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RPE65 and the Accumulation of Retinyl Esters in Mouse Retinal Pigment Epithelium[†]

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Abstract

The RPE65 protein of the retinal pigment epithelium (RPE) enables the conversion of retinyl esters to the visual pigment chromophore 11-*cis* retinal. Fresh 11-*cis* retinal is generated from retinyl esters following photoisomerization of the visual pigment chromophore to all-*trans* during light detection. Large amounts of esters accumulate in *Rpe65*^{-/-} mice, indicating their continuous formation when 11-*cis* retinal generation is blocked. We hypothesized that absence of light, by limiting the conversion of esters to 11-*cis* retinal, would also result in the build-up of retinyl esters in the RPE of wild type mice. We used HPLC to quantify ester levels in organic extracts of the RPE from wild type and *Rpe65*^{-/-} mice. Retinyl ester levels in Sv/129 wild type mice that were dark adapted for various intervals over a 4-week period were similar to those in mice raised in cyclic light. In C57BL/6 mice however, which contain less Rpe65 protein, dark adaptation was accompanied by an increase in ester levels compared to cyclic light controls. Retinyl ester levels were much higher in *Rpe65*^{-/-} mice compared to wild type and kept increasing with age. The results suggest that the RPE65 role in retinyl ester homeostasis extends beyond enabling the formation of 11-*cis* retinal.

Graphical abstract

In the absence of RPE65, retinyl esters accumulate in the mouse retinal pigment epithelium

[†]This article is a part of the Special Issue dedicated to Dr. Wolfgang Gärtner on the occasion of his 65th birthday.

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INTRODUCTION

Rhodopsin, the photopigment of vertebrate rod photoreceptor cells, contains 11-*cis* retinal as its light-sensitive moiety (1, 2). Absorption of light by the retinyl chromophore isomerizes it to all-*trans*, activating rhodopsin, and initiating a cascade of biochemical reactions that culminate in a change in cell membrane potential, and hence the conversion of light to an electrical signal (3, 4). The photoisomerization of the chromophore from 11-*cis* to all-*trans* destroys rhodopsin. Thus, the ability of the photoreceptor cell to continue detecting light requires the regeneration of rhodopsin. This regeneration process involves two steps: one, the release of all-*trans* retinal by the photoactivated rhodopsin, leaving the apo-protein opsin, and, two, the formation of rhodopsin from opsin and fresh 11-*cis* retinal (5–7). Fresh 11-*cis* retinal is supplied to rod photoreceptors from the adjacent cells of the retinal pigment epithelium (RPE) (8), where it is generated by Rpe65 with retinyl esters as substrate (9–11). The precursor to retinyl esters is all-*trans* retinol (vitamin A), which reaches RPE from both its basal and apical sides. At the basal side, the source for all-*trans* retinol is the circulation (12), while at the apical side it is the photoreceptor outer segments (13), where it is generated from the reduction of the all-*trans* retinal released by photoactivated rhodopsin (14, 15). In the RPE, all-*trans* retinol is converted to retinyl esters by lecithin:retinol acyltransferase (LRAT) (16–18).

Since 11-*cis* retinal is required for the detection of light, defects in its synthesis are associated with significant loss of visual function, including blindness (19). Defects in RPE65 in particular, the protein enabling the conversion of retinyl esters to 11-*cis* retinol, are linked with Leber Congenital Amaurosis (20–23). Interestingly, lack of the RPE65 protein, as in *Rpe65*^{−/−} mice, is associated not just with the inability to form 11-*cis* retinal, but also with a large accumulation of retinyl esters in the RPE (21, 24). This accumulation indicates that there is a continuous influx of all-*trans* retinol into the RPE from the circulation. This continuous influx generates retinyl esters, which, in the absence of Rpe65 and conversion to 11-*cis* retinol, accumulate.

Given this continuous influx of all-*trans* retinol into the RPE, we investigated whether rearing wild type mice in the absence of light, thereby reducing the requirement for 11-*cis* retinal synthesis, would also result in accumulation of retinyl esters. We examined ester accumulation in two strains of wild type mice, Sv/129 and C57BL/6, the latter of which contain significantly less amounts of the Rpe65 protein (25). We measured levels of retinyl esters with HPLC analysis of RPE organic extracts. We found that rearing Sv/129 mice in the absence of light for up to one month did not result in a detectable increase of RPE retinyl ester levels. In the case of C57BL/6 mice however, absence of light resulted to an increase in retinyl ester levels compared to cyclic light reared controls. These increases in retinyl ester levels were much smaller than those observed in *Rpe65*^{−/−} animals. The results suggest that Rpe65, apart from enabling the formation of 11-*cis* retinol, may play additional roles in RPE retinyl ester homeostasis.

