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All-*trans* retinal levels and formation of lipofuscin precursors after bleaching in rod photoreceptors from wild type and *Abca4*$^{-/-}$ mice

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Abstract

The accumulation of lipofuscin in the cells of the retinal pigment epithelium (RPE) is thought to play an important role in the development and progression of degenerative diseases of the retina. The bulk of RPE lipofuscin originates in reactions of the rhodopsin chromophore, retinal, with components of the photoreceptor outer segment. The 11-*cis* retinal isomer is generated in the RPE and supplied to rod photoreceptor outer segments where it is incorporated as the chromophore of rhodopsin. It is photoisomerized during light detection to all-*trans* and subsequently released by photoactivated rhodopsin as all-*trans* retinal, which is removed through reduction to all-*trans* retinol in a reaction requiring metabolic input in the form of NADPH. Both 11-*cis* and all-*trans* retinal can form lipofuscin precursor fluorophores in rod photoreceptor outer segments. Increased accumulation of lipofuscin has been suggested to result from excess formation of lipofuscin precursors due to buildup of all-*trans* retinal released by light exposure. In connection with this suggestion, the Abca4 transporter protein, an outer segment protein defects in which result in recessive Stargardt disease, has been proposed to promote the removal of all-*trans* retinal by facilitating its availability for reduction. To examine this possibility, we have measured the outer segment levels of all-*trans* retinal, all-*trans* retinol, and of lipofuscin precursors after bleaching by imaging the fluorescence of single rod photoreceptors isolated from wild type and *Abca4*$^{-/-}$ mice. We found that all-*trans* retinol and all-*trans* retinal levels increased after bleaching in both wild type and *Abca4*$^{-/-}$ rods. At all times after bleaching, there was no significant difference in all-*trans* retinal levels between the two strains. All-*trans* retinol levels were not significantly different between the two strains at early times, but were lower in *Abca4*$^{-/-}$ rods at times longer than 20 min after bleaching. Bleaching in the presence of lower metabolic substrate concentrations resulted in higher all-*trans* retinal levels and increased formation of lipofuscin precursors in both wild type and *Abca4*$^{-/-}$ rods. The results show that conditions that result in buildup of all-*trans* retinal levels result in increased generation of lipofuscin precursors in both wild type and *Abca4*$^{-/-}$ rods. The results are consistent with the proposal that Abca4 facilitates the reduction of all-*trans*

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retinal to retinol; absence of Abca4 however does not appear to be associated with higher all-*trans* retinal levels compared to wild type.

Keywords

photoreceptor; retinal; retinol; NADPH; ABCA4; lipofuscin; fluorescence

1. INTRODUCTION

Lipofuscin, a complex mixture of fluorescent pigments, accumulates with age in the lysosomes of post-mitotic cells (Katz and Robison, 2002), including the retinal pigment epithelial (RPE) cells in the eye (Delori et al., 1995a; Delori et al., 2001; Feeney, 1978; Wing et al., 1978). Lipofuscin has a range of deleterious effects on cell and tissue function (Eldred and Lasky, 1993; Lakkaraju et al., 2007; Rozanowska et al., 1998; Sparrow et al., 2000; Vives-Bauza et al., 2008), and its accumulation is thought to play a role in degenerative diseases of the retina, including Age-related Macular Degeneration (AMD) (Sparrow and Boulton, 2005; Winkler et al., 1999) and Stargardt disease (Delori et al., 1995b; Weng et al., 1999). Although the exact composition of lipofuscin is not known, *bis*-retinoid compounds, which are derived from the condensation of two molecules of retinal with other cellular components (Sparrow et al., 2012), have been identified as major constituents of RPE lipofuscin. A2E is the best characterized *bis*-retinoid lipofuscin component to date (Ben-Shabat et al., 2002; Parish et al., 1998).

Lipofuscin fluorophores originate in the photoreceptor outer segments (Boyer et al., 2012; Katz et al., 1986), and, through the daily phagocytosis of the outer segments by the RPE, enter the RPE lysosomal compartment where they accumulate after additional processing. Reactions of the visual pigment chromophore play a pivotal role in lipofuscin formation: suppressing the generation of the visual pigment chromophore 11-*cis* retinal results in loss of most of RPE lipofuscin (Katz and Redmond, 2001). Rhodopsin, the visual pigment of rod photoreceptors, utilizes 11-*cis* retinal as the chromophore to detect light (Ebrey and Koutalos, 2001). Light detection is initiated by an active rhodopsin photointermediate generated through the photoisomerization of the 11-*cis* chromophore of rhodopsin to all-*trans*. Photoactivated rhodopsin triggers a cascade of reactions culminating to a change in membrane potential, which constitutes the light response. Photoisomerization destroys rhodopsin, which is then regenerated via a two-step process: first, all-*trans* retinal is released, leaving the apo-protein opsin, and, second, opsin binds freshly supplied 11-*cis* retinal and re-forms rhodopsin. The released all-*trans* retinal is reduced within the rod outer segment to all-*trans* retinol, which is then transported to the RPE where it is recycled to make 11-*cis* retinal (Lamb and Pugh, 2004; Saari, 2000; Tang et al., 2013). The reduction of all-*trans* retinal to retinol is catalyzed by the enzyme retinol dehydrogenase RDH8 (Chen et al., 2012; Maeda et al., 2005) and requires metabolic input in the form of NADPH (Adler et al., 2014; Futterman et al., 1970). 11-*Cis* and all-*trans* retinal can both generate lipofuscin precursorsin rod outer segments, as evidenced by measurements of single cell fluorescence (Boyer et al., 2012), as well as of *bis*-retinoids (Ben-Shabat et al., 2002; Liu et al., 2000; Mata et al., 2000; Quazi and Molday, 2014). In the absence of 11-*cis* retinal generation, and

therefore absence of both 11-*cis* and all-*trans* retinal in rod outer segments, no *bis*-retinoid formation is detected in RPE or neural retina (Boyer et al., 2012; Wu et al., 2009). The residual fluorescence signal detected in RPE cells and in rod outer segments of *Rpe65*$^{-/-}$ mice is much smaller and has a different emission spectrum from that of lipofuscin that forms with intact 11-*cis* retinal generation. Thus, it appears that the bulk of RPE lipofuscin originates from reactions of 11-*cis* and all-*trans* retinal with outer segment components.

The significant lipofuscin fluorescence and *bis*-retinoid levels measured in dark-reared wild type and *Abca4*$^{-/-}$ animals (Boyer et al., 2012; Lenis, 2016; Ueda et al., 2016) suggest that 11-*cis* retinal is a major contributor to lipofuscin generation. The extent of all-*trans* retinal contribution is not as clear, especially in view of the observation that lipofuscin levels in dark-reared animals are at least the same or even higher than those in cyclic-light-reared ones (Boyer et al., 2012; Ueda et al., 2016). It is important to note however, that photodegradation of *bis*-retinoids lowers lipofuscin levels in cyclic-light-reared mice (Ueda et al., 2016), blunting the observable contribution of all-*trans* retinal. Focusing on the reactions in rod outer segments and the origins of lipofuscin, the degree to which all-*trans* retinal contributes to lipofuscin generation will depend on the pathways that facilitate its removal after its release from photoactivated rhodopsin.

