Involvement of Endoplasmic Reticulum Stress in All-Trans-Retinal-Induced Retinal Pigment Epithelium Degeneration

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ABSTRACT

Excess accumulation of endogenous all-trans-retinal (atRAL) contributes to degeneration of the retinal pigment epithelium (RPE) and photoreceptor cells, and plays a role in the etiologies of age-related macular degeneration (AMD) and Stargardt’s disease. In this study, we reveal that human RPE cells tolerate exposure of up to 5 μM atRAL without deleterious effects, but higher concentrations are detrimental and induce cell apoptosis. atRAL treatment significantly increased production of intracellular reactive oxygen species (ROS) and up-regulated mRNA expression of Nrf2, HO-1, and c-GCSh within RPE cells, thereby causing oxidative stress. ROS localized to mitochondria and endoplasmic reticulum (ER). ER-resident molecular chaperone BiP, a marker of ER stress, was up-regulated at the translational level, and meanwhile, the PERK-eIF2α-ATF4 signaling pathway was activated. Expression levels of ATF4, CHOP, and GADD34 in RPE cells increased in a concentration-dependent manner after incubation with atRAL. Salubrinal, a selective inhibitor of ER stress, alleviated atRAL-induced cell death. The antioxidant N-acetylcysteine (NAC) effectively blocked RPE cell loss and ER stress activation, suggesting that atRAL-induced ROS generation is responsible for RPE degeneration and is an early trigger of ER stress. Furthermore, the mitochondrial transmembrane potential was lost after atRAL exposure, and was followed by caspase-3 activation and poly (ADP-ribose) polymerase cleavage. The results demonstrate that atRAL-driven ROS overproduction-induced ER stress is involved in cellular mitochondrial dysfunction and apoptosis of RPE cells.

Key words: all-trans-retinal; retinal pigment epithelium; endoplasmic reticulum; reactive oxygen species; mitochondrial dysfunction.

The visual cycle, also known as the retinoid cycle, is an indispensable metabolic process responsible for continuous generation of 11-cis-retinal from all-trans-retinal (atRAL) (Fig. 1A) in vertebrate retina (Rando, 2001). As a highly reactive aldehyde-bearing agent as well as a potent photosensitizer, atRAL can permeabilize and kill cells (Maeda et al., 2009; Masutomi et al., 2012; Sparrow et al., 2010). Upon exposure to light, 11-cis-retinal in the retina is converted to atRAL, and careful management of the latter is achieved by photoreceptor and retinal pigment epithelial (RPE) cells in a functional visual cycle. atRAL is cleared from photoreceptors via the ATP-binding cassette transporter ABCA4 and retinol dehydrogenases (RDHs). ABCA4 transports or flips the Schiff base condensation product of atRAL and phosphatidylethanolamine across the disc membrane into the
cytoplasm of the photoreceptor outer segment where atRAL is converted to all-trans-retinol by means of RDHs (Chen et al., 2012a; Molday et al., 2009). All-trans-retinol is then transferred to the RPE where it is ultimately converted back to 11-cis-retinal. RDH8, a retinoid reductase in photoreceptors, is capable of clearing the bulk of atRAL released from photoactivated rhodopsin after light exposure (Chen et al., 2012a). As they are critical to atRAL clearance, deficiency of either RDH8 or ABCA4 typically leads to retinal degeneration. Indeed, mice with a double knock-out of the RDH8 and ABCA4 genes exhibit accumulations of atRAL condensation products, photoreceptor loss, and RPE dystrophy in the retina at an early age (Maeda et al., 2008). The buildup of excess atRAL is a likely cause of these changes (Chen et al., 2012b; Maeda et al., 2009).

Cells of the RPE form a monolayer between photoreceptor outer segments and the choroid, and are firmly attached to Bruch’s membrane (BrM). Important functions of RPE include nourishment of photoreceptor cells and daily phagocytosis of shed photoreceptor outer segments, enabling photoreceptors to maintain excitability and achieve self-renewal. The RPE also sustains function of the choriocapillaris, a specialized capillary bed that lies beneath the RPE on the opposite side of BrM (Ford et al., 2011). In light of the active metabolism of photoreceptor cells, the integrity of the choriocapillaris and the RPE is essential for meeting metabolic demands of photoreceptors and maintaining the normal physiologic function of the eyes. RPE is especially vulnerable to damage owing to its phagocytosis of photoreceptor outer segments, anatomic location, and high metabolic activity (He et al., 2008). Diffusion of atRAL from photoreceptor outer segments, the phagocytization of photoreceptor outer segments by RPE cells, and the fact that most atRAL-eliminating RDHs are found in photoreceptor cells rather than in the RPE (Parker and Crouch, 2010), can all contribute to excessive and dangerous atRAL accumulation in the RPE.