MATERIALS AND METHODS

Mice were from established colonies at the Medical University of South Carolina. Wild type Sv/129 and C57BL/6 mice were originally obtained from Harlan Laboratories (Indianapolis, IN); breeding pairs of *Rpe65*^{-/-} mice were kindly provided by Dr. M. Redmond (NIH). *Rpe65*^{-/-} mice were reared in cyclic light and were 0.5 – 8 months old. Wild type mice were 8 – 12 weeks old. For cyclic light reared controls, wild type mice were kept in cyclic light (6:00 – 18:00; light intensity at cage level during the day part of the cycle 130 – 170 lux) until sacrifice at either 8 weeks or 12 weeks of age. For absence of light, wild type mice were reared in the dark from 8 weeks of age for 1, 2, 4, 7, 14 or 28 days prior to sacrifice. They were kept in ventilated cabinets, and exposed to dim red light only when checking on their health and for cage changes. Animals were euthanized by carbon dioxide asphyxiation followed by cervical dislocation. All animal protocols were approved by the Institutional Animal Care and Use Committee of the Medical University of South Carolina and followed recommendations from the Panel on Euthanasia of the American Veterinary Medical Association.

All tissue manipulations were performed under dim red light. Cyclic light reared mice were not dark-adapted before sacrifice in order to minimize changes in retinyl ester levels that might occur as a result of the shift from light-adapted to a dark-adapted state; however, sacrifice and subsequent dissection and procedures were performed in a dark room. Total time in the dark for cyclic light reared mice was no more than 20 minutes. Following sacrifice, eyes were enucleated and hemisected at the level of the ora serrata under a mammalian physiological solution (in mmol/L: 130 NaCl, 5 KCl, 0.5 MgCl, 2 CaCl₂, 25 hemisodium-HEPES, 5 glucose, pH = 7.40). The retina was removed in dark adapted mice, but not in cyclic light reared mice. Since esters are not present in the neural retina (see, for example, (26); as well as our own observations), removing the neural retina does not have a significant impact on the measurements. The eyecups were then stored frozen at –80 °C protected from light.

Extraction procedures followed methods similar to those described previously (27, 28) and were performed under dim red light. Two eyecups per sample were homogenized, using a glass homogenizer, in 1 mL of hexane and 500 µL of distilled water. The sample was homogenized until no large chunks of tissue could be seen, then vortexed briefly, and centrifuged for 3 minutes in a clinical tabletop centrifuge (Centrifuge Model 228, Fisher Scientific, Pittsburgh, PA). The organic top layer was removed and stored in an amber vial and the aqueous bottom layer was transferred by pipette back into the homogenizer. This extraction procedure was repeated three times in total per sample. For each sample, the combined organic layers were dried under a stream of argon gas and then stored at –80 °C protected from light. For chromatography, samples were re-suspended in 100 µL hexane. Data were corrected for the extraction efficiency of the procedure. This was estimated separately by carrying out the same homogenization and extraction procedure with neural retina samples (that do not contain any esters) to which known quantities of retinyl palmitate (Sigma-Aldrich, St. Louis, MO) had been added. The efficiency estimated in this way was 60%, in agreement with previous reports (29).

HPLC analysis was performed under dim red light using a mobile phase of 11.2% ethyl acetate, 2.0% isopropanol, 1.4% octanol, 85.4% hexane, in a Waters 515 HPLC (Waters Corp., Milford, MA) with an Alltech Lichrospher Silica 60 column. The flow rate was 1 mL per minute. Absorption spectra were recorded with a Waters 996 Photodiode Array Detector. The retinyl ester peak was determined by retention time and absorption spectrum through comparison to a retinyl palmitate standard (Sigma-Aldrich, St. Louis, MO). The amount of retinyl ester in each sample was determined from the Area Under the Curve (AUC) for the peak at $\lambda = 325$ nm, using an extinction coefficient of $51,800 \text{ M}^{-1} \text{ cm}^{-1}$ (30).

For determination of ester isomer ratio, the retinyl ester peak was collected after HPLC, dried under argon, and saponified in 1.5 mL 0.06N KOH in ethanol at 50°C for 30 min. Following saponification, the sample was once again extracted with hexane and water, dried, and analyzed by HPLC (17). Ester isomers were quantified by their respective retinol isomer peaks.

Flat mounting of RPE for imaging retinyl ester fluorescence was carried out as described (31). Color photographs were taken on a Zeiss Axioplan 2 microscope (Carl Zeiss, Thornwood, NY) using a $63\times$ oil immersion objective ($\text{NA} = 1.4$) with a Nikon D200 (Nikon, Inc., Melville, NY) digital camera. Fluorescence was excited with 360 nm light and the emission was collected >420 nm.

