Interphotoreceptor Retinoid-Binding Protein Mitigates Cellular Oxidative Stress and Mitochondrial Dysfunction Induced by All-trans-Retinal

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PURPOSE. Point and null mutations in interphotoreceptor retinoid-binding protein (IRBP) cause retinal dystrophy in affected patients and IRBP-deficient mice with unknown mechanism. This study investigated whether IRBP protects cells from damages induced by all-trans-retinal (aRAL), which was increased in the Irbp−/− retina.

METHODS. Wild-type and Irbp−/− mice retinal explants in buffer with or without purified IBRP were exposed to 800 lux light for different times and subjected to retinoid analysis by high-performance liquid chromatography. Purity of IRBP was determined by Coomassie Brilliant Blue staining and immunoblot analysis. Cellular damages induced by aRAL in the presence or absence of IRBP were evaluated in the mouse photoreceptor-derived 661W cells. Cell viability and death were measured by 3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and TUNEL assays. Expression and modification levels of retinal proteins were determined by immunoblot analysis. Intracellular reactive oxygen species (ROS) and nitric oxide (NO) were detected with fluorogenic dyes and confocal microscopy. Mitochondrial membrane potential was analyzed by using JC-1 fluorescent probe and a flow cytometer.

RESULTS. Content of aRAL in Irbp−/− retinal explants exposed to light for 40 minutes was significantly higher than that in wild-type retinas under the same light conditions. All-trans-retinal caused increase in cell death, tumor necrosis factor activation, and Adam17 upregulation in 661W cells. NADPH oxidase-1 (NOX1) upregulation, ROS generation, NO-mediated protein S-nitrosylation, and mitochondrial dysfunction were also observed in 661W cells treated with aRAL. These cytotoxic effects were significantly attenuated in the presence of IRBP.

CONCLUSIONS. Interphotoreceptor retinoid-binding protein is required for preventing accumulation of retinal aRAL, which causes inflammation, oxidative stress, and mitochondrial dysfunction of the cells.

Keywords: visual cycle, IRBP, all-trans-retinal, retinal degeneration, photoreceptor

The light-sensitive chromophore of the visual pigments in cone and rod photoreceptors is 11-cis-retinal (11cRAL). It keeps the visual pigments in an inactive conformation and functions as a molecular switch for activating opsins in response to light stimulation. When light hits the visual pigments, its energy converts 11cRAL to all-trans-retinal (aRAL), inducing structural rearrangement and activation of opsins. The activated opsins then trigger the phototransduction that converts light signal into electrical and neural signals in the photoreceptors. Both 11cRAL and aRAL possess a highly reactive aldehyde group and are the precursors of A2E,1,2 a cytotoxic byproduct of the retinoid visual cycle necessary for regenerating 11cRAL.3 Several studies have suggested that aRAL is a critical pathogenic factor in light-induced retinal degeneration and retinopathy of mouse model.4 In addition, A2E and aRAL dimer have been suggested to contribute to the pathology of Stargardt disease.5 These studies indicate that preventing excess accumulation of aRAL and 11cRAL is important for maintaining retinal health.

Interphotoreceptor retinoid-binding protein (IRBP) expressed by photoreceptors is known to bind with 11cRAL, aRAL, 11-cis-retinol (11cROL), and all-trans-retinol (aROL).6–9 This 140-kDa secretory glycoprotein with four homologous modules is the most abundant soluble protein in the interphotoreceptor matrix (IPM).10,11 The high abundance implicates its important role in protecting, solubilizing, and detoxifying retinoids in the IPM and photoreceptors. In ex vivo experiments, IRBP has been shown to promote release of 11cRAL from the retinal pigment epithelium (RPE)12,13 and aROL from the neural retina after photobleaching of the visual pigments.14–16 Irbp−/− mice display severe retinal degeneration and vision impairment.17–21

