

Retinal Neuroprotection against Ischemia-Reperfusion Damage Induced by Postconditioning

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PURPOSE. Retinal ischemia may provoke blindness. There is no effective treatment against retinal ischemic damage. The authors investigated whether brief intermittent ischemia applied during the onset of reperfusion (i.e., postconditioning) protects the retina from ischemia-reperfusion damage.

METHODS. Ischemia was induced by increasing intraocular pressure (120 mm Hg for 40 or 60 minutes). Five minutes after reperfusion, animals underwent 3, 7, or 10 cycles of 1-minute ischemia/1-minute reperfusion or 7 minutes of ischemia. In other experiments, seven ischemia-reperfusion cycles were applied 10, 30, and 60 minutes or 24 hours after ischemia. A group of animals received intraperitoneal injections of cycloheximide (CHX) 1 minute before or 6 hours after postconditioning. Seven or 14 days after ischemia, animals were subjected to electroretinography and histologic analysis.

RESULTS. Seven ischemia-reperfusion cycles applied 5 minutes after reperfusion afforded significant functional protection in eyes exposed to ischemia-reperfusion injury. A marked reduction in retinal thickness and an increase in Müller cell glial fibrillary acidic protein (GFAP) levels were observed in ischemic retinas, whereas postconditioning preserved retinal structure and reduced GFAP levels in Müller cells. Postconditioning initiated between 5 and 60 minutes after reperfusion protected against ischemic injury. Retinal protection depended on the number of ischemia-reperfusion cycles. One 7-minute pulse applied 5 minutes after ischemia induced significant protection against ischemic damage. Retinal protection induced by postconditioning was reversed by CHX (injected 1 minute before but not 6 hours after postconditioning).

CONCLUSIONS. These results indicate that postconditioning significantly protected retinal function and histology from ischemia-reperfusion injury through a mechanism that involved de novo synthesis of protein. (*Invest Ophthalmol Vis Sci.* 2009; 50:3922-3930) DOI:10.1167/iovs.08-3344

Retinal ischemia induces irreversible morphologic and functional changes that result in blindness. Ischemic retinopathy develops when retinal blood flow is insufficient to match the metabolic needs of the retina, one of the highest oxygen-

consuming tissues. At the cellular level, retinal ischemic injury consists of a self-reinforcing destructive cascade involving several mechanisms, such as depolarization, calcium influx, oxidative stress, and increased glutamatergic stimulation,¹ among others. In addition, reperfusion with oxygenated blood after ischemia has the potential to aggravate ischemic damage, an effect known as reperfusion injury.

At present, there is no effective treatment strategy to protect the retina from ischemia-reperfusion damage. Several obstacles have limited the development of neuroprotective agents for retinal ischemia. First, there is the issue of timing; a drug would have to be given within a reasonable time window of the ischemic event. Second, all attempts to date have been characterized by incomplete protection and the risk for non-specific effects, including toxicity.

An alternative approach to protect the retina from ischemic damage is to use endogenously available means of neuroprotection.² It is possible to activate an endogenous protection that prevents ischemia-reperfusion injury by ischemic preconditioning (IPC).³ First described in 1986 as a paradoxical form of cardioprotection,⁴ IPC is now acknowledged as a potent endogenous protection inducing tolerance against ischemia in several organs, including the retina.³ IPC requires a brief period of ischemia that does not produce any significant damage. Preconditioning will turn on as yet incompletely described factors that result in tolerance to the subsequent severely damaging ischemic event. Harnessing endogenous neuroprotection has the potential advantages of specificity, avoidance of side effects, and perhaps even enhanced protection by the use of endogenous properties of the tissue (for a review, see Ref. 5). In fact, it was shown that IPC affords the retina a greater degree of functional protection against ischemic damage than any known neuroprotective agent.³

Although IPC confers robust neuroprotection in different *in vitro* and *in vivo* models of ischemia,⁵ its translational relevance is limited by the fact that the IPC stimulus must be applied 24 or more hours before the onset of harmful ischemia. Another endogenous form of cardioprotection in which a short series of repetitive cycles of brief ischemia-reperfusion were applied immediately at the onset of reperfusion, termed postconditioning, has been reported more recently. This procedure reduces myocardial injury to an extent comparable to that of IPC.⁶ In addition, Zhao et al.⁷ first documented that ischemic postconditioning reduced cerebral ischemia-reperfusion injury. Other groups further confirmed the effectiveness of postconditioning in the central nervous system.⁸⁻¹¹ Given that the effectiveness of postconditioning at the retinal level has not been previously studied and that postconditioning may have translational relevance to therapeutic strategies for retinal ischemia treatment, the aim of the present study was to examine whether postconditioning induces functional and histologic protection against retinal ischemia-reperfusion injury.

MATERIALS AND METHODS

Animals

Male Wistar rats (average weight, 300 ± 50 g) were housed in a standard animal room with food and water *ad libitum* under controlled

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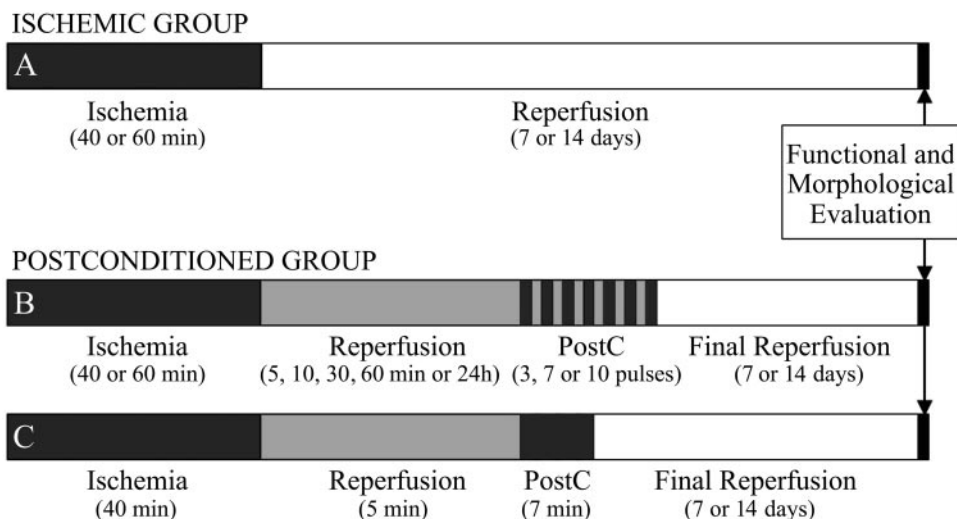


FIGURE 1. (A–C) Experimental groups and protocols for retinal functional and histologic studies. ERGs were assessed at 7 or 14 days after ischemia, whereas histologic studies were performed 14 days after ischemia.