All reagents were of analytical grade; organic solvents were HPLC grade. Experiments were carried out in triplicate.

RESULTS AND DISCUSSION

The quantity of retinyl esters in the RPE of Sv/129 and C57BL/6 wild type mice reared in the absence of light was measured over 28 days. For comparison, the levels of retinyl esters in the RPE of *Rpe65*^{-/-} mice of different ages were also measured. Fig. 1 shows chromatograms of organic RPE extracts from C57BL/6 and *Rpe65*^{-/-} mice, illustrating the large accumulation of esters in the latter. In Sv/129 wild type mice, the quantity of RPE retinyl esters remained fairly constant over the period of 28 days in the dark, 40 – 50 pmol. The increase of 0.54 ± 0.32 pmol/day indicated by the linear regression (Fig. 2A) was not significant ($p = 0.1$). Also, the levels of retinyl esters after 28 days in the dark were not different from the levels of cyclic light reared animals of the same age (Fig. 2A). In C57BL/6 mice however, the levels of esters in the dark increased at a rate of 3.5 ± 0.7 pmol/day ($p = 0.0045$), reaching ~160 pmol after 28 days, significantly higher ($p = 0.0063$) than the levels in cyclic light reared animals (Fig. 2B). The levels of retinyl esters in cyclic light reared C57BL/6 mice were similar to those of Sv/129, and furthermore did not change significantly between 8 and 12 weeks of age (Fig. 2A and 2B). For both strains, there was a transient increase in retinyl ester levels after one day in the dark (Fig. 2A and 2B). For comparison, levels of retinyl esters were much higher in *Rpe65*^{-/-} mice reared in cyclic light, reaching more than 1.5 nmol by 2 months of age and increasing linearly with age at a rate of 0.88 ± 0.03 nmol/month ($p < 0.001$) (Fig. 3A). The large accumulation of retinyl esters in *Rpe65*^{-/-} mice is also evident in fluorescence micrographs of flat-mounted RPE tissue where the retinyl ester droplets can be readily visualized (Fig. 3B). The increase in retinyl

ester levels observed in C57BL/6 mice was not due to the specific increase in a particular isomer: the isomeric composition was the same for mice reared for 4 weeks in the dark as in age-matched cyclic light reared animals (Fig. 4).

The results of Fig. 2 for cyclic light reared wild type mice are in broad agreement with previous reports that show an RPE retinyl ester content ranging from ~70 pmol/eye (32) to ~150 pmol/eye (26). This is several-fold lower than the amount of rhodopsin of 500–600 pmol in the dark-adapted retina (32, 33). Exposure to light can destroy large fractions of rhodopsin, even close to 100%, which then needs to be regenerated. In such cases, an amount of esters that far exceeds the available stores needs to be made available for the synthesis of fresh 11-*cis* retinal. This additional amount of esters may be generated from the recycling of all-*trans* retinol generated in the photoreceptor outer segments from the reduction of all-*trans* retinal released by photoactivated rhodopsin (see (5) for a thorough discussion), however all-*trans* retinol influx into the RPE from the choroidal circulation is likely to contribute. Apart from the need to regenerate the rhodopsin bleached by light, 11-*cis* retinal is also needed to form rhodopsin from the opsin generated on a daily basis as part of the rod outer segment renewal process. In this daily process, approximately 10% of a rod outer segment is renewed, with the distal tip being phagocytosed by the RPE, while a newly synthesized portion is added at the base (34, 35). Thus, the amounts of 11-*cis* retinal needed for the regeneration of the newly formed opsin are comparable to the level of esters present in the RPE. In addition, the 11-*cis* retinal entering the RPE as part of phagocytosed rhodopsin could also be recycled and used (36).

In view of the large amounts of retinyl esters that need to be converted to 11-*cis* retinal in the presence of light, the fairly stable levels of retinyl esters in wild type Sv/129 mice in cyclic light as well as during 4 weeks in the dark (Fig. 2A) suggest that formation and storage of retinyl esters is tightly regulated in the RPE. This regulation is impaired in the absence of Rpe65, resulting in a steady increase in retinyl ester levels (Fig. 3). As demonstrated previously by Qtaishat et al (26) using radioactively labeled retinol, this increase in retinyl esters is due to a substantial influx of all-*trans* retinol from the circulation, which however is not counterbalanced by an efflux from the RPE. From their studies, they suggested that the 11-*cis* retinoids generated by Rpe65 may be the factor regulating the efflux of retinol. Our measurements do not provide the same level of detail as those of Qtaishat et al, but they do extend to longer times in the dark. At these longer times, a build-up of retinyl esters becomes detectable after a 2-week period in the C57BL/6 mice (Fig. 2B). This is again consistent with an insufficiently counterbalanced influx of all-*trans* retinol from the circulation. Since C57BL/6 mice contain much less Rpe65 protein than Sv/129 (25), but do generate 11-*cis* retinoids, our results point to Rpe65 playing a role in mediating the efflux of all-*trans* retinol from the RPE.

