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All-trans-retinal induces Bax activation via DNA damage to mediate retinal cell apoptosis

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Abstract

The current study investigates the cellular events which trigger activation of proapoptotic Bcl-2-associated X protein (Bax) in retinal cell death induced by all-*trans*-retinal (atRAL). Cellular events which activate Bax, such as DNA damage by oxidative stress and phosphorylation of p53, were evaluated by immunochemical and biochemical methods using ARPE-19 cells, 661W cells, cultured neural retinas and a retinal degeneration model, *Abca4*^{-/-}*Rdh8*^{-/-} mice. atRAL-induced Bax activation in cultured neural retinas was examined by pharmacological and genetic methods. Other Bax-related cellular events were also evaluated by pharmacological and biochemical methods. Production of 8-OHdG, a DNA damage indicator, and the phosphorylation of p53 at Ser 46 were detected prior to Bax activation in ARPE-19 cells incubated with atRAL. Light exposure to *Abca4*^{-/-}*Rdh8*^{-/-} mice also caused the above mentioned events in conditions of short term intense light exposure and regular room lighting conditions. Incubation with Bax inhibiting peptide and deletion of the Bax gene partially protected retinal cells from atRAL toxicity in cultured neural retina. Necrosis was demonstrated not to be the main pathway in atRAL mediated cell death. Bcl-2-interacting mediator and Bcl-2 expression levels were not altered by atRAL *in vitro*. atRAL-induced oxidative stress results in DNA damage leading to the activation of Bax by

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phosphorylated p53. This cascade is closely associated with an apoptotic cell death mechanism rather than necrosis.

Keywords

Retina; visual cycle; all-trans-retinal; oxidative stress; DNA damage; p53; Bcl-2-associated X protein; apoptosis

1. Introduction

All-*trans*-retinal (atRAL) is one of the major byproducts in the visual cycle, and is produced following photo-isomerization of the visual chromophore 11-*cis*-retinal. To maintain vision, 11-*cis*-retinal has to be regenerated via the visual cycle which is a sequential enzymatic process occurring between photoreceptor outer segments and the retinal pigment epithelium (RPE) (Palczewski, 2010). Impairments in the visual cycle can cause disturbances in visual function and lead to retinal degeneration (Travis et al., 2007). atRAL is cleared from photoreceptors by ATP-binding cassette transporter (ABCA4) and all-*trans*-retinol dehydrogenase (RDH). ABCA4 transports atRAL from the disc lumen back into the cytoplasm (Molday, 2007). RDH8 is a major all-*trans*-RDH in the retina (Maeda et al., 2007), catalyzing the reduction of atRAL to all-*trans*-retinol. Mutations in *ABCA4* are found in patients with Stargardt's disease (Allikmets et al., 1997), cone-rod dystrophy (Cremers et al., 1998), and recessive retinitis pigmentosa (Martinez-Mir et al., 1998; Zhang et al., 2005). Heterozygous *ABCA4* mutations increase the risk of developing age-related macular degeneration (Allikmets, 2000). Mice carrying a double knock-out of the *Abca4* gene and the *Rdh8* gene displayed pathological delay of atRAL clearance after light exposure resulting in light-induced retinal degeneration (Maeda et al., 2008). Of note, these double knockout mice develop retinal degeneration similar to human macular degeneration with accumulation of atRAL condensation products such as A2E. In previous studies, the cascade of signaling in retinal degeneration by atRAL has been partially studied (Chen et al., 2012; Maeda et al., 2009). We showed that NADPH oxidase can be activated by an increase in intracellular calcium, $[Ca^{2+}]_i$ via the phospholipase C (PLC)/ inositol 1,4,5-triphosphate (IP_3) pathway, resulting in overproduction of reactive oxygen species (ROS) (Chen et al., 2012; Chen et al., 2013a; Maeda et al., 2009).

Toxic effects of atRAL also promote mitochondrial damage which leads to mitochondrial-associated apoptosis (Maeda et al., 2009). atRAL-induced cell death in ARPE-19 cell was attenuated by co-incubating with Bcl-2-associated X protein (Bax)-inhibiting peptide (BIP) (Maeda et al., 2009), suggesting a connection between Bax activation and cell death. Bax is a proapoptotic member of the Bcl-2 family which normally resides in the cytosol and is translocated to mitochondria when cells are under apoptotic stress (Wolter et al., 1997). Bax induces opening the mitochondrial permeability transition pore, which promotes the release of cytochrome C followed by an apoptotic cascade (Jurgensmeier et al., 1998). BIP, a cell-penetrating penta peptide, has a unique function in both binding Bax and inhibiting Bax activation proceeding apoptosis thus protecting cells from Bax-mediated cell death (Li et al., 2007). BIP was designed based on the Bax binding domain of Ku70, which is a multifunctional protein involved in DNA repair and in the regulation of apoptosis (Gomez et

al., 2007). Several studies have demonstrated that the mitochondrial apoptosis pathway is regulated by members of the Bcl-2 protein family (Bordone et al., 2012; Cottet and Schorderet, 2008, 2009; Hahn et al., 2004; Hahn et al., 2003; Hamann et al., 2009; Maeda et al., 2009) and could be involved in some types of retinal degeneration. Bax-induced apoptosis was shown to be responsible for progressive loss of rods in *Rpe65* deficient mice, a model of Leber congenital amaurosis (Cottet and Schorderet, 2008; Hamann et al., 2009), and was also reported in light-induced retinal degeneration (Bordone et al., 2012; Hahn et al., 2004; Maeda et al., 2009).