conditions of humidity and temperature ($21^{\circ}\text{C} \pm 2^{\circ}\text{C}$) and under a 12-hour light/12-hour dark lighting schedule (lights on at 7:00 AM). All animal procedures were in strict accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Ischemia Methodology

Animals were anesthetized with ketamine hydrochloride (150 mg/kg) and xylazine hydrochloride (2 mg/kg) administered intraperitoneally. After topical instillation of proparacaine, the anterior chamber of each eye was cannulated with a 30-gauge needle connected to a pressurized bottle filled with sterile normal saline solution. Retinal ischemia was induced by increasing intraocular pressure (IOP) to 120 mm Hg, as previously described.¹² The increased IOP was maintained for exactly 40 or 60 minutes. With this maneuver, complete ocular ischemia was produced, characterized by the loss of electroretinogram (ERG) b-waves and the cessation of flow in retinal vessels, determined by funduscopic examination. During and after (before rats were returned to the animal house) the experiments, the animals were kept normothermic with heated blankets. All rats were allowed at least 7 days of reperfusion after sustained ischemia. A few animals in which cataracts developed because of lens injury were not used any further in the experiments.

Postconditioning Protocol

Rats were assigned to different groups, as depicted in Figure 1. Eyes were subjected to ischemia by increasing IOP to 120 mm Hg for 40 or 60 minutes except for the sham group. In the sham group, animals were anesthetized and cannulated without raising IOP; this procedure did not affect retinal function or histology compared with intact eyes. Control eyes were subjected to ischemia only, without any further interruption of reperfusion (Fig. 1A). For the postconditioned group, reperfusion was established for 5 minutes, after which eyes were submitted to increased IOP (120 mm Hg) for 1 minute and to reperfusion for 1 minute. This maneuver was performed 3, 7, or 10 times. The sham postconditioning procedure did not affect retinal function or histology compared with results in ischemic eyes. In another set of experiments, eyes were subjected to seven cycles of 1-minute ischemia/1-minute reperfusion 10, 30, 60 minutes, or 24 hours after ischemia (Fig. 1B). In addition, 5 minutes after 40-minute ischemia, a group of animals was submitted to 7 minutes of ischemia (Fig. 1C).

Electroretinography

Electroretinographic activity was assessed before (preischemia) and 7 or 14 days after ischemia, as previously described.¹² Briefly, after 6 hours of dark adaptation, rats were anesthetized under dim red illumination. Phenylephrine hydrochloride and tropicamide were used to

dilate the pupils, and the cornea was intermittently irrigated with balanced salt solution to maintain the baseline recording and to prevent keratopathy. Rats were placed facing the stimulus at a distance of 20 cm. All recordings were completed within 20 minutes, and animals were kept warm during and after the procedure. A reference electrode was placed through the ear, a grounding electrode was attached to the tail, and a gold electrode was placed in contact with the central cornea. A 15-W red light was used to enable accurate electrode placement. This maneuver did not significantly affect dark adaptation and was switched off during the electrophysiological recordings. ERGs were recorded from both eyes simultaneously, and 10 responses to flashes of unattenuated white light (5 ms, 0.2 Hz) from a photic stimulator (light-emitting diodes) set at maximum brightness ($350 \text{ cd} \cdot \text{s/m}^2$ without filter) were amplified, filtered (1.5 Hz low-pass filter, 1000 high-pass filter, notch activated), and averaged (Akonic BIO-PC, Buenos Aires, Argentina). The a-wave was measured as the difference in amplitude between the recording at onset and the trough of the negative deflection, and the b-wave amplitude was measured from the trough of the a-wave to the peak of the b-wave. Runs were repeated three times at 5-minute intervals to confirm consistency. Mean values from each eye were averaged, and the resultant mean value was used to compute the group means a- and b-wave amplitude \pm SE. Mean peak latencies and peak-to-peak amplitudes of the responses from each group of rats were compared. Baseline (preischemic) recordings were taken at least 1 day before treatment.

Histologic Evaluation

Fourteen days after ischemia, rats were killed and their eyes were immediately enucleated, immersed for 24 hours in a fixative containing 4% formaldehyde in 0.1 M phosphate buffer (pH 7.2), and embedded in paraffin. Eyes were sectioned ($5 \mu\text{m}$) along the vertical meridian through the optic nerve head. Microscopic images were digitally captured with a microscope (Eclipse E400 [Nikon, Tokyo, Japan]; 6-V halogen lamp, 20 W, equipped with a stabilized light source) and a (Coolpix s10; Nikon) camera. Sections were stained with hematoxylin and eosin and were analyzed by masked observers. The average retinal thickness (in micrometers) for each eye was measured, and the number of cells in the ganglion cell layer (GCL) was calculated by linear cell density (cells per $200 \mu\text{m}$). No attempt was made to distinguish cell types in the GCL for enumeration of cell number. Measurements ($\times 400$) were obtained at 1 mm dorsal and ventral from the optic disc. For each eye, results obtained from four separate sections were averaged, and the mean of 10 eyes was recorded as the representative value for each group.