Previous studies have demonstrated that reactive oxygen species (ROS) production, which can be induced by atRAL and mediated through NAPDH oxidase, is involved in the degeneration of photoreceptors and RPE (Chen et al., 2012b). There is evidence to indicate that adverse effects of atRAL include DNA damage, Bax activation, caspase activation, and mitochondria-associated cell death (Maeda et al., 2009; Sawada et al., 2014), but the precise sequence of molecular events underlying RPE degeneration remains unclarified.

A growing body of research suggests that endoplasmic reticulum (ER) stress is associated with the pathogenesis of several eye diseases such as retinitis pigmentosa, glaucoma, and...
ER stress can develop in response to various stimuli including glucose starvation, hypoxia, defective protein secretion/degradation, and oxidative stress. ROS are thought to play an important role in ER damage (Naranmandura et al., 2012). Disruption of ER function results in the buildup of unfolded and misfolded proteins in the ER lumen, which in turn initiates the unfolded protein response (UPR) which activates changes in three transmembrane receptors (i.e., eukaryotic translation initiation factor 2-alpha kinase 3 [PERK], inositol-requiring enzyme 1α [IRE1α], and activating transcription factor 6 [ATF6]) and adaptive responses to maintain cellular homeostasis and promote cell survival. Nevertheless, when ER injury persists and adaptive responses to maintain cellular homeostasis and promote cell survival are overwhelmed, ER stress becomes pathological and initiates cell death (Ruegg et al., 2008). ER stress in the retina has been shown to be associated with retinal degenerative disorders including age-related macular degeneration (AMD) (Lin et al., 2007; Zode et al., 2011). Disruption of ER function results in the buildup of unfolded and misfolded proteins in the ER lumen, which in turn initiates the unfolded protein response (UPR) which activates changes in three transmembrane receptors (i.e., eukaryotic translation initiation factor 2-alpha kinase 3 [PERK], inositol-requiring enzyme 1α [IRE1α], and activating transcription factor 6 [ATF6]) and adaptive responses to maintain cellular homeostasis and promote cell survival. Nevertheless, when ER injury persists and adaptive responses to maintain cellular homeostasis and promote cell survival are overwhelmed, ER stress becomes pathological and initiates cell death (Ruegg et al., 2008).

In the present study, we evaluated intracellular ROS generation and oxidative stress, as well as subcellular localization of ROS, in atRAL-loaded human RPE cells. We also investigated whether ER stress occurred in atRAL-treated RPE cells, and whether it was involved in atRAL-induced RPE cell apoptosis. We were able to elucidate the relationships between atRAL-associated ROS overproduction, ER stress, and mitochondrial dysfunction in RPE cells. Since excessive accumulation of endogenous atRAL can affect RPE, our findings provide more insightful understanding of atRAL toxicity in human RPE cells and retinopathies characterized by delayed atRAL clearance such as AMD and recessive Stargardt’s disease.

**MATERIALS AND METHODS**

Materials Thapsigargin (Tg) and atRAL were purchased from Sigma-Aldrich (St. Louis, Missouri). Salubrinal was obtained from Santa Cruz Biotechnology (Santa Cruz, California). N-acetylcysteine (NAC), a prodrug of L-cysteine which is a precursor to the biologic antioxidant glutathione, was purchased from Beyotime (Haimen, China). atRAL was prepared as a stock dimethyl sulfoxide (DMSO) solution (20 mM) that was stored in a refrigerator at −20°C. The stock solution was diluted with fresh medium to achieve desired final concentrations. A human RPE cell line (ARPE-19) that displays the differentiated phenotype of RPE cells, containing marker proteins specific for RPE cells, and displaying the differentiated phenotype of RPE cells, was maintained in Dulbecco’s Modified Eagle’s medium with 10% fetal calf serum (HyClone, Logan, Utah) in a humidified incubator at 37°C and 5% carbon dioxide (CO2). Cells were divided 1:3 at every passage and passages 2–8 following cell resuscitation were used in the study. Cells seeded in 6- or 96-well cell culture plates (Nunc, Shanghai, China) attained 70%–80% confluence and were used for subsequent experiments.

