E amounts. Excess buffer was removed by centrifuging the solutions in a tabletop centrifuge (14,000 rpm; Eppendorf Centrifuge 5415C; Eppendorf, AG, Hamburg, Germany). The RPE-choroid tissue pellets were homogenized in 1.5 ml of phosphate buffered saline with 3 ml of 1:1 chloroform/methanol; 1 ml of chloroform, and 1 ml of methylene chloride were subsequently added to the homogenate. After mixing, the homogenate was centrifuged in a tabletop clinical centrifuge, and the organic phase was collected and dried under argon.

HPLC experiments were repeated three times. For each experiment, a dried extract from two to six eyecups was suspended in methanol with 0.1% trifluoroacetic acid (TFA) and analyzed with a Waters 1525 binary HPLC (Waters Corp., Milford, MA) using a reverse phase gradient from 85% acetonitrile with 0.1% TFA and 15% water with 0.1% TFA to 100% acetonitrile/TFA. Absorbance was monitored by a Waters 2998 PDA detector, and the A2E peak was determined by retention time and absorbance spectrum through comparison with a synthetic A2E standard (10). The peak was collected under dim red light and subjected to LC-MS/MS to confirm identification and for quantification of A2E by published methods (31). HPLC experiments with retinas separated from the RPE were carried out the same way.

**Rod Outer Segment Membrane Preparation**—Mouse rod outer segment membranes were prepared from isolated mouse retinas with a sucrose density flotation method (32). The supernatant containing the rod outer segment membranes and the pellet were collected separately and processed for HPLC analysis.

**Fluorescence Imaging of Isolated Rods**—Single, living isolated rod photoreceptors were obtained and imaged as described previously (33). Lipofuscin-like fluorescence was measured with 490 nm excitation and collecting emission >515 nm. Retinal fluorescence was measured with 360 nm excitation and collecting emission >420 nm. All-trans- and 11-cis-retinal were delivered using 1% bovine serum albumin (BSA) as carrier. Experiments were carried out at 37°C. Fluorescence emission spectra of isolated rod outer segments were measured in the SP2 Leica laser scanning confocal microscope, at room temperature. Isolated cells were applied to poly-l-lysine-coated (0.01%; diluted with distilled water from 0.1% solution, Sigma) superfrost microscope slides (Fisherbrand, Fisher), allowed to settle for 2–5 min, and then covered with coverslips. Fluorescence spectra were measured using the same method as for lipofuscin granule spectra but with the pinhole fully open. All preparation and measurements were performed under dim red light. All reagents were of analytical grade; organic solvents were HPLC grade.

**Statistical Analysis**—Statistical significance was tested with analysis of variance. In the figures, statistically significant differences are indicated with an asterisk.

**RESULTS**

**Lipofuscin Is Present in the RPE of Dark-reared Animals**—Fluorescence images (excitation 450–490 nm) of RPE from dark-reared wild type and Abca4−/− animals reveal the presence of the characteristic orange lipofuscin granules (Fig. 1, A and B). The fluorescence emission spectra of these granules collected with excitation 488 nm (Fig. 1D) peak at ~610 nm. Although the number and size of granules increased with age, the color of the granules and their emission spectra were essentially the same for animals reared in cyclic light and continuous darkness and did not depend on age or the strain for 129/sv, C57BL/6, and Abca4−/− mice.

Interestingly, the RPE from cyclic light-reared Rpe65−/− mice, which do not produce 11-cis-retinal and hence do not form visual pigments, also showed accumulation of fluorescent granules (Fig. 1C). However, the color of the fluorescence of...
Increased linearly with age in both cyclic light- and dark-reared BU/MP in 12-month-old mice. Similarly, eyecup fluorescence increased at a rate of 8.7/12-month-old cyclic light-reared animals. In dark-reared animals, eyecup fluorescence was 10.7/12-month-old wild type 129/sv mice (Fig. 2A). The number of fluorescent granules increased with age, but their emission spectrum did not change appreciably.

