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Involvement of glutamate in retinal protection against ischemia/reperfusion damage induced by post-conditioning

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Abstract

Retinal ischemia could provoke blindness and there is no effective treatment against retinal ischemic damage. Brief intermittent ischemia applied during the onset of reperfusion (i.e., post-conditioning) protects the retina from ischemia/reperfusion injury. Multiple evidences support that glutamate is implicated in retinal ischemic damage. We investigated the involvement of glutamate clearance in post-conditioning-induced protection. For this purpose, ischemia was induced by increasing intra-ocular pressure for 40 min, and 5 min after reperfusion, animals underwent seven cycles of 1 min/1 min ischemia/reperfusion. One, three, or seven days after ischemia, animals were subjected to electroretinography and histological analysis. The functional and histological protection induced by post-conditioning was evident at 7 (but not 1 or 3) days post-ischemia. An increase in Müller cell glial fibrillary acidic protein (GFAP) levels was observed at 1, 3, and 7 days after ischemia, whereas post-conditioning reduced GFAP levels of Müller cells at 3 and 7 days post-ischemia. Three days after ischemia, a significant decrease in glutamate uptake and glutamine synthetase activity was observed, whereas post-conditioning reversed the effect of ischemia. The intravitreal injection of supraphysiological levels of glutamate mimicked electroretinographic and histological alterations provoked by ischemia, which were abrogated by post-conditioning. These results support the involvement of glutamate in retinal protection against ischemia/reperfusion damage induced by post-conditioning.

Keywords: glutamate, glutamine, ischemia, post-conditioning, retina.

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Retinal ischemia induces irreversible 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 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, among others [reviewed by Osborne *et al.* (2004)]. In addition, reperfusion with oxygenated blood after ischemia also has the potential to aggravate ischemic damage, an effect known as reperfusion injury.

Although at present there is no effective treatment, it is possible to activate an endogenous protection mechanism that prevents from retinal ischemia/reperfusion (I/R) damage by ischemic pre-conditioning (IPC) (Roth *et al.* 1998; Roth 2004). IPC requires a brief period of ischemia applied before ischemic injury, which does not produce any significant damage *per se*, and triggers yet incompletely described mechanism(s) that result in tolerance to the subsequent severely damaging ischemic event [reviewed by Gidday (2006)]. It was shown that IPC affords the retina a greater degree of functional protection against ischemic damage than any known neuroprotective agent (Roth *et al.* 1998).

Although IPC confers robust neuroprotection in different *in vitro* and *in vivo* models of ischemia, 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 ischemic protection,

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Abbreviations used: ERG, electroretinogram; GCL, ganglion cell layer; GFAP, glial fibrillary acidic protein; I/R, ischemia/reperfusion; IOP, intra-ocular pressure; IPC, ischemic pre-conditioning; IPL, inner plexiform layer; OPs, oscillatory potentials; PostC, post-conditioning.

in which a short series of repetitive cycles of brief I/R are applied immediately at the onset of reperfusion, termed post-conditioning (PostC), has been reported in several tissues (Na *et al.* 1996; Zhao *et al.* 2006; Pignataro *et al.* 2008). We have recently shown that seven I/R (1 min/1 min) cycles applied 5 min after the reperfusion onset, afford significant histological and functional protection in eyes exposed to I/R injury (Fernandez *et al.* 2009).

Glutamate is the main excitatory neurotransmitter in the retina but it is toxic when present in excessive amounts. Thus, an appropriate clearance of synaptic glutamate is required for the normal function of retinal excitatory synapses and for prevention of neurotoxicity. Glial cells, mainly astrocytes and Müller cells, surround glutamatergic synapses, and express glutamate transporters and the glutamate-metabolizing enzyme, glutamine synthetase (Riepe and Norenburg 1977; Sarthy and Lam 1978). Glutamate is transported into glial cells and amidated by glutamine synthetase to the non-toxic amino acid glutamine. Glutamine is then released by the glial cells and taken up by neurons where it is hydrolyzed by glutaminase to form glutamate again, completing the retinal glutamate/glutamine cycle (Thoreson and Witkovsky 1999; Poitry *et al.* 2000). In this way, the neurotransmitter pool is replenished and glutamate neurotoxicity is prevented.

Several lines of evidence strongly support the involvement of glutamate in retinal ischemic damage. In that sense, it was shown that electrophysiological and neuronal damage following ischemia resembles that caused by exposure to glutamate (Iversen 1991; Ikeda *et al.* 1992). Moreover, glutamate antagonists or inhibitors of glutamate release prevent retinal ischemic damage (Mosinger *et al.* 1991; Izumi *et al.* 2003), and retinal ischemia induces a significant increase in glutamate release (Neal *et al.* 1994; Cazevieille and Osborne 1997). The aim of the present work was to analyze the involvement of glutamate in retinal ischemic protection provided by PostC.

Materials and methods

Animals and tissues

Animal studies were conducted under the guidelines established by the School of Medicine, University of Buenos Aires. Male *Wistar* rats (average weight, 300 ± 50 g) were housed in a standard animal room with food and water *ad libitum* under controlled conditions of humidity and temperature (21 ± 2°C). The room was lighted by fluorescent lights that were turned on and off automatically every 12 h (on from 6.00 AM to 6.00 PM). All animal use procedures were in strict accordance with the NIH Guide for Care and Use of Laboratory Animals.

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 both eyes was cannulated with a 30-gauge needle connected to a pressurized bottle filled with sterile normal saline solution. Retinal ischemia was induced by increasing intra-ocular pressure (IOP) to 120 mmHg for exactly 40 min, as previously described (Fernandez *et al.* 2009). With this maneuver, complete ocular ischemia was produced, characterized by cessation of flow in retinal vessels, determined by funduscopic examination. During and after (before returning rats to the animal house) the experiments, animals were kept normothermic with heated blankets. Few animals in which cataracts developed because of lens injury were not used any further in the experiments.

Post-conditioning protocol

Eyes were subjected to ischemia by increasing IOP to 120 mmHg for 40 min except for the sham group, in which animals were anesthetized and cannulated without raising IOP. Control eyes were subjected to ischemia only, without any further interruption of reperfusion. For the post-conditioned group, reperfusion was established for 5 min after which eyes were subjected to seven cycles of I/R (1 min ischemia/1 min reperfusion), as previously described (Fernandez *et al.* 2009). A group of animals was subjected to PostC, 5 min after an intravitreal injection of glutamate.

