Molecular Steps Involved in Light-Induced Oxidative Damage to Retinal Rods

Gian Carlo Demontis, Biancamaria Longoni, and Pier Lorenzo Marchiafava

PURPOSE. To define the molecular mechanism underlying light-induced oxidative damage to retinal photoreceptors.

METHODS. Oxidative stress was induced in isolated rod photoreceptors by bright 470- to 490-nm light and monitored by measuring the conversion of dihydrorhodamine 123 to rhodamine, with fluorescence microscopy. The effect of the wave length on oxidant generation was investigated by applying prebleaching stimuli of either 485- or 520-nm light before the bright 470- to 490-nm light. The role of internal messengers in photooxidative stress and membrane damage by bright 470- to 490-nm light was investigated by patch-clamp recording.

RESULTS. Constant illumination with bright 470- to 490-nm light caused a rapid increase in generation of oxidants, which peaked after approximately 60 seconds, and a decrease in membrane resistance, eventually producing irreversible membrane damage. The time course and extent of oxidant generation were not affected by the absence of intracellular guanosine triphosphate (GTP) or adenosine triphosphate (ATP), suggesting that oxidative stress and membrane damage induced by 470- to 490-nm light do not require coupling to a GTP-binding protein. Prebleaching exposure to 520-nm light suppressed oxidative stress and membrane damage by subsequent application of bright 470- to 490-nm light, and the extent of suppression increased with prebleaching duration.

CONCLUSIONS. Oxidative stress and damage induced in rods in response to 470- to 490-nm light require rhodopsin activation, but not visual transduction steps downstream of active rhodopsin. Prebleaching with 485- or 520-nm light has a different effect on the level of a transient rhodopsin intermediate required for lipid peroxidation by 470- to 490-nm light. (Invest Ophtalmol Vis Sci. 2002;43:2421–2427)

Retinal rod and cone photoreceptors are cells specialized in light detection and transduction into an electrical signal. However, animals exposed to high levels of ambient light may become permanently blind because of extensive loss of retinal photoreceptors. Whereas the molecular steps of the phototransduction cascade have been detailed and described by quantitative models in rods, a much less exhaustive account is available at present for the mechanisms of light-induced damage.

The effects of bright light are associated with increased lipid peroxidation, which causes apoptosis through the downregulation of NF-κB mRNA and its protein in 661-W cells and in mouse photoreceptor cells or through the downregulation of the antiapoptotic protein Bcl-2 in amphibian rods. However, the molecular mechanisms that cause lipid peroxidation in response to intense light are presently unknown.

Apoptotic cell death has also been reported in rods with increased susceptibility to light-induced damage as a result of specific mutations in the rhodopsin gene, which prolong the life of activated rhodopsin (metarhodopsin II [MII]). In addition, it has recently been reported that eyes of mice with no rhodopsin, as a result of a defective synthesis of 11-cis-retinal by pigmented epithelial cells, are protected against light-induced damage. Furthermore, a null mutation in the rhodopsin kinase gene, which also prolongs the life of MII, has been reported to increase the sensitivity to light-induced damage through an apoptotic mechanism. These observations suggest that both lipid peroxidation and prolonged MII life may lead to light-induced apoptosis in rods. However, the possible relationship between active rhodopsin and lipid peroxidation has not so far been clarified, despite extensive investigation in vivo models. Indeed, this failure may be traced to the lack of a suitable model for investigating, in isolated rods, the relationship between lipid peroxidation, membrane damage, and rhodopsin activation that occurs in light-induced damage.

The general purpose of the present work was to investigate at the single-cell level the relationship between activation of rhodopsin and lipid peroxidation in the mechanism of light-induced damage. We used fluorescence microscopy of dihydrorhodamine 123 (DHR123; Molecular Probes, Inc., Eugene, OR) conversion to fluorescent rhodamine (RHO123) by oxidants, to monitor the time course and the subcellular localization of oxidant generation in rods of frog eyes. The role of the visual cascade in light-induced oxidative stress was investigated by patch-clamp recording in the whole-cell mode, which allows both intracellular washout of critical components of the phototransduction cascade and monitoring of the effect of oxidative stress on membrane permeability.