In this study, we examined a sequence of cellular events which lead to Bax activation in ARPE-19 cells, 661W cells, cultured mouse neural retinas and *Abca4*^{-/-}*Rdh8*^{-/-} mice in order to investigate atRAL toxicity in RPE and photoreceptor cells. DNA damage and the subsequent phosphorylation of p53 contributed to Bax activation, which could be one of the major cell death pathways induced by atRAL.

2. Materials and methods

2.1. Animals

Abca4^{-/-}*Rdh8*^{-/-} mice and *Bax*^{-/-} mice were generated and genotyped as previously described (Maeda et al., 2008). C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor Maine). All mice in this study were housed in the animal facility at the School of Medicine, Case Western Reserve University, where they were regularly maintained in a 12 h light (~10 lux) /12 h dark cycle environment. All animal procedures and experiments were approved by the Case Western Reserve University Animal Care Committees and conformed to both the recommendations of the American Veterinary Medical Association Panel on Euthanasia and the Association of Research for Vision and Ophthalmology.

2.1. Induction of light damage

Light damage was induced in mice by fluorescent light exposure at 10,000 lux (150 W spiral lamps, Commercial Electric) for 30 min in a white bucket as previously described (Maeda et al., 2012). The mice were dark-adapted overnight and pupils were dilated with 1% tropicamide eye solution in prior to light exposure.

2.2. Measurement of spectral-domain optical coherence tomography (SD-OCT)

SD-OCT (Bioptigen, Research Triangle Park, NC) was employed for *in vivo* retinal imaging of mice as previously described (Maeda et al., 2012).

2.3. Materials and chemical synthesis

All-*trans*-retinal (atRAL), receptor interacting peptide1 kinase inhibitor, necrostatin-1 (Nec-1) and p53 inhibitor, pifithrin- α were obtained from Sigma-Aldrich (St. Louis, MO). Bax inhibiting peptide (BIP) having the sequence, VPTLK (>95% purity) was synthesized by Biopeptide Co., Inc. (San Diego, CA, USA). Stock solution of atRAL (10 mM) was prepared with cell-culture grade Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich). Final volume of atRAL solution in the cell culture medium solution was less than 0.3% in this study.

2.4. Lactate Dehydrogenase (LDH) Colorimetric Assay

LDH measurements were conducted using LDH-Cytotoxicity Colorimetric Assay Kit II (BioVision, Milpitas, CA) on cultured ARPE-19 cells and 661W cells in 96 well plates (10^4 cells/ well) following manufacturer's instructions. Optical density values from the 96-well plate at 450 nm wavelength were measured with a microplate reader (Multiscan FC Microplate Reader, Fisher Scientific Inc., Pittsburgh, PA, USA).

2.5. WST-1 assay

WST-1 assay was conducted with Cell Proliferation Reagent WST-1 (Roche Applied Science, Indianapolis, IN) on ARPE-19 cells cultured in 96-well plates (1×10^4 cells/ well) following manufacturer's instructions. Optical density values from the 96-well plate at 450 nm wavelength were measured with a microplate reader (Multiscan FC Microplate Reader, Fisher Scientific Inc., Pittsburgh, PA, USA).

2.6. In vitro detection of Bax activation

ARPE-19 cells in 24 well cell plates (1×10^5 cells/well) were treated with atRAL at a final concentration of 10 – 30 μ M in the cell culture medium for the indicated time with or without five mar Bax inhibiting peptide, BIP. Cells were washed in cold PBS and kept on ice after incubation. Cells were then fixed for 30 min with 4% paraformaldehyde prior to permeabilization with 0.5% Triton X at room temperature. Bax activation was detected via immunocytochemistry (ICC) with anti-Bax (6A7), a mouse monoclonal antibody at a dilution of 1:100 (BD Pharmingen, San Diego, CA, USA). Cy-3 conjugated anti-mouse IgG antibody was the secondary antibody and was used at a dilution of 1:200. The signal intensity of activated Bax was monitored with an inverted fluorescence microscope (Leica DMI 6000B).

2.7. In vitro detection of phosphorylated p53 at Ser46

ARPE-19 cells were cultured in 24 well plates (1×10^5 cells/well) and atRAL was added at a final concentration of 30 μ M then incubated for 30 min. Phosphorylation of p53 at Ser46 was detected by ICC with anti-phosphorylated p53 (Ser46) specific antibody (rabbit polyclonal, at 1:50 dilution, Abcam, Cambridge, MA). Cy-3 conjugated anti-rabbit IgG antibody was used as the secondary antibody. Signal intensity measurements of phosphorylated p53 were also performed.

2.8. In vitro detection of 8-OHdG

ARPE-19 cells in 24 well plates (1×10^5 cells/well) were incubated with atRAL at the indicated concentration (10 – 30 μ M) for 30 min. The marker of DNA injury by oxidative stress, 8-hydroxyhydroguanine (8-OHdG) was monitored using ICC. Cells were incubated for 1 h with anti-8-OHdG antibody at a concentration of 10 μ g/ml (Japan Institute for the Control of Aging, Fukuroi, Japan). Cy-3 conjugated anti-mouse IgG antibody was used as the secondary antibody.

2.9. Mitochondria-selective Staining

ARPE-19 cells in 96 well plates (1×10^4 cells/well) were incubated with Mitochondrion-Selective Probes, MitoTracker Orange CMTMRos (Invitrogen, Grand Island, NY) at 100 nM for 30 min.