The removal of all-*trans* retinal has been proposed to be facilitated by the ABCA4 transporter protein, defects in which are associated with recessive Stargardt disease. Defects in ABCA4 also result in increased accumulation of lipofuscin in the RPE (Boyer et al., 2012; Weng et al., 1999), as well as increased levels of lipofuscin precursors in rod photoreceptor outer segments (Boyer et al., 2012). In terms of function, ABCA4 is known to catalyze the translocation of phosphatidylethanolamine and its Schiff bases with 11-*cis* and all-*trans* retinal, from the lumen to the cytosolic side of the rod outer segment membrane disks (Quazi et al., 2012). Thus, loss of ABCA4 function would result in trapping of all-*trans* retinal in the disk lumen, preventing its access to RDH8 and reduction to all-*trans* retinol. The build-up of all-*trans* retinal would result in increased formation of lipofuscin precursors in the rod outer segments followed by increased deposition of lipofuscin in the RPE of *Abca4*$^{-/-}$ mice.

Because of the cytotoxicity of lipofuscin, conditions that can promote its formation, such as the lack of the Abca4 transporter protein or the buildup of all-*trans* retinal, have received a lot of attention. Previous studies have relied on biochemical measurements, which cannot distinguish between the all-*trans* retinal that has been released from photoactivated rhodopsin and the one that is still covalently bound to it (in the form of the photointermediates metarhodopsin II and III) – in biochemical measurements all of the all-*trans* retinal is extracted and quantified, not just the released. Here, we have used fluorescence imaging to measure all-*trans* retinol, all-*trans* retinal, and lipofuscin precursors in single rod photoreceptors isolated from the retinas of wild type Sv/129 and *Abca4*$^{-/-}$ mice. We have used these measurements to compare the appearance and removal of the all-*trans* retinal generated by light in wild type and *Abca4*$^{-/-}$ rods. We have also examined whether the increased accumulation of all-*trans* retinal brought about by limited availability of metabolic substrate results in increased formation of lipofuscin precursor fluorophores.

2. MATERIALS AND METHODS

2.1 Animals

Wild type Sv/129 and *Abca4*$^{-/-}$ transgenic mice originated from established colonies at the Medical University of South Carolina. Sv/129 mice were originally obtained from Harlan Laboratories (Indianapolis, IN); breeding pairs of *Abca4*$^{-/-}$ animals were generous gifts of Dr. G.H. Travis. The background strain of the *Abca4*$^{-/-}$ animals was Sv/129. 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 followed National Institutes of Health guidelines for care and use of Laboratory animals. Animals were reared in cyclic light, 12-h light cycle (06:00–18:00); light intensity at cage level during the light cycle was 130 – 170 lux, measured with a light meter (LM631; Meterman Test Tools, Everett, WA).

2.2 Fluorescence imaging of isolated rods

For experiments, 2–3 months old animals were dark-adapted overnight and sacrificed under dim red light. Single isolated rod photoreceptor cells were obtained and imaged at 37 °C in physiological solution on a Zeiss Axiovert 100 epifluorescence microscope, as described previously (Chen et al., 2009). Both metabolically intact rods (with attached ellipsoids) and metabolically compromised broken off rod outer segments (ROS) were used (Chen and Koutalos, 2010). The physiological solutions used for experiments contained different concentrations of glucose or glutamine as metabolic substrates. For all-*trans* retinal and all-*trans* retinol measurements, fluorescence was excited by 340 and 380 nm light with emission collected >420 nm (Chen et al., 2012). For lipofuscin precursor measurements, fluorescence was excited with 490 nm light and emission collected >515 nm (Boyer et al., 2012). Data were collected and analyzed using Slidebook (Intelligent Imaging Innovations, Denver, CO).

2.3 Separation of the fluorescence signals of all-*trans* retinol and all-*trans* retinal

It is not straightforward experimentally to separate the fluorescence signals of all-*trans* retinol and all-*trans* retinal by using different excitation filters. With glass optics, 340 nm is the lowest wavelength that can be used, which is only the isosbestic point for retinal (absorption λ_{max}~380 nm) and retinol (absorption λ_{max}~325 nm) (Chen et al., 2012). Because of this constraint, fluorescence measurements were carried out with 340 and 380 nm excitation light (emission >420 nm), providing the fluorescence intensities Fex-340 and Fex-380, respectively. The fluorescence signals of retinol and retinal both contribute to Fex-340 and Fex-380. If Fex-λ is the rod outer segment fluorescence excited with light of wavelength λ (λ = 340 or 380 nm), and Fex-λ(ROL) and Fex-λ(RAL) the contributions of retinol and retinal fluorescence, respectively, then

$$\text{Fex-}\ \lambda = \text{Fex-}\ \lambda(\text{ROL}) + \text{Fex-}\ \lambda(\text{RAL}) \quad (1)$$

The contributions of retinol and retinal are proportional to the respective extinction coefficients e-λ(ROL) and e-λ(RAL) at wavelength λ, the fluorescence quantum yields

Q(ROL) and Q(RAL) (which do not depend on wavelength), and their outer segment concentrations [ROL] and [RAL]. That is,

$$\text{Fex-}\ \lambda(\text{ROL}) \propto \varepsilon\text{-}\ \lambda(\text{ROL}) \times \text{Q}(\text{ROL}) \times [\text{ROL}] \quad (2a)$$

$$\text{Fex-}\ \lambda(\text{RAL}) \propto \varepsilon\text{-}\ \lambda(\text{RAL}) \times \text{Q}(\text{RAL}) \times [\text{RAL}] \quad (2b)$$

To estimate the contributions Fex-λ(ROL) and Fex-λ(RAL) of retinol and retinal to the overall outer segment fluorescence signal Fex-λ, it is necessary to know the relative fluorescence quantum yield Q(ROL)/Q(RAL). This however is not known with sufficient accuracy (Chen et al., 2012), hence in general it is not possible to separate the contributions of retinol and retinal using the measurements of Fex-340 and Fex-380. It is only in special cases where the contributions can be separated by simplifying Eq. 1.

One special case obtains under physiological metabolic conditions for metabolically intact rods, that is, in the presence of 5 mM glucose as metabolic substrate, when >70–80% of the all-*trans* retinal released after bleaching is converted to all-*trans* retinol. Under these conditions, because e-340(ROL) ≈ e-340(RAL), Q(ROL)/Q(RAL) ≥ 5, and [ROL]/[RAL] ≥ 3–4, all-*trans* retinal contributes less than 10% of the fluorescence excited by 340 nm light. So,

$$\text{Fex-}\ 340 \approx \text{Fex-}\ 340(\text{ROL}) \quad (3)$$

that is, Fex-340, the fluorescence excited by 340 nm light, is mostly due to all-*trans* retinol and can be used as a measure of its level. On the other hand, Fex-380, the fluorescence excited by 380 nm light, contains significant contributions by both retinol and retinal, even in this case. These contributions can however be separated using Eq. 3 and the fluorescence intensity ratio

$$\text{Rex}(\text{ROL}) = \text{Fex-}\ 340(\text{ROL}) / \text{Fex-}\ 380(\text{ROL}) = 6.95 \quad (4)$$

the value of which has been determined experimentally previously (Adler et al., 2014; Chen et al., 2012). From Eq. 1,

$$\text{Fex-}\ 380(\text{RAL}) = \text{Fex-}\ 380 - \text{Fex-}\ 380(\text{ROL}) \quad (5)$$

Substituting from Eqs. 3 and 4, and rewriting Eq. 3, we obtain

$$\text{Fex- } 380(\text{RAL}) \approx \text{Fex- } 380 - \text{Fex- } 340/\text{Rex}(\text{ROL}) \quad (6a)$$

$$\text{Fex- } 340(\text{ROL}) \approx \text{Fex- } 340 \quad (6b)$$

Eqs. 6a and 6b provide separate measures of the retinal and retinol levels. It should be emphasized that these equations hold only under the specific conditions of metabolically intact rods in the presence of 5 mM glucose.

2.4 Statistical analysis

Error bars denote SEM. Linear regression was used to test for statistical significance.

