The Effect of Ischemic Preconditioning on Light-Induced Photoreceptor Injury

Robert J. Casson, John P. M. Wood, Jose Melena, Glyn Chidlow, and Neville N. Osborne

PURPOSE. To determine whether ischemic preconditioning (IPC) upregulates certain retinal survival factors and to assess the protective effect of retinal IPC against light-induced photoreceptor degeneration.

METHODS. Albino rats underwent IPC induced by raising the intraocular pressure in one eye to 120 mm Hg for 5 minutes. The fellow eye underwent sham treatment. Basic fibroblast growth factor (bFGF), ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), glial fibrillary acidic protein (GFAP), and Bcl-2 were measured after 6 and 48 hours, by the reverse transcription–polymerase chain reaction and immunoblot analysis. Other preconditioned rats received 48 hours of photic injury (2000 lux) 24 hours after IPC. The a- and b-wave amplitudes of the flash electoretinograms were measured 5 days later, followed by analysis of rhodopsin mRNA levels and histology. The influence of adenosine A1 receptor blockade was assessed.

RESULTS. bFGF, GFAP, and Bcl-2 were upregulated after IPC. BDNF was not upregulated. The marked reduction of the a- and b-wave amplitudes and the structural injury to the photoreceptors induced by the photic insult were significantly reduced by IPC. The protection afforded by IPC was not influenced by adenosine A1 antagonism.

CONCLUSIONS. IPC upregulates bFGF, GFAP, and Bcl-2 and protects photoreceptors against light-induced injury. These factors may be involved in the protective response. (Invest Ophthalmol Vis Sci. 2003;44:1348–1354) DOI:10.1167/iovs.02-0368

Ischemic preconditioning (IPC) refers to the phenomenon whereby a brief period of ischemia protects a tissue against a subsequent severe ischemic insult. This effect has been demonstrated in myocardium,5–3 brain,4,5 and, more recently, in retina.6–11 Roth et al.6 reported that IPC affords the retina a greater degree of functional protection against an ischemic insult than any known neuroprotective agent, making IPC in the retina a particularly attractive area for further research. Although some requirements for the production of IPC in the retina have been established,8–11 the mechanisms involved in this conditioning response remain poorly understood.

Other types of preconditioning injury (mechanical injury,3,13 bright light,3 and sectioning of the optic nerve13) have been shown to protect the retina against light-induced injury. There is considerable evidence implicating trophic factors, particularly basic fibroblast growth factor (bFGF), in all these conditioning responses. Faktorovich et al.12 noted that subretinal insertion of a dry needle provides localized protection against photoreceptor degeneration in the Royal College of Surgeons (RCS) rat and suggested that this may be due to release of endogenous bFGF. Wen et al.16 later showed that bFGF and ciliary neurotrophic factor (CNTF) mRNAs are upregulated in the retina after subretinal incision and proposed that the retina mounts an endogenous neuroprotective response by upregulating trophic factors after an injury. Similarly, others have shown an upregulation of bFGF and CNTF after photic injury14,17 and after axotomy,18,19 indicating that the preconditioning injury may evoke generalized protective responses, independent of the nature of the stimulus.

We hypothesized, therefore, that retinal IPC may also upregulate endogenous survival factors and predicted that, if this were so, IPC would afford protection against light-induced injury.

METHODS

Treatment of Animals

Procedures used in this study conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Home Office in the United Kingdom. Adult female Wistar rats (weight, 200–250 g) housed in a 12-hour light–dark cycle were used for all experiments. Food and water were provided ad libitum. Anesthesia was achieved with a combination of intramuscular 4 mL/kg fentanyl citrate (Hypnorm; 0.315 mg/mL) and 10 mg/mL fluanisone (both from Janssen Pharmaceutica, Beerse, Belgium) and 4 mL/kg diazepam. Animals were killed by an overdose of intraperitoneal pentobarbitone.