2.10. Immunohistochemistry

Immunohistochemistry was performed as previously described (Maeda et al., 2005b). Anti-Bax (6A7) antibody (BD Biosciences, San Jose, CA) and anti-phosphorylated p53 (Ser46) specific antibody (Abcam Cambridge, MA) were used at 1:100 dilution, and 8-OHdG (JalCA, Fukuiro, Japan) at 10 μ g/ml as primary antibodies. Anti-rhodopsin mouse monoclonal antibody (1D4) is a generous gift from Dr. R. S. Molday (University of British Columbia, Vancouver, CA). Subsequently, frozen mouse retinal sections were incubated with Cy-3 conjugated secondary antibody, DAPI (Invitrogen, Carlsbad, CA) and peanut agglutinin conjugated with Alexa 488 (Invitrogen, Carlsbad, CA) to visualize targeted molecules, nuclei and cone sheath respectively.

2.11. Immunoblotting

Cell extracts were lysed in ice-cold lysis buffer (150 mM NaCl, 1 mM EDTA, 0.2% NP-40 and 20 mM Tris-HCl, pH7.5) containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and 1 mM phenylmethanesulfonyl fluoride (Sigma-Aldrich). Protein from each sample was transferred onto Immobilon-P Membrane (Millipore, Bedford, MA) after SDS-PAGE gel electrophoresis. The membranes were incubated in 1% BSA solution containing anti-Bcl-2-interacting mediator (Bim) rabbit polyclonal antibody (Cell Signaling Technology, Boston, MA), anti-Bcl-2 mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-ERK1/2 rabbit polyclonal (Abcam), and anti-phospho-p38 rabbit polyclonal (Cell signaling) following the manufacturer's instructions. Targeted molecules were visualized with alkaline phosphatase (Promega, Madison, WI) at a dilution of 1: 5,000. Anti- β -actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and Anti- β -tubulin antibody (obtained from DSHA at University of IOWA) were used for loading controls.

2.12. Ex-vivo cytotoxicity assay in cultured neural retina

Cultured neural retina from 6-week-old animals was prepared as previously described (Chen et al., 2013b). Eyes enucleated from mice were washed in a penicillin-streptomycin solution (50 unit/ml) (Sigma-Aldrich) and soaked in Hanks-balanced salt solution (Hyclone, Waltham, MA). Eye cups were flattened by slitting the peripheral side. Sterilized filter paper (5×5 mm) was placed on the neural retina side of the flattened-eye cup and the paper was carefully peeled away from the RPE/choroid. Each retina on the paper was put into a single well of a 12-well plate with 0.5 ml of DMEM containing 10% FCS and incubated for 16 h at 37°C. Retinas were further incubated with or without 30 μ M atRAL for 6 h at 37°C after washing with 0.5 ml of DMEM containing 10% FCS twice. LDH assay was performed to determine amounts of retinal cell death (BioVision, Mountain View, CA). The percentage of cytotoxicity was calculated as [(a retina with atRAL – a retina without atRAL) / (lysis control – a retina without atRAL)] $\times$ 100.

2.13. Luciferase reporter assay for p53 expression pathway

ARPE 19 cells in 96-well plate (1×10^4 cells/well) containing cell culture media supplemented with 10% FCS, 1mM sodium pyruvate, 1% non-essential amino acids, and 1% penicillin-streptomycin liquid. When ARPE 19 cells reached 90% confluency, the cells were transfected with 75 ng/well of pF5A [CMV/p53-Nluc/Neo] vector (Promega, Madison, WI) and 0.3 μ l/well of Lipofectamine 2000 (Invitrogen, Grand Island, NY) in Opti-MEM® I Reduced Serum Media (Invitrogen, Grand Island, NY). After transfection, cells were treated with 1% DMSO, 10 μ M and 30 μ M of *all-trans*-retinal for the following durations: 5, 10, 20, 30, and 60 min. The luciferase assay was performed according to the Promega Nano-Glo™ Luciferase Assay System Instruction Manual. Luminescence was measured using FlexStation 3 microplate reader (Molecular Devices, Downingtown, PA), and the luciferase expression level of each treatment group was averaged from three replicates.

2.14. Statistical analyses

Data representing the means $\pm$ S.D. for the results of at least three independent experiments were compared by the one way ANOVA-test.

3. Results

3.1. Sequential molecular events of retinal cell death by *all-trans*-retinal

We examined a sequence of events induced by atRAL. First, atRAL was incubated with ARPE-19 cells at concentrations of 10, 20 and 30 μ M for 16 h to determine whether atRAL is associated with retinal cell death. Co-incubation with atRAL displayed a dose-dependent toxicity which included the morphological disruption of ARPE-19 cells (Fig. 1A) and a decrease in their viability was observed to be time-dependent measured by LDH assay (Fig. 1B). atRAL showed higher toxicity when compared to other aldehydes which exist in the human body, such as acrolein (Jia et al., 2007) and acetaldehyde (Garaycochea et al., 2012) (Suppl. Fig. 1). To ascertain the relationship between the mitochondria-associated cellular events involved in atRAL mediated cell death, time course analysis of $[Ca^{2+}]_i$, ROS generation and Bax activation were conducted. Changes of $[Ca^{2+}]_i$ after incubation with 30 μ M of atRAL were monitored using the $[Ca^{2+}]_i$ indicator, Fluo-3 AM (Suppl. Fig. 2A, 2B). The signal intensity of Fluo-3 AM rapidly increased within one minute after the addition of atRAL to ARPE-19 cells (Suppl. Fig. 2B) and this increased level was maintained for 10 minutes. The signal intensity was carefully measured from cells in the field; however, the data might not be conclusive due to an increase in the background signal which could come from calcium leakage from damaged cells. In addition, we demonstrated that an increase in $[Ca^{2+}]_i$ could serve as a triggering event in Bax activation by a pharmacological approach with 2-Aminoethyl diphenylborinate (2-APB), which can prevent increase of $[Ca^{2+}]_i$ by blocking endoplasmic reticulum calcium ATPase pumps. 2-APB treatment could maintain cell viability significantly in both ARPE-19 and 661W after co-incubation with atRAL (Suppl. Fig. 3A, 3B). Removal of extracellular calcium by the addition of EGTA failed to maintain cell viability in ARPE19 cells incubated with atRAL (data not shown). These observations suggest that increased $[Ca^{2+}]_i$ can be involved in the cell death process induced by atRAL. The generation of reactive oxygen species (ROS) was visualized by using the ROS probe, 2',7'-dichlorofluorescein diacetate (DCF-DA) (Suppl. Fig. 2C). ROS generation