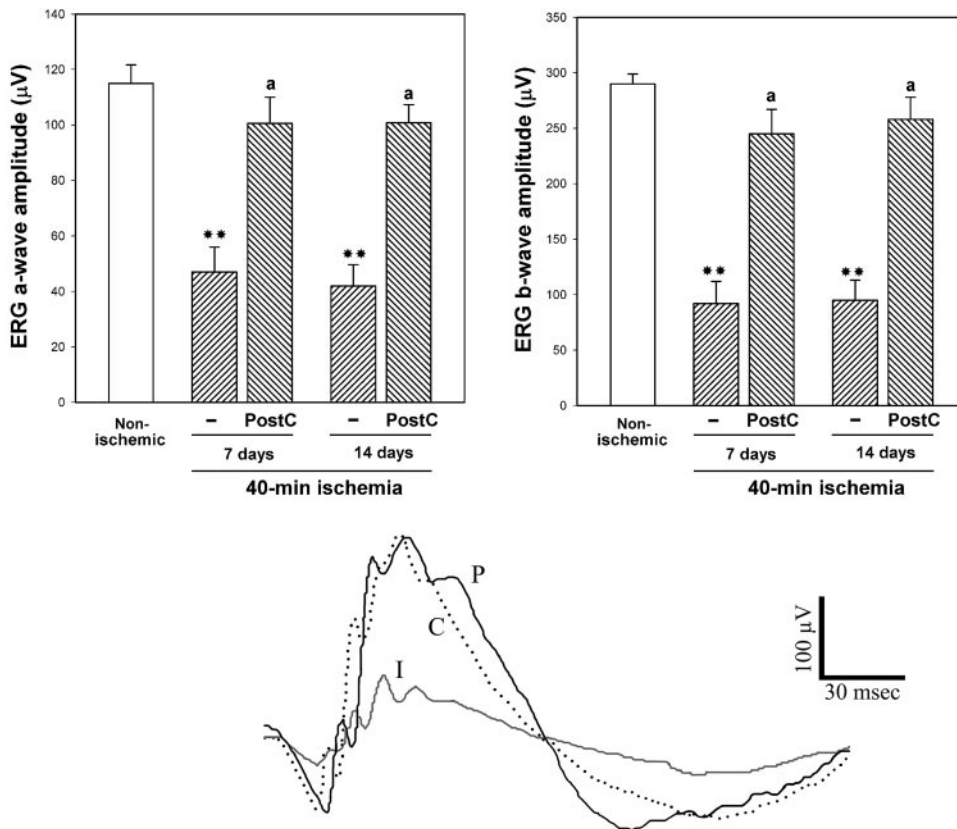


FIGURE 2. Scotopic ERGs from rats subjected to 40-minute ischemia without (in one eye) or with (in the contralateral eye) seven cycles of ischemia-reperfusion applied 5 minutes after ischemia. ERGs were registered 7 or 14 days after ischemia. *Top:* average amplitudes of scotopic ERG a- and b-wave. Significant decreases in amplitude (but not latency) of ERG a- and b-waves were observed in eyes exposed to 40-minute ischemia without postconditioning (- PostC). In post-conditioned eyes, significant preservation of ERG a- and b-wave amplitude was observed at 7 and 14 days after ischemia. Data are mean \pm SE ($n = 10$ eyes/group). ** $P < 0.01$ vs. nonischemic values; ^a $P < 0.01$ vs. ischemic eyes without PostC (Tukey test). *Bottom:* representative scotopic ERG traces from a nonischemic eye (C), from an eye subjected to 40-minute ischemia without PostC (I), and from an eye subjected to 40-minute ischemia and 5 minutes after ischemia exposed to seven cycles of ischemia-reperfusion (P).

Immunohistochemical Studies

Antigen retrieval was performed by heating (90°C), for 30 minutes, unstained sections immersed in citrate buffer (pH 6.3) and then pre-incubated with 2% normal horse serum, 0.1% bovine serum albumin (BSA), and 0.4% Triton X-100 in 0.01 M PBS for 1 hour. For immunodetection of glial cells, sections were incubated overnight at 4°C with a mouse monoclonal anti-vimentin antibody (1:100; Dako, Carpinteria, CA) or a mouse monoclonal anti-GFAP antibody conjugated to Cy3 (1:1200; Sigma Chemical Co., St. Louis, MO). To analyze plexiform layers, a mouse monoclonal anti-synaptophysin antibody (1:100; Dako) was used. Some sections were treated without primary antibodies to confirm specificity. An anti-mouse secondary antibody conjugated to Alexa Fluor 568 (1:500; Molecular Probes, Eugene, OR) was used to detect vimentin and synaptophysin. After immunostaining, nuclei were stained with the fluorescent dye Hoechst 33342 (5 $\mu\text{g}/\text{mL}$ in 1% dimethyl sulfoxide) and were placed under a microscope (BX50; Olympus, Tokyo, Japan) for observations.

Ischemic Postconditioning Inhibitor

To analyze the involvement of de novo synthesis of proteins, animals received cycloheximide (CHX; 0.4 mg/kg intraperitoneally) 1 minute before or 6 hours after seven ischemia-reperfusion cycles.

Statistical Analysis

Statistical analysis of results was made by two-way analysis of variance (ANOVA) followed by Tukey test, as stated. Parametric tests were used throughout because data met the requirements for those tests (no significant differences in standard deviations among groups or data populations that followed a Gaussian distribution).

RESULTS

Figure 2 depicts the effect of postconditioning on retinal ischemic damage induced by increasing IOP to 120 mm Hg for 40

minutes. Five minutes after 40-minute ischemia, rats were submitted to seven cycles of 1-minute ischemia/1-minute reperfusion, and ERG was assessed 7 or 14 days after ischemia. Average amplitudes of ERG a- and b-waves in nonischemic eyes or 7 or 14 days after 40-minute ischemia with or without postconditioning and representative scotopic ERG traces from rat eyes submitted to these treatments are shown in Figure 2. Ischemia for 40 minutes and reperfusion for 7 or 14 days induced a significant ($P < 0.01$) decrease in ERG a- and b-wave amplitude, whereas their latencies remained unchanged. In eyes submitted to postconditioning, the ischemia-reperfusion-induced decrease in a- and b-wave amplitude was completely reversed at 7 or 14 days after ischemia. No significant differences were observed between nonischemic eyes and eyes submitted to postconditioning without ischemia or between ischemia without postconditioning and with sham postconditioning (data not shown).

Figure 3 shows representative photomicrographs of the rat retina. Figure 3A (inset magnified in Fig. 3B) shows a representative photomicrograph of nonischemic retinas. Fourteen days after 40-minute ischemia, typical histopathologic features of ischemic damage were observed in the retina (Figs. 3C, D) showing marked reduction in total retinal thickness. In addition, ischemia caused the frequent formation of folds with loss of photoreceptors (Fig. 3E) and a significant decrease in cell counts in the GCL. Postconditioning (seven cycles of 1-minute ischemia/1-minute reperfusion) significantly preserved retinal structure, as shown in Table 1 and Figure 3.

The protective effect of postconditioning against ischemia-reperfusion injury was also evident when ischemia was induced by increasing IOP up to 120 mm Hg for 60 minutes. Average amplitudes of scotopic ERG a- and b-waves before ischemia or 7 days after 60-minute ischemia in eyes with or without postconditioning are depicted in Figure 4A, and representative scotopic ERG traces from rats submitted to these