3. RESULTS

3.1 Levels of all-*trans* retinol and all-*trans* retinal after bleaching in single isolated rods from wild type and *Abca4*$^{-/-}$ mice

The outer segment fluorescence of a dark-adapted rod photoreceptor excited by UV light increases after bleaching. This increase in fluorescence is due to the all-*trans* retinal and all-*trans* retinol generated by bleaching. In a metabolically intact wild type rod photoreceptor with access to non-limiting metabolic substrate concentrations, the intensity of the fluorescence excited by 340 nm light, Fex-340, is much higher than that excited by 380 nm, Fex-380 (Fig. 1A). The high Fex-340/Fex-380 ratio reflects the conversion of the greater part of the all-*trans* retinal released after bleaching to all-*trans* retinol, a conversion that is made possible by the generation of the requisite quantities of NADPH. Fig. 1B shows the values of the rod outer segment fluorescence excited by 340 and 380 nm, Fex-340 and Fex-380, measured at different times after bleaching. From the values of Fex-340 and Fex-380, the fluorescence signals due to all-*trans* retinal, Fex-380(RAL), and all-*trans* retinol, Fex-340(ROL), were calculated using Eqs. 6a and 6b, for each time point after bleaching. Fig. 2 shows that a metabolically intact *Abca4*$^{-/-}$ rod photoreceptor also converts the greater part of the all-*trans* retinal released after bleaching to all-*trans* retinol. Fig. 3 compares the fluorescence signals due to the all-*trans* retinol and all-*trans* retinal generated after bleaching in Sv/129 (n = 12) and *Abca4*$^{-/-}$ (n = 13) rods. There was no detectable difference in the all-*trans* retinal levels between wild type (○) and *Abca4*$^{-/-}$ (●) rods at all times after bleaching. There was also no difference in the all-*trans* retinol levels at early times (up to 10 min) after bleaching. At later times however (longer than 20 min), all-*trans* retinol levels were consistently lower in *Abca4*$^{-/-}$ (▲) rods compared to wild type (△), although this reduction in levels was statistically significant only at 20 min ($p = 0.028$; Student's t-test).

3.2 Lipofuscin precursor formation after bleaching in single isolated rods from wild type and *Abca4*$^{-/-}$ mice

The outer segments of rod photoreceptors from wild type and *Abca4*$^{-/-}$ mice contain fluorophores that originate from reactions of 11-*cis* and all-*trans* retinal, and their fluorescence emission spectrum (490 nm light excitation) is similar to that of lipofuscin. We refer to them as lipofuscin precursors and their fluorescence is measured by exciting with 490 nm light and collecting the emission >515 nm (Boyer et al., 2012). At the same time, after bleaching a dark-adapted rod the extent of the conversion of the all-*trans* retinal released from photoactivated rhodopsin to all-*trans* retinol can be measured from the Fex-340/Fex-380 fluorescence ratio. Then, the Fex-340/Fex-380 ratio can be used as a measure of the accumulation of all-*trans* retinal after bleaching: the lower the ratio, the higher the accumulation of all-*trans* retinal. Thus, by measuring these different fluorescence signals, we can examine whether accumulation of all-*trans* retinal results in increased formation of lipofuscin precursors. For metabolically intact rod photoreceptors in the presence of 5 mM glucose, the lipofuscin precursor fluorescence at 30 min after light exposure (Fig. 4A and Fig. 4C, □) is similar to that in the dark-adapted cell (Fig. 4A and Fig. 4C, ■), in agreement with previous work (Boyer et al., 2012)). Under these conditions, there is also limited accumulation of all-*trans* retinal released from photoactivated rhodopsin: it is quantitatively reduced to all-*trans* retinol, as denoted by the high value of the Fex-340/Fex-380 ratio (Fig. 4A and Fig. 4C, □), in agreement with previous work (Adler et al., 2014)). In the absence of metabolic substrate, at 30 min after light exposure the Fex-340/Fex-380 ratio was lower (Fig. 4B and Fig. 4C, ◦), indicating the accumulation of all-*trans* retinal; at the same time, lipofuscin precursor fluorescence (Fig. 4B and Fig. 4C, ◦) increased substantially compared to the dark-adapted cell (Fig. 4B and Fig. 4C, ◆). Low concentrations of metabolic substrate have previously been shown to result in accumulation of all-*trans* retinal (Adler et al., 2014). Measuring the fluorescence of lipofuscin precursors in the same cells before and after bleaching, provided the relation between the accumulation of all-*trans* retinal and the generation of lipofuscin precursors (Fig. 5). In wild type Sv/129 rods, limiting the availability of metabolic substrate increased the accumulation of all-*trans* retinal, and at the same time increased the levels of lipofuscin precursors after bleaching (Fig. 5, ○; a, 5 mM glucose, n = 9 cells; b, 0.5 mM glutamine, n = 7; c, 50 μM glucose, n = 17; d, 50 μM glutamine, n = 16; e, 0 substrate, n =12; f, broken-off rod outer segments, n =11).. The basal levels of lipofuscin precursor fluorescence in dark-adapted rods, before bleaching, did not vary significantly across the different conditions and were averaged for all wild type rods (Fig. 5, ●, n = 72 cells). Similar results were obtained in *Abca4*$^{-/-}$ rods, (Fig. 5, △; a, 5 mM glucose, n = 9 cells; b, 0.5 mM glutamine, n = 8; c, 50 μM glucose, n = 8; d, 50 μM glutamine, n = 9; e, 0 substrate, n =9; f, broken-off rod outer segments, n =7). The basal levels of lipofuscin precursor fluorescence in dark-adapted *Abca4*$^{-/-}$ rods, before bleaching, did not vary significantly across the different conditions and were averaged (Fig. 5, ▲, n = 50 cells); as expected, they were higher than the basal levels in wild type rods. Thus, after light exposure, lipofuscin precursor fluorescence increased with increased accumulation of all-*trans* retinal in rods from both Sv/129 wild type (Fig. 5, ○; $p < 0.001$, linear regression) and *Abca4*$^{-/-}$ mice (Fig. 5, △; $p < 0.002$, linear regression).

4. DISCUSSION

We have used fluorescence imaging of single photoreceptor cells to measure the levels of all-*trans* retinol, all-*trans* retinal, and lipofuscin precursors in real time in a living cell and at physiological concentrations. Such single cell fluorescence measurements have important advantages as well as limitations. One important advantage is that of measuring the all-*trans* retinal that has been released from photoactivated rhodopsin, while biochemical measurements measure the total all-*trans* retinal, including the one that is still covalently bound in the form of the photointermediates metarhodopsin II and III, or as a Schiff base with phosphatidylethanolamine. One important limitation of fluorescence measurements is that because the measuring light bleaches the photopigment, meaningful measurements can be carried out only following a full bleach. Another important limitation is that the fluorescence signal does not by itself uniquely identify the fluorophores. This particular limitation has been addressed in previous experiments that included measurements of emission spectra, which support the assignment of the UV-excited fluorescence signals to all-*trans* retinol and retinal (Chen et al., 2012) and the 490 nm-excited signal to retinal-derived lipofuscin precursors (Boyer et al., 2012). The fluorescence signals excited by 340 and 380 nm, Fex-340 and Fex-380, as well as their intensity ratio, have been used before to partially separate the signals from all-*trans* retinal and all-*trans* retinol and estimate the conversion of the former to the latter (Adler et al., 2014; Chen et al., 2012). Using this assay, experiments with $Rdh8^{-/-}$ and $Rdh12^{-/-}$ mice showed that lack of the Rdh8 or Rdh12 dehydrogenase enzymes suppresses the conversion of all-*trans* retinal to all-*trans* retinol in the compartment that lacks a dehydrogenase (Chen et al., 2012). In metabolically intact rods and in the absence of metabolic limitations, the signal due to all-*trans* retinol dominates the fluorescence measured with 340 and 360 nm excitation and therefore can be measured reliably. Moreover, because all of all-*trans* retinol in the rod outer segment is virtually free (Wu et al., 2006) the pool giving rise to the fluorescence signal is the same with the one measured biochemically. Comparison of fluorescence and biochemical measurements of all-*trans* retinol shows that they are in good agreement ((Blakeley et al., 2011; Chen et al., 2012), see also (Adler et al., 2014)). However, due to the reasons given above, such comparison between fluorescence and biochemical measurements is not possible for all-*trans* retinal. In addition, and in contrast to all-*trans* retinol, there is no fluorescence signal that is dominated by all-*trans* retinal, which necessitates calculating its signal through a subtraction procedure (Eq. 6a). This introduces a level of uncertainty in the fluorescence measurement of all-*trans* retinal. In contrast to all-*trans* retinol and all-*trans* retinal, the chemical identity of the lipofuscin precursor fluorophores is unclear. They are likely to be *bis*-retinoids, but all-*trans* retinal Schiff bases may contribute to the fluorescence signal as well. Direct comparisons with biochemical experiments are lacking, with an important difference being that precursors form rapidly in cells, while *bis*-retinoids form very slowly *in vitro*.

