Rapamycin Inhibits Light-Induced Necrosome Activation Occurring in Wild-Type, but not RPE65-Null, Mouse Retina

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Purpose. Both photodamage and aberrant visual cycle contribute to disease progress of many retinal degenerative disorders, whereas the signaling pathways causing photoreceptor death remain unclear. Here we investigated the effects of intense photo-stress on (1) necrosome activation in wild-type and RPE65-null mice, (2) interaction of p62/Sequestosome-1 with the necrosome proteins, and (3) the effects of rapamycin on photodamage-induced necrosome activation and retinal degeneration in wild-type mice.

Methods. Dark-adapted *rd*12 mice and 129S2/Sv mice with or without rapamycin treatment were exposed to 15,000 lux light for different times. Expression levels and subcellular localization of proteins were determined through immunoblot and immunohistochemical analyses. Cone sheaths were stained with peanut agglutinin. Correlation between photoreceptor degeneration and receptor-interacting protein kinase-1 (RIPK1) expression was assessed with Spearman's correlation analysis. Protein-protein interaction was analyzed by immunoprecipitation.

Results. Intense light caused rod and cone degeneration accompanied by a significant increase in RIPK1-RIPK3 expressions, mixed lineage kinase domain-like protein phosphorylation, damage-associated molecular patterns protein release, and inflammatory responses in wild-type mouse retina. The same intense light did not induce the necrosome activation in *rd*12 retina, but it did in *rd*12 mice that received 9-*cis*-retinal supply. RIPK1 expression levels are positively correlated with the degrees of rod and cone degeneration. Photodamage upregulated expression and interaction of the p62 autophagosome cargo protein with the necrosome proteins, whereas rapamycin treatment attenuated the light-induced necrosome activation and photoreceptor degeneration.

Conclusions. Necrosome activation contributed to photodamage-induced rod and cone degeneration. The visual cycle and autophagy are the important therapeutic targets to alleviate light-induced retinal necroptosis.

Keywords: retinal photodamage, RPE65, necroptosis, p62, autophagy, receptor-interacting protein kinase (RIPK), mixed lineage kinase domain-like protein (MLKL)

Photoreceptor degeneration is a common cause of vision loss in patients with age-related macular degeneration (AMD), Stargardt disease (STGD), and other retinal dystrophies. Extensive studies showed that aberrant visual cycle plays a critical pathogenic role in the development or progress of these macular or retinal degenerative diseases. For example, mutations in the visual cycle genes for RPE65 isomerase, lecithin:retinol acyltransferase, or interphotoreceptor retinoid-binding protein have been linked to Leber congenital amaurosis and retinitis pigmentosa.[1–4] Excessive early accumulation of the visual cycle–derived bisretinoids are the main pathogenic factors for the development of STGD (a juvenile form of macular degeneration) caused by mutations in the retinal-specific ATP-binding cassette transporter A4.[5,6] Although a small amount of the bisretinoids may not cause a significant photoreceptor damage in the young healthy population, the visual cycle byproducts gradually accumulate with aging as the major components of the retinal lipofuscin, an important pathologic hallmark implicated in the development and progression of geographic atrophy of AMD.[7,8] Clinical and epidemiologic studies suggest that intense light exposure to the retina is a risk cofactor for development or progression of AMD and STGD.[9,10] Light also promoted photoreceptor death in the *rd*10 mice caused by a mutation in *Phosphodiesterase-6b*.[11] Both the retinal susceptibility to light-induced degeneration and the accumulation rates of the visual cycle–derived bisretinoids are positively correlated with the rates of the visual cycle in mice.[12–15]

In an early study, Noell et al.[16] found that vitamin A deficiency protected the retina against light-induced damage in rats. Recent studies have identified rhodopsin as the initiator of rod degeneration induced by light damage.[17,18] Deficiency in rhodopsin kinase that deactivates photoactivated rhodopsin resulted in a significant increase in susceptibility of rod degeneration induced by light.[17] Conversely, *Rpe65*$^{-/-}$ mice lacking 11-*cis* retinal chromophore that functions as a molecular switch for photoactivating rhodopsin showed a significant protection of photoreceptors against light damage.[18] The contents of rhodopsin per rod outer segment length are positively correlated with the retinal susceptibility to light-induced degeneration.[19]

RPE65 isomerase that catalyzes the rate-limiting step of the visual cycle necessary for recycling the 11-*cis* retinal chromophore has been identified as a genetic modifier of the light-induced retinal degeneration in mice. Its higher expression levels or activities accelerated the regeneration rates of rhodopsin and are associated with an increase in both retinal susceptibility to light-induced degeneration and the accumulation rates of the visual cycle-derived cytotoxic bisretinoids.[12–15] Conversely, pharmacological reduction of RPE65 function protected retinal photoreceptors from light-induced degeneration.[20–22]

Intense light exposure has been shown to cause oxidative stress in the retina by producing singlet oxygen (one of the reactive oxygen species) from bisretinoids and by generating superoxide through activation of the neural nitric-oxide synthase.[23–25] Retinal light damage in rodents is therefore an important animal model for retinal degeneration induced by photo-oxidative stress and is used as the model to study the pathogenic mechanisms of photoreceptor death in STGD and AMD. Excessive formation of the cytotoxic retinoids such as all-*trans* retinal and A2E is a common pathogenic factor involved in the mechanism of retinal photodamage, STGD and AMD. Because light-induced retinal degeneration is an acute pathology, it can synchronize the biochemical reactions, some of which are involved in the signaling pathways of photoreceptor death in STGD and AMD. Light-induced retinal degeneration is also often used to test the efficacy of therapeutic interventions in protection of the retina against photo-oxidative damage.