was detected 10 min after incubation with 30 μ M of atRAL and reached a plateau level 15 min after atRAL incubation (Suppl. Fig. 2D). Bax activation was detected by activated Bax-specific antibody (6A7) in ARPE-19 cells incubated with 30 μ M of atRAL (Fig. 1C). Bax activation was detected 10 min after incubation with 30 μ M of atRAL and attained a plateau level 1 h after atRAL exposure (Fig. 1D). To ensure the suitability of this approach in monitoring Bax activation, Bax activation was evaluated in ARPE-19 cell which were pre-treated with Bax inhibiting peptide (BIP) (Suppl. Fig. 4). Attenuation of Bax activation was observed (Suppl. Fig. 4A) and cell viability was preserved in cells treated with BIP (Suppl. Fig. 4B). We also evaluated that Bax activation in 2-APB treated cells after co-incubation with atRAL. Bax activation was significantly attenuated in both ARPE-19 and 661W by 2-APB treatment (Suppl. Fig. 3B). This data implies that we can monitor Bax activation in atRAL treated retinal cells efficiently, thus supporting our previous results (Maeda et al., 2009). Overall, these observations indicate that the sequential cellular events in ARPE-19 cells exposed to atRAL occur in the following order: 1. Elevation of $[Ca^{2+}]_i$ concentration, 2. ROS generation and 3. Bax activation.

3.2. Assessment of a potential molecular pathway for Bax activation in retinal cell death mediated by atRAL

It is known that Bax activation is regulated by Bcl-2 family members and several types of cellular stress including oxidative stress and necrosis (Kim et al., 2006). We showed that Bax activation was observed following ROS generation *in vitro* (Fig. 1 and Suppl. Fig. 2). However, it remained unclear what type of cellular events conjoin ROS generation and Bax activation, and what role other Bcl-2 family members play in Bax activation. To address these issues, we further examined the time course of cellular events regulating Bax activation.

Detection of DNA Damage and p53 Phosphorylation at Ser46—It is reported that DNA damage can be induced by ROS generation, which results in Bax activation via p53 activation (Bishayee et al., 2013; Smeenk et al., 2011). We assessed ROS driven DNA damage and p53-mediated activation of Bax *in vitro* in ARPE-19 cells. First, DNA damage of ARPE-19 cells was monitored using ICC with 8-hydroxyhydroguanine (8-OHdG) after incubation with atRAL. The signal of 8-OHdG was detected in the cytoplasmic space of ARPE-19 cells at 30 min after incubation with 30 μ M of atRAL (Fig. 2A), and the signal intensity increased in both the nucleus and cytoplasmic space in a dose-dependent manner (Fig. 2B). Next, to examine if p53 activation via phosphorylation at Ser46 is involved in atRAL mediated cell death, we conducted ICC with a specific antibody for phosphorylated p53 at Ser46. The signals showing phosphorylation of p53 at Ser46 were increased in both the cytoplasmic space and nucleus of ARPE-19 cells at 30 min after atRAL incubation at a concentration of 30 μ M (Fig. 3A left panel). The signals representing phosphorylated p53 and mitochondria were co-localized in the cytoplasmic space of cells treated with atRAL at the rate of $71.5 \pm 10.1\%$ (Yellow circle, Fig. 3A left panel). In contrast, such signal was not observed in cells treated with DMSO (right panel). Of note, the increase of signal intensities measuring DNA damage and post-translational modification of p53 attained plateau levels after 30 min observation (data not shown). Next, we assessed changes in the expression level of p53 in ARPE-19 cells using a luciferase reporter assay. ARPE-19 cells were

transfected with pF5A [CMV/p53-Nluc/Neo] vector, which enabled us to monitor p53 expression quantitatively by measuring luciferase activity in cells. The luminescence in transfected cells increased robustly until 10 min after incubation with 10 and 30 μ M of atRAL and continuously increased in a milder fashion and almost plateaued 30 min after incubation (Fig. 3B). We examined involvement of cellular events which can be related with p53 activation, such as extracellular-signal-regulated kinases (ERK1/2) and p38 with immunoblot analyses. Increase in phosphorylation levels of both ERK1/2 and p38 were observed (Fig. 3C). To further confirm the involvement the effects of a p53 inhibitor, pifithrin- α , on Bax activation in ARPE-19 cell death induced by atRAL was examined. Pifithrin- α decreased Bax activation levels more than 50% compared to cells of the non-treated group (Fig. 3D). Cell viability was also maintained following pifithrin- α treatment in a dose-dependent manner (Fig. 3E). These observations indicate that phosphorylation of p53 can induce Bax activation resulting in attenuation of cell viability.

