Mechanism of All-trans-retinal Toxicity with Implications for Stargardt Disease and Age-related Macular Degeneration*  

Yu Chen‡, Kiichiro Okano‡, Tadao Maeda‡§, Vishal Chauhan‡§, Marcin Golczak‡, Akiko Maeda‡, and Krzysztof Palczewski‡

From the Departments of‡Pharmacology and§Ophthalmology, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106–4965

Background: High levels of all-trans-retinal (atRAL) are associated with photoreceptor degeneration.

Results: atRAL promotes NADPH oxidase-mediated overproduction of intracellular reactive oxygen species.

Conclusion: A cascade of signaling events is demonstrated to underlie the action of atRAL in photoreceptor degeneration in mice.

Significance: Mechanistic elucidation of atRAL-mediated photoreceptor degeneration is essential for understanding the molecular pathogenesis of Stargardt disease and other types of retinal degeneration.

Compromised clearance of all-trans-retinal (atRAL), a component of the retinoid cycle, increases the susceptibility of mouse retina to acute light-induced photoreceptor degeneration. Abca4−/−/Rdh8−/− mice featuring defective atRAL clearance were used to examine the one or more underlying molecular mechanisms, because exposure to intense light causes severe photoreceptor degeneration in these animals. Here we report that bright light exposure of Abca4−/−/Rdh8−/− mice increased atRAL levels in the retina that induced rapid NADPH oxidase-mediated overproduction of intracellular reactive oxygen species (ROS). Moreover, such ROS generation was inhibited by blocking phospholipase C and inositol 1,4,5-trisphosphate-induced Ca2+ release, indicating that activation occurs upstream of NADPH oxidase-mediated ROS generation. Because multiple upstream G protein–coupled receptors can activate phospholipase C, we then tested the effects of antagonists of serotonin 2A (5-HT2A, R) and M3-muscarinic (M3, R) receptors and found they both protected Abca4−/−/Rdh8−/− mouse retinas from light-induced degeneration. Thus, a cascade of signaling events appears to mediate the toxicity of atRAL in light-induced photoreceptor degeneration of Abca4−/−/Rdh8−/− mice. A similar mechanism may be operative in human Stargardt disease and age-related macular degeneration.

To sustain vision, all-trans-retinal (atRAL), released from light-activated visual pigments, including rhodopsin, must be continuously isomerized back to its 11-cis isomer (1). This process occurs by a sequence of reactions catalyzed by membrane-bound enzymes of the retinoid cycle located in rod and cone photoreceptor cell outer segments and the retinal pigmented epithelium (RPE) (2–5). Regeneration of rhodopsin requires 11-cis-retinal (11-cis-RAL) supplied from the RPE, but cone pigments are also regenerated in cone-dominant species by a separate “cone visual cycle” (6–8). A high flux of retinoids through the retinoid cycle, as occurs during intense light exposure, can cause elevated levels of toxic retinoid intermediates, especially atRAL, that can induce photoreceptor degeneration (9). Toxic effects of atRAL include caspase activation and mitochondrial-associated cell death (10), but the precise sequence of molecular events that leads to photoreceptor degeneration remains to be clarified.

Even in the presence of a functional retinoid cycle, A2E, a retinal dimer, and other toxic atRAL condensation products (11–13) accumulate with age (14). These compounds are fluorescent biomarkers of aberrant atRAL metabolism (15). Patients affected by retinal degeneration in age-related macular degeneration, Stargardt disease, or some other retinal diseases feature abnormal accumulation of these atRAL condensation products (16). Mice carrying a double knock-out of the Rdh8 gene, which encodes one of the main enzymes that reduces atRAL in rod and cone outer segments (17), and the Abca4 gene (18, 19), which encodes the transporter of atRAL from the inside to the outside of disc membranes, rapidly accumulate atRAL condensation products and manifest RPE/photoreceptor dystrophy at an early age (20). The similarity of this retinopathy to human age-related macular degeneration makes these Abca4−/−/Rdh8−/− mice invaluable for research aimed at ameliorating this devastating blinding disease (10, 21). Mutations in ABCA4 can cause Stargardt macular degeneration (22), cone-cotransplant, DCF-DA, 2,7'-dichlorofluorescein diacetate; DHE, dihydroethidium; DPI, diphenyleneiodonium; GPCR, G protein–coupled receptor; IP3R, inositol 1,4,5-trisphosphate; IP3; IP3 receptor; M3, R, M3-muscarinic receptor; OCT, optical coherence tomography; ONH, optic nerve head; ONL, outer nuclear layer; PLC, phospholipase C; Ret-NH2, retinalamine; ROS, reactive oxygen species; RPE, retinal pigmented epithelium; ERG, electoretinogram.

