Preconditioning with Bright Light Evokes a Protective Response against Light Damage in the Rat Retina

Changdong Liu, Min Peng, Alan M. Laties, and Rong Wen

Departments of Ophthalmology and Cell and Developmental Biology, University of Pennsylvania, School of Medicine, Philadelphia, Pennsylvania 19104

Constant exposure to bright light induces photoreceptor degeneration and at the same time upregulates the expression of several neurotrophic factors in the retina. The question is whether the induced neurotrophic factors protect photoreceptors. We used a preconditioning paradigm to show that animals preconditioned with bright light became resistant to subsequent light damage. This paradigm consisted of a 12–48 hr preexposure, followed by a 48 hr “rest phase” of normal cyclic lighting. The greatest protection was achieved by a 12 hr preexposure. Preconditioning induces a prolonged increase in two endogenous neurotrophic factors: basic fibroblast growth factor (bFGF) and ciliary neurotrophic factor (CNTF). It also stimulates the phosphorylation of extracellular signal-regulated protein kinases (Erks) in both photoreceptors and Müller cells. These findings indicate that exposure to bright light initiates two opposing processes: a fast degenerative process that kills photoreceptors and a relatively slower process that leads to the protection of photoreceptors. The extent of light damage, therefore, depends on the interaction of the two processes. These results also suggest a role of endogenous bFGF and CNTF in photoreceptor protection and the importance of Erk activation in photoreceptor survival.

Key words: photoreceptor; Müller cell; light damage; degeneration; bFGF; CNTF; Erk; retina; rat

Noell and coworkers reported in 1966 that unremitting exposure to visible light, even at low irradiance levels, induced photoreceptor degeneration in albino rats. Although the exact mechanism is still not fully understood, this model of induced photoreceptor degeneration has been used widely to study the capability of photoreceptor protection by antioxidants (Organisciak and Winkler, 1994), neurotrophic factors (Faktorovich et al., 1992; LaVail et al., 1992), and agents that induce endogenous neurotrophic factors (Wen et al., 1996).

Recent studies provide evidence that endogenous neurotrophic factors protect photoreceptors from degeneration. For example, photoreceptors near a wound site are protected from light damage (Faktorovich et al., 1992; Wen et al., 1995). The injury-induced photoreceptor protection is accompanied by a dramatic increase in the expression of basic fibroblast growth factor (bFGF) and ciliary neurotrophic factor (CNTF) surrounding the wound (Wen et al., 1995). These findings lead to a hypothesis that the retina responds to injury by upregulating neurotrophic factors to protect retinal cells and to accelerate repair and wound healing (Wen et al., 1995). Consistent with the hypothesis are the observations that retinal bFGF and CNTF are elevated in animals undergoing inherited retinal degenerations (Gao and Hollyfield, 1995) or light damage (Steinberg et al., 1995; Gao and Hollyfield, 1996).

Despite the upregulation of neurotrophic factors, severe loss of photoreceptors occurs in inherited or light-induced retinal degeneration. This raises a question as to whether these endogenous factors really protect photoreceptors. One possible explanation is that these factors do protect photoreceptors to some extent. In their absence photoreceptor loss would be more severe, yet in the presence of progressive photoreceptor degeneration, such protective protection is hard to demonstrate.

In the present work we used a preconditioning paradigm to detect the putative protection. The preconditioning consisted of exposure to bright light, followed by a “rest phase” in normal cyclic light. Animals so preconditioned displayed a remarkable resistance to light damage. The preconditioning resulted in a prolonged expression of bFGF and CNTF in the retina. It also activated extracellular signal-regulated protein kinases (Erks) in both photoreceptors and Müller cells. These findings provide evidence that exposure to bright light evokes a response that protects photoreceptors and suggest that neurotrophic factors likely mediate the protection.

MATERIALS AND METHODS

Animals and light exposure. Male Sprague Dawley rats (2–3 months old) were used in all experiments. Animals were kept in a 12:12 hr light/dark cycle at an in-cage illumination of <10 candelas (1 cd = 10.76 lux) for at least 7 d before the experiments. Preexposure to bright light was performed in a constant-light room of white fluorescent light in which the in-cage illuminance was 115–130 cd. Photoreceptor degeneration (light damage) was induced by exposing animals to the same intensity continuously for 7 d. The in-cage temperature was kept at 20–22°C.