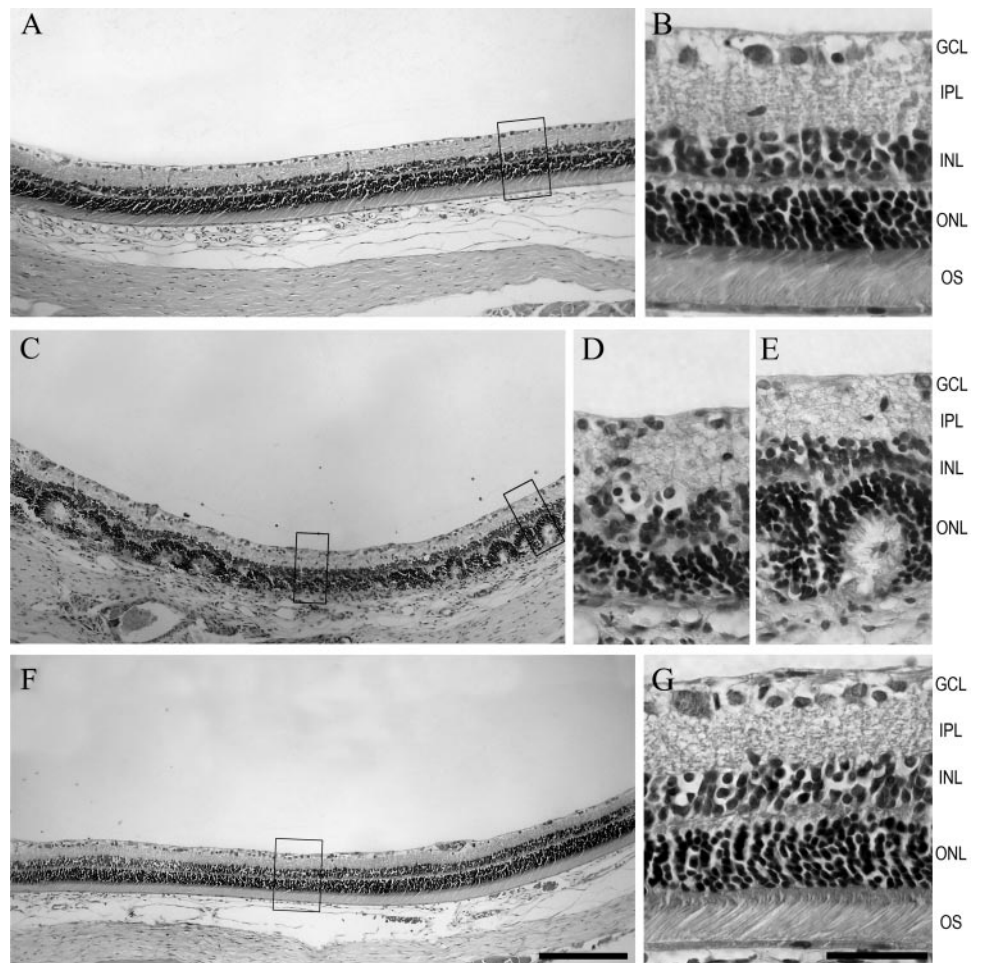


FIGURE 3. Representative photomicrographs showing histologic appearance of the nonischemic group (A, detail in B), ischemic eyes 14 days after 40 minutes of ischemia without postconditioning (PostC) (C, details in D and E), and ischemic eyes submitted to seven cycles of ischemia-reperfusion 5 minutes after ischemia (F, detail in G). Severe retinal damage is shown in eyes submitted to ischemia without PostC, whereas in postconditioned eyes, retinal structure was notably preserved. Scale bars: (F) 200 μm ; (G) 50 μm . GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer, OS, outer segment.

treatments are shown in Figure 4B. Ischemia for 60 minutes and reperfusion for 7 days induced further decreases in ERG a- and b-wave amplitudes compared with damage induced by 40-minute ischemia ($P < 0.01$). Similar results were observed 14 days after ischemia (data not shown). Seven cycles of 1-minute ischemia/1-minute reperfusion applied 5 minutes after 60-minute ischemia significantly reduced the electroretinographic dysfunction at both periods after ischemia. The protective effect of postconditioning was also evident at the histologic level, as shown in the lower panel of Figure 4. Compared with nonischemic retinas (Fig. 4C), retinas submitted to 60-minute ischemia showed profound structural alter-

tations (Fig. 4D), whereas postconditioning significantly reduced the decrease in total retinal thicknesses induced by 60-minute ischemia (Fig. 4E; Table 1). Widespread retinal disorganization in ischemic eyes without postconditioning rendered impossible quantitative cell counts in the GCL.

To further analyze the morphologic protection induced by postconditioning against ischemia-reperfusion damage, retinal immunoreactivity for glial fibrillary acidic protein (GFAP), vimentin, and synaptophysin was analyzed 14 days after 40-minute ischemia without or with postconditioning induced by seven cycles of 1-minute ischemia/1-minute reperfusion. In nonischemic retinas, astrocytes localized in the nerve fiber

TABLE 1. Total Retinal Thicknesses and GCL Cell Counts in Retinas from Nonischemic Eyes (Control) and Eyes Submitted to 40- or 60-Minute Ischemia without or with PostC

Treatment	Total Retinal Thickness (μm)	GCL Cell Count (cell number/200 μm)
Control	133.8 \pm 2.5	14.2 \pm 0.6
40-minute ischemia	96.6 \pm 7.7*	7.3 \pm 0.9*
40-minute ischemia + PostC	119.2 \pm 3.2†	10 \pm 0.6†
60-minute ischemia	34.5 \pm 4.3*	—
60-minute ischemia + PostC	99.7 \pm 3.6*‡	8.3 \pm 2.4§

Data are mean \pm SEM; $n = 10$ retinas/group. PostC (postconditioning) significantly preserved retinal histology from damage induced by both periods of ischemia.

* $P < 0.01$ vs. nonischemic (control) eyes.

† $P < 0.01$ vs. 40-minute ischemia without PostC.

‡ $P < 0.01$ vs. 60-minute ischemia without PostC.

§ $P < 0.05$ vs. nonischemic (control) eyes (Tukey test).