* This work was supported, in whole or in part, by National Institutes of Health Grants EY009339, EY021126, EY019031, EY019880, and P30 EY11373. This work was also supported by the Research to Prevent Blindness Foundation and the Ohio Lions Eye Research Foundation.

‡ A John H. Hord Professor of Pharmacology. To whom correspondence should be addressed: Dept. of Pharmacology, School of Medicine, Case Western Reserve University, 10900 Euclid Ave., Cleveland, OH 44106-4965. Tel.: 216-368-4631; Fax: 216-368-1300; E-mail: lcxp65@case.edu.

§ Ophthalmology, School of Medicine, Case Western Reserve University, Cleveland, Ohio.
rods (23), or recessive retinitis pigmentosa (24, 25). Heterozygous mutations in ABOA increase the risk of developing age-related macular degeneration as well (16).

Abca4−/−Rdh8−/− mice, which exhibits markedly delayed clearance of atRAL after photobleaching and serves as a model of cone and rod retinal degeneration (10, 21), allowed us to examine in greater detail the molecular pathways involved in the pathogenesis of this retinopathy. Oxidative stress is a major mechanism contributing to photoreceptor cell death in animal models of retinal degeneration, including light-induced retinopathy (26, 27). Tightly regulated low levels of reactive oxygen species (ROS) are needed to mediate physiological functions, including cell survival, growth, differentiation, and metabolism. But excessive production of ROS can damage macromolecules, including DNA, proteins, and lipids (28). Thus, aberrant ROS generation constitutes a major mechanism of pathological cell death.

NADPH oxidase is the main enzymatic source of superoxide and hydrogen peroxide (29), and its product ROS, which is involved in retinal degeneration (30, 31). Rac1, an essential component of the NADPH oxidase complex, is implicated in light-induced retinal degeneration, because Rac1 deficiency partially protects photoreceptor cells against photo-oxidative insult (30). Treatment with the NADPH oxidase inhibitor apocynin (1-(4-hydroxy-3-methoxyphenyl)ethanone (APO)) (32) can protect BALB/c mice from developing light-induced retinal degeneration (30). Moreover, APO is effective in preventing cone cell death in a mouse model of retinitis pigmentosa (31). These findings imply that, by causing oxidative stress, NADPH oxidase is mechanistically involved in the pathogenesis of some types of retinal degeneration.

Although atRAL stimulates the production of superoxide via NADPH oxidase (33, 34), there are observations that such stimulation is not the result of a direct interaction between atRAL and this enzyme (35). PLC activation reportedly occurs prior to NADPH oxidase-dependent ROS production in atRAL-treated neutrophils suggesting that products of PLC enzymatic activity, diacylglycerols and inositol 1,4,5-trisphosphate (IP3), could be the intermediates involved in this pathway (33). IP3 promotes release of Ca2+ from the endoplasmic reticulum into the cytosol through binding to an intracellular IP3-receptor, IP3R (36). This signaling pathway may underlie the previously unexplained observation that atRAL causes a rapid increase in intracellular Ca2+ (10). Ca2+ signaling has also been reported to increase ROS production by NADPH oxidase (37). Because PLC is typically activated by G protein-coupled receptors (GPCRs) coupled to Gα protein (38), specific GPCRs could affect overall PLC activation, thus mediating atRAL-induced toxic effects.

Results from cell culture experiments indicate that atRAL-induced generation of ROS can be mediated through NADPH oxidase. We further investigated the in vivo signaling mechanisms that mediate the action of atRAL in causing ROS production and light-induced photoreceptor degeneration. The results indicate that PLC activation and the resulting second messenger IP3 contribute to atRAL-induced NADPH oxidase activation. The toxic action of atRAL was also diminished by blocking serotonin 2A (5-HT2A) or M3-muscarinic (M3) receptors, implicating GPCR participation in the overall process. These observations raise the possibility that certain types of retinal degeneration could be prevented by therapies selectively targeting transient sequestration (buffering) of elevated atRAL, antagonizing a subset of GPCRs, or inhibiting PLC, IP3R, or NADPH oxidase, alone or in combination.

