Retinal Pigment Epithelium Cell Death Is Associated With NLRP3 Inflammasome Activation by All-trans Retinal

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PURPOSE. Visual (retinoid) cycle anomalies induce aberrant build-up of all-trans retinal (atRAL), which is a cause of RPE atrophy in Stargardt disease type 1 and age-related macular degeneration. NLR family pyrin domain containing 3 (NLRP3) inflammasome activation is implicated in the etiology of age-related macular degeneration. Here, we elucidated the relationship between NLRP3 inflammasome activation and atRAL-induced death of RPE cells.

METHODS. Cellular toxicities were assessed by MTS or MTT assays. Expression levels of mRNAs and proteins were determined by quantitative reverse transcription–polymerase chain reaction, Western blotting, or enzyme-linked immunosorbent assay. Fluorescence microscopy was used to examine intracellular signals. Ultrastructural features of organelles were examined by transmission electron microscope.

RESULTS. Abnormal accumulation of atRAL was associated with a significant increase in the proportion of human ARPE-19 cells exhibiting features of apoptosis and Caspase-3/gasdermin E (GSDME)-mediated pyroptosis. These cells also exhibited elevated expression of NLRP3, ASC, cleaved Caspase-1/poly ADP-ribose polymerase (PARP)/Caspase-3/GSDME, interleukin-1β (IL-1β), and IL-18, as well as NLRP3 inflammasome-related genes (IL18 and IL18). After exposure of human ARPE-19 cells to excess atRAL, reactive oxygen species (ROS) (including mitochondrial ROS) and cathepsins released from lysosomes transmitted signals leading to NLRP3 inflammasome activation. Suppressing the production of ROS, NLRP3 inflammasome, Caspase-1, cathepsin B, or cathepsin D protected ARPE-19 cells against atRAL-associated cytotoxicity. Damage to mitochondria, lysosomes, and endoplasmic reticulum in atRAL-exposed ARPE-19 cells was partially alleviated by treatment with MCC950, a selective NLRP3 inflammasome inhibitor.

CONCLUSIONS. Aberrant build-up of atRAL promotes the death of RPE cells via NLRP3 inflammasome activation.

Keywords: all-trans retinal, atRAL, NLRP3, retinal pigment epithelium, RPE, visual (retinoid) cycle

The processing of stimuli in the visual (retinoid) cycle involves close cooperation between the retinal pigment epithelium (RPE) and photoreceptors. During the visual (retinoid) cycle, all-trans retinal (atRAL) is generated after light-mediated bleaching of rhodopsin, and the conversion of atRAL back into 11-cis retinal is essential for proper phototransduction.1 In atRAL metabolism, ABCA4 (encoded by the Abca4 gene) functions as an active transporter of atRAL by translocating all-trans N-retinylidene-phosphatidylethanolamine (NR-PE), a Schiff-base conjugate of atRAL and PE, from the inside to the outside of disc membranes localized in the outer segments of photoreceptors.2 Dissociation of all-trans NR-PE produces atRAL and PE in the photoreceptor cytoplasm, where atRAL is reduced, generating vitamin A, primarily via retinol dehydrogenase 8 (RDH8 [encoded by the Rdb8 gene]).3 Because both ABCA4 and RDH8 are critical for atRAL clearance, a deficiency in either of these proteins leads to aberrant accumulation of atRAL in photoreceptors and the RPE, potentially resulting in retinal degeneration due to atRAL-mediated cell permeabilization and death.3,4 Indeed, Abca4 and Rdb8 double knockout (DKO) (Abca4−/−/Rdb8−/−) mice exhibit the primary features of Stargardt disease type 1 (STGD1) and age-related macular degeneration (AMD),5,6 as well as early photoreceptor loss and RPE dystrophy.3,5 The NLRP3 inflammasome is a multiprotein complex that has been implicated in the pathogenesis of AMD.7,8 The NLRP3 inflammasome is activated by extracellular and intracellular danger signals and subsequently activates Caspase-1, provokes inflammatory responses via production of mature interleukin-1β (IL-1β) and IL-18, and induces pyroptosis via cleavage of...
gasdermin family proteins. However, whether aberrant accumulation of atRAL activates NLRP3 inflammasome in RPE cells and the potential relationship to RPE cell death remain unclear. In the present study, we further elucidated the mechanisms underlying the death of RPE cells associated with the excess accumulation of atRAL.