Histological evaluation of photoreceptor preservation. Animals were killed by CO₂ overdose, immediately followed by vascular perfusion with mixed aldehydes (LaVail and Battelle, 1975). Eyes were embedded in an Epon/Araldite mixture and sectioned at 1 μm thickness to display the entire retina along the vertical meridian of the eye (LaVail and Battelle, 1975). Photoreceptor preservation was assessed by light microscopy, using a scoring system to account for the well known nonuniform distribution of light damage across the retina and, in each retinal region, the number of surviving photoreceptor nuclei as well as the condition of the inner and outer segments of photoreceptors. The system used a five point scale, with the score for normal retina being five and the most severe loss of photoreceptors being one (Wen et al., 1996). Each tissue section was...
assessed independently by three scientists equally familiar with the scoring criteria.

**RNA preparation and Northern blot analysis.** Retinas were dissected, snap-frozen in liquid nitrogen, and stored at \(-80^\circ\text{C}\). Total RNA was obtained from pooled retinas with an RNeasy Total RNA Kit (Qiagen, Chatsworth, CA) according to the manufacturer’s instructions. Northern blot analyses were performed as previously described (Wen et al., 1995). Briefly, total RNA (20 \(\mu\text{g}\) of each sample) was electrophoresed on 1% agarose formaldehyde gels and transferred to a nylon membrane (Hybond-N, Amersham, Arlington Heights, IL). Blots were prehybridized for 4 hr at 50°C. Random primed \(^{32}\)P-labeled cDNA probes for rat bFGF (gift of Dr. A. D. Baird, Whittier Institute for Diabetes and Endocrinology, La Jolla, CA; (Shimazaki et al., 1988)), rat CNTF (gift of Dr. N. Y. Ip, Regeneron Pharmaceuticals, Tarrytown, NY (Stöckli et al., 1989)), or rat 18s RNA (gift of Dr. D. Schlessinger, Washington University, St. Louis, MO; (Bowman et al., 1981)) were added to the hybridization buffer (10 \(\text{cpm/ml}\)) and hybridized at 50°C overnight. Blots were exposed to a Storage Phosphor Screen (Molecular Dynamics, Sunnyvale, CA), and data were digitized by scanning the phosphor screen with a PhosphorImager System (Molecular Dynamics).

**Protein preparation and immunoblotting analysis.** Retinas were dissected, snap-frozen in liquid nitrogen, and stored at \(-80^\circ\text{C}\). Pooled retinas were homogenized, and total protein (100 \(\mu\text{g/lane}\)) was electrophoresed on polyacrylamide gels and transferred to nitrocellular membranes (Bio-Rad Labs, Hercules, CA). Blots were examined by immunoblotting analysis, using the following antibodies: rabbit anti-bFGF polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), chicken anti-CNTF polyclonal antibodies (Promega, Madison, WI), rabbit anti-p44/42 phospho–Erk polyclonal antibodies (Promega), and rabbit anti-p44/42 Erk polyclonal antibodies (New England Biolabs, Beverly, MA). Signals were visualized with an ECL kit (Amersham, Arlington Heights, IL) and recorded on Hyperfilm (Amersham).

**Immunocytochemistry.** Eyes, removed from 4% paraformaldehyde-perfused animals, were cryoprotected with 20% sucrose, frozen in Tissue-Tek OCT compound (Miles, Elkhart, IN) in powdered dry ice, and stored at \(-80^\circ\text{C}\). Cryosections of 10 \(\mu\text{m}\) were cut through the entire retina along the vertical meridian and thaw-mounted onto Super Frost Plus glass slides (Fisher Scientific, Pittsburgh, PA). For immunostaining, sections were rinsed in PBS and permeabilized with 0.1% Triton X-100 for 30 min. The sections were incubated with blocking solution (10% goat serum in PBS) for 1 hr, followed by a 2 hr incubation with anti-phospho–Erk (1:800 dilution) antibodies at room temperature. Immunoreactivity was visualized with an ABC kit (Vector Laboratories, Burlingame, CA) and a TSA-Direct kit (NEN-Life Science Products, Boston, MA), according to the manufacturers’ instructions.