Intravitreal injections

Animals were anesthetized as described. A drop of proparacaine (0.5%) was administered in each eye for local anesthesia. With a Hamilton syringe (Hamilton, Reno, NV, USA) and a 30-gauge needle, 4 μL of 0.3 M glutamate [estimated final concentration 20 mM, considering a vitreous volume of 60 μL (Sumioka *et al.* 2000)] in sterile pyrogen-free saline was injected into one eye of anesthetized rats, while an equal volume of vehicle (saline solution) was injected in the fellow eye. Injections were applied at 1 mm of the limbus and the needle was left in the eye for 60 s; this small volume prevented the increase in IOP and volume loss. The dose of glutamate was selected on the basis of the report by Sisk and Kuwabara (1985). Retinal function and histology were examined 7 days after intravitreal injections of vehicle or glutamate.

Electroretinography

Electroretinographic activity was assessed before (pre-ischemia), and 1, 3, or 7 days after ischemia, as well 7 days after an intravitreal injection of vehicle or glutamate, as previously described (Fernandez *et al.* 2009). Briefly, after 6 h 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 min 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. Electroretinogram (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

were amplified, filtered (1.5 Hz low-pass filter, 1000 Hz 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 with 5-min 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 ± SEM. The mean peak latencies and peak-to-peak amplitudes of the responses from each group of rats were compared.

Oscillatory potentials (OPs) were assessed as previously described (Moreno *et al.* 2005a). Briefly, the same photic stimulator with a 0.2 Hz frequency and filters of high (300 Hz) or low (100 Hz) frequency were used. The amplitudes of the OPs were estimated by measuring the heights from the baseline drawn between the troughs of successive wavelets to their peaks. The sum of three OPs was used for statistical analysis. Baseline (pre-ischemic) ERG recordings were taken at least 1 day before treatment. In a group of animals, ERG and OPs were assessed 7 days after an intravitreal injection of glutamate without or with PostC.

Histological evaluation

Rats were killed and their eyes were immediately enucleated, immersed for 24 h in a fixative containing 4% formaldehyde in 0.1 M phosphate buffer (pH 7.2), and embedded in paraffin. Eyes were sectioned (5 μm) along the vertical meridian through the optic nerve head. Microscopic images were digitally captured with a Nikon Eclipse E400 microscope (illumination: 6-V halogen lamp, 20 W, equipped with a stabilized light source) via a Nikon Coolpix s10 camera (Nikon, Abingdon, VA, USA). Sections were stained with hematoxylin and eosin and analyzed by masked observers. The total retinal, inner plexiform layer (IPL), and inner nuclear layer thickness (in μm) was measured for each eye and the number of cells in the ganglion cell layer (GCL) was counted along 200 μm for each section. No attempt was made to distinguish cell types in the GCL for enumeration of cell number. Measurements (×400) were obtained at 1 mm dorsal and ventral from the optic disk. For each eye, results obtained from four separate sections were averaged and the mean of five eyes was recorded as the representative value for each group.

Immunohistochemical studies

Antigen retrieval was performed by heating (90°C) for 30 min unstained sections immersed in citrate buffer (pH 6.3) and then pre-incubated with 2% normal horse serum, 0.1% bovine serum albumin, and 0.4% Triton X-100 in 0.01 M phosphate-buffered saline for 1 h. For immunodetection of glial cells, sections were incubated overnight at 4°C with a mouse monoclonal anti-glial fibrillary acidic protein (GFAP) antibody conjugated to Cy3 (1 : 1200; Sigma Chemical Co., St Louis, MO, USA). Some sections were treated without primary antibodies to confirm specificity. An Olympus BX50 microscope (Olympus, Tokyo, Japan) was used for microscopic observations.

L-[^{3}H]-glutamate and L-[^{3}H]-glutamine uptake assessment

The influx of L-[^{3}H]-glutamate and L-[^{3}H]-glutamine was assessed in a crude synaptosomal fraction of rat retinas, as previously described (Moreno *et al.* 2005b). Retinas were homogenized (1 : 9 w/v) in 0.32 M sucrose containing 1 mM $MgCl_2$, and centrifuged at 900 *g* for 10 min at 4°C. Nuclei-free homogenates were further centrifuged at 30 000 *g* for 20 min. The pellet was immediately resuspended in buffer HEPES–Tris, containing 140 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose (adjusted to pH 7.4 with Tris base), and aliquots (100–300 μg protein/100 μL) were incubated with 100 μL of L-[^{3}H]-glutamate or L-[^{3}H]-glutamine (500 000–800 000 dpm/tube, specific activity: 17.25 and 51 Ci/mmol, respectively). After 5 min, amino acid uptake was terminated by adding 4 mL of ice cold HEPES–Tris buffer. The mixture was immediately poured onto Whatman GF/B filters (Whatman Inc., Piscataway, NJ, USA) under vacuum. The filters were washed twice with 4 mL aliquots of ice-cold buffer and the radioactivity on the filters was counted in a liquid scintillation counter. Non-specific uptake of L-[^{3}H]-glutamate or L-[^{3}H]-glutamine into synaptosomes was assessed by adding an excess of glutamate or glutamine (10 mM), respectively.

Glutamine synthetase assessment

Each retina was homogenized in 200 μL of 10 mM potassium phosphate, pH 7.2. Glutamine synthetase activity was assessed as described (Moreno *et al.* 2005b). Reaction mixtures contained 150 μL of retinal homogenates and 150 μL of a stock solution (100 mM imidazole-HCl buffer, 40 mM $MgCl_2$, 50 mM β-mercaptoethanol, 20 mM ATP, 100 mM glutamate, and 200 mM hydroxylamine, adjusted to pH 7.2). Tubes were incubated for 15 min at 37°C. The reaction was stopped by adding 0.6 mL of ferric chloride reagent (0.37 M $FeCl_3$, 0.67 M HCl, and 0.20 M trichloroacetic acid). Samples were placed for 5 min on ice. Precipitated proteins were removed by centrifugation, and the absorbance of the supernatants was read at 535 nm against a reagent blank. Under these conditions, 1 μmol of γ-glutamylhydroxamic acids gave an absorbance of 0.340. Glutamine synthetase specific activity was expressed as μmoles of γ-glutamylhydroxamate per hour per milligram of protein.

