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Interphotoreceptor Retinoid-Binding Protein Protects Retinoids from Photodegradation

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Abstract

Retinol degrades rapidly in light into a variety of photoproducts. It is remarkable that visual cycle retinoids can evade photodegradation as they are exchanged between the photoreceptors, retinal pigment epithelium and Müller glia. Within the interphotoreceptor matrix, all-*trans* retinol, 11-*cis* retinol and retinal are bound by interphotoreceptor retinoid-binding protein (IRBP). Apart from its role in retinoid trafficking and targeting, could IRBP have a photoprotective function? HPLC was used to evaluate the ability of IRBP to protect all-*trans* and 11-*cis* retinols from photodegradation when exposed to incandescent light (0 to 8,842 $\mu\text{W}/\text{cm}^2$); time periods of 0 – 60 min, and bIRBP: retinol molar ratios of 1:1 to 1:5. bIRBP afforded a significant prevention of both all-*trans* and 11-*cis* retinol to rapid photodegradation. The effect was significant over the entire light intensity range tested, and extended to the bIRBP: retinol ratio 1:5. In view of the continual exposure of the retina to light, and the high oxidative stress in the outer retina, our results suggest IRBP may have an important protective role in the visual cycle by reducing photodegradation of all-*trans* and 11-*cis* retinols. This role of IRBP is particularly relevant in the high flux conditions of the cone visual cycle.

INTRODUCTION

Vision begins with the 11-*cis* to all-*trans* photoisomerization of retinal bound to opsin (1). This event initiates a series of transient intermediates, the last of which is unstable resulting in the release of all-*trans* retinal, which is reduced to all-*trans* retinol (2, 3). All-*trans* retinol then leaves the outer segment and crosses the interphotoreceptor matrix (IPM) to access the

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Supplementary figures 1 and 2 and Tables 1 and 2 can be found online, on DOI:xxxx

retinal pigment epithelium (RPE) or Müller glia in the rod and cone visual cycles, respectively (4, 5). Through its melanosomes and antioxidant systems, the RPE provides a protective redox environment for those biochemical steps in the visual cycle taking place in its cytoplasm (6–8). The RPE produces 11-*cis* retinal that can be used directly for visual pigment regeneration. In contrast, the Müller cells release 11-*cis* retinol into the IPM that cones, but not rods, are able to oxidize to 11-*cis* retinal for pigment regeneration (9–11). Thus, both all-*trans* and 11-*cis* retinols, along with 11-*cis* retinal are continually exchanged between the rods, cones, RPE, and Müller cells. As these retinoids cross the IPM, they are exposed to incident light, oxygen, and reactive oxygen species (12). Although Parker et al. (2011) reported retinol degradation by UV light (360 nm) upon a short exposure period of 30S (at 10 cd/m² or 1.46 μ W/cm²; (13)), it is not known how the labile visual cycle retinoids are protected from such photodegradation during this obligatory intercellular trafficking.

Within the IPM, visual cycle retinoids are bound by interphotoreceptor retinoid-binding protein (IRBP) (14, 15). Mutation of IRBP has been associated with a form of autosomal recessive retinitis pigmentosa (16) possibly due to decreased secretion of functionally active protein (17). The absence of IRBP in transgenic *IRBP*^{-/-} mice results in an early onset photoreceptor degeneration with disorganization of the outer segments (18). It is not understood how the absence of IRBP leads to outer segment degeneration.

Although it is the most abundant soluble protein in the IPM, IRBP's role in the visual cycle is far from clear (19). IRBP was initially thought to only provide a shuttle for visual cycle retinoids crossing the matrix (15, 20, 21). Previous studies reported that 11-*cis* retinal (22, 23) and all-*trans* retinol (24) release from the RPE and rod outer-segments depends strongly on IRBP. However, its presence may not be an absolute requirement for transfer of visual retinoids between the RPE and retina (25), dark adaptation (26), cone regeneration (27), or transfer of vitamin A between liposomes (28). More than 20 years ago, while looking for a vehicle to effectively deliver retinoids to isolated photoreceptors, Crouch *et al* (1992) observed that IRBP can protect all-*trans* retinol *in vitro* from isomerization and oxidation (29). This protective property, which is potentially critical to the function of the visual cycle and stability of the retina, has not received adequate attention (30, 13, 19). We therefore further investigate this protective function with highly purified bovine IRBP (bIRBP). A preliminary description of our study has been presented in abstract form at annual meetings of the Association for Vision Research (Sung et al. Invest. Ophthalm. Vis. Sci [1592, 2012]; Tsin AT, et al. Invest. Ophthalm. Vis. Sci [3765, 2013]).