Our data indicate that bright 470- to 490-nm light induces the generation of oxidants in both the inner (IS) and outer segments (OS) of rods, although by means of different mechanisms. In the OS, rhodopsin isomerization appears to be a common molecular step in both visual transduction and light-induced oxidative stress. Activated rhodopsin may act in rods by increasing the chances of lipid peroxidation and membrane damage by 470- to 490-nm light without requiring the guanosine triphosphatase (GTP)- and adenosine triphosphatase (ATP)-dependent steps of the phototransduction cascade.

METHODS

Preparation

Monitoring of oxidant generation by confocal or conventional fluorescence microscopy and patch-clamp recording were conducted, either on isolated retinal rod OS or in OS with the distalmost portion of the inner segment, the ellipsoid, entirely filled with mitochondria (IS), occasionally attached (OS±IS). The size of the rod fragments, which
were obtained from adult frogs (Rana pipiens) dark-adapted for 24 hours as already described.12 was unrelated to the data obtained. For monitoring of lipid peroxidation, the preparation included rods with their cell bodies and nuclear regions, in addition to OS and IS+OS. Animals were reared and killed in accordance with institutional rules for care and handling of experimental animals and in adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Light Stimuli**

Long-wavelength blue light stimuli used for inducing oxidative stress in OS or IS+OS were obtained with band-pass filters for both confocal fluorescence microscopy (constant in the region of 465–495 nm) and conventional fluorescence microscopy (constant in the region 470–490 nm). For prebleaching stimuli with conventional fluorescence microscopy, the transillumination source (a 12-V, 100-W halogen lamp) was filtered with narrow band-pass filters (Linos Photonics [formerly Spindler & Hoyer], Goettingen, Germany) at 485 ± 10 or 520 ± 10 nm. These prebleaching stimuli are thought to have effective singlet to isomerizing rhodopsin.

**Confocal Fluorescence Microscopy**

DHR123 was prepared as a 10 mM stock solution in dimethyl sulfoxide (DMSO). It was brought to its final concentration (10 or 20 μM) in saline, the composition of which was, in millimolar: NaCl, 120; KCl, 2.6; CaCl₂, 3; MgCl₂, 1.5; HEPES, 10; and glucose, 12 [pH 7.6]. After 20 minutes’ incubation with DHR123 at 4°C, cells were observed at 20°C under very dim red light with an inverted microscope (Eclipse 300; Nikon, Tokyo, Japan) equipped with a laser scanning confocal system (Radiance Plus; Bio-Rad, Hercules, CA).

The light from the microscope’s fluorescent lamp provided the phototoxicative stimulus, after band-pass filtering with a cube, whose emission was constant in the region of 465 to 495 nm (465–to-495-nm light). Confocal images were collected at selected times by turning on the Argon laser (λ, 488 nm, 1.4 mW/cm²) at minimum (6%) energy for 2 seconds and using an HQ 515/30 emission filter (Bio-Rad). The laser source provided approximately 2.06 × 10⁶ photons/μm² per second. For 2-second tests applied every 20 seconds, this adds up to approximately 16 × 10⁶ photons/μm² over 1 minute, to be compared with a total of 480 × 10⁶ photons/μm² per minute provided by the phototoxicative stimulus. After analog-to-digital conversion, images were analyzed by computer (Photoshop 5.0; Adobe Systems, Inc., San Diego, CA).

Considering that DHR123 is mostly used for monitoring reactive oxygen species at the mitochondrial level, we verified in preliminary experiments its reactivity with lipid peroxides. DHR123 was incubated with 2,2'-azobis(2-amidinopropane) (ABAP) in the presence of linoleic acid at 37°C. In these conditions, lipid peroxides generated from the reaction of ABAP decomposition products with linoleic acid oxidize DHR123 to fluorescent RHO123. The sensitivity of DHR123 to oxidation by lipid peroxides is also in general agreement with a recent report.14

**Conventional Fluorescence Microscopy**

Conventional fluorescence microscopy was performed (Orthoplan; Leitz, Wetzlar, Germany), as previously described.6,12 The oxidative stimulus consisted of filtered light from the microscope’s fluorescent lamp (HBO 100 W; Osram, Munich, Germany), filtered with a Plömpak K3 cube; Leitz) with an excitation band-pass filter, ranging from 470 to 490 nm (470- to 490-nm light). The fluorescent signal emitted from RHO123 was visualized with a suppression long-pass filter at 515 nm. Incubation with DHR and fluorescence measurements were performed in the same conditions reported for confocal microscopy.