Ischemia Methodology

After anesthesia, both pupils were dilated with cyclopentolate (1%), the rat was placed in a stereotactic frame, and a 30-gauge needle, attached to an elevated sterile isotonic saline reservoir, was inserted into the anterior chamber. This produced an intraocular pressure (IOP) of 120 mm Hg, inducing retinal ischemia, as evidenced by immediate whitening of the fundus. The ischemia was maintained for exactly 5 minutes, and the fellow eye received an identical paracentesis with a 30-gauge needle, but the IOP was not elevated. Chloramphenicol ointment was applied to both eyes after the procedure, and the rat was kept warm during recovery, before it was returned to the animal house. A few animals in which a cataract developed (due to inadvertent lens injury) were not used any further in the experiments.

Light-Induced Injury

Twenty-four hours after the preconditioning ischemia, rats were placed separately in cages and exposed to evenly distributed bright light. The floor of each cage was illuminated by approximately 2000 lux. The temperature inside each cage was maintained at 24°C, and the animals had free access to food and water. The rats were placed under these conditions at the same time of day for each experiment and returned to their normal housing after 48 hours of constant light exposure. Five days later, the flash ERG was recorded.

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**Ischemic Preconditioning Inhibitors**

To assess the effect of a known inhibitor of the protection afforded by IPC, rats were divided into groups and received no drug, dimethyl sulfoxide (DMSO) vehicle, or an A1 adenosine receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 4.5 mg/kg). These agents were administered intraperitoneally 15 minutes before the induction of ischemia.

**Assessment of Retinal mRNA Levels**

The levels of cyclophilin, rhodopsin, bFGF, glial fibrillary acidic protein (GFAP), CNTF, and brain-derived neurotrophic factor (BDNF) mRNAs present in the retina 6 and 48 hours after IPC were determined with a semiquantitative reverse transcription-polymerase chain reaction technique (RT-PCR), as described previously. Briefly, total RNA was isolated, and first strand cDNA synthesis performed on 2 μg DNA-treated RNA. The individual cDNA species were amplified in a 10-μL reaction, containing the 2-μL cDNA aliquot, PCR buffer (10 mM Tris-HCl [pH 8.3] and 50 mM KCl), 4 mM MgCl₂, 200 μM of each dNTP, 4 ng/μL of both the sense and antisense primers, and 2.5 U Taq polymerase. Reactions were initiated by incubating at 94°C for 10 minutes and PCRs (94°C for 15 seconds; 52°C, 55°C, or 56°C for 30 seconds; 72°C for 30 seconds) performed for a suitable number of cycles followed by a final extension at 72°C for 3 minutes. Intereperiment variations were avoided by performing all amplifications in a single run. The oligonucleotides primer pairs and their annealing temperatures are shown in Table 1. The PCR products of all primer pairs yielded single bands corresponding to the expected molecular weights, which are also shown in Table 1. PCR reaction products were separated on 1.5% agarose gels using ethidium bromide for visualization. The relative abundance of each PCR product was determined by digital analysis of gel photographs on computer (Labworks software; Ultra-violet Products). For each protein of interest, the level of expression in the preconditioned eyes was compared to the expression in the fellow eye. A one-way ANOVA was used to determine whether there was a significant difference in these ratios between the various groups and a Tukey HSD test was used for post hoc comparisons. Student’s paired t tests (or Wilcoxon signed-rank tests if the data were not normally distributed) were used to compare independent groups.

**Electrophoresis and Immunoblot Analysis**

Electrophoresis was performed with a midget vertical slab system (LKB, Gaithersburg, MD). Ten percent polyacrylamide gels containing 0.1% SDS were used as the separating gels. Electrophoretic transfer of proteins to nitrocellulose sheets (pore size, 0.45 μm) occurred overnight at 0.1 A as described by Towbin et al., and the immunoprobing procedure was performed as previously described.42 Immunoblots were recorded and analyzed on computer (Labworks software; Ultra-violet Products). For each protein of interest, the level of expression in the preconditioned eyes was compared to the expression in the fellow sham-treated eyes. For semiquantitative analysis, this ratio was then compared to an internal standard protein ratio (protein kinase Ca [PKCa]), which was assumed to be unaffected by the IPC.