**EXPERIMENTAL PROCEDURES**

**Animals**—Abca4−/−Rdh8−/− mice, generated and genotyped as previously described (20), were used when they reached 4 to 5 weeks of age. Eight- to 12-week-old BALB/c mice were obtained from Jackson Laboratory (Bar Harbor, ME). All mice were housed in the Animal Resource Center at the School of Medicine, Case Western Reserve University, where they were routinely maintained in a 12-h light (less than 10 lux)/12-h dark cycle environment. For bright light exposure experiments, mice were dark-adapted for 24 h prior to illumination at 10,000 lux (150-watt spiral lamp, Commercial Electric) for either 30 min (Abca4−/−Rdh8−/− mice) or 2 h (BALB/c mice). Abca4−/−Rdh8−/− mouse pupils were dilated with 1% tropicamide prior to light exposure, whereas BALB/c mice did not require pupil dilation before such exposure. Analyses of retinal structural and functional changes were performed 7 days after bright light exposure. All animal-handling procedures and experiments were approved by the Institutional Animal Care and Use Committee at Case Western Reserve University.

**Chemicals**—atRAL was purchased from Toronto Research Chemicals, Inc. (Toronto, Canada). All-trans-retinoic acid (atRA), apocynin (APO), diphenyliodonium (DPI), 2-aminoethoxydiphenyl borate (2-APB), ketanserin, and 8-hydroxy-N,N-dipropyl-2-aminotetralin (8-OH-DPAT) were obtained from Sigma. Pregabalain was synthesized by Ricerca Bioscience LLC (Concord, OH). A2E (39) and all-trans-retinylamine (Ret-NH2) were synthesized as previously described (40). U-73122 was purchased from Calbiochem (Gibbstown, NJ). Ritanserin and 1,1-dimethyl-4-diphenylacetoxyperipenidinium iodide (4-DAMP) were purchased from Tocris (Ellisville, MO).

**In Vitro Detection and Quantification of Intracellular ROS**—ARPE19 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, low glucose) supplemented with 10% fetal bovine serum. The ROS probes, 2’,7’-dichlorofluorescein diacetate (DCF-DA, Sigma) or dihydroethidium (DHE, Invitrogen) were added in DMSO at a concentration of 400 nm (final solvent concentration, 1% v/v) after indicated pretreatments and incubated at 37 °C for 10 min before cells were thoroughly washed in phosphate-buffered saline. ROS signals were subsequently observed at the same exposure setting under an inverted fluorescence microscope (Leica DMI 6000 B). Fluorescence quantification was performed with Metamorph imaging software (Molecular Devices, Downington, PA). Thresholds corresponding to fluorescent signals were set from the images, and average fluorescence intensities were recorded for statistical analyses.

**In Vivo Detection of ROS**—The ROS probe, DHE, at a dose of 20 mg/kg body weight in 25 μl of DMSO, was administered to Abca4−/−Rdh8−/− mice via intraperitoneal injection 30 min prior to light exposure. Eye cups obtained after removing the cornea, lens, and vitreous body from enucleated eye globes 3 h
post light illumination were fixed in 4% paraformaldehyde. Cryosections were prepared from fixed eye cups and cut at 12-μm thickness for microscopic assessment of ROS fluorescence in the retina using ImageJ (National Institutes of Health).

**Mouse Treatments**—Ret-NH₂ and pregabalin were administered by gavage to 24-h dark-adapted mice at a dose of 100 mg/kg body weight 2 h before illumination. All other experimental compounds were given to 24 h dark-adapted mice by intraperitoneal injection through a 28-gauge needle at 24 h and 1 h prior to bright light exposure. Tested compounds and their doses were as follows: APO, 50 mg/kg body weight; DPI, 1 mg/kg body weight; U-73122, 6.25 mg/kg body weight; 2-APB, 2.5 mg/kg body weight; ketanserin, 1.25 mg/kg body weight; ritanserin, 3.75 mg/kg body weight; 8-OH-DPAT, 10 mg/kg body weight; and 4-DAMP, 6.25 mg/kg body weight. The gavage volume was 100 μl per treatment. The injected volume of the injected drug did not exceed 50 μl per animal. Ret-NH₂ was prepared in soybean oil. Pregabalin and 8-OH-DPAT were dissolved in water. All other drugs were dissolved in DMSO.

