Rhodopsin-Mediated Blue-Light Damage to the Rat Retina: Effect of Photoreversal of Bleaching

Christian Grimm,[1] *Andreas Wenzel,*[1] *Theodore P. Williams,*[2] *Pascal O. Rol,*[1] *Farhad Hafezi,*[1] *and Charlotte E. Remé*[1]

Purpose. Acute white-light damage to rods depends on the amount of rhodopsin available for bleaching during light exposure. Bleached rhodopsin is metabolically regenerated through the visual cycle involving the pigment epithelium, or photochemically by deep blue light through photoreversal of bleaching. Because photoreversal is faster than metabolic regeneration of rhodopsin by several orders of magnitude, the photon catch capacity of the retina is significantly augmented during blue-light illumination, which may explain the greater susceptibility of the retina to blue light than to green light. However, blue light can also affect function of several blue-light–absorbing enzymes that may lead to the induction of retinal damage. Therefore, this study was conducted to test whether rhodopsin and its bleaching intermediates play a role in blue-light–induced retinal degeneration.

Methods. Eyes of anesthetized rats and mice that did or did not contain rhodopsin were exposed to green (550 ± 10 nm) or deep blue (403 ± 10 nm) light for up to 2 hours. Rats with nearly rhodopsinless retinas were obtained by bleaching rhodopsin in animals with inhibited metabolic rhodopsin regeneration—that is, under halothane anesthesia. In addition, *Rpe65*$^{-/-}$ mice that are completely without rhodopsin were used to test the susceptibility to blue-light damage of a rodent retina completely devoid of the visual pigment. Effects of illumination on photoreceptor morphology were assessed 24 hours or 10 days thereafter by morphologic and biochemical methods.

Results. Exposure to blue light resulted in severe retinal damage and activation of the transcription factor AP-1 in rats. In contrast, green light had no effect. When rhodopsin was almost completely bleached by short-term green-light exposure while metabolic regeneration (but not photoreversal) was prevented by halothane anesthesia, blue-light exposure induced distinct lesions in rat retinas. When both metabolic rhodopsin regeneration and photoreversal of bleaching were almost completely inhibited, blue-light exposure caused only very moderate lesions. When mice without rhodopsin were exposed to blue light, no damage occurred, in contrast to wild-type control mice.

Conclusions. Short time exposure to blue light has deleterious effects on retinal morphology. Because damage was observed only in the presence of the visual pigment, blue-light–induced retinal degeneration is rhodopsin mediated. Absorption of blue light by other proteins is not sufficient to induce light damage. Photoreversal of bleaching, which occurs only in blue but not in green light, increases the photon-catch capacity of the retina and may thus account for the difference in the damage potential between blue and green light. (*Invest Ophthalmol Vis Sci.* 2001;42:497–505)

Excessive exposure to visible light causes photochemical lesions in the retina of vertebrates.[1] The damaging light is absorbed by rhodopsin[2] and the amount of bleachable rhodopsin available during light exposure, determined by the rate of rhodopsin regeneration after bleaching, is a major factor influencing susceptibility to light damage. The rate of rhodopsin regeneration sets the number of photons that are absorbed per unit of time. This number is critical for the induction of photoreceptor apoptosis in mice. Therefore, mouse strains with slow metabolic rhodopsin regeneration are more resistant to light damage than mouse strains with fast regeneration kinetics.[3] An almost complete depletion of docosahexanoic acid (DHA) distinctly reduces the regeneration rate of rhodopsin and prevents light-induced lesions[4] even though the amount of rhodopsin in dark-adapted eyes is increased.[5] After light absorption, the damaging stimulus is transformed into an intracellular death signal and transmitted to downstream effectors such as AP-1.[6,7] Induction of c-Fos containing AP-1 activity is an essential step specifically for light-induced apoptosis of photoreceptors in mice.[6–8] This does not exclude that phototransduction mechanisms such as altered Ca^{2+} levels may also be involved in photoreceptor apoptosis induced by light, as evidenced by experiments in arrestin knockout animals.[9] In addition, exposure to high levels of light may cause lipid peroxidation[10,11] which, similar to the formation of free radicals, is discussed controversially as a cofactor and/or causative agent in light damage. Our earlier studies, however, were unable to demonstrate lipid peroxidation or an enhanced damage susceptibility after dietary increase of retinal polyunsaturated fatty acids in short-term experiments with white light.[12]

In rats, exposure to excessive levels of white light causes apoptotic cell death, not only of photoreceptors,[13] but, with a short delay, also of the retinal pigment epithelium (RPE).[14]

Different wavelengths and different intensities of light have specific physical properties that can differently affect biologic molecules. There is a broad spectrum of light-damage experiments in which different parameters are applied so that strict comparisons are almost impossible (for review see Reference 15). In line with this diversity, several action spectra of light damage have been recorded for the retina. Williams and Howell[16] reported an action spectrum that closely resembled the absorption spectrum of rhodopsin, suggesting that light of a wavelength of approximately 500 nm would be most effective in inducing light damage. In other studies, however, light of shorter wavelengths generally caused the most severe damage.[17–20] To date, molecular mechanisms by which visible light of short wavelength damages the retina are unknown. It has been hypothesized that blue-light damage may occur by photosensitizing, oxygen-dependent processes that may affect photoreceptors and RPE similarly.[21,22] In support of this view,

From the [1]Department of Ophthalmology, University Eye Clinic, Zurich, Switzerland; and the [2]Department of Biological Science, Florida State University, Tallahassee.

Supported by the Swiss National Science Foundation; the E. & B. Grimmke Foundation, Germany; SUVA-Forschungsfonds, Switzerland; and Paolo Baiocchi, Intercast, Parma, Italy.

Submitted for publication June 7, 2000; revised August 17 and September 29, 2000; accepted October 6, 2000.

Commercial relationships policy: N.

Corresponding author: Christian Grimm, Department of Ophthalmology, University Eye Clinic, Frauenklinikstrasse 24, CH-8091 Zurich, Switzerland. cgrimm@opht.unizh.ch

the application of antioxidants such as dimethylthiourea results in a partial protection of the retina against light damage.[23]

Several chromophores have been suggested that may be involved in blue-light damage. Cytochrome oxidase is inhibited in the rat retina after exposure to blue light, suggesting reduced retinal metabolism after light exposure.[24–26] Prostaglandin G/H synthase, in contrast, is activated after absorption of blue light, leading to peroxidation in the retina and to the production of superoxide radicals that may have the capacity to injure retinal tissue[27] and induce apoptosis. Furthermore, the identification of a blue and ultraviolet light-absorbing opsin in the RPE[28] renders it possible that excessive absorption of light by pigment epithelial cells could create a death signal that is transmitted to the photoreceptors.

In white light, the primary chromophore for light damage is rhodopsin.[2] It has been shown that the rate of metabolic rhodopsin regeneration in the visual cycle that involves the transport of the chromophore to and from the RPE with isomerization and redox steps,[29,30] sets the photon-catch capacity of a retina and is a major determinant in susceptibility to light damage.[3] However, bleached rhodopsin may be regenerated, not only metabolically, but also photochemically. In vitro, visible light of short wavelength (blue light) can restore activatable rhodopsin by a process called photoreversal of bleaching.[31] Recently, we have demonstrated that photoreversal of bleaching by blue light also occurs in the living rat eye.[32] Because the photochemical reversal is extremely fast, it significantly increases the photon-catch capacity of rhodopsin in photoreceptors during a given light exposure. This may explain the greater susceptibility of the retina to blue light than to light of longer wavelength.