The significance of IRBP function in human retinal health and vision is reflected by the fact that mutations in IRBP are associated with retinal diseases and vision impairment. Recently, two nonsense mutations (Y510X and E1152X) of IRBP have been identified in children with high myopia and retinal dystrophy.22 In addition, a missense mutation (D1080N)
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has been found in adults with retinitis pigmentosa (RP). The RP-associated missense mutation has been shown to abolish secretion of IRBP in culture cells, suggesting that the mutation results in loss of IRBP in the patients’ IPM. Optical coherence tomography and electoretinography have shown that all affected patients with any of the mutations exhibit abnormal retinal structure and severe impairment of both cone and rod visual function, suggesting that IRBP is required for normal retinal development or for maintaining retinal structure and function. We recently have shown that tumor necrosis factor-α (TNF-α), a potent proinflammatory cytokine that can induce cellular apoptosis and necrosis, is increased several-fold in the Irbp−/− retina and IPM. However, the molecular mechanisms leading to upregulation and activation of TNF in Irbp−/− retina remain unknown. In this study, we first investigated the effect of IRBP deficiency on accumulation of atRAL in the retina. We then analyzed the pathologic roles of atRAL in induction of TNF activation, oxidative stress, mitochondrial dysfunction, and cell death. At the same time, we also investigated protective role of IRBP against atRAL-induced cytotoxicity in the mouse photoreceptor-derived 661W cells.

**METHODS**

**Animals and Ex Vivo Experiments**

Wild-type (WT) 129S2/Sv (Charles River Laboratories, Wilmington, MA, USA) and Irbp−/− mice were maintained in 12-hour cyclic light at ~30 lux. The Irbp−/− mice were originally generated by Liou et al. In this study, we used Irbp−/− mice with the 129S2/Sv genetic background. All animal experiments followed the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the protocols were approved by the Institutional Animal Care and Use Committee. For retinal ex vivo experiments, we isolated retinas from 5-week-old dark-adapted WT and Irbp−/− mice and placed each in 100 µl serum-free Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 20 mM HEPES buffer (pH 7.4). We added 4 µl purified IRBP, which is similar to IRBP concentration in rat IPM, into the media of WT retinal explants. After exposure to white light at 800 lux for 10, 20, or 40 minutes, retinoids were analyzed as described below.

**High-Performance Liquid Chromatography Analysis of Retinoids**

We converted atRAL and I1cRAL to syn- and anti-retinoloxime isomers by using 150 mM hydroxyamine before extraction of retinoids from retinas. Retinoids were extracted with hexane and analyzed by normal-phase HPLC as previously described.

In brief, retinoids in hexane extracts were evaporated, redissolved in 100 µl hexane, and separated on a silica column (Zorbax-Sil 5 µm; 250 × 4.6 mm; Agilent Technologies, Santa Clara, CA, USA) by gradient (0.2%-10% dioxane in hexane at 2.0 ml/min flow rate) elution of mobile phase in an Agilent 1100 HPLC system.

**Expression and Purification of IRBP**

The 293T-LC cells grown in DMEM containing 10% fetal bovine serum (FBS) were transfected with IRBP-expression plasmid (pIRBP) or pRK5 mock vector, as described previously. One day after transfection, we replaced the media with fresh serum-free or 1% FBS-containing DMEM and incubated an additional 16 hours. The media were centrifuged at 20,000g for 30 minutes. Presence of IRBP in the serum-free media of the pIRBP-transfected (IRBP-medium) or pRK5-transfected (mock-medium) cells was analyzed by Coomassie Brilliant Blue (CBB) staining and immunoblot analysis using an antibody against IRBP. The IRBP-medium and mock-medium were then concentrated with Amicon Ultra 100-KD molecular-weight cutoff (Millipore, Billerica, MA, USA). To purify IRBP from the 1% FBS-containing media, we precipitated IRBP with 16% saturated ammonium sulfate. The precipitates were resolved in 50 mM Tris-HCl buffer (pH 7.5) containing EDTA-free protease inhibitors, 150 mM NaCl, and 0.1 mM dithiothreitol, and were incubated with concavalin A (ConA) Sepharose 4B (GE Healthcare, Pittsburgh, PA, USA). After washing three times, IRBP was eluted with the buffer containing 10% methyl a-D-mannopyranoside (Sigma-Aldrich Corp., St. Louis, MO, USA). Purity of IRBP was analyzed by CBB staining and immunoblot analysis.

