Inhibition of the visual cycle by A2E through direct interaction with RPE65 and implications in Stargardt disease

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Stargardt disease (STGD) is the major form of inherited juvenile macular degeneration. Pyridinium bis-retinoid A2E is a major component of lipofuscin which accumulates in retinal pigment epithelium (RPE) cells in STGD and contributes to the disease pathogenesis. However, the precise role of A2E in vision loss is unclear. Here we report that A2E efficiently inhibits RPE65 isomerohydrolase, a key enzyme in the visual cycle. Subretinal injection of A2E significantly inhibited retinoid isomerohydrolase activity in mice. Likewise, A2E also inhibited isomerohydrolase activity in cells coexpressing RPE65, lecithin retinol acyltransferase (LRAT), and cellular retinaldehyde-binding protein. In vitro isomerohydrolase activity assays confirmed that A2E inhibited enzymatic activity of recombinant RPE65 in a concentration-dependent manner, but did not inhibit LRAT activity. The inhibition type for isomerohydrolase was found to be reversible and competitive with $K_i = 13.6 \mu\text{M}$. To determine the direct interaction of A2E with RPE65 protein, fluorescence binding assays were performed. As shown by fluorimetric titration, binding of purified RPE65 with A2E enhanced the bis-retinoid fluorescence. Consistently, the fluorescence of RPE65 decreased upon incubation with A2E. Both of these experiments suggest a direct, specific binding of A2E to RPE65. The binding constant for A2E and purified RPE65 was calculated to be 250 nM. These results demonstrate that A2E inhibits the regeneration of 11-*cis* retinal, the chromophore of visual pigments, which represents a unique mechanism by which A2E may impair vision in STGD.

Stargardt disease (STGD) is the most common inherited form of recessive juvenile macular degeneration (estimated incidence 1:10,000) (1, 2). STGD patients experience a loss of high-resolution vision and central vision (3, 4). The gene defective in STGD, ABCA4 (ABCR), encodes ATP-binding retinal transporter specifically expressed in the rims of rod and cone outer segment discs (5–7). ABCR transports *N*-retinylidene-phosphatidylethanolamine across the lipid bilayer from the intradiscal to the cytoplasmic side of the disc membrane, which promotes the removal of *all-trans* retinal from photoreceptor outer segments by increasing the availability of substrate for *all-trans* retinol dehydrogenase (8–10). The hallmark of STGD is the accumulation of cellular debris (lipofuscin) which consists of a complex mixture of pigments in the retinal pigment epithelium (RPE) (11, 12). One of the major components of lipofuscin is the fluorescent bis-retinoid A2E and its isomers (13, 14). A2E is considered to be nondegradable, because it is resistant to enzymatic processing and accumulates with age in normal eyes. The accumulation of A2E-laden lipofuscin is significantly increased in the RPE of STGD patients compared to age-matched controls (15, 16). A2E is formed through the multistep reaction of *all-trans* retinal with abundant phospholipid (phosphatidylethanolamine) in the outer segment membranes of photoreceptors (15, 17). It has been shown that A2E can potentially produce a number of toxic effects on RPE cells: destabilizing membranes (18, 19), inhibiting respiration in mitochondria (20), increasing blue-light photodamage (21, 22), impairing lysosomal acidification (23), and impairing degradation of phospholipids from phagocytosed

outer segments (24). The oxidative damage of A2E under blue-light irradiation has been shown to cause DNA fragmentation in cultured RPE cells (25). It was also suggested that A2E oxidation products may trigger the complement system, which predisposes the macula to disease and contributes to chronic inflammation (26). However, it is presently unclear which of the aforementioned potentially toxic effects of A2E is responsible for the pathogenesis of STGD.