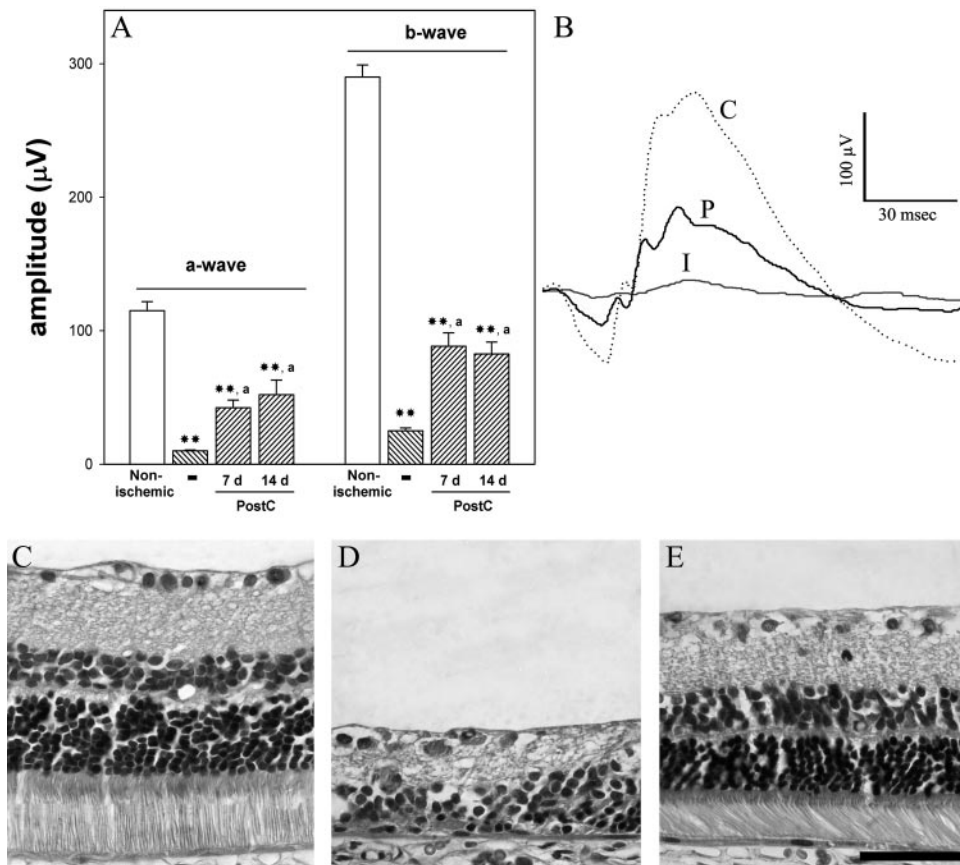


FIGURE 4. Protective effect of post-conditioning (PostC) on retinal injury induced by 60-minute ischemia. **(A)** Average amplitudes of scotopic ERG a- and b-waves. In eyes submitted to 60-minute ischemia, PostC (seven cycles of ischemia-reperfusion, 5 minutes after 40 minute-ischemia) induced a significant increase in the amplitude of ERG a- and b-wave amplitude assessed 7 and 14 days after 60-minute ischemia compared with ischemic eyes without PostC. Shown are means of a- and b-wave amplitude assessed 7 days after 60 minute-ischemia, which did not differ from those obtained in ischemic eyes without PostC and those obtained 14 days after ischemia (not shown). Data are mean \pm SE ($n = 10$ eyes/group). ** $P < 0.01$ vs. nonischemic eyes; ^a $P < 0.01$ vs. ischemic eyes without PostC (Tukey test). **(B)** Representative scotopic ERGs of nonischemic eyes (C) and eyes exposed to 60-minute ischemia without (D) or with PostC (P) and assessed 7 days after ischemia. *Lower:* representative photomicrographs showing nonischemic eyes (C) and eyes 14 days after 60 minutes of ischemia without (D) or with PostC (E). Scale bar, 50 μ m.

layer and the GCL layer were weakly GFAP immunopositive (Fig. 5A), whereas 40-minute ischemia induced an increase in retinal GFAP levels in astrocytes and spread to glial processes from the inner limiting membrane to the outer retina associated with activated Müller cells (Fig. 5B). Postconditioning decreased the levels of GFAP in Müller cells to those observed in nonischemic retinas (Fig. 5C). No differences in vimentin immunoreactivity were noted among experimental groups (Figs. 5D–F). However, disorganization of Müller cells and their processes spanning the retina was observed in retinas submitted to 40-minute ischemia. Intense immunostaining for synaptophysin was observed in both plexiform layers from nonischemic retinas (Fig. 5G), whereas in retinas submitted to 40-minute ischemia, weak immunolabeling was observed in the inner plexiform layer, and no staining was observed in the outer plexiform layer (Fig. 5H). Synaptophysin-like immunoreactivity did not differ between nonischemic and postconditioned retinas (Fig. 5I).

To analyze the influence of the number of ischemia-reperfusion cycles on the protective effect of postconditioning, eyes were exposed to 3, 7, or 10 cycles of 1-minute ischemia/1-minute reperfusion, applied 5 minutes after 40-minute ischemia, and ERGs were assessed 7 days after ischemia (Fig. 6). Similar protection was achieved by 7 and 10 ischemia-reperfusion cycles (1 minute/1 minute), whereas 3 cycles were ineffective. In addition, as also shown in Figure 6, a 7-minute ischemia interval applied 5 minutes after 40 minutes of ischemia significantly protected the retina from ischemia-reperfusion damage.

Figure 7 depicts the analysis of the time window of post-conditioning-induced protection against retinal damage. Post-conditioning induced by seven ischemia-reperfusion cycles (1 minute/1 minute) was effective at inducing retinal protection when applied 5, 10, 30, and 60 minutes (but not 24 hours) after

40-minute ischemia in a time-dependent manner. For the ERG a-wave, maximal protection was observed when postconditioning was applied 5 or 10 minutes after ischemia, whereas for the ERG b-wave, the maximal protective effect of postconditioning was observed at 5, 10, and 30 minutes after ischemia.

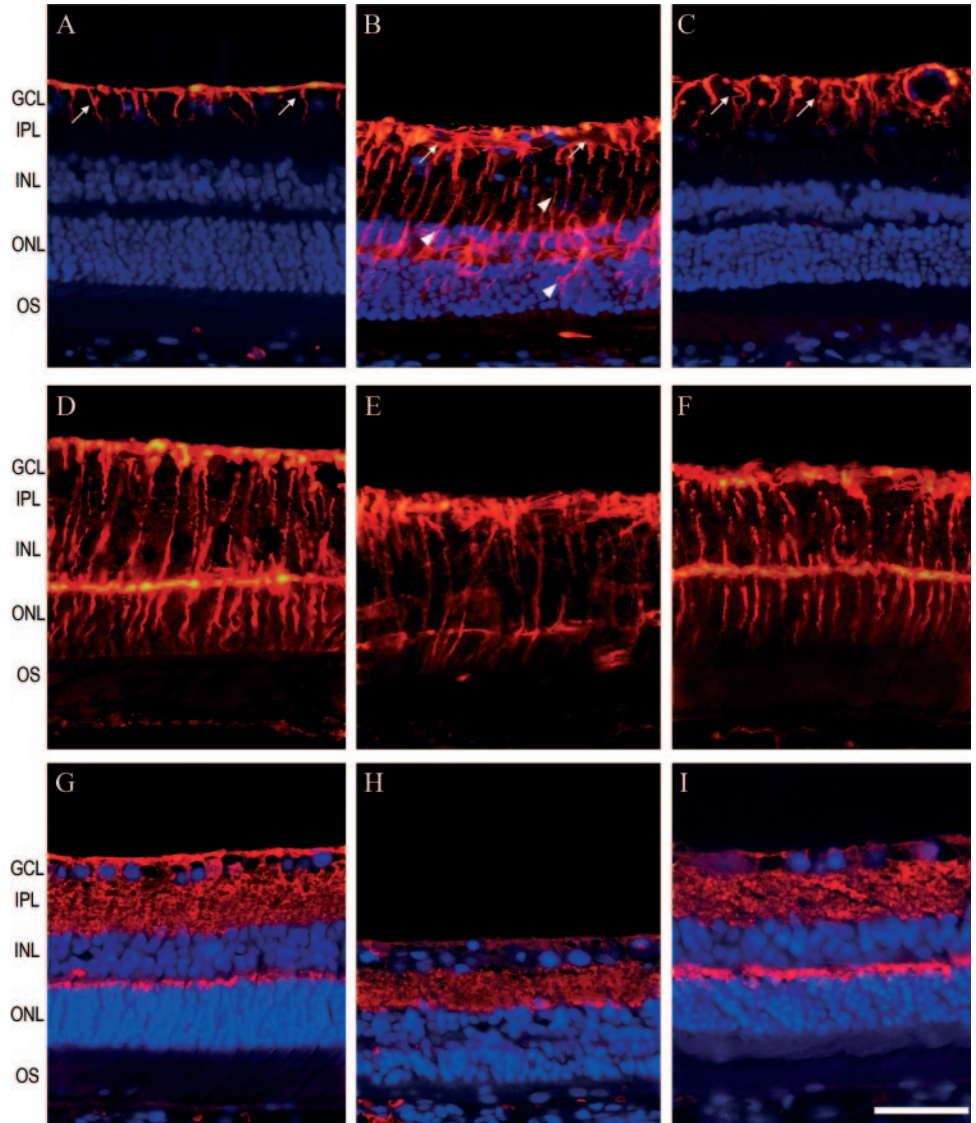
The effect of CHX on postconditioning-induced protection against ischemia-reperfusion damage is shown in Figure 8. When CHX was injected 1 minute before the application of seven cycles of 1-minute ischemia/1-minute reperfusion (i.e., 4 minutes after the onset of reperfusion), complete attenuation in the recovery of the ERG a- and b-wave was observed in postconditioned eyes. When CHX was injected 6 hours after ischemia, no changes in the ERG recovery induced by postconditioning were observed, as shown in the upper and middle panels of Figure 8. CHX did not change the ERGs in nonischemic eyes (data not shown), and CHX injected 4 minutes or 6 hours after reperfusion onset did not affect the ERGs in eyes submitted to ischemia without postconditioning. Moreover, CHX injected 1 minute before postconditioning (Fig. 8E) abrogated the histologic protection induced by postconditioning, whereas CHX injected 6 hours after postconditioning was ineffective (Fig. 8G). CHX did not affect retinal alterations induced by ischemia (Figs. 8D, F).

