Resveratrol Prevents Light-Induced Retinal Degeneration via Suppressing Activator Protein-1 Activation

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Light damage to the retina accelerates retinal degeneration in human diseases and rodent models. Recently, the polyphenolic phytoalexin resveratrol has been shown to exert various bioactivities in addition to its classical antioxidant property. In the present study, we investigated the effect of resveratrol on light-induced retinal degeneration together with its underlying molecular mechanisms. BALB/c mice with light exposure (5000-lux white light for 3 hours) were orally pretreated with resveratrol at a dose of 50 mg/kg for 5 days. Retinal damage was evaluated by TdT-mediated dUTP nick-end labeling, outer nuclear layer morphometry, and electroretinography. Administration of resveratrol to mice with light exposure led to a significant suppression of light-induced pathological parameters, including TdT-mediated dUTP nick-end labeling-positive retinal cells, outer nuclear layer thinning, and electroretinography changes. To clarify the underlying molecular mechanisms, the nuclear translocation of activator protein-1 subunit c-fos was evaluated by enzyme-linked immunosorbent assay, and the retinal activity of sirtuin 1 was measured by deacetylase fluorometric assay. Retinal activator protein-1 activation, up-regulated following light exposure, was significantly reduced by application of resveratrol. In parallel, retinal sirtuin 1 activity, reduced in animals with light damage, was significantly augmented by resveratrol treatment. Our data suggest the potential use of resveratrol as a therapeutic agent to prevent retinal degeneration related to light damage.

Resveratrol (3,5,4′-trihydroxystilbene), one of dietary polyphenols found in red wine and grape skin, is known to have an antioxidant effect for reducing cardiovascular events. This mechanism may contribute to the “French paradox,” which refers to a phenomenon that the French suffer a relatively low incidence of cardiovascular diseases, despite taking high-caloric and high-fat diet. Moreover, resveratrol has been reported to exhibit various bioactivities including anti-tumorigenic, anti-angiogenic, and neuroprotective effects. Recently, sirtuin 1, a known regulator of aging, has proven to be activated by resveratrol. Indeed, administration of resveratrol extended life spans of yeast, Caenorhabditis elegans, Drosophila melanogaster, short-lived fish Nothobranchius furzeri and mice fed with a high-fat diet.

We have shown that resveratrol is anti-inflammatory in the eye. In the murine model of endotoxin-induced retinal inflammation, resveratrol functioned dually as a SIRT1 activator and an antioxidant agent, both of which led to the deactivation of a nuclear factor-κB, the major redox-sensitive transcription factor that promotes the expression of various inflammation-related genes. Also in recent ex vivo data, resveratrol exerted vasodilative effects on the retina through the activation of nitric oxide synthase and potassium channels. Although these reports on the eye focused mainly on vascular changes, it remains to be determined if resveratrol is protective of retinal neurons.

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Retinal neurodegenerative diseases such as retinitis pigmentosa and age-related macular degeneration are significant causes of severe vision loss and blindness. Increasing evidence has suggested that light damage to the retina accelerates human retinal degeneration, which leads to the concept of photaging of the eye. Apoptotic cell death of photoreceptors is an essential feature shared by both human diseases and rodent models of light-induced retinal degeneration. Clinically, no therapeutic strategy has been established so far that hampers the development of retinal degeneration or restores the visual function. Therefore, investigation into photoreceptor apoptosis may provide a clue to prevent blindness due to retinal degeneration. Recently, in addition to the classically known pathway involving caspases, various chemical mediators are shown to play key roles in apoptotic cell death. These apoptosis-related molecules include apoptosis inducing factor, poly-(ADP-ribose) polymerase-1, endonuclease G and activator protein (AP)-1. Light-induced retinal degeneration is an established animal model for studying visual cell death by apoptosis. Herein, we report the preventive effect of resveratrol on the pathogenesis of light-induced retinal degeneration together with underlying molecular mechanisms.

Materials and Methods

Animals

BALB/c male mice (Clea, Tokyo, Japan) at the age of 6 weeks were used in the present study. Animals were housed in plastic cages in a climate-controlled animal facility and kept under dim cyclic light (5 lux, 12 hours on/off) in our institution, except where otherwise indicated. All animal experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Treatment with Resveratrol

Mice were orally administered with vehicle (6.67% dimethyl sulfoxide in PBS) or resveratrol (Sigma-Aldrich, St. Louis, MO) at the dose of 50 mg/kg body weight (BW) by using a gastric intubation daily for 5 days until light exposure. The dose of 50 mg/kg BW is equivalent to that applied to protect against neuronal injury associated with cerebral ischemia-reperfusion in mice. 