**Cell Viability Assay**

The 661W cells, a mouse photoreceptor-derived cell line, were kindly provided by Muayyad R. Al-Ubaidi at the University of Oklahoma and were maintained as described previously. For cell viability assay, the cells at a density of 5 × 10⁴ cells/well in a 96-well plate were incubated with the indicated concentration of atRAL in serum-free medium with or without IRBP for 24 hours. Cell viability was determined by using a CellTiter™ AQueous assay kit (Promega, Madison, WI, USA) as described previously. Briefly, the culture media were replaced with 95 µl fresh media containing 5 µl 3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) solution. After 1 hour, the absorbance at 490 nm was measured by using a microplate reader (Molecular Device, Sunnyvale, CA, USA).

**TUNEL Assay**

Detection and quantification of cell death at single-cell level was determined by TUNEL assay using the in situ cell death detection kit (Roche, Indianapolis, IN, USA), according to the manufacturer’s protocol. Briefly, 661W cells on glass coverslips (5 × 10⁵ cells/well in six-well plate) were treated with 1.8 µM atRAL, washed with phosphate-buffered saline (PBS), fixed with 4.0% paraformaldehyde in PBS for 15 minutes, and then permeabilized with 0.5% Triton X-100 in PBS for 10 minutes. After incubation with the mixture of enzyme solution and label solution for 1 hour at 37°C, fluorescent signals were captured with a Zeiss LSM710 Meta confocal microscope (Oberkochen, Germany) with an ×20 objective lens.

**Measurement of Intracellular Reactive Oxygen Species (ROS) and Nitric Oxide (NO)**

The cellular ROS and NO were measured by using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma-Aldrich Corp.) and 4,5-diaminofluorescein diacetate (DAF-2DA) (Sigma-Aldrich Corp.), respectively. 661W cells in a Corning Costar 96-well black clear-bottom plate at a density of 5 × 10⁵ cells/well were incubated with 1.8 µM atRAL in the presence or absence of IRBP for 2 hours, and then with 20 µM DCFH-DA or 10 µM DAF-2DA for 20 minutes. After washing with PBS twice, the relative fluorescence unit was measured at an excitation wavelength of 485 nm and an emission wavelength of 528 nm with a fluorescence microplate reader (Molecular Device). The cells incubated in the same condition were used for capturing fluorescent signals with a Zeiss LSM710 Meta confocal microscope with an ×20 objective lens. Each image
was collected under the same system settings (e.g., the same laser intensity and scan speed).

**Preparation of Cell Lysates and Mitochondrial and Nuclear Fractions**

661W cells incubated with aRAl in serum-free media with or without IRBP were washed with ice-cold PBS containing protease and phosphatase inhibitors and incubated with lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 1% SDS, 1% Nonidet P-40, 1 mM EDTA, 1 mM dithiothreitol, 200 mM benzamidine-HCl) for 30 minutes on ice. After centrifugation at 16,000 g for 20 minutes at 4°C, the supernatant was used as the cell lysates. Mitochondrial and nuclear fractions were prepared from 661W cells by using the Cell Fractionation Kit (Abcam, Cambridge, MA, USA), according to the manufacturer’s protocol.

**Analysis of Mitochondrial Membrane Potentials**

661W cells were incubated with 1.8 μM aRAl in fresh serum-free medium, in IRBP-medium, or in mock-medium for 6 hours. After washing with PBS, the cells were incubated with 2.5 μg/mL 5,5′,6,6′-tetrachloro-1,1,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (Thermo Fisher Scientific, Waltham, MA, USA) for 20 minutes. JC-1 is a green fluorescent cationic carbocyanine dye that enters the negatively charged mitochondrial matrix and forms J-aggregates that exhibit red fluorescence. After washing with PBS, the green fluorescent JC-1 monomers and the red fluorescent J-aggregates in the cells were detected with a Gallios Flow Cytometer (Beckman Coulter, Brea, CA, USA). A total of 30,000 cells were acquired. The data were analyzed by using the Kaluza Analysis Software and displayed in histogram of JC-1 green fluorescence and in dot plot of J-aggregate red fluorescence (y-axis) against JC-1 green fluorescence (x-axis).