**MATERIALS AND METHODS**

**Reagents**

atRAL, Mito-TEMPO, Ca-074-Me, pepstatin A, anti-ZO-1, and AC-YVAD-CMK were purchased from Sigma-Aldrich (St. Louis, MO, USA). MCC950 was purchased from Selleck (Shanghai, China). CV09 was provided by the Xianming Deng Laboratory (School of Life Sciences, Xiamen University, Xiamen City, Fujian, China). NAC was purchased from Aladdin (Shanghai, China). MitoTracker, LysoTracker, ER-Tracker, and anti-β-actin (catalog no. 30102ES40) were obtained from Yeasen Corporation (Shanghai, China). The following antibodies were used: anti-GAPDH (catalog no. 51748, RRID: AB_10622025; Cell Signaling Technology, Danvers, MA, USA), anti-NLRP3 (Cryo-2, catalog no. AG-20B-0014, RRID: AB_2490202; AdipoGen, Liestal, Switzerland), anti-cleaved Caspase1 (human, catalog no. AG-20B-0068, RRID: AB_2490257; AdipoGen), anti-IL-1β (catalog no. AB221842, RRID: AB:2715505; Cell Signaling Technology), anti-cleaved Caspase-3 (catalog no. 9664, RRID: AB:2070042; Cell Signaling Technology), anti-cleaved poly ADP-ribose polymerase (PARP) (catalog no. 5625, RRID: AB:10699459; Cell Signaling Technology), anti-cathepsin B (D1C7V, catalog no. 31718, RRID: AB:2687580; Cell Signaling Technology), anti-IL-18 (catalog no. D120851; BBI Life Sciences, Shanghai, China), anti-cathepsin D (catalog no. ab75852, RRID: AB:1523267; Abcam, Cambridge, MA, USA), anti-gasdermin D (GSDMD) (catalog no. ab209845, RRID: AB:2783550), anti-DFA5 (gasdermin E [GSDME]) (catalog no. ab215191, RRID: AB:2757000; Abcam), anti-ASC (catalog no. sc-22514-R, RRID: AB:2174874; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-RPE65 (catalog no. 17939-1-AP, RRID: AB_2285290; Proteintech, Wuhan, Hubei, China).

**Culture of ARPE-19 Cells**

ARPE-19 human RPE cells were purchased from FuDan IBS Cell Center (Shanghai, China) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Shanghai, China) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Beijing, China), penicillin (100 units/ml), and streptomycin (100 units/ml) in a humidified incubator with 5% CO2 at 37°C.

**MTS Assay**

ARPE-19 cells were incubated with serial dilutions of atRAL (0, 5, 10, 15, 20, and 40 µM) for 3, 6, and 12 hours. Cytotoxicity of atRAL was assayed using an MTS assay, performed by adding 20 µl of CellTitre 96 AQueous One Solution reagent (Promega, Madison, WI, USA) directly to cells contained in 96-well plates, followed by incubation for 5 hours; absorbance at 490 nm was measured by a 1510 Multiskan GO spectrophotometer. The number of intact or hollow mitochondria was determined using a modified MTT colorimetric assay. Briefly, after cells were treated with 15 µM atRAL and AC-YVAD-CMK (0, 25, 50, or 100 µM), NAC (0, 1.25, 2.5, or 5 µM), Mito-TEMPO (0, 25, 50, or 100 µM), Ca-074-Me (0, 5, or 10 µM), or pepstatin A (0, 50, or 100 µM) for 6 or 12 hours, 10 µl of MTT solution was added to 100 µl of culture medium in each well to achieve a final concentration of approximately 0.1 mg/ml. After incubation for 4 hours at 37°C, the solution was removed, and 100 µl of DMSO was added to each well. After mixing by oscillation for 10 minutes, the optical density was measured at 570 nm by using the 1510 Multiskan GO spectrophotometer. Cell viability is presented as the proportion of the control optical density.

**Transmission Electron Microscopy (TEM)**

ARPE-19 cells were incubated with 15 µM atRAL for 1 and 3 hours, and control cells were treated with DMSO alone for 3 hours. Alternatively, ARPE-19 cells were cultured with 15 µM atRAL and 100 µM MCC950 for 1 and 3 hours. Before atRAL exposure, ARPE-19 cells were pretreated with MCC950 (100 µM) for 1 hour. Cells were trypsinized, pelleted by centrifugation at 1200 rpm for 5 minutes, and immediately fixed in PBS containing 2.5% glutaraldehyde (vol/vol), followed by incubation overnight at 4°C. After dehydration, pellets were embedded in Embed 812 resin and cut into thin sections for electron microscopy (JEOL2100FC; Jeol Ltd., Tokyo, Japan). The number of intact or hollow mitochondria was determined for at least three different fields at each time point, and the percentage was calculated as the number of hollow mitochondria divided by total number of mitochondria in each field. To measure the thickness of the ER, the distance between two membranes of the ER lumen was determined at five different locations in each TEM photograph by using ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA); at least three photographs for each time point were examined.