MATERIALS AND METHODS

This research has been approved by The University of Texas at San Antonio and SUNY Buffalo Biosafety Committees, and the Buffalo Veterans Affairs Medical Center Research & Development committee. HPLC grade solvents were purchased from Fisher Scientific Co. (Springfield, NJ). All other chemicals were of the highest purity, and obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. Retinoids were handled under dim red light, and stored at -80°C under argon. Working stocks of all-*trans* retinol were prepared in 100% ethanol. 11-*cis* retinal was obtained in a pure crystalline form from Dr. Rosalie Crouch (Department of Ophthalmology, Medical University of South Carolina, USA). 11-

cis retinol was prepared from 11-*cis* retinal using sodium borohydride (31) and its purity confirmed by HPLC and absorbance spectroscopy.

Purification of IRBP

Our strategy to purify bIRBP was modified from that of others (30). All steps were carried out at 4°C in the presence of 0.5 mM DTT and protease inhibitors. Adult bovine retinas were collected under dim red light (WL Lawson Co., Lincoln, NE), and held at -80°C until use. Retinas were thawed in PBS (2 mM potassium phosphate, 7 mM sodium phosphate, 13.4 mM KCl, 136 mM NaCl, pH 7.4) containing 0.5 mM phenylmethylsulfonyl fluoride, and centrifuged briefly at $2,000 \times g$ to collect retinas. The retinas were re-suspended in fresh PBS and soaked for an additional 10 min with gentle agitation, followed by centrifugation at $3,000 \times g$ for 10 min. The pooled washes were centrifuged at $20,000 \times g$ for 30 min and incubated with a 50% Concanavalin A Sepharose 4B slurry (GE Healthcare, Piscataway, NJ) for 4 hrs with continuous gentle agitation in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM CaCl_2 , 1 mM MgCl_2 , and 1 mM MnCl_2 . The bIRBP was eluted overnight in 10% methyl α -D-mannopyranoside, 50 mM Tris-HCl pH 7.5.

The bIRBP was subjected to a Q high performance sepharose column (GE Healthcare, Piscataway, NJ) equilibrated with 20 mM Tris-HCl pH 7.5, 50 mM NaCl on an Äkta Fast Protein-Liquid Chromatography system. It was then eluted at 600 mM NaCl with a linear gradient. The bIRBP was concentrated to 5 mL and subjected to size exclusion chromatography (Sephacryl S-300HR; running buffer, 20 mM Tris-HCl pH 7.5, and 100 mM NaCl). The protein was further purified on a second QHP column and eluted with a NaCl gradient with a final purity of ~98% based on SDS-PAGE. The concentration of purified bIRBP was determined by absorbance spectroscopy and amino acid analysis. HPLC showed that the purified IRBP contained less than 1.7% endogenous retinols. All experiments were performed using a stock solution of 13.45 μM bIRBP in 20 mM Tris, 100 mM NaCl, pH 7.5.