In the present study, we tested whether rhodopsin might be involved in blue-light–mediated damage to the rodent retina by using several experimental paradigms, including photoreversal of bleaching, inhibition of metabolic rhodopsin regeneration, and transgenic mice without rhodopsin.

Methods

Animals

All experiments conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and to the guidelines of the Veterinary Authority of Zurich. Male albino Sprague–Dawley rats, 10 to 12 weeks old, were maintained for 3 to 7 weeks in 3-lux cyclic light, light/dark (L/D = 12 hours/12 hours). Mice were reared in 60-lux cyclic light (L/D = 12 hours/12 hours). They were bred on a mixed SV129/C57BL/6 genetic background and were either homozygous wild-type ($Rpe65^{+/+}$) or mutant (knockout; $Rpe65^{-/-}$) for the *Rpe65* gene. $Rpe65^{-/-}$ mice do not contain rhodopsin but possess a near normal retinal morphology for up to 7 weeks after birth.[2,33]

Light Exposure

Rats were dark adapted overnight (16 hours) and anesthetized with a mixture of ketamine (75 mg/kg) and xylazine (23 mg/kg) or with halothane (oxygen flow, 0.8 l/min, evaporator at position 1.5). Left eyes of anesthetized rats were kept moist with Methocel (CibaVision, Hergiswil, Switzerland) and exposed either to deep blue light (403 ± 10 nm) or to green light (550 ± 10 nm) for up to 120 minutes. Mice were dark adapted overnight (16 hours) and their pupils dilated with Cyclogyl (1%; Alcon, Fort Worth, TX) and phenylephrine (5%). They were then anesthetized with halothane. During anesthesia, mice were kept on a heating pad (set at 40°C) that was covered with a tissue. After a postexposure period of 24 hours in darkness, animals were killed for analysis or were put back into cyclic light for an additional 9 days. The light exposure system consisted of a xenon arc reflector lamp (230 V, 50 Hz, 120 W; Intralux MDR 100, Volpi; Schlieren, Switzerland) with interference filters to eliminate UV and infrared (IR) radiation and a liquid fiberoptic light guide (8 mm in diameter) to the animal's eye. The optical system included a switch holder for blue (403 nm, bandwidth ±10 nm) or green (550 nm, bandwidth ±10 nm) interference filters (Fig. 1).

Intensities of exposure were at 3.1 mW/cm^2 (Ganzfeld) or 33 mW/cm^2 (spot) for 403-nm blue light and 8.7 mW/cm^2 (Ganzfeld) or 47 mW/cm^2 (spot) for 550-nm green light. Potential thermal effects of the illumination had to be excluded by the usage of an interference filter in our system that prevented light above 700 nm from passing through the fiberoptic light guide. This was particularly important to prevent heating of the second interference filter (narrow-banded blue or green filter) mounted in the optical system (Fig. 1). For prebleaching of rhodopsin, a green-light intensity of 700 μW/cm^2 was used.

Microscopy and Damage Assessment

In dim red light, eye cups of enucleated rat eyes were prepared and immersed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) at 4°C. After 3 to 5 hours of fixation, the superior and inferior central retina adjacent to the optic nerve was trimmed, and fixation was continued in the same medium as before at 4°C overnight.[12] Mouse eyes were enucleated, fixed overnight at 4°C in the glutaraldehyde-cacodylate buffer, and the superior and inferior central areas of the retinas were trimmed.[34] Retinal tissue was washed in cacodylate buffer, postfixed in osmium tetroxide for 1 hour, dehydrated in increasing ethanol concentrations, and embedded in Epon 812. For light microscopy, 0.5-μm sections were stained with methylene blue and analyzed by microscope (Axiophot; Zeiss, Oberkochen, Germany). Extent of light damage was assessed by counting pyknotic nuclei in a 200-μm segment of the inferior central retina (the most affected region in our setup). More than 80% pyknotic nuclei: very severe damage (+++); 40% to 80% pyknotic nuclei: severe damage (++); 5% to 40% pyknotic nuclei: damage (+); below 5% pyknotic nuclei: no damage (−).

TUNEL Assay

Eyes were fixed in 2% paraformaldehyde for 2 hours at 4°C followed by dehydration and paraffin embedding. TdT-mediated dUTP nick-end labeling (TUNEL) was performed with modifications using an in situ cell death detection kit (Boehringer–Mannheim, Mannheim, Germany) on 5-μm paraffin sections. DNA strand breaks were labeled with fluorescein and visualized with a fluorescein isothiocyanate (FITC) filter, as described.[34]

DNA Fragmentation Analysis

Retinas were removed rapidly through a slit in the cornea and frozen in liquid nitrogen. Retinal tissue was homogenized in 1 ml of 10 mM Tris (pH 8), 10 mM EDTA, and 10 mM NaCl. Sodium dodecyl sulfate

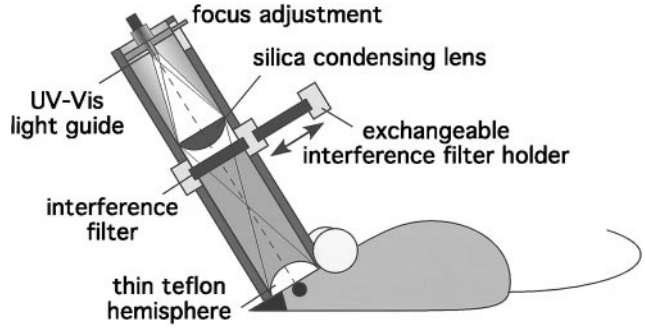

Figure 1. Illumination device. Light generated by a xenon short-arc reflector lamp with interference filters to eliminate UV and IR radiation was condensed by a silica lens and uniformly distributed on the cornea by a thin Teflon hemisphere. To evenly illuminate the retinas, the device was placed directly above the moisturized left eye of an anesthetized animal.

(SDS) was added to a final concentration of 0.5%, and proteins were digested with proteinase K (0.2 mg/ml) at 37°C for 16 hours. Fresh proteinase K was added (0.2 mg/ml) and incubation continued for 2 hours at 50°C. The mixture was extracted once with phenol-chloroform-isoamylalcohol (25:24:1) and twice with chloroform-isoamylalcohol (24:1). NaCl (final concentration, 300 mM) and EtOH (2.5 volumes) were added, and DNA was precipitated overnight at −20°C. After centrifugation for 10 minutes at 4000*g* (4°C), DNA was washed once with 70% EtOH and air dried for 1 hour at room temperature. TE (10 mM Tris [pH 8] and 1 mM EDTA) was added (100 μl per retina), and DNA was allowed to rehydrate for 2 days at 4°C. RNA was digested by the addition of 20 μg RNase A and incubation at 37°C for 1 hour. DNA concentration was determined by reading optical density at 260 nm (OD_{260}). Total DNA (10 μg) was analyzed on a 1.5% agarose gel, stained with ethidium bromide, visualized at 254 nm and compared with a 100-bp DNA ladder (Pharmacia Biotech, Uppsala, Sweden).

Electrophoretic Mobility Shift Assay

For electrophoretic mobility shift assay (EMSA), nuclear extracts were prepared as described previously for mice.[8] Briefly, one retina was homogenized in 400 μl of 10 mM HEPES-KOH (pH 7.9), 1 mM β-mercaptoethanol, and 1 mM dithiothreitol (DTT) in the presence of protease inhibitors. After incubation on ice for 10 minutes the homogenate was vortexed for 10 seconds and centrifuged. The pellet was resuspended in 50 μl of 20 mM HEPES-KOH (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM β-mercaptoethanol, and 1 mM DTT in the presence of protease inhibitors and incubated on ice for 10 minutes. Cellular debris was removed by centrifugation at 23,000*g* for 30 minutes at 4°C. Protein concentrations were determined using the Bradford protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as standard.