**Analysis of cell viability.** Cytotoxicity was assessed by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Solabio, Beijing, China). Briefly, after incubations of ARPE-19 cells with chemicals for indicated time intervals, 20 μl of MTT solution was added to 200 μl of culture medium in each well to achieve a final concentration of 0.5 mg/ml. Following 4 h of incubation at 37°C, the solution was removed and 150 μl DMSO were added to each well. The resulting solutions, after oscillating for 10 min, were spectrophotometrically measured at 490 nm. Cell viability was presented as a proportion of control optical density. The half maximal inhibitory concentration (IC50) of atRAL after exposure to ARPE-19 cells was calculated with SPSS software (SPSS Inc, Chicago, Illinois).

Annexin-V/propidium iodide flow cytometry analysis. Flow cytometric assays to monitor apoptosis using Annexin-V/propidium iodide (PI) staining (BioVision, Mountain View, California) were performed according to the manufacturer’s instructions with minor modifications. Briefly, after incubation with atRAL (0, 5, 10, 15, or 20 μM) for 6 h, ARPE-19 cells were harvested and washed twice with ice-cold phosphate-buffered saline (PBS). Annexin V-fluorescein isothiocyanate (FITC) and PI were added to the cell suspension and incubated at room temperature for 10 min in the dark. For each sample, at least 104 cells were analyzed using a Cytomics FC 500 flow cytometer (Beckman Coulter, Brea, California).

**Measurement of ROS in RPE cells.** The production of total ROS was tested using a dichloro-dihydro-fluorescein diacetate (DCFH-DA) staining assay (Beyotime). ARPE-19 cells seeded on 96- or 6-well culture plates were maintained in a humidification incubator for 24 h and then exposed to atRAL (0, 5, 10, 15, or 20 μM) for 6 h. Cells were then washed with PBS, incubated with JC-1 for 30 min at 37°C, and photographed under an inverted fluorescence microscope (BX51W1-FV1000, Olympus, Japan). Alternatively, cells were harvested, washed with PBS, and immediately analyzed using the flow cytometer.

**Localization of ROS in RPE cells.** ARPE-19 cells seeded in a 35-mm glass-bottomed dish (Shengyou Biotechnology, Hangzhou, China) were maintained in a humidification incubator for 24 h and then exposed to 0 or 15 μM atRAL for 6 h. The ROS fluorescent probe DCFH-DA was excited by an argon ion laser. ER-Tracker and Mito-Tracker were used to label ER and mitochondria, respectively. Confocal micrographs were obtained using a Cytomics FC 500 flow cytometer (Beckman Coulter, Brea, California).
TABLE 1. The Sequences of Primers for Real-Time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
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<tr>
<td>Nrf2</td>
<td>Nrf2-F</td>
<td>5'-CGTATGCAACAGGACATTG-3'</td>
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<td></td>
<td>Nrf2-R</td>
<td>5'-ACTTGGTGGGTCTTCTGTG-3'</td>
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<td>HO-1-F</td>
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<td>HO-1-R</td>
<td>5'-AACGCTTACGCGCCAGCTAAAG-3'</td>
</tr>
<tr>
<td>γ-GCSH</td>
<td>γ-GCSH-F</td>
<td>5'-TTCAGAGGACATGATGAC-3'</td>
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<tr>
<td></td>
<td>γ-GCSH-R</td>
<td>5'-GATATGGCTGGATTCTTCTCT-3'</td>
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<tr>
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<td>BiP-F</td>
<td>5'-TTTCATTCCAGGAGGAT-3'</td>
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<td>BiP-R</td>
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<td>GRP94-F</td>
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<td>GRP94-R</td>
<td>5'-TGGGTTGCTGGTTCTTCTCC-3'</td>
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<tr>
<td>ATF4</td>
<td>ATF4-F</td>
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<tr>
<td></td>
<td>ATF4-R</td>
<td>5'-TTCTGATGGCTGCGTTCTCTCC-3'</td>
</tr>
<tr>
<td>CHOP</td>
<td>CHOP-F</td>
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<td>CHOP-R</td>
<td>5'-GACCTGCAAGAGGCTGTC-3'</td>
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<td>GADD34</td>
<td>GADD34-F</td>
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</tr>
<tr>
<td></td>
<td>GAPDH-R</td>
<td>5'-GGCTGCTGCTGGGTCCATTG-3'</td>
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emission was detected by a 560-nm long-pass filter. Confocal scanning parameters were established to ensure that cells from wells without the probes did not display a fluorescent signal. The cells exposed to DCFH-DA produced a green signal, whereas cells exposed to ER-Tracker or Mito-Tracker produced a red signal.