L-[^{3}H]-glutamate and L-[^{3}H]-glutamine release

For release studies, crude synaptosomal fractions were incubated for 30 min at 37°C with [^{3}H]-glutamate or L-[^{3}H]-glutamine (1 000 000–1 500 000 dpm/retina) in 500 μL of HEPES–Tris buffer. Synaptosomal fractions were washed by centrifugation in fresh buffer to remove the excess of radioligand, and incubated for 10 min with gentle shaking in 500 μL of the same buffer or in a high K^+ buffer (50 mM) in which osmolarity was conserved by equimolar reduction of Na^+ concentration. Synaptosomes were centrifuged, pellets were digested with hyamine hydroxide, and radioactivity in the medium and that incorporated into the tissue were determined in a scintillation counter. Fractional release was calculated as the ratio: radioactivity released/total radioactivity uptake by the tissue. Greater than 80% and 85% of the released radioactivity was identified as authentic glutamate or glutamine, respectively, by TLC.

Glutaminase activity assessment

Glutaminase activity was assessed as described (Moreno *et al.* 2005b). Each retina was homogenized in 100 μL of 0.1% Triton X-100 in 7.5 mM Tris HCl, pH 8.8. The assay mixture contained 20 μL of retinal homogenate (200–400 μg of proteins), 20 mM

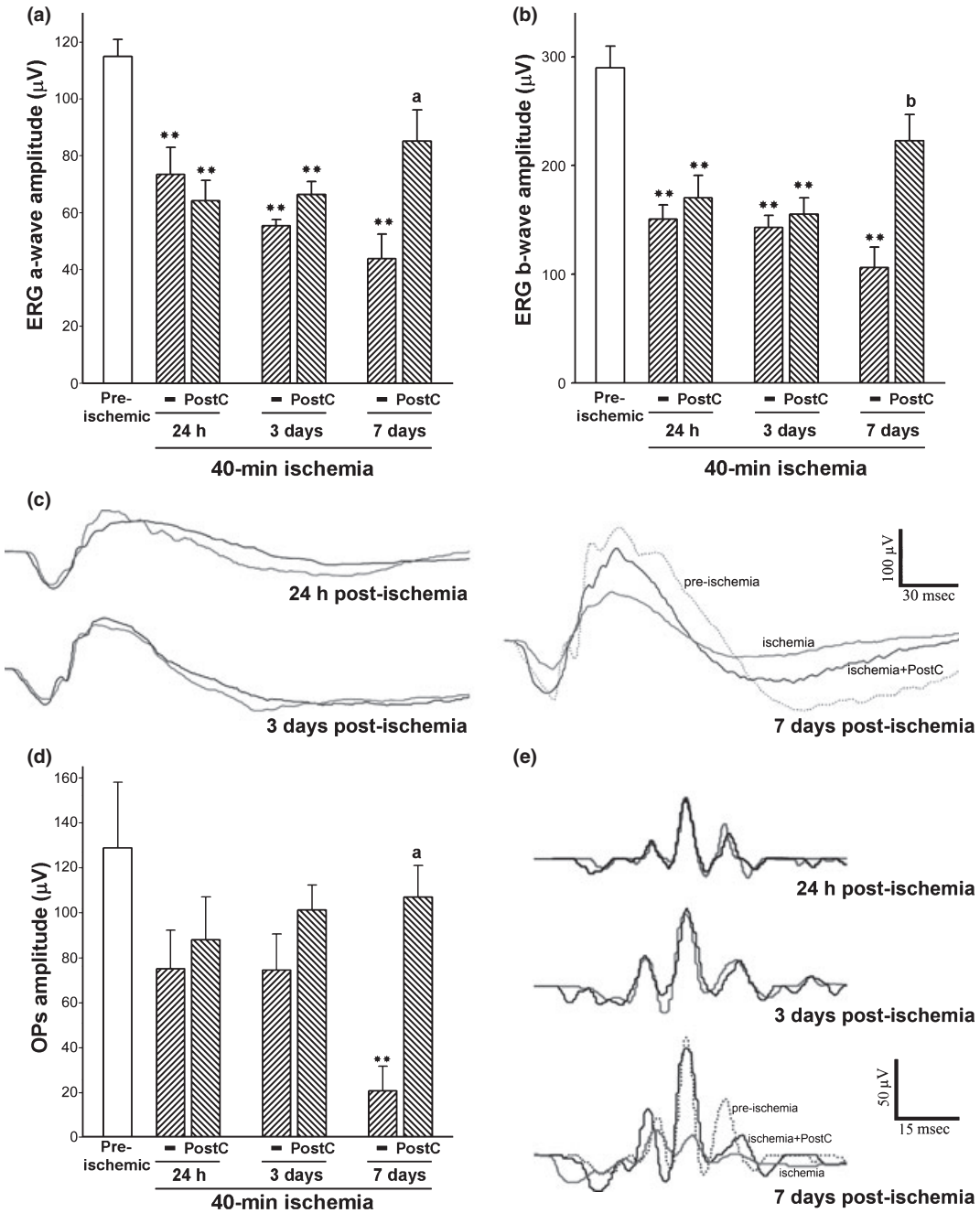

Fig. 1 Temporal course of the electroretinographic protection induced by PostC. Eyes were subjected to 40-min ischemia without or with PostC, and ERGs and OPs were assessed at 1, 3, or 7 days after ischemia. At all these time points, ischemia induced a significant decrease in ERG a- and b-wave amplitude when compared with pre-ischemic values, whereas PostC significantly reversed the effect of I/R only at 7 days post-ischemia. The differences in sum of scotopic ERG OP amplitudes between ischemic eyes and ischemic eyes with PostC were significant at 7 days post-ischemia. Panel a: ERG a-wave amplitude; panel b: ERG b-wave amplitude; panel C: representative scotopic ERG traces from a pre-ischemic eye (dotted line) from an eye subjected to ischemia without PostC (gray line) and from an eye subjected to ischemia with PostC (black line) at different time post-ischemia; panel d: average amplitudes of OPs assessed before ischemia or 1, 3 and 7 days post-ischemia without or with PostC; panel e: representative OPs traces from a pre-ischemic eye (dotted line) from an eye subjected to ischemia without PostC (gray line) and from an eye subjected to ischemia with PostC (black line), at different intervals post-ischemia. For panels a, b, and d, data are the mean ± SEM ($n = 10$ animals per group), $^{**}p < 0.01$ versus pre-ischemic values. a: $p < 0.05$, b: $p < 0.01$ versus ischemia by Tukey's test.