DISCUSSION

The present results indicate, for the first time, that repetitive cycles of briefly interrupted reperfusion performed at the onset of full reperfusion (postconditioning) significantly protected retinal function and histology from ischemia-reperfusion injury through a mechanism that involves the de novo synthesis of proteins.

Postconditioning was originally described by Zhao et al.,⁶ who showed that three cycles of 30-second reperfusion/30-

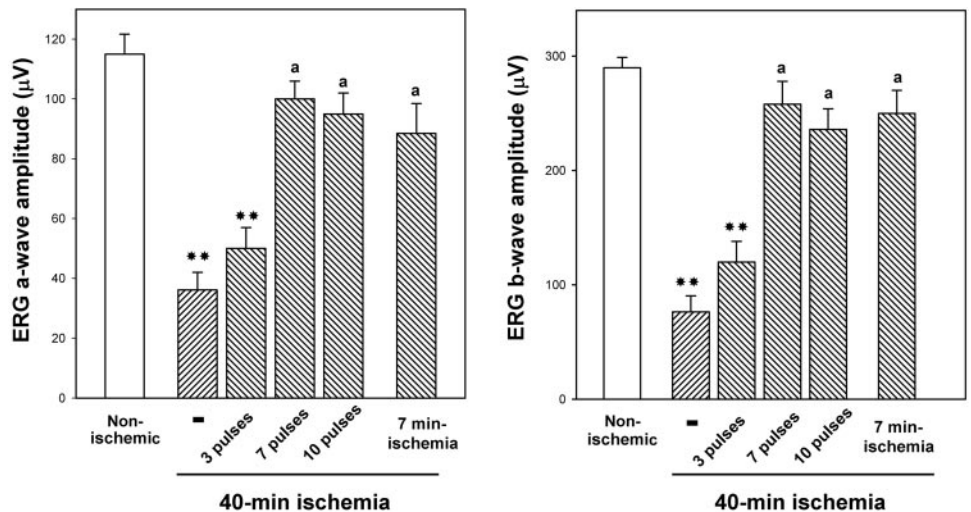
FIGURE 5. Immunohistochemical detection of GFAP, vimentin, and synaptophysin in nonischemic eyes or eyes 14 days after 40 minutes of ischemia without or with postconditioning (PostC; 7 ischemia-reperfusion cycles, 5 minutes after 40 minutes of ischemia). *Top:* immunohistochemistry staining for GFAP (red). In ischemic eyes an intense GFAP (+) immunoreactivity was observed in astrocytes (arrows) and Müller cell bodies and their processes (arrowheads, B). PostC reduced GFAP immunoreactivity (C), showing only few positive astrocytes similar to control eyes (A). *Middle:* immunohistochemistry for vimentin (red). No differences in the intensity of the vimentin-immunoreactivity were observed among nonischemic eyes (D) or ischemic eyes without (E) or with (F) PostC, but in ischemic eyes, a high disorganization of Müller processes was observed (E). *Bottom:* immunohistochemical staining for synaptophysin (red). Intense immunostaining was observed in both plexiform layers from nonischemic eyes (G), whereas in eyes submitted to 40-minute ischemia without PostC (H), weak immunolabeling was observed in the IPL, and no staining was observed in the OPL. Synaptophysin-like immunoreactivity was similar between nonischemic and postconditioned eyes (I). *Top and bottom:* cell nuclei were counterstained with Hoechst 33342 (blue). Scale bar, 50 μ m.



second reocclusion preceding full reperfusion confers cardioprotection against ischemia-reperfusion damage. Subsequent studies confirmed the cardioprotective effects of postconditioning in vivo and in vitro.¹³⁻¹⁵

In addition to cardiac protection, postconditioning, a simple and harmless method, provides protection against ischemia-reperfusion injury to several organs, such as liver¹⁶ and kidney.¹⁷ Moreover, Zhao et al.⁷ demonstrated that ischemic

FIGURE 6. Influence of the number of ischemia-reperfusion cycles on the electroretinographic protection induced by postconditioning (PostC). Significant protection was induced by the application of 7 or 10 cycles of 1-minute ischemia/1-minute reperfusion 5 minutes after 40-minute ischemia, whereas three cycles were ineffective. In eyes submitted to 7-minute ischemia 5 minutes after 40-minute ischemia, significant reversal of the decrease in the ERG a- and b-wave amplitudes induced by 40-minute ischemia was also observed. Data are mean \pm SE ($n = 10$ eyes/group). ** $P < 0.01$ vs. nonischemic values; ^a $P < 0.01$ vs. ischemic values without PostC (Tukey test).