RPE Lipofuscin Levels Increase with Age Regardless of Light Exposure—Because the distribution of lipofuscin fluorescence is not necessarily homogeneous across the RPE, we measured lipofuscin levels by collecting the fluorescence from a whole flat-mounted eyecup. Eyecup fluorescence increased approximately linearly with age in both cyclic light- and dark-reared wild type 129/sv mice (Fig. 2A). In 1-month-old cyclic light-reared animals, eyecup fluorescence was 10.7 ± 1.2 BU/MP. This fluorescence accumulated steadily with age at a rate of 5.6 ± 0.9 BU/MP/month to reach 62.2 ± 5.5 BU/MP in 12-month-old cyclic light-reared animals. In dark-reared animals, fluorescence increased at a rate of 8.7 ± 2.1 BU/MP/month, from 17.8 ± 0.2 BU/MP in 1-month-old to 88.3 ± 7.9 BU/MP in 12-month-old mice. Similarly, eyecup fluorescence increased linearly with age in both cyclic light- and dark-reared Abca4−/− mice (Fig. 2B), with rates of 11.6 ± 1.7 BU/MP/month and 15.7 ± 1.7 BU/MP/month, respectively. These rates were both substantially higher than those for wild type mice. The eyecup fluorescence levels in 1-month-old animals were 3.9 ± 0.3 and 3.6 ± 0.2 BU/MP for dark- and cyclic light-reared, respectively, both lower than those in wild type. By 12 months, the levels increased to 190.2 ± 18.5 and 142.1 ± 4.4 BU/MP, much higher than wild type. In Rpe65−/− animals, fluorescence levels showed only a modest increase with age, from 7.0 ± 0.6 at 1 month to 28.0 ± 1.9 BU/MP at 6 months (Fig. 2C). Then they declined slightly to 21.3 ± 1.0 BU/MP by 12 months. For most ages, the levels of fluorescence in Rpe65−/− mice were much lower than in wild type and in Abca4−/− animals (Fig. 2C).

Substantial RPE fluorescence levels were also found in C57BL/6 mice, which have a much slower rate of rhodopsin regeneration than 129/sv due to the presence of a Rpe65 L450M variant (34). The levels were 171.3 ± 10.6 and 163.4 ± 39.4 BU/MP in 12-month-old cyclic light- and dark-reared mice, respectively (Fig. 2D). The data for the cyclic light-reared C57BL/6 mice have been re-plotted from Ref. 35 for comparison.

RPE A2E Content Increases with Age Regardless of Light Exposure—The intensity of lipofuscin fluorescence does not necessarily reflect the mass of accumulated material. Because the overall composition of lipofuscin is not known, we have used a major as well as the best characterized component, A2E, as an indicator of mass accumulation. In addition, the A2E fluorescence emission spectrum is similar to that of lipofuscin, and a specific model for its generation has been proposed.

Chromatograms of organic extracts of RPE-choroid tissue from dark-reared 129/sv (Fig. 3A) and Abca4−/− (Fig. 3B) animals show the presence of large amounts of A2E, as judged by the absorption spectrum and comparison with the chromatogram of a synthetic standard (Fig. 3C). To confirm that the compound is A2E, the HPLC fraction eluting between 10 and 11 min was collected and analyzed with LC-MS/MS. A 592 m/z peak characteristic for A2E is present and upon fragmentation gives for both 129/sv (Fig. 3D) and Abca4−/− (Fig. 3E) patterns that are identical to that for synthetic A2E (Fig. 3F). The prominent 418 ion can also be used to accurately quantify A2E in the HPLC fraction (31). The total amount of A2E determined from the 418 ion was ~40–50% of the amount determined from chromatographic absorption spectroscopy. This proportion was the same for extracts from dark-reared and cyclic light-reared animals and did not change significantly with age.