EMSAs were performed as described.[35] Briefly, two oligonucleotides were annealed to form a double-stranded DNA coding for an AP-1–specific (5′-AAG CAT GAG TCA GAC AC-3′) DNA-binding sequence (TPA response element; TRE). The annealed oligos were labeled using polynucleotide kinase (Boehringer–Mannheim) and ^{32}P-γATP (Hartmann Analytic, Braunschweig, Germany). For EMSA, 2 to 5 μg (5 μl) protein of nuclear extract was incubated on ice for 20 minutes with 19 μl of 5 mM $MgCl_2$, 0.1 mM EDTA, 0.75 mM DTT, 7.5% glycerol, and 0.05% NP-40 containing 24 μg BSA and 2 μg poly d (I-C) (Boehringer–Mannheim). Radiolabeled oligonucleotide (1 μl) was added, and incubation was continued for another 20 minutes. Protein-DNA complexes were resolved at 150 V on a 6% native polyacrylamide gel using 0.25× TBE as running buffer and visualized on x-ray film.

Results

Retinal Damage after Short-Term Exposure to Blue Light

To determine the threshold for photoreceptor apoptosis induced by blue or green light, rats were exposed for 30, 60, 90, or 120 minutes with the Ganzfeld apparatus. A thin Teflon hemisphere ensured a near uniform illumination of the rat retina (Fig. 1) but reduced the energy output of the light source (as measured at the level of the cornea) by almost a factor of 10.

Illumination with 3.1 mW/cm^2 of 403-nm blue light for 30 minutes caused vesiculation of rod outer segments in the central region of the inferior part of the retina, as observed at 24 hours after exposure (Fig. 2B, white arrowheads). In most experiments, the superior retina was less affected by the light exposure (not shown). The reasons for the topologic differences are not clear but may be intrinsic to our experimental setup. Similar vesiculation was detected after illumination for 60 minutes, but, in addition, some pyknotic nuclei (white arrows) and apoptotic bodies (black arrows) were formed in the outer nuclear layer (ONL). Obviously, the threshold for blue-light–mediated damage was passed after 60 minutes of illumination (Fig. 2C). The number of cells containing pyknotic nuclei increased dramatically after illumination for 90 (Fig. 2D) and 120 (Fig. 2E) minutes, with no photoreceptor cells surviving the blue-light illumination, as judged 10 days after exposure (Fig. 2F). Cells of the pigment epithelium had also degenerated and were completely removed at this time point. Strikingly, rod outer segment (ROS) vesiculation was virtually absent in retinas that received a 90- or 120-minute exposure (Figs. 2D, 2E; see also Fig. 4, Table 1 and discussion).

In contrast, cells of the retina did not show any signs of damage after exposure to 550 nm green light, even when the energy level (8.7 mW/cm^2) was elevated almost threefold above that of blue-light illumination (3.1 mW/cm^2). At 24 hours after illumination for 120 minutes, the retina appeared indistinguishable from dark control samples (Figs. 2A, 2G; Table 1). Similarly, the retina and the RPE appeared intact, even 10 days after green-light exposure, excluding that green light induces cell death after a delay (Fig. 2H).

At 24 hours after exposure, blue-light, but not green-light, exposure resulted in the production of a DNA ladder after gel electrophoresis of genomic DNA (Fig. 3A) and the formation of TUNEL-positive nuclei (Fig. 3B). Both markers are indicative of ongoing apoptosis, and therefore, necrotic cell death may be excluded from blue-light–mediated retinal degeneration.

To reach higher energy levels of light, we removed the Ganzfeld device. This allowed the exposure to 33 mW/cm^2 of blue or 47 mW/cm^2 of green light, respectively. Under these conditions, 10 minutes of exposure to blue light were sufficient to induce condensation of most photoreceptor nuclei (Fig. 4A) resulting in TUNEL-positive staining in the affected area (data not shown). However, light damage was less uniformly distributed across the retina. We noticed strong hot spots of light damage within sharply delineated regions (Fig. 4C and data not shown), which may have been caused by focal exposure of certain retinal areas. Of note, areas that contained almost exclusively pyknotic nuclei did not show ROS vesiculations (Figs. 4A, 4C). In contrast, ROS vesiculation was very prominent in immediately adjacent areas that contained very few nuclei with condensed chromatin (Fig. 4C). Similar observations were made when the exposure duration was varied (Fig. 2). Most important, however, light damage was qualitatively the same as with the Ganzfeld system. Blue light caused severe retinal degeneration (Fig. 4A), whereas green light did not affect any cell type of the retina, even at the high energy level of 47 mW/cm^2 (Fig. 4B).

Essential Role of Rhodopsin in Blue-Light–Induced Photoreceptor Apoptosis

Preservation of Retinal Morphology by the Absence of Bleachable Rhodopsin in Rat Eyes. Bleaching of rhodopsin was done by the illumination with low-level green light (700 μW/cm^2) for 1 hour. Such a long exposure, in contrast to a 5-minute exposure[32] (see later description), not only caused complete bleaching but also allowed the conversion of all bleaching intermediates to products that were no longer photoreversible by blue light (data not shown). However, to achieve this complete bleaching, rats had to be anesthetized with halothane which almost completely prevents metabolic rhodopsin regeneration.[36] After complete bleaching, rhodopsin was regenerated to 37% ± 4.4% of the dark value after 30 minutes recovery in darkness (n = 5 retinas) and 83.3% ± 6.1% after 120 minutes (n = 5 retinas). However, in animals anesthetized with halothane, the values after 30 minutes and 120 minutes were only 7.6% ± 1.8% (n = 4 retinas) and 8.35% ± 2.3% (n = 4 retinas), respectively. Consequently, our prebleaching protocol in animals anesthetized with halothane