Photodegradation assays

Retinol in the presence or absence of bIRBP was exposed to light ranging from 0 to 8,842 $\mu\text{W}/\text{cm}^2$ (250–900 nm) in an aluminum light chamber utilizing a 100 Watt 1170 lumen incandescent A19 bulb with a soft white finish (General Electric). Light intensity was adjusted to specified light levels using a L-246 illuminometer (Sekonic, White Planes, NY). All samples were exposed to dim red light (for transfer during lab analysis) and/or bright light (for photodegradation) in borosilicate glass (Fisher Scientific 12×75mm; Cat # 14-961-26) test tubes in a test tube rack over an ice tray. The temperature of the solution was measured by a thermometer and it was maintained at 22°C, by cooling fans which pulled heat outside of the box, throughout the experimental period. The light source was located at the side of the box 18 cm from samples, which received light from all directions due to light reflections from the chamber's aluminum lining. The spectral irradiance from the light source was measured by the output of a PTI QuantaMaster 500 spectrophotometer, calibrated with a SpectraPhysics power meter to obtain power in Watts (For details, see Fig. S1 legend). Our light source delivered 8,842 $\mu\text{W}/\text{cm}^2$ (250–900 nm; at 4,000 Lux intensity) which include 8.6 $\mu\text{W}/\text{cm}^2$ of UV light (250–400 nm) to retinol samples (see Table S1).

Retinols were transferred and processed for HPLC analyses under dim red light. In this report, “dark” or “darkness” conditions refer to no additional light stimuli other than dim red light. Each sample was extracted three times with an equal volume of cold ethanol and two volumes of hexane followed by centrifugation at $1500 \times g$ at 5°C for 3 min. Samples were then dried under nitrogen, and immediately analyzed using an HPLC system equipped with a Zorbax normal phase Rx-SIL $5 \mu\text{m}$, $4.6 \text{ mm} \times 250 \text{ mm}$ column (Agilent Technologies, Santa Clara, CA, USA) and on-line 2996 photo-diode array detector run by Empower software (Waters Technologies, Milford, MA, USA). The HPLC column was subjected to 10% dioxane in hexane at a flow rate of 1.0 mL min^{-1} and the eluent monitored at 318 nm. Retinols were identified by comparison with retention times of authentic standards and their online UV spectra (with absorbance maxima at 318 and 325 nm for 11-*cis* and all-*trans* retinols, respectively). Quantification was achieved by comparing retinol peak areas to those in calibration curves obtained from authentic standards. Most experiments were carried out at a 1:1 molar ratio of bIRBP to retinol. To prepare such solutions, $1.34 \times 10^{-3} \mu\text{moles}$ of all-*trans*, or 11-*cis* retinol in ethanol was dried under nitrogen and re-dissolved in $9.0 \mu\text{L}$ of ethanol. Bovine IRBP ($1.34 \times 10^{-3} \mu\text{moles}$ in $100 \mu\text{L}$), or buffer alone, was added and quickly dispersed in $351 \mu\text{L}$ of degassed buffer (20 mM Tris, 100 mM NaCl, pH 7.5) to yield a final volume of $460 \mu\text{L}$ (containing $2.9 \mu\text{M}$ of retinol and/or bIRBP). The sample groups were then exposed to $7,780 \mu\text{W/cm}^2$ or maintained under dim red light for 0 and 30 min. Sample groups were: dark, no bIRBP; dark, with bIRBP; light, no bIRBP; and light, with bIRBP (Fig. 1).

For the time course studies, $100 \mu\text{L}$ was removed from each sample at various time intervals under $7,780 \mu\text{W/cm}^2$ (Figs. 2, 3). To study the specificity of IRBP’s protective activity, bIRBP, bovine serum albumin (BSA), water-soluble α -tocopherol analog Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Cayman Chemical, Ann Arbor) and thioredoxin each were added to retinol samples in 1 mL buffer solution (9 mM of retinol; in 1:1 Molar ratio) in a fused quartz cuvette with 1 cm path length. Time-dependent decrease in retinol absorbance within the cuvette over 60 min. was monitored directly by scanning spectrophotometry following the decrease in retinol absorbance at 325 nm (Fig. 3B).

For experiments on the effect of bIRBP at different light intensities, samples were exposed to $5,894 \mu\text{W/cm}^2$, $7,780 \mu\text{W/cm}^2$, or $8,842 \mu\text{W/cm}^2$ for 30 min, and $200 \mu\text{L}$ aliquots removed for HPLC analysis (Fig. 4). To determine the effect of various stoichiometric bIRBP/retinol ratios, samples were prepared at molar ratios of 1:1, 1:2 and 1:5 and $200 \mu\text{L}$ aliquots were removed for HPLC after 30 min. exposure to $7,780 \mu\text{W/cm}^2$ (Fig. 5).