The amount of A2E increased in cyclic light- and dark-reared 129/sv mice from 0.4 ± 0.3 and 2.6 ± 0.2 pmol/eye at 1 month to 12.9 ± 1.9 and 11.4 ± 1.3 pmol/eye at 12 months. A2E accumulated in a linear fashion with rates of 1.2 ± 0.2 and 0.8 ± 0.1 pmol/eye/month in cyclic light- and dark-reared animals, respectively (Fig. 4A). In Abca4−/− mice, the levels of A2E were higher than in wild type at all ages, in both cyclic light- and dark-reared animals, beginning from 7.5 ± 4.6 and 4.0 ± 1.3 pmol/eye at 1 month, reaching 79.2 ± 14.7 and 70.0 ± 17.0 at 12 months, respectively. A2E accumulated with age at significantly higher rates in Abca4−/− than in wild type, 6.2 ± 0.9 (cyclic light) and 5.6 ± 0.7 (dark) pmol/eye/month (Fig. 4B).
A2E was undetectable in RPE samples from cyclic light-reared Rpe65/H11002/H11002 mice of 1–12 months of age. For reporting purposes, a nominal level of A2E was measured by integrating the chromatogram intensity from 10 to 11 min, covering the retention time of the synthetic A2E standard (Fig. 4 C). A2E in these samples was undetectable even with LC-MS/MS, which can detect femtomole levels.

Significant A2E accumulation takes place in cyclic light- and dark-reared C57Bl/6 mice as well, reaching 9.6 ± 0.9 and 7.8 ± 0.4 pmol/eye, respectively, at 12 months of age (Fig. 4 D). The data for the cyclic light-reared C57BL/6 mice have been replotted from Ref. 35 for comparison.

Lipofuscin-like Fluorophores in Rod Outer Segments Originate from 11-cis-Retinal—Because lipofuscin is expected to originate in the outer segments of rod photoreceptors, we examined isolated rods for the presence of lipofuscin-like fluorophores. Fluorescence images (excitation 490 nm; emission >515 nm) of dark-adapted rod photoreceptors show the presence of fluorophores in the outer segment (Fig. 5, A and B). The intensity of this fluorescence is the same in the outer segments of intact cells and broken off rod outer segments and independent of whether the animals were reared in cyclic light or in darkness. The fluorescence intensity was higher in Abca4/H11002/H11002 than wild type (p < 0.002), and higher in wild type than in Rpe65/H11002/H11002 (p < 0.05) rod outer segments (Fig. 5 B). The emission spectra of this fluorescence signal are consistent with those of the lipofuscin granules found in the RPE of the respective strains (Fig. 6). Because the fluorophores responsible for this signal are present in the outer segments of dark-adapted cells from dark-reared animals, it is unlikely that their source is all-trans-retinal; 11-cis is a much more likely candidate. Indeed, addition of moderate (5 μM) concentrations of 11-cis-retinal to intact wild type rod photoreceptors resulted in an increase in outer segment fluorescence compared with the addition of all-trans (p < 0.04) (Fig. 5 C). The addition of all-trans-retinal did not result in a significant increase in outer segment fluorescence (Fig. 5 C).

The fluorescence emission spectra of dark-adapted rod outer segments from cyclic light- and dark-reared wild type and
Abca4−/− animals were the same and peaked ∼610 nm (Fig. 6, A and B). Rod outer segments from Rpe65−/− mice had low levels of fluorescence (Fig. 5B), and their fluorescence emission spectrum was shifted to lower wavelengths, peaking ∼550 nm (Fig. 6D). Addition of all-trans- or 11-cis-retinal to broken off Rpe65−/− rod outer segments resulted in a large increase in fluorescence (Fig. 6C), with a shift in the fluorescence emission spectrum to longer wavelengths, with a peak ∼610 nm (Fig. 6D).

