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Mitochondria-derived Reactive Oxygen Species Mediate Blue Light–induced Death of Retinal Pigment Epithelial Cells¶

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ABSTRACT

Throughout the lifetime of an individual, light is focused onto the retina. The resulting photooxidative stress can cause acute or chronic retinal damage. The pathogenesis of age-related macular degeneration (AMD), the leading cause of legal blindness in the developed world, involves oxidative stress and death of the retinal pigment epithelium (RPE) followed by death of the overlying photoreceptors. Evidence suggests that damage due to exposure to light plays a role in AMD and other age-related eye diseases. In this work a system for light-induced damage and death of the RPE, based on the human ARPE-19 cell line, was used. Induction of mitochondria-derived reactive oxygen species (ROS) is shown to play a critical role in the death of cells exposed to short-wavelength blue light (425 ± 20 nm). ROS and cell death are blocked either by inhibiting the mitochondrial electron transport chain or by mitochondria-specific antioxidants. These results show that mitochondria are an important source of toxic oxygen radicals in blue light–exposed RPE cells and may indicate new approaches for treating AMD using mitochondria-targeted antioxidants.

INTRODUCTION

The free radical theory of aging (1) states that changes in biological function over time are due to cumulative cellular damage caused by reactive oxygen species (ROS). This theory is supported by studies showing progressive, even exponential, accumulation of ROS-damaged proteins, lipids and nucleic acids as cells and organisms age (2). Oxidative injury to cells is associated with several diseases, including Alzheimer's disease (3), amyotrophic lateral sclerosis (4), muscular dystrophy (5), Parkinson's disease (6), age-related macular degeneration (AMD) (7) and cataract (8). These diseases are late- or slow-onset diseases in which damage accumulates over time, similar or parallel to the aging process. Damage to mitochondria or an underlying mitochondrial dysfunction has been demonstrated in such processes (9), implicating mitochondria as the generators or the targets of oxidative stress.

AMD is a disease of late onset, rare in individuals under the age of 55 years. AMD is the leading cause of severe visual impairment in developed countries, affecting and disabling 10% of individuals over the age of 65 years (10). The pathogenesis of the disease is poorly understood, and to date, there is no efficient cure or prevention method. Still, damage to and death of the retinal pigment epithelium (RPE), postmitotic cells that line the retina and function in its maintenance, is a crucial and perhaps a triggering event in AMD (11). One mechanism of RPE damage is light exposure. Visible light (400–700 nm wavelength) has been known for some time to induce cell death both *in vivo* and *in vitro* (12,13). Most of the UV and infrared light is blocked by the cornea and the lens (14). Blue light, *i.e.* visible light of short wavelengths, can induce cell death at an intensity 10-fold lower than that required for longer-wavelength green light (15). Oxidative stress is suggested as the mechanism for visible light–induced damage (16). It has been shown that blue light can induce chromatin breaks *in vitro* and that the frequency of breaks could be reduced by antioxidants (17). But it is also important to note that both *in vitro* and *in vivo*, some antioxidants do not provide cells with the expected protection from light-induced death. For instance, ascorbate (vitamin C) provides incomplete retinal protection for albino rats exposed to blue light (18). In addition, a recent clinical trial testing the efficacy of antioxidant supplementation in people with AMD found only a moderate benefit (7).

In this study, aimed at helping to understand light-induced damage to the RPE, we studied light of 405–445 nm for three reasons. First, blue light of 400–500 nm has been shown to damage RPE *in vivo* (15) and *in vitro* (19,20). Second, short-wavelength light is more reactive than is longer-wavelength visible light. Third, the RPE is laden with mitochondria, and the cytochromes of the respiratory chain all absorb light significantly within the 410–440 nm range. Cytochrome absorption of blue light inhibits the function of isolated cytochromes *in vitro* (21) and inhibits cytochrome *c* oxidase activity *in vitro* (22) and *in vivo* (23).

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Abbreviations: AMD, age-related macular degeneration; DHE, dihydroethidium; DMEM, Dulbecco modified Eagle medium; DMSO, dimethyl sulfoxide; ETC, electron transport chain; mtDNA, mitochondrial DNA; PDTC, pyrrolidinedithiocarbamate; ROS, reactive oxygen species; RPE, retinal pigment epithelium.