**Thiobarbituric Acid Reactive Species Assay**

Thiobarbituric acid reactive species (TBARS), an indicator of lipid peroxidation,15 were assayed as previously described.6 The light source was a 12-V, 100-W halogen lamp with the infrared component (>700 nm) filtered out. The dish with the cell suspension was placed over ice, and the TBARS assay was performed after an illumination time of 10 minutes. Similar results were observed after 15 minutes of illumination. In a typical experiment, the suspension with OS, IS+OS and rods was split into three identical aliquots to obtain the same protein content. One aliquot was kept in darkness, the second was exposed to bright light, and the third was exposed to light in the presence of the antioxidant Trolox, an analogue of vitamin E (Hoffmann-LaRoche, Basel, Switzerland). All aliquots were kept on ice until assay. Results are thus expressed as the increase compared with darkness (i.e., the internal control).

**Electrophysiological Recording**

Patch pipettes were drawn on a two-stage horizontal puller (BB-CH, Mecanex, Nyon, Switzerland) from 100 μL hemotocrit borosilicate tubes (Brand GMBH, Wertheim, Germany) to a bubble number of approximately 1.5 to 2.0, and their resistance in saline solution was between 1 and 2 MΩ, when filled with an intracellular solution, the composition of which was ([in millimolar]): KCl, 110; NaCl 7; MgCl₂, 1.5; EDTA 2; EGTA 2; and HEPES 5 (pH 7.2 with KOH).

A 100-μL aliquot of isolated rods was added to the recording chamber placed on the stage of an inverted microscope (Diaphot; Nikon) and whole-cell recording was started after a gigaseal was formed, in accordance with standard methods.11 Data were recorded with an amplifier (EPC-7; List Electronics, Germany), and filtered at 20 Hz with a four-pole Butterworth filter, unless differently specified. Data were digitized (LabMaster A/D Board; Axon Instruments, Foster City, CA) at a sampling rate of 500 Hz, unless differently specified, and stored online on the hard disk of a 486-MHz microprocessor-based computer.

Direct illumination with the bright light was in the form of a 20-μm diameter spot, illuminating the OS, away from the area of the membrane contacted by the pipette, to avoid unspecific effects on the lipid–glass seal.

**RESULTS**

**Properties of Light-Induced Lipid Peroxidation in Retinal Rods**

As shown in Figure 1, bright light (LIGHT) increased 6.1 ± 0.4-fold compared with darkness (DARK), with the level of
Figure 2. Imaging of light-induced oxidative stress by confocal microscopy. (A) Confocal imaging of DHR123 oxidation by light at the beginning of light application. (B, C, D) Changes in fluorescence after 20, 40, and 60 seconds (numbers, top right) of steady illumination with 465- to 495-nm light. Imaging was obtained at selected times by turning on (2-second) the laser light at minimum energy. (D, dotted areas) ROIs (966 pixels) in the inner (I.S.) and the outer (O.S.) segments, respectively. (E) Relative increase in fluorescence measured over the ROI. Fluorescence counts were normally distributed for both IS and OS up to 60 seconds. After 100 seconds of steady illumination, the distribution of fluorescence in the OS was skewed, suggesting saturation of the imaging system. The relative increase in fluorescence was computed in accordance with the relationship: Fluorescence increase, \( F_t = (F_t - F_0)/F_0 \), with \( F_0 \) and \( F_t \) being the average fluorescence over ROI at time \( t \) and time 0, respectively. \( F_t \) was 24.5 and 9.2 counts/pixel for IS and OS, respectively. (F) Photometric recordings of DHR123 oxidation to fluorescent RHO123 were obtained with conventional fluorescence microscopy during steady illumination with 470- to 490-nm light. Two OS were illuminated with either unattenuated (0.0) or half-attenuated stimuli (0.5).