**Immunoblot Analysis**

Proteins were separated in an 8%, 10%, or 12% polyacrylamide gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto an Immobilon-P membrane (Millipore). The membrane was incubated sequentially in blocking buffer, primary antibody, and horseradish peroxidase–conjugated anti-mouse or rabbit IgG secondary antibody. Antibodies against β-actin (Sigma-Aldrich Corp.), IRBP, TNF (Abcam), Adam17 (Chemicon, Billerica, MA, USA), S-nitrosocysteine (Sigma-Aldrich Corp.), apoptosis inducing factor (AIF) (Abcam), NADPH oxidase-1 (NOX1; GeneFex, Irvine, CA, USA), and PARP1 (Cell Signaling Technology, Danvers, MA, USA) were used as the primary antibodies. Immunoblots were visualized and quantified as described.

**Statistical Analysis**

Data were expressed as means ± standard deviation (SD) from at least three separate experiments unless otherwise indicated. Data were analyzed by using 1-way analysis of variance, followed by each pair of Student’s t-tests for multiple comparisons. All analyses were done with Microsoft Excel software (Microsoft, Redmond, WA, USA).

**RESULTS**

**Accumulation of aRAL in Irbp−/− Retina Exposed to Light**

Since IRBP has been shown to play an important role in protection and transportation of retinoids, we tested if aRAL accumulates in IRBP−/− retina after long-time photobleaching of the visual pigments. To do this experiment, we purified IRBP from pIRBP-transfected 293T-LC cell media by a ConA affinity chromatography (Fig. 1A). We then placed dark-adapted WT and IRBP−/− retinas in IRBP-containing or not containing buffer, which mimics WT and IRBP−/− IPMs, respectively. After exposure to bright light for different times, we analyzed retinoids in the retinas. Since IRBP−/− mice had retinal degeneration, we expressed the levels of aRAL as the percentage of total retinoids. As shown in Figure 1B, aRAL in WT retina decreased as light-exposure time increased. The amounts of aRAL in WT retina exposed to light for 40 minutes was significantly lower than those in WT retina exposed to light for 10 minutes (P < 0.01). However, the amounts of aRAL in IRBP−/− retina were not greatly changed during 10 to 40
minutes of light-exposure time (Fig. 1B). As a result, the contents of a RAL in Irbp/C0/C0 retina exposed to light for longer times were significantly higher than those in WT retina (Figs. 1B, 1C).

IRBP Alleviated Cell Death Induced by a RAL

Since a RAL possesses a highly reactive aldehyde group, we tested the effects of a RAL on cell viability. We incubated 661W cells with a series of increasing concentration (0–5 μM) of a RAL for 24 hours. As shown in Figure 2A, cell viability was significantly decreased by a RAL treatment in a dose-dependent manner. The estimated IC50 was approximately 1.8 μM. To examine the effect of IRBP on the a RAL-mediated inhibition of cell viability, we performed the cell viability assay with 1.8 μM a RAL as in (G). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). TUNEL-positive nuclei were indicated with arrows. Scale bars: 50 μm.

IRBP Prevents Cellular Damages Induced by a RAL

In a previous study, we have shown that TNF is dramatically increased in soluble fraction of the Irbp−/− IPM. We therefore tested whether a RAL can promote production of soluble TNF (sTNF), the active form of TNF, in 661W cells. We incubated the cells with a series of increasing concentration of a RAL in serum-free media. Immunoblot analysis showed that the level of sTNF in the cells treated with 1.8 μM a RAL was approximately 2-fold of that in control cells (Fig. 3A). To test if IRBP inhibits the a RAL-mediated...
activation of TNF, we incubated 661W cells with 1.8 μM aRAL, either in fresh serum-free medium, IRBP-medium, or mock-medium. As shown in Figure 3B, the aRAL-mediated elevation of sTNF was markedly suppressed by IRBP-medium, but not by mock-medium.