3.3. Impact of Bax activation in retinal cell death of cultured neural retinas

To assess atRAL toxicity to photoreceptor cells, cultured neural retinas were dissected from 6-week-old *Bax*^{-/-} and WT (*Bax*^{+/+}) mice. The WT retinas were incubated with BIP at 200 μ M for 16 h whereas those from *Bax*^{-/-} mice were incubated in medium without BIP. Then these retinas were co-incubated with 30 μ M of atRAL for 8 h. After the incubation, the morphology and cytotoxicity of the retinal tissues were evaluated by immunohistochemistry and LDH assay. Retinal morphology was well maintained in BIP-treated retinas and *Bax*^{-/-} retinas, even though the alignment of the outer nuclear layer and outer segments were notably disrupted in the vehicle control group (Fig. 4A). The cytotoxicity of BIP-treated retinas and *Bax*^{-/-} retinas decreased to $20.2 \pm 5.4\%$ and $3.8 \pm 3.1\%$ respectively whereas cytotoxicity in vehicle control group was $36.2 \pm 11.3\%$ (Fig. 4B). From these observations, Bax activation appears to be one of the major pathways involved in retinal cell death induced by atRAL. As another model of photoreceptor cells, we conducted atRAL toxicity assay with the cell line 661W (al-Ubaidi et al., 1992). Cell viability of 661W cells were maintained until 2 h incubation with atRAL. The viability of 661W was decreased to $51.1 \pm 2.4\%$ (Suppl. Fig. 5), whereas viability of ARPE-19 cells was maintained as $89.3 \pm 2.9\%$ after 4 h incubation with 30 μ M of atRAL (Fig. 1A, 1B). Bax activation in 661W cells after atRAL treatment was also assessed by ICC using 6A7 antibody. The signal intensity was increased in atRAL treated cells, though the fold-change was lower compared to those in ARPE-19 cells due to lower specificity of the antibody in the 661W mouse cell line compared to the human derived ARPE-19 line (data not shown). These observations support the hypothesis that Bax activation could be involved in both type of retinal cells, photoreceptor and RPE cells.

3.4. Involvement of DNA damage, phosphorylated p53 at Ser46, and Bax activation in a mouse model of retinal degeneration mediated by atRAL

These molecular events which were induced by atRAL *in vitro* were assessed in a retinal degeneration mouse model, the *Abca4*^{-/-}*Rdh8*^{-/-} mouse. *Abca4*^{-/-}*Rdh8*^{-/-} mice have been observed to exhibit light-induced retinal degeneration with excess accumulation of atRAL (Maeda et al., 2008). The morphological changes of retinas after intense light exposure at 10,000 lux for 30 min were monitored by *in vivo* spectral domain optical coherent

tomography (SD-OCT) (Fig. 5). At 1 h after intense light exposure, decrease in the thickness of the outer nuclear layer (ONL) with higher intensity of signals were observed in SD-OCT images from *Abca4*^{-/-}*Rdh8*^{-/-} mice, whereas such changes were not found in SD-OCT images obtained from WT mice at 1 h after same intense light exposure and *Abca4*^{-/-}*Rdh8*^{-/-} mice without light exposure. The signal intensity of 8-OHdG, phosphorylated p53 at Ser46 and Bax activation increased in ONL and inner segments (IS) of *Abca4*^{-/-}*Rdh8*^{-/-} retinas whereas signals of these molecules were not obvious in light-exposed wild type mice and *Abca4*^{-/-}*Rdh8*^{-/-} mice without light exposure (Fig. 5). Similar molecular events were observed in *Abca4*^{-/-}*Rdh8*^{-/-} mice at 6 months of age at milder levels compared to those after intense light exposure (Suppl. Fig. 6). Given these observations, Bax activation could be induced via p53 phosphorylation triggered by oxidative stress produced by ROS generation caused by accumulation of atRAL *in vivo* as well.

3.5. Participations of other cellular events for activation or deactivation of Bax and necrotic pathway in retinal cell death induced by atRAL

Bcl-2 and Bim (Bcl-2-interacting mediator) are known to play a major role in the regulation of Bax (Czabotar et al., 2009). To examine if Bim and Bcl-2 are involved in Bax activation in atRAL-induced cell death, expression levels of these molecules were compared in ARPE-19 cells at 60 min after atRAL treatment by immunoblot analyses using anti-Bim or anti-Bcl-2 antibody. The expression levels of these molecules did not change in the presence of atRAL (Fig. 6A, 6B), suggesting that Bax activation was not governed by these two molecules under this experimental condition.

Necrosis is one of the major cell death pathways and is involved in retinal cell death mediated by various causes (Murakami et al., 2011). Since Bax can be activated during necrosis (Capon et al., 2012), we examined the involvement of necrosis using a pharmacological approach to further understand retinal cell death by mediated by atRAL. ARPE-19 cells and WT cultured mouse neural retinas were pre-treated with Necrostatin-1 (Nec-1), which is commonly used as an inhibitor of necrosis (Dong et al., 2012; Trichonas et al., 2010), and protective effects Nec-1 on atRAL cell toxicity was evaluated. Notably, no apparent protective effects of Nec-1 were observed, whereas BIP treatment prevented ARPE-19 cells from atRAL-induced death in a dose-dependent manner (Fig. 6C). Similarly, atRAL-induced death was not prevented by Nec-1 treatment in cultured neural retinas of WT mice (Fig. 6D).

4. Discussion

In order to understand the pathological mechanisms of atRAL mediated retinal cell death, we examined molecular events using pharmacological approaches in our previous studies (Chen et al., 2012; Maeda et al., 2009). In these studies, we reported that NADPH oxidase-mediated ROS generation via PLC/IP₃/Ca²⁺ signaling and apoptotic cell death via Bax activation. However, the molecular events that bridge the gap between ROS generation and Bax activation in atRAL mediated cell death remain unclear.