However, the signaling pathways leading to photoreceptor death in photodamaged retina remain controversial. In some studies, the caspase-dependent apoptosis has been suggested to result in photoreceptor loss in light damaged retinas,[26,27] while other studies showed that light damage caused retinal degeneration in a caspase-independent mechanism.[28,29] Expression levels of several key pro-apoptotic (*Bad*, *Bax*, *Casp3*) and anti-apoptotic (*Bcl2*, *Bcl2l*) genes were similar in wild-type (WT) and *Rpe65*$^{-/-}$ mouse retinas exposed to intense light.[18] These data suggest that cell death pathways other than the caspase 3-dependent apoptosis are involved in the light-induced WT retinal degeneration and the protection of the *Rpe65*$^{-/-}$ retina against light damage.[18] More recent studies suggest that multiple cell death mechanisms participate in photo-oxidative stress–induced photoreceptor death.[30,31]

Identifying an important cell death pathway or the relationships between different cell death mechanisms in photodamaged retinas may provide useful information for the development of therapeutic strategy alleviating vision loss in patients with photoreceptor degenerative disease associated with aberrant visual cycle. In this study, we first investigated activation of the necroptotic pathway in the retinas of WT and *Rpe65*^{rd12} (*rd*12) mice exposed to intense light. We then analyzed expression and interaction of the p62 autophagosome cargo protein with the necrosome proteins, and the effects of rapamycin on the necrosome activation and degeneration of rod and cone photoreceptors in WT mice exposed to intense light. We found that intense photo-stress activated the necrosomes in WT, but not RPE65-null, mouse retinas. Our results also suggest that in addition to necroptosis, autophagy impairment also contributed to the light-induced necrosome activation and degeneration of rods and cones in WT retina.

Methods

Animals

WT 129S2/Sv strain (Charles River Laboratories, Wilmington, MA, USA) and *Rpe65*^{rd12} (*rd*12) (Jackson Laboratories, Bar Harbor, ME, USA) mice were maintained under controlled conditions of 21° to 25°C and a 12-hour light (~30 lux)/dark cycle with free access to standard chow diet and water. The *rd*12 mice have been backcrossed to 129S2/Sv strain for three generations. All experiments on animals were performed on both sexes of mice in accordance with the Association for Research of Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research and the protocols approved by the Institutional Animal Care and Use Committee for LSU Health Sciences Center.

Light Exposure and Chemical Treatment

After dilation of the pupils with a drop of 1% tropicamide under dim red light (Kodak Wratten 1A; Kodak, Rochester, NY, USA), dark-adapted WT and *rd*12 mice were exposed to 15,000 lux of white fluorescent light for different time (0.5 ~ 3 hours). The mice were then kept in darkness for another one or two days. We found that light exposure for two hours caused retinal degeneration in WT mice. Except where noted, we exposed WT mice to the intense light for two hours in most experiments. For rapamycin (T1537; TargetMol, Wellesley Hills, MA, USA) treatment, WT mice were injected intraperitoneally with 1.25 mg of rapamycin in dimethyl sulfoxide (DMSO) or DMSO per kilogram body weight once daily for two weeks, starting at postnatal day 14. For 9-*cis*-retinal (9*c*RAL) treatment, *rd*12 mice were injected intraperitoneally with 2.5 mg 9*c*RAL (dissolved in 10% ethanol and 10% bovine serum albumin in saline) per mouse and were kept in darkness for 12 hours before being exposed to intense light for two hours.

Immunoblot Analysis

Protein concentrations of the retinal tissues homogenized in the radioimmunoprecipitation assay buffer containing phenylmethylsulfonyl fluoride and phosphatase inhibitor (Bio-Rad, Hercules, CA, USA) were determined using a bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Proteins (10-30 µg/sample) were denatured in the Laemmli buffer for 10 minutes at 70°C (or room temperature for opsins), separated in a polyacrylamide gel, and transferred to an Immobilon-P membrane (Millipore, Burlington, MA, USA) by electrophoresis. The membrane was incubated in blocking buffer, primary antibody, and the horseradish peroxidase–conjugated secondary antibody

against mouse or rabbit IgG (7076 and 7074; Cell Signaling Technology, Danvers, MA, USA). Immunoblots were visualized with the ECL Prime Western blotting detection reagent and ImageQuant LAS 4000. The primary antibodies used are for RIPK1 (610458; BD Bioscience, San Jose, CA, USA); MLKL (SAB2103620), M-opsin (AB5405), Tubulin (T2200) (Millipore); RIPK3 (95702), phospho-MLKL (pMLKL, 37333) (Cell Signaling Technology); p62 (sc-48402), S-opsin (sc-14363) (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA), or rhodopsin (ab5417; Abcam, Cambridge, MA, USA).