glutamine, 0.2–0.5 μCi L-[^{3}H]-glutamine, and 45 mM potassium phosphate, pH 8.2, in a total volume of 100 μL. Tubes were incubated for 1 h at 30°C, with gentle agitation. The reaction was stopped by adding 1 mL of cold 20 mM imidazole, pH 7.0. Samples were briefly centrifuged, and the supernatants were applied to 0.6 × 3.5 cm beds of anion exchange resin (Dowex, AG1-X2, 200–400 mesh hydroxide form, Bio-Rad Laboratories, Hercules, CA, USA) previously charged with 1 M HCl and washed with water. The reaction substrate was removed with 6 mL of imidazole buffer, which was discarded and the reaction product was eluted with 3 mL of 0.1 M HCl. Aliquots of this fraction were mixed with scintillation cocktail for measurement of radioactivity. Blanks were determined from samples lacking retinal homogenates. Glutaminase specific activity was expressed as μmoles of glutamate per mg protein per hour. Protein content was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Statistical analysis

Statistical analysis of results was made by a two-way ANOVA followed by a Tukey's test, as stated.

Results

The temporal course of the electroretinographic protection induced by PostC on retinal I/R damage provoked by increasing IOP to 120 mm Hg for 40 min was studied. The average amplitudes of ERG a- and b- waves as well as representative scotopic ERG traces from rat eyes subjected to these treatments are shown in Fig. 1. One, 3, and 7 days after the reperfusion onset, a significant decrease in ERG a- and b-wave amplitude when compared with pre-ischemic values was observed, without changes in their latencies. PostC did not induce any significant protection at 1 or 3 days after ischemia, whereas 7 days after ischemia, a significant reversion of the decrease in both parameters was observed in eyes subjected to PostC. No significant differences were observed between pre-ischemic values in animals which were anesthetized and cannulated without raising IOP, and those from eyes subjected to PostC without ischemia or between ischemia without PostC and with sham PostC (data not shown). The sum of scotopic ERG OPs amplitudes was not significantly affected by ischemia at 1 or 3 days, whereas a significant decrease in this parameter was observed 7 days after ischemia, which was reversed by PostC, as shown in Fig. 1.

Figure 2 shows representative photomicrographs of rat retinas subjected to ischemia without or with PostC. One or 3 days after ischemia, the morphology of ischemic retinas (Fig. 2b and c, respectively) did not differ from non-ischemic retinas (Fig. 2a) or retinas subjected to ischemia followed by PostC (Fig. 2f and g, respectively). Seven days after ischemia (Fig. 2d), typical histopathological features of ischemic damage were observed, showing marked reduction

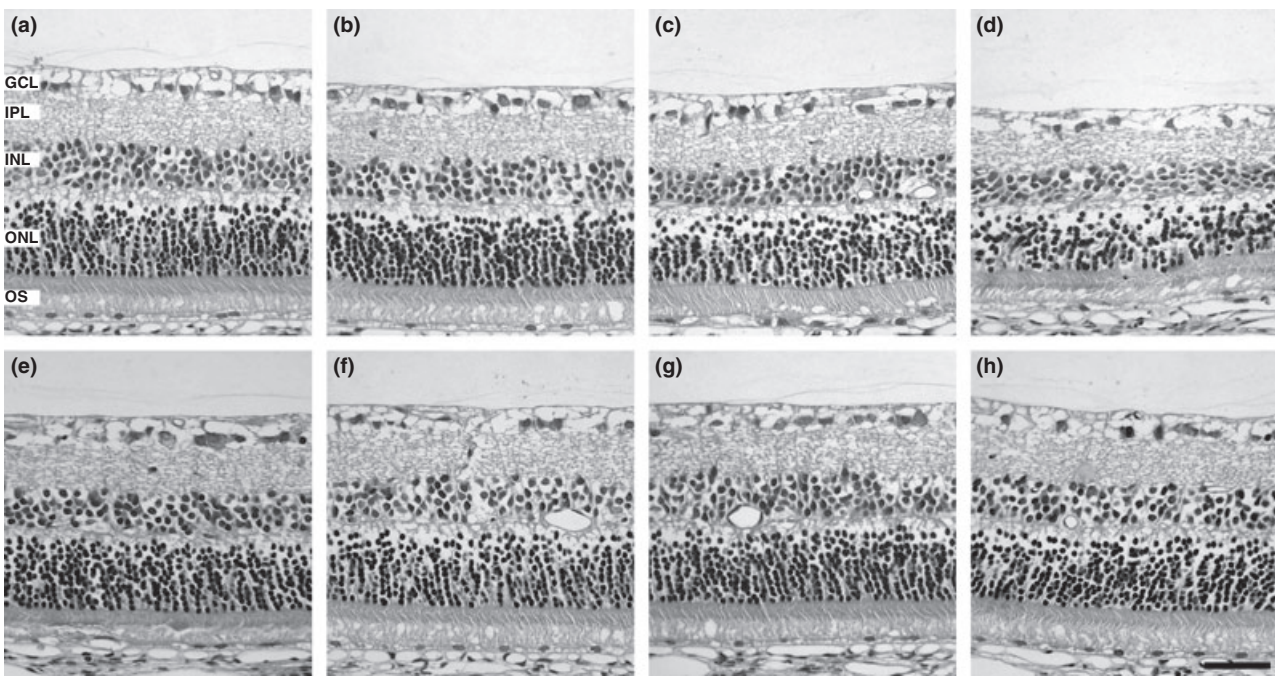

Fig. 2 Representative photomicrographs showing histological appearance of the non-ischemic control retinas (a), ischemic retinas without PostC examined 24 h (b), 3 (c) or 7 (d) days after 40 min of ischemia, retinas examined 7 days after PostC without ischemia (e), or ischemic retinas with PostC examined 24 h (f), 3 (g) or 7 (h) days after 40 min of ischemia. Seven days (but not 24 h or 3 days) after ischemia, a severe retinal damage was observed, whereas in eyes subjected to PostC, the retinal structure was notably preserved. Scale bar = 50 μm. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer, OS, outer segment of photoreceptors.