To address these issues, we conducted a series of experiments. First, we determined the time line of molecular events involved in cell death *in vitro* by atRAL with range of 10-30 μM (Fig. 1 and Suppl. Fig. 2). Of note, 30 μM of atRAL corresponds to the amount of bleaching less than 0.5% of rhodopsin molecules after photon absorption *in vivo* which can be attained in the living retina (Nickell et al., 2007). Our results showed that Bax activation occurred immediately after ROS generation via PLC/IP₃/Ca²⁺ signaling. From this result, we hypothesized that molecular events triggering Bax activation are induced by excess ROS generation. To support this hypothesis, we examined whether DNA damage induced p53 phosphorylation, known to activate Bax, occurs in atRAL induced cell death (Smeenk et al., 2011). DNA damage (Fig. 2) and p53 phosphorylation (Fig. 3) occurred fully within 30 min after atRAL incubation, both of which proceed Bax activation. Increased levels of 8-OHdG indicate the existence of DNA damage after ROS production by incubation with atRAL, suggesting that atRAL induces DNA damage through ROS generation in the cell. Following DNA damage, p53 functions as a transcription factor stimulating apoptosis-inducing gene expression (Benchimol, 2001). Post-transcriptional modifications of p53 are critical for the activation of p53 and its ability to turn on target genes (Xu, 2003). When DNA damage is too severe to be repaired, Ser46 kinase is activated and induces phosphorylation of p53 at Ser46 causing a change in p53 conformation (Oda et al., 2000). Phosphorylated p53 at Ser46 has a stronger affinity for promoters of proapoptotic genes when compared to promoters of cell-cycle-arrest related or DNA repair-related genes (Oda et al., 2000). The current study demonstrated that a p53 inhibitor attenuated Bax activation and provided protection to retinal cells exposed to atRAL (Fig. 3), whereas p53 inhibition was not as potent in preventing retinal cell death when compared to directly inhibiting Bax with BIP. The differences observed in cell viability when different inhibitors are used suggest the existence of other factors besides p53 being able to activate Bax, such as activation of several types of GPCRs, expressed on the plasma membrane of cells (Chen et al., 2012; Chen et al., 2013a). Of note, activation of ERKs and p38, which can activate p53 via DNA damage (Lu and Xu, 2006) and ROS generation (Kralova et al., 2008) respectively was observed in atRAL mediated cell death (Fig. 3B), which can support our hypothesis about p53 activation process in this study.

Next, we evaluated the impact of Bax activation in retinal cells, involving RPE and photoreceptor cell death with ARPE-19, mouse cultured neural retinas and 661W cells respectively. It is known that Bax activation can be involved in retinal degeneration related to the visual cycle (Hamann et al., 2009; Maeda et al., 2009). In this study, we demonstrated that BIP attenuated Bax activation and protected ARPE-19 cells from apoptosis *in vitro* (Suppl. Fig. 4), which is consistent with our previous study (Maeda et al., 2009). Given these results, we addressed this issue using pharmacological and genetic approaches on an *ex-vivo* cultured retinal tissue system as a model for photoreceptor cells. Pre-treatment with BIP significantly attenuated the toxic effects of atRAL in retinal tissue (Fig. 4A). In addition, deletion of Bax further decreased cytotoxicity of atRAL (Fig. 4A, 4B). We also investigated involvement of [Ca²⁺]_i increase as a triggering event in Bax activation by a pharmacological approach with 2-Aminoethyl diphenylborinate (2-APB), which can prevent increase of [Ca²⁺]_i by blocking endoplasmic reticulum calcium-ATPase pumps. 2-APB treatment could maintain cell viability significantly in both ARPE-19 and 661W after co-

incubation with atRAL (Suppl. Fig. 3A and B). Removal of extracellular calcium by the addition of EGTA failed to maintain cell viability in ARPE19 cells incubated with atRAL (data not shown). These observations suggest that a rise in $[Ca^{2+}]_i$ can be involved in the cell death process induced by atRAL. We also evaluated that Bax activation in 2-APB treated cells after co-incubation with atRAL. Bax activation was significantly attenuated in both ARPE-19 and 661W by 2-APB treatment (Suppl. Fig. 3B). Of note, involvement of $[Ca^{2+}]_i$ increase by atRAL was evidenced *in vivo* using the retinal degeneration mouse model, *Rdh8^{-/-}ABCA4^{-/-}* mice in our previous study (Chen et al., 2012).

Finally, we showed these molecular events occur in the retinal cell death process via atRAL accumulation *in vivo* using an animal model of retinal degeneration, *Rdh8^{-/-}Abca4^{-/-}* mice (Fig. 5 and Suppl. Fig. 6). Our results presented here suggests that apoptotic cell death mediated by Bax activation could be one of the major molecular events involved in this mouse model. Since Bax is known to be involved in the induction of necrosis as well as apoptosis, it was our interest to determine whether atRAL-induced Bax activation resulted in apoptosis or/and necrosis of retinal cells (Murakami et al., 2011). Cell viability was examined using RIP1 kinase inhibitor, Nec-1 to explore necrotic cell death modality. Recent studies have shown RIP kinases linked with ROS generation during the necrosis process. RIP3 which is activated by RIP1 causes mitochondrial ROS production when stimulated by the Krebs cycle and oxidative phosphorylation (Zhang et al., 2009). Notably, Nec-1 did not show any protective effects in atRAL mediated retinal cell death both *in vitro* and *ex-vivo* in this study (Fig. 6C, 6D). Furthermore, we previously showed that caspase inhibitor rescued retinal cells from atRAL, suggesting that apoptosis is the main type of cell death triggered by atRAL (Maeda et al., 2009). A potential explanation of this result is that retinal cell death involving atRAL occurs acutely in this study whereas necrosis is more frequently involved in chronic cell death. Therefore because of the rapidity of cell death it seems unlikely that necrosis is the main cell death pathway in this study. Furthermore, expression of Bcl-2-interacting mediator (Bim) and Bcl-2 showed no obvious changes after incubation with atRAL. Bim and Bcl-2 may not have a major role in the regulation of Bax activation in retinal cell death mediated by atRAL. Further studies identifying the messengers activating Bax in atRAL-treated retinal cells are warranted.