RNA extraction, complementary DNA synthesis, and quantitative real-time polymerase chain reaction. Total cellular RNA was purified using TRIzol reagent (Invitrogen Inc, Carlsbad, California), and complementary DNA (cDNA) was synthesized using a ReverTra Ace qPCR RT kit (TOYOBO, Japan). Quantitative real-time polymerase chain reaction (qPCR) was performed using Brilliant SYBR Green qPCR Master Mix (Takara, Japan). The qPCR cycling consisted of 40 cycles of amplification of the template cDNA with primer annealing at 60°C and was carried out on a CFX96 qPCR detection system (Bio-Rad, USA). The sequences of primers (Shanghai Sunny Biotechnology, Shanghai, China) used for qPCR are listed in Table 1. The amplification efficiencies of each gene did not amplify and exhibited a CT value more than 33. Each qPCR was performed on at least 3 different experimental samples, and each sample was assessed in triplicate. The relative expression level of each target gene was calculated using the 2^−ΔΔCt method (Livak and Schmittgen, 2001), and was normalized with that of reference gene GAPDH.

Western blot analysis. Immunoblotting was performed using the following primary antibodies: mouse anti-transcriptional factor C/EBP homologous protein (CHOP), rabbit anti-BiP, rabbit anti-phospho-eukaryotic initiation factor 2 (eIF2α), rabbit anti-caspase-3, rabbit anti-cleaved poly (ADP-ribose) polymerase (PARP), mouse anti-ß-tubulin (Cell Signaling Technology, Danvers, Massachusetts), and rabbit anti-phospho-PERK (Santa Cruz Biotechnology). Cellular proteins were extracted with radioummunoprecipitation assay lysis buffer (Beyotime) and the protein concentration was determined with an enhanced bicinchoninic acid protein assay kit (Beyotime). Extracted proteins were applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) denaturing gels. After electrophoresis, proteins on the gel were transferred to a nitrocellulose blotting membrane, which was then blocked in 5% non-fat milk for 2 h at room temperature. Each membrane was incubated overnight at 4°C with the specific primary antibody and an IRDye 800CW donkey anti-rabbit or an IRDye 680RD donkey anti-mouse secondary antibody (LI-COR Biosciences, Lincoln, Nebraska) for 2 h at room temperature. The protein bands were visualized and quantified by an Odyssey Infrared Imaging system (LI-COR Biosciences) with the use of 2-color fluorescence detection at 700 and 800 nm.

Statistical analysis. All experiments were performed at least 3 times. Data were analyzed by 1-way analysis of variance, followed by the Newman-Keuls test for multiple comparisons. A P value of less than 0.05 was accepted as significant. The statistical and graphical software GraphPad Prism (Version 5, GraphPad Software, Inc, La Jolla, California) was used for all analyses.

RESULTS

atRAL-Induced Apoptosis in RPE Cells

As shown in Figure 1B, atRAL decreased RPE cell survival as a function of both exposure time and atRAL concentration. The IC50s for atRAL after incubations of 3, 6, or 12 h were 17.59, 16.16, and 13.75 μM, respectively. Additionally, morphologic changes of ARPE-19 cells were observed following atRAL exposure (Supplementary Fig. 1). Based on morphologic images and MTT data, the test concentration of 5 μM atRAL appeared to produce no cytotoxicity in ARPE-19 cells; however, concentrations starting from 10 μM caused extensive changes in cell morphology (round, shrunken), and 15 and 20 μM atRAL significantly decreased cell viability by approximately 50% and 80%, respectively, after 6 h of exposure (Fig. 1B and Supplementary Fig. 1). Annexin V/PI staining revealed Annexin V+/PI− and Annexin V+/PI+ cells, indicating cell death by both early- and late-stage apoptosis. After incubation with atRAL for 6 h, the percentage of Annexin V positive cells (apoptotic cells) was elevated in a concentration-dependent manner (Fig. 1C).
FIG. 2. atRAL provokes ROS generation and oxidative stress in RPE cells. A, Intracellular ROS, 6 h after exposure of cells to 0–15 μM atRAL, was visualized by fluorescence microscopy. B, Levels of intracellular ROS, 6 h after incubation of cells with 0–20 μM atRAL, were measured by flow cytometry (a). Data are calculated as fold increase in fluorescence in treated cells compared with that of untreated cells (b). C, The mRNA expression levels of oxidative stress-related genes Nrf2 (a), HO-1 (b), and γ-GCS (c) in cells untreated or treated with 5–20 μM atRAL for 6 h were quantified by qPCR. Expression levels were normalized to GAPDH mRNA levels, and expressed as fold increase in treated cells versus control cells. Each value represents mean ± SEM of 3 independent experiments, each performed in triplicate. D, Intracellular ROS levels, 6 h after incubating cells with 15 μM atRAL in the absence or presence of 2.5 mM NAC were measured by flow cytometric assay (a) and were also presented as fold increase in treated cells compared with control cells (b). E, Cell viability, 6 h after introducing 0–5 mM NAC and 15 μM atRAL to cultures, was probed by MTT assay. Each value represents mean ± SEM (n = 3–6). *P < 0.05; ***P < 0.001.
AtRAL Provoked ROS Generation and Oxidative Stress in RPE Cells
After atRAL-treated ARPE-19 cells were incubated with the fluorescent probe DCFH-DA, fluorescence microscopy imaging indicated that fluorescence intensity, which reflects ROS levels in the cytoplasm, increased with increasing atRAL exposure concentrations (Fig. 2A). To further corroborate the ROS overproduction, we also measured ROS generation by flow cytometry. Compared with control cells, the quantity of intracellular ROS did not significantly change in cells treated with 5 μM atRAL, but was significantly elevated in cells treated with 10 or 20 μM atRAL (increases of approximately 2- or 4.3-fold, respectively) (Fig. 2B, a and b).