**Histopathology**

Anesthetized rats were transcardially perfused with 50 mL 10 mM phosphate-buffered saline, followed by 4% paraformaldehyde. The eyes were enucleated and immersion fixed for 1 hour in 4% paraformaldehyde, transferred to 10% neutral-buffered formalin overnight and processed for routine paraffin-embedded sections on an automated tissue processor (Shandon Pathcen; Thermo Shandon Inc, Pittsburgh, PA). Eyes were embedded sagittally and 5-μm serial sections including the optic nerve were cut using a rotary microtome (Microm HM 330; McBay Instruments, Chatsworth, CA) and stained with hematoxylin and eosin.

**Statistical Analysis**

The a- and b-wave amplitudes were expressed as a percentage of baseline, and, to compare independent groups, the mean of the ratios between paired eyes (one eye received IPC and the fellow received sham treatment) were calculated and used as the unit of analysis. A one-way ANOVA was used to determine whether there was a significant difference in these ratios between the various groups and a Tukey honest significant difference (HSD) test was used for post hoc comparisons. Student’s paired t tests (or Wilcoxon signed-rank tests if the data were not normally distributed) were used to compare independent groups.

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**Table 1. RT-PCR Primer Sequences for the Amplified mRNAs**

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<th>mRNA</th>
<th>Primer Sequences</th>
<th>Size (bp)</th>
<th>Annealing Temperature (°C)</th>
<th>Accession Number</th>
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<td>Cyclophilin</td>
<td>5'-TGGTCACCCACCGTGTTCG-3' and 5'-GTCACCCCGTGTTCG-3'</td>
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<td>Rhodopsin</td>
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<td>55</td>
<td>Z46957</td>
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<tr>
<td>GFAP</td>
<td>5'-ATTCGGGCTCCTCTGCTTCCTC-3' and 5'-GCCTATCGCCCTGCTGTCTG-3'</td>
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<td>55</td>
<td>U03700</td>
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<td>bFGF</td>
<td>5'-GCCTTCACCCCGGCACTCAAGG-3' and 5'-GCACACTCCTCCTGATGGACACAA-3'</td>
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<td>55</td>
<td>M22427</td>
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<tr>
<td>CNTF</td>
<td>5'-TGGCTAGAAAGGAAGATTGCT-3' and 5'-ACGAAGGCTGATGGAGACACT-3'</td>
<td>468</td>
<td>56</td>
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<td>BDNF</td>
<td>5'-ACGGGGGCTGGTGAGCAGAT-3' and 5'-GTCTATCTATGAAACGCGCGC-3'</td>
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<td>55</td>
<td>M61178</td>
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<td>Bcl-2</td>
<td>5'-TTGCGCTTCTTCTTGGATGAGT-3' and 5'-TACGTGTATGGAACT-3'</td>
<td>331</td>
<td>52</td>
<td>L14680</td>
</tr>
</tbody>
</table>

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parametric assumptions were not met) were used to compare the mean mRNA and protein ratios (between preconditioned and sham-treated eyes) of the factors of interest with the mean ratio of the internal standard (no post hoc comparisons were made). A one-way ANOVA was used to determine whether a significant change in mRNA level of each gene-product occurred over time, and specific comparisons were made with a post hoc Tukey HSD test. All statistical determinations were performed on computer (SPSS for Windows, ver. 10; SPSS Inc., Chicago, IL) and all data are expressed as mean ± SEM. A $P < 0.05$ was considered statistically significant.

**RESULTS**

**Effect of IPC and Drug Treatments on Light-Induced ERG Changes**

IPC caused a transient but complete loss of the ERG, which rapidly recovered after the cessation of ischemia and returned to baseline levels 48 hours later (Fig. 1). There was no significant difference in the a-wave ($P = 0.84$) or b-wave ($P = 0.76$) amplitudes between sham-treated eyes and untreated eyes after exposure to light. (Fig. 2). Light treatment caused a marked attenuation of both the a- and b-waves; however, the preconditioned eyes were less affected (representative ERGs are shown in Fig. 3).

Figure 4 shows the effect of IPC on the a- and b-wave amplitudes compared with sham treatment (expressed as a ratio between paired eyes) and the influence of DPCPX on the effect. IPC significantly attenuated the reduction in the amplitude of the a-wave ($P = 0.033$) and b-wave ($P = 0.003$) compared with the sham-treated eyes and was not affected by DPCPX or its vehicle.