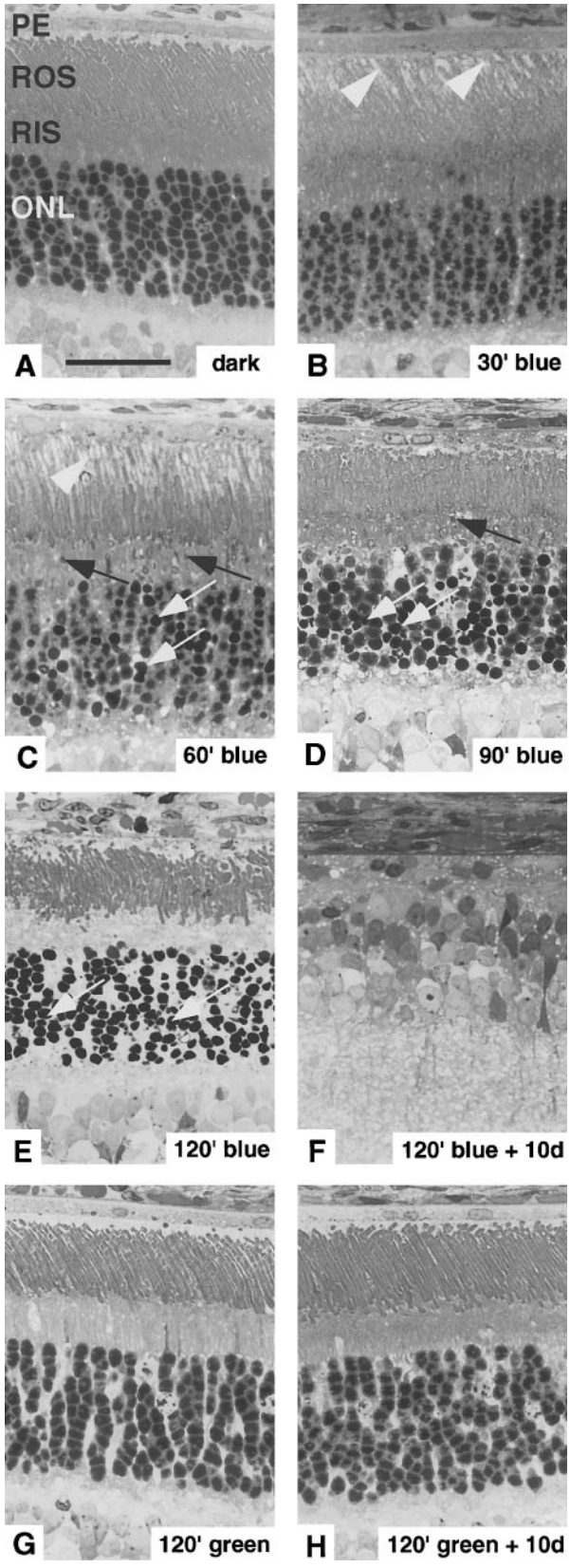

FIGURE 2. Light microscopy of retinal sections from rats that were not (**A**) or that were (**B** through **H**) exposed to light with the Ganzfeld device, using light and time of illumination indicated (blue light: 403 nm, 3.1 mW/cm^2 intensity; green light: 550 nm, 8.7 mW/cm^2 intensity). (**A**) Retinal section from a dark-adapted control animal. (**B** through **E**) Retinal morphology at 24 hours after illumination at an intensity of 3.1 mW/cm^2. (**F**) Retinal morphology 10 days after illumination (+ 10d). (**G**, **H**) Morphology of the retina 24 hours and 10 days after illumination. Retinas of rats exposed to green light or kept in darkness displayed comparable photoreceptor morphology. Blue light induced predominantly ROS vesiculation (*white arrowheads*) when exposure time was 30 or 60 minutes (**B**, **C**) but increasingly caused the formation of pyknotic nuclei (*white arrows*), whereas ROS vesiculations decreased when exposure duration was increased (**C**: <10% pyknotic photoreceptor nuclei; **D**: ~50% pyknotic nuclei; **E**: >90% pyknotic nuclei). (**C**) Mixture of ROS vesiculation, pyknotic nuclei, and apoptotic bodies (*black arrows*) was observed. (**F**) Degenerated photoreceptor cell layer and the RPE at +10 days. In contrast, green-light exposure did not affect photoreceptors or the pigment epithelium. (**G**, **H**) Representative sections of two to three independent experiments are shown. RIS, rod inner segments. Scale bar, 25 μm.

resulted in the virtual absence of bleachable rhodopsin from rat eyes during the subsequent illumination with doses of blue light that have been shown to damage the retina in the normal situation (Fig. 2). At the end of the 1-hour preillumination period with green light (700 μW/cm^2), the interference filter was switched to allow illumination with high-energy blue light (3.1 mW/cm^2; Ganzfeld) and exposure continued for additional 2 hours under halothane.

Control animals were anesthetized and illuminated with the same protocol, except that no green prebleaching was performed, and animals remained in darkness during the first hour of halothane anesthesia instead. Therefore, rhodopsin was present at normal levels at the beginning of the blue-light illumination in the control animals. Retinas almost devoid of rhodopsin showed only very moderate photoreceptor apoptosis (Fig. 5C; most affected area is shown; Table 1), in contrast to retinas of the control animals that contained almost exclusively pyknotic photoreceptor nuclei after the treatment with blue light (Fig. 5B; Table 1).

Preservation of Retinal Morphology by the Absence of Rhodopsin in Mouse Eyes. In our second approach we used mice without rhodopsin due to the knockout of the *Rpe65* gene. RPE65 is a protein specifically expressed in the RPE and essential for the reisomerization of all-*trans* retinol to the 11-*cis* configuration needed to reconstitute rhodopsin after bleaching.[33] Because these mice express the apoprotein opsin, they display an almost regular retinal morphology with only slightly less densely packed ROS (Fig. 5F). They are therefore well suited for investigation of the role(s) of rhodopsin in light damage.[2,33] Control mice were wild type for *Rpe65* and had standard levels of rhodopsin (not shown) and normal retinal morphology (Fig. 5D). All mice were anesthetized with halothane and exposed to very high levels of blue light (403 nm; 33 mW/cm^2; spot) for up to 45 minutes. Control wild-type mice exposed for 30 minutes displayed severe retinal damage and photoreceptor apoptosis 24 hours after blue-light exposure (Fig. 5E; Table 1). In marked contrast, the retina of *Rpe65*$^{-/-}$ mice appeared intact with no apoptotic photoreceptors detectable, although light exposure was for 45 minutes even (Fig. 5G; Table 1). This strongly suggests that blue-light–induced photoreceptor apoptosis is rhodopsin mediated.

Extension of the exposure period to 1 hour resulted in another type of blue-light damage that affected the RPE. This damage was independent of rhodopsin, because it also occurred in rhodopsin-less *Rpe65*$^{-/-}$ mice. However, it is important to note that the photoreceptors still did not show condensation of nuclear chromatin (data not shown). The nature of these injuries in the RPE remains unknown for our experimental conditions but must involve mechanisms different from the ones for light-induced photoreceptor apoptosis. This is further supported by preliminary observations made in c-*fos*$^{-/-}$ mice. These mice not only are protected against white-light damage[7]

TABLE 1. Summary of Experimental Paradigms

Animal	Light	Duration (min)	Anesthesia	Rhodopsin Regeneration	Comment	Damage*
Rat	Blue	120	Ketamine/xylazine	Photoreversal: yes Metabolic: yes		+++ (Fig. 2)
Rat	Green	120	Ketamine/xylazine	Photoreversal: no Metabolic: yes	Green-light of long wavelength does not photoreverse bleaching intermediates	− (Fig. 2)
Rat	Green+blue	5 + 60–120	Ketamine/xylazine	Photoreversal: yes Metabolic: yes	Photoreversal by blue light restores rhodopsin from bleaching intermediates	++ (Fig. 6)
Rat	Dark+blue	60 + 120	Halothane	Photoreversal: yes Metabolic: no	Almost complete inhibition of metabolic regeneration by halothane; photoreversal occurs by blue-light	++ (Fig. 5)
Rat	Green+blue	60 + 120	Halothane	Photoreversal: no Metabolic: no	Retinas almost completely depleted of rhodopsin; nearly no metabolic regeneration; no bleaching intermediates available for photoreversal	− (+) (Fig. 5)
Mouse (wild-type)	Blue	30	Halothane	Photoreversal: yes Metabolic: no	Almost complete inhibition of metabolic regeneration by halothane	+++ (Fig. 5)
Mouse ($Rpe65^{-/-}$)	Blue	45	Halothane	Photoreversal: no Metabolic: no	No rhodopsin in the retina	− (Fig. 5)

Green + Blue, Exposure to green light (550 nm) was immediately followed by exposure to blue light (403 nm).