Table 1 Effect of PostC on the histological damage induced by I/R or glutamate

Treatment	Total retinal thickness (μm)	Inner plexiform layer (μm)	Inner nuclear layer (μm)	GCL cell count (cell number/200 μm)
Non-ischemic	144.2 ± 3.8	31.9 ± 2.2	19.9 ± 6.1	14.9 ± 0.9
Ischemia	119.1 ± 5.4**	22.2 ± 2.8*	12.5 ± 4.9	7.5 ± 1.6**
Ischemia + PostC	149.3 ± 3.5[a]	32.2 ± 1.9[b]	18.1 ± 8.9	13.7 ± 0.9[a]
Glutamate	129.4 ± 3.0**	20.8 ± 2.5**	21.2 ± 4.6	6.4 ± 1.1**
Glutamate + PostC	150.6 ± 3.5[c]	34.7 ± 2.2[c]	22.6 ± 4.8	12.9 ± 1.3[c]

PostC, post-conditioning; GCL, ganglion cell layer; I/R, ischemia/reperfusion.
Total retinal and inner layer thicknesses and GCL cell count in non-ischemic eyes, or eyes subjected to I/R or glutamate intravitreal injections without or with PostC. Similar results were observed in non-ischemic eyes and eyes intravitreally injected with vehicle (data not shown). PostC significantly reversed the histological damage induced by 40-min ischemia and intravitreal injection of glutamate. Data are mean ± SEM, *n*: 10 retinas/group. *$p < 0.05$ and **$p < 0.01$ versus non-ischemic eyes (or vehicle-injected eyes).
[a]$p < 0.01$ versus ischemic eyes; [b]$p < 0.05$ versus ischemic eyes; [c]$p < 0.01$ versus glutamate injected eyes by Tukey's test.

in the total retinal thickness and decrease in GCL cell number (Table 1), whereas PostC (2H) significantly preserved retinal structure at this time point, as shown in Table 1.

To further analyze the morphological protection induced by PostC against I/R damage, retinal immunoreactivity for GFAP was analyzed at several time points after ischemia without or with PostC. In non-ischemic retinas, astrocytes localized in the nerve fiber layer and GCL were weakly GFAP-immunopositive (Fig. 3a), whereas 24 h (Fig. 3b), 3 days (Fig. 3c), and 7 days (Fig. 3d) after 40-min ischemia

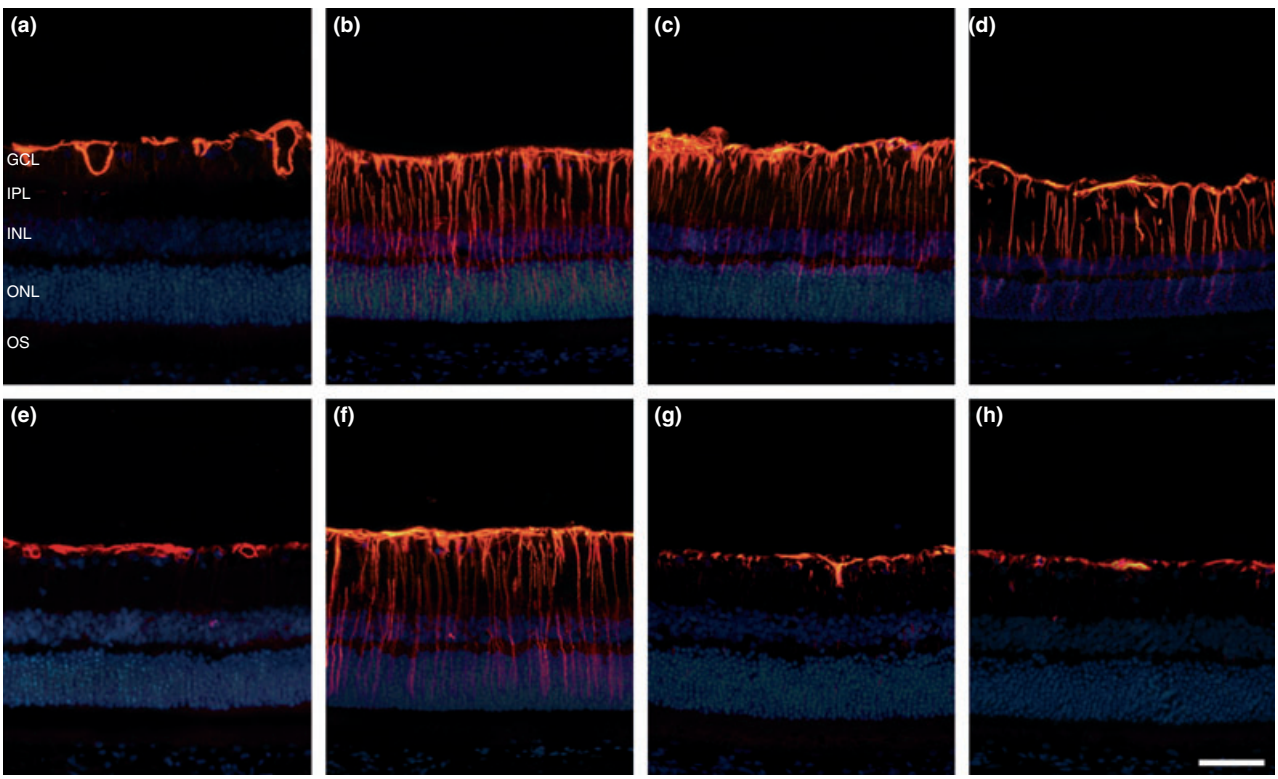

Fig. 3 Immunohistochemical detection of GFAP in non-ischemic control retinas (a), ischemic retinas without PostC examined 24 h (b), 3 (c) or 7 (d) days after 40-min ischemia, retinas examined 7 days after PostC without ischemia (e), or ischemic retinas with PostC examined 24 h (f), 3 (g) or 7 (h) days after 40 min of ischemia. In ischemic retinas, an intense GFAP (+) immunoreactivity was observed in astrocytes and Müller cell bodies and their processes, at all time points. At 3 and 7 days post-ischemia, PostC reduced GFAP immunoreactivity showing only few positive astrocytes similar to control retinas. Scale bar = 50 μm. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer, OS, outer segment of photoreceptors.