Such a role for Rpe65 would involve making retinyl esters available for conversion back to all-*trans* retinol. The enzymes catalyzing the conversion are likely to be retinyl ester hydrolases (37–39) (REH in Fig. 5), and it is conceivable that the hydrolase activity of Rpe65 may be playing a role as well. Another enzyme that has been suggested to catalyze the conversion of retinyl esters back to all-*trans* retinol is LRAT (17) – but see (39). Because RPE retinyl ester hydrolases can hydrolyze both 11-*cis* and all-*trans* retinyl esters, their

involvement would be consistent with the finding that the increase in retinyl esters in the dark is not due to a particular isomer (Fig. 4). Retinyl esters are known to accumulate in storage droplets (40–42), which are readily visible in the RPE of *Rpe65*^{−/−} mice ((40, 43); Fig. 3B). RPE65 however does not localize to these storage droplets, the retinosomes (40), so it is unlikely to be acting on that pool of esters; it is rather more likely to be acting on the esters present in the internal membranes. In the presence of light, the high rate of ester utilization for 11-*cis* retinal synthesis would not allow significant accumulation in storage droplets. Another possibility for the lack of ester accumulation in cyclic-light-reared animals would be signaling by RGR, which has been shown to mediate a light-dependent decrease in retinyl esters in *Rpe65*^{−/−} mice (44). Our results provide no mechanistic information on whether a particular form of Rpe65 (membrane-associated or soluble (45, 46)) is involved, or whether the light-triggered translocation of Rpe65 (47) is related to the process.

To summarize, our results show that under cyclic light conditions, the levels of retinyl esters remain fairly stable in the RPE of Sv/129 and C57BL/6 mice. Dark adaptation for up to 4 weeks does not affect retinyl ester levels in Sv/129 mice, but does result in a significant increase in C57BL/6 mice, which contain less amounts of the Rpe65 protein. A much larger increase in retinyl ester levels is observed in mice that lack Rpe65. The results suggest that Rpe65, in addition to enabling the formation of 11-*cis* retinol from retinyl esters, may also be facilitating the availability of esters for their conversion back to all-*trans* retinol, which can then leave the RPE (Fig. 5).

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Figure 1.

Chromatograms (325 nm) of organic extracts of RPE from *Rpe65^{-/-}* and C57BL/6 mice. *Rpe65^{-/-}* mice were 2 months old; C57BL/6 were reared in cyclic light for 8 weeks and then in dark for 14 days. Insets are absorption spectra of the peaks denoted by the star (*).

Figure 2. Levels of retinyl esters in Sv/129 (A) and C57BL/6 (B) wild type mice reared in cyclic light (□) and in the absence of light (■). Each point represents the average of three experiments. Dotted lines represent linear regressions to the dark data points with slopes of 0.54 ± 0.32 pmol/day ($p = 0.1$) (A) and 3.5 ± 0.7 pmol/day ($p = 0.0045$) (B). The line connecting the two cyclic light points in (B) is for guiding the eye. Error bars represent SEM.

Figure 3.

Accumulation of retinyl esters in the RPE of *Rpe65*^{-/-} mice. (A) Organic RPE extracts show large increase in retinyl ester levels with age in *Rpe65*^{-/-} mice (●). Each point represents the average of three experiments. Dotted line is a linear regression to the points with a slope of 0.88 ± 0.03 nmol/month ($p < 0.001$). Error bars represent SEM. Levels of esters from C57BL/6 mice at 8 and 12 weeks of age in cyclic light and after 28 days in the dark (□, ■) are re-plotted from Fig. 2B for comparison. (B) Color image of the fluorescence (excitation 360 nm; emission > 420 nm) emitted by a flat-mounted RPE from a 6 month old cyclic light-reared *Rpe65*^{-/-} animal. The light blue color of the droplets is characteristic of retinyl ester fluorescence.

Figure 4.

The isomeric composition of RPE retinyl esters is the same in cyclic light reared and dark reared C57BL/6 mice. Experiments in triplicates; error bars represent SEM.

Figure 5.

Diagram describing a putative additional role for Rpe65. The protein enables the mobilization of retinyl esters, facilitating their conversion back to all-*trans* retinol, which can leave the RPE cell. Such a role would allow for the removal of retinyl esters by converting them back to all-*trans* retinol and account for the accumulation of large amounts of esters in the absence of Rpe65. Abbreviations: LRAT, lecithin:retinol acyltransferase; REH, retinyl ester hydrolase.