Immunohistochemistry and Peanut Agglutinin (PNA) Staining

Retinal cryosections were prepared from the dorsal-ventral midline of mouse. Briefly, enucleated mouse eyeballs were fixed overnight with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). After removing cornea and lens, eyecups were immersed in 15% sucrose in 0.1 M PB for two hours, in 30% sucrose in 0.1 M PB for two hours, and then in a 1:1 mixture of 30% sucrose and Optimal Cutting Temperature medium (Sakura Finetek USA, Inc., Torrance, CA, USA) for two hours at 4°C. After embedding eyecups in Optimal Cutting Temperature medium, 20-µm–thick sections were cut on a Shandon Cryotome SME cryostat (Thermo Fisher Scientific, Waltham, MA, USA). The sections were immunostained with the primary antibodies listed in the method of immunoblot analysis and the Alexa Fluor 488- or Alexa Fluor 555-conjugated goat anti-rabbit or anti-mouse IgG secondary antibodies (A11034 and A21424; Thermo Fisher Scientific, Inc.) as described previously.[32] Nuclei were counterstained with 4′-6′-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, Inc. St. Louis, MO, USA). For PNA staining, retinal sections were incubated with 50 µg/mL fluorescein-tagged PNA (Vector Laboratories, Inc., Burlingame, CA, USA) for one hour at room temperature. After mounting slides with Fluoromount-G (SouthernBiotech, Birmingham, AL, USA), images were captured with a Zeiss LSM710 Meta confocal microscope with an objective lens at magnification ×20 or oil-immersion lens at magnification × 40. PNA-positive cone numbers and Rho-positive outer segment (OS) length were determined after full scanning using an Olympus BX61VS microscope (Olympus, Tokyo, Japan) equipped with VS-ASW FL software.

Immunoprecipitation (IP)

Retinas were lysed in a 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, and protease inhibitors. The retinal lysates adjusted to 1 mg/mL protein were preincubated with mouse IgG (Cell Signaling Technology) or a mouse monoclonal antibody against RIPK1 or p62 (Santa Cruz Biotechnology). After gentle rocking overnight at 4°C, Dynabeads Protein G (Thermo Fisher Scientific) was added into the lysates-antibody mixture, and incubated for four hours at 4°C. Tubes containing the mixture were placed on the magnet for one minute, and the supernatants were removed. After being washed three times with washing buffer, the immunoprecipitates bound to the beads were mixed with elution buffer and the NuPAGE LDS Sample Buffer containing the NuPAGE Reducing Agent (Thermo Fisher Scientific) and heated for 10 minutes at 70°C. After being spun in a centrifuge, the supernatants were subjected to immunoblot analysis.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was performed according to the manufacture′s protocols on a SpectraMax 190 Microplate Reader (Molecular Devices, San Jose, CA, USA). We used commercial ELISA kits for high mobility group box-1 (HMGB1) (EKF57808; Biomatik, Wilmington, DE, USA), tumor necrosis factor-α (TNF- α) (MTA00B; R&D Systems, Inc., Minneapolis, MN, USA) and interleukin-1 α (IL-1α) (ab199076; Abcam.Com, Boston, MA, USA) to measure the concentrations of these proteins in the vitreous humor, interphotoreceptor matrix, or retinal homogenates of mice.

Statistical Analysis

All data were presented as mean ± standard deviation (SD) of three or more independent experiments. Statistical analysis of data was performed using Graphpad Prism Software Version 6.0 (Graphpad Software, La Jolla, CA, USA). The significance of difference was determined by one-way analysis of variance with the post hoc Dunnett's test. The significance threshold was set at 0.05 for all statistical tests. Asterisks in all figures denote significant differences between control and test groups (*$P < 0.05$, **$P \leq 0.01$, ***$P < 0.006$). Error bars show SD ($n \geq 3$).

Results

RIPK1 in WT, but not rd12, Mouse Retina was Upregulated by Photodamage

RIPK1 is a serine/threonine protein kinase containing a death domain that interacts with the TNF receptor-associated death domain protein. Recent studies showed that RIPK1 is involved in the signaling pathways linked to photoreceptor death in various models of retinal degeneration.[33–35] To know whether RIPK1 is involved in photodamage-induced photoreceptor degeneration, we analyzed expression of opsins and RIPK1 in the retinas of WT mice exposed to intense light for different times. Rhodopsin immunohistochemistry and PNA staining of the cone sheaths showed that the lengths of the rod and cone OS, as well as the thickness of the outer nuclear layers, were reduced as the light-exposing time increased (Fig. 1A). In contrast, expression levels of RIPK1 in the retina were upregulated as the light exposing time increased (Fig. 1B). Spearman's correlation analysis showed that expression levels of RIPK1 are negatively correlated with both the PNA-positive cone OS number ($R^2 = 0.6952$, $P < 0.0001$) and the rhodopsin-positive rod OS length ($R^2 = 0.7229$, $P < 0.0001$) in a photodamage-dependent fashion (Figs. 1C, 1D).

To confirm whether cones are degenerated in photodamaged retinas, we analyzed expression levels of M- and S-opsins in control and light-exposed mouse retinas. Immunohistochemical and immunoblot analyses revealed that both M- and S-cones are significantly reduced in mice exposed to 15,000 lux light for two hours (Figs. 1E, 1F).

