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Research article

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

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, 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).

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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 precursors in 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 et al., 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