**RNA Interference Assay**

siRNAs designed for knock down of the human NLRP3 gene were synthesized by GenePharma (Shanghai, China). The sequences of the siRNA targeting NLRP3 were GUGCAUU GAAGACAGGAAUTT (sense) and AUUCCGUCUCAAUG CACTT (antisense). The sequences of the siNC were UUUCUGCAAGUGUCACGUTT (sense) and AGUGUAGAC GUGCGGAGAATT (antisense). ARPE-19 cells (3,5 × 105 cells/well) were cultured in 6-well culture plates for 12 hours and then transfected with 100 pmol of siNLRP3 or siNC in DMEM by using 10 µl of Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions. At 6-hours posttransfection, the transfection medium was replaced with DMEM supplemented with 10% fetal bovine serum, and the cells were incubated for an additional 36 hours. Subsequently, cells were treated with 15 µM atRAL for 6 hours, after which cell lysates and culture supernatants were prepared.

**Treatment With NAC, Mito-TEMPO, Ca-074-Me, or Pepstatin A**

ARPE-19 cells seeded in 96- or 6-well culture plates were maintained in a constant-temperature incubator at 37°C for 24 hours. The cytoprotective effect of the NLRP3 inflammasome inhibitors MCC950 or CV09 in atRAL-treated ARPE-19 cells was determined using MTS assay. Cells were seeded in 96-well plates for 2 days and then pretreated with MCC950 (100 µM) or CV09 (25 µM) for 1 hour. Cells were then treated for 6 hours with 15 µM atRAL. CellTitre 96 AQueous One Solution reagent (20 µl) was added directly to the culture medium. After incubation for an additional 3 hours, absorbance was recorded at 490 nm by using the 1510 Multiskan GO spectrophotometer.
hours and then treated with NAC for 2 hours or Ca-074-Me, pepstatin A, or Mito-TEMPO for 1 hour. Cells were then treated for 6 or 12 hours with 15 μM atRAL in the absence or presence of various concentrations of NAC, Mito-TEMPO, Ca-074-Me, or pepstatin A.

Caspase-1 Activity Assay

Caspase-1 activity was assessed using a FAM-FLICATM Polycaspase Assay Kit (Immunochemistry Technologies, Blooming- ton, MN, USA) according to the manufacturer’s instructions. ARPE-19 cells were treated with 15 μM atRAL for 6 hours, incubated with the FLICA probe FAM-YVAD-FMK for 30 minutes, and then washed three times with PBS. Images were acquired using a Nikon ECLIPSE TE2000-U fluorescence microscope (Tokyo, Japan).

Treatment With the Caspase-1 Inhibitor AC-YVAD-CMK

ARPE-19 cells were seeded in 96-well culture plates and maintained in a constant temperature incubator at 37°C for 24 hours. Prior to atRAL treatment, cells were treated with AC-YVAD-CMK (0, 25, 50, or 100 μM) for 30 minutes. Cells were then treated with 15 μM atRAL for 6 hours in the absence or presence of AC-YVAD-CMK.

Immunofluorescence

ARPE-19 cells seeded in 6-well culture plates were fixed in 5% paraformaldehyde for 10 minutes. The paraformaldehyde was removed, and the plates were washed with PBS. Fixed cells were treated with blocking buffer containing 2% BSA in PBS for 1 hour on ice. Plates were subsequently incubated overnight at 4°C with indicated primary antibodies at 1:200 dilutions. After washing with PBS, secondary antibodies were applied, and signals were visualized using an Olympus FV1000 confocal microscope (Tochigi, Japan).

Acridine Orange Staining

At 3, 6, and 12 hours after treatment with 15 μM atRAL, ARPE-19 cells were incubated with 5 μg/ml of acridine orange for 15 minutes. After removal of the medium, cells were washed with PBS three times, and stained cells were examined under the confocal microscope at an excitation wavelength of 488 nm. Red fluorescence (emission peak at approximately 650 nm) was observed in the lysosomal compartments, and green fluorescence (emission peak at 530–550 nm) was detected in the cytosolic and nuclear compartments.