IRBP Inhibited Adam17 Upregulation Induced by aRAL

Since TNF-α converting enzyme (TACE)/Adam17 metalloprotease has been shown to mediate cleavage of membrane-bound pro-TNF in the post-Golgi,34 we tested the effect of aRAL on expression levels of Adam17 in 661W cells. Immunoblot analysis showed that protein levels of Adam17 (approximately 100 kDa) were increased 50% in the cells treated with 1.8 μM aRAL for 4 to 6 hours (Fig. 3C). This upregulation of Adam17 was significantly attenuated by incubation of aRAL with IRBP-medium (Fig. 3D). To test if Adam17 is upregulated in the Irbp−/− retina and RPE, we compared expression levels of Adam17 in WT and Irbp−/− tissues. Immunoblot analysis showed that Adam17 was increased at least 2-fold in the Irbp−/− retina and RPE (Figs. 3E, 3F).

IRBP Suppressed aRAL-Induced Production of ROS in 661W Cells

To analyze the mechanism by which aRAL causes cell damage, we tested if aRAL stimulates ROS generation in 661W cells. DCFH-DA is a cell-permeable fluorogenic probe. Reactive oxygen species in the cells convert DCFH-DA to highly fluorescent DCF. We incubated DCFH-DA with 661W cells treated with 0 to 1.8 μM aRAL in the presence or absence of IRBP. Confocal microscopy showed that the DCF fluorescent signal was dramatically increased in the cells treated with aRAL in fresh medium, as compared to the control cells maintained in fresh medium without aRAL (Figs. 4A, 4B). The DCF fluorescent signal was significantly decreased in the cells treated with aRAL in the IRBP-medium, but not in the mock-medium (Figs. 4A, 4B). We then analyzed expression of NOX1, a catalytic subunit of the superoxide-generating NADPH oxidase, in 661W cells. Immunoblot analysis showed that aRAL stimulated expression of NOX1 in a time-dependent manner (Fig. 4C). To test if IRBP suppresses the aRAL-induced upregulation of NOX1, we incubated 661W cells with aRAL in the IRBP-medium or mock-medium. Immunoblot analysis showed that the IRBP-medium, but not mock-medium, significantly reduced expression levels of NOX1 in the cells (Fig. 4D). To confirm this result in vivo, we analyzed expression levels of NOX1 in the retina and RPE of WT and Irbp−/− mice. As shown in Figures 4E and 4F, NOX1 was increased more than 2-fold in the Irbp−/− retina and RPE compared to WT retina and RPE.

IRBP Attenuated aRAL-Induced Production of NO

Since NO also plays an important role in induction of oxidative condition,35 we tested if aRAL increases intracellular NO level in 661W cells. To detect NO we used DAF-2DA, a fluorescent detector of NO in living cells. Confocal pictures showed that the fluorescent signal (red) of DAF in 661W cells was dramatically increased in the cells treated with aRAL in fresh medium, as compared to the control cells maintained in fresh medium without aRAL (Figs. 5A, 5B). The DCF fluorescent signal was significantly decreased in the cells treated with aRAL in the IRBP-medium, but not in the mock-medium (Figs. 5A, 5B). To confirm these results, we analyzed expression levels of NOX1 in the retina and RPE of WT and Irbp−/− mice. As shown in Figures 4E and 4F, NOX1 was increased more than 2-fold in the Irbp−/− retina and RPE compared to WT retina and RPE.

FIGURE 3. Interphotoreceptor retinoid-binding protein attenuated TNF activation triggered by aRAL. (A) Immunoblot analysis showing increase of sTNF in 661W cells treated with aRAL for 6 hours. Histogram shows relative immunoblot intensities of sTNF normalized by actin. (B) Immunoblot analysis of sTNF in 661W cells treated with aRAL in serum-free medium, mock-medium, or IRBP-medium. Histogram shows relative immunoblot intensities of sTNF. *P < 0.05 indicates significant differences between cells treated with aRAL in mock-versus IRBP-medium. (C) Immunoblot analysis of Adam17 in 661W cells treated with aRAL for indicated times. Histogram shows normalized intensities of Adam17 immunoblots. (D) Immunoblot analysis of Adam17 in 661W cells treated with aRAL in the indicated media. (E, F) Immunoblot analysis of Adam17 in the retina (E) and RPE (F) of WT and Irbp−/− mice. **P < 0.01 indicates significant differences between WT and Irbp−/− tissues. Error bars denote SD (n = 3).
IRBP Prevents Cellular Damages Induced by aRAL