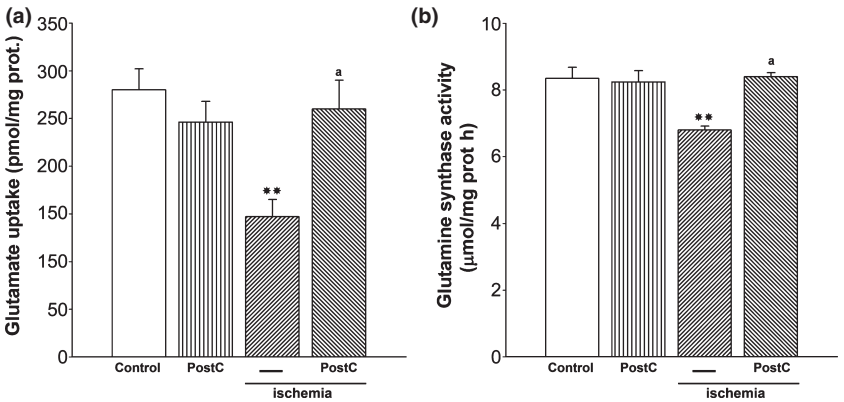

Fig. 4 Effect of ischemia and PostC on retinal glutamate uptake (panel a) and glutamine synthetase activity (panel b). Three days after ischemia, a significant decrease in glutamate uptake and glutamine synthetase activity was observed in ischemic retinas, whereas PostC reversed the effect of ischemia. No differences in these parameters were observed between non-ischemic eyes and eyes subjected to PostC without ischemia. Data were mean ± SEM (n = 10–12 animals per group). **$p < 0.01$ versus non-ischemic retinas; a: $p < 0.01$ versus ischemic retinas without PostC, by Tukey's test.

Table 2 Effect of ischemia without or with post-conditioning (PostC) on glutamate and glutamine release, and glutaminase activity

	Glutamate release (% fractional release)		Glutamine release (% fractional release)		Glutaminase activity (μmol glutamate/mg protein per hour)
Treatment	Basal	High K^+	Basal	High K^+	
Non-ischemic	59.6 ± 1.9	78.9 ± 1.5**	57.3 ± 1.6	81.0 ± 0.8**	1.98 ± 0.31
PostC	62.0 ± 2	79.0 ± 2.0**	56.6 ± 2.1	82.3 ± 1.9**	1.84 ± 0.13
Ischemia	61.3 ± 1.9	80.0 ± 2.0**	60.2 ± 1.6	78.0 ± 1.4**	2.12 ± 0.28
Ischemia + PostC	62.4 ± 2.9	80.3 ± 1.3**	57.5 ± 0.7	76.8 ± 2.6**	1.85 ± 0.16

High K^+ (50 mM) induced a significant release of both [^{3}H]-glutamine and [^{3}H]-glutamate. PostC alone, 40-min ischemia, and ischemia plus PostC did not affect glutamate and glutamine release or glutaminase activity. Data are means ± SEM (n = 6 animals/group). **$p < 0.01$ versus basal release by Tukey's test.

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 was observed. After 3 days (but not 24 h) of ischemia, PostC decreased the levels of GFAP in Müller cells to those observed in non-ischemic retinas (Fig. 3f and g, respectively). After 7 days of ischemia, a similar pattern to that obtained 3 days after ischemia was observed (Fig. 3h).

Figure 4 shows L-[^{3}H]-glutamate uptake and glutamine synthetase activity in retinas from eyes subjected to ischemia without or with PostC, assessed 3 days after ischemia. These parameters significantly decreased in ischemic eyes when compared with non-ischemic eyes. PostC which did not affect these parameters in non-ischemic eyes significantly reversed the effect of ischemia.

Table 2 shows the effect of PostC on retinal glutamine and glutamate release and glutaminase activity. No changes in these parameters were observed among the experimental groups.

The effect of intravitreal injections of glutamate with or without PostC on scotopic ERG and OPs is shown in Fig. 5. The injection of glutamate induced a significant decrease in the ERG a- and b-wave amplitude (but not their latencies) as well on the sum of OPs when compared with vehicle-injected eyes, whereas seven I/R cycles applied 5 min after glutamate injections significantly abrogated the effect of glutamate. The effect of an intravitreal injection of glutamate without or with PostC on retinal histology is depicted in Fig. 5. Seven days after the injection, glutamate (Fig. 5f) induced a significant reduction in the total retinal and IPL thickness and a significant decrease in the GCL cell number when compared with vehicle injected eyes (Table 1), whereas PostC reversed the effect of glutamate.

Discussion

The foregoing results indicated that ischemia induced significant alterations in the retinal glutamate/glutamine