A2E, a positively charged retinoid quaternary amine, is a byproduct of the visual cycle and incomplete digestion of phagocytosed photoreceptor outer segments in RPE cells (15, 27). The visual cycle is a series of enzymatic reactions in the retina and RPE which serves to regenerate 11-*cis* retinal, the chromophore of the visual pigments (28). The limiting step of the visual cycle is the isomerization/hydrolysis of *all-trans* retinyl ester to 11-*cis* retinol, a precursor of 11-*cis* retinal (29). Previously, it has been shown that positively charged retinoids, such as retinyl amine, inhibit the isomerization of retinoids in the visual cycle (30). However, the potential effect of A2E on the visual cycle has not been investigated previously.

Here, we present evidence that the positively charged bis-retinoid A2E potentially inhibits isomerohydrolase in the visual cycle. This inhibition could cause a disruption of 11-*cis* retinal supply to the retina and result in photoreceptor dysfunction. Because it is established that RPE65 is the isomerohydrolase of the visual cycle (31–33), we hypothesized that A2E may inhibit the isomerization step of the visual cycle through direct interaction with RPE65. Using fluorescence titration of purified RPE65 with A2E, we determined the binding constant and the binding stoichiometry between RPE65 and A2E. Our findings reveal a unique mechanism for the pathogenesis of STGD and explain why macular functioning is particularly affected by A2E.

Results

A2E Inhibits Retinoid Isomerization in the Visual Cycle in Mouse Eyes.

To find out if A2E inhibits the isomerization step of the retinoid visual cycle in vivo, we injected 1 μL of 30 mM A2E into the subretinal space of BALB/c mice. Control mice received 1- μL injections of vehicle alone. BALB/c mice were selected because they have higher endogenous isomerase activity due to higher abundance of RPE65 in their eyes (34). After injection, the mice were kept in the dark for 24 h and then killed. Eyes were enucleated, dissected, RPE-containing eyecups were homogenized, and the total homogenates were used for the isomerase assay.

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sion when they bound to retinoid-binding protein, compared to unbound retinoids (38). As measured by fluorescence at 576 nm, mixing A2E with RPE65 resulted in a marked enhancement in fluorescence of A2E (Fig. 5A). As a negative control, we used ribonuclease A which does not bind A2E. As expected, no increase in the A2E fluorescence was detected upon mixing A2E with the negative control protein (Fig. 5A).

The binding of retinol to retinol-binding protein is known to result in energy transfer to the bound retinol from the excited tryptophanyl residues which are located adjacent to the binding site (39). We have also measured the direct binding of A2E with RPE65 using the tryptophane fluorescence. A typical fluorescence titration curve of RPE65 with A2E showed that the titration of RPE65 with A2E could be followed by observing the quenching of the protein fluorescence, and the binding para-

meters can be also derived using mass law equation. A representative titration curve of RPE65 with A2E is given in Fig. 5C. It should be pointed out that the inner filter effect as a result of absorbance of A2E was very small at these concentrations of ligand and protein and thus can be neglected. The data averaged from four titration curves derived an apparent dissociation constant of $K_d = 250 \pm 30$ nM. The apparent calculated number of binding sites suggests that a 1:1 molecular complex was formed. Thus, as expected, RPE65 bound to A2E with high affinity.

Discussion

The detailed sequence of the initiation and pathogenesis of STGD has not been elucidated. Multifocal electroretinography (ERG) demonstrated that the ERG signal in the foveal region was completely flat in patients with early STGD (40). However, there was no convincing explanation why the cone-rich macula responsible for high-resolution vision is particularly affected in STGD. In this study, we propose a pathogenic mechanism for STGD. Our studies show that bis-retinoid A2E, which is accumulated in the RPE of STGD patients, has a potent inhibitory effect on retinoid isomerization, a key step of the visual cycle. This inhibition may result in a deficient supply of 11-*cis* retinal chromophore, particularly for the macula which has higher demand for chromophore recycling. Lack of chromophore could destabilize opsin protein and subsequently lead to photoreceptor degeneration (41). The data presented here clearly demonstrate that micromolar concentrations of A2E inhibited isomerization/hydrolysis of *all-trans* retinyl esters to 11-*cis* retinol catalyzed by RPE65, but did not affect the retinyl ester synthesis by LRAT. This suggests that A2E selectively inhibits the isomerization step of the visual cycle catalyzed by RPE65.