A previous study demonstrated that the *Rpe65*$^{-/-}$ mouse retina is protected from light-induced degeneration.[18] To know whether RPE65 is required for the upregulation of RIPK1 in photodamaged retinas, we exposed the intense light to WT and *rd*12 mice for zero or two hours. Immunoblot analysis showed that intense light induced a dramatic increase of RIPK1 expression in the WT retinas, but not in *rd*12 retinas (Fig. 1G). This result suggests that

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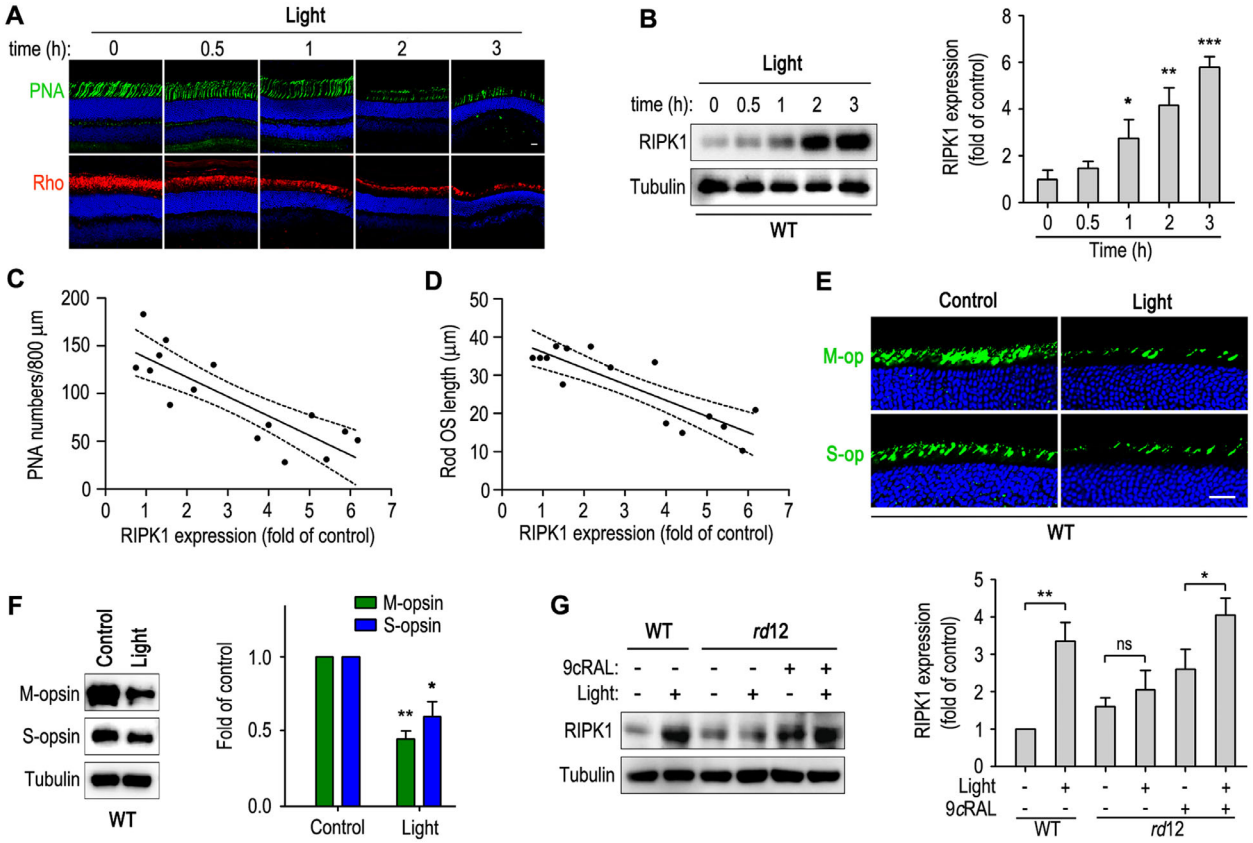

Figure 1. Positive correlation between light-induced photoreceptor degeneration and RIPK1 expression in WT mouse retina. (**A**) Cone sheathes and rod outer segments in the superior retinas of mice exposed to 15,000 lux light for the indicated time were stained with PNA or rhodopsin antibody. Cell nuclei were stained with DAPI. *Scare bar:* 20 μm. (**B**) Immunoblot and its quantitative (right panel histogram) analysis of RIPK1 in the retinas exposed to the intense light for the indicated time. Intensities of the RIPK1 immunoblot bands were normalized to the bands of tubulin. (**C, D**) Spearman's correlation analysis showing negative correlation of the RIPK1 expression levels with the PNA-positive cone numbers (**C**) and with the rod OS lengths (**D**). (**E, F**) Immunohistochemistry and immunoblot analysis of M-opsin (M-op) and S-opsin (S-op) in the retinas exposed to the intense light for 0 (control) or two hours (light). Histogram on the right panel in **F** shows the relative immunoblot intensities of cone opsins normalized to the tubulin immunoblots. (**G**) Immunoblot and its quantitative (histogram) analysis of RIPK1 in the retinas of WT mice and *rd*12 mice treated with 9-*cis*-retinal or saline. Mice were exposed to intense light for zero or two hours. *Asterisks* in all figures denote significant differences between control and test groups (*$P < 0.05$, **$P \leq 0.01$, ***$P < 0.006$). *Error bars*: SD ($n \geq 3$).

the light-sensitive 11-*cis*-retinal chromophore provided by the RPE65-dependent visual cycle is needed to upregulate RIPK1 in the light exposed retinas. To confirm this possibility, we injected 9-*cis*-retinal, a functional isochromophore,[36] into *rd*12 mice before exposing the mice to the same intensity light for zero or two hours. Immunoblot analysis revealed that the intense light exposure resulted in a significant increase of RIPK1 in the *rd*12 mice received 9-*cis*-retinal treatment (Fig. 1G).