The identity of the fluorophores with the lipofuscin-like emission spectra has not been established, but they are clearly by-products of retinal. HPLC analysis of organic extracts of retinas separated from the RPE show the presence of several species absorbing >400 nm, including A2E, in tissues from both cyclic light- and dark-reared animals (Fig. 7, A and B). The identity of A2E was confirmed from its absorbance spectrum and with tandem mass spectrometry (Fig. 7C and D). Most of the A2E in the retina was present in the rod outer segments, as >85% (measured per mg of protein) of it was found in the rod outer segment membrane fraction of a sucrose density centrifugation of homogenates of isolated retinas. A detailed characterization of the levels of A2E in the retina and their change with age was not carried out. The 129/sv retina A2E levels measured with HPLC were lower than those found in the RPE, 1.7 ± 0.2 pmol/retina (3-month-old cyclic light-reared animals). Higher levels of A2E, 5.9 ± 1.0 pmol/retina (3-month-old cyclic light-reared animals), were found in retinas from 3-month-old cyclic light-reared animals (Fig. 7A). Higher levels of A2E, 5.9 ± 1.0 pmol/retina (3-month-old cyclic light-reared animals), were found in retinas from 3-month-old Rpe65−/− (cyclic light-reared), 129/sv (dark-reared), and Abca4−/− (dark-reared) mice. Fluorescence images are shown at the same scaling. Bars, 5 μm. B, outer segment lipofuscin-like fluorescence levels in dark-adapted metabolically intact rods (Intact) and metabolically compromised broken off rod outer segments (ROS) from dark-reared (D) and cyclic light-reared (CL) mice. C, exposure of dark-adapted metabolically intact rods to 5 μm all-trans- or 11-cis-retinal for 5 min. All experiments were at 37 °C. Numbers of cells are shown within each column. Error bars represent standard errors.

Abca4−/− animals. Similar levels, 1.3 ± 0.1 pmol/retina (129/sv) and 4.7 ± 1.2 pmol/retina (Abca4−/−), were found in 3-month-old dark-reared animals. The substances absorbing >400 nm were present in the retinas of both cyclic light- and dark-reared animals and in both wild type and Abca4−/− animals. They were not present, however, in extracts from Rpe65−/− tissues, either retina or RPE, corroborating their retinoid origin (Fig. 7A); the peaks appearing in these chromatograms show absorbance peaks <350 nm. It is unlikely that the presence of these substances in retina extracts represents contamination from the RPE, as was most evident by comparing the HPLCs at 510 nm (supplemental Fig. 1); their relative amounts differ between the retina and the RPE, and there are peaks detected in one but not the other.

Lipofuscin-like Fluorophores in Rod Outer Segments Can Form from All-trans-retinal—Fig. 6C demonstrates that lipofuscin-like fluorophores can form in rod outer segments upon the addition of exogenous all-trans-retinal. We examined whether these fluorophores can also form from the all-trans-retinal released from photoactivated rhodopsin after light excitation. In the outer segments of metabolically intact rod photoreceptors, most of the endogenously released all-trans-retinal is converted to all-trans retinol (33, 36); in the metabolically compromised broken off rod outer segments, however, it is not (37).
The fluorescence images in Fig. 8A show that lipofuscin-like fluorophores form in a wild type broken off rod outer segment following exposure to light. By contrast, there is no detectable formation of these fluorophores in the outer segment of a metabolically intact rod, where retinol forms instead (Fig. 8A). The emission spectra of the fluorescence of bleached broken off rod outer segments are the same as those obtained with additions of exogenous all-trans-retinal shown in Fig. 6D (data not shown). Fig. 8B shows the kinetics of the increase in lipofuscin-like fluorescence in wild type broken off rod outer segments and the lack of any detectable increase in the outer segments of metabolically intact rods. Interestingly, the same results are obtained with rods from Abca4−/− mice (Fig. 8C), with no detectable fluorescence increase in metabolically intact rods. Compared with wild type, the Abca4−/− cells had higher overall levels of fluorescence (see also Fig. 5B). The rate of fluorescence increase in broken off rod outer segments after bleaching was 0.11 ± 0.02 units/min for Abca4−/−, almost three times as large as the rate of 0.04 ± 0.01 units/min for wild type.