RESULTS

bIRBP protects retinol from photodegradation

We began by asking whether bIRBP could protect all-*trans* and 11-*cis* retinols from light mediated photolysis. After incubation in buffer in dim red light for 30 min., all-*trans* retinol and 11-*cis* retinol were reduced by $31.1 \pm 2.4\%$ and by $18.4 \pm 9.08\%$ respectively (Fig. 1). In contrast, the presence of IRBP prevented a significant reduction in retinol concentration. However, when the samples were exposed to $7,780 \mu\text{W/cm}^2$ for 30 min., all-*trans* and 11-*cis* retinol were reduced by $67.5 \pm 1.9\%$ and $84.2 \pm 1.3\%$, respectively (Fig. 1). The presence of

bIRBP reduced the photodegradation to only $20.9 \pm 5.5\%$ reduction for all-*trans* retinol and $18.7 \pm 1.2\%$ reduction for 11-*cis* retinol (Fig. 1). These levels are comparable to those observed in “dark only” samples (Fig. 1), suggesting that bIRBP is strongly protective against retinol photodegradation.

We next characterized the time course of the degradation. HPLC was used to analyze the retinoid composition in the presence or absence of bIRBP over 30 min. at $7,780 \mu\text{W}/\text{cm}^2$. Fig. 2A, B shows representative chromatograms of the photodecomposition of all-*trans* and 11-*cis* retinols, respectively, at different time points. In panel A, the amount of all-*trans* retinol in the presence of bIRBP (horizontal blue arrowheads) fell only slightly over the 30 min. interval. A smaller peak (not labeled) eluting before all-*trans* retinol grew slightly during this time. This peak is consistent with 13-*cis* retinol by its absorbance spectrum and elution position. In the absence of bIRBP, the quantity of all-*trans* retinol fell rapidly (slanted red arrowheads). A 13-*cis* retinol peak was not detected in the absence of bIRBP. It is possible that this small 13-*cis* retinol build up was not sufficiently stable in the absence of bIRBP to allow its detection. Fig. 2B shows a similar protective activity of bIRBP for 11-*cis* retinol. Bovine IRBP prevented the rapid disappearance of the 11-*cis* retinol peak (horizontal blue vs. slanted red arrowheads) over time. A smaller peak corresponding to all-*trans* retinol typically eluted after 11-*cis* retinol in both the presence and absence of bIRBP. The increased all-*trans*/11-*cis* retinol ratio in the absence of bIRBP hints to a potential protective effect on the isomeric state of 11-*cis* retinol. Finally, the data indicate that 11-*cis* retinol was more susceptible to photodegradation than all-*trans* retinol (Fig. 2C). Further studies will be needed to determine the mechanism(s) of IRBP's ability to protect 11-*cis* and all-*trans* retinol from photodegradation.

To address the specificity of bIRBP's protective capacity, we compared the rate of all-*trans* retinol photodegradation in buffer alone with that in the presence of bIRBP, BSA, thioredoxin, or vitamin E analog Trolox. In the dark, even in buffer alone, little degradation was evident (red and black HPLC peaks, Fig. 3A insert). However, in the presence of light, all-*trans* retinol rapidly degraded (filled diamonds, Fig. 3A and Fig. 3B). In contrast, little decrease in retinol absorbance occurred when bIRBP was present (filled squares, Fig. 3B). IRBP's protective effect was greater than that which could be provided by BSA, thioredoxin, or Trolox. The molecular basis for this specificity is presently unknown.

Effect of light intensity and IRBP: retinol stoichiometry on retinol photodegradation and IRBP protection

In the absence of bIRBP, only 40%, 32%, and 19% of all-*trans* retinol remained after 30 min exposure to $5,894 \mu\text{W}/\text{cm}^2$, $7,780 \mu\text{W}/\text{cm}^2$, or $8,842 \mu\text{W}/\text{cm}^2$, respectively, and this light effect was determined to be statistically significant (Fig. 4A). Under the same conditions 11-*cis* retinol also exhibited a light-dependent photodegradation (29%, 17%, and 8%, Fig. 4B). However, in the presence of bIRBP, an average of 83.7% and 76.3% of all-*trans* and 11-*cis* retinol respectively remained under these light conditions.