Light Exposure

Light exposure experiments were performed as described previously with slight modification. Mice were exposed to 5000 lux of white light for 3 hours from 9:00 AM in a dedicated exposure box having stainless mirrors at the lateral side and floors (Tinker-N, Kyoto, Japan). The box contained a white fluorescent lamp (FHD100ECW; Panasonic, Osaka, Japan) and an air conditioner to maintain the temperature inside as 23°C. Before light exposure, mice were dark adapted for 12 hours. The pupils were dilated with a mixed solution of 0.5% tropicamide and 0.5% phenylephrine (Mydrin-P; Santen, Osaka, Japan) just before light exposure. Immediately after 3-hour exposure to light (at 12:00), the mice were carried under dim cyclic light (5 lux, 12 hours on/off).

TdT-Mediated dUTP Nick-End Labeling

Mice were anesthetized with pentobarbital sodium (70 mg/kg BW) and perfused with 10 ml of PBS 48 hours after the start of light exposure. Subsequently, eyes were enucleated and fixed in 4% paraformaldehyde overnight at 4°C. After fixation, tissues were processed and embedded in an optimal cutting temperature compound (Tissue-Tek; Sakura Finetek, Torrance, CA) for cryosections. Six 10-μm cryosections from the optic nerve were prepared, and TdT-mediated dUTP nick-end labeling (TUNEL) was performed using the ApopTag Red apoptosis detection kit (Chemicon, Temecula, CA) according to the manufacturer’s protocol. Nuclei were stained with 10-μg/ml Hoechst bisbenzimide 33258 (Sigma-Aldrich). Fluorescence images were obtained using Axiol Imager (Carl Zeiss, Oberkochen, Germany), and TUNEL-positive cells were counted in the outer nuclear layer (ONL), composed exclusively of photoreceptor cell bodies.

Measurement of ONL Thickness

Mice were anesthetized with pentobarbital sodium (70 mg/kg BW), perfused with 10 ml of PBS 4 days, 1 week or 2 weeks after the start of light exposure, and the eyes were then enucleated. Paraffin-embedded retinal sections (3 μm) were prepared and stained with hematoxylin and eosin. ONL thickness was measured at each 0.2–0.3 mm point from the optic nerve head to the most peripheral area using ImageJ software (National Institutes of Health, Bethesda, MD).

Electroretinography (ERG)

ERG analysis was performed as previously described, 19–23 4 days, 1 week or 2 weeks after light exposure. Mice were anesthetized with pentobarbital sodium (70 mg/kg BW) and placed on a heating pad that maintained their body temperature at 35–36°C throughout the experiment. The pupils were dilated with a mixed solution of 0.5% tropicamide and 0.5% phenylephrine (Mydrin-P; Santen). The ground electrode was a subcutaneous needle in the tail and the reference electrode was placed subcutaneously between the eyes. The active contact lens electrodes (Mayo, Inazawa, Japan) were placed on the cornea. Recordings were performed with PowerLab system 2/25 (AD Instruments, New South Wales, Australia). Responses were differentially amplified and filtered through a digital bandpass filter ranging from 0.313 to 1000 Hz to yield a- and b-waves. Light pulses of 800 cds/m² and 4 ms duration were delivered via a commercial Ganzfeld stimulator (Ganzfeld System SG-2002; LKC Technologies, Gaithersburg, MD). The amplitude of the
a-wave was measured from the baseline to the trough of the a-wave, and the amplitude of the b-wave was determined from the trough of the a-wave to the peak of the b-wave. The implicit time of the a- and b-waves was measured from the onset of stimuli to the peak of each wave.

**Enzyme-Linked Immunosorbent Assay for c-fos after Nuclear Extraction**

Three hours after light exposure, mice were sacrificed with an overdose of anesthesia and the eyes were immediately enucleated. The retina was carefully isolated and homogenized in 50 μL of hypotonic buffer (10 mmol/L HEPES-KCl, 1 mmol/L β-mercapto-ethanol, 1 mmol/L di-thiothreitol). After incubation on ice for 10 minutes, the homogenate was vortexed for 10 seconds and centrifuged. The supernatant was discarded and the pellet was resuspended in 100 μL lysis buffer in the presence of protease inhibitors, and incubated on ice for 10 minutes. Cellular debris was removed by centrifugation at 15,000 rpm for 15 minutes at 4°C, and 10 μg protein was subjected to enzyme-linked immunosorbent assay for the c-fos subunit of AP-1. Activation of c-fos was determined by measuring the c-fos protein level in the nuclear extracts with the AP-1 c-fos Transcription Factor Assay kit (Active Motif, Carlsbad, CA) according to the manufacturer’s instruction. The tissue sample concentration was calculated from a standard curve and corrected for protein concentration evaluated by the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