* +++, More than 80% pyknotic photoreceptor nuclei; ++, 40–80% pyknotic nuclei; − (+), approximately 5% pyknotic nuclei; −, no damage.

but also did not show photoreceptor apoptosis after blue-light exposure (data not shown). However, similar to the $Rpe65^{-/-}$ mice, c-$fos^{-/-}$ mice showed damage to the RPE after high-energy blue-light exposure (data not shown).

Blue-Light–Induced Retinal Damage after a Pre-Bleach of Rhodopsin: Effect of Photoreversal of Bleaching

To test whether photograph regeneration would supply sufficient rhodopsin molecules for light damage to occur, we exposed rat eyes to 5 minutes of green light (8.7 mW/cm^2). After this prebleaching, which reduced rhodopsin to 5.8% ± 1% (n = 3), eyes were exposed to blue light for 60 (Fig. 6A), 90 (Fig. 6B), or 120 minutes (Fig. 6C) at an intensity of 3.1 mW/cm^2. In control experiments, green-light illumination continued for 120 minutes without switching to blue light (Fig. 6D). Blue light induced photoreceptor apoptosis in a dose-dependent manner, whereas green light did not affect retinal morphology. Of note, light damage induced by blue light after pre-exposure to green light was qualitatively indistinguishable from damage without a preceding green-light bleaching (compare Figs. 2 and 6). Because the switch from green to blue light occurred after 5 minutes, presumed intermediates of the bleaching process were still present and could be photoreversed to rhodopsin by the following blue-light exposure,[32] in contrast to the experiment shown in Figure 5.

Induction of AP-1 by Blue-Light Exposure

Characteristic features of rhodopsin-mediated white-light damage include the induction of the intrinsic apoptotic cell death program through activation of the transcription factor AP-1.[6,8] Similarly, blue-light illumination induced internucleosomal fragmentation of genomic DNA (Fig. 3A) and positive TUNEL staining of photoreceptor nuclei (Fig. 3B). Both markers indicate that cell death occurs by apoptosis. Furthermore, blue light, but not green light, strongly induced DNA binding activity of the transcription factor AP-1 in nuclei of rat retinas. Induction was fast and occurred as early as 30 minutes after the end of exposure but was especially strong at 6 hours after lights off (Fig. 7). This time course matches the timing of AP-1 activation after white-light exposure in mice.[8]

DISCUSSION

Rhodopsin Mediated Photoreceptor Apoptosis Induced by Blue Light

Illumination with excessive white light induces apoptosis of photoreceptors, which leads to retinal degeneration. The degenerative process is initiated through the absorption of pho-

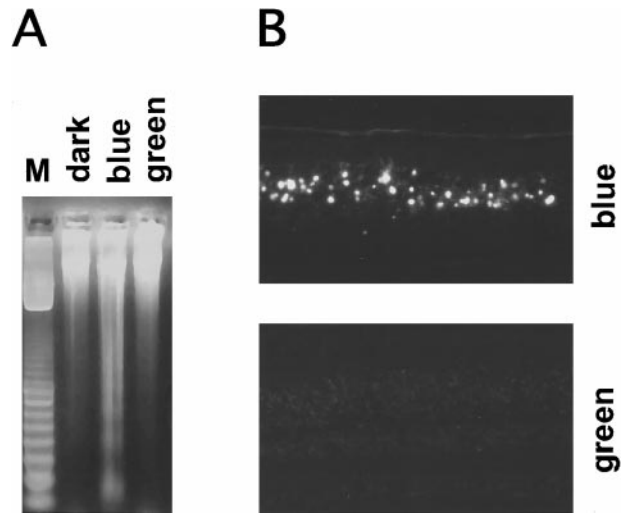

FIGURE 3. Illumination with blue light but not with green light induces DNA strand breaks indicating apoptotic cell death. (**A**) Formation of a DNA ladder in agarose gel electrophoresis 24 hours after blue-light (120 minutes, 3.1 mW/cm^2, Ganzfeld) but not after green-light (120 minutes, 8.7 mW/cm^2, Ganzfeld) illumination. (**B**) TUNEL staining of retinas 24 hours after blue-light (*top*, 120 minutes, 3.1 mW/cm^2, Ganzfeld) or green-light (*bottom*, 120 minutes, 8.7 mW/cm^2, Ganzfeld) illumination. Positive staining is exclusively found after blue-light illumination.

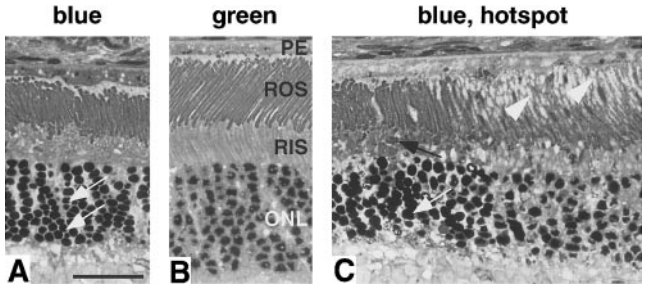

FIGURE 4. Light microscopy of retinal sections from rats that were exposed for 10 minutes to blue light of 33 mW/cm^2 (**A**, **C**) or green light of 47 mW/cm^2 (**B**) without the Ganzfeld device. Illumination with blue light resulted in hot spots of retinal light damage (**A**, >95% pyknotic photoreceptor nuclei) with sharp boundaries (**C**), whereas green light did not damage the retina (**B**). Representative sections (central inferior retina) of two to four independent experiments are shown. Abbreviations, symbols, and scale bar are as in Figure 2.

tons by the visual pigment rhodopsin[2] and involves activation of the downstream effector and transcription factor AP-1.[6,7] A critical determinant of the susceptibility of a retina to white-light damage is the rate at which rhodopsin is metabolically regenerated after bleaching.[3] Because the regeneration rate determines the availability of bleachable rhodopsin during illumination, it sets the number of photons that are absorbed per time unit. This number is critical for the induction of photoreceptor apoptosis in mice. Therefore, mouse strains with a slow metabolic rhodopsin regeneration are more resistant to light damage than mouse strains with fast regeneration kinetics.[3]

Several lines of evidence strongly indicate that blue-light–induced photoreceptor cell death is also rhodopsin mediated. As in white light, cell death of photoreceptors after blue-light exposure occurred through apoptosis, and blue-light damage involved activation of AP-1. The time course of this activation resembled the one seen after exposure to white light.[8] Most important, however, rat retinas almost completely devoid of rhodopsin showed strongly reduced light damage after blue-light exposure, and mouse photoreceptors without rhodopsin were completely protected against blue-light exposure. Therefore, other blue-absorbing molecules, such as prostaglandin synthase[27] and cytochrome oxidase,[24,26] may play only minor roles as primary light receptors in the mediation of photoreceptor apoptosis by blue light. Similarly, we assume that lipid peroxidations and production of free radicals by light do not suffice to induce immediate photoreceptor death in the absence of rhodopsin. Alternatively, formation of such molecules could depend on the absorption of large numbers of photons by rhodopsin and thus be secondary effects. Therefore, we suggest, that rhodopsin is the main chromophore necessary for blue-light–induced retinal degeneration. Intracellular signaling pathways may be very similar in blue- and white-light conditions.