Retinyl ester, a substrate for RPE65 isomerohydrolase, is insoluble in water, which historically hindered its study in biochemical assays. Recently, we developed a unique isomerohydrolase assay based on incorporation of the retinyl ester in liposomes (35). This assay allowed us to directly measure the conversion of *all-trans* retinyl palmitate to 11-*cis* retinol in the presence of A2E and calculate the inhibition constant. The inhibition of this reaction was found to be competitive and reversible, with an apparent inhibition constant calculated to be 13.6 μ M.

Next, we analyzed the effect of physiological concentrations of A2E on the isomerohydrolase reaction in the intracellular environment. Because known RPE cell lines do not produce RPE65 protein in cell culture, we expressed RPE65 in 293A-LRAT cells with an adenovirus vector. The concentration dependence of A2E inhibition was nonlinear, probably because addition of low concentrations of A2E in the cell culture media caused limited cellular uptake of A2E. Alternatively, a significant amount of A2E may have been bound in lysosomes and could not reach RPE65, which is predominantly expressed in endoplasmic reticulum. However, a significant decline in isomerohydrolase activity was observed within the range of 25–50 μ M in A2E concentration, and the reaction was almost completely inhibited by A2E at 75 μ M. Previously, it has been shown that treatment of cultured RPE cells with 25–50 μ M A2E leads to a level of intracellular A2E accumulation that is comparable to that identified in human RPE of healthy donors 58–79 y of age (24). We conclude that these concentrations of A2E can significantly reduce the 11-*cis* retinol synthesis in the eye and may worsen vision in elderly people.

Although the mouse model of STGD is currently known to accumulate A2E in the RPE, there is little information about the effect of A2E on the visual cycle *in vivo* (42). *Abcr*^{-/-} mice, a model of STGD, lack the retinoid ABCA4 transporter and show a delayed dark adaptation, which correlates with A2E accumulation in the RPE, suggesting that the visual cycle is disturbed (42). However, knockout of a gene may also have an effect on the visual cycle independent of A2E. Therefore, to analyze the effect

Fig. 5. Fluorescence measurement of binding of A2E to RPE65. (A) Binding of A2E to purified chicken RPE65. Fluorescence emission spectra of 1 μ M A2E in PBS, 0.1% CHAPS or after the addition of 1 μ M RPE65 or 1 μ M RNase A (control). Excitation peaked at 400 nm. (B) Titration of chicken RPE65 with A2E as measured by the increase in fluorescence intensity of A2E. Excitation was recorded at wavelength 400 nm, emission at wavelength 576 nm. The titration system consisted of 2 ml of 0.1 μ M of RPE65 (●) or 0.1 μ M pancreatic ribonuclease A (□) in 0.1% CHAPS, PBS buffer, pH 7.4. (C) Titration of chicken RPE65 with A2E as measured by the quenching of protein fluorescence. Excitation was recorded at wavelength 278 nm, emission at wavelength 340 nm. The titration system consisted of 2 mL of 0.1 μ M of RPE65 in 0.1% CHAPS in PBS, pH 7.4 (mean $\pm$ SD, $n = 4$).

of A2E on the visual cycle, we injected A2E into the subretinal space of wild-type BALB/c mice. Our results showed that A2E significantly decreased the isomerization reaction in the mouse eye. It is worth mentioning that mice do not have a macula and subretinal injection cannot deliver A2E to entire RPE, which may explain the fact that the inhibitory effect of A2E on the visual cycle in mouse eye is not as potent as in the human eye with STGD.