**Deacetylase Fluorometric Assay for SIRT1**

Three hours after light exposure, the animals were sacrificed with an overdose of anesthesia and the eyes were immediately enucleated. The retina was carefully isolated and placed into 100 μL of lysis buffer and then sonicated. The lysate was centrifuged at 15,000 rpm for 15 minutes at 4°C. The activity of SIRT1 in the supernatant was determined with SIRT1/Sir2 Deacetylase Fluorometric Assay kits (CycLex, Ina, Japan) according to the manufacturer’s protocols.

**Statistical Analysis**

All results were expressed as mean ± SD. The values were processed for statistical analyses (Mann-Whitney test), and differences were considered statistically significant at \( P < 0.05 \).

**Results**

**Suppression of Light-Induced Apoptotic Cell Death with Resveratrol**

To unravel the effect of resveratrol on light-induced apoptotic cell death of photoreceptors, TUNEL assay was performed (Figure 1). Under dim cyclic light (5 lux, 12 hours on/off), the number of TUNEL-positive cells in ONL was negligible (only a few cells, if any). Light exposure to vehicle-treated mice induced apoptotic cell death in ONL (Figure 1A) with the number of 863.4 ± 201.0 cells/section, which was significantly reduced to 680.5 ± 98.0 cells/section by application with resveratrol.

**Suppression of Light-Induced ONL Thinning with Resveratrol**

To evaluate the effect of resveratrol on light-induced histological damage to the retina, ONL thickness was analyzed at three time points, ie, 4 days (Figure 2, A–E), 1 week (data not shown) and 2 weeks (Figure 2, F–J) after light exposure. Light exposure to vehicle-treated mice substantially induced apoptotic cell death in ONL (Figure 1A) with the number of 863.4 ± 201.0 cells/section, which was significantly reduced to 680.5 ± 98.0 cells/section by application with resveratrol.
Suppression of Light-Induced Retinal Dysfunction with Resveratrol

To investigate the effect of resveratrol on light-induced retinal dysfunction, ERG analysis was performed at three evaluation points of 4 days (Figure 3, A, C, and E), 1 week (data not shown), and 2 weeks (Figure 3, B, D, and F) after light exposure. Light exposure to vehicle-treated mice at 4 days (Figure 2, B and G) altered with resveratrol application (Figure 2, B and G). Accordingly, the protective effect of resveratrol pretreatment on light-induced ONL thinning (Figure 2D, blue in Figure 2E) proved to be narrowly maintained at least until 2 weeks (Figure 2I, blue in Figure 2J), although the light-damaged ONL at 2 weeks (Figure 2H, green in Figure 2J) substantially and progressively became thinner than that at 4 days (Figure 2C, green in Figure 2E) following light exposure.

Figure 2. Suppression of light-induced ONL thinning with resveratrol. Data obtained at four days (A–E) and two weeks (F–J) after light exposure. A–D and F–I. Representative images of H&E staining for retinal sections. E and J. Quantification of ONL thickness (arrowheads in A–D and F–I). Compared to vehicle-treated mice receiving no light exposure (A and F), light exposure to vehicle-treated mice (C and H) led to significant (red versus green in E and J) reduction of ONL thickness, which was significantly (green versus navy in E and J) recovered by application with resveratrol (D and I). Scale bars = 50 μm. n = 6 to 12. *P < 0.05; **P < 0.01.

Figure 3. Suppression of light-induced retinal dysfunction with resveratrol. Data obtained at four days (A, C, and E) and two weeks (B, D, and F) after light exposure. A and B. Representative ERG wave responses. Quantification of the amplitude of a-wave (C and D) and b-wave (E and F), both of which were significantly recovered by application with resveratrol. n = 6 to 12. *P < 0.05; **P < 0.01.
4 days led to significant ($P < 0.01$ for both, Figure 3, A, C, and E) reduction of the amplitude of a-wave (88.4 ± 17.6 μV, Figure 3C) and b-wave (190.2 ± 63.0 μV, Figure 3E) as compared to vehicle-treated mice receiving no light exposure (301.6 ± 76.1 μV and 644.4 ± 234.0 μV for a-wave and b-wave, respectively). Systemic administration of resveratrol to light-exposed mice significantly ($P < 0.01$ for both, Figure 3, A, C, and E) recovered the reduction of the amplitude of a-wave (166.1 ± 39.9 μV, Figure 3C) and b-wave (435.9 ± 157.4 μV, Figure 3E) as compared to vehicle treatment to light-exposed animals (88.4 ± 17.6 μV and 190.2 ± 63.0 μV for a-wave and b-wave, respectively).