However, because high-energy blue light could damage cells of the RPE in a rhodopsin-independent way (data not shown), the molecules and mechanisms described above may be relevant for this latter type of lesion. Other molecules that strongly absorb light in the blue range include lipofuscin and melanin.[37,38] Although melanin may not have an essential role in light damage,[39,40] lipofuscin, or its main component A2E, may be a candidate for the mediation of light damage to the RPE. Both, cell toxicity[41] and inhibition of mitochondrial function by A2E are increased by light exposure.[42] However, rhodopsin, one of the precursors thought to be involved in the formation of A2E is missing in *Rpe65*$^{-/-}$ mice.[43] It is unlikely that these mice contain A2E in the RPE and therefore that A2E may be involved in the mediation of blue-light damage to the RPE in the *Rpe65*$^{-/-}$ mice. An alternative explanation for the observed RPE damage would be thermal effects. However, long-wavelength and heat-creating light was eliminated in our illumination system by the use of an interference filter (see the Materials and Methods section). Clearly, the analysis of blue-light–mediated damage to the RPE needs further investigations. With *Rpe65*$^{-/-}$ and c-*fos*$^{-/-}$ mice, both of which resist blue-light damage to the photoreceptors but not to the RPE, there are two suitable model systems used to analyze molecular and cellular mechanisms of light-induced RPE damage in vivo without the interference of photoreceptor cell death.

Effect of Photoreversal of Bleaching on Light-Induced Retinal Degeneration

To restore bleachable rhodopsin, a retina exposed to blue light does not depend on the slow metabolic regeneration in the visual cycle alone. Blue light, by the extremely rapid process of photoreversal,[31] can photochemically regenerate rhodopsin in vivo from long-lived bleaching intermediates, probably metarhodopsin MII.[32,44] Although blue light is less efficiently absorbed by rhodopsin than light at approximately 500 nm, photoreversal by blue light nevertheless dramatically increases the photon-catch capacity of the retina[32] and thus increases its susceptibility to light damage. Therefore, photoreversal of bleaching may indeed play a significant role in mediating dam-

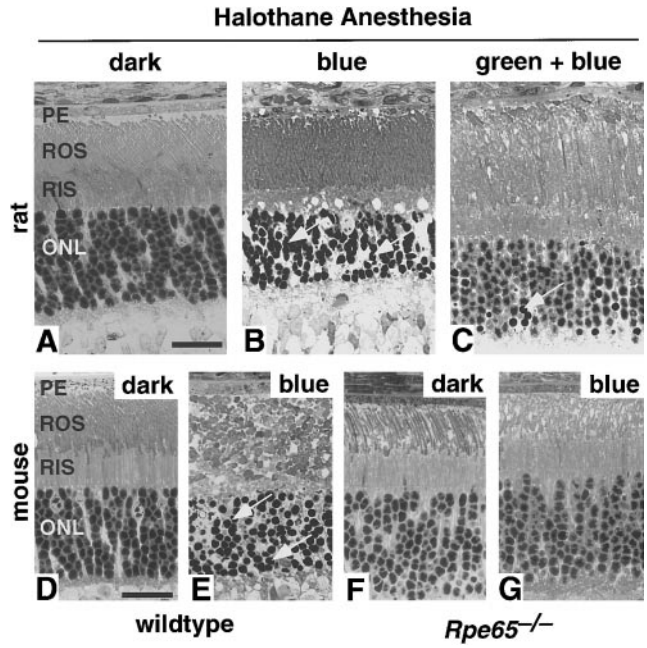

FIGURE 5. Blue-light damage is rhodopsin mediated. (**A**) Retina of a control (dark-adapted) rat. (**B**) Anesthetized rats were kept in darkness for 1 hour followed by a 2-hour exposure to light (3.1 mW/cm^2; Ganzfeld). Retinal tissue was prepared at 24 hours in darkness after the end of illumination. Pyknotic nuclei appeared in more than 95% of the photoreceptors. (**C**) Rats with eyes illuminated for 1 hour with green light (700 μW/cm^2; Ganzfeld) followed by a 2-hour exposure to blue light (3.1 mW/cm^2; Ganzfeld). Approximately 5% of nuclei were pyknotic. (**D** through **G**) Retinal tissue of mice that contained normal levels of rhodopsin (**D**, **E**) or that were completely without visual pigment (*Rpe65*$^{-/-}$, **F**, **G**). Anesthetized mice were kept in darkness for 45 minutes (**D**, **F**) or were exposed to high-energy blue light (403 nm; 33 mW/cm^2; spot) for 30 minutes (**E**, >95% pyknotic nuclei) and 45 minutes (**G**, no pyknotic nuclei detected). Retinal tissue was prepared after a recovery period of 24 hours in darkness. Representative sections (central inferior retina) of two to three independent experiments are shown. Abbreviations, symbols and scale bar as in Figure 2.

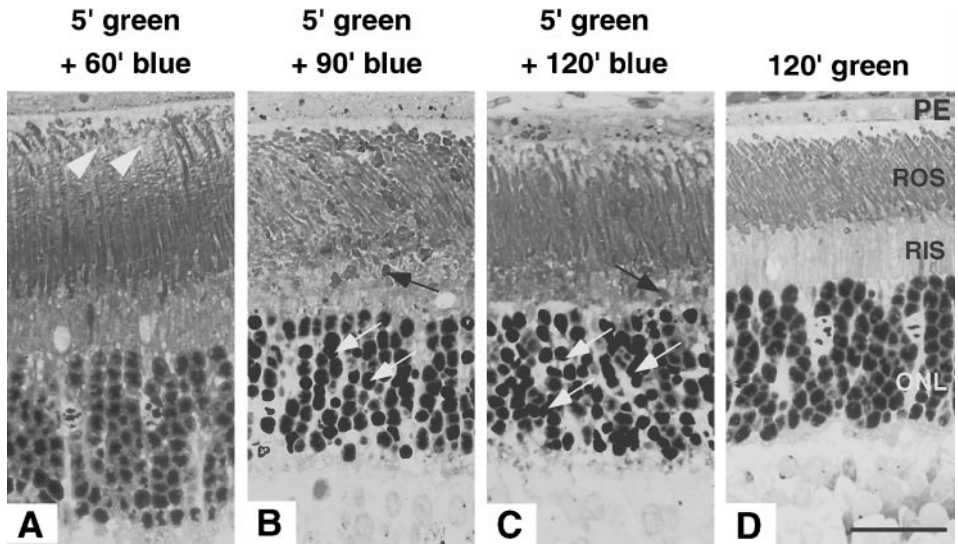

FIGURE 6. Light microscopy of retinal sections from rats that were exposed for the times shown to green-light (8.7 mW/cm^2; Ganzfeld) followed by blue-light (3.1 mW/cm^2; Ganzfeld) illumination. (**A**) ROS vesiculation but no pyknotic photoreceptor nuclei; (**B**) approximately 50% pyknotic nuclei; (**C**) approximately 80% pyknotic nuclei; (**D**) no pyknotic nuclei (control illumination: 8.7 mW/cm^2; Ganzfeld). Distinct apoptosis was seen (**B**, **C**), whereas threshold lesions (**A**) appeared as ROS vesiculations. Abbreviations, symbols, and scale bar are as in Figure 2.

age by visible light of short wavelength. When rhodopsin was prebleached to near completion with nondamaging light (green), immediate exposure to blue light was capable of restoring sufficient rhodopsin for the absorption of enough photons required for light damage to occur. However, this appeared to be possible only when reversible intermediates of the bleaching process were still present in photoreceptors, suggesting that the effect is specific and depends on rhodopsin.