Similar toxicity was observed in atRAL and 9-*cis*-retinal treated cells *in vitro* probably because these molecules share the same highly reactive aldehyde group (Maeda et al., 2009). Importantly, in physiological conditions, free forms of all-*trans*-retinal or other retinal aldehydes can exist in many human tissues. Produced 11- and 9-*cis*-retinal bind to carrier proteins such as CRALBP and IRBP to transport from RPE cells to photoreceptors. After transportation to photoreceptors, 9-*cis* or 11-*cis* retinal form a Schiff-base with rhodopsin and cone-opsin. Released atRAL from photo-activated rhodopsin is immediately reduced to a less toxic form, all-*trans*-retinol, by retinoid dehydrogenases in the retina, such as RDH8 (Maeda et al., 2005a), RDH12 (Maeda et al., 2006) and RDH10 (Wu et al., 2004). This evidence indicates that such retinal aldehydes do not have toxicity to the retina in the presence of a functional retinoid and cycle under physiological conditions.

In conclusion, this study demonstrates that atRAL mediated retinal cell death is associated with an increase in ROS production which results in DNA damage leading to the

phosphorylation of Bax by p53 at Ser 46 (Figure 7). This cascade ultimately contributes to apoptotic but not necrotic retinal cell death.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ABCA4	ATP-binding cassette transporter
atRAL	all- <i>trans</i> -retinal
Bax	Bcl-2-associated X protein
Bim	Bcl-2-interacting mediator
BIP	Bcl-2-associated X protein (Bax)-inhibiting peptide
ERK	Extracellular signal-regulated kinases
ICC	immunocytochemistry: intracellular calcium, $[Ca^{2+}]_i$
IP₃	inositol 1,4,5-triphosphate
LDH	Lactate Dehydrogenase
Nec-1	necrostatin-1
PLC	phospholipase C
RDH	all- <i>trans</i> -retinol dehydrogenase
RPE	retinal pigment epithelium
ROS	reactive oxygen species
SD-OCT	spectral-domain optical coherence tomography
8-OHdG	8-hydroxyhydroguanine

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Research Highlights

1. We examine role of Bax activation in retinal cell death by all-trans-retinal.
2. DNA damage can be caused by excess amount of all-*trans*-retinal in retinal cells.
3. p53 phosphorylation can activate Bax following DNA damage.
4. Inhibition of Bax activation can results in amelioration of retinal cell death.
5. These cellular events can be observed in light induced retinal damage *in vivo*.

Figure 1. Decreased cell viability and Bax activation are induced by all-*trans*-retinal in ARPE-19 cells

(A) Light microscope images of ARPE-19 cells after incubation with all-*trans*-retinal (atRAL). ARPE-19 cells were incubated with indicated concentrations of atRAL for 16 h at 37°C. Severity of ARPE-19 cell death was dose-dependent. (B) Cell viability was evaluated in ARPE-19 cells after atRAL treatment with LDH assay. Cell viability was significantly attenuated dose-dependently compared to those of vehicle treatment only. (C) Bax activation in ARPE-19 cells by 30 μM of atRAL treatment was visualized by immunocytochemistry using a specific antibody against activated Bax (6A7). (D) The fluorescence intensity of activated Bax increased in a time-dependent manner. Error bars indicate SE. Each experiment was done in triplicate at each time point. *, #, ^p, $P < 0.05$ compared with data obtained from DMSO, or *, $P < 0.05$ compared with data at 0 min incubation, $n > 3$.

Figure 2. Detection of DNA damage induced by all-*trans*-retinal in ARPE-19 cells
 (A) Accumulation of 8-hydroxyhydroguanine (8-OHdG) in ARPE-19 cells after 30 μM of atRAL treatment. (B) Intensity of 8-OHdG increased significantly in a dose-dependent manner with increasing concentration of atRAL compared to those of vehicle treatment only. Bars in B indicate SD. Experiments were done in triplicate. *, $P < 0.05$, $n > 3$.

Figure 3. Roles of p53 in retinal cell death by all-trans-retinal

(A) Location of phosphorylated p53 in ARPE-19 cells after 30 μ M of atRAL treatment was demonstrated by immunohistochemistry using an antibody against p53 phosphorylated at Ser46 (green, left panel). The signal of phosphorylated p53 in ARPE-19 cells was colocalized with signals showing nuclei (DAPI, blue) and mitochondria (Mitotracker, red in the yellow circle). (B) Gene expression levels of p53 were evaluated by luciferase reporter assay with ARPE-19 cells transfected with pF5A [CMV/p53-Nluc/Neo] vector. Levels of p53 gene expression were significantly increased 5 min after atRAL incubation, no significant difference between 10 and 30 μ M of atRAL concentration was found. (C) Protein expression of phosphorylated-ERK1, phosphorylated-ERK2 and phospho-p38 was examined in cell extracts from ARPE-19 cells with and without 30 μ M atRAL treatment by immunoblot. (D) Bax activation by p53 inhibitor, Pifithrin- α , in ARPE-19 cell treated with 30 μ M of atRAL was decreased by 50% when compared to the vehicle control. (E) Cell viability increased 1.7-fold following 5 μ M of Pifithrin- α treatment under incubation with 30 μ M of atRAL. DAPI nuclear staining: blue, phosphorylated p53 staining with specific antibody: green, mitochondrial staining with mitotracker: red in A. Bars in B, C and D indicate SD (n=3). *, #, P<0.05 compared with data at 0 min incubation, or #, P<0.05 compared with vehicle control, n>3.