Similarly, light exposure to vehicle-treated mice at 2 weeks led to significant ($P < 0.01$ for both, Figure 3, B, D, and F) reduction of the amplitude of a-wave (56.6 ± 28.9 μV, Figure 3D) and b-wave (134.6 ± 175.4 μV, Figure 3F) as compared to vehicle-treated mice receiving no light exposure (366.6 ± 58.1 μV and 590.5 ± 157.6 μV for a-wave and b-wave, respectively). Systemic administration of resveratrol to light-exposed mice significantly ($P < 0.01$ for a-wave and $P < 0.05$ for b-wave, Figure 3, B, D, and F) recovered the reduction of the amplitude of a-wave (122.7 ± 37.5 μV, Figure 3D) and b-wave (342.0 ± 188.7 μV, Figure 3F), as compared to vehicle treatment to light-exposed animals (56.6 ± 28.9 μV and 134.6 ± 175.4 μV for a-wave and b-wave, respectively).

No significant ($P > 0.05$ for each) differences between vehicle or resveratrol were detected in implicit time of a-wave or b-wave at each time point (4 days, 1 week or 2 weeks; data not shown).

**Suppression of Light-Induced AP-1 Activation with Resveratrol**

To clarify pro-apoptotic signal transduction involved in resveratrol-induced suppression of photoreceptor apoptosis (Figure 1) followed by retinal degeneration (Figure 2) and dysfunction (Figure 3), AP-1 activation was examined by measuring the c-fos level in nuclear extracts from the retina (Figure 4). Light exposure to vehicle-treated mice significantly ($P < 0.01$) induced retinal AP-1 activation (16.8 ± 3.2 ng/mg) as compared to vehicle-treated mice receiving no light exposure (6.2 ± 2.8 ng/mg). Systemic application of resveratrol to light-exposed mice significantly ($P < 0.01$) suppressed retinal AP-1 activation (10.2 ± 2.2 ng/mg) as compared to vehicle treatment to light-exposed animals (16.8 ± 3.2 ng/mg). In contrast, administration of resveratrol to mice with no light exposure (6.3 ± 3.6 ng/mg) did not alter ($P > 0.05$) physiological baseline levels of c-fos (6.2 ± 2.8 ng/mg).

**Suppression of Light-Induced SIRT1 Deactivation with Resveratrol**

To reveal a possible role played by SIRT1 in resveratrol-induced suppression of the apoptotic signaling pathway (Figure 4), retinal SIRT1 activity was analyzed by deacetylase fluorometric assay (Figure 5). Light exposure to vehicle-treated mice led to significant ($P < 0.01$) reduction of retinal SIRT1 activity (83.9 ± 9.4% of control) as compared to vehicle-treated mice receiving no light exposure (100 ± 6.7% serving as control). Systemic administration of resveratrol to light-exposed mice significantly ($P < 0.05$) reversed retinal SIRT1 activity (99.4 ± 5.7% of control) as compared to vehicle treatment to light-exposed animals (83.9 ± 9.4% of control). In contrast, administration of resveratrol to mice with no light exposure (99.8 ± 14.3% of control) did not change ($P > 0.05$) physiological baseline levels of SIRT1 (100 ± 6.7%).

**Discussion**

The present study reveals, for the first time to our knowledge, the protective effect of resveratrol on light-induced retinal degeneration together with underlying molecular mechanisms. Resveratrol application to light-exposed mice ameliorated several retinal parameters including apoptotic cell death (Figure 1), anatomical structure (Figure 2), and functional change (Figure 3) induced by light damage. As possible molecular mechanisms, retinal AP-1 activation (Figure 4) and SIRT1 deactivation (Figure 5), both of which resulted from light exposure, were inhibited by pretreatment with resveratrol. Resveratrol is
one of polyphenolic phytoalexins contained abundantly in red wine, grape skin, and peanut skin. Resveratrol has been shown to have various bioactivities including antioxidative, anti-inflammatory, anti-tumorigenic, anti-angiogenic, neuroprotective and vasodilative effects. Notably, it was reported that resveratrol exerts an anti-aging or lifespan-extending action through the activation of SIRT1. In addition to its anti-aging property, SIRT1 has been shown to suppress AP-1 activity, suggesting the validity of the currently shown molecular pathway through which resveratrol protected from light-induced retinal degeneration.