**RESULTS**

**Preconditioning protects photoreceptors from light damage**

The preconditioning paradigm is illustrated schematically in Figure 1. For preconditioning, animals were preexposed for 12, 24, or 48 hr to an intensity of 115–130 cd. On termination of the preexposure, animals were returned to normal cyclic light conditions for a “rest phase” of 48 hr. To induce light damage, we placed preconditioned animals to constant light of 115–130 cd for 7 d immediately after the rest phase. Controls received constant light of 115–130 cd for 7 d without any preconditioning.

Figure 2 shows representative sections of superior retinas from a normal animal (Fig. 2A) and animals that received constant light exposure (115–130 cd) for 7 d without (Fig. 2B) or with (Fig. 2C) a 1 hr preconditioning. Severe photoreceptor degeneration was observed in animals without preconditioning (Fig. 2B). In these retinas the outer nuclear layer (ONL), where photoreceptor nuclei reside, was reduced from 10–11 rows of nuclei in normal animals (Fig. 2A) to one to two rows (Fig. 2B). There was almost a complete absence of photoreceptor inner segments, and the remaining outer segments formed large rounded or oblong profiles (Fig. 2B). In the preconditioned animals, however, the photoreceptor degeneration was much less severe (Fig. 2C). There were, on average, five to seven rows of photoreceptor nuclei in the ONL. The inner segments were present, although shorter than normal. The outer segments were better preserved; many also showed rounded and oblong profiles (Fig. 2C).

The degree of photoreceptor preservation was scored. In animals without preconditioning the degree of photoreceptor preservation after 7 d of constant light exposure is 1.10 ± 0.17 (mean ± SD, \(n = 12\)). In animals given a 12 hr preconditioning, the score is 3.17 ± 0.22 (\(n = 6\)) and 2.96 ± 0.21 (\(n = 12\)) for those that received a 24 hr preconditioning. The score for animals of 48 hr preconditioning is 1.91 ± 0.52 (\(n = 12\)) (Fig. 3).

The lesser protection in animals that received longer preconditioning suggests that some light damage resulted from the preexposure. To assess the possible light damage induced by preconditioning itself, we exposed animals to 115–130 cd for 12, 24, or 48 hr and returned them to normal cyclic light for 9 d (to match the 2 d of the rest phase and the 7 d of constant light exposure). Figure 4 shows representative superior retinas from these animals. For those receiving a 12 hr exposure, there was no obvious morphological change in their photoreceptors (Fig. 4A). The outer and inner segments of photoreceptors appeared similar to those of normal animals (see Fig. 2A). No measurable loss of photoreceptor nuclei was found in the ONL (Fig. 4A). In contrast, 24 hr of exposure led to disorganization...
of the outer segments and enlargement of their tips. There was measurable shortening of the inner segments, and the ONL was reduced by one to two rows of cell nuclei (Fig. 4B). Significant light damage was evident in retinas after 48 hr of exposure (Fig. 4C). The outer segments of photoreceptors exhibited rounded and oblong profiles. There was a significant shortening of the inner segments. Moreover, the ONL was reduced to six to seven rows of nuclei. In 24 and 48 hr exposed animals, the damage to the inferior retinas was slightly greater than that superiorly (data not shown).

**Figure 2.** Protection of photoreceptors by 12 hr preconditioning. A, Normal retina (superior region) of a rat kept in cyclic light. The photoreceptor outer segments (OS) are opposed to the retinal pigment epithelium (RPE), distinct photoreceptor inner segments (IS) are present, and the outer nuclear layer (ONL) consists of 10–11 rows of photoreceptor cell nuclei. B, Retina of a rat exposed to 7 d of constant light without preconditioning (superior region). The ONL is reduced to one to two rows of nuclei; the inner segments are missing or are reduced to short stumps; the few remaining outer segments are in the form of large rounded or oblong profiles. C, Superior retina of a rat that received a 12 hr preconditioning and then was exposed to 7 d of constant light. The ONL shows six to seven rows of nuclei; the inner segments are shorter than normal, and the outer segments, although better preserved than in B, are disorganized, many showing rounded and oblong profiles. Toluidine blue stain was used. OPL, Outer plexiform layer; INL, inner nuclear layer. Scale bar, 20 μm.