Light-Induced Activation of the Necrosomes in WT, but not rd12, Mouse Retina

Previous studies suggest that non-apoptotic signals contribute to retinal degeneration induced by photodamage and aberrant visual cycle.[28,33,37,38] To know whether necroptosis is involved in light-induced retinal degeneration, we analyzed activation of the necrosomes in photodamaged retinas. Immunoblot analysis showed that RIPK3 expression and pMLKL were markedly increased in the WT retinas exposed to 15,000 lux light for two hours (Fig. 2A). Immunohistochemistry revealed that both RIPK1 and RIPK3 are dramatically upregulated in the photoreceptors and colocalized to the inner segments of photoreceptors in photodamaged retinas (Fig. 2B). Consistent with this observation, immunoprecipitation of RIPK1 showed enhanced interaction of RIPK1 with both RIPK3 and pMLKL in the retinas exposed to intense light (Fig. 2C).

We next investigated whether the necrosome is activated in the *rd*12 mouse retinas exposed to 15,000 lux light for two hours. As shown in Figure 2D, neither RIPK3 upregulation nor phosphorylation of MLKL were significantly induced in the light exposed *rd*12 mouse retinas. This observation and the result shown in Figure 1G suggest that intense light activated the necrosomes in WT, but not in *rd*12, mouse retina through a pathway involving the light-sensitive visual pigments. In all experiments thereafter we used only WT mice.

The damage-associated molecular patterns (DAMPs) are the danger signals released by stressed cells undergoing necrosis that promote the inflammatory responses. To confirm whether necrosis or necroptosis was induced in the photodamaged WT mouse retina, we analyzed release

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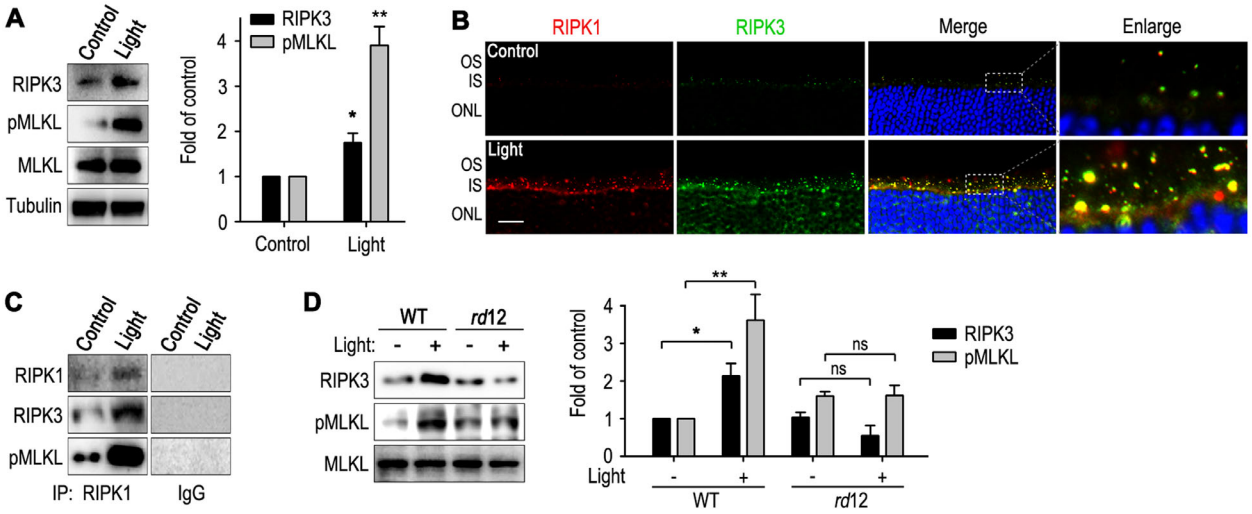

Figure 2. Intense light stress activated the necrosomes in WT but not *rd*12 retinas. (**A**) Immunoblot analysis of RIPK3, phospho-MLKL (pMLKL), and total MLKL in control and light exposed WT retinas. Histogram in the right panel shows the relative intensities of the RIPK3 and pMLKL immunoblots normalized to the tubulin bands. (**B**) Immunohistochemical analysis of RIPK1 and RIPK3 in control and light-exposed WT retinas. (**C**) Immunoprecipitation (IP) of RIPK1 in the WT retinas. The co-IP proteins were detected with antibodies against RIPK3, pMLKL, or RIPK1. (**D**) Immunoblot analysis of RIPK3, pMLKL, and MLKL in the retinas of WT and *rd*12 mice exposed or not exposed to 15,000 lux light. Histogram in the right panel shows the relative intensities of the RIPK3 and pMLKL immunoblot bands normalized to the tubulin bands.