FIGURE 4. Interphotoreceptor retinoid-binding protein suppressed aRAL-induced production of ROS. (A) Cells treated with aRAL in fresh serum-free, mock- or IRBP-medium were incubated with the DCFH-DA fluorogenic probe. Reactive oxygen species–induced fluorescence was visualized by confocal microscopy. Scale bars: 50 μm. (B) Relative fluorescence intensities in the cells shown in (A). Similar results were observed in three independent experiments. **P < 0.01 indicates significant differences between cells treated with aRAL in mock-versus IRBP-medium. (C) Immunoblot analysis showing increase of NOX1 in the cells treated with aRAL for indicated times. Histogram shows relative intensities of NOX1 immunoblots normalized by actin. Error bars denote SD (n = 3). (D) Immunoblot analysis of NOX1 in 661W cells treated with aRAL either in serum-free, mock- or IRBP-medium for 2 hours. Normalized intensities of NOX1 immunoblots are shown in the histogram. (E, F) Immunoblot analysis (E) and relative immunoblot intensities (F) of NOX1 in the retina and RPE of WT and Irbp−/− mice. **P < 0.01 indicates significant differences between WT and Irbp−/− tissues.

IRBP Mitigated Mitochondrial Dysfunction Induced by aRAL

Oxidative stress has been shown to cause mitochondrial dysfunction.36 We therefore analyzed mitochondrial membrane potentials by using a cell-permeable JC-1 dye, a mitochondrial potential indicator that exists either as a green fluorescent monomer at depolarized membrane potentials or as a red fluorescent J-aggregate at hyperpolarized membrane potentials. As shown in Figures 6A and 6B, the populations of cells with green fluorescence were increased in the cells treated with aRAL in fresh medium (20% ± 1.2%) or mock-medium (23% ± 1.0%), as compared to the untreated control cells (3.9% ± 0.1%) or the cells treated with aRAL in IRBP-medium (4.6% ± 0.8%).

We then further analyzed the mitochondrial integrity by detecting AIF in mitochondrial and nuclear fractions. Apoptosis inducing factor is normally localized to mitochondria, but when mitochondrion is damaged, it moves to the cytosol and the nucleus.37 Qualitative immunoblot analysis showed that AIF was increased in the nuclear fraction of 661W cells treated with aRAL in fresh medium or mock-medium, as compared to the control cells and the cells treated with aRAL in IRBP-medium (Figs. 6C, 6D). In contrast, AIF was decreased in the mitochondrial fraction of 661W cells treated with aRAL in fresh medium or mock-medium, as compared to the control cells and the cells treated with aRAL in IRBP-medium (Figs. 6C, 6E).

DISCUSSION

Using purified IRBP and retinal explants of WT and Irbp−/− mice, we demonstrated that IRBP is essential for preventing accumulation of aRAL in the retina under bright light conditions. Furthermore, we found that IRBP plays an important role in protection of the mouse photoreceptor-derived 661W cells from TNF activation, oxidative stress, and mitochondrial dysfunction caused by aRAL. These findings may reflect a mechanism of IRBP function in retinal neuroprotection.

The well-known biochemical function of IRBP is its high-affinity binding with retinoids involved in the visual cycle. Although all retinoid isomers can bind with IRBP, the major composition of retinoids bound to IRBP changes depending on light condition. In dark-adapted eyes, 11cRAL increases while aROL decreases. In light-exposed eyes, however, aRAL and aROL increases, whereas 11cRAL decreases.7,8 Retinoids decompose rapidly into a number of products, including their aldehyde form, retinals.9 Interphotoreceptor retinoid-binding protein has been shown to protect retinol from isomerization, oxidation, and photodegradation.9,38 In this study, we observed that the content of aRAL in Irbp−/− retinal explants exposed to light for 40 minutes was significantly higher than that in WT retinas under the same light conditions (Fig. 1). Since IRBP
promotes the release of arROL from isolated retina after photobleaching of the visual pigments.\textsuperscript{14,15} The higher content of arRAL in the Irbp\textsuperscript{−/−} retina could be due to oxidation of arROL retained in photoreceptors by retinol dehydrogenases (RDHs) and/or by photothermal oxidation.