All-*trans* and 11-*cis* retinal have both been shown to generate lipofuscin precursor fluorophores and *bis*-retinoids when added to rod outer segments and outer segment membranes (Ben-Shabat et al., 2002; Boyer et al., 2012; Quazi and Molday, 2014). In rod outer segments *in situ*, lipofuscin precursors could be generated from the all-*trans* retinal

released by photoactivated rhodopsin following light absorption, or from the 11-*cis* retinal provided by the RPE for the regeneration of opsin to rhodopsin. Abca4 has been shown to facilitate the translocation of both all-*trans* and 11-*cis* retinal, in the form of their Schiff bases with phosphatidylethanolamine, N-retinylidene-phosphatidylethanolamine (NRPE), from the lumen to the cytosolic side of disk membranes. On the cytosolic side of disk membranes, both all-*trans* and 11-*cis* retinal can be cleared by Rdh8, the rod outer segment retinol dedydrogenase enzyme. Although Rdh8 is specific for the all-*trans* isomer of retinal, phosphatidylethanolamine catalyzes the isomerization of 11-*cis* retinal to all-*trans*, converting 11-*cis* retinal into a substrate for Rdh8 as well (Quazi and Molday, 2014). On this basis, Abca4 has been proposed to allow for the faster clearance of any excess all-*trans* or 11-*cis* retinal. This mechanism can readily explain the higher levels of lipofuscin precursors present in the outer segments of rod photoreceptors from *Abca4*$^{-/-}$ mice, and the large accumulation of lipofuscin and *bis*-retinoids in the RPE of *Abca4*$^{-/-}$ mice and of Stargardt patients with defects in ABCA4.

This same mechanism would also predict that, following light exposure, higher levels of all-*trans* retinal would accumulate in *Abca4*$^{-/-}$ rod outer segments compared to wild type. This prediction is not borne out by the experiments shown in Fig. 3, which find no difference in the all-*trans* retinal levels between wild type and *Abca4*$^{-/-}$ rods. Fig. 3 does show somewhat lower levels of all-*trans* retinol in *Abca4*$^{-/-}$ rods at later times after bleaching. These results are in good agreement with our previous biochemical and fluorescence measurements of all-*trans* retinol formation kinetics in the retinas of *Abca4*$^{-/-}$ and wild type mice (Blakeley et al., 2011). They are however, only in partial agreement with the original report that, following light exposure, there is an elevation of all-*trans* retinal and a decline in all-*trans* retinol in *Abca4*$^{-/-}$ retinas compared to wild type (Weng et al., 1999). A factor complicating a direct comparison between the different studies is that in the original report the mice were anesthetized. A possible explanation that could reconcile the present experiments with those in the original report is that the elevated all-*trans* retinal levels found in the *Abca4*$^{-/-}$ originate solely from NRPE, that is, from its Schiff base with phosphatidylethanolamine. This would also explain the reduction in the all-*trans* retinol levels observed in both studies. Following the proposal in the initial study (Weng et al., 1999), this explanation posits that some of the all-*trans* retinal released by photoactivated rhodopsin forms a relatively stable NRPE in the disk lumen. This NRPE is not in rapid equilibrium with free all-*trans* retinal, it is trapped in the disk lumen, and can initiate *bis*-retinoid formation. In wild type rods, Abca4 translocates NRPE from the lumen to the cytosolic side of the disk, where the NRPE is hydrolyzed and all-*trans* retinal is reduced by Rdh8 – this process eliminates the trapped NRPE and limits *bis*-retinoid formation. In the absence of Abca4, NRPE is trapped in the disk lumen, giving rise to higher levels of total extracted all-*trans* retinal. Lack of Abca4 does not affect all-*trans* retinol formation at early times, but makes some difference at later times (Fig. 3), as some of the released all-*trans* retinal that has not been reduced ends up trapped as NRPE in the lumen. Lack of Abca4 also does not affect the levels of free all-*trans* retinal, which is at equilibrium with all-*trans* retinol but not with NRPE. The portion of all-*trans* retinal handled by Abca4 is that which is trapped as NRPE in the lumen of the disk. Judging from the reduction in all-*trans* retinol levels in *Abca4*$^{-/-}$ rods at later times (Fig. 3), this portion appears to be no more than 20–30%, in agreement with previous estimates

(Lamb and Pugh, 2004). Thus, any accumulation of all-*trans* NRPE in *Abca4*$^{-/-}$ rods, although it can lead to excess formation of *bis*-retinoids, is not associated with accumulation of free all-*trans* retinal. However, such an excess formation of *bis*-retinoids in metabolically intact *Abca4*$^{-/-}$ rods was not detected in the experiments shown in Fig. 5, as the levels of lipofuscin precursor fluorophores following bleaching in 5 mM glucose are not significantly increased compared to those in the dark-adapted state (see also (Boyer et al., 2012)). A possible explanation is that this increase in lipofuscin precursor levels is below the resolution of the measurements.

The same argument, applied in the case of 11-*cis* retinal, can also account for the higher levels of lipofuscin fluorescence and *bis*-retinoids found in dark-reared *Abca4*$^{-/-}$ mice compared to wild type. Although 11-*cis* retinal is a poor substrate for Rdh8, rod outer segment lipids catalyze its isomerization to all-*trans*, allowing its reduction (Quazi and Molday, 2014). However, any 11-*cis* retinal that flows into the rod outer segment and does not end up binding to opsin, may be trapped in the disk lumen as a Schiff base with phosphatidylethanolamine and initiate *bis*-retinoid formation. Abca4 will translocate the 11-*cis* retinal Schiff base from the lumen to the cytosolic side of the disk, facilitating the eventual removal of 11-*cis* retinal (Quazi and Molday, 2014). Lack of Abca4 will result in the trapping of the 11-*cis* retinal Schiff base, diminished clearance of 11-*cis* retinal, and higher accumulation of lipofuscin compared to wild type.