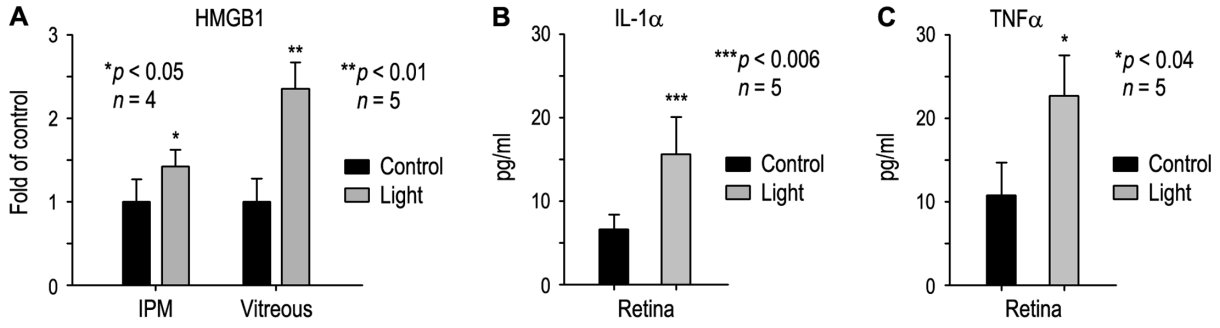

Figure 3. Elevation of danger signal protein and inflammatory cytokines in photodamaged retina. HMGB1 in the vitreous and the interphotoreceptor matrix (IPM) (**A**), IL-1α and TNF-α in the retinal homogenates (**B, C**) of control and intense light exposed WT mice were detected by ELISA.

of HMGB1, a key DAMP protein promoting retinal inflammation response,[39] in the control and light-exposed mice. ELISA data showed that the amounts of HMGB1 released into the vitreous and the interphotoreceptor matrix were significantly increased in photodamaged mice, as compared to control mice (Fig. 3A). In addition, TNFα and IL-1α that induce cell death and/or inflammation were increased approximately twofold in the photodamaged retinas, as compared to control retinas (Figs. 3B, 3C).

Light-Induced Upregulation p62 That Interacts With the Necrosome Proteins

The ubiquitin-binding protein p62/Sequestosome-1 has been known to interact with RIPK1.[40] We investigated whether photodamage alters the abundances of p62, RIPK1 and other necrosome proteins in the retinas. Both immunoblot and immunohistochemical analyses showed that p62 was increased in the photodamaged retinas, as compared to control retinas (Figs. 4A–C). Double-immunostaining showed that the population of p62 colocalized with RIPK3 and phospho-MLKL were increased in photodamaged retinas (Figs. 4B, 4C). Consistent with this observation, immunoprecipitation of p62 revealed its strong interaction with the RIPK1-RIPK3 necrosome proteins (Fig. 4D).

Rapamycin Mitigated Necrosome Activation and Photoreceptor Death

Because p62 plays an important role in the autophagic degeneration of proteins,[41] our data (Fig. 4) may have linked the necrosomes to defective autophagy in the photodamaged retina. We therefore tested the effects of rapamycin that induces autophagy on the light-induce necrosome activation and photoreceptor degeneration in WT mice. We treated mice with rapamycin before intense light exposure. Immunoblot analysis showed that MLKL activation and the

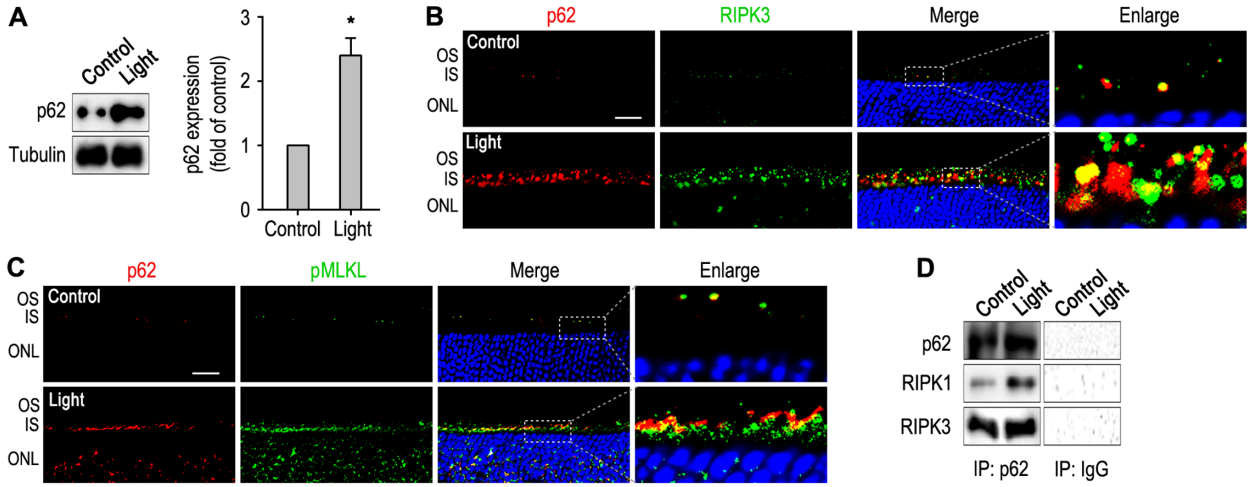

Figure 4. Interaction of p62 with RIPK3 and pMLKL in the retina. (**A**) Immunoblot and its quantitative (histogram) analysis show increase of p62 in photodamaged retina. (**B, C**) Immunohistochemistry shows increase and colocalization of p62 with RIPK3 (**B**) and pMLKL (**C**) in the photoreceptor IS of photodamaged mice. (**D**) Interaction of p62 with RIPK1 and RIPK3 was analyzed by immunoprecipitation assay.