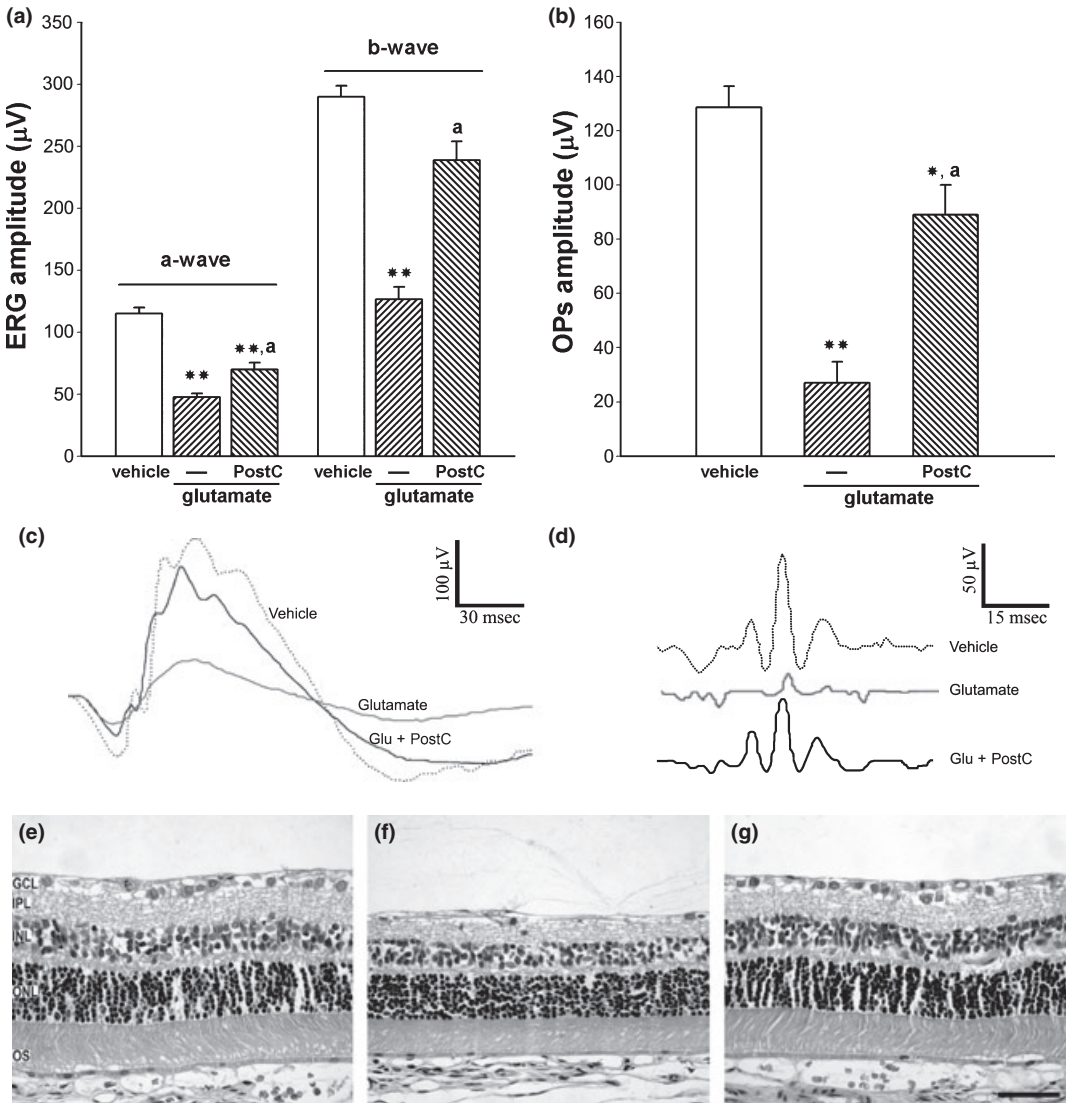

Fig. 5 Effect of an intravitreal injection of glutamate without or with PostC on retinal function and histology. Eyes were intravitreally injected with vehicle or glutamate. Seven I/R cycles were applied 5 min after injections, and the ERG was recorded 7 days after injections. Glutamate induced a significant decrease in the ERG a- and b-wave amplitude, and OP sum, whereas PostC significantly reversed the effect of glutamate. Panel a: average amplitudes of scotopic ERG a- and b-wave; Panel b: average amplitudes of OPs. Shown are means ± SEM ($n = 10$ animals/group). *$p < 0.05$ and **$p < 0.01$ versus vehicle-injected eyes; a: $p < 0.01$ versus glutamate-injected eyes by Tukey's test. Representative scotopic ERGs and OPs traces from eyes injected with glutamate without or with PostC are shown in Panel c and d, respectively. Representative photomicrographs showing histological appearance of retinas obtained 7 days after injection of vehicle (e), glutamate (f), and an eye injected with glutamate and subjected to PostC (g). Glutamate induced a decrease in total retinal and the inner plexiform layer thickness and a decrease in GCL cell number, whereas PostC preserved retinal morphology from glutamate damage. Scale bar = 50 μm. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer, OS, outer segment of photoreceptors.

cycle activity, whereas PostC reversed the effect of ischemia on glutamate clearance. In a previous report, we showed that PostC (seven I/R cycles, 1 min/1 min) provided significant functional and histological protection against I/R injury (Fernandez *et al.* 2009). In that sense, we have demonstrated that PostC induces significant electroretinographic protection both 7 and 14 days after ischemia, and histological protection 14 days after ischemia.

To acquire insight into the mechanism(s) involved in the retinal protection induced by PostC, a more detailed temporal course of this phenomenon was studied. The time–course of post-ischemic neuronal damage in the retina was believed to

be similar to that observed in other regions of the CNS. It appears that a 'maturation' phenomenon occurs, whereby ischemic damage, at least according to histological criteria, becomes more evident with increasing times following ischemia (Rosenbaum *et al.* 2001). It was demonstrated that the functional (ERG) and conventional histological changes after ischemia progressed over time and that ERG disturbance predated morphologic changes (Rosenbaum *et al.* 2001). The present results further supported that the scotopic ERG a- and b-wave amplitudes (but not OPs) were more sensitive indicators of retinal I/R damage, because these functional measurements revealed injury at a time when retinal morphology appeared relatively normal (e.g., 24 h and 3 days post-ischemia).

The origin of OPs has not been definitively determined but OPs are generally thought to originate from feedback neural pathways in the inner retina, especially around the IPL and mainly from amacrine cells, although ganglion cells and bipolar cells may contribute to some parts of the OPs. (Heynen *et al.* 1985; Wachtmeister 1998; Rangaswamy *et al.* 2006). Moreover, OPs are known to be strongly dependent on retinal circulation (Wachtmeister 1998). Contrary to our expectations, however, the sum of OPs significantly decreased only 7 days after ischemia. We do not have any clear explanation for this result. Although a high variability could have influenced the OP data, this result could suggest that the retinal activity from the inner retinal layers (OPs) was affected by ischemia later than the middle and outer layers (the ERG b- and a-waves). Only at 7 days (but not 24 h or 3 days) post-ischemia, a significant functional (sum of OPs and ERG a- and b-wave amplitude) protection was observed in post-conditioned eyes.