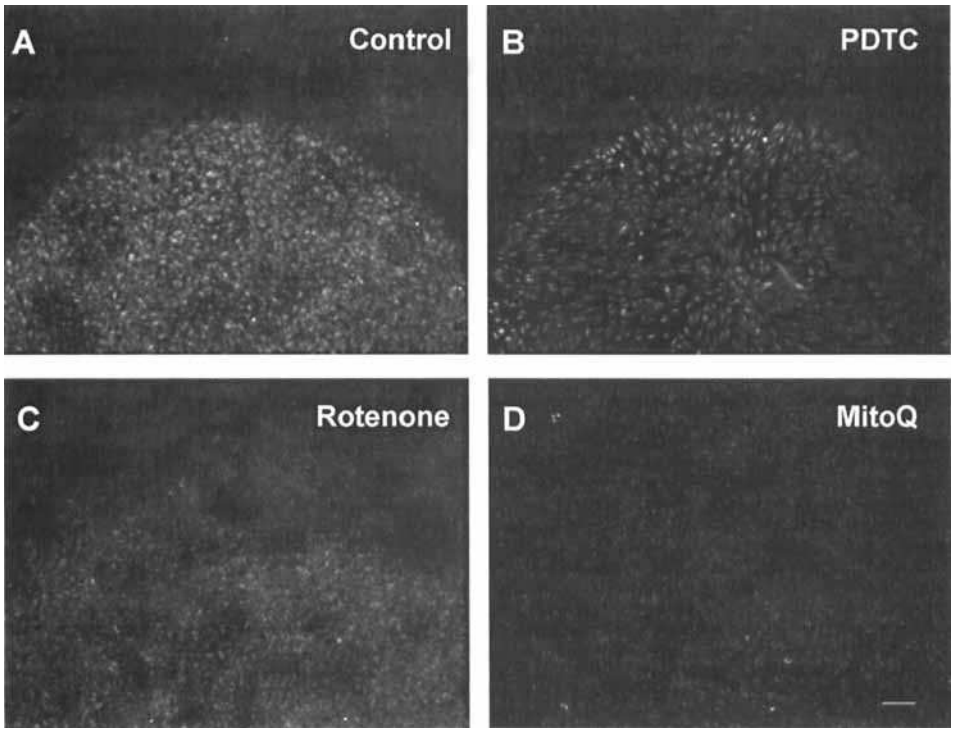

Figure 1. Mitochondria-specific antioxidants inhibit blue light–induced ROS production. DHE fluorescence detects ROS in ARPE-19 cells exposed to a spot of blue light. A: DMSO control. The top half of a circular illumination spot labels with DHE. As expected, the cells outside the circular illumination spot do not label. B: PDTC treatment. C: Rotenone treatment. D: MitoQ treatment. Scale bar, 100 μm.

Light-mediated inhibition of cytochrome function *in vivo* could produce ROS because inhibition of electron transport produces ROS (24). Thus, we hypothesized that the interaction of blue light with RPE mitochondrial cytochromes might be a source of ROS.

Under normal physiological conditions the major sources of ROS are the mitochondria (25), but the source of ROS under various pathological conditions is not clear. Short-wavelength visible light has been suggested to elicit ROS by targeting flavins and, specifically in the visual system, by reacting with pigmented components of lipofuscin (26–29). In this work we show that short-wavelength blue light induces ROS production in the mitochondria of an RPE cell line, resulting in cell death, which is prevented by a potent antioxidant targeted into the mitochondria. This suggests that protecting mitochondria from blue light–induced stress could slow down the deterioration of aging retinas.

MATERIALS AND METHODS

Cell culture and blue light treatment. ARPE-19 cells were grown to confluence in Dulbecco modified Eagle medium (DMEM)–F12 medium supplemented with 10% fetal calf serum, as described by Dunn *et al.* (30), on either six-well culture plates or chamber slides. Their response to light varies in relation to confluence and age of the culture because ARPE-19 cells continue to divide and compact even after reaching confluence. To control for this, all cells were light treated 24 h after reaching confluence. Light treatment was provided through the 20× objective of a Nikon TE300 inverted fluorescence microscope equipped with a 100 W mercury lamp. An interference filter for 425 ± 20 nm (Chroma Technology, Brattleboro, VT) was used to provide short-wavelength 10 mW/mm^2 blue light. This light exposure did not heat the medium: the temperature in the wells did not rise at all even after 7 min of illumination.