In contrast, when green light is used for exposure, supply of unbleached rhodopsin exclusively depends on metabolic regeneration in the visual cycle. Thus, in green light it would take much longer time to absorb deleterious amounts of photons than in blue light. Our results show that even short periods of blue-light illumination induced photoreceptor apoptosis, whereas green light had no effect on retinal morphology. Nevertheless, we cannot exclude that illumination with green light in the range of several hours or days would eventually lead to retinal damage in our experimental system, provided that metabolic regeneration is functioning. Indeed, other experimental setups use green light to induce retinal damage.[40,45] However, four major differences between the setup used here and that used by others should be considered: 1) We used narrow-band green light of high intensity (in milliwatts), whereas others used broad-band green light of low intensity (in microwatts) containing a small proportion of light below a wavelength of 500 nm.[1,45] Green light, as applied in the present study, is not able to photoreverse rhodopsin in vivo.[32] Broad-band green light reaching below a wavelength of 500 nm, however, may enable some photoreversal. 2) We used short-term exposure in contrast to the long-term exposure used in other studies. 3) In the present study, anesthetized rats were exposed in contrast to free-moving animals used by others, and consequences of anesthesia for light damage have not been studied in full detail yet. 4) Light exposure resulted in damage primarily of the inferior hemisphere of the retina comparable to some forms of retinitis pigmentosa, whereas other studies point to the superior hemisphere as the more sensitive region for light damage (for review, see Reference 21). This again demonstrates differences between the individual experimental setups.

The phenomenon of photoreversal of bleaching may explain why light of shorter wavelength is generally more damaging to the retina than light of longer wavelengths. Because white light contains light of all wavelengths, we suggest that the emission spectrum of white light bulbs is highly critical, not only for the induction of retinal damage in laboratory animals, but also for safety in human eyes.

ROS Vesiculation as Indicator of Photoreceptor Survival?

Illumination of a retina with blue light for 30 minutes caused vesiculation of ROS observed 24 hours after lights off. The absence of pyknotic nuclei suggests that the cells did not activate the apoptotic death program. Increasing the illumination time to 60 minutes resulted in similar ROS vesiculations, but in addition, several photoreceptor nuclei appeared condensed, indicating ongoing apoptosis. Extending the illumination time to 90 and 120 minutes increased the number of pyknotic nuclei dramatically. In these tissues, no ROS vesiculation was detected (Figs. 2B through 2E). This phenomenon was especially pronounced in experiments that were performed without the Ganzfeld device (Fig. 4). In this situation, we observed hot spots of light damage with sharp borderlines to the neighboring tissue. It was striking that in areas with almost exclusively pyknotic nuclei, no ROS vesiculation was observed, whereas in areas immediately adjacent, ROS vesiculation was very pronounced, but almost no pyknotic nuclei were detectable. This observation suggests that ROS vesiculations and the formation of pyknotic nuclei could be mutually exclusive. Moderate light levels, which do not induce cell death, would cause ROS vesiculations that will be repaired,[46] whereas high light levels would cause pyknotic nuclei and cell death. The induction of programmed cell death may therefore prevent ROS vesiculation or vice versa.

In earlier studies we found release of arachidonic acid (AA), DHA, and leukotriene B_4, after light exposure (reviewed in Reference 15). We do not know whether they are involved in the vesiculation of ROS or the apoptosis of photoreceptors. Nevertheless, we might speculate that at moderate levels, these mediators could destabilize membranes leading to ROS vesiculation but might induce apoptosis if they surpass a critical concentration. However, molecular mechanisms leading to either ROS vesiculations or apoptosis induction remain to be elucidated.

CONCLUSIONS

These data show that blue-light–induced lesions were rhodopsin mediated. Levels of bleachable rhodopsin during light exposure play a pivotal role in susceptibility to retinal light

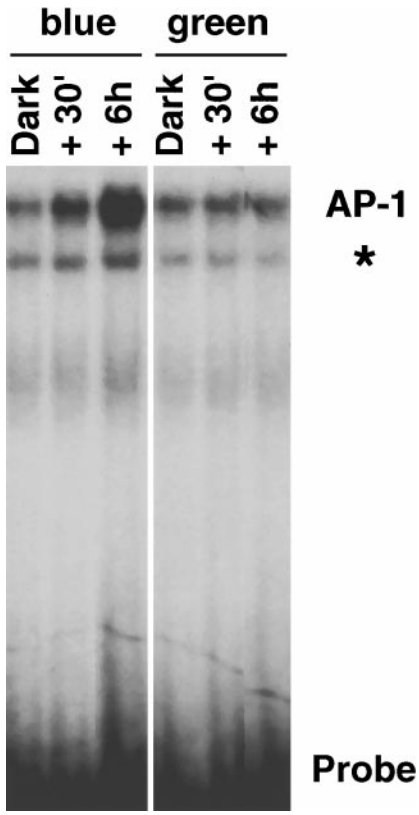

FIGURE 7. Induction of AP-1 DNA-binding activity after blue-light illumination. Eyes were not illuminated (dark) or were exposed to blue (10 minutes, 33 mW/cm^2, spot) or green (120 minutes, 47 mW/cm^2, spot) light. Nuclear extracts from retinal cells were prepared at the indicated time points after illumination and were incubated with radiolabeled oligonucleotides (probe) representing an AP-1–binding site. Complexes were resolved on native polyacrylamide gels. *Nonspecific band (Reference 8 and data not shown).

damage. Blue light is readily absorbed by rhodopsin and by intermediates of the bleaching process.[32] Thus, it efficiently photoregenerates the visual pigment. As a result, the photon absorption capacity is dramatically increased in the retina during blue-light exposure, and consequently the susceptibility to light-induced cell death is significantly augmented. Photoreversal of rhodopsin bleaching may thus be an important molecular mechanism underlying blue-light damage to the retina. We also hypothesize that the blue-light component may significantly increase the deleterious effect of white light for photoreceptor cell viability after light exposure.

Acknowledgments

The authors thank Coni Imsand, Dora Greuter, and Gaby Hoegger for skilled technical assistance and Michael Redmond for providing Rpe65 knockout mice.