RNA Extraction and Quantitative RT-PCR Analysis

Total RNA was extracted from cells using TRIzol reagent (Yeasen) and reverse transcribed using a 10-μl oligo (dT) system (TOYOBO, Osaka, Japan). The following genes were targeted: Human Rpe65 (NM_000329.2, AGAGGTACTGACAATGGGCTT [forward] and GCCCCTATGACAGACAT [reverse]), human Rbp1 (NM_000320.4, CATTGAAGCTGGCTACCCTG [forward] and ATTCTCCAGGAGCTTCTCCA [reverse]), human Ac7b (NM_0011101.3, AGAAAACTTGCCAGCACACC [forward] and AGAGGGCTACGAGGATAGCA [reverse]), human NLRP3 (NM_001243133.1, CCTGTATGGAGTGCCACCC [forward] and TCAACCAGCCTCGGAGGAG [reverse]), Human IL1B (NM_000576.2, AGCTGATGGCCCTAAACAGA [forward] and GCATCTCTGACGGTGGTCC [reverse]), and Human IL18 (NM_001562.3, CACCCCGGACCATATTATT [forward] and TCTGGGACACTTCTCTGAAA [reverse]).

Western Blot Analysis

Cells were lysed using a protein lysis buffer. Immunoblotting was then performed using primary antibodies against NLRP3 (1:500), ASC (1:500), Caspase-1 (1:500), IL-1β (1:500), IL-18 (1:1000), GSDMD (1:1000), GSDME (1:1000), cleaved PARP (1:500), cleaved Caspase-3 (1:500), GAPDH (1:2000), and β-actin (1:2000), followed by the addition of horseradish peroxidase-labeled secondary antibodies. Alternatively, cell culture supernatants were collected and mixed with 750-μl methanol. Chloroform was added at a ratio of 1:5, and the mixture was centrifuged at 12,000 rpm for 10 minutes at 4°C. The interlayer was collected and washed using 750 μl of methanol and then centrifuged. Precipitates were dried and dissolved in loading buffer. Quantification was performed using Quantity One software (version 4.6.2; Bio-Rad, Hercules, CA, USA). GAPDH or β-actin was used as a loading control.

Enzyme-Linked Immunosorbent Assay (ELISA)

Cell culture supernatants were added directly to wells of 96-well microplates precoated with anti-human IL-1β or IL-18. ELISA (human IL-1β Valukine ELISA kit, R&D Systems, Minneapolis, MN, USA; human IL-18 platinum ELISA kit, eBioscience, Carlsbad, CA, USA) was performed according to the manufacturer’s instructions. Absorbance was read at 620 nm by using the 1510 Multiskan GO spectrophotometer.

Statistical Analyses

Statistical analyses and data plotting were performed using GraphPad Prism software, version 5.0 (La Jolla, CA, USA). Values are expressed as mean ± standard error of the mean (SEM) of at least three independent experiments. The Mann-Whitney U test was used to compare differences between groups. A P value less than 0.05 was considered statistically significant.

RESULTS

Cytotoxicity of atRAL in ARPE-19 Cells

Expression of mRNAs for the RPE cell-specific markers Rbp1 and Rpe65 as well as RPE65 protein (Supplementary Fig. S1) confirmed the similarity of ARPE-19 cells to human RPE cells in vitro. As illustrated in Supplementary Figure S2, treatment with atRAL induced time- and concentration-dependent decreases in the viability of ARPE-19 cells. Half maximal inhibitory concentration (IC50) values for atRAL incubated with ARPE-19 cells for 3, 6, and 12 hours were 39.54, 18.11, and 14.21 μM, respectively. In addition, incubation of ARPE-19 cells with atRAL for 6 hours at concentrations of 15, 20, and 40 μM resulted in significant decreases in cell viability of approximately 37%, 58%, and 93%, respectively. Based on the results of these cytotoxicity tests, ARPE-19 cells were treated with 15-μM atRAL in subsequent experiments.