The antioxidant response element (ARE)-driven HO-1 and γ-GCSH genes up-regulations, which are mediated by nuclear factor E2-related factor 2 (Nrf2), represent important cellular adaptive survival responses to various cellular stresses, especially oxidative stress. qPCR analysis to assess mRNA expression levels of Nrf2, HO-1, and γ-GCSH revealed that expression of Nrf2 and γ-GCSH was significantly increased after incubation with 20 μM atRAL (Fig. 2C, a and c), but was not affected by lower atRAL concentrations (5 and 10 μM). The mRNA expression level of HO-1 was increased in a concentration-dependent manner after treatment with 10 and 20 μM atRAL (approximately 3- and 13.5-fold of that in the untreated group, respectively) (Fig. 2C, b).

NAC is an antioxidant that can quench free radicals (Cao et al., 2009). To test whether NAC could attenuate atRAL-induced cell death, we examined intracellular ROS level and cell viability in the presence or absence of NAC. Inclusion of 2.5 mM NAC in RPE cells treated with 15 μM atRAL dramatically reduced ROS production when compared with cells treated with atRAL in the absence of NAC (Fig. 2D, a and b). Additionally, MTT assay results showed that, after 6 h of incubation with atRAL in the presence of NAC, the decreases in cell viability due to atRAL exposure were significantly counteracted by NAC (Fig. 2E).

Subcellular Localization of ROS Generated Following Exposure of RPE Cells to atRAL
Confocal microscopic imaging of fluorescently probed cells revealed that ROS-associated fluorescence dispersively distributed in the cytoplasm (Fig. 3A, white arrow head) and partially colocalized within mitochondria (Mito-Tracker), as indicated by yellow fluorescence (Fig. 3A, white arrow), suggesting that ROS generated by atRAL treatment could cause a direct oxidative damage to mitochondria. Massive ROS fluorescence was
atRAL Increased the Expression of BiP in RPE Cells

mRNA expression of BiP, a gene that codes for the ER-resident molecular chaperone BiP (an ER stress marker), was not significantly elevated in human RPE cells after incubation with atRAL for 6 h (Fig. 4A); there was, however, a slight upward trend in expression with increasing atRAL concentration (a 0.24-fold increase at the 20 μM group in comparison with that in the control). By contrast, after thapsigargin (Tg, an inhibitor of ER Ca2+ uptake), which serves as the ER stress positive control, was incubated with ARPE-19 cells for 6 h, BiP expression was increased by approximately 22-fold versus the control group without causing obvious cell death. Furthermore, Western blot analysis showed that BiP protein was concentration-dependently increased in cells treated with 0–20 μM atRAL for 6 h (Fig. 4B), and increased following treatment with 15 μM atRAL and incubation for 0.5, 1, 3, or 6 h (Fig. 4C).

Activation of the PERK-eIF2α-ATF4 Signaling Pathway in RPE Cells by atRAL

Under physiological conditions, the ER-resident molecular chaperone BiP binds with PERK and inhibits its phosphorylation (Sano and Reed, 2013). When unfolded proteins accumulate during ER stress, PERK dissociates from BiP and is autophosphorylated, and thus inhibits the global protein synthesis (Lai et al., 2007). In the current study, Western blot analysis showed that phosphorylated PERK was up-regulated after treatment with 5 and 10 μM atRAL for 6 h but decreased after treatment with 15 and 20 μM (Fig. 5A), and expression of phospho-eIF2α was up-regulated with increasing atRAL concentration, and the effect was statistically significant at a concentration of 20 μM atRAL (Fig. 5A). Moreover, expression of phospho-PERK and phospho-eIF2α exhibited an increase after short (0.5 and 1 h) incubations with 15 μM atRAL, but showed a trend to decrease from 3 to 6 h (Fig. 5B).