**Preconditioning induces prolonged expression of bFGF and CNTF**

Previous work showed that, among several neurotrophic factors, only the expression of bFGF and CNTF was induced by exposure to constant light (Steinberg et al., 1995). We therefore examined the mRNA and protein expression of these two neurotrophic factors. Northern blotting analysis was used to determine the mRNA expression of bFGF and CNTF. Retinas from animals exposed to bright light for 12 hr were collected at 0, 0.5, 1, 2, 4, 7,
or 10 d after exposure. A major 7.0 kb transcript was detected by using probes complementary to mRNA encoding for bFGF (Fig. 5, top). A significant increase in bFGF mRNA was present by the end of the exposure (day 0); the maximum was reached within 1 d after exposure. Although the expression then slowly declined, it was still at a relatively high level 10 d after exposure. CNTF mRNA was detected as a single band of 1.2 kb (Fig. 5, middle). An increase in CNTF mRNA was first detectable 0.5 d after exposure, reaching its peak in 4 d. The level of 18s rRNA was assessed as a control for RNA loading (Fig. 5, bottom).

The protein expression of bFGF and CNTF was determined by immunoblotting analyses. bFGF protein was detected as three bands at 24, 22.5, and 18 kDa. Its expression showed a progressive increase in CNTF mRNA was detected as a single band of 1.2 kb (Fig. 5, middle). An increase in CNTF mRNA was first detectable 0.5 d after exposure, reaching its peak in 4 d. The level of 18s rRNA was assessed as a control for RNA loading (Fig. 5, bottom).

Preconditioning induces phosphorylation/activation of Erk in photoreceptors and Müller cells

We next investigated the activation state of Erks by immunoblotting, using anti-phospho–Erk antibodies to recognize specifically the dually phosphorylated form of Erk1 (p44) and Erk2 (p42). As shown in Figure 7 (top), a dramatic increase in Erk phosphorylation was observed immediately after a 12 hr exposure to 115–130 cd, which lasted at least 12 hr. The protein levels of the two Erks were not altered by the exposure (see Fig. 6, bottom).

The induced phospho–Erks in the retina were localized by immunocytochemistry. As shown in Figure 8, in the normal retina only a few cells in the inner nuclear layer were phospho-Erk-positive; at least one could be identified as a Müller cell, its typical radial processes positively stained (Fig. 8A). After a 12 hr exposure to 115–130 cd, some inner segments of photoreceptors stained positively (Fig. 8B, arrowheads). In addition, there was a dramatic increase in phospho–Erk-positive cell bodies in the inner nuclear layer (Fig. 8B). In most of these cells, positive immunoreactivity also was detected in processes that extended to the inner and the outer limiting membranes, identifying them as Müller cells (Fig. 8B).

DISCUSSION

We have used a preconditioning paradigm to reveal a remarkable ability of the retina to mount a protective response for photoreceptors. The preconditioning paradigm is composed of two parts: a short preexposure to bright light that initiates the protective response and a 48 hr “rest phase” under normal cyclic light that allows the protective response to develop. Retinas so preconditioned, exhibit a substantial resistance to light damage. These results indicate that exposure to bright light initiates two opposing processes in the retina: a degenerative process that kills photoreceptors and a protective response that protects them. The time courses of the two processes are quite different. The degenerative one is faster, causing accelerated death of photoreceptors in the first 2 d of exposure, especially during the second day of exposure. The protective response is slower and requires 2 d to develop fully. Thus, during the first 2 d of continuous exposure to bright light (115–130 cd in the present work), massive photoreceptor death occurs before the protective response is fully developed.

Since its discovery in 1966, continued research efforts have provided a wealth of information on light damage (for review, see Organisciak and Winkler, 1994; Rapp, 1995). It already is known that susceptibility to light damage relates to light intensity of the rearing environment of animals (Penn and Anderson, 1994). The relatively low light damage susceptibility of animals raised in bright lighting environment has been explained in part by increased antioxidant levels found in retinas of these animals (Penn et al., 1987). Recently, the photoreceptor protection properties of several neurotrophic factors, including bFGF and CNTF, have been clearly demonstrated against light damage in rats, as well as in the RCS (Royal College of Surgeons) rats bearing an inherited photoreceptor degeneration (Faktorovich et al., 1990, 1992; La-Vail et al., 1992). In addition, upregulation of endogenous bFGF and CNTF observed in mechanically injured retina is believed to be responsible for injury-induced photoreceptor protection (Wen et al., 1995). Increased bFGF or CNTF expression also is found in inherited and induced retinal degenerative animal models (Gao and Hollyfield, 1995, 1996; Steinberg et al., 1995). In the present work we show that a 12 hr exposure to bright light induces a large and prolonged increase in bFGF and CNTF expression. Moreover, significant elevation of the two proteins coincides with the 2 d “rest phase” of the preconditioning paradigm. Together, these findings strongly suggest that endogenous neurotrophic factors take part in the preconditioning-induced photoreceptor protection.