To characterize the dependence of photodegradation on the bIRBP: retinol stoichiometry, samples at various ratios (0:1, 1:1; 0:2, 1:2; and 0:5, 1:5) were exposed to $7,780 \mu\text{W}/\text{cm}^2$ for 30 min. Fig. 5A shows that when the bIRBP: retinol was 1:1, 79.1% of all-*trans* retinol

remained at the end of the 30 min. When the bIRBP: retinol ratio was 1:2, 78.7% all-*trans* retinol remained. However, at a 1:5 bIRBP: retinol ratio, only 60.5% all-*trans* retinol remained in the sample. This constitutes a significant reduction (~20%) from the level observed in the 1:1 and 1:2 ratios. Fig. 5B, shows that at a 1:1 bIRBP: retinol ratio 81.3% of 11-*cis* retinol remained in the sample, and at 1:2 bIRBP: retinol ratio 81.2% 11-*cis* retinol was recovered by HPLC analysis. Although the amount of 11-*cis* retinol remaining in the 1:5 bIRBP: retinol sample was reduced to 72.4% (Fig. 5B), the level of reduction from 1:1 and 1:2 did not attain statistical significance. Nevertheless, the noted “drop off” of bIRBP’s protective effect at 1:5 bIRBP: retinol ratio is consistent with bIRBP’s ligand-binding stoichiometry.

DISCUSSION

The classic study of Boettner and Wolter (1962), established that direct and scattered light between 300–2000 nm reaches the retina surface, and includes an amount of UVA/UVB light that is not filtered out by the lens and cornea (32). The ability of UV light to reach the retina could allow for retinoid photodegradation as the λ_{max} for all-*trans* and 11-*cis* retinol is 318 and 325 nm respectively. Its ability to protect retinol from photodegradation *in vitro* suggests a new function for IRBP in the rod and cone visual cycles.

UV light in our system was likely responsible for the rapid degradation of retinol in the aqueous solutions in our present study. Parker *et al* (2011) reported light exposure of 10 cd/m² (or 1.46 $\mu\text{W}/\text{cm}^2$ at 360 nm for 30 S) resulted in an 83% loss of retinol (10 μM) in buffer (13). In comparison, the total UV irradiance in our light source ranges from 1.4–8.6 $\mu\text{W}/\text{cm}^2$ (after attenuation by borosilicate glass, see Table S1). Thus, the power density of the UV component of our light source falls within a range used by Parker *et al* for UV degradation of retinol. Furthermore, our exposure time of 30 min. far exceeds the short 30 S in their study. We conclude that our light source contains sufficient UV irradiance for retinol photodegradation. [Note: Although infrared (IR) light also IR reaches the retina (32), and some retinoids do absorb IR (33), it is unlikely that low energy IR photons can account for the observed retinoid decomposition]. Results from additional experiments using a UV or a UV/VIS filter also provided direct evidence to show that retinol degradation observed in our present study was attributable to the UV component of our light source (see Fig. S2). Temperature was maintained at 22°C in all test samples throughout the course of our experiments (Table S2). No significant reduction in retinol was observed in samples exposed to dim light at all time points. Thus we conclude that the large reduction of retinols upon light exposure was attributable to photo- (rather than thermal-) degradation. In comparison to BSA, thioredoxin and vitamin E analog trolox, bIRBP showed higher activity towards protecting all-*trans* retinol from photodegradation and this protection is time and concentration-dependent for both all-*trans* and 11-*cis* retinol (Fig. 3).