Preparation and analysis of Rho^0 cells lacking a respiratory chain to test the role of the respiratory chain in blue light–induced ROS production. To generate Rho^0 RPE cells, ARPE-19 cells were grown in low amounts of ethidium bromide–containing medium for 10 weeks, eliminating mitochondrial DNA (mtDNA) so that the cells lacked a functional respiratory chain. The medium was supplemented with uridine and pyruvate as described by King and Attardi (31). For respiration analysis 10 million parental ARPE-19 cells or 10 million Rho^0 cells were trypsinized and resuspended in 3 mL of DMEM–F12 with 10% fetal calf serum and analyzed in a respirometer as described by Gottlieb *et al.* (32). Where indicated, 1 m*M* KCN was added to stop respiration. To demonstrate lack of mtDNA, 1 μg of total cell DNA from either parental ARPE-19 cells or ARPE-Rho^0 cells was digested with *pvu*II to linearize the circular mitochondrial genome, which was separated by 0.7% agarose gel electrophoresis and tested by Southern analysis with a probe for the mtDNA D-loop.

Chemical detection of ROS production and cell death. ARPE-19 cells were grown to confluence in six-well plates and then blue light treated for 1 min. Thirty minutes before blue light exposure, 5 m*M* dihydroethidium (DHE; Molecular Probes, Eugene, OR) was added to enable detection of intracellular ROS after light exposure. When DHE is oxidized to ethidium, it binds DNA and fluoresces red (33). Throughout the experiments cells beyond the circular field of irradiation in a given culture served as a convenient nonilluminated negative control. Pyrrolidinedithiocarbamate (PDTC; 40 μm), an antioxidant, 5 μ*M* MitoQ, a mitochondria-targeted antioxidant (34), or 5 μL dimethyl sulfoxide (DMSO) (used as a control because it is the solvent for MitoQ and PDTC) was also added to selected cultures 30 min before illumination. When used, 10 μ*M* rotenone was added to the culture 30 s before light treatment, and the medium was replaced with rotenone-free medium immediately after illumination. Rotenone binds reversibly to the ND1 component at the distal end of Complex I of the respiratory chain and can partially inhibit succinate-supported ROS production (production of ROS downstream of ND1) (35,36). Rotenone was promptly removed after light treatment to avoid its toxicity and because it is also known to increase nicotinamide adenine dinucleotide (reduced form)–supported ROS production (from the proximal portion of Complex I). Cells were analyzed by fluorescence microscopy 15 min after light treatment for the presence of ethidium in the nuclei as described in the next paragraph.

To analyze cell death due to blue light illumination, cells were grown on four-well chamber slides (LabTek) for 24 h after achieving confluence, and 45 min before illumination 5 μ*M* MitoQ or DMSO only was added. Cells were then blue light treated for 5 min. Twenty-four hours after illumination cells were analyzed for death with an Annexin V–propidium iodide detection system (Roche, Indianapolis, IN). For all fluorescence imaging, cells were analyzed with a Nikon TE-300 microscope (Nikon, Tokyo, Japan) and SpotRT Slider camera (Diagnostic Instruments, Inc., Sterling Heights, MI), using ImagePro Plus software, version 4.1 (Media Cybernetics, Silver Spring, MD). For DHE fluorescence a Cy3 (green