Protection of photoreceptors by preconditioning exposure resembles “conditioning lesion” effects previously recognized in the brain and the peripheral nervous system. An initial (conditioning) lesion facilitates tissue recovery from a subsequent (test) lesion (McQuarrie et al., 1977; Nieto-Sampedro et al., 1984; Perez-Polo et al., 1990). The accelerated recovery from the second lesion has been attributed to an increase in synthesis and the secretion of
Figure 4. Light damage by preexposure. A, Superior retina of a rat received a 12 hr exposure. Outer and inner segments of photoreceptors appear similar to normal control (see Fig. 2A). There is no apparent loss of photoreceptor nuclei in the ONL. B, Superior retina from a rat of 24 hr exposure. Outer segments of photoreceptors are disorganized, and their tips are enlarged. Inner segments are shortened. The ONL is reduced by one to two rows of cell nuclei. C, Retina of a rat exposed to constant light for 48 hr. Outer segments of photoreceptors clearly are damaged, showing rounded and oblong profiles. The inner segments are shortened significantly. The ONL is reduced to six to seven rows of nuclei. Abbreviations, staining, and scale bar are the same as in Figure 1.
neurotrophic factors stimulated by the conditioning lesion (Nieto-Sampedro and Cotman, 1985), such as bFGF (Finklestein et al., 1988; Frautschy et al., 1991; Logan et al., 1992) or CNTF (Ip et al., 1993).

Many neurotrophic factors, including NGF, BDNF, and bFGF, exert their effects by interacting with receptor tyrosine kinases and by activating the Ras/Raf/MAPK (mitogen-activated protein kinase) pathway. Activation of Erks is well recognized as an essential step in the Ras/MAPK cascade (Davis, 1993; Nishida and Gotoh, 1993; Leevers and Marshall, 1995). Some stress signals also use this signaling pathway for Erk activation. For example, ultraviolet irradiation induces phosphorylation of Erks in HeLa cells via growth factor receptors and a Ras-dependent pathway (Sachsenmaier et al., 1994). H$_2$O$_2$ rapidly induces Erk phosphorylation in National Institutes of Health 3T3 and PC 12 cells also via a Ras-dependent pathway (Guyton et al., 1996). In addition, inhibition of H$_2$O$_2$-induced Erk activation greatly increases the susceptibility of cells to H$_2$O$_2$ toxicity (Guyton et al., 1996).

Figure 5. Expression of bFGF and CNTF mRNAs after 12 hr exposure. A Northern blot was hybridized with probes for bFGF and rehybridized with probes for CNTF. A major 7.0 kb transcript was detected in all lanes (top). The same blot was rehybridized with probes for CNTF mRNA. CNTF transcript was detected as a 1.2 kb band (middle). The time after the 12 hr preexposure is indicated at the top of each lane. The blot finally was rehybridized with probes for 18s rRNA (bottom).

Figure 6. Expression of bFGF and CNTF protein after 12 hr exposure. Immunoblotting analyses were performed to assess bFGF and CNTF proteins after a 12 hr preexposure. Three isoforms of bFGF protein of 24, 22.5, and 18 kDa were detected. CNTF protein was found as a single band at 26 kDa. The time after the 12 hr preexposure is indicated at the top of each lane.

Figure 7. Phosphorylation of Erks after 12 hr exposure. Immunoblotting analyses were performed to detect Erk phosphorylation in the retina. Dually phosphorylated Erks (pp-Erk) or Erk proteins (Erk) were detected as two bands at 44 and 42 kDa. The time after the 12 hr preexposure is indicated at the top of each lane.
by the similarity of induced expression of neurotrophic factors by exposure to bright light (Steinberg et al., 1995) and by mechanical injury (Wen et al., 1995). Further investigation of such a mechanism could well have therapeutic potential for degenerative disorders of photoreceptors.

REFERENCES