Phosphorylation of eIF2α also allows for preferential transcription of UPR-dependent genes such as ATF4. Important targets driven by ATF4 are CHOP and GADD34 (growth arrest and DNA damage-inducible 34). Transcription levels of ATF4 (Fig. 5C) and CHOP (Fig. 5D) as measured by qPCR were significantly up-regulated after incubation with atRAL (10, 15, or 20 μM) for 6 h. Western blot analysis showed that CHOP was raised in a concentration-dependent manner after atRAL treatment for 6 h (Fig. 5E). Since CHOP-mediated activation of GADD34 promotes protein dephosphorylation of eIF2α that reverses translational inhibition (Novoa et al., 2001), we also tested the mRNA expression level of GADD34. As depicted in Figure 5F, GADD34 expression was up-regulated with increasing of atRAL concentrations.

Clearance of Intracellular ROS Relieved ER Stress and Inhibition of ER Stress Ameliorated atRAL Cytotoxicity

To better understand atRAL-mediated ER stress in ARPE-19 cells, we partially scavenged the ROS using 5 mM antioxidant NAC. As shown in Figure 6A, 15 μM atRAL remarkably induced ER stress, which increased the mRNA expression of ATF4 and CHOP. NAC, however, significantly reversed the mRNA expression of CHOP in RPE cells (Fig. 6A, b), but could not suppress ATF4 up-regulation (Fig. 6A, a). Salubrinal, an established ER stress inhibitor, can rescue cells from tunicamycin-induced apoptosis by inhibiting eIF2α dephosphorylation (Boyce et al., 2005). We then investigated whether the inhibition of ER stress with salubrinal could protect atRAL-treated cells from colocalized with ER (ER-Tracker), as characterized by yellow fluorescence (Fig. 3B, white arrow).
Our data demonstrated that, after incubation with 40, 80, and 100 μM salubrinal, cell viability of atRAL-treated cells increased from 50.87% to 61.68%, 63.04%, and 70.86%, respectively.

**atRAL Caused Mitochondrial Dysfunction and Caspase-Dependent Apoptosis**

Results of staining with the ΔΨm sensing dye JC-1 revealed that control cells exhibited an intact ΔΨm whereas atRAL treatment induced ΔΨm loss in a concentration-dependent manner, as indicated by a shift in JC-1 fluorescence from aggregate (red) to monomer (green) (Fig. 7A). To confirm this finding, we further assessed the fluorescence of JC-1 with flow cytometry and found that, compared with the untreated cells (4.6%), the number of cells with low ΔΨm was not significantly increased following 5 μM atRAL treatment (6.2%), but was apparently elevated after 10 μM (22.3%), 15 μM (62.5%), and 20 μM (74.5%) atRAL exposure for 6 h (Fig. 7B). Additionally, we also observed a concentration-dependent up-regulation of cleaved fragment of caspase-3, which was accompanied by a reduction of pro-caspase-3 (Fig. 7C). To further identify the activation of the caspase cascade, Western blot analysis was employed to detect cleaved PARP, one of the caspase-3 downstream effectors. As depicted in Figure 7C, cleaved PARP was concentration-dependently elevated after 6 h of atRAL treatment.

**DISCUSSION**

Although retinals including 11-cis-retinal and atRAL are essential to vision, they can be chemically reactive and toxic...
RPE cell loss (Figs. 1B and 1C). As an endogenous compound in the retina, atRAL is generally eliminated by reduction to all-trans-retinol by RDHs such as RDH11, or the formation of a series of retinal-derived adducts that are mostly trapped in the RPE lysosomes (Parker and Crouch, 2010; Sparrow et al., 2010), explaining why a low concentration of atRAL can be tolerable to the RPE. Photoreceptor and RPE cells display an inherent mechanism for clearing free atRAL, but when the latter accumulates to a critical concentration within RPE cells, it may eventually initiate cell apoptosis (Fig. 1C) and exert a deleterious effect on the RPE (Figs. 2A and 2B).

Treatments of cultured RPE cells with atRAL in our experiments simulate the in vivo conditions in which acute and intensive light exposure or disruption of atRAL clearance induce excessive deposition of this retinoid in the RPE (Maeda et al., 2009).