**OCT**—Ultra-high resolution SD-Optical Coherence Tomography (OCT, Bioptigen, Research Triangle Park, NC) was used for *in vivo* imaging of mouse retinas. Mice were anesthetized by intraperitoneal injection of a mixture consisting of ketamine (6 mg/ml) and xylazine (0.44 mg/ml) diluted with 10 mM sodium phosphate, pH 7.2, and 100 mM NaCl given at a dose of 20 μl/g body weight. Pupils were dilated with 1% tropicamide prior to imaging. Four frames of OCT images acquired in the B-mode were averaged for presentation.

**Histology and Immunohistochemistry**—Retinal histology and immunohistochemistry were performed as previously described (41). Briefly, eye cups freed of cornea, lens, and vitreous body were fixed in 2% glutaraldehyde/4% paraformaldehyde and processed for Epon embedding. Sections of 1-μm thickness were cut and stained with toluidine blue for histological examination under a light microscope. Immunohistochemical analysis was performed on 12-μm thick cryosections prepared from 4% paraformaldehyde-fixed eye cups. Collected cryosections were stained with DAPI and subjected to examination for rhodopsin, and with pea-nut agglutinin for cone sheaths.

**ERGs**—All ERG procedures were performed by published methods (41). For single-flash recording, the duration of white light flash stimuli (from 20 μs to 1 ms) was adjusted to provide...
a range of illumination intensities (from 3.7 to 1.6 log cd/s/m²). Three to five recordings were made at sufficient intervals between flash stimuli (from 3 s to 1 min) to allow recovery from any photobleaching effects.

Retinoid Analyses—Extraction, derivatization, and separation of retinoids were performed, and 11-cis-retinal content was analyzed by HPLC by procedures previously described (41).

Statistical Analyses—Results were averaged from at least three independent experiments. Data were expressed as means ± S.E., and statistical analyses were performed using the student’s t test for p value calculations.

RESULTS

atRAL Stimulates Intracellular ROS Production through NADPH Oxidase—To determine the effect of atRAL on retinal ROS production, we incubated ARPE19 cells, an immortalized human RPE-like cell line susceptible to atRAL-induced cell death, with atRAL followed by examination with a ROS probe. As shown in Fig. 1A, atRAL exposure significantly elevated intracellular ROS production prior to massive cell death in a dose-dependent manner. Because the probe used, DCF-DA, is highly selective for H₂O₂ and hydroxyl radicals, intracellular ROS levels were also examined by another commonly used ROS probe, DHE, which is especially sensitive to superoxide. Consistently, the intracellular ROS signal-identified DHE probe was markedly increased in ARPE19 cells treated with 30 μM atRAL (Fig. 1B), a concentration that reproducibly caused excessive ARPE19 cell death as reported previously (10). The same concentration of atRAL would be produced by a ~1% bleach of rhodopsin under physiological conditions. Interestingly, atRAL-related metabolic products such as all-trans-retinol (atROL), all-trans-retinoic acid (atRA), and A2E did not induce overproduction of intracellular ROS (supplemental Fig. S1). The difference between atRAL and the other retinoids in triggering intracellular ROS production could explain the difference in their effect on inducing cell death, because neither atROL, atRA, nor A2E induced cell death at the concentrations examined (10).

NADPH oxidase is the primary catalyst involved in atRAL-stimulated superoxide production by neutrophils (33, 34). To examine the role of NADPH oxidase in retinal cells, we added APO, a widely used NADPH oxidase inhibitor that interrupts NADPH oxidase complex assembly, to ARPE19 cells together with atRAL. As shown in Fig. 1C, APO treatment inhibited atRAL-induced intracellular ROS generation and enhanced ARPE19 cell survival (supplemental Fig. S2). Taken together, these results indicate that NADPH oxidase is required for atRAL-induced ROS production in ARPE19 cells, a finding that implies involvement of NADPH oxidase-mediated ROS generation in atRAL-induced retinal cell death.