In the present study, resveratrol application led to significant suppression of TUNEL-positive cells in the ONL 2 days after light exposure (Figure 1). The increase in the number of TUNEL-positive cells in the ONL following light exposure is a well-known landmark of photoreceptor apoptosis in light-induced retinal degeneration. The current finding that resveratrol reduced light-induced apoptotic cell death in the retina is supported in part by previous data showing the anti-apoptotic activity of resveratrol in vitro under oxidative stress. To investigate the sequential events following photoreceptor apoptosis, we measured ONL thickness 4 days, 1 week, and 2 weeks after light exposure. The ONL, composed exclusively of photoreceptor cell bodies, is a known target of light-induced retinal degeneration. In accordance with the data on apoptotic cell death (Figure 1), resveratrol pre-treatment to light-exposed mice resulted in significant suppression of ONL thinning (Figure 2). To confirm these histological data (Figures 1 and 2), we analyzed retinal function by using ERG (Figure 3). Light exposure is known to cause damage to retinal function represented by ERG. Light-induced suppression of both a-wave and b-wave in ERG was reversed by application with resveratrol (Figure 3), suggesting that the amelioration of retinal dysfunction is attributed to resveratrol-mediated suppression of ONL injury (Figures 1 and 2). The long-term protection of resveratrol from light-induced retinal damages (Figures 2 and 3) was confirmed to last at least until 2 weeks following light exposure. The present study is the first to show the neuroprotective effect of resveratrol on retinal degeneration.

To further confirm the currently observed inhibitory effect of resveratrol on histological and functional damage to the retina (Figures 1–3), we investigated underlying molecular mechanisms (Figures 4 and 5). Of several important pro-apoptotic pathways, we focused on AP-1, the major transcription factor that regulates the intracellular signals for cell cycle, differentiation, and apoptosis. AP-1 is typically a heterodimer that consists of the c-fos and c-jun subunit proteins. It has been previously shown that c-fos is essential for light-induced apoptosis of photoreceptors. Mice lacking c-fos were resistant to light damage and exhibited a significant decrease in light-induced apoptotic cell death and subsequent ONL thinning as compared to wild-type controls. In our present data (Figure 4), c-fos levels were elevated in the nuclear extracts from the retina of light-exposed mice and significantly reduced by application with resveratrol. This is consistent with a previous report showing the inhibitory effect of resveratrol on c-fos expression in the murine model of skin cancer. Accordingly, the neuroprotective effect of resveratrol on light-induced retinal degeneration and dysfunction (Figures 1–3) is attributable at least in part to the modulation of AP-1 that is causally linked to light-induced apoptosis of photoreceptors.

In addition to its suppressive effect on AP-1 activation, resveratrol is a known activator of the histone deacetylase SIRT1, a key modulator for extending the lifespan of several species including rodents. It has been recently revealed that in murine fibroblasts SIRT1 plays an inhibitory role in the transcriptional activity of AP-1 by targeting (directly binding to) c-jun. In the present study, resveratrol application to light-exposed mice significantly recovered retinal SIRT1 activity, which was decreased due to light damage (Figure 5). The reverse relationship between AP-1 (Figure 4) and SIRT1 (Figure 5) are comparable with and explained by the recent in vitro data showing the involvement of SIRT1 in AP-1 deactivation. Taken together, light exposure caused retinal SIRT1 deactivation (Figure 5) together with AP-1 activation (Figure 4), leading to photoreceptor apoptosis (Figure 1) and subsequent retinal degeneration (Figure 2) and dysfunction (Figure 3), all of which were reversed by the SIRT1 activator resveratrol (Figures 1–5).

Clinically, resveratrol is now under phase-II investigations for cancer and diabetes. Moreover, resveratrol has received orphan-drug designation for MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, stroke-like episodes) syndrome by the U.S. Food and Drug Administration. So far, no major safety concern has been reported. At present, there is no established treatment for retinal neurodegenerative diseases, to which light damage is causally linked, including retinitis pigmentosa and age-related macular degeneration. Our present data provide molecular evidence of the potential validity of resveratrol supplementation as a therapeutic strategy to prevent retinal degeneration related to light damage.

References