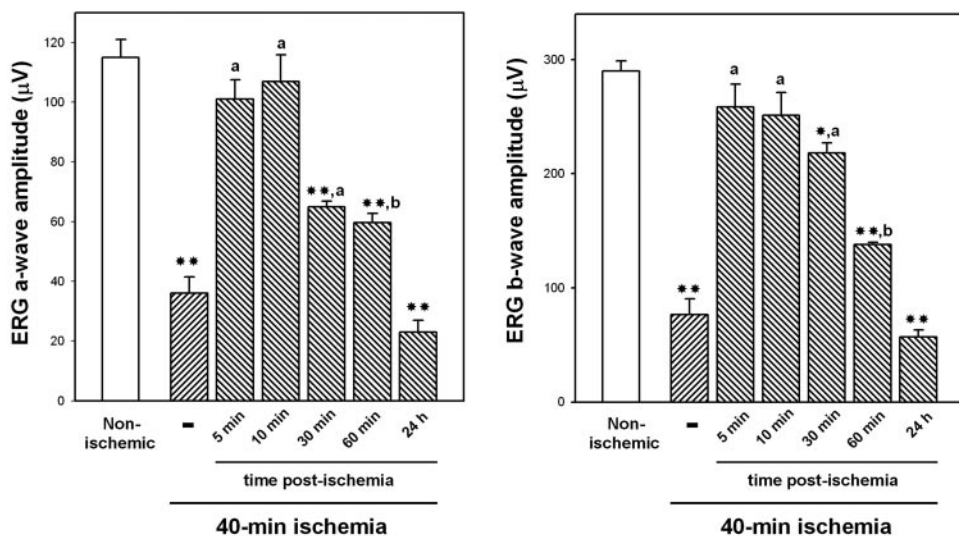


FIGURE 7. Influence of the time elapsed after ischemia on the electroretinographic protection induced by postconditioning (PostC). Eyes were submitted to seven ischemia-reperfusion cycles at different periods after 40-minute ischemia. PostC afforded significant protection when applied 5, 10, 30, or 60 minutes (but not 24 hours) after ischemia in a time-dependent manner. Data are mean \pm SE ($n = 10$ eyes/group). * $P < 0.05$; ** $P < 0.01$ vs. nonischemic values; ^a $P < 0.01$; ^b $P < 0.05$ vs. ischemic values without PostC (Tukey test).

postconditioning reduces cerebral ischemic damage. Our results indicate that the early moments of reperfusion are critical in the pathogenesis of retinal ischemic injury and that manipulation of this early reperfusion phase could reduce retinal ischemic damage.

Using a reproducible model of ischemia, we showed herein that seven cycles of 1-minute ischemia/1-minute reperfusion

applied 5 minutes after 40-minute ischemia significantly protected the retina from ischemia-reperfusion damage. Compared with nonischemic values, the ERG a- and b-wave amplitudes were strongly reduced at 7 or 14 days after 40-minute ischemia. Moreover, ischemia-reperfusion induced profound histologic alterations. Postconditioning reversed the functional and histologic consequences of retinal ischemia.

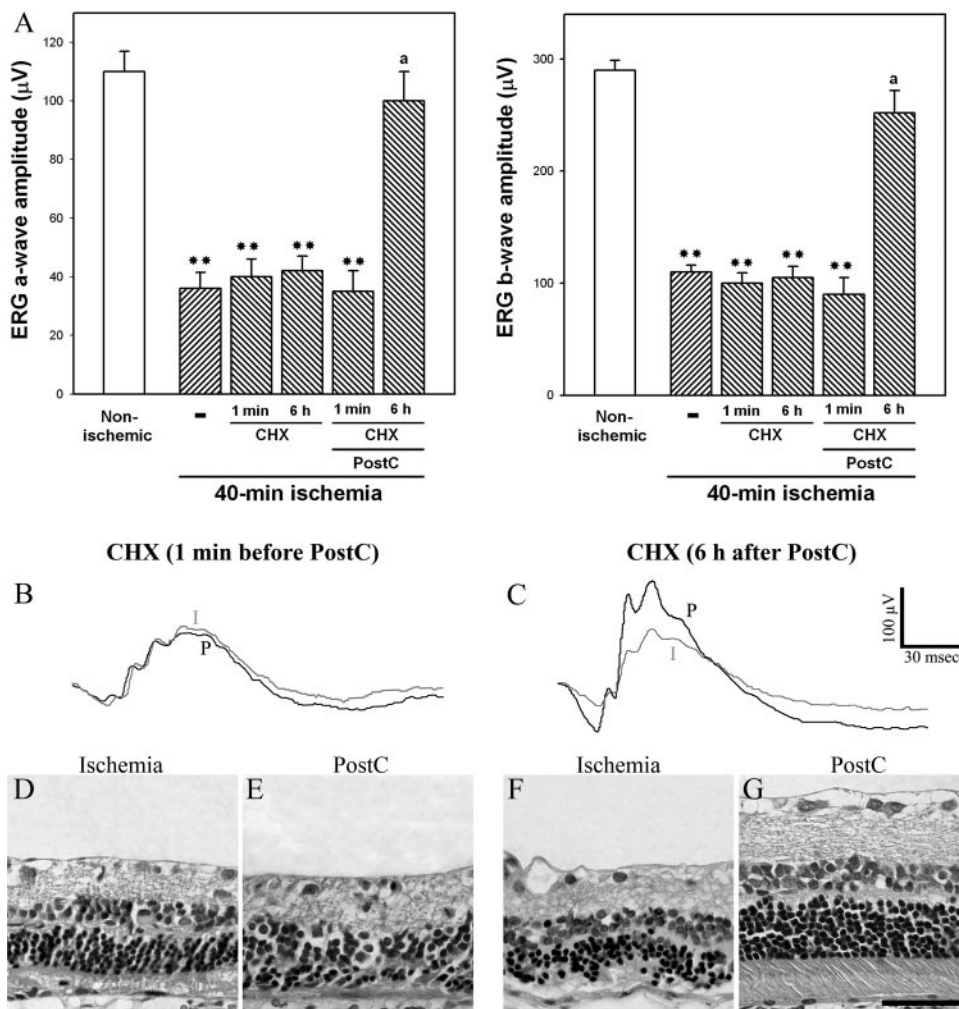


FIGURE 8. Effect of CHX on functional and histologic protection induced by postconditioning (PostC). Eyes were submitted to seven ischemia-reperfusion cycles applied 5 minutes after 40-minute ischemia. CHX was injected 1 minute before or 6 hours after PostC, and the ERG was recorded 7 days after ischemia. (A) Average amplitudes of scotopic ERG a- (left) and b-wave (right). When CHX was injected 1 minute before (but not 6 hours after) PostC, complete reversal of the electroretinographic protection induced by PostC was observed. Data are mean \pm SE ($n = 10$ eyes/group). ** $P < 0.01$ vs. nonischemic eyes; ^a $P < 0.01$ vs. ischemic eyes without PostC (Tukey test). (B) Representative scotopic ERGs from an ischemic eye without (I) or with (P) PostC in an animal injected with CHX 1 minute before PostC. (C) Representative scotopic ERG from ischemic eye without (I) or with (P) PostC in an animal injected with CHX 6 hours after PostC. *Bottom:* representative photomicrographs of eyes submitted to ischemia without (D) or with (E) PostC in an animal injected with CHX 1 minute before or 6 hours after PostC (F and G, respectively). Retinal protection induced by PostC was abrogated by the injection of CHX 1 minute before PostC (E), but not 6 hours after PostC (G).