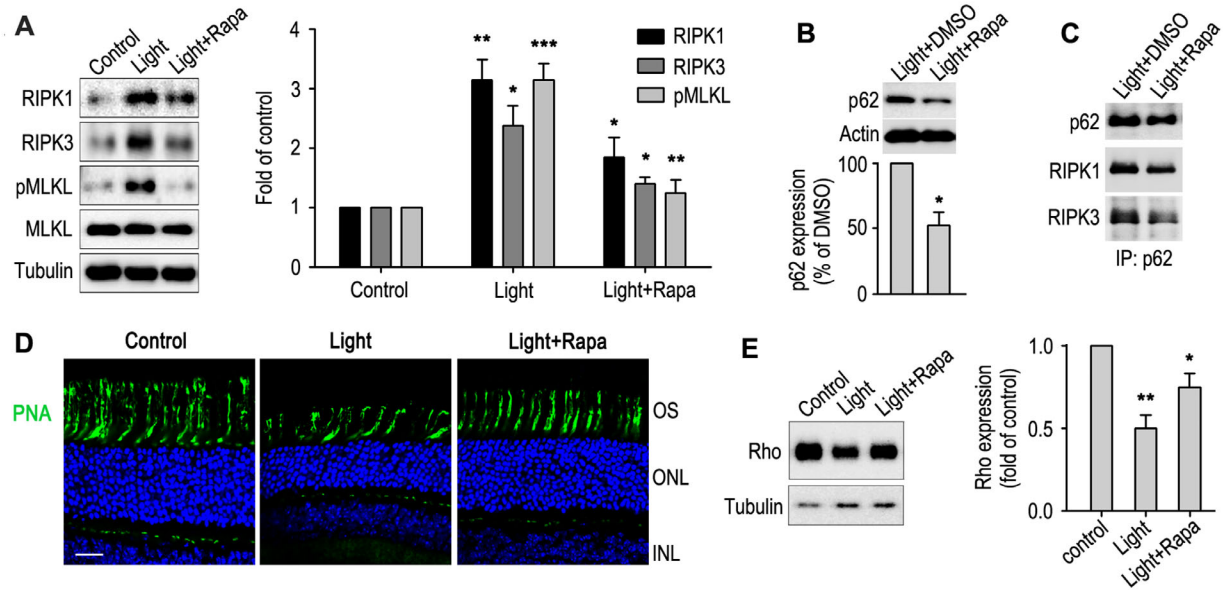

Figure 5. Rapamycin alleviated light-induced necrosome activation and retinal degeneration. (**A**) Immunoblot analysis of light-induced necrosome activation in the retinas of WT mice treated with rapamycin (Light + Rapa) or vehicle. Histogram is showing the relative immunoblot intensities of RIPK1, RIPK3 and pMLKL normalized to the tubulin immunoblots. (**B**) Immunoblot analysis of p62 in the retinas of DMSO- or rapamycin-treated mice exposed to intense light for two hours. (**C**) Interaction between p62 and the necrosome proteins in the retinas of mice with the indicated treatments was analyzed by immunoprecipitation assay. (**D**) PNA-staining of cone sheathes in the superior retinas of mice with or without rapamycin treatment. The mice were exposed or not exposed to 15,000 lux light for two hours. Cell nuclei were stained with DAPI. (**E**) Immunoblot analysis of rhodopsin in the retinas of mice with the same treatments as in (D).

expression levels of RIPK1 and RIPK3 in the rapamycin-treated mouse retinas were clearly lower than those in the DMSO-treated control mice under the same photodamage conditions (Fig. 5A). Expression levels of p62, as well as RIPK1 and RIPK3 proteins bound to p62, were also significantly reduced in the rapamycin-treated mice, as compared to DMSO-treated mice (Figs. 5B, 5C), suggesting that autophagy promoted degradation of the necrosome proteins. Fluorescence images of PNA-staining showed an increase in the numbers and lengths of the cone OS in the rapamycin-treated mice (Fig. 5D). The abundance of rhodopsin in rapamycin-treated mice was also greater than that in DMSO-treated mice under the same photodamage conditions (Fig. 5E).

Discussion

Identification of the signaling pathways of photoreceptor death caused by photo-oxidative stress may provide important information of therapeutic targets for some forms of macular and retinal degenerative diseases. In the present study, we provided evidence that necrosome activation and

impaired autophagy contributed to the light-induced degeneration of rods and cones in WT mice. Under the same photodamage conditions, RPE65 deficiency showed significant resistance against light-induced necrosome activation in the retina, whereas exogenous 9-*cis*-retinal restored the susceptibility to light-induced activation of the necrosome in RPE65-null mice.

The photon energy, which is necessary for initiating the phototransduction, causes photo-oxidative stress in the retina. In previous studies, we established a photodamage protocol that causes retinal degeneration in the 129S2 mouse strain.[14,15] Necroptosis has never been analyzed using the protocol. In the present study, we first investigated the relationships between retinal degeneration and RIPK1 expression in mice exposed to 15,000 lux white light for different times. We observed that the numbers of rods and cones were progressively decreased, whereas the expression levels of RIPK1 were gradually increased in a light exposure time–dependent manner. The Spearman correlation analysis further quantitatively validated a strong negative correlation between RIPK1 expression levels and cone numbers and rod OS lengths (Fig. 1). These results suggest that RIPK1 contributed to the degeneration of rods and cones in photodamaged retina.