It has previously been shown that in metabolically intact rod photoreceptors from wild type mice, and in the presence of adequate metabolic substrate, the all-*trans* retinal released from photoactivated rhodopsin is quantitatively converted to all-*trans* retinol (Chen and Koutalos, 2010; Chen et al., 2012), and there is no significant increase in lipofuscin precursor levels (Boyer et al., 2012). On the other hand, in broken-off rod outer segments, which have no access to NADPH and cannot reduce all-*trans* retinal (Chen et al., 2012), there is a significant increase in lipofuscin precursor levels following light exposure (Boyer et al., 2012). The experiments shown in Fig. 5 extend these results to intermediate levels of all-*trans* retinal accumulation. This was achieved by supplying the cells with low metabolic substrate concentrations and in this way suppressing the generation of NADPH to different extents. For both wild type and *Abca4*$^{-/-}$ rods, higher levels of all-*trans* retinal are associated with higher formation of lipofuscin precursors. For rod photoreceptors *in situ*, this would suggest that perturbations in metabolic activity during light exposure would result in increased formation of lipofuscin. The results cannot exclude the possibility that some lipofuscin precursors form from all-*trans* retinal even in the presence of adequate metabolic substrate, at levels that are not resolved by the present measurements. Indeed, and as discussed above, in intact *Abca4*$^{-/-}$ rods in the presence of 5 mM glucose, the results are consistent with some increase in the levels of lipofuscin precursors after bleaching originating from all-*trans* retinal trapped in the form of NRPE; but this increase is not detected in the experiments shown in Fig. 5. It is important to emphasize that the increased formation of lipofuscin precursors shown in Fig. 5 is due to the inadequate supply of NADPH brought about by the metabolic perturbations, which result in the accumulation of all-*trans* retinal. The higher levels of lipofuscin precursors found in *Abca4*$^{-/-}$ rods compared to wild type (and seen clearly in Fig. 5) do not originate from lack of NADPH and are not associated with any accumulation of all-*trans* retinal.

CONCLUSIONS

The present work shows that lack of Abca4 does not affect the formation of all-*trans* retinol at early times after light exposure; at later times however, all-*trans* retinol levels are slightly lower in *Abca4*$^{-/-}$ rods, consistent with some of the all-*trans* retinal being unavailable for reduction. Following light exposure, there is no accumulation of all-*trans* retinal in *Abca4*$^{-/-}$ rods compared to wild type, likely because all-*trans* retinal is trapped as a Schiff base or forms *bis*-retinoids. In both *Abca4*$^{-/-}$ and wild type rods, exposure to low metabolic substrate concentrations results in accumulation of all-*trans* retinal, which is associated with formation of higher levels of lipofuscin precursors.

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References

Adler L 4th, Chen C, Koutalos Y. Mitochondria contribute to NADPH generation in mouse rod photoreceptors. J Biol Chem. 2014; 289:1519–28. [PubMed: 24297174]

Ben-Shabat S, Parish CA, Vollmer HR, Itagaki Y, Fishkin N, Nakanishi K, Sparrow JR. Biosynthetic studies of A2E, a major fluorophore of retinal pigment epithelial lipofuscin. J Biol Chem. 2002; 277:7183–90. [PubMed: 11756445]

Blakeley LR, Chen C, Chen CK, Chen J, Crouch RK, Travis GH, Koutalos Y. Rod outer segment retinol formation is independent of Abca4, arrestin, rhodopsin kinase, and rhodopsin palmitylation. Invest Ophthalmol Vis Sci. 2011; 52:3483–91. [PubMed: 21398289]

Boyer NP, Higbee D, Currin MB, Blakeley LR, Chen C, Ablonczy Z, Crouch RK, Koutalos Y. Lipofuscin and N-retinylidene-N-retinylethanolamine (A2E) accumulate in retinal pigment epithelium in absence of light exposure: their origin is 11-cis-retinal. J Biol Chem. 2012; 287:22276–86. [PubMed: 22570475]

Chen C, Blakeley LR, Koutalos Y. Formation of all-trans retinol after visual pigment bleaching in mouse photoreceptors. Invest Ophthalmol Vis Sci. 2009; 50:3589–95. [PubMed: 19264891]

Chen C, Koutalos Y. Rapid formation of all-trans retinol after bleaching in frog and mouse rod photoreceptor outer segments. Photochem Photobiol Sci. 2010; 9:1475–9. [PubMed: 20697621]

Chen C, Thompson DA, Koutalos Y. Reduction of all-trans-retinal in vertebrate rod photoreceptors requires the combined action of RDH8 and RDH12. J Biol Chem. 2012; 287:24662–70. [PubMed: 22621924]

Delori FC, Dorey CK, Staurenghi G, Arend O, Goger DG, Weiter JJ. In vivo fluorescence of the ocular fundus exhibits retinal pigment epithelium lipofuscin characteristics. Invest Ophthalmol Vis Sci. 1995a; 36:718–29. [PubMed: 7890502]

Delori FC, Goger DG, Dorey CK. Age-related accumulation and spatial distribution of lipofuscin in RPE of normal subjects. Invest Ophthalmol Vis Sci. 2001; 42:1855–66. [PubMed: 11431454]

Delori FC, Staurenghi G, Arend O, Dorey CK, Goger DG, Weiter JJ. In vivo measurement of lipofuscin in Stargardt's disease--Fundus flavimaculatus. Invest Ophthalmol Vis Sci. 1995b; 36:2327–31. [PubMed: 7558729]

Ebrey T, Koutalos Y. Vertebrate photoreceptors. Prog Retin Eye Res. 2001; 20:49–94. [PubMed: 11070368]

Eldred GE, Lasky MR. Retinal age pigments generated by self-assembling lysosomotropic detergents. Nature. 1993; 361:724–6. [PubMed: 8441466]

Feeney L. Lipofuscin and melanin of human retinal pigment epithelium. Fluorescence, enzyme cytochemical, and ultrastructural studies. Invest Ophthalmol Vis Sci. 1978; 17:583–600. [PubMed: 669890]

Futterman S, Hendrickson A, Bishop PE, Rollins MH, Vacano E. Metabolism of glucose and reduction of retinaldehyde in retinal photoreceptors. J Neurochem. 1970; 17:149–56. [PubMed: 4395446]

Katz ML, Drea CM, Eldred GE, Hess HH, Robison WG Jr. Influence of early photoreceptor degeneration on lipofuscin in the retinal pigment epithelium. Exp Eye Res. 1986; 43:561–73. [PubMed: 3792460]

Katz ML, Redmond TM. Effect of Rpe65 knockout on accumulation of lipofuscin fluorophores in the retinal pigment epithelium. Invest Ophthalmol Vis Sci. 2001; 42:3023–30. [PubMed: 11687551]

Katz ML, Robison WG Jr. What is lipofuscin? Defining characteristics and differentiation from other autofluorescent lysosomal storage bodies. Arch Gerontol Geriatr. 2002; 34:169–84. [PubMed: 14764321]

Lakkaraju A, Finnemann SC, Rodriguez-Boulan E. The lipofuscin fluorophore A2E perturbs cholesterol metabolism in retinal pigment epithelial cells. Proc Natl Acad Sci U S A. 2007; 104:11026–31. [PubMed: 17578916]

Lamb TD, Pugh EN Jr. Dark adaptation and the retinoid cycle of vision. Prog Retin Eye Res. 2004; 23:307–80. [PubMed: 15177205]

Lenis TL, Jiang Z, Sarfare S, Le A, Eddington S, Bok D, Molday RS, Nusinowitz S, Redmond M, Travis GH, Radu RA. Elucidating the role of Retinal Pigment Epithelium (RPE)-specific ABCA4 in Stargardt disease. ARVO Meeting Abstracts. 2016; 57:3177.