**DISCUSSION**

Lipofuscin and A2E Accumulate in RPE in the Absence of Light Exposure—The results of Figs. 1–4 demonstrate that lipofuscin and A2E accumulate in the RPE of wild type and Abca4-deficient mice in the absence of significant light exposure. The amounts of A2E (pmol/eye) measured in this study are in broad agreement with the amounts reported previously from wild type or Abca4−/− animals reared in cyclic light (20, 21, 38). Unfortunately, no direct comparison with previous studies is possible for total eyecup levels of lipofuscin, as it is difficult to compare fluorescence measurements across different studies utilizing different procedures and instruments. Nevertheless, the present results are in qualitative agreement
with the previous observations of RPE lipofuscin fluorescence increasing with age (20, 23). The striking observation in this study is that absence of light exposure did not affect significantly the levels and rates of lipofuscin and A2E accumulation for a time period of up to 12 months (Figs. 2 and 4). This observation held for the 129/sv and C57BL/6 as well as for the Abca4−/− mice. This finding is inconsistent with the proposed models for lipofuscin and A2E formation (21, 22) and directly contradicts previous reports of a lack of A2E level increases in animals reared in darkness (21, 38).

A possible explanation for the discrepancy is the underestimation of the A2E levels in dark-reared animals previously, because the complexity of the chromatograms of RPE extracts can hamper the quantification of A2E from its absorbance. In this study, selected monitoring of a fragment (MS/MS) ion was used to confirm the identity of A2E and provide an independent quantification method with tandem mass spectra (Fig. 3). For estimating lipofuscin accumulation, previous studies relied on fluorescence measurements from selected representative areas. The distribution of lipofuscin fluorescence does not appear to be homogeneous over the whole eyeball (see images in Ref. 39), and observations under high magnification reveal variable granule accumulation even in neighboring cells (data not shown). Because of this variability, the total eyeball fluorescence is a better measure of overall lipofuscin accumulation. An important concern with the use of total eyeball fluorescence instead of a selected area is tissue damage during processing. Care was exercised during eyeball dissection and processing to limit any damage to the edge. The effects of any tissue damage are expected to contribute no more than 20% on the reported values of lipofuscin fluorescence (see “Experimental Procedures”).

Lipofuscin and A2E Accumulate in the RPE of Mice of Different Strains—In accordance with previous reports (20, 21, 23), this study found that lipofuscin (Fig. 2C) and A2E levels (Fig. 4, A and B) increased severalfold faster with age in the RPE of Abca4−/− mice than wild type. We have extended this observation to include measurement of lipofuscin and A2E accumulation in dark-reared animals. Specifically, in 129/sv wild type animals, eyeball fluorescence increased at a rate of 5.6–8.7 BU/MP/month, and in Abca4−/− with a rate about two times higher, 11.6–15.7 BU/MP/month. For A2E, the rate of increase was 0.8–1.2 pmol/eye/month in 129/sv, but almost six times higher, 5.6–6.2 pmol/eye/month in Abca4−/−. The lack of correspondence in the rates of increase of lipofuscin and A2E when comparing the two strains is not necessarily surprising. Although the emission spectrum of A2E is similar to that of lipofuscin, it represents only a single component. In addition, the intensity of lipofuscin fluorescence does not necessarily reflect the mass of accumulated material, and finally, it is likely that total eyeball fluorescence includes contributions from nonlipofuscin fluorophores.

This incongruence between lipofuscin and A2E levels is also seen when comparing the two wild type strains, 129/sv and C57BL/6 (Figs. 2D and 4D); although eyeball fluorescence is higher in C57BL/6 animals, the A2E levels are lower. The lower A2E levels in the C57BL/6 mice compared with 129/sv (Fig. 4D) are in some sense consistent with the slower rate of generation of 11-cis-retinal in these animals (34, 40). This difference, however, is not proportional to the severalfold difference in the Rpe65 enzyme activity (40).