Attenuation was controlled by a calibrated neutral-density filter. The unattenuated stimulus intensity was \( 7.98 \times 10^6 \) photons/\( \mu m^2 \) per second. Because the stimulus and the excitation light are the same, attenuation of the stimulus also attenuates the fluorescent signal recorded. To correct for reduced intensity of the fluorescence emitted, after verification of linearity in the ratio between excitation and emission intensity, the record labeled 0.3 was scaled by a factor of 2 before plotting.

TBARS in retinal preparations consisting of OS, IS+OS, and intact rods, indicating the ability of light to increase lipid peroxidation. In the presence of the antioxidant Trolox (LIGHT+TROLOX) the TBARS level decreased to 0.5 ± 0.2 times the dark value, suggesting that lipid peroxidation also occurs in darkness. The effects of both light and light + Trolox are significantly different from those of darkness (\( P < 0.01; \) one-way ANOVA).

**Imaging of Light-Induced Oxidative Stress in Retinal Rods**

The subcellular localization of lipid peroxidation was investigated by confocal microscopy of DHR123, which is oxidized by lipid peroxides to fluorescent RHO123. \(^{14}\) (see the Methods section). In Figure 2, confocal microscopy images of time-dependent formation of RHO123 during continuous illumination of an OS+IS sample with 465- to 495-nm light are illustrated in Figures 2A to 2D. At the beginning of light application (Fig. 2A, 0 sec), the IS was appreciable, whereas the OS was barely visible. After 20 seconds of illumination (Fig. 2B) both OS and IS were clearly discernible, and the fluorescence further increased after 40 (Fig. 2C) and 60 seconds of light exposure (Fig. 2D).

Changes in the fluorescence intensity over regions of interest (ROIs), indicated in Figure 2D in both IS and OS samples by the white dotted ellipsoids, are shown in Figure 2E. Average ROI luminosity (normalized to fluorescence intensity at time 0) over IS (Fig. 2E, open circles) reached a plateau at a level approximately 1.5 times basal. In contrast, the average fluorescence over the ROIs of OS (Fig. 2E, filled circles) increased 6.5 times basal. A comparable result was obtained in three additional experiments, with an average relative increase in fluorescence of 2.0 ± 0.5- and 6.8 ± 0.6-fold, in the IS and OS, respectively. Control experiments on the effects of the laser light were performed in three independent experiments in the absence of 465- to 495-nm light, by measuring DHR123 oxidation with 2 seconds of laser illumination every 20 seconds. In these conditions, the relative increases in fluorescence in the inner and outer segment were 1.1 ± 0.2- and 1.7 ± 0.3-fold, respectively. These results suggest that the changes in DHR123 oxidation observed on exposure to steady 465- to 495-nm light may not be ascribed to the 2-second illumination with the attenuated laser light.

The kinetics and the intensity-dependence of the generation of light-induced oxidants in the OS were investigated by conventional fluorescence microscopy, and the results are illustrated in Figure 2F. Traces plot the time-course of the increase in fluorescence induced in an OS by steady illumination with 470- to 490-nm light delivering \( 7.98 \times 10^6 \) (0.0) and \( 3.95 \times 10^6 \) (0.5) photons/\( \mu m^2 \) per second (calculated at 480 nm), respectively. At both intensities, after an initial slow increase the fluorescence steeply increased to a peak level, which was followed by the decay of fluorescence with the brightest light used (also Fig. 3B).
Role of Rhodopsin in Light-Induced Oxidative Stress in the Outer Segment of Frog Rods

The role of rhodopsin in the oxidative stress induced by 470- to 490-nm light was investigated with prebleaching stimuli of different wavelengths but similar effectiveness in causing rhodopsin isomerization. The oxidative response to bright 470- to 490-nm light was measured after a 1-minute application of either 485- or 520-nm prebleaching stimuli (photon fluxes of 1.02 × 10⁶ or 1.04 × 10⁶ photons/μm² per second, respectively). The rationale behind this is that if rhodopsin has an exclusive role in light-induced oxidative stress, then a similar oxidative response to the bright 470- to 490-nm light would be expected from rods conditioned by a 1-minute prebleaching with either 520- or 485-nm light. However, data in Figure 4A...
higher in response to 485-nm (filled circles) than to 525-nm (open circles) light, with slopes of 1.09 pW/sec and 0.25 pW/sec, respectively. These results suggest that similar levels of isomerized rhodopsin produce oxidative responses that critically depend on stimulus wavelength.