References

1. Noell WK, Walker VS, Kang BS, Berman S. Retinal damage by light in rats. *Invest Ophthalmol.* 1966;5:450–473.
2. Grimm C, Wenzel A, Hafezi F, et al. Protection of Rpe65-deficient mice identifies rhodopsin as mediator of light-induced retinal degeneration. *Nat Genet*. 2000;25:63–66.
3. Wenzel A, Remé CE, Williams TP, Hafezi F, Grimm C. The Rpe65 Leu450Met mutation increases retinal resistance against light-induced degeneration by slowing rhodopsin regeneration. *J Neurosci.* In press.
4. Bush RA, Reme CE, Malnoe A. Light damage in the rat retina: the effect of dietary deprivation of N-3 fatty acids on acute structural alterations. *Exp Eye Res.* 1991;53:741–752.
5. Bush RA, Malnoe A, Reme CE, Williams TP. Dietary deficiency of N-3 fatty acids alters rhodopsin content and function in the rat retina. *Invest Ophthalmol Vis Sci.* 1994;35:91–100.
6. Wenzel A, Grimm C, Marti A, et al. c-Fos controls the "private pathway" of light-induced apoptosis of retinal photoreceptors. *J Neurosci*. 2000;20:81–88.
7. Hafezi F, Steinbach JP, Marti A, et al. The absence of c-fos prevents light-induced apoptotic cell death of photoreceptors in retinal degeneration in vivo. *Nat Med.* 1997;3:346–349.
8. Hafezi F, Marti A, Grimm C, Wenzel A, Remé CE. Differential DNA binding activities of the transcription factors AP-1 and Oct-1 during light-induced apoptosis of photoreceptors. *Vision Res.* 1999;39:2511–2518.
9. Chen J, Simon MI, Matthes MT, Yasumura D, LaVail MM. Increased susceptibility to light damage in an arrestin knockout mouse model of Oguchi disease (stationary night blindness). *Invest Ophthalmol Vis Sci.* 1999;40:2978–2982.
10. Handelman GJ, Dratz EA. The role of antioxidants in the retina and retinal pigment epithelium and the nature of prooxidant-induced damage. *Adv Free Radic Biol Med.* 1986;2:1–89.
11. Kayatz P, Heimann K, Schraermeyer U. Ultrastructural localization of light-induced lipid peroxides in the rat retina. *Invest Ophthalmol Vis Sci.* 1999;40:2314–2321.
12. Remé CE, Malnoe A, Jung HH, Wei Q, Munz K. Effect of dietary fish oil on acute light-induced photoreceptor damage in the rat retina. *Invest Ophthalmol Vis Sci.* 1994;35:78–90.
13. Reme CE, Grimm C, Hafezi F, Marti A, Wenzel A. Apoptotic cell death in retinal degenerations. *Prog Retinal Eye Res.* 1998;17:443–464.
14. Hafezi F, Marti A, Munz K, Reme CE. Light-induced apoptosis: differential timing in the retina and pigment epithelium. *Exp Eye Res.* 1997;64:963–970.
15. Remé CE, Hafezi F, Munz K, Reinboth J. Light damage to retina and pigment epithelium. In: Marmor MF, Wolfensberger TJ, eds. *The Retinal Pigment Epithelium*: *Current Aspects of Function and Disease.* New York: Oxford; 1998:563–586.
16. Williams TP, Howell WL. Action spectrum of retinal light-damage in albino rats. *Invest Ophthalmol Vis Sci.* 1983;24:285–287.
17. Rapp LM, Tolman BL, Dhindsa HS. Separate mechanisms for retinal damage by ultraviolet-A and mid-visible light. *Invest Ophthalmol Vis Sci.* 1990;31:1186–1190.
18. Ham WT Jr, Mueller HA, Ruffolo JJ Jr, Clarke AM. Sensitivity of the retina to radiation damage as a function of wavelength. *Photochem Photobiol.* 1979;29:735–743.
19. van Norren D, Schellekens P. Blue-light hazard in rat. *Vision Res.* 1990;30:1517–1520.
20. Rapp LM, Smith SC. Morphologic comparisons between rhodopsin-mediated and short-wavelength classes of retinal light damage. *Invest Ophthalmol Vis Sci.* 1992;33:3367–3377.
21. Organisciak DT, Winkler BS. Retinal light damage: practical and theoretical considerations. *Prog Retinal Eye Res.* 1994;13:1–29.
22. Crockett RS, Lawwill T. Oxygen dependence of damage by 435 nm light in cultured retinal epithelium. *Curr Eye Res.* 1984;3:209–215.
23. Organisciak DT, Darrow RM, Jiang YI, Marak GE, Blanks JC. Protection by dimethylthiourea against retinal light damage in rats. *Invest Ophthalmol Vis Sci.* 1992;33:1599–1609.
24. Chen E, Soderberg PG, Lindstrom B. Cytochrome oxidase activity in rat retina after exposure to 404 nm blue-light. *Curr Eye Res.* 1992;11:825–831.
25. Chen E. Inhibition of enzymes by short-wave optical radiation and its effect on the retina. *Acta Ophthalmol Suppl.* 1993;208:1–50.
26. Chen E. Inhibition of cytochrome oxidase and blue-light damage in rat retina. *Graefes Arch Clin Exp Ophthalmol.* 1993;231:416–423.
27. Hanna N, Peri KG, Abran D, et al. Light induces peroxidation in retina by activating prostaglandin G/H synthase. *Free Radic Biol Med.* 1997;23:885–897.

28. Hao W, Fong HK. Blue and ultraviolet light-absorbing opsin from the retinal pigment epithelium. *Biochemistry.* 1996;35:6251–6256.
29. Corson DW, Crouch RK. Physiological activity of retinoids in natural and artificial visual pigments. *Photochem Photobiol.* 1996; 63:595–600.
30. Rando RR. Polyenes and vision. *Chem Biol.* 1996;3:255–262.
31. Williams TP. Photoreversal of rhodopsin bleaching. *J Gen Physiol.* 1964;47:679–689.
32. Grimm C, Remé CE, Rol PO, Williams TP. Blue-light's effects on rhodopsin: photoreversal of bleaching in living rat eyes. *Invest Ophthalmol Vis Sci.* 2000;41:3984–3990.
33. Redmond TM, Yu S, Lee E, et al. Rpe65 is necessary for production of 11-cis-vitamin A in the retinal visual cycle. *Nat Genet.* 1998;20: 344–351.
34. Hafezi F, Abegg M, Grimm C, et al. Retinal degeneration in the rd mouse in the absence of c-fos. *Invest Ophthalmol Vis Sci.* 1998; 39:2239–2244.
35. Marti A, Jehn B, Costello E, et al. Protein kinase A and AP-1 (c-Fos/JunD) are induced during apoptosis of mouse mammary epithelial cells. *Oncogene.* 1994;9:1213–1223.
36. Keller CB, Grimm C, Wenzel A, Remé CE. Halothane slows rhodopsin regeneration after bleaching and prevents light induced retinal damage in BALB/c mice [ARVO Abstract]. *Invest Ophthalmol Vis Sci.* 2000;41(4):**S**598. Abstract nr 3176.
37. Docchio F, Boulton M, Cubeddu R, Ramponi R, Barker PD. Age-related changes in the fluorescence of melanin and lipofuscin granules of the retinal pigment epithelium: a time-resolved fluorescence spectroscopy study. *Photochem Photobiol.* 1991;54:247–253.
38. Eldred GE, Katz ML. Fluorophores of the human retinal pigment epithelium: separation and spectral characterization. *Exp Eye Res.* 1988;47:71–86.
39. Hoppeler T, Hendrickson P, Dietrich C, Remé C. Morphology and time-course of defined photochemical lesions in the rabbit retina. *Curr Eye Res.* 1988;7:849–860.
40. Gorgels TGM, Van Norren D. Two spectral types of retinal light damage occur albino as well as in pigmented rat: no essential role for melanin. *Exp Eye Res.* 1998;66:155–162.
41. Wassell J, Davies S, Bardsley W, Boulton M. The photoreactivity of the retinal age pigment lipofuscin. *J Biol Chem.* 1999;274:23828–23832.
42. Suter M, Remé CE, Grimm C, et al. Age-related macular degeneration: the lipofuscin component A2E detaches pro-apoptotic proteins from mitochondria and induces apoptosis in mammalian pigment epithelium cells. *J Biol Chem.* In press.
43. Weng J, Mata NL, Azarian SM, et al. Insights into the function of Rim protein in photoreceptors and etiology of Stargardt's disease from the phenotype in abcr knockout mice. *Cell.* 1999;98:13–23.
44. Cone RA. Early receptor potential: photoreversible charge displacement in rhodopsin. *Science.* 1967;155:1128–1131.
45. Organisciak DT, Li M, Darrow RM, Farber DB. Photoreceptor cell damage by light in young Royal College of Surgeons rats. *Curr Eye Res.* 1999;19:188–196.
46. Szczesny PJ, Munz K, Remé CE. Light damage in the rat retina: patterns of acute lesions and recovery. In: Pleyer U, Schmidt K, Thiel HJ, eds. *Cell and Tissue Protection in Ophthalmology*. Stuttgart: Hippokrates Verlag: 1995:163–175.