Figure 4. Pharmacologic and genetic inhibition of Bax activation in cultured neural retina mediated by atRAL

(A) Morphology of the photoreceptor layer (ONL and OS) in 6-week-old *Bax*^{-/-} retina was preserved compared to WT retina at 6 h after 30 μM of atRAL treatment. (B) Protective effects of Bax inhibition by BIP and deletion of Bax gene from atRAL toxicity at 30 μM concentration were examined with whole retinal tissue of WT mice and *Bax*^{-/-} mice in *ex-vivo* respectively using LDH assay. WT retina treated with BIP showed significant decreases in cell toxicity compared to the vehicle control. Cell toxicity in whole neural retina of *Bax*^{-/-} mice was significantly attenuated compared to those of WT retina treated with atRAL. Error bars indicate SD. Each experiment was done in triplicate at each time point. Blue: DAPI nuclear staining, red: rhodopsin staining with 1D4, green: cone sheath staining with PNA. ONL: outer nuclear layer, OS: outer segment. Bars in B indicate 20 μm. *, P<0.05, n=3.

Figure 5. Assessment of Bax activation and relative events in light-induced retinal degeneration in *Abca4*^{-/-}*Rdh8*^{-/-} mice

4–6 weeks old *Abca4*^{-/-}*Rdh8*^{-/-} and WT mice were exposed to 10,000 lux light for 30 min. SD-OCT images at B-scan liner mode were compared, which were obtained 1 h after light exposure in superior retina 500 μ m away from optic nerve head (Top panels). Intensity of signals and decrease of thickness were observed in the outer nuclear layer of *Abca4*^{-/-}*Rdh8*^{-/-} mice with light exposure [light (+)] whereas no apparent changes were observed in WT mice with light exposure [light (+)] and *Abca4*^{-/-}*Rdh8*^{-/-} mice without light exposure [light (-)]. 8-OHdG, phosphorylated p53 (phos-p53) and activated Bax (Activ-Bax) were examined in photoreceptor layer of *Abca4*^{-/-}*Rdh8*^{-/-} mice by immunohistochemistry using specific antibodies. The signals of 8-OHdG were increased in outer nuclear layer (ONL) and inner segment (IS) of *Abca4*^{-/-}*Rdh8*^{-/-} mice [light (+)]. Phosphorylated p53 at Ser46 was detected in ONL and weakly in IS of *Abca4*^{-/-}*Rdh8*^{-/-} mice [light (+)]. Bax activation was observed in both ONL and IS in *Abca4*^{-/-}*Rdh8*^{-/-} mice [light (+)]. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; IS, inner segments. Bars indicate 10 μ m.

Figure 6. Evaluation of other molecules for activation or deactivation of Bax and necrotic pathway in retinal cell death by atRAL

Expression levels of Bim (A) and Bcl-2 (B) were examined in cell extracts from ARPE-19 cells with and without 30 μM of atRAL treatment. Levels of protein expression in these two molecules were not changed in ARPE-19 cells at 30 min after incubation with 30 μM of atRAL. (C) The protective effect of receptor interacting peptide1 kinase inhibitor, necrostatin-1 (Nec-1) against retinal cell death by atRAL was tested in *in vitro* and *ex-vivo*. Cell viability was evaluated at 8 h after treatment with 30 μM of atRAL by WST-1 assay in ARPE-19 cells pretreated with Nec-1. (D) Cell viability was not preserved in ARPE-19 cells following Nec-1 treatment whereas dose-dependent protective effects were observed in those treated with BIP. (E) Cell toxicity was evaluated at 8 h after treatment with 30 μM of atRAL by LDH assay in the neural retinas from WT pretreated with 100 μM of Nec-1 or 400 μM of BIP prior to atRAL treatment. No changes in cytotoxicity were observed in retinal tissue with Nec-1 treatment whereas those treated with BIP decreased to less than 30% of those of vehicle control. Immunoblot analyses were conducted three times independently. *, $P < 0.05$, $n > 3$.

Figure 7. Diagram of cellular events involved in Bax mediated cell death by atRAL

atRAL is a major byproduct of the retinoid cycle with cellular toxicity. Any events, which induce delay of atRAL clearance from photoreceptors, cause excess accumulation of atRAL resulting in mitochondria mediated apoptotic cell death. In atRAL mediated cell death pathway, following cellular events can be involved. Excess levels of atRAL can activate G protein coupled receptors (GPCR), which increase $[Ca^{2+}]_i$ levels via PLC/IP₃ signaling. Increase of $[Ca^{2+}]_i$ cause ROS production via NADPH oxidase activation, which can be attenuated by 2-APB. Next, ROS can induce DNA damage resulting in p53 phosphorylation. Finally, phosphorylated p53 can activate Bax, resulting in mitochondrial apoptotic cell death. Bax activation can be reduced by Bax inhibitory peptide.