The neural retina and RPE contain a large amount of retinoids. In dark-adapted eye, at least 80\% of total retinoids are arRAL, and 60\% of total retinoids in the neural retina are arRAL.\textsuperscript{18} The neural retina and RPE contain large amounts of arRAL and localized to the neural retina, 18 mainly in photoreceptors. Following exposure to bright light, which causes large photobleaching of the visual pigments, at least 80\% of total retinoids are a

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\caption{Interphotoreceptor retinoid-binding protein attenuated arRAL-induced production of NO in 661W cells. (A) Cells treated with arRAL in fresh serum-free, mock-, or IRBP-medium were incubated with DAF-2DA fluorescence detector of NO for 20 minutes. Nitric oxide was visualized by confocal microscopy. \textit{Scale bars:} 20 μm. (B) Relative DAF intensities in the cells shown in (A). Similar results were observed in three independent experiments. **P < 0.01 indicates significant differences between cells treated with arRAL in mock- versus IRBP-medium. (C) S-nitrosylated proteins in the cells treated with arRAL for indicated times were detected by immunoblot analysis with an antibody against S-nitroso-cysteine. (D) Normalized relative immunoblot intensities of S-nitrosylated proteins shown in (C). (E) Immunoblot analysis of S-nitrosylated proteins in the cells treated with arRAL in serum-free, mock-, or IRBP-medium. (F) Relative immunoblot intensities of S-nitrosylated proteins shown in (E). **P < 0.01 indicates significant differences between cells treated with arRAL in mock- versus IRBP-medium.
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oxidative and nitrosative stresses by damaging macromolecules.\textsuperscript{35,50} In the present study, we observed that a

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\caption{Interphotoreceptor retinoid-binding protein attenuated arRAL-induced production of NO in 661W cells. (A) Cells treated with arRAL in fresh serum-free, mock-, or IRBP-medium were incubated with DAF-2DA fluorescence detector of NO for 20 minutes. Nitric oxide was visualized by confocal microscopy. \textit{Scale bars:} 20 μm. (B) Relative DAF intensities in the cells shown in (A). Similar results were observed in three independent experiments. **P < 0.01 indicates significant differences between cells treated with arRAL in mock- versus IRBP-medium. (C) S-nitrosylated proteins in the cells treated with arRAL for indicated times were detected by immunoblot analysis with an antibody against S-nitroso-cysteine. (D) Normalized relative immunoblot intensities of S-nitrosylated proteins shown in (C). (E) Immunoblot analysis of S-nitrosylated proteins in the cells treated with arRAL in serum-free, mock-, or IRBP-medium. (F) Relative immunoblot intensities of S-nitrosylated proteins shown in (E). **P < 0.01 indicates significant differences between cells treated with arRAL in mock- versus IRBP-medium.
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oxidant that also causes cellular damage by reacting directly with macromolecules.\textsuperscript{35,50} In the present study, we observed