Eyecup fluorescence increases modestly with age up to 6 months in Rpe65−/− animals. The fluorescence levels in this strain are much lower than those in wild type and Abca4−/− animals (Fig. 2C), in agreement with the undetectability of lipofuscin fluorescence reported previously (22). This fluorescence is clearly different from that observed in the other strains and is associated with background tissue fluorescence and with fluorescent granules with a blue-shifted emission spectrum (Fig. 1, C and E). It is not clear whether the granules observed in the Rpe65−/− eyecups are related to lysosomes in the same way as those in the wild type and Abca4−/− animals. A2E is virtually undetectable in the RPE tissues of these cyclic light-reared Rpe65−/− animals at all ages, again pointing out the lack of correspondence between eyecup fluorescence and a chemically defined lipofuscin component.

The differences in lipofuscin and A2E levels between the different strains along with the lack of correspondence between lipofuscin and A2E changes make even more striking the agreement in those levels for cyclic light- and dark-reared animals of the same strain. These observations strongly reinforce the lack of a major effect on lipofuscin and A2E levels by exposure to light.

Effects of Light Exposure on Lipofuscin and A2E Levels—Exposure to light during cyclic light-rearing has no discernible effect on the emission spectrum of lipofuscin granules either. Interestingly, bright light exposure of all-trans-retinal PDE and A2E results in an increase in the intensity of their fluorescence (41). Such changes may be associated with the phototoxicity of lipofuscin and A2E, and with the generation of reactive photoproducts (42). However, it appears that they do not result in obvious changes in the RPE of the animal models examined here, for which the fluorescence intensity and spectrum of lipofuscin do not change appreciably with time of exposure to light. A possible explanation is that there are significant differences in the chemical composition of lipofuscin between cyclic light- and dark-reared animals, which are not reflected in the lipofuscin fluorescence intensity and spectrum and do not include A2E. The similarity in the levels of A2E between cyclic light- and dark-reared animals should also be interpreted with caution, as the overall metabolism of A2E (and the possibility of its clearance) is not well understood.

Retinoid Origins of Lipofuscin and A2E—Lipofuscin granule spectra from all strains that generate 11-cis-retinal are very similar, with a peak at ~610 nm, regardless of age and rearing conditions. These spectra are essentially the same as those reported previously for lipofuscin and A2E (39). Absence of 11-cis-retinal results in a substantially shifted emission spectrum with a peak at ~550 nm. In addition, no compounds with significant absorbance >400 nm, such as A2E, were detected in RPE extracts from Rpe65−/− mice. This suggests that the characteristic orange color and emission spectrum of RPE lipofuscin might be indicative of the contributions of retinoid metabolites. This is strongly supported by the results from the experiments with isolated rods and retinas. We followed previous work that has identified lipofuscin and A2E precursors in retinas (27, 43, 44). The fluorescence levels of dark-adapted Rpe65−/− rods were lower than those from strains with intact
11-cis-retinal generating machinery, and their fluorescence emission spectra were shifted to lower wavelengths, similar to the spectra of the RPE granules of the same strain. However, the fluorescence emission spectra of dark-adapted wild type and Abca4−/− rods were similar to those of the RPE granules of their strains, peaking at ~610 nm. In addition, there were no compounds with significant absorbance of >400 nm detected in the extracts from Rpe65−/− retinas. Comparing the other two strains, the higher lipofuscin-like fluorescence intensity in Abca4−/− cells was commensurate with the higher levels of lipofuscin and A2E accumulating in their RPE. Overall, the results from the three strains lend further support to the present model for the origins of RPE lipofuscin from components that are generated from retinal in the outer segment, which are then introduced to the RPE through phagocytosis.