NADPH Oxidase Mediates Light-induced ROS Production in Abca4−/−Rdh8−/− Mouse Retina—To further test the observation that atRAL induces ROS overproduction through
NADPH oxidase in vivo, the ROS probe DHE was administered to Abca4−/−Rdh8−/− mice 30 min before light exposure at 10,000 lux for 30 min. This regimen was selected because this illumination intensity caused marked photoreceptor degeneration in Abca4−/−Rdh8−/− mice, whereas WT controls manifested no obvious morphological changes (supplemental Fig. S3). Compared with the ROS signal detected in the outer nuclear layer (ONL) of Abca4−/−Rdh8−/− mice unexposed to light, a strong ROS signal was recorded in the ONL of retinas from light-exposed and vehicle-treated Abca4−/−Rdh8−/− mice (Fig. 2). When APO was administered 1 h prior to illumination, these APO-treated double mutant mice displayed substantially
Mechanism of All-trans-retinal Toxicity

Inhibition of NADPH Oxidase Protects Abca4−/−Rdh8−/− Mouse Retina against Acute Light-induced Photoreceptor Degeneration—To directly examine if atRAL-induced NADPH oxidase-mediated ROS production is mechanistically implicated in acute light-induced photoreceptor degeneration, we treated Abca4−/−Rdh8−/− mice with APO, DPI, or vehicle control (DMSO) 1 h prior to light exposure at 10,000 lux for 30 min. The effect of NADPH oxidase inhibitor treatment was assessed 7 days after illumination. Although OCT scans revealed significantly disrupted photoreceptor structures in DMSO-treated mice, OCT of both APO-treated (Fig. 3A, panel a) and DPI-treated (data not shown) mice exhibited well preserved retinal morphology. This observation was confirmed by retinal histological examination. Retinas from DMSO-treated mice manifested prominent structural disorder with shortened lengths of photoreceptor outer/inner segments, markedly decreased cell numbers in the ONL, and increased pyknosis of photoreceptor nuclei. This morphology contrasted sharply with the nearly intact retinal morphology manifested by APO-treated (Fig. 3A, panel b) or DPI-treated mice (Fig. 3B, panel a). Immunohistochemical examination for rhodopsin in rod photoreceptor outer segments and peanut agglutinin-labeling of cone cell matrix sheaths were also performed. These images revealed abundant and organized expression of rhodopsin and peanut agglutinin in APO-treated (Fig. 3A, panel c) or DPI-treated (data not shown) mice, in sharp contrast to the residual pattern of rhodopsin and peanut agglutinin staining detected in DMSO-treated mice. Quantification of ONL thickness after DAPI staining revealed that both APO (Fig. 3A, panel d) and DPI (Fig. 3B, panel b) pretreatment greatly preserved photoreceptors compared with DMSO pretreatment. These results support the notion that NADPH oxidase-mediated ROS generation is mechanistically implicated in the action of atRAL during light-induced photoreceptor degeneration.

Involvement of PLC/IP3/Ca2+ Signaling in Light-induced atRAL-mediated Photoreceptor Degeneration—To test the hypothesis that PLC/IP3/Ca2+ signaling is involved in the cascade of events related to atRAL toxicity in light-induced photoreceptor degeneration, we pretreated Abca4−/−Rdh8−/− mice with the selective PLC inhibitor, U-73122 (43), prior to light exposure. In contrast to Abca4−/−Rdh8−/− mice pretreated with DMSO that reproducibly manifested severe histological photoreceptor degeneration, Abca4−/−Rdh8−/− mice pretreated with U-73122 exhibited markedly less light-induced photoreceptor damage (Fig. 4A, panel a) and ONL thickness measurements provided further evidence of a protective effect (Fig. 4A, panel b). These results strongly support the involve-
Mechanism of All-trans-retinal Toxicity