**Discussion**

The present study indicates that oxidative stress occurs in both the IS and OS of retinal rods, although by different mechanisms. In particular, light-induced oxidative stress and damage in the OS result from a novel, unconventional action of rhodopsin, which acts without coupling to a GTP-binding protein.

**Cation Influx and Oxidative Stress in the IS of Retinal Rods**

Data in Figure 1 suggest the occurrence of lipid peroxidation both in light and in darkness. The imaging of oxidative stress illustrated in Figure 2A provides direct evidence for the generation in darkness of oxidants by showing IS fluorescence at the beginning of illumination.

The steady sodium influx that takes place in darkness through the light-sensitive channels and the Na⁺-Ca²⁺,K⁺ exchanger must be balanced by the operation of Na⁺,K⁺-adenosine triphosphatase (ATPase), which imposes a high metabolic load on rods. This metabolic load is mostly satisfied through the oxidative metabolism that takes place in mitochondria, which are compartmentalized in the IS. Considering that the respiratory chain of mitochondria is a well-known source of reactive-oxygen species, the oxidative stress that occurs in the IS in darkness is likely to result from the metabolic cost of extrusion of cations.

In general agreement with this hypothesis, the increase in IS fluorescence during illumination with bright 470- to 490-nm light (Figs. 2A–D) is associated with an increase in inward current, from -35 to -600 pA (Fig. 3B). This increase in inward current during exposure to bright light is thought to cause a metabolic load, increased mitochondrial activity, and oxidant generation, which in turn enhances the fluorescence in the IS. This is in agreement with the idea that oxidative stress in the inner segment, either in darkness or during illumination, is of metabolic origin and associated with the mitochondrial respiration that provides the energy required for ion transport.

**Rhodopsin Isomerization and Oxidative Stress in the Outer Segment of Retinal Rods**

Data in Figures 2A through 2D indicate that light causes a relative increase in oxidative stress that is larger in the OS than in the IS of rods, suggesting a role for molecular components that is specific to the OS. The oxidative stress occurring in the IS is unlikely to be of metabolic origin, because mitochondria are compartmentalized in the IS. The probe sensitivity to lipid peroxides suggests that the increased fluorescence in the OS during illumination may mirror lipid peroxidation. For instance, light-induced isomerization of rhodopsin may cause G-protein–mediated activation of phospholipase A₂ and release of arachidonic acid, which is further metabolized to lipid peroxides. However, this hypothesis is not supported by the different oxidative responses to prebleaching stimuli that generate similar amounts of isomerized rhodopsin. It is possible that the previously reported increase in phospholipase A₂ activity in response to illumination is a consequence, rather than a cause, of light-induced oxidative stress. Direct evidence against the role of phospholipase A₂ or other biochemical steps activated by isomerized rhodopsin is provided by the data in Figure 3B, which show that the oxidative stress and damage induced by the 470- to 490-nm light in the OS do
not require ATP- and GTP-dependent steps of the phototransduction cascade downstream of rhodopsin.

Several mechanisms may cause oxidative stress in the OS in response to 470- to 490-nm light without involving transductional steps downstream of activated rhodopsin. For instance, 470- to 490-nm light may act by a direct action on polyunsaturated fatty acids that are specific for the OS and especially susceptible to peroxidation, such as docosahexaenoic acid. Although polyunsaturated lipids’ peak absorbance is far from the stimulus wavelength that induces oxidative stress (470–490 nm), light of 485 nm is thought to be more effective than that of 520 nm. In addition, direct action of 470- to 490-nm light on bulk polyunsaturated lipids should be independent of rhodopsin isomerization. In agreement with this hypothesis, the results in Figure 4A show that the 485-nm prebleaching stimulus was more effective in causing oxidative stress than that of 520 nm. However, the suppression of the oxidative response (Fig. 4A) after a 1-minute 520-nm prebleaching argues against the hypothesis that 470- to 490-nm light may cause peroxidation of bulk lipids in the disc membrane by a rhodopsin-independent mechanism.