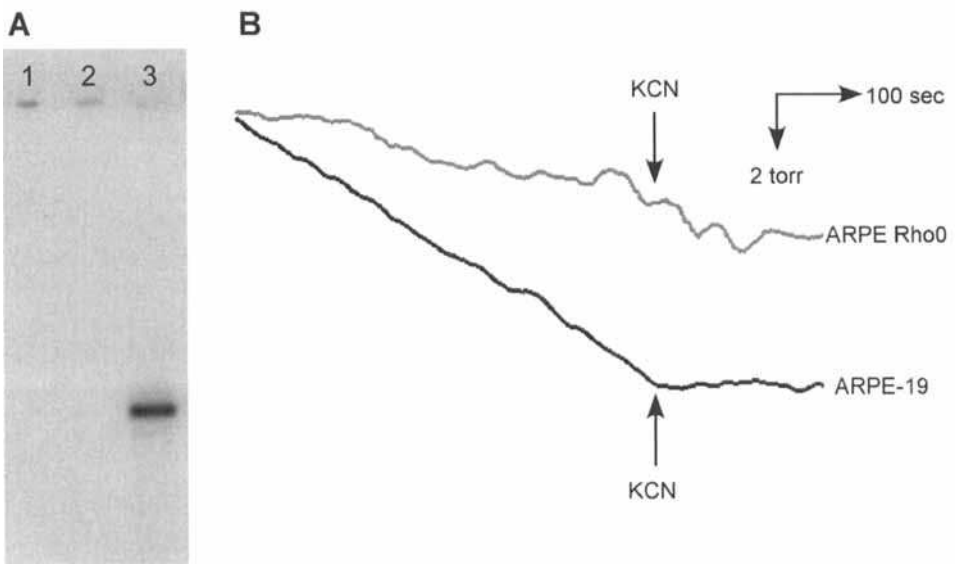

Figure 2. ARPE-Rho^0 cells do not have mtDNA and do not have a functioning respiratory chain. A: Southern analysis of ARPE-19 cells and ARPE-Rho^0 cells. DNA was extracted 2 months after beginning treatment with ethidium bromide (lane 1) or 1 month after treatment was stopped (lane 2). The mtDNA probe detects full-length, 16.5 kb linearized mtDNA only in the parental ARPE-19 cells' DNA (lane 3) but not in that of the Rho^0 cells (lanes 1 and 2). B: A respirometer was used to measure oxygen consumption of ARPE-19 and ARPE-Rho^0 cells. KCN was added to demonstrate that for ARPE-19 cells the drop in oxygen tension in the chamber was due to oxygen consumption at Complex IV of the respiratory chain, whereas the aberrant changes in oxygen levels in the ARPE-Rho^0 cells were KCN insensitive.

excitation–red emission) filter set (Chroma Technology) was used. For annexin–propidium iodide experiments annexin fluorescence was imaged with a Cy2 filter set (blue excitation–green emission) and propidium iodide fluorescence with a Cy3 filter set. Individual Cy2 and Cy3 images were then merged using ImagePro Plus software. "Filter bleed" was ruled out because of the spatial separation of the Cy2 and Cy3 signals: annexin labels the membrane, and propidium iodide labels the nucleus. Within each experiment comparable images were acquired using identical exposure parameters across wells.

RESULTS

Irradiation of ARPE-19 cells with short-wavelength visible light results in oxidative stress

We used the well-characterized human RPE cell line ARPE-19 to investigate the effects of short-wavelength visible light on cells. These cells do not contain the pigments melanin, xanthophylls or lipofuscin (30). To test for ROS production by light in our system, we treated them with blue light for 1 min. The cells received short treatments with high-intensity light to enable analysis of ROS production. The spot of illuminated cells was DHE positive 15 min after irradiation, whereas the surrounding cells were negative (Fig. 1A). The cells in this control well were exposed to DMSO before illumination because it is the solvent for the drugs described below. Incubating the cells with PDTC, an ROS scavenger and metal chelator (37), before light exposure reduced the DHE signal (Fig. 1B), suggesting that the fluorescence was due to ROS production. Blue light was unable to induce DHE fluorescence in the absence of cells (not shown).

Blue light–induced ROS are produced in the mitochondria

To test whether the ROS were produced by the mitochondrial electron transport chain (ETC), rotenone was added to the DHE-containing medium. By blocking the ETC, rotenone significantly reduced the DHE staining in light-treated cells (Fig. 1C), indicating that much of the light-induced ROS were produced by the mitochondria. To further investigate whether ROS were generated in the mitochondria, cells were incubated with MitoQ, a synthetically modified ubiquinone targeted to the mitochondrial matrix, which has been shown to protect cells from hydrogen peroxide–induced death (34), for 45 min before illumination. Treatment with MitoQ blocked illumination-induced DHE staining, indicating that most of the ROS are produced in the mitochondria (Fig. 1D).

Blue light–induced ROS production requires the mitochondrial respiratory chain

To further address the hypothesis that 425 nm light induces ROS production in the respiratory chain, we generated a Rho^0 subline from ARPE-19 cells. The lack of mtDNA in the ARPE-Rho^0 cell line was confirmed by Southern analysis using a probe for the mitochondrial D-loop region (Fig. 2A). The inability of ARPE-Rho^0 cells to respire was confirmed by oxygen consumption analysis in a respirometer (Fig. 2B). In Rho^0 cells the slow decrease in oxygen tension in the respirometer chamber persisted even after the addition of cyanide, a potent inhibitor of Complex IV of the respiratory chain; thus, the decrease was not caused by Complex IV–dependent respiration. Once the ARPE-Rho^0 cell line was established, the cells were subjected to 1 min of blue light treatment after incubation with DHE. ARPE-Rho^0 cells showed markedly reduced staining with DHE when compared with parental ARPE-19 cells (Fig. 3), except for individual cells with strong label that were also present beyond the light spot and were probably due to pathological processes unrelated to light exposure. These results support our hypothesis that the majority of 425 nm–induced ROS are produced in the mitochondria and, together with evidence that rotenone reduces blue light–induced ROS production, suggest that blue light–induced ROS production requires electron transport within the respiratory chain.