Our observations raise the important question as to what may be the mechanism(s) responsible for IRBP’s ability to protect all-*trans* retinol from photodegradation. Retinol’s broad absorption spectrum with peak at ~325 nm allows it to readily absorb UVA and B light resulting in $\pi \rightarrow \pi^*$ transitions producing singlet excited states (34). Little is known about the chemistry of retinol photodecomposition, and almost nothing about how retinoid-

binding proteins could protect retinoids from such destruction. We anticipate that the mechanism of IRBP's protective effects could be related to the retinoid being bound to a protein. It is likely that the excitation energy could be transferred to the protein residue rescuing the retinoid from degradation. Recently, bIRBP was shown to have significant thiol-dependent free radical scavenging activity (30). Ongoing X-ray crystallographic analysis coupled with functional studies will further uncover the mechanism(s) of IRBP's protective activity.

Although highly labile *in vitro* and prone to photodegradation, little is known about how visual cycle retinoids are protected from light *in vivo* especially when outside the RPE. In such environments, retinol is susceptible to being oxidized to retinal, which can quickly degrade into various isomers, but also to anhydrovitamin A, retinoid epoxides, peroxides, and a number of cleavage products (35–37). This is not unique to retinols, as a variety of natural retinoids including retinoic acid, retinyl acetate, and retinal palmitate are highly susceptible to photodecomposition. Oxygen is thought to promote this decomposition in most (38–40, 35), but not all studies (41), and some pathways are likely oxygen independent. Oxygen is significantly less active in promoting degradation in the absence of light (35). Previous studies have shown that photoisomerization and photodegradation of retinoids occur within the UVA and UVB range (42, 36, 43, 41), and are required to initiate retinyl palmitate and retinol breakdown with aeration (reactive oxygen species) strongly enhancing the decomposition (39, 40). The transient nature of many of the breakdown products has limited our understanding of the decomposition process. Although pharmacological strategies to retard photodegradation are sought (44), little is known about how retinoids are naturally protected at tissue sites that receive light exposure. For carotenoids, the dietary precursor of retinoids, the lack of end groups enhances the stability. Retinoid binding proteins may play an important role. An early observation in the characterization of serum retinol-binding protein was its ability to prevent the decomposition of all-*trans* retinol (45). The mechanism of this activity is unknown.

An emerging picture is that protecting all-*trans* and 11-*cis* retinols from photodecomposition may be central to the function of IRBP. In their study of the translocation of all-*trans* retinol between liposomes, Ho *et al.* (1989) made the surprising observation that bIRBP not only does not accelerate the transfer, but slows it down (28). The authors considered whether this was due to decomposition of the retinol during the transfer. However, they found that in contrast to retinol in buffer, or bound to BSA where retinol degraded rapidly; retinol bound to IRBP was stable. These observations challenged the prevailing view that IRBP functions simply to solubilize visual cycle retinoids in the aqueous milieu of the IPM. The concept was extended with HPLC studies showing that bIRBP can protect the oxidative and isomeric state of all-*trans* and 11-*cis* retinols (29, 13). The mechanism of this protective effect remains largely unknown. The activity appears to be contained within each of the IRBP structural modules (46). X-ray crystallography studies combined with site directed mutagenesis and molecular modeling studies identified a hydrophobic cavity within each of the IRBP modules. These homologous binding sites are not identical suggesting that the binding domains are tailored for different retinoids, other ligands, or different functions. The saturation of bIRBP's protective effect at a 1:5 bIRBP: retinol molar ratio (Fig. 5) is

consistent with its containing 2–4 retinol-binding sites (14, 19). Purified bIRBP inhibits oxidation of 2,2'-azinobis [3-ethylbenzothiazoline -6- sulfonate] by metmyoglobin, and this free radical scavenging activity is significantly higher compared to that of ovalbumin, thioredoxin, and vitamin E analog Trolox (Sun D, et al. Invest. Ophthalm. Vis. Sci. Abstract [1592, 2012]). In support of such a role, the outer segments of *IRBP*^{-/-} mice accumulate 4-hydroxy-2-nonenal and acrolein biomarkers for oxidative damage.