The data in Figure 4A support the possibility that rhodopsin plays a key role in the oxidative stress induced by 470- to 490-nm light. One hypothesis is that light-induced oxidative stress in the OS is associated with double bond breaking of 11-cis-retinal during cis-trans isomerization. In the case of free retinal, this photochemical reaction has been reported to cause the formation of oxygen free radicals. An alternative hypothesis is that a transient state of active rhodopsin may promote the peroxidation by 470- to 490-nm light of a subset of lipids tightly bound to active rhodopsin, before decaying to inactive intermediates. In this case, the complex formed by lipids with active rhodopsin may absorb the 470- to 490-nm light that causes the peroxidation of lipids in the complex. The idea implicit in this hypothesis is that rhodopsin activation affects a subset, rather than the bulk, of membrane lipids. Both hypotheses have weaknesses. A problem with oxygen radical formation during retinal isomerization is that it is unknown whether oxygen may access the rhodopsin pocket, where retinal is enclosed, during the photochemical reaction that occurs on a time scale of picoseconds. An additional problem with this hypothesis is that the formation of the superoxide radical from a retinal Shiff base during irradiation with light above 455 nm, in the presence of reduced nicotinamide (NADH), has a low quantum yield. However, it is presently unknown whether the polyunsaturated lipid–rhodopsin complex may absorb effectively at 470 to 490 nm.

An additional problem with the hypothesis of the lipid–rhodopsin complex is the nature of the transient form of active rhodopsin. The life span of the active rhodopsin intermediate that is necessary, but not sufficient, for 470-to 490-nm light-induced lipid peroxidation and membrane damage may provide a clue to its identification. The first state of isomerized rhodopsin with a lifetime of seconds is MII. MII decays to metarhodopsin III (MIII), with a time constant of approximately 100 seconds, and to free retinal and opsin with a time constant of approximately 15 minutes. MIII absorption peaks at 465 nm and may play a causative role in the oxidative stress induced in the OS by absorbing 470- to 490-nm light. However, the hypothesis is not consistent with three observations. First, the absorption of 470- to 490-nm light would shift MII–MIII equilibrium toward MIII, thus reducing MIII levels. Second, the time course of MIII formation is slower than the oxidative response induced by 470- to 490-nm light. Third, an increase, rather than a suppression, of oxidative stress should be expected after 520 ± 10-nm prebleaching stimuli that favor formation of MII. Data in Figure 4A, documenting such suppression, are not consistent with a role for MIII as a chromophore involved in photooxidative stress by 470- to 490-nm light.

Based on kinetic considerations, however, MII is a good candidate for the rhodopsin intermediate promoting peroxidation of OS lipids by 470- to 490-nm light. Furthermore, rhodopsin isomerization may cause the formation of a carbonyl group of a subset of membrane phospholipids closely associated with rhodopsin, perhaps a consequence of conformational modifications leading to volume changes on MII formation. The bending or stretching of the double bonds of lipids closely associated with MII, by modifying the energy level, may shift their absorption peak toward longer wavelengths and increase the susceptibility to lipid peroxidation by 470- to 490-nm light. However, this point is rather speculative at this stage and requires further investigation.

As a final remark, we note that Trolox, the water-soluble analogue of vitamin E, prevents light-induced lipid peroxidation in isolated rods. The water-soluble antioxidant ascorbate has been reported to protect photoreceptors of either dark-reared or cyclic-light–reared rats from exposure to bright light. It has been proposed that ascorbate protects the eye from the irreversible type I form of light damage in dark-reared rats. Although ascorbate protection suggests that oxidative stress plays a key role in light-induced damage, other evidence is controversial. For example, vitamin E does not protect photoreceptors from damage induced by the exposure to bright cyclic light, and ascorbate appears to shift light damage to the type II form typical in cyclic-light–reared animals. In the future, the cellular approach may clarify the protective role of antioxidants against specific forms of light-induced damage.

**Acknowledgments**

The authors thank Giovanna Bresciani, Emilio Cardaci, and Gino Bertolini for skilled technical support.

**References**