The presence of lipofuscin precursors in the retina, specifically in rod outer segments, is in agreement with previous reports (21, 26, 27). It has previously been shown that these precursors could form after bleaching, and A2E in particular was shown to form in retinas after the addition of exogenous all-trans-retinal (26). As pointed out previously (26), phospholipase D, which is present in rod outer segments (45), would cleave A2PE to generate A2E.

The amounts of A2E and other lipofuscin precursors found in the retina are fairly substantial when compared with the amounts found accumulated in the RPE. Keeping in mind that ~10% of mouse rod outer segments is being phagocytosed daily by the RPE (46), comparison of the amounts of A2E found in the retina and the accumulated amounts in the RPE would indicate that A2E and probably other lipofuscin precursors are eliminated even in dark-reared animals. It is not clear whether such elimination reflects nonspecific degradation of the compounds or proceeds via a specialized lysosomal process.

**All-trans-retinal Is Unlikely to be a Major Source of Lipofuscin and A2E**—Chronic light exposure had no effect on the levels of RPE lipofuscin and A2E or on the levels of lipofuscin-like fluorescence in dark-adapted rod outer segments. This striking result indicates that all-trans-retinal is not a necessary intermediate for lipofuscin and A2E formation, as proposed in current models. The present results do not of course exclude the formation of lipofuscin and A2E from all-trans-retinal under conditions of cyclic light. However, the similarity in the A2E levels between cyclic light- and dark-rearing conditions suggests that any contribution of all-trans-retinal cannot be a major one. The lack of an effect of light exposure would be consistent with the clearance of all-trans-retinal generated by light through its reduction to all-trans-retinol in the photoreceptor outer segments (33) and the lack of significant formation of lipofuscin-like fluorophores in metabolically intact rod outer segments (Fig. 8). The results with broken off rod outer segments after light exposure (Fig. 8) suggest that the conditions under which all-trans-retinal could make a significant contribution would involve limitations in the availability of NADPH. Such limitations might perhaps occur under continuous bright light that would generate large amounts of all-trans-retinal or in pathological conditions that involve defects in the NADPH-generating pathways.

**11-cis-Retinal Is the Likely Source of Lipofuscin and A2E**—Because the continuous generation of 11-cis-retinal is required for lipofuscin and A2E formation, and the present data argue against an essential role for all-trans-retinal, this would leave 11-cis-retinal, free or as part of rhodopsin, as source. Free 11-cis-retinal, which flows into photoreceptors even under dark-rearing conditions for the purposes of outer segment renewal (47), is a likely source. This is especially so in view of the presence of lipofuscin-like fluorophores in the dark-adapted rods of dark-reared animals (Figs. 5 and 6), as well as of A2E along with other compounds of retinoid origin in retina extracts (Fig. 7). The possibility was demonstrated by the formation of lipofuscin-like fluorophores upon addition of moderate concentrations of exogenous 11-cis-retinal to metabolically intact dark-adapted rods (Fig. 5C). The result should not be too surprising, because 11-cis-retinal is not a good substrate for the rod photoreceptor retinol dehydrogenase enzyme, which is specific for the all-trans-isomer (48). This dehydrogenase activity and its specificity account for the lack of an effect by the same concentration of all-trans-retinal (Fig. 5C).

The pathway of lipofuscin formation involving 11-cis-retinal would of course operate under light conditions as well, because fresh 11-cis-retinal would continuously flow into the rod outer segment for rhodopsin regeneration (Fig. 9).

The results do not exclude contributions to RPE lipofuscin and A2E by rhodopsin, which contains 11-cis-retinal. In this case, rhodopsin would enter the RPE lysosomal compartment through the daily phagocytosis of the rod outer segments (49); 11-cis-retinal would then be released during lysosomal digestion and would form bis-retinoid adducts with PE that would eventually end up as A2E through a pathway similar to the one that has been described for all-trans-retinal.