Involvement of $G_q$-coupled Receptors in Light-induced atRAL-mediated Retinal Degeneration—$5\text{-HT}_{2A}$R has been suggested to be involved in NADPH oxidase activation (45). Additionally, chronic or acute $5\text{-HT}_{2A}$R activation causes considerable reduction in 5-HT$_{1A}$R activity (46–49). The 5-HT$_{1A}$R is involved in light-induced photoreceptor degeneration, because selective 5-HT$_{1A}$R agonists protect the rat retina against photo-oxidative stress (50). Moreover, 5-HT$_{2A}$R activates PLC (54), and PLC activation is involved in the in vivo action of atRAL (Fig. 4). Therefore we hypothesized that increased functionality of the 5-HT$_{2A}$R could contribute to the pathogenesis of light-induced photoreceptor degeneration in Abca4$^{-/-}$Rdh8$^{-/-}$ mice. To test this hypothesis, Abca4$^{-/-}$Rdh8$^{-/-}$ mice were treated with selective 5-HT$_{2A}$R antagonist ketanserin (51) prior to light exposure. A substantial protective effect of ketanserin against light-induced photoreceptor degeneration was observed compared with DMSO pretreatment (Fig. 5A, panels a and b). A similar observation was made when Abca4$^{-/-}$Rdh8$^{-/-}$ mice were treated with another selective 5-HT$_{2A}$R antagonist, ritanserin (52) (supplemental Fig. S5). A role for 5-HTRs in light-induced atRAL-mediated retinal degeneration in Abca4$^{-/-}$Rdh8$^{-/-}$ mice is additionally supported by the protective effect of the 5-HT$_{1A}$R agonist, 8-OH-DPAT (53) (supplemental Fig. S6).

Considering that PLC can be activated by multiple $G_q$-coupled receptors, we tested whether 5-HT$_{2A}$R is the only GPCR involved in atRAL-induced PLC activation. Interestingly, the M$_3$R antagonist, 4-DAMP (54), also preserved retinal morphology in Abca4$^{-/-}$Rdh8$^{-/-}$ mice challenged by acute light exposure (Fig. 5B, panels a and b), supporting the idea that multiple $G_q$-coupled receptors could be activated to mediate the effect of atRAL on PLC activation in light-induced photoreceptor degeneration.

Involvement of these mechanisms in light-induced atRAL-mediated photoreceptor degeneration was also shown by improved retinal function of light-challenged Abca4$^{-/-}$Rdh8$^{-/-}$ mice after pretreatment with several pharmacological agents that protected against histological damage. As indicated in Fig. 6, compared with light-challenged WT control and Abca4$^{-/-}$Rdh8$^{-/-}$ mice without light exposure, light-challenged Abca4$^{-/-}$Rdh8$^{-/-}$ mice pretreated with DMSO exhibited decreased amplitudes of both a-waves and b-waves, indicating marked impairment of their retinal function. The protective effect of these treatments on retinal function was observed by increased a-wave and b-wave amplitudes compared with those observed in DMSO-treated Abca4$^{-/-}$Rdh8$^{-/-}$ mice. Data presented above were derived from studies with Abca4$^{-/-}$Rdh8$^{-/-}$ mice, a genetically modified animal model with deficiencies in atRAL transport and clearance owing to targeted deletion of the Rdh8 and Abca4 genes. To determine if the mechanisms proposed were merely secondary to genetic modification or arose from some unidentified off-target effects in Abca4$^{-/-}$Rdh8$^{-/-}$ mice, we further tested our hypotheses in light-challenged BALB/c mice, a classic model of light-induced photoreceptor degeneration. Compared with unexposed con-

FIGURE 6. Retinal function in Abca4$^{-/-}$Rdh8$^{-/-}$ mice is substantially preserved by several different treatments. Scotopic ERGs were recorded and both a-waves (top) and b-waves (bottom) were plotted to evaluate retinal function in Abca4$^{-/-}$Rdh8$^{-/-}$ mice 7 days after they were pretreated with the indicated compounds. Compared with WT mice exposed to bright light (Light_WT) and Abca4$^{-/-}$Rdh8$^{-/-}$ mice without light exposure (No light), light exposure at 10,000 lux for 30 min significantly impaired retinal function as indicated by decreased a-wave and b-wave amplitudes in mice treated with DMSO vehicle control (Light_DMSO). Compounds showing a protective effect against this light-induced retinopathy included ketanserin, ritanserin, 4-DAMP, U-73122, 2-APB, APO, and DPI.

ment of PLC activation in light-induced atRAL-mediated photoreceptor degeneration.