No evident changes in retinal morphology were observed in eyes without or with PostC at 24 h or 3 days post-ischemia, whereas 7 days after ischemia typical histopathological features of ischemic damage were observed in ischemic retinas which were not observed in ischemic eyes subjected to PostC. Taken together, these results indicate that an end-stage for PostC protection (e.g., a time point in which both histological and functional protection were evident) occurred 7 days after ischemia. Therefore, studies aimed at identifying the mechanisms involved in PostC should be performed at an earlier time point than 7 days. For this purpose, a period of 3 days after ischemia was chosen. At this time point, no morphological and functional differences were observed between non-post-conditioned and post-conditioned eyes, but a clear difference was observed in retinal Müller cells, as shown by GFAP immunolabeling. GFAP up-regulation is a hallmark of reactive astrocytes (Pekny and Nilsson 2005) and retinal pathology modulates its expression (Bignami and Dahl 1979; Wu *et al.* 2003). Müller cells that do not express GFAP under physiological conditions are known to express GFAP in pathological situations (Erickson *et al.* 1987; Osborne *et al.* 1991). I/R provoked a significant alteration in Müller cells, as shown by an increase in GFAP immunoreactivity at 1, 3, and 7 days after ischemia. At 3 and 7 (but not 1) days post-ischemia, GFAP immunolabeling in Müller cells markedly decreased in eyes subjected to PostC. As Müller cells played a relevant role in retinal glutamate clearance, the glutamate/glutamine cycle activity was assessed 3 days after ischemia in non-post-conditioned and post-conditioned retinas.

The link between excitotoxicity and retinal ischemic damage had received considerable experimental support. However, to our knowledge, the glutamate/glutamine cycle activity was not previously studied in ischemic retinas. The present results indicated that retinal glutamate uptake and glutamine synthetase activity were susceptible to I/R and that these parameters could be involved in retinal protection against I/R injury induced by PostC.

Glutamate transporters are responsible for maintaining low synaptic glutamate concentrations. The present results showed a significant decrease in retinal glutamate uptake 3 days after ischemia. In agreement, immunocytochemical studies showed a decrease in glutamate uptake by Müller cells under ischemia (Napper *et al.* 1999; Barnett *et al.* 2001). While these studies did not assess changes in the functional capacity of glutamate transporters, our results demonstrated a removing glutamate disability in retinas from ischemic eyes which was significantly improved by PostC.

The synaptically released glutamate is taken up into Müller cells where glutamine synthetase converts it into glutamine. As Müller cells rapidly convert glutamate to glutamine, the driving force for glutamate uptake will be stronger in these cells than in neurons which have much higher intracellular free glutamate concentrations (Pow and Robinson 1994). In fact, although glutamate uptake is controlled by the expression and post-translational modifications, physiological measurements suggest that glutamate uptake may also depend on its metabolism (Gegelashvili and Schousboe 1998; Tanaka 2000). The present results showed a significant decrease in retinal glutamine synthetase activity in ischemic retinas which was reversed in eyes subjected to PostC. Therefore, the changes in glutamine synthetase activity could account for the changes in glutamate uptake observed under these experimental conditions. Notably, PostC *per se* (in non-ischemic retinas) did not change glutamate uptake and glutamine synthetase activity which is in agreement with the fact that this maneuver *per se* did not affect retinal function and histology.

After the reaction is catalyzed by glutamine synthetase, glutamine is released from Müller cells and can be a precursor for neuronal glutamate. As shown herein, glutamine uptake and release, glutaminase activity, as well as glutamate release did not change 3 days after ischemia without or with PostC. However, the decrease in glutamate synthetase activity and glutamine uptake in ischemic retinas by themselves could contribute synergically and/or redun-

dantly to an excessive increase in glutamate synaptic level, whereas the enhancement of glutamate transporter and glutamine synthetase activity by PostC might protect from retinal damage resulting from I/R injury. In agreement with these results, it was reported that the suppression of retinal glutamate transporter activity significantly enhanced retinal neurodegeneration induced by ischemia (Bull and Barnett 2004). Moreover, a significant decrease in extracellular glutamate levels during the ischemic insult was observed 3 days after the induction of cortical ischemic tolerance by spreading depression (Douen *et al.* 2002).

The fact that glutamate clearance impaired by I/R on day 3 was resorted by PostC, whereas PostC did not provide any protection at this time point, could suggest that the process of retinal protection induced by PostC started after (but not simultaneously with) the restoration of glutamate levels. In this scenario, the restoration of glutamate levels induced by PostC 3 days after ischemia could initiate a process of both retinal function and histology rescue which was completed at 7 days post-ischemia.

At retinal level, glutamate uptake and glutamine synthetase are mainly localized in Müller's cells (Derouiche and Rauen 1995). Therefore, the results showing the PostC-induced reversion of ischemic effect on GFAP immunolabeling, glutamate uptake, and glutamine synthetase activity, point at Müller cells as being putative target for the protective effect of PostC on I/R damage, albeit the involvement of other cellular type(s) cannot be ruled out.

These results support that ischemic damage can be mediated by an increase in retinal synaptic glutamate concentrations and that PostC protects the retina through a decrease in this parameter. Two necessary (although not sufficient) conditions are needed to prove this hypothesis: (i) high concentrations of glutamate should provoke a retinal damage similar to that induced by I/R and (ii) PostC should protect retinal histology and function from glutamate toxicity. The results presented herein support this hypothesis. Indeed, supraphysiological concentrations of glutamate induced a functional (ERG a- and b-wave and OP amplitude) and histological damage similar to that provoked by ischemia, and PostC protected the retina from the deleterious effects of high concentrations of glutamate.

While the exact mechanisms of PostC protective components are yet to be clearly defined, in all likelihood, PostC is a multifactorial process requiring the interaction of numerous signals, second messengers, and effector mechanisms. Thus, without excluding the activation of other transduction pathways described for PostC in the CNS, such as reduction of oxidative stress (Zhao *et al.* 2003; Halkos *et al.* 2004), inhibition of c-Jun NH_2-terminal kinase/P38 activities (Sun H *et al.* 2006), promotion of extracellular regulated kinase 1/2 activity (Yang *et al.* 2004) and ε protein kinase C phosphorylation (Zatta *et al.* 2006), and Akt phosphorylation (Zhao 2007) among others, these results support that reducing glutamate synaptic levels could represent a crucial step in retinal protection induced by PostC.

Post-conditioning can be a promissory resource in the treatment of retinal I/R injury. Although the mechanism(s) involved in retinal PostC deserve to be further examined, the demonstration that glutamate clearance may be involved in retinal protection induced by PostC, could contribute to find new therapeutic strategies for retinal ischemic diseases.

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