Roth et al.³ reported that IPC is more effective at decreasing retinal ischemic injury than any previously reported pharmacologic treatment. However, despite the high effectiveness of IPC for retinal protection, its use as a clinical strategy is limited because the onset of retinal ischemia is unpredictable, in contrast to the onset of reperfusion, which may be predictable.

The present results indicated that postconditioning induced a complete recovery from ischemia-reperfusion damage as seen on ERG. Postconditioning did not merely delay retinal dysfunction, it prevented it because the recovery seen on ERG was a sustained effect noted up to 14 days after ischemia. In addition, the beneficial effects of postconditioning were observed at a histologic level because significant preservation of retinal morphology was observed in ischemic retinas submitted to postconditioning. The structural preservation of the retina induced by postconditioning was further confirmed by the analyses of GFAP, vimentin, and synaptophysin immunoreactivities. GFAP upregulation is a hallmark of reactive astrocytes,¹⁸ and retinal pathology modulates its expression.^{19,20} Müller cells that do not express GFAP under physiological conditions are known to express GFAP in pathologic situations.^{21,22} Vimentin immunoreactivity indicated no apparent changes in the number of Müller cells among treatments. However, 40-minute ischemia provoked a significant alteration in Müller cells, as shown by an increase in GFAP immunoreactivity that was not observed in ischemic eyes submitted to postconditioning. Synaptophysin immunoreactivity has been extensively used as a quantitative measure of presynaptic terminals.^{23,24} Synaptophysin is an abundant vesicular protein thought to modulate neurotransmission through its association with other synaptic proteins, and it may regulate vesicle biogenesis and recycling.^{25,26} The decrease in synaptophysin immunoreactivity in the outer plexiform layer from ischemic retinas suggests that retinal ischemia provoked an impaired synaptic function and loss of neurotransmission, particularly between the outer and middle retina, that was significantly protected in postconditioned eyes.

As described by Ettaiche et al.,²⁷ retinal dysfunction was dependent on the time of hypertension because greater functional and histologic injury was provoked by 60-minute ischemia than by 40-minute ischemia. Notwithstanding, postconditioning was significantly effective even against more pronounced ischemic damage, such as that induced by 60-minute ischemia.

The neuroprotective effect was not significantly improved with extension of postconditioning algorithm to 10 cycles, but it was not evident with three cycles, which is in agreement with the findings of Yang et al.,²⁸ who showed that increasing the number of cycles of postconditioning did not increase the amount of salvaged myocardium.

Several studies on postconditioning suggest that ischemia-reperfusion intermittency is a critical aspect in the protective effect of postconditioning against ischemic damage in different tissues. However, results reported herein indicated that a single 7-minute period of ischemia (which could be a simpler maneuver than the applications of shorter pulses) were as effective as seven cycles of 1-minute ischemia/1-minute reperfusion. In agreement with this result, Pignataro et al.⁹ have recently shown that a single 10-minute postconditioning ischemia was effective at reducing infarction volume induced by 100 minutes of occlusion of the middle cerebral artery.

The temporal profile of efficacy of postconditioning stimulus has been studied in different tissues. In the heart, it was shown that three 10-second cycles of reperfusion-reocclusion applied in the first minute provides optimal protection, but delaying postconditioning for even 1 minute eliminates protection.^{29,30} Results in the brain are different. Optimal protection was obtained with a single 10-minute period of reocclusion

administered 10 minutes after 100 minutes of middle cerebral artery occlusion.⁹ In addition, it was shown that three cycles of 15-second reperfusion/15-second reocclusion applied immediately after reperfusion or after 45-second reperfusion had neuroprotective effects in the hippocampus CA1 layer, whereas the protective effect was lost when the 15-second reocclusion was applied 60 seconds after reperfusion, demonstrating that the timing of application of reocclusion is critical. Therefore, the retinal neuroprotection observed with postconditioning starting even 60 minutes after the onset of reperfusion was unexpected. Hence, it seems that the application timing of postconditioning for protection against retinal ischemia may be not as critical as that for protection in other tissues, a discrepancy that may be attributable to the tissue-dependent differences with respect to ischemia-reperfusion injury. This delayed protection could increase the potential translational advantages of retinal postconditioning because 60 minutes could be a long enough interval to allow therapeutic manipulation after an appropriate diagnosis of a retinal ischemic process. Notably, Ren et al.³¹ have recently reported that when executed 3 hours and 6 hours (but not 12 hours) after stroke, delayed postconditioning reduces infarct size. Given that a longer time window for the application of postconditioning better reproduces the clinical scenario for an ischemic event, a more detailed temporal course for the effectiveness of retinal postconditioning deserves to be further examined. Experiments are now planned to address this issue.

To acquire insight into the mechanisms involved in retinal protection induced by postconditioning, the effect of CHX injected at different intervals after ischemia was examined. CHX did not affect the functional damage induced by ischemia-reperfusion, but when CHX was injected 1 minute before seven cycles of ischemia-reperfusion, it significantly reduced the protective effect of postconditioning. Conversely, the inhibition of protein synthesis 6 hours after postconditioning was ineffective in changing the protective effects of postconditioning, suggesting that transcriptional changes and the respective changes in gene expression should occur between 1 hour and 6 hours of recovery from ischemia. In agreement with these results, it was demonstrated in pyramidal CA1 neurons in the hippocampus that CHX injected simultaneously with postconditioning suppresses the beneficial effect of postconditioning against ischemia, but it was ineffective when injected 5 hours after postconditioning.⁸

In conclusion, the results reported herein demonstrate a highly protective effect of postconditioning against retinal ischemic injury. This histologic and functional protection depended on the degree of ischemia, the number of ischemia-reperfusion cycles, the de novo synthesis of protein, and the onset time of postconditioning and was even effective when applied 60 minutes after the onset of reperfusion.

Visual disability or blindness may follow retinal ischemia. Although investigators have studied the ability of exogenous agents to treat retinal ischemic damage, thus far none of these strategies is completely effective. Although the mechanisms involved in retinal postconditioning deserve to be further examined, the demonstration that innocuous manipulation of the retina, even when applied after the ischemic insult, is able to dramatically reduce ischemic damage could contribute to the discovery of new therapeutic strategies for ischemic retinal diseases.

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