To further validate the involvement of PLC/IP$_3$/Ca$^{2+}$ signaling in atRAL-mediated photoreceptor degeneration in vivo, 2-APB, an antagonist of IP$_3$/IP$_{3}$R-mediated Ca$^{2+}$ release (44), was administered to Abca4$^{-/-}$Rdh8$^{-/-}$ mice prior to light exposure. Retinal morphological examination revealed that 2-APB pretreatment significantly preserved retinal morphology after illumination compared with DMSO pretreatment (Fig. 4B, panel a). Further, 2-APB pretreatment reduced ROS production in light-exposed Abca4$^{-/-}$Rdh8$^{-/-}$ mouse photoreceptors to a level comparable to that observed in photoreceptors of mice without light exposure (Fig. 4B, panel b). Thus, IP$_3$-mediated Ca$^{2+}$ elevation is mechanistically associated with atRAL-induced ROS production during light-induced photoreceptor degeneration. Taken together, our results demonstrate that the PLC/IP$_3$/Ca$^{2+}$ pathway acts upstream of light-induced atRAL-mediated ROS generation and subsequent photoreceptor degeneration.
trol mice, BALB/c mice exposed to intense light exhibited severe photoreceptor degeneration indicated by disrupted retinal histology (Fig. 7A), decreased ocular 11-cis-RAL content (supplemental Fig. S7), and impaired retinal function (Fig. 7B). In contrast, pharmacological pretreatment targeting each proposed mechanism displayed significant protection of photoreceptors against acute light-induced degeneration as assessed by morphological (Fig. 7A), biochemical (supplemental Fig. S7), and functional tests (Fig. 7B).

DISCUSSION

Although atRAL is cytotoxic in cultured cells and associated with light-induced photoreceptor cell death in vivo (21), the involved mechanisms remain to be clarified. atRAL induces high levels of superoxide in neutrophils via NADPH oxidase, the primary enzymatic source of generated superoxide (29). Experimental results described here identify a series of intrinsically linked events, including the participation of GPCRs, PLC/IP$_3$/Ca$^{2+}$ signaling, and NADPH oxidase-mediated ROS production, which are responsible for the pathogenesis of atRAL-mediated light-induced retinal degeneration in Abca$^{	ext{+/−}}$Rdh$^{	ext{−/−}}$ mice, a model for rod/cone degeneration. We further show that these mechanisms could play a role in the pathogenesis of photo-oxidative retinal degeneration in BALB/c mice as well.

atRAL has recently emerged as a critical player in the pathogenesis of retinal degeneration through its association with photoreceptor cell death (10, 55). However, how this retinoid exerts its toxic effects during retinal degeneration has not been previously investigated in vivo. The present study revealed that atRAL rapidly induced ROS overproduction in cultured RPE-like cells prior to cell death. This effect was also observed in the retinas of Abca$^{	ext{+/−}}$Rdh$^{	ext{−/−}}$ mice after bright light exposure sufficient to cause prominent photoreceptor cell death in vivo, suggesting that atRAL release upon rhodopsin photobleaching

FIGURE 7. Light-induced retinal degeneration in BALB/c mice. Twelve-week-old BALB/c mice were dark-adapted followed by indicated pharmacological treatments via intraperitoneal injection 1 h prior to their exposure to white light at 10,000 lux for 2 h. All experimental evaluations were carried out 7 days later. Controls either without light exposure (No light) or with DMSO vehicle treatment followed by light exposure (Light_DMSO) were included for all analyses. A, retinal thin sections examined under light microscopy (63×) after toluidine blue staining. Scale bar, 20 μm on all panels. B, retinal function assessed by scotopic ERG in BALB/c mice 7 days after the indicated pretreatments.
is involved in ROS production. Consistent with this hypothesis, treatment of mice with Ret-NH₂, a retinal scavenger and retinoid cycle inhibitor, and the primary amine-containing pregabalin that buffers atRAL significantly reduced light-induced ROS production in the ONL.

Oxidative stress is a major mechanism contributing to photoreceptor cell death in various animal models of retinal degeneration, including acute light-induced retinopathy. This is supported primarily by the protective effect of antioxidants in animal models of retinal degeneration and by the observation that photoreceptor cell death induced by light exposure involves overproduction of superoxide. NADPH oxidase has only recently been implicated as the enzymatic source of ROS in the ONL.

Mechanism of All-trans-retinal Toxicity

Increased functionality of Gₛ-coupled GPCRs is involved in mediating atRAL toxicity during light-induced photoreceptor degeneration; however, the mechanism remains to be clarified (black arrow with dotted line). Activation of Gₛ-coupled GPCRs causes activation of PLC/IP₃/Ca²⁺ signaling, which in turn leads to NADPH oxidase-mediated ROS production and photoreceptor degeneration (black arrows). Pharmacological interventions targeting Gₛ-coupled GPCRs, PLC/IP₃/Ca²⁺, and NADPH oxidase protect the photoreceptor from light-induced, atRAL-mediated degeneration (red bars).