**FIGURE 1.** The electroretinogram flattened during a 5-minute period of ischemia and rapidly recovered after cessation of ischemia, returning to baseline appearance by 48 hours.

**FIGURE 2.** The a- and b-wave amplitudes of untreated eyes measured 5 days after 48 hours of photic injury were not significantly different from sham-treated eyes ($P = 0.84$ and 0.76, respectively; unpaired t-test).

**FIGURE 3.** Representative electroretinograms recorded 5 days after 48 hours of photic injury. (A) Sham-treated eye; (B) fellow preconditioned eye. Both electroretinograms were reduced in amplitude, but the preconditioned eye exhibited better preservation of the wave forms.
Effect of IPC on Light-Induced Histopathologic Changes

The most noticeable differences between preconditioned and sham-treated eyes occurred in the central retina. In this region there was a marked loss of photoreceptors and a decrease in the thickness of the outer nuclear layer in both the preconditioned and the sham-treated eyes, but this photoreceptor loss was clearly less marked in the preconditioned eyes (Fig. 5).

mRNA Expression after 5 Minutes of Retinal Ischemia

Six hours after IPC, GFAP, and bFGF mRNA levels were already significantly greater than the housekeeping gene, cyclophilin ($P = 0.025$ and 0.011, respectively; Fig. 6). After 48 hours, GFAP and bFGF were still significantly upregulated ($P = 0.043$ and 0.026, respectively), but after 5 and 10 days had returned to levels that were not significantly different from cyclophilin.CNTF, bcl-2, and BDNF were not significantly affected ($P = 0.09, 0.12, and 0.39$, respectively; Fig. 6). GFAP levels reduced over time, but the bFGF level was the only one that changed significantly. The level at 6 hours was significantly greater than the level at 5 or 10 days ($P = 0.038$ and 0.045, respectively; Fig. 6).

Rhodopsin mRNA Levels

Five days after photic injury, the rhodopsin mRNA levels in preconditioned eyes were significantly greater than the levels in the fellow sham-treated eyes ($P = 0.019$; Fig. 7).

Protein Expression after 5 Minutes of Retinal Ischemia

After 2 days, the expression of bFGF, GFAP, and Bcl-2 was significantly greater than the expression of PKCa ($P = 0.006$, Fig. 8).

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IPC is a potent neuroprotective mechanism against subsequent ischemic injury. The effect has been described in various tissues, including the retina. Recent in vivo experiments have shown that IPC provides functional protection (attenuation of ERG changes) after prolonged retinal ischemia, although the two independent reports to date differ considerably in the degree of functional protection afforded. Roth et al. reported that the window of opportunity between the IPC and subsequent severe ischemia was 1 to 168 hours. The maximal response was reported to occur between 24 and 72 hours; hence, we chose a period of 48 hours between the IPC and the midpoint of the light-induced injury. Although some mechanisms involved in retinal IPC, which are common to IPC in other tissues, have been established, the phenomenon remains poorly understood.

Various neurotrophic factors, cytokines, and growth factors (including bFGF), collectively known as survival factors, protect neurons against different forms of injury. Exogenous bFGF, delivered intravitreally, delays photoreceptor degeneration in the RCS rat and affords protection to light-injured photoreceptors. Endogenous trophic factors are upregulated after various types of nonischemic conditioning injury: mechanical injury to the retina, preconditioning with light, and optic nerve transection protect photoreceptors against light-induced injury. In all cases, bFGF is upregulated after the conditioning injury. Hence, there is considerable evidence that bFGF is involved in a generalized endogenous neuroprotective response.