atRAL Activates NLRP3 Inflammasome in ARPE-19 Cells

Incubation of ARPE-19 cells with 15 μM atRAL resulted in a significant time-dependent increase in the level of IL1B mRNA (Fig. 1A), and clear increases in the level of IL18 mRNA were observed at 6 and 12 hours (Fig. 1A; Table). Immunoblot analyses indicated time-dependent increases in the levels of NLRP3 and ASC protein in lysates of atRAL-treated ARPE-19 cells (Fig. 1B). Cleaved Caspase-1, which functions downstream of NLRP3 inflammasome signaling, accumulated in the
**Figure 1.** atRAL activates NLRP3 inflammasomes and induces pyroptosis and apoptosis in ARPE-19 cells. (A) Relative abundance of *IL1B* and *IL18* mRNAs in ARPE-19 cells exposed to 15 μM atRAL for 1, 3, 6, and 12 hours. Control cells were incubated with DMSO alone for 12 hours. Data were obtained from three cell culture replicates. (B) Immunoblot analysis of NLRP3, ASC, cleaved Caspase-1, pro-IL-1β, pro-IL-18, and β-actin in cell lysates as well as IL-1β and IL-18 in culture supernatants of ARPE-19 cells treated with 15 μM atRAL for 1, 3, 6, and 12 hours. Molecular weight markers (kD) are shown to the left of the blots. The full-length blot for NLRP3 is presented in Supplementary Figure S4. (C) ELISA analysis of...
secreted IL-1β and IL-18 in culture supernatants of ARPE-19 cells treated with 15 μM atRAL for 1, 3, 6, and 12 hours. Data were obtained from four cell culture replicates. (B) Immunofluorescence staining of NLRP3 in ARPE-19 cells treated with 15 μM atRAL for 6 hours. Note that cells were pretreated with AC-YVAD-CMK for 30 minutes. (C) Immunoblot analysis of GSDMD, GSDME, cleaved GSDMD, cleaved GSDME, and GAPDH in cell lysates as well as cleaved Caspase-1 in culture supernatants of ARPE-19 cells treated with 15 μM atRAL for 1, 3, 6, and 12 hours. Molecular weight markers (kD) are indicated to the left of the blots. (D) Immunoblot analysis of cleaved PARP, cleaved Caspase-3, and GAPDH in ARPE-19 cells after 1, 3, 6, and 12 hours of treatment with 15 μM atRAL. Molecular weight markers (kD) are shown to the left of the blots. (E) Immunoblot analysis of NLRP3 in ARPE-19 cells treated with 15 μM atRAL for 6 hours. Note that cells were pretreated with AC-YVAD-CMK for 30 minutes. (F) Immunoblot analysis of GSDMD, GSDME, cleaved GSDMD, cleaved GSDME, and GAPDH in cell lysates as well as cleaved Caspase-1 in culture supernatants of ARPE-19 cells treated with 15 μM atRAL for 1, 3, 6, and 12 hours. Molecular weight markers (kD) are indicated to the left of the blots. The full-length blot for cleaved Caspase-1 is presented in Supplementary Figure S4. (G) Caspase-1 activation in ARPE-19 cells treated with 15 μM atRAL for 6 hours. Caspase-1 activity was visualized in green using the FLICA probe FAM-YVAD-FMK. Control cells were examined 6 hours after treatment with 15 μM atRAL and 50 μM Caspase-1-specific inhibitor AC-YVAD-CMK. Note that cells were pretreated with 50 μM AC-YVAD-CMK for 30 minutes. (H) MTT analysis of the survival of ARPE-19 cells treated with 15 μM atRAL and various concentrations of AC-YVAD-CMK (0, 25, 50, and 100 μM) for 6 hours. Note that cells were pretreated with AC-YVAD-CMK for 30 minutes. Data were obtained from three cell culture replicates. *P < 0.05, **P < 0.01, and ***P < 0.001. All data are shown as the mean ± SEM.

Table. Profiles of Chemokines and Cytokines Induced by atRAL in ARPE-19 Cells

<table>
<thead>
<tr>
<th>Classification</th>
<th>Name</th>
<th>Gene Expression (Folds of Change)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemokines</td>
<td>IL-8</td>
<td>6.170 ± 0.009***</td>
</tr>
<tr>
<td></td>
<td>MCP-1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>MIG</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>MIP-1α</td>
<td>0.857 ± 0.048</td>
</tr>
<tr>
<td></td>
<td>MIP-1β</td>
<td>-</td>
</tr>
<tr>
<td>Cytokines</td>
<td>IL-1β</td>
<td>6.680 ± 0.097***</td>
</tr>
<tr>
<td></td>
<td>IL-18</td>
<td>1.110 ± 0.032*</td>
</tr>
<tr>
<td></td>
<td>IL-2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>IL-6</td>
<td>0.816 ± 0.042</td>
</tr>
<tr>
<td></td>
<td>IL-10</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>IL-17A</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>IL-22</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>TNF-2</td>
<td>1.007 ± 0.030</td>
</tr>
<tr>
<td></td>
<td>G-CSF</td>
<td>0.929 ± 0.019</td>
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<tr>
<td></td>
<td>IFN-γ</td>
<td>-</td>
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<td></td>
<td>IFN-α</td>
<td>-</td>
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</tbody>
</table>