IRBP's ability to prevent the formation of cytotoxic retinoid decomposition products during the visual cycle may be critical to the health of the retina. In the rod and cone visual cycles, all-*trans* retinol and 11-*cis* retinol released from the outer segments, and Müller cells, respectively are susceptible to photodegradation in the subretinal space. Their photodecomposition products are known to be cytotoxic (47). The toxicity of retinal may be enhanced by further photodegradation (48). Furthermore, all-*trans* retinal was recently shown to promote NADPH oxidase-mediated overproduction of intracellular reactive oxygen species (49). Finally, defective clearance of all-*trans* retinal from the outer segment results in the formation of bisretinoid lipofuscin compounds known to trigger Stargardt's disease and age related macular degeneration (50–52).

The cone visual cycle presents a number of challenges to protecting 11-*cis* retinol from photodecomposition. First, 11-*cis* retinol may diffuse from the Müller cell villi to the cone outer segments (53), which due to the separation afforded by the inner segments, is a significantly longer distance to travel compared to 11-*cis* retinal's journey from the RPE to the rod outer segments. Second, the higher retinoid flux associated with the cone visual cycle compared to that of the rods suggests a longer cumulative extracellular trafficking time and hence a higher photodegradation risk. Finally, the 11-*cis* isomeric configuration increases retinol's photoinstability compared to all-*trans* retinol.

Transgenic mice lacking IRBP may provide insight into its photoprotective role. The cone electroretinogram is reduced in *IRBP*^{-/-} mice although cone densities and opsin levels are reportedly similar to wild-type controls (54). A retinoid deficiency may play a role as supplementation with 9-*cis* retinal produces a significant ERG recovery (54). Although cones and rods may degenerate at similar rates, cones are more functionally affected, and show a greater reduction in outer segment length (55). The mechanism for the disproportionate reduction in cone function, and outer-segment length are not understood. A "mistrafficking" of cone opsins has been proposed. We would hypothesize that cytotoxic photodecomposition products of all-*trans* and 11-*cis* retinols, and 11-*cis* retinal may play a role in compromising the integrity of the cone outer segments.

A role for IRBP in protecting the retina from cytotoxic visual cycle photodecomposition products has not received adequate attention. The function of IRBP is controversial, as not all studies have demonstrated a clear dependence of visual cycle physiology on the presence of IRBP (28, 25, 27). These studies have provided important insight to the older proposal that IRBP being simply a binding protein which facilitates the diffusion of retinol across the interphotoreceptor matrix is not nearly the whole story. Our data taken together with that of others are consistent with an emerging picture that a shuttle function *per se* is not as central to the role of IRBP as is a function in buffering free retinoids, and protecting these

compounds from photodecomposition during their intercellular trafficking. These functions may have particular importance in the role of IRBP in the cone cycle, and the pathogenesis of retinal degenerations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Bovine Interphotoreceptor retinoid-binding protein (bIRBP) protects retinol from photodegradation. 2.9 μM all-*trans* retinol (atROL) and 11-*cis* retinol (11cROL) were incubated for 30 min in the presence of light (7,780 $\mu\text{W}/\text{cm}^2$), or dim red light (dark), with or without 2.9 μM bIRBP. The retinoids remaining in sample were extracted and quantified by HPLC. A significant reduction of atROL and 11cROL without bIRBP was observed between dark and light only samples (t-test, $p < 0.01$). Conversely, the addition of bIRBP significantly protected both isomers from photodegradation (Light only vs. Light + IRBP; t-test, $p < 0.01$). Error bars represent $\pm$ SEM ($N = 3-5$).

Figure 2.