Increased functionality of Gₛ-coupled GPCRs is involved in mediating atRAL toxicity during light-induced photoreceptor degeneration (black arrow with dotted line). Activation of Gₛ-coupled GPCRs causes activation of PLC/IP₃/Ca²⁺ signaling, which in turn leads to NADPH oxidase-mediated ROS production and photoreceptor degeneration (black arrows). Pharmacological interventions targeting Gₛ-coupled GPCRs, PLC/IP₃/Ca²⁺, and NADPH oxidase protect the photoreceptor from light-induced, atRAL-mediated degeneration (red bars).
Mechanism of All-trans-retinal Toxicity

activation may also enhance intracellular Ca\(^{2+}\) levels by increasing the sensitivity of the endoplasmic reticulum to IP\(_3\), thereby promoting Ca\(^{2+}\) release from these intracellular stores. The rise in Ca\(^{2+}\) levels could be abolished by treatment with the NADPH oxidase inhibitor, DPI, or by a deficiency of Rac1 in these cells (37). NADPH inhibitors and antagonists of PLC/IP\(_3\)/Ca\(^{2+}\) signaling had similar effects in protecting retinas from atRAL-mediated degeneration, implying that these mechanisms are involved in the same signaling pathway.

The PLC pathway is activated by multiple GPCRs coupled to G\(_q\) protein, suggesting that GPCRs could mediate the effect of atRAL on PLC activation. Among known pharmacologically distinct GPCRs associated with PLC activation, 5-HT\(_{2A}\)R is an excellent candidate for activating PLC, although little previous data exists regarding its involvement in light-induced retinal degeneration. 5-HT\(_{2A}\)R expression is readily detectable in the retina and 5-HT\(_{2A}\)R activation mainly leads to elevations in cytosolic Ca\(^{2+}\) through PLC activation (57). Our results further demonstrate that atRAL-mediated PLC activation during light-induced retinal degeneration could result from upstream activation of multiple GPCRs, such as 5-HT\(_{2A}\)R and M\(_{1}\)R, that employ PLC/IP\(_3\)/Ca\(^{2+}\) as their primary intracellular signaling pathway (58). However, further studies are required to elucidate the mechanism of G\(_q\)-coupled GPCR activation in the context of atRAL-mediated, light-induced retinal degeneration.

Collectively, these findings demonstrate that atRAL toxicity in light-induced retinal degeneration could be mediated through a signaling cascade implicating GPCRs, PLC/IP\(_3\)/Ca\(^{2+}\) signaling, and NADPH oxidase (Fig. 8). Pharmacological interventions targeting these mechanisms can provide novel therapeutic strategies for treating blinding retinal disorders such as Stargardt disease and age-related macular degeneration.

Acknowledgments—We thank Dr. Zhiqian Dong for expert handling of mice, Dr. Satomi Shioste and Dr. Kaede Ishikawa for help with the treatment regimes, Satsumi Roos for block preparation and plastic sectioning, and Hiroko Matsuyama for retinoid analyses. We also thank Dr. L. T. Webster, Jr., Dr. Jack Saari, Dr. Michael E. Maguire, and members of the Palczewski laboratory for critical comments on the manuscript.

Note Added in Proof—During the review of our manuscript, we came upon a recently published paper (59), which indicates that unsaturated fatty acids activate PLC/IP\(_3\)/Ca\(^{2+}\) signaling through GPCR activation and induce ROS overproduction in TM4t mouse mammary tumor cells. This complex mechanism highlights the effect of unsaturated fatty acids on apoptosis. Given that all-trans-retinal shares common properties with unsaturated fatty acids with respect to stimulating superoxide production and activating PLC signaling, this paper corroborates our findings of the effect of all-trans-retinal on GPCR, PLC signaling, and ROS generation.

REFERENCES

17. Rattner, A., Smallwood, P. M., and Nathans, J. (2000) Identification and characterization of all-trans-retinol dehydrogenase from photoreceptor outer segments, the visual cycle enzyme that reduces all-trans-retinal to all-trans-retinol. J. Biol. Chem. 275, 11034–11043
Autosomal recessive retinitis pigmentosa and cone-rod dystrophy caused by splice site mutations in the Stargardt’s disease gene ABCR. Hum Mol Genet 7, 355–362