Liu J, Itagaki Y, Ben-Shabat S, Nakanishi K, Sparrow JR. The biosynthesis of A2E, a fluorophore of aging retina, involves the formation of the precursor, A2-PE, in the photoreceptor outer segment membrane. J Biol Chem. 2000; 275:29354–60. [PubMed: 10887199]

Maeda A, Maeda T, Imanishi Y, Kuksa V, Alekseev A, Bronson JD, Zhang H, Zhu L, Sun W, Saperstein DA, Rieke F, Baehr W, Palczewski K. Role of photoreceptor-specific retinol dehydrogenase in the retinoid cycle in vivo. J Biol Chem. 2005; 280:18822–32. [PubMed: 15755727]

Mata NL, Weng J, Travis GH. Biosynthesis of a major lipofuscin fluorophore in mice and humans with ABCR-mediated retinal and macular degeneration. Proc Natl Acad Sci U S A. 2000; 97:7154–9. [PubMed: 10852960]

Parish CA, Hashimoto M, Nakanishi K, Dillon J, Sparrow J. Isolation and one-step preparation of A2E and iso-A2E, fluorophores from human retinal pigment epithelium. Proc Natl Acad Sci U S A. 1998; 95:14609–13. [PubMed: 9843937]

Quazi F, Lenevich S, Molday RS. ABCA4 is an N-retinylidene-phosphatidylethanolamine and phosphatidylethanolamine importer. Nat Commun. 2012; 3:925. [PubMed: 22735453]

Quazi F, Molday RS. ATP-binding cassette transporter ABCA4 and chemical isomerization protect photoreceptor cells from the toxic accumulation of excess 11-cis-retinal. Proc Natl Acad Sci U S A. 2014; 111:5024–9. [PubMed: 24707049]

Rozanowska M, Wessels J, Boulton M, Burke JM, Rodgers MA, Truscott TG, Sarna T. Blue light-induced singlet oxygen generation by retinal lipofuscin in non-polar media. Free Radic Biol Med. 1998; 24:1107–12. [PubMed: 9626564]

Saari JC. Biochemistry of visual pigment regeneration: the Friedenwald lecture. Invest Ophthalmol Vis Sci. 2000; 41:337–48. [PubMed: 10670460]

Sparrow JR, Boulton M. RPE lipofuscin and its role in retinal pathobiology. Exp Eye Res. 2005; 80:595–606. [PubMed: 15862166]

Sparrow JR, Gregory-Roberts E, Yamamoto K, Blonska A, Ghosh SK, Ueda K, Zhou J. The bisretinoids of retinal pigment epithelium. Prog Retin Eye Res. 2012; 31:121–35. [PubMed: 22209824]

Sparrow JR, Nakanishi K, Parish CA. The lipofuscin fluorophore A2E mediates blue light-induced damage to retinal pigmented epithelial cells. Invest Ophthalmol Vis Sci. 2000; 41:1981–9. [PubMed: 10845625]

Tang PH, Kono M, Koutalos Y, Ablonczy Z, Crouch RK. New insights into retinoid metabolism and cycling within the retina. Prog Retin Eye Res. 2013; 32:48–63. [PubMed: 23063666]

Ueda K, Zhao J, Kim HJ, Sparrow JR. Photodegradation of retinal bisretinoids in mouse models and implications for macular degeneration. Proc Natl Acad Sci U S A. 2016; 113:6904–9. [PubMed: 27274068]

Vives-Bauza C, Anand M, Shirazi AK, Magrane J, Gao J, Vollmer-Snarr HR, Manfredi G, Finnemann SC. The age lipid A2E and mitochondrial dysfunction synergistically impair phagocytosis by retinal pigment epithelial cells. J Biol Chem. 2008; 283:24770–80. [PubMed: 18621729]

Weng J, Mata NL, Azarian SM, Tzekov RT, Birch DG, Travis GH. Insights into the function of Rim protein in photoreceptors and etiology of Stargardt's disease from the phenotype in abcr knockout mice. Cell. 1999; 98:13–23. [PubMed: 10412977]

Wing GL, Blanchard GC, Weiter JJ. The topography and age relationship of lipofuscin concentration in the retinal pigment epithelium. Invest Ophthalmol Vis Sci. 1978; 17:601–7. [PubMed: 669891]

Winkler BS, Boulton ME, Gottsch JD, Sternberg P. Oxidative damage and age-related macular degeneration. Mol Vis. 1999; 5:32. [PubMed: 10562656]

Wu Q, Chen C, Koutalos Y. All-trans retinol in rod photoreceptor outer segments moves unrestrictedly by passive diffusion. Biophys J. 2006; 91:4678–89. [PubMed: 17012326]

Wu Y, Fishkin NE, Pande A, Pande J, Sparrow JR. Novel lipofuscin bisretinoids prominent in human retina and in a model of recessive Stargardt disease. J Biol Chem. 2009; 284:20155–66. [PubMed: 19478335]

Highlights

- Fluorescence imaging of retinal, retinol, and lipofuscin precursors in single rods
- Accumulation of light-generated free all-*trans* retinal is unaffected by lack of Abca4
- Metabolic limitations result in all-*trans* retinal accumulation
- Metabolic limitations lead to increased formation of lipofuscin precursors

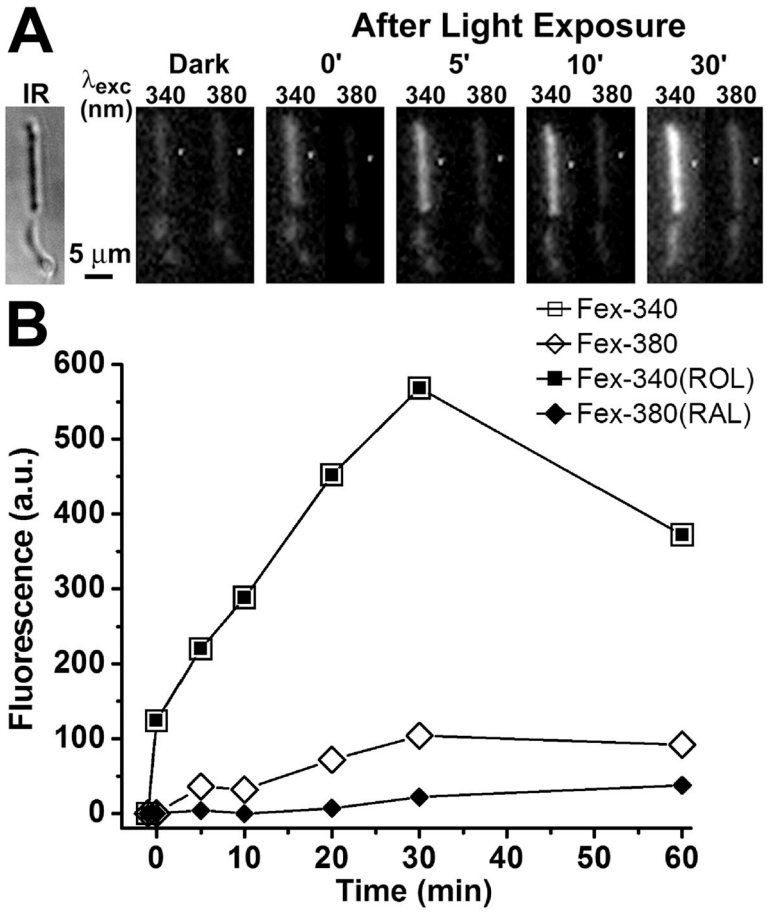

Figure 1.

Measurements of fluorescence of all-*trans* retinol and all-*trans* retinal in a metabolically intact rod photoreceptor isolated from a dark-adapted Sv/129 wild type mouse (2 month old). The fluorescence signals due to retinol and retinal can be separated by exciting rod outer segment fluorescence with 340 and 380 nm light and collecting emission >420 nm. (A) Infrared (IR) and fluorescence images of the cell before (Dark) and at different times after bleaching; to facilitate comparisons, the fluorescence images of the cell are shown with the same intensity scaling. Bleaching was carried out with long-wavelength (>530 nm) light for 1 min. (B) Intensities of the outer segment fluorescence excited by 340 and 380 nm light, and the fluorescence signals due to outer segment all-*trans* retinol, Fex-340(ROL), and all-*trans* retinal, Fex-380(RAL), at different times after bleaching. All-*trans* retinol is responsible for all of the fluorescence excited by 340 nm. Bleaching was carried out between t = −1 and t = 0 min. Experiment at 37 °C.