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\caption{Interphotoreceptor retinoid-binding protein attenuated arRAL-induced production of NO in 661W cells. (A) Cells treated with arRAL in fresh serum-free, mock-, or IRBP-medium were incubated with DAF-2DA fluorescence detector of NO for 20 minutes. Nitric oxide was visualized by confocal microscopy. \textit{Scale bars:} 20 μm. (B) Relative DAF intensities in the cells shown in (A). Similar results were observed in three independent experiments. **P < 0.01 indicates significant differences between cells treated with arRAL in mock- versus IRBP-medium. (C) S-nitrosylated proteins in the cells treated with arRAL for indicated times were detected by immunoblot analysis with an antibody against S-nitroso-cysteine. (D) Normalized relative immunoblot intensities of S-nitrosylated proteins shown in (C). (E) Immunoblot analysis of S-nitrosylated proteins in the cells treated with arRAL in serum-free, mock-, or IRBP-medium. (F) Relative immunoblot intensities of S-nitrosylated proteins shown in (E). **P < 0.01 indicates significant differences between cells treated with arRAL in mock- versus IRBP-medium.
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retina, whereas the amount of TNF in the Irbp\textsuperscript{−/−} IPM is more than 10-fold higher than that in WT IPM,\textsuperscript{25} suggesting that secreted sTNF is increased in the Irbp\textsuperscript{−/−} IPM. It is known that the secreted sTNF, the more active form of TNF, is derived from the transmembrane-anchored TNF precursor through the TACE/Adam17-mediated proteolytic process in the post-Golgi and plasma membrane.\textsuperscript{34,45} In this study, we observed that Adam17 was upregulated in 661W cells by arRAL treatment (Fig. 3C). Consistent with this result, sTNF, but not TNF precursor, was significantly increased in arRAL-treated 661W cells (Fig. 3A). Both sTNF and Adam17 were reduced when the cells were treated with arRAL in the presence of IRBP (Figs. 3B, 3D). These results suggest that arRAL is one of the pathogenic factors that induced upregulation of TNF and Adam17 in the Irbp\textsuperscript{−/−} retina and RPE (Figs. 3E, 3F) and that Adam17 upregulated by arRAL promoted secretion of sTNF into the Irbp\textsuperscript{−/−} IPM.

Reactive oxygen species and NO are important intracellular messengers that regulate cellular function, survival, and differentiation.\textsuperscript{46,47} Reactive oxygen species induce upregulation of Adam17,\textsuperscript{48} and NO activates Adam17.\textsuperscript{49} We found that arRAL stimulated the generation of ROS and NO in 661W cells (Figs. 4A, 4B, 5A, 5B). These observations suggest that ROS and NO are involved in upregulation and activation of Adam17 in 661W cells treated with arRAL.

Excessive generation of ROS and/or NO induces cellular oxidative and nitrosative stresses by damaging macromolecules important for cellular structure, function, and survival.\textsuperscript{55,56} NOX1 plays a critical role in generation of ROS. Reaction between superoxide and NO produces peroxynitrite, a strong oxidant that also causes cellular damage by reacting directly with macromolecules.\textsuperscript{55,56} In the present study, we observed
that NOX1 expression (Fig. 4C) and protein S-nitrosylation were significantly increased in 661W cells treated with aRAL (Fig. 5C). In addition, both aRAL (Fig. 1) and NOX1 (Figs. 4E, 4F) were increased in the Irbp/C0/+/C0 ocular tissues. Consistent with these results, IRBP inhibited the aRAL-mediated NOX1 upregulation and protein S-nitrosylation (Figs. 4D, 5E, 5F).

Since oxidative and nitrosative stresses are pathologic mechanisms involved in rod and cone photoreceptor degeneration in retinal degenerative diseases,35,51–53 our results suggest that IRBP plays an important role in protection of photoreceptors from oxidative stress through suppression of aRAL accumulation and its thiol-dependent antioxidant activity.54

In a previous study,18 we have shown that dark rearing could not prevent degeneration of photoreceptors in Irbp/−/− mice. This observation suggests that IRBP has an unidentified function(s) necessary for photoreceptor survival. Expression of IRBP in the developing retina implicates a potential role for IRBP in retinal development and/or maturation.6,58 Wisard et al.21 have found that the size and weight of Irbp/C0/+/C0 mouse eyes are greater than those of WT mice. The excessive ocular enlargement starts between P7 and P10. Although the mechanism of how IRBP regulates ocular development remains
unknown, it is known that the IPM, where IRBP accumulates, contains numerous growth and neurotrophic factors that regulate cell proliferation, differentiation, and survival.\(^{10,60}\) The significant increase of TNF in the \(Irbp^{−/−}\) IPM suggests that IRBP is required for maintaining IPM homeostasis essential for retinal neurodevelopment and function. Both aRfαL-mediated cellular damages and aberrant regulation of extracellular signaling proteins in the IPM may contribute to retinal degeneration in \(Irbp^{−/−}\) mice. Identification of IRBP-interacting protein in the IPM is therefore important for understanding the molecular mechanism of IRBP function and may contribute to the development of therapeutic interventions for retinal dystrophy caused by IRBP mutations.

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