Cells were treated with 15 μM atRAL for 6 hours, after which the relative abundance of mRNAs for various chemokines and cytokines was determined by quantitative RTPCR. “−” indicates no expression. Data were obtained from three cell culture replicates. *P < 0.05, **P < 0.01, and ***P < 0.001. All data are presented as the mean ± SEM.

atRAL Damages Mitochondria, Lysosomes, and ER

Confocal microscopy was used to examine the subcellular localization of atRAL in ARPE-19 cells. atRAL is a fluorophore that emits green fluorescence when excited at 405 nm. ARPE-19 cells were incubated with 15 μM atRAL for 3 hours and then stained with the red fluorescent dyes MitoTracker, LysoTracker, and ER-Tracker. The presence of yellow spots indicated that atRAL diffused into the mitochondria, lysosomes, and ER of the ARPE-19 cells (Fig. 3A).

When examined under TEM, normal mitochondria have two membrane layers, with the inner membrane folding to form cristae (Fig. 3B). After 1 hour of incubation with 15 μM atRAL, mitochondria exhibited various ultrastructural alterations, such as a lucent matrix and disorganized cristae. Three hours after

Collectively, these findings indicate that atRAL induces the death of RPE cells at least in part via the NLRP3 inflammasome signaling pathway.

All-trans Retinal Activates NLRP3 Inflammasome

Importantly, the results of CellTiter 96 AQueous One Solution Cell Proliferation (MTS) assay showed that treatment with either 100 μM MCC950 or 25 μM CY09 effectively rescued ARPE-19 cells from the cytotoxic effects of 15 μM atRAL (Fig. 2D). Notably, the results of CellTiter 96 AQueous One Solution Cell Proliferation (MTS) assay showed that treatment with either 100 μM MCC950 or 25 μM CY09 effectively rescued ARPE-19 cells from the cytotoxic effects of 15 μM atRAL (Fig. 2D). Collectively, these findings indicate that atRAL induces the death of RPE cells at least in part via the NLRP3 inflammasome signaling pathway.
treatment, approximately 30% of the mitochondria became hollow, with only the outer membranes remaining (Figs. 3B, 3C). Lysosomes are single-layer, membrane-bound vesicular structures that appear dark under TEM. In atRAL-treated ARPE-19 cells, vacuoles appeared 1 hour after stimulation, and at 3 hours, abnormal lysosomes exhibiting multiple membrane-like structures began to accumulate within the cells (Fig. 3B). By contrast, treatment with 15 μM atRAL had a minimal effect on the ER at 1 hour, but abnormal structures and increased thickness of the ER were observed after 3 hours (Figs. 3B, 3D).

Treatment with the selective NLRP3 inflammasome inhibitor MCC950 (100 μM) attenuated the atRAL induced damage to the mitochondria, lysosomes, and ER in ARPE-19 cells after 1 or 3 hours of exposure (Fig. 3B).

**Reactive Oxygen Species (ROS) Induced by atRAL Activate NLRP3 Inflammasome**

As ROS are potent initiators of NLRP3 inflammasome signaling, we examined whether ROS are associated with
atRAL-induced NLRP3 inflammasome activation in ARPE-19 cells. Immunoblot analysis demonstrated that levels of NLRP3, ASC, and cleaved Caspase-1 in cell lysates and amounts of mature IL-1β and IL-18 in culture supernatants were reduced in ARPE-19 cells treated with 15 μM atRAL for 6 hours in the presence of 2.5 mM N-acetyl-cysteine (NAC), a global ROS scavenger (Fig. 4A). ELISA analysis revealed that treatment with 2.5 mM NAC significantly decreased the levels of mature IL-1β and IL-18 in culture supernatants of atRAL-treated ARPE-19 cells (Fig. 4B). MTT assay demonstrated that at concentrations ranging from 1.25 to 5 mM, NAC rescued ARPE-19 cells against atRAL-induced cytotoxicity (Fig. 4C).

Mitochondria are the primary source of ROS. We have previously reported that intracellular ROS induced by atRAL are generated in part by mitochondria. Mito-TEMPO was used to specifically characterize the involvement of mitochondrial ROS (mtROS) in atRAL-induced NLRP3 inflammasome activation in ARPE-19 cells. As expected, Western blot analysis showed that treatment with 50 μM Mito-TEMPO reduced the levels of NLRP3, ASC, and cleaved Caspase-1 in cell lysates and amounts of mature IL-1β and IL-18 in culture supernatants of atRAL-treated ARPE-19 cells (Fig. 5A). ELISA analysis revealed that treatment with 50 μM Mito-TEMPO decreased the levels of mature IL-1β and IL-18 in culture supernatants of atRAL-treated ARPE-19 cells (Fig. 5B). Confocal imaging in the horizontal plane indicated reduced Caspase-1 activity in ARPE-19 cells treated with 15 μM atRAL and 50 μM Mito-TEMPO (Fig. 5C). Notably, the results of the MTT assay revealed that treatment with Mito-TEMPO at concentrations ranging from 25 to 100 μM attenuated the atRAL-induced death of ARPE-19 cells (Fig. 5D).