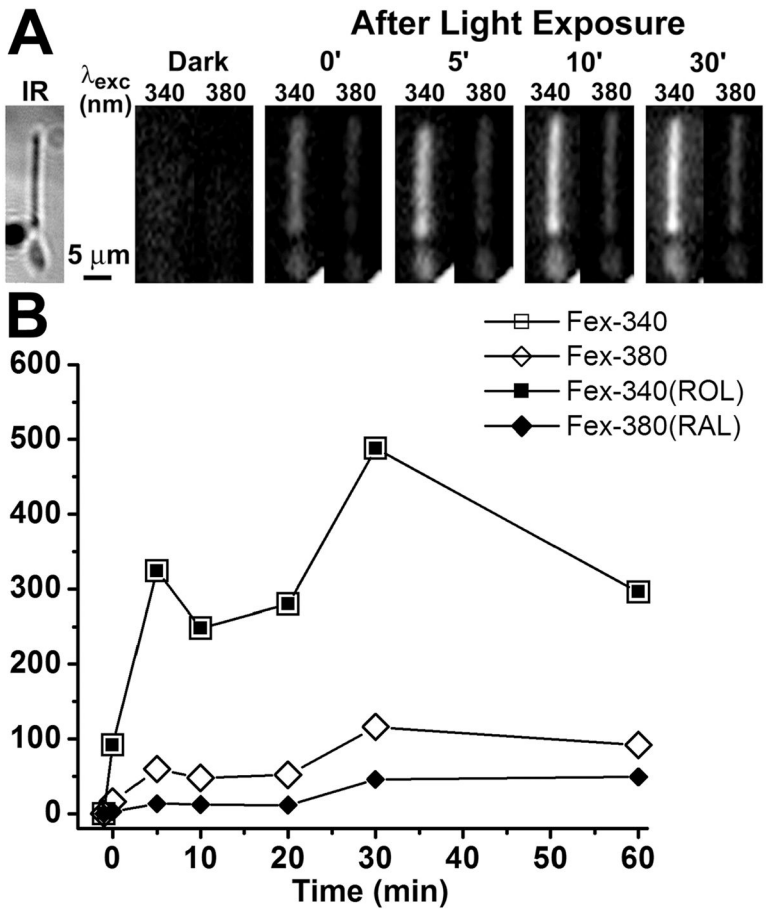

Figure 2.

Measurements of fluorescence of all-*trans* retinol and all-*trans* retinal in a metabolically intact rod photoreceptor isolated from a dark-adapted *Abca4*$^{-/-}$ mouse (2 month old). (A) Infrared (IR) and fluorescence images of the cell before (Dark) and at different times after bleaching; to facilitate comparisons, the fluorescence images of the cell are shown with the same intensity scaling. Bleaching was carried out with long-wavelength (>530 nm) light for 1 min. (B) Intensities of the outer segment fluorescence excited by 340 and 380 nm light, and the fluorescence signals due to outer segment all-*trans* retinol, Fex-340(ROL), and all-*trans* retinal, Fex-380(RAL), at different times after bleaching. All-*trans* retinol is responsible for all of the fluorescence excited by 340 nm. Bleaching was carried out between t = −1 and t = 0 min. Experiment at 37 °C.

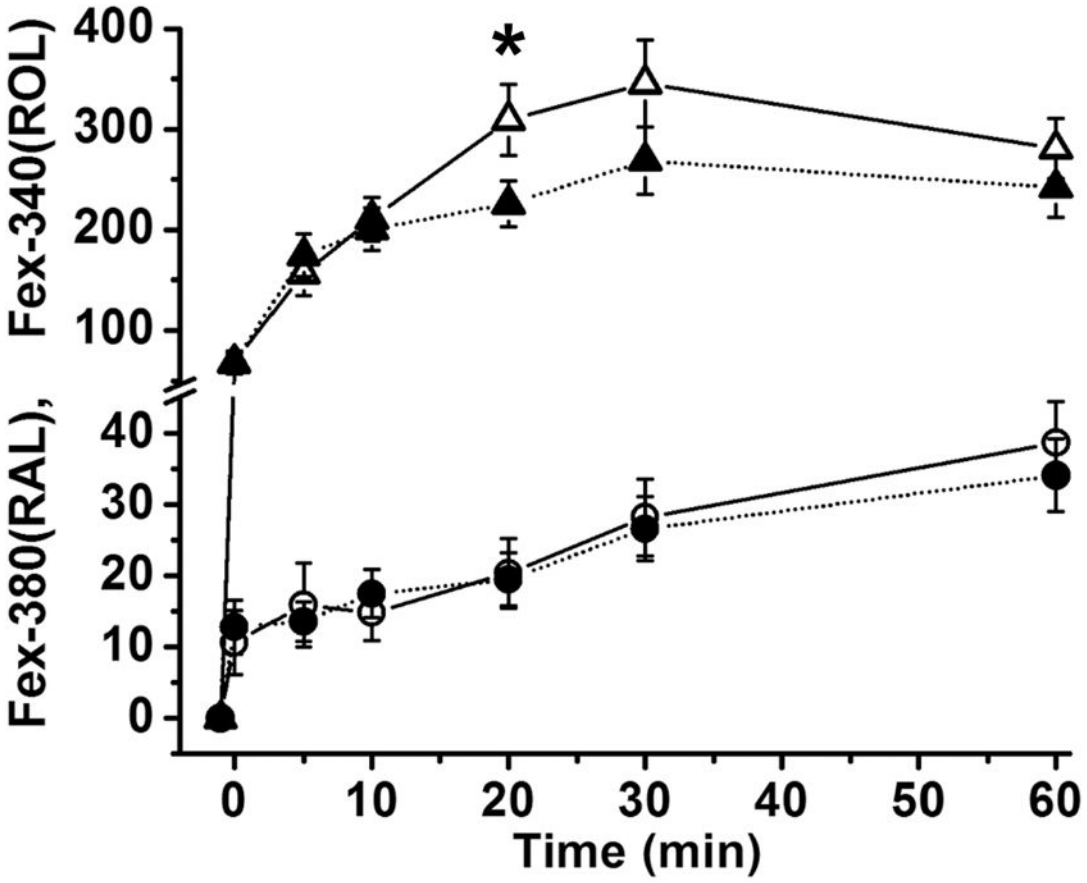

Figure 3.

The kinetics of all-*trans* retinal and all-*trans* retinol in metabolically intact rod photoreceptors isolated from dark-adapted Sv/129 wild type and *Abca4*$^{-/-}$ mice. All-*trans* retinol and all-*trans* retinal levels were measured from their contributions, Fex-340(ROL) and Fex-380(RAL), to the fluorescence excited by 340 and 380 nm, as shown in Figs. 1 and 2. Dark-adapted isolated cells were bleached with long-wavelength (>530 nm) light for 1 min (between t = −1 and t = 0 min). Fluorescence due to all-*trans* retinol was the same for Sv/129 wild type (△, n = 12) and *Abca4*$^{-/-}$ cells (▲, n = 13) at early times after bleaching, but was significantly lower in *Abca4*$^{-/-}$ cells at 20 min. The fluorescence due to all-*trans* retinal measured simultaneously was the same for Sv/129 wild type (○) and *Abca4*$^{-/-}$ (●) rods at all times after bleaching. Mice were 2 months old. Error bars represent standard errors. Experiments at 37 °C.