To investigate if A2E inhibition of the isomerohydrolase reaction occurs via direct binding to RPE65, we used fluorescence titration of purified RPE65 by A2E. Fluorescent techniques are well suited for measurement of binding of retinoids to retinoid-binding proteins because retinoids have high fluorescence. The fluorescence intensity of retinoids is known to be significantly higher in nonpolar than in polar solvents (43). The retinoid-binding site of RPE65 represents a tunnel covered by hydrophobic residues (44) and therefore, mimics nonpolar media. As expected, A2E fluorescence was enhanced upon binding to RPE65 as a result of transfer from the polar milieu of water to the nonpolar environment of the protein binding site. The emission spectrum of A2E was shifted to shorter wavelengths when bound to RPE65. A similar blue shift was observed previously for retinol-binding to the Ov20 protein from the parasitic nematode (45). This spectrum change shows that binding of A2E can be easily detected, and the binding constant can be determined. Conversely, the binding of retinoids to the protein can be followed by quenching the fluorescence emission of protein tryptophan residues which occurs as a result of the energy transfer to the bound ligand. Previously, this method has been used to study the binding of retinyl ester to bovine RPE65 (46). We used both of these methods to titrate RPE65 with A2E, and a high affinity binding was observed ($K_d = 250$ nM). Furthermore, the binding of A2E to RPE65 is specific, because no binding was observed for the negative control protein, RNase A.

A2E reaches the level of 61 pmol per 0.5×0.5 cm in RPE of STGD patients, which corresponds to an average concentration greater than 0.5 mM within RPE cells (15, 47). This value significantly exceeds the inhibitory concentrations of A2E observed in this study. Thus, even if as little as 2% of total intracellular A2E is bound to RPE65 in RPE cells, this can drastically reduce retinol isomerization rate and consequently, visual pigment regeneration. This will lead to dysfunction of photoreceptors and impairment of vision even if photoreceptor cells are not degenerated. None of the previously described potentially toxic effects of A2E accumulation explain why the degeneration primarily occurs in the macula rather than other parts of the retina. The macula is an area of the retina which contains a high density of cone photoreceptors and is used in daylight vision. Therefore, the macula, compared to the peripheral retina, has a high demand for the 11-*cis* retinal chromophore to efficiently regenerate the visual pigment to maintain a fast and constant light response. Recently, it has been shown that the isomerization activity in the central retina is four times higher than that in the peripheral retina in the macaque eye (48). This increased isomerization activity correlates well with the higher content of RPE65 (48) and higher concentration of retinyl palmitate (49) in the RPE adjacent to macular region. Therefore, inhibiting RPE65 isomerohydrolase and decreasing the supply of 11-*cis* retinal in the macular region has more severe impacts on the central vision. Indeed, vitamin A deprivation causes slow dark adaptation and photoreceptor degeneration in vivo and features STGD symptoms (50, 51). It has been also demonstrated that the RPE65 deficiency leads to cone opsin mislocalization and early cone degeneration in the central portion of mouse retina (41, 52). Cone survival was restored after the subretinal delivery of 11-*cis* retinal (52). This finding suggests that insufficient supply of 11-*cis* retinal chromophore can accelerate cone degeneration in the central retina. Prolonged inhibition of RPE65 by A2E may cause local starvation of

11-*cis* retinal chromophore in the central retina and consequently lead to photoreceptor degeneration.

In summary, we have demonstrated that A2E may affect the visual cycle by inhibiting RPE65 isomerohydrolase activity and thereby decreasing the supply of 11-*cis* retinal to the photoreceptors in the macular region of the retina, leading to disrupted visual function. As a consequence, vision can be impaired independent of, and prior to, photoreceptor degeneration. We propose that this represents a unique mechanism for STGD initiation and progression.

Methods

Synthesis of A2E. A2E was synthesized and purified by reversed phase HPLC as previously described (14). We did not separate A2E from iso-A2E, because these compounds are found in a similar ratio in eyes that are dissected in the dark, suggesting they are in equilibrium in vivo.