Nrf2 is an important transcription factor that binds to ARE and its up-regulation signifies an important cellular adaptive survival response to multiple cellular stimuli including oxidative stress (Nguyen et al., 2003). ARE controls the expression of genes whose protein products (e.g., HO-1 and γ-GCS) are crucial in the detoxification and elimination of reactive oxidants. In this regard, the up-regulation of Nrf2, HO-1, and γ-GCSs suggests that excessive accumulation of atRAL resulted in the overproduction of intracellular ROS and induced oxidative stress in the RPE (Fig. 2C). Moreover, NAC attenuates atRAL-induced oxidative stress (Fig. 2D) and improves cell survival (Fig. 2E), thereby indicating that the induction of oxidative stress and the activation of oxidative stress downstream pathways are responsible for RPE degeneration.

Our findings demonstrated that ROS were generated and dispersively distributed in RPE cell cytoplasm, whereas some of ROS aggregated and localized to mitochondria and ER (Figs. 3A and 3B). A previously published study revealed that atRAL could directly act on and elicit a poisonous effect in mitochondria by inhibiting mitochondrial oxidation and, beyond a certain concentration uncoupling oxidative phosphorylation (Maeda et al., 2009). The ability of atRAL to permeabilize mitochondrial membrane could result in the leakage of important enzymes and thereby induce mitochondrial dysfunction. In addition, atRAL-induced ROS generation in the cytoplasm directly attacked organelles and induced severe cellular injury. Any impairment of electron transfer through the mitochondrial respiratory chain would lead to incomplete reduction of oxygen and consequently result in ROS formation (Naranmandura et al., 2011; Wang et al., 2004), which explains why ROS was localized within the mitochondria. Evidence presented here indicated that atRAL-induced ROS overproduction in mitochondria was likely another way to directly induce mitochondrial dysfunction. Similarly, intracellular ROS could directly induce damage in the ER. In the ER, ROS can be generated during the process of protein oxidative folding (e.g., the formation of disulfide bonds), which is driven by the membrane-associated flavoprotein Ero1α (Sevier and Kaiser, 2008; Song et al., 2008). Ero1α dysfunction may lead to aberrant production of ROS (Fig 3B), and obstruction of the proper protein folding by ROS. Our data suggest that excessive ROS generation mediated by atRAL could cause oxidative damage to mitochondria and potentially disrupt mitochondrial homeostasis. In addition, ER may also be a target cellular organelle for atRAL-associated ROS.

The disruption of ER homeostasis may cause ER stress, which has been implicated in the pathogenesis of a broad range of diseases (Sano and Reed, 2013). When excessive unfolded proteins accumulate in the ER, BiP is released from the ER-resident protein (PERK, IRE1, or ATF6) to facilitate the
folding/assembly of amassed proteins (Shinkai et al., 2010). BiP is not only critical for cell survival under such stress, but also widely used as a marker of ER stress (Dou et al., 2012). Our results show that the transcription level of BiP, however, is not very sensitive to atRAL-induced stress in the RPE, compared with the ER stress positive control Tg which increased BiP expression by 22-fold without significant inhibition of cell viability (Fig. 4A). Furthermore, we examined the mRNA expression level of an additional ER chaperone, GRP94 (Shinkai et al., 2010), and found that the GRP94 gene was not affected by atRAL exposure (Supplementary Fig. 2). An earlier report showed that the mRNA level of BiP did not change under slight ER stress (Naranmandura et al., 2012), but this may not explain the phenomenon observed in our experiments. By contrast, the protein level of BiP exhibited a significant increase (Figs. 4B and 4C), suggesting that atRAL increased the translation levels rather than the transcription levels of ER chaperones like BiP.