MitoQ protects ARPE-19 cells from blue light–induced death

ARPE-19 cells illuminated with 425 nm light showed signs of damage and death that correlated with the duration of light treatment. When cells were illuminated for 5 min, cell death was detected in the treated spot 24 h later (see below).

To test whether ROS were the mediators of light-induced death of ARPE-19 cells, untreated cells and cells treated with MitoQ were illuminated with 425 nm light for 5 min. Phase-contrast photographs taken 24 h after illumination show that blue light–exposed cells undergo cell shrinkage with partial detachment from the tissue culture plate (Fig. 4A). In contrast, cells supplemented

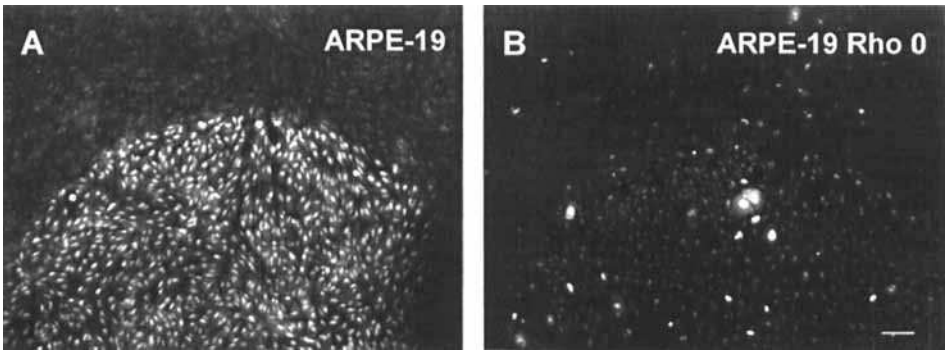

Figure 3. Blue light does not induce ROS production in Rho^0 cells. Cells were incubated with DHE for 30 min before illumination with 425 nm light for 1 min, and pictures were taken 15 min later. The spots of ROS-positive cells seen in the ARPE-Rho^0 field were present both outside and within the illumination field and therefore are not due to the light treatment. A: ARPE-19 cells. B: ARPE-Rho^0 cells. Scale bar, 100 μm.

with MitoQ appear more normal (Fig. 4D). We further analyzed the cells for staining with Annexin-V (green fluorescence) and propidium iodide (red fluorescence), markers of cell death, 24 h after irradiation. Annexin-V binds to phosphatidyl serine on the plasma membrane during cell death, and propidium iodide stains the nuclei of dead cells that have lost plasma membrane integrity (38). MitoQ significantly rescued the light-treated cells from death (compare untreated cells in Fig. 4B,C with treated cells in Fig. 4E,F). This demonstrates not only that ROS are produced in mitochondria after irradiation with 425 nm light, but also that these ROS are the cause of cell death and inhibiting them prevents light-induced cellular damage.

DISCUSSION

This work provides evidence that blue light induces ROS production through the ETC. Blue light–induced DHE fluorescence, primarily a measure of superoxide production, was reduced when the ETC was blocked with rotenone or when mtDNA, which encodes components of the ETC, was eliminated in Rho^0 cells. Supplementing ARPE-19 cells with MitoQ, a ubiquinone selectively targeted to the mitochondria, significantly reduced blue light–induced ROS production and protected the cells from death.

The rotenone-mediated reduction in blue light–induced ROS provides evidence that the ETC generates ROS when cells are exposed to blue light. Because rotenone binds reversibly to the ND1 component at the distal end of Complex I of the respiratory chain, but can only partially inhibit succinate-supported ROS production (production of ROS downstream of ND1) (35,36), some ROS may still be produced by blue light interaction with the partially inhibited ETC. This may explain why rotenone is less effective at reducing blue light–induced ROS than MitoQ, which can quench ROS produced by the ETC.