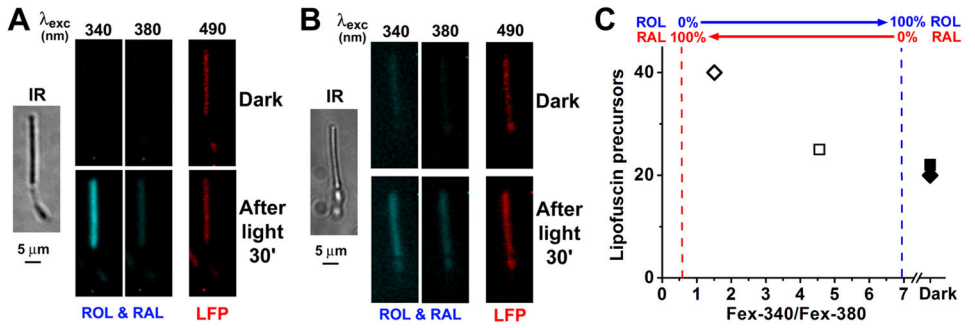

Figure 4.

Measurement of the fluorescence of all-*trans* retinol, all-*trans* retinal, and of lipofuscin precursors in metabolically intact rod photoreceptors isolated from dark-adapted Sv/129 wild type mice. Fluorescence signals due to retinol and retinal (ROL & RAL) were obtained by exciting rod outer segment fluorescence with 340 and 380 nm light and collecting emission >420 nm. Fluorescence signals due to lipofuscin precursors (LFP) were obtained with 490 nm excitation and collecting emission >515 nm. IR, infrared images of the cells; to facilitate comparisons, the fluorescence images of the cells before (Dark) and at 30 min after bleaching are shown with the same intensity scaling. Bleaching was carried out with long-wavelength (>530 nm) light for 1 min. (A) 5 mM glucose present as metabolic substrate. (B) No metabolic substrate present. (C) Relationship between lipofuscin precursor levels and all-*trans* retinal accumulation in the two cells. All-*trans* retinal accumulation was measured as the Fex-340/Fex-380 ratio. The value Fex-340/Fex-380 = 0.55 corresponds to 100% retinal-0% retinol; Fex-340/Fex-380 = 6.95 corresponds to 0% retinal-100% retinol. Initial levels of lipofuscin precursors were similar in the dark-adapted cells (5 mM glucose, ■; 0 glucose,◆). 30 min after bleaching, in 5 mM glucose, there was quantitative conversion of all-*trans* retinal to all-*trans* retinol and virtually no increase in the level of lipofuscin precursors (□); in 0 glucose, there was accumulation of all-*trans* retinal and a substantial increase in the level of lipofuscin precursors (◇). Experiments at 37 °C.

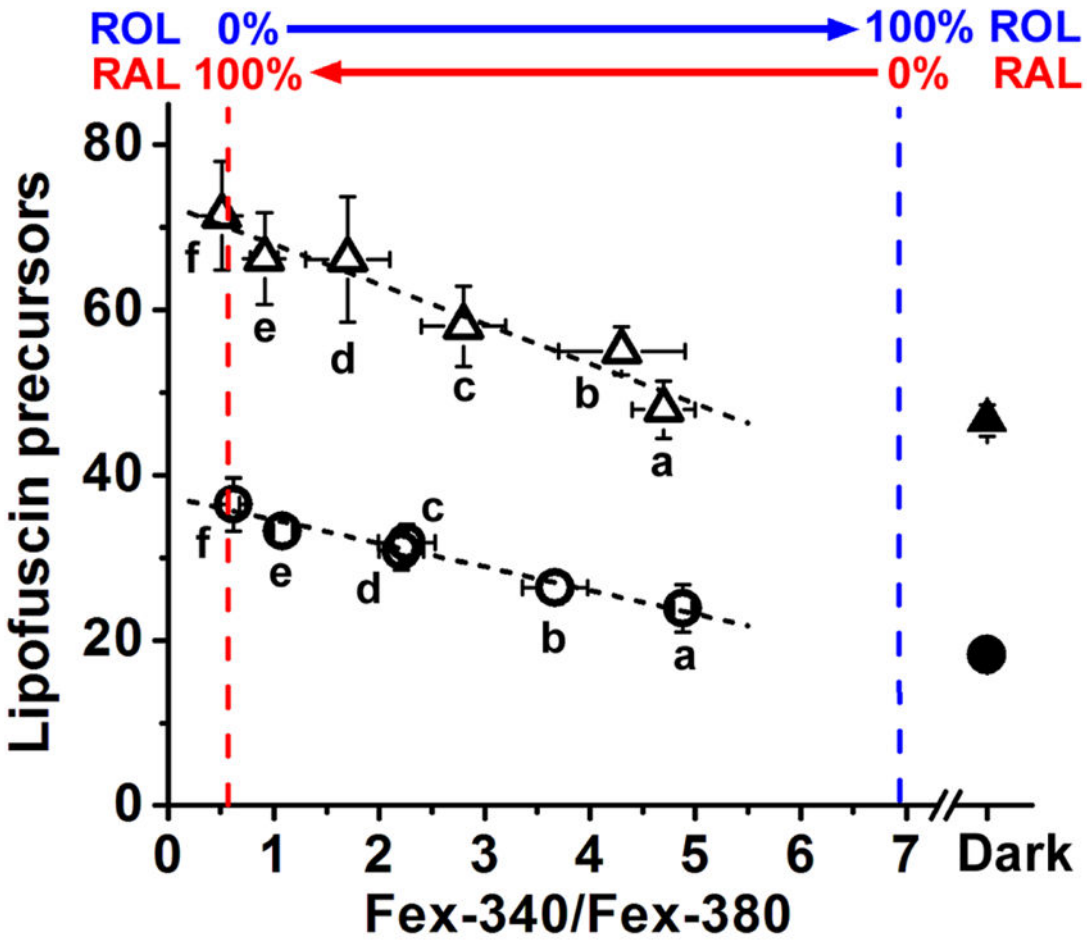

Figure 5.

Increased levels of all-*trans* retinal after bleaching result in increased formation of lipofuscin precursors in rod photoreceptors isolated from Sv/129 (○, ●; n = 72 cells total) and *Abca4*$^{-/-}$ (△, ▲; n = 50 cells total) mice. Lipofuscin precursor fluorescence was measured in isolated dark-adapted rods (●, ▲); subsequently, the cells were bleached with long-wavelength (>530 nm) light for 1 min and rod outer segment fluorescence signals from all-*trans* retinol, all-*trans* retinal and lipofuscin precursors measured at 30 min after bleaching (○, △). Retinol and retinal fluorescence were obtained by exciting with 340 and 380 nm light and collecting emission >420 nm. Lipofuscin precursor fluorescence was obtained with 490 nm excitation and collecting emission >515 nm. All-*trans* retinal accumulation was measured as the Fex-340/Fex-380 ratio. The value Fex-340/Fex-380 = 0.55 corresponds to 100% retinal-0% retinol; Fex-340/Fex-380 = 6.95 corresponds to 0% retinal-100% retinol. Different levels of all-*trans* retinal were brought about by using conditions that suppress the supply of NADPH to the rod outer segment. Conditions were: a, 5 mM glucose; b, 0.5 mM glutamine; c, 50 μM glucose; d, 50 μM glutamine; e, no substrate; f, broken-off rod outer segments. Numbers of cells were: for wild type; a, n = 9; b, n = 7; c, n = 17; d, n = 16; e, n =12; f, n =11; for *Abca4*$^{-/-}$; a, n = 9 cells; b, n = 8; c, n = 8; d, n = 9; e, n =9; f, n =7.

Experiments at 37 °C. Error bars denote standard errors. The dashed lines through the data points represent linear regression lines.