**Reexamination of the Role of the Abca4 Transporter**—None of the experiments presented here provide support for the current model for Abca4 function (17, 50), and some are directly inconsistent with it. These include the presence of lipofuscin
precursors in the rod outer segments of dark-adapted dark-reared wild type and Abca4+/− mice, as well as the absence of a detectable increase in lipofuscin precursors in metabolically intact Abca4−/− rods after bleaching. Most important, chronic light exposure had no effect on the levels of RPE lipofuscin and A2E in Abca4−/− mice. Abca4 has been proposed to facilitate the clearance of the all-trans-retinal generated by light by translocating the Schiff base of the all-trans-retinal with PE from the intradiscal side to the cytoplasmic side; on the cytoplasmic side, all-trans-retinal can be converted to all-trans-retinol by retinol dehydrogenase and removed from the cell. In this model, absence of Abca4 would result in the accumulation of all-trans-retinal and formation of bis-retinoid adducts like A2E. The model would predict that in the absence of light exposure, the absence of Abca4 would not make a difference to the generation of A2E. The present results directly invalidate this model for Abca4 and are in line with other recent experiments showing that the formation of all-trans-retinol after light exposure is not affected by the lack of Abca4 (36).

The higher levels of lipofuscin precursors in rods, along with the high levels of lipofuscin and A2E accumulation in the absence of Abca4 even in dark-reared animals, suggest that Abca4 has an important role in retinoid processing, albeit not one that involves specifically all-trans-retinal.

The results are consistent with Abca4 facilitating the sequestration of an 11-cis-retinal complex, which would form in both dark and light conditions from free 11-cis-retinal arriving from the RPE. The experiments described here provide no direct data on the direction in which Abca4 would be translocating its substrate. They are consistent with it operating in the same direction as other ABCA transporters (30), i.e. moving the substrate away from the cytoplasmic side (51, 52). The results, however, have invalidated the need for the translocation of NRPE from the intradiscal to the cytosolic side of the disk membrane. Such a direction of translocation of 11-cis-NRPE, from the cytosolic to the intradiscal side, would provide protection of the cytosolic enzymatic machinery from the reactive retinaldehyde. Such protection would be especially needed against 11-cis-retinal, which is not processed by the rod outer segment retinol dehydrogenase (Fig. 5C) (48). The results do not exclude all-trans-NRPE being an important substrate for Abca4 as well. Translocation of all-trans-NRPE to the intradiscal side would be important under conditions of excessive buildup of all-trans-retinal when reduction by Rdh8, the rod outer segment retinol dehydrogenase, cannot keep up. Such a function would be consistent with the observation that mice deficient in both Abca4 and Rdh8 show much more prominent retinal degeneration and are more susceptible to retinal toxicity than either of the strains deficient in only one (53, 54). Fig. 9 presents a scheme that incorporates this model for Abca4 along with the pathways proposed to generate lipofuscin.

The model of Fig. 9 does not provide an explanation for the higher levels of lipofuscin and bis-retinoids found in Abca4−/− animals compared with wild type. One possible explanation could be the higher levels of PE found in the rod outer segments of Abca4−/− animals (17), which would promote the formation of more bis-retinoid adducts. Such an explanation is supported by the formation of more lipofuscin-like fluorophores in Abca4−/− broken off rod outer segments after bleaching (Fig. 8). Another possibility, for which there is presently no evidence, would be a more complex processing of NRPE by Abca4.

In summary, the results presented demonstrate that the formation of lipofuscin and the lipofuscin component A2E in the RPE do not require the generation of all-trans-retinal by exposure to light. Instead they suggest that lipofuscin originates from free 11-cis-retinal, which is continuously supplied to the rod photoreceptor for rhodopsin regeneration and outer segment renewal. The results further suggest that Abca4 may well function in the same direction as other ABCA transporters, and that its physiological role involves the translocation of 11-cis- and perhaps all-trans-NRPE.

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