We find that MitoQ, a ubiquinone derivative targeted to mitochondria by covalent attachment to a lipophilic cation (34), protects ARPE-19 cells from blue light–induced ROS production and from death. MitoQ is taken up by mitochondria, and there the quinone is reduced to a quinol by the action of the respiratory chain. The quinol is the active antioxidant and is converted to the quinone on detoxifying ROS. However, the quinone is rapidly recycled to the active quinol by the respiratory chain. Because of this self-regenerating capacity, MitoQ is a potent antioxidant. In addition to ARPE-19 cells exposed to blue light, MitoQ also protects other cell types from mitochondrial oxidative stress–induced death. Friedreich ataxia fibroblasts, which have increased mitochondrial oxidative stress resulting from a deficiency of frataxin, are also protected by MitoQ (39). Our data on ARPE-19 cells suggest that MitoQ might protect RPE cells *in vivo* from light-induced oxidative damage. This hypothesis could be tested because oral administration of MitoQ to mice results in delivery to the brain in addition to other tissues (40).

Although other pigments present in the eye, namely rhodopsin, lipofuscin and melanin, can be the mediators of light-induced damage (27–29,41–43), and the absence of lipofuscin and melanin from the cultured ARPE-19 cells used in our studies prevents us from assessing the relative importance of these pigments in blue

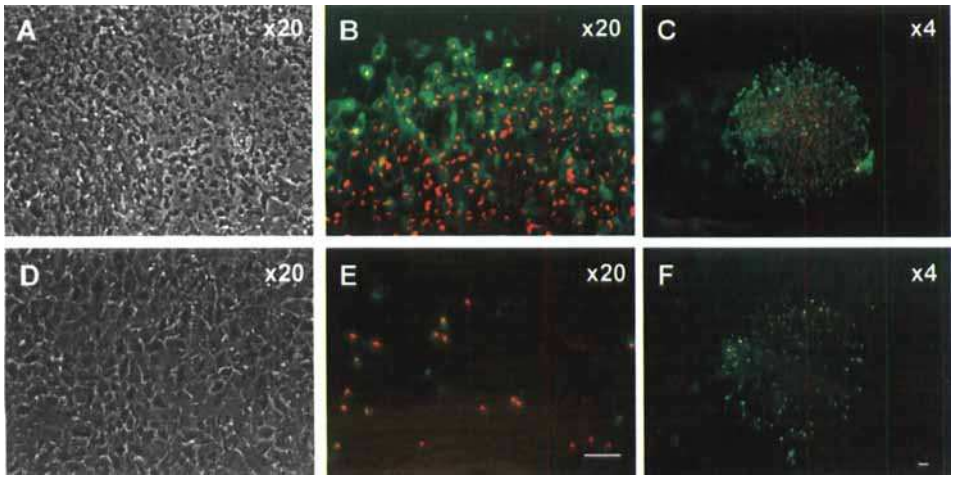

Figure 4. MitoQ protects ARPE-19 cells from light-induced death. ARPE-19 cells were illuminated with a circular spot of blue light for 5 min and analyzed 24 h later with Annexin-V (green) and with propidium iodide (red) to detect dead cells. A–C: Control ARPE-19 cells. D–F: ARPE-19 cells incubated with 5 μ*M* MitoQ 45 min before irradiation. Cells outside the illuminated circle are negative for both annexin and propidium iodide as expected. Brightfield images show condensed, dying cells in the absence of MitoQ (A), in contrast to normal morphology in the presence of MitoQ (D). Microscope objective for photography is indicated (4×, 20×). The entire illuminated circle and the surrounding non-illuminated cells are shown in 4× magnification images. Scale bars, 100 μm.

light–mediated toxicity, our results suggest that mitochondrial cytochromes are strongly affected by blue light. *In vivo*, this could lead to mitochondria impairment and cell dysfunction over time. Part of this impairment could result from accumulated ROS-mediated mtDNA mutations, which are associated with aging and age-related diseases such as Alzheimer's disease (44,45). *In vitro*, oxidative stress causes mutations in RPE mtDNA (46). Accumulation of mtDNA damage would be particularly significant in RPE because of its high metabolic rate and because it does not regenerate; many RPE cells live for the lifetime of an individual, providing ample time to accumulate mutations.

Our results with cultured RPE cells indicate that blue light can induce ROS and cell death through interaction with the mitochondria's ETC. These results justify *in vivo* studies to determine whether MitoQ or other mitochondria-targeted antioxidants might protect against blue light damage to the RPE. It is hoped that patients with AMD may one day benefit from targeted antioxidants to preserve mitochondrial function and to help halt the progression of the disease.

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