The first event in initiating the phototransduction is absorption of light energy by the 11-*cis*-retinal chromophore bound with rod and cone opsins. To know if the chromophore is required to cause the light-induced upregulation of RIPK1, we exposed RPE65-deficient mice to 15,000 lux light for two hours. Unlike WT mice, *rd*12 mice did not display a significant increase in RIPK1 expression after exposing to intense light. However, RIPK1 expression was markedly upregulated in the retina of *rd*12 mice that have received 9-*cis*-retinal (9*c*RAL) supply before the same intense light exposure (Fig. 1G). These results suggest that the chromophore photoisomerization–mediated phototransduction contributed to the RIPK1 upregulation in the retina. We also observed that 9cRAL-treatment itself resulted in a remarkable increase of RIPK1 in the *rd*12 retina before intense light exposure, possibly because of the cytotoxic effect of 9*c*RAL that possesses a highly reactive aldehyde group. In addition, consistent with the published data,[35] the expression level of RIPK1 in the *rd*12 retina was higher than that in WT retina (Fig. 1G). In mouse models of Leber congenital amaurosis caused by shortage of 11-*cis*-retinal supply, opsins are misfolded,[42–44] which might induce upregulation of RIPK1 in the chromophore-deficient retina.

Because RIPK1 is a hinge molecule that links both apoptosis and necroptosis,[35 45] we analyzed activation of the canonical necroptotic signaling in the retina and photoreceptors. Immunoblot and immunohistochemical analyses revealed that two-hour intense light exposure resulted in an obvious increase in MLKL phosphorylation and expression of both RIPK1 and RIPK3 in the retina and photoreceptor inner segments (IS) (Figs. 2A, 2B). It is generally known that RIPK1 recruits RIPK3 to form the necrosomes and then activate MLKL via its phosphorylation to cause necroptotic cell death. Elevation of the phospho-MLKL and RIPK1-RIPK3 punctate signals in the IS (Figs. 2B, 2C) reveals activation of the necroptotic pathway in the photoreceptors of WT mice exposed to intense light.

Consistent with the result of *rd*12 mice shown in Figure 1G, two-hour intense light exposure did not induce a significant RIPK3 upregulation and MLKL phosphorylation in the *rd*12 retina (Fig. 2D). The results shown in Figures 1 and 2 together suggest that intense light promoted necrosome formation and activation of the necroptotic pathway in the WT retina, but not in the RPE65-null retina.

A previous study on dsRNA-mediated retinal degeneration showed that activation of the necroptotic pathway leads to the DAMP-mediated inflammatory responses.[34] Consistent with this study, we observed that retinal photodamage promoted release of HMGB1, a key DAMP protein, into the vitreous humor and interphotoreceptor matrix (Fig. 3A). HMGB1 has been recognized as a molecular marker of necroptosis. It is located in the cell nuclei under physiological condition and is released by necrotic, but not apoptotic, cells.[46] HMGB1 is also a potent mediator of inflammation.[46] We observe a clear increase of IL-1α and TNFα in the photodamaged retinas (Figs. 3B, 3C). Increase in these pro-inflammatory cytokines could be associated in part with HMGB1-mediated inflammation. Because necroptosis often causes inflammation response, our data suggest that necroptosis is involved in light-induced acute retinal degeneration. However, our data do not rule out the possibility that apoptosis is also involved the light-induced retinal denegation.

It has been known that cell death occurs through necroptosis when ubiquitin-binding protein p62 associates with RIPK1 that recruits the necrosomes.[47] We analyzed expression, colocalization and interaction of p62 with the necrosome proteins in normal and light damaged retinas. Our results showed an obvious increase in the abundance of p62 protein in the intense light exposed retina; the increased p62 was colocalized with RIPK3 and phospho-MLKL in the photoreceptor IS (Fig. 4). Immunoprecipitation of p62 further confirmed its interaction with the necrosome proteins and activated MLKL. p62 directly binds with both ubiquitinated proteins and Atg8/LC3 in autophagosomes, thereby facilitating autophagic degradation of the ubiquitinate protein aggregates.[41] In agreement with this, our results (Figs. 5B, 5C) suggest that interaction of p62 with RIPK1 and RIPK3 may link the necrosome protein complexes, including the MLKL oligomers to the autophagosomes for lysosomal degradation, which in turn protect photoreceptors from light-induced degeneration. Because p62 itself also undergoes autophagic degradation,[41] upregulating transcription of p62 may be important to mitigate light-induced retinal necroptosis through enhancing autophagic degradation of the necrosomes.

Autophagy not only serves to protect retinal cell survival and function, but also contributes to cell death.[48–51] To know whether autophagy has protective effects against light-induced necrosome activation and photoreceptor death, we treated mice with rapamycin before performing retinal photodamage. As an inhibitor of mTOR, rapamycin has been shown to preserve photoreceptor survival and function in several retinal degeneration models, including *Rdh8*$^{-/-}$/*Abca4*$^{-/-}$ double knockout (excessive all-*trans*-retinal accumulation), mitochondrial oxidative phosphorylation-deficient, NaIO3 (a strong oxidant)-administrated and moderately photo-stressed mice.[52–54] We observed that rapamycin treatment before intense light exposure partially prevented the photodamage-induced necrosome activation, as well as rod and cone degeneration (Fig. 5). These data suggest that pharmacological induction of autophagy in the retina provides anti-necroptotic effects in photo-oxidative stress-induced retinal degeneration, possibly by promoting the degradation of the necrosome protein complexes via the autophagy-lysosome pathway.

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