Effect of time and bIRBP on the rate of retinol photodegradation. 2.9 μM all-*trans* retinol (atROL), or 11-*cis* retinol (11cROL) were exposed to 7,780 $\mu\text{W}/\text{cm}^2$ of light for 0, 10, 20 and 30 min in the presence (horizontal blue arrowheads) or absence (slanted red arrowheads) of 2.9 μM bIRBP. HPLC wavelength was monitored at 318 nm. A) Chromatograms of HPLC analysis comparing the amount of all-*trans* retinol in buffer alone (slanted red arrowheads) with that in the presence (horizontal blue arrowheads) of bIRBP. The small peak eluting before all-*trans* retinol corresponds to 13-*cis* retinol. The green dashed line aligns the retention time of atROL retention time of all HPLC chromatograms. B) Representative HPLC chromatograms of 11-*cis* retinol incubations in presence (horizontal blue arrowheads) and absence (slanted red arrowheads) of bIRBP. The peak eluting after 11-*cis* retinol corresponds to all-*trans* retinol. The green dashed lines align the retention times of 11-*cis* retinol and all-*trans* retinol in all HPLC chromatograms. C) Percent all-*trans* retinol (solid lines with filled symbols) and 11-*cis* retinol (dashed lines with open symbols) remaining after light exposure in the presence (blue lines) and absence (red lines) of bIRBP. A two-way ANOVA was statistically significant for time [(F (3, 12) = 81.75, $P < 0.0001$)] and bIRBP treatment [(F (1, 12) = 148.2, $P < 0.0001$)] for atROL, and time [(F (3, 12) = 89.13, $P < 0.0001$)] and bIRBP treatment [(F (1, 12) = 118.3, $P < 0.001$)] for 11cROL. Bonferroni posttests to test differences between groups were significant for all treatment times of both isomers (10 min, $P < 0.05$; 20 min and 30 min, $P < 0.01$). Error bars equal $\pm$ SEM (N = 3).

Figure 3.

Time-dependent photodegradation of all-*trans* retinol and protection by bIRBP, BSA, Trolox (water soluble vitamin E derivative), and thioredoxin. A) Light induced all-*trans* retinol degradation in buffer was monitored by HPLC in a manner similar to that described for results in Fig. 2 above. All-*trans* retinol (1.8 pmoles in ethanol) was dried and solubilized in mobile phase, and analyzed by HPLC (red). This is compared to the same amount of retinol re-dissolved in buffer and kept in the dark for 30 min before hexane extraction and HPLC analysis (black; recovery was ~100% with minimal generation of 13-*cis* retinol). Photodiode array spectrum confirms the eluted peak as all-*trans* retinol (vertical arrow). The blue HPLC elution chromatograms show the rapid degradation of all-*trans* retinol with the generation of the 13-*cis* isomer. Open and filled circles represent duplicate determinations of all-*trans* retinol as a function of time. Calibration of HPLC chromatogram: vertical line, 0.015, and 0.005 absorbance units for the dark and light data respectively, horizontal line = 1 min. B) Degradation of 9 μ M retinol in buffer in a quartz cuvette (see Methods section for details) was monitored in a spectrophotometer by absorbance change at 325 nm over time with bIRBP (filled square), BSA (open diamond), Trolox (filled triangle), thioredoxin (Thrx; open triangle) in buffer or buffer alone (filled diamond). IRBP, BSA, Trolox, and Thrx were at a 1:1 molar ratio with retinol.

Figure 4.

Dependence of retinoid degradation on light intensity and bIRBP. A) All-*trans* retinol and B) 11-*cis* retinol were exposed to 5,894 $\mu\text{W}/\text{cm}^2$, 7,780 $\mu\text{W}/\text{cm}^2$ or 8,842 $\mu\text{W}/\text{cm}^2$ for 30 min with and without bIRBP. Following light treatment, retinoids were extracted, and quantified by HPLC. A one-way ANOVA showed a statistically significant light effect in the absence of bIRBP for atROL [(F(1,8) = 25.39, P = 0.0012)] and 11cROL [(F(2,8) = 22.76, P = 0.0016)]. Error bars represent $\pm$ SEM (N = 3).

Figure 5.

Dependence of A) all-*trans* retinol and B) 11-*cis* retinol photodegradation on the bIRBP: retinoid molar ratio. Various ratios of bIRBP and retinoid (0:1,1:1; 0:2, 1:2; 0:5 1:5; respectively) were exposed to 7,780 $\mu\text{W}/\text{cm}^2$ for 30 min, followed by retinoid extraction, and quantification by HPLC. bIRBP: atROL (1:5 molar ratio): atROL percent remaining was significantly lower than that from bIRBP: atROL (1:2 molar ratio) (t-test, $P < 0.05$). Error bars represent $\pm$ SEM ($N = 3-5$).