atRAL-Induced Lysosomal Degeneration Activates NLRP3 Inflammasome

The acidic environment of lysosomes sustains the activity of cathepsins, proteases that degrade proteins and other intracellular wastes. In ARPE-19 cells treated with 15 μM atRAL for 3, 6, and 12 hours and then stained with acridine orange, the number of cells exhibiting only green fluorescence increased in a time-dependent manner (Fig. 6A), which indicated a decrease in the number of intracellular acidic compartments.

Previous studies have reported that cathepsins can trigger NLRP3 inflammasome activation when released into the cytoplasm after lysosome rupture.20 Cathepsin B (CatB) and cathepsin D (CatD) normally exhibit a compartment-restricted staining pattern. However, diffusion of both CatB and CatD into the cytoplasm was observed in ARPE-19 cells treated with 15 μM atRAL for 6 hours (Fig. 6B), indicative of leakage of these proteases from lysosomes (Figs. 3B, 6B). To determine whether atRAL-induced leakage of lysosomal cathepsins into the cytoplasm contributes to NLRP3 inflammasome activation in ARPE-19 cells, cells were treated with 10 μM Ca-074-Me and 50 μM pepstatin A to inhibit the activity of CatB and CatD, respectively. Western blot analysis demonstrated that treatment with Ca-074-Me or pepstatin A resulted in decreased levels of NLRP3, ASC, and cleaved Caspase-1 in cell lysates and significantly reduced levels of mature IL-1β and IL-18 in culture supernatants of atRAL-treated ARPE-19 cells (Figs. 6C, 6D). ELISA analysis revealed that treatment with Ca-074-Me or pepstatin A diminished the secretion of mature IL-1β and IL-18 into culture supernatants of atRAL-treated ARPE-19 cells (Figs. 6E, 6F). Furthermore, the results of the MTT assay indicated that treatment with either Ca-074-Me (5 and 10 μM) or...
pepstatin A (50 and 100 μM) protected ARPE-19 cells against the effects of atRAL-induced cytotoxicity in cells treated for 6 and 12 hours (Figs. 6G, 6H).

**DISCUSSION**

Inside photoreceptors of a single mouse eye, the concentration of rhodopsin was estimated to be nearly 5 mM in disc membranes and 8 mM in the cytoplasm of outer segments. After rhodopsin is bleached by light, atRAL is released in large amounts and can accumulate in the retina when its clearance is disrupted. Accordingly, we concluded that the concentration of atRAL used in our experiments could be physiologically significant. Although normally generated, atRAL is a toxic intermediate of the visual (retinoid) cycle, and it can induce apoptosis in ARPE-19 cells via mitochondrial dysfunction, DNA damage, and ER stress. An increasing body of evidence suggests that bisretinoid adducts derived from atRAL constitute the primary components of RPE lipofuscin, and they have been identified as potential pathogenic factors in STGD1 and AMD. Moreover, ROS are reported to have the ability to potentially activate NLRP3 inflammasome. Indeed, in the present study, we showed that NAC and Mito-TEMPO treatment rescued ARPE-19 cells from atRAL-induced cytotoxic effects via attenuation of ROS-mediated NLRP3 inflammasome activation (Figs. 4, 5). In addition to deleterious effects on mitochondria, we also found that atRAL adversely affected lysosomes of ARPE-19 cells by inducing the translocation of cathepsins into the cytosol (Figs. 3B, 6A, 6B), thus activating NLRP3 inflammasome and ultimately inducing cell death (Figs. 6C–H).

atRAL also induced pyroptosis in our study (Figs. 1E, 1F). Previous studies revealed that pyroptosis is mediated by cleavage of GSDMD by Caspase-1 or cleavage of GSDME by Caspase-3. Interestingly, the levels of cleaved Caspase-1 were increased in both cell lysates (Fig. 1B) and culture supernatants (Fig. 1F) of atRAL-treated ARPE-19 cells. Caspase-1 activity was found to be critical for induction of cell death (Figs. 1G, 1H), but no cleaved GSDMD was detected (Fig. 1F).
More recently, Kerur et al.\textsuperscript{32} reported that GSDMD mediates Alu RNA-induced RPE degeneration, and similar to our study, they did not detect cleaved GSDMD.\textsuperscript{32} In contrast, cleavage of GSDME was detected in our study following atRAL-mediated activation of Caspase-3 (Figs. 1E, 1F), suggesting that atRAL induces pyroptosis in ARPE-19 cells via Caspase-3/GSDME activation.