In Vitro Isomerohydrolase Assay. The 293A-LRAT cells stably expressing LRAT were infected by adenovirus expressing chicken RPE65 at a multiplicity of infection (MOI) 100 and used as a source of RPE65 as described previously (35). *All-trans* [11,12-³H]-retinol in ethanol (1 mCi/mL, 52 Ci/mmol, Perkin Elmer) was dried under argon and resuspended in the same volume of dimethyl formamide (DMF). For each reaction, 2 μ L of the nondiluted *all-trans* [11,12-³H]-retinol (1 mCi/mL, 52 Ci/mmol, Perkin Elmer) in DMF and 62 μ g of cell lysate coexpressing LRAT and RPE65 or the homogenate of two BALB/C mouse eyecups were added into 200 μ L of a reaction buffer (10 mM 1,3-bis [tris(hydroxymethyl)-methylamino]propane, pH 8.0, 100 mM NaCl) containing 0.5% BSA and 25 μ M CRALBP (34, 53). Alternatively, the retinyl ester-containing liposomes (250 μ M lipids, 3.3 μ M *all-trans* retinyl palmitate) and either 250 μ g of total proteins of cell lysates or 25 μ g of the purified RPE65 were used as substrate and the enzyme. Liposomes and purified RPE65 were prepared as described previously (35). After 2-h incubation in the dark at 37 °C, retinoids generated were extracted with 300 μ L of cold methanol and 300 μ L of hexane. The upper organic phase was collected and analyzed by normal phase HPLC as described (34). Each form of retinoids was identified based on comparison to retention times of known retinoid standards. Nonlinear regression analysis of v -versus- $[S]$ data was used to calculate V_{max} (apparent) and K_m (apparent) in the absence and in the presence of inhibitor. The inhibition constant for A2E was calculated from the following equation: $K_i = [I]/(K_m/K_m - 1)$ where $[I]$ = concentration of inhibitor, K_m = Michaelis constant in the absence of inhibitor, K_{mi} = Michaelis constant in the presence of inhibitor (36).

Intracellular Retinoid Isomerohydrolase Assay. HEK293A-LRAT cells were transfected with pcDNA6 vector expressing CRALBP. Six hours after transfection, the cells were infected with Ad-RPE65 expressing chicken RPE65 at MOI 100. Fifteen hours after infection, the cells were treated with various concentrations of A2E in culture media for 6 h, and then 2 μ M of *all-trans* retinol was added and incubated for an additional 2 h. The cells were harvested, lysed by sonication, and retinoids were extracted as described previously (34). Saponification of retinyl esters was performed as described elsewhere (54). The products of saponification were analyzed by HPLC as described previously (34).

Fluorescence Assays. Two methods were used to monitor binding between RPE65 and A2E. In the first method, A2E dissolved in ethanol was gradually added to 2 mL solution of 0.1 μ M RPE65 in PBS, 0.1% CHAPS, pH 7.4, and thoroughly mixed. After 20 min incubation at 20 °C, emission fluorescence of A2E was measured with excitation at 400 nm and emission at 576 nm. Final volume of A2E addition was less than 1% of the total sample volume. RNase A protein at concentration 0.1 μ M in the same buffer was used as a negative control for binding. We also measured RPE65 intrinsic tryptophan fluorescence quenching upon binding with A2E. Intrinsic tryptophan fluorescence of RPE65 (0.1 μ M concentration) was measured in the PBS, 0.1% CHAPS, pH 7.4, at the same incubation conditions as above. Excitation was at 278 nm, and emission was recorded at 340 nm. All measurements were performed using quartz cuvettes with a path length of 1.0 cm on a PC-1 spectrofluorimeter. Excitation and emission slit widths were 2 mm. All emission fluorescence values are means of at least three measurements. Data analyses were performed using Prism 5.0 (GraphPad Software), statistical software. In each titration experiment, total fluorescence emission intensity was corrected for background counts obtained at the same concentrations of A2E for each experimental point. The binding constant (K_d) and the number of binding sites (n) were calculated by nonlinear regression fitting of the

experimentally derived α values using the equation (38) $P\alpha = R\alpha/n(1 - \alpha) - K_d/n$ where P = total protein concentration, $\alpha = (F_{\max} - F)/(F_{\max} - F_0)$, n = number of independent binding sites, R = total A2E concentration at each addition of ligand, K_d = dissociation constant, $F_{\max}$ = fluorescence intensity at saturation, and F_0 = initial fluorescence intensity.

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