ER stress can be activated by PERK phosphorylation, and the subsequent phosphorylation of eIF2α, ultimately resulting in a general inhibition of protein translation (Lai et al., 2007). atRAL exposure produced concentration- and time-dependent changes in the phosphorylation of PERK and eIF2α in RPE cells (Figs. 5A and 5B). In mammalian cells, as an acute response to ER stress, the rapid phosphorylation of eIF2α is unable to efficiently initiate translation, consequently leading to the attenuation of global protein synthesis (Lai et al., 2007; Rutkowski and Kaufman, 2007). Besides eIF2α, PERK can also phosphorylate Nrf2 and thus prevent oxidative stress by the promotion of antioxidant genes such as HO-1 (Fig. 2C) (Cullinan and Diehl, 2004). Phosphorylation of eIF2α inhibits global protein synthesis, while promoting transcriptional up-regulation of ATF4 (Fig. 5C). ATF4 activates the transcription of genes involved in amino acid metabolism, glutathione biosynthesis, and protection against oxidative stress (Malabanan and Khachigian, 2010). ATF4 also up-regulates GADD34 (Fig. 5F) to dephosphorylate eIF2α and thus ends the ER stress-induced translational arrest (Szegezdi et al., 2006). Overexpression of GADD34, however, can initiate or enhance apoptosis because it permits translation of mRNAs that encode proapoptotic proteins. Besides, ATF4 also up-regulates genes (e.g., CHOP and ATF3) that contribute to cell-cycle arrest and trigger programmed cell death in case various UPR-induced mechanisms fail to alleviate ER stress (Magne et al., 2011). CHOP expression was remarkably elevated in atRAL-treated RPE cells (Figs. 5D and 5E). Previous studies
showed that CHOP mediated the activation of Ero1α expression during ER stress (Song et al., 2008) and thus aggravated the accumulation of ROS in ER within stressed cells, consistent with our present finding (Fig. 3B). Furthermore, CHOP can induce apoptosis via a direct inhibition of Bcl-2 transcription and induction of Bim expression (Dou et al., 2012; McCullough et al., 2001; Puthalakath et al., 2007). Previous research has also revealed that ER stress-activated caspase 4 initiated cell apoptosis (Dou et al., 2012). In contrast, the use of an ER stress inhibitor, salubrinal, significantly attenuated atRAL-induced RPE cell viability loss (Fig. 6B). We concluded that ER stress-mediated cell apoptosis might participate in atRAL-induced RPE dysfunction.

Partial quenching of intracellular ROS with the antioxidant NAC significantly suppressed atRAL-induced CHOP expression (Fig. 6A, b) but could not attenuate atRAL-activated ATF4 up-regulation (Fig. 6A, a), probably because ATF4 plays a pivotal role in the cellular adaptive response under stress (Magne et al., 2011) and maintains its relatively high expression level, which is critical for cell survival. The data confirmed that atRAL-mediated ROS overproduction is an early event in initiating ER stress-induced RPE cell apoptosis, and the clearance of ROS with antioxidants may be an effective strategy to protect cells against excessive atRAL in the retina.

atRAL-treated cells manifested an apparent mitochondrial ΔΨm loss. As described above, besides its possibly direct poisonous effect, atRAL-induced ROS generation in the cytoplasm could damage mitochondria, and atRAL-mediated ROS overproduction in mitochondria may be an alternative way to directly induce mitochondrial dysfunction. Furthermore, a previously reported work demonstrated that ER stress could induce mitochondrial ROS generation and potentially perturb mitochondrial homeostasis (Dou et al., 2012). ER stress-related molecular events, like up-regulated CHOP (Figs. 5D and 5E), contributed to ER stress-related mitochondrial dysfunction (Dou et al., 2012; McCullough et al., 2001; Puthalakath et al., 2007). This represents an indirect or an upstream effect of the mitochondrial injury arising from atRAL. Mitochondrial membrane permeability disruption, accompanied by the release of cytochrome c, is a critical mitochondrial apoptotic event, ultimately promoting programmed cell death by activating apoptotic cascades (Dou et al., 2012). Our results reflect that atRAL induced RPE cell apoptosis via the caspase-dependent mitochondrial apoptotic pathway (Fig. 7C).
Collectively, although atRAL is indispensable for vision, excessive accumulation of this molecule within RPE cells caused cytotoxicity. Our study demonstrated the important role of ROS generation induced by atRAL in RPE cell apoptosis. Since ROS clearance by the antioxidant NAC alleviated RPE cell death (Figs. 2D and 2E), the protection of RPE cells against injury caused by atRAL could be, at least in part, accomplished by quenching free radicals. Importantly, our results revealed that ROS-mediated mitochondrial dysfunction plays a decisive role in RPE cell death. Aberrant ROS generated in mitochondria directly perturbed mitochondrial homeostasis, whereas ROS-initiated ER stress ultimately impairs mitochondrial function indirectly, thus activating the caspase-dependent mitochondrial apoptotic pathway (Fig. 8). This study expands our understanding of atRAL toxicity in human RPE cells and retinopathies such as Stargardt’s disease and AMD, which are characterized by delayed clearance of atRAL, and could open avenues toward target therapies on alleviating deleterious effects of atRAL in cellular organelles.

SUPPLEMENTARY DATA
Supplementary data are available online at http://toxsci.oxfordjournals.org.

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