Considered collectively, our data suggest that aberrant accumulation of atRAL in RPE cells resulting from anomalies in the visual (retinoid) cycle occurs earlier in NLRP3 inflammasome activation than the accumulation of atRAL-derived bisretinoid A2E. As illustrated in Figure 7, atRAL accumulating in RPE cells damages both the mitochondria and lysosomes. ROS (including mtROS) and leakage of the lysosomal proteases CatB/CatD into the cytosol activate NLRP3 inflammasome and induce increased expression of ASC and cleaved Caspase-1 with the secretion of the proinflammatory cytokines IL-1\textbeta and IL-18, ultimately resulting in pyroptosis or apoptosis. This is the first report describing a relationship between atRAL-induced death of RPE cells and NLRP3 inflammasome activation resulting from aberrant accumulation of atRAL beyond a critical threshold level. The results of our study enhance the understanding of both atRAL-associated toxicity in human RPE cells and retinopathies characterized by disrupted clearance of atRAL, such as STGD1 and AMD. Suppression of NLRP3 inflammasome activation could, thus, ameliorate RPE degeneration resulting from the overaccumulation of atRAL.
**Figure 6.** atRAL destabilizes lysosomes to activate NLRP3 inflammasome in ARPE-19 cells. (A) Acridine orange staining of ARPE-19 cells treated with 15 μM atRAL for 3, 6, and 12 hours. Cells treated with DMSO served as a vehicle control. DNA/RNA was fluorescently labeled as green and acidic lysosomal compartments as red. White asterisks indicate cells with only green staining, suggestive of lysosomal alkalization. (B) Immunofluorescent staining of CatB (red) and CatD (red) in ARPE-19 cells treated with 15 μM atRAL for 3 and 6 hours. (C) Immunoblot analysis of NLRP3, ASC, pro-Caspase-1, cleaved Caspase-1, pro-IL-1β, pro-IL-18, and β-actin in cell lysates as well as IL-1β and IL-18 in culture supernatants of ARPE-19 cells treated with 15 μM atRAL for 6 hours with or without 10 μM Ca-074-Me. Note that cells were pretreated with Ca-074-Me for 1 hour. The full-length blot for NLRP3 is presented in Supplementary Figure S9. (D) Immunoblot analysis of NLRP3, ASC, pro-Caspase-1, cleaved Caspase-1, pro-IL-1β, pro-IL-18, and β-actin in cell lysates as well as IL-1β and IL-18 in culture supernatants of ARPE-19 cells treated with 15 μM atRAL for 6 hours in the presence or absence of 50 μM pepstatin A. Note that cells were pretreated with pepstatin A for 1 hour. The full-length blot for NLRP3 is presented in Supplementary Fig. S9. (E) ELISA analysis of secreted IL-1β and IL-18 in culture supernatants of ARPE-19 cells treated with 15 μM atRAL...
for 6 hours with or without 10 μM Ca-074-Me. Note that cells were pretreated with Ca-074-Me for 1 hour. Data were obtained from four cell culture replicates. (F) ELISA analysis of secreted IL-1β and IL-18 in culture supernatants of ARPE-19 cells treated with 15 μM atRAL for 6 hours in the presence or absence of 50 μM pepstatin A. Note that cells were pretreated with pepstatin A for 1 hour. Data were obtained from four cell culture replicates. (G) MTT assay of the survival of ARPE-19 cells treated with 15 μM atRAL and the CatB-specific inhibitor Ca-074-Me (5 and 10 μM) for 6 and 12 hours. Data were obtained from six cell culture replicates. Note that cells were pretreated with Ca-074-Me for 1 hour. For Western blotting, molecular weight markers (kD) are indicated to the left of the blots. *P < 0.05, **P < 0.01, and ***P < 0.001. All data are expressed as the mean ± SEM.

**FIGURE 7.** Model of atRAL-induced NLRP3 inflammasome activation in RPE cells. After entering RPE cells, atRAL damages and impairs the function of mitochondria and lysosomes. Subsequently, released ROS (including mtROS) and leakage of lysosomal proteases (CatB/CatD) into the cytosol result in NLRP3 inflammasome activation, secretion of the pro-inflammatory cytokines IL-1β and IL-18, pyroptosis, and apoptosis.

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**References**