We found an upregulation of bFGF within 6 hours of IPC, increased levels during the period of light-induced injury, and a return to normal levels by day 5. The temporal sequence of this response is consistent with the window of opportunity known to exist for retinal IPC, providing circumstantial evidence that bFGF may be involved in the response. Valter et al. have reported that hypoxia stimulates bFGF expression in the RCS rat but that hypoxia-induced damage overwhelms any protection afforded by this or other factors. However, in normal albino rats, hypoxia did not upregulate bFGF, but did afford protection against light-induced injury. Grimm et al. very recently reported that systemic hypoxia in mice briefly upregulates erythropoietin and bFGF mRNA and that hypoxic preconditioning attenuates light-induced photoreceptor injury. The therapeutic window in this paradigm was less than 16 hours. Complete retinal ischemia may be a more robust conditioning injury than hypoxia and may initiate a more prolonged response from the retina than systemic hypoxia.

GFAP is widely used as an indicator of glial cell response to neuronal injury. Our laboratory and others have reported its upregulation after retinal injury. Cao et al. noted the close temporal relationship between upregulation of GFAP and bFGF after a mechanical injury to the adult rat retina. Similarly, the time course of GFAP mRNA upregulation after IPC was similar to that of bFGF, suggesting that activated Müller cells may play a role in endogenous neuroprotection by producing trophic factors.

**DISCUSSION**

**FIGURE 6.** mRNA ratio between preconditioned and sham-treated eyes at various time points (n = 8 at 6 hours and 48 hours and n = 4 at 5 and 10 days). After 6 hours, GFAP and bFGF were significantly upregulated in the preconditioned eyes compared with the housekeeping gene (cyclophilin; P = 0.025 and 0.011, respectively). After 48 hours, GFAP and bFGF mRNA were still significantly upregulated (P = 0.043 and 0.026, respectively). The other gene product levels were not significantly different from that of cyclophilin. The bFGF mRNA level was significantly greater at 6 hours than at 5 or 10 days (P = 0.038 and 0.048, respectively). *P < 0.05.

**FIGURE 7.** Comparison of rhodopsin mRNA levels between preconditioned and sham-treated retinas 5 days after photic injury. (A) Representative PCR gel. Odd-numbered lanes are from preconditioned eyes and even-numbered lanes are from the sham-treated fellow eyes. (B) Mean mRNA levels in the preconditioned and sham-treated eyes. The rhodopsin mRNA was significantly reduced in the sham-treated eyes compared with the preconditioned eyes (P = 0.016), whereas the housekeeping gene product (cyclophilin) was not affected by ischemic preconditioning. *P < 0.05.
Adenosine receptors are thought to be involved in the IPC response in myocardium and have also been implicated in cerebral IPC. More recently, they have been shown to play important roles in retinal IPC. We observed that retinal IPC provided a partial functional protection against light-induced injury and that this protective effect was not influenced by an adenosine A1 antagonist. The dose, timing, and route of delivery of the adenosine A1 antagonist that we tested were identical with that used in a previous study in which the effect of IPC against ischemia was significantly reduced. The lack of inhibition by an adenosine A1 antagonist suggests that adenosine A1 receptors are not involved in the photoreceptor protection against light-induced injury.

Although the molecular events that underlie light-induced photoreceptor apoptosis are not completely understood, Bcl-2 is an important antiapoptotic factor in several retinal degenerations. We found a significantly increased expression of Bcl-2 protein after IPC. The mRNA, however, was not elevated at either 6 or 48 hours after IPC. The antibody that we used to detect Bcl-2 was directed at the activated protein, and increased expression of the active form may arise without increased expression of mRNA. The relationship between the increased expression of bFGF and Bcl-2 is unclear and may represent a distinct IPC-induced protective mechanism.

It is possible that the upregulation of bFGF plays no role in the IPC-induced protective response and that it is simply an irrelevant bystander. We have not shown that the inhibition of bFGF or other survival factors influences the response. To our knowledge, however, this has never been shown. Perhaps because, in practice, this is very difficult to do, and the notion that trophic factors delivered to the retina provide protection but the identical factors produced endogenously do not seems very unlikely.

In conclusion, IPC affords protection to the photoreceptors against light-induced injury and unlike the protection afforded against subsequent ischemia, does not involve adenosine A1 receptors; however, the increased expression of bFGF, GFAP, and Bcl-2 may be a component of the protective response. A better understanding of the complex retinal response to a conditioning injury, including IPC, and the endogenous protective mechanisms that are evoked, may have therapeutic implications for certain retinal diseases.

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**References**


