Glutamate Elevation in Rabbit Vitreous During Transient Ischemia-Reperfusion

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Purpose: To investigate the response of the retina to an ischemic insult, we measured the levels of glutamate in the vitreous of rabbit eyes.

Methods: Ocular ischemia was induced in the vitreous of rabbit eyes by simultaneous ligation of the optic nerve, ciliary arteries, and extraocular muscles for 45 minutes. Contralateral eyes were subjected to a conjunctival peritomy to serve as sham-operated controls. Reperfusion was achieved by releasing the ligature. Eyes were enucleated at the end of the ischemic period or 15, 45, or 75 minutes after reperfusion.

Results: Analysis of the vitreous free amino acids showed a significant increase in glutamate levels in the operated eyes at the end of the ischemic period \( (P < .001) \) and after 15 minutes of reperfusion \( (P < .05) \) as compared with the contralateral, sham-operated eyes. Glutamine levels in the vitreous were unchanged throughout the study.

Conclusions: These results show that glutamate, which is considered to be derived from synaptic release of the retinal neurons or accumulation due to a deterioration of glutamate uptake or a degradation system in the retina, was transiently elevated in the vitreous.

Key Words: Glutamate, glutamine, ischemia, retina, vitreous.
rior segments were carefully removed together with the vitreous attached at the base. Then approximately 200 mg of the core vitreous was sampled.

In the ischemia-reperfusion model study, ocular ischemia was induced by optic nerve ligature. Pupils were dilated by topical mydriatics. After making a 360° conjunctival peritomy, the optic nerve, ciliary arteries and veins, and extraocular muscles were simultaneously ligated with 4-0 silk threads in the right eye. Cessation of retinal and choroidal blood flow was confirmed by ophthalmoscopy. After 45 minutes, the ligatures were released and reperfusion was allowed for 15, 45, or 75 minutes. Reperfusion of the blood flow was confirmed by ophthalmoscopy. Eyes in which reperfusion did not occur were excluded. Eyes that were subjected to ischemia-reperfusion were defined as operated eyes. Left eyes were subjected to a conjunctival peritomy and used and defined as sham-operated controls. Each group consisted of six animals. At the end of an ischemic period, and 15, 45, or 75 minutes after reperfusion, eyes were enucleated, and rabbits were sacrificed. The sampled vitreous was stored in a frozen condition until analysis.

Amino Acid Analysis

Measurement of the levels of free amino acids in the vitreous was carried out according to the following method. Briefly, approximately 20 mg of vitreous was sonicated in 200 μL of 70% ethanol in an iced bath. Samples were lyophilized, alkalinized with triethylamine, and reacted with phenylisothiocyanate (PITC). The resulting PITC derivatives were applied to an HPLC Pico-Tag system (Millipore, Milford, MA, USA). Concentrations of glutamate and glutamine were calculated and expressed as nmol/mg (wet weight) of vitreous. In each group, values were averaged, and standard errors (SE) were calculated.

Histology of Retinas

From each group, one eye was selected randomly for the evaluation of retinal morphology. Retinas were excised from the eye cup and were fixed with 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer. Retinas were then dehydrated with ethanol and were embedded in epoxy. One-micrometer-thick sections were prepared and were placed on gelatin-coated slides. Sections were stained with toluidine blue and were observed by light microscopy.

Statistical Analysis

Concentrations of the amino acids in operated and sham-operated control eyes were statistically compared with a paired t-test. Those of operated and normal control eyes were compared by a one-way analysis of variance (ANOVA). After comparison by ANOVA among the five groups, post-hoc multiple comparison tests were performed with Fisher’s protected least significant difference (PLSD) test. A level of P < .05 was considered statistically significant.

Results

Under normal conditions, mean levels of glutamate and glutamine in the rabbit vitreous were less than 22.8 and 350.3 nmol/mg of vitreous, respectively. There was no significant difference between right and left eyes in the normal control group.

The relationship between time course and concentrations of the two amino acids is given in Figures 1A,B. Glutamate levels in the vitreous of sham-operated control eyes in each group did not differ significantly from those of normal controls. Glutamate levels in the vitreous of operated eyes that had been enucleated at the end of ischemia and after 15 minutes of reperfusion showed a statistically significant elevation, as compared with sham-operated control eyes (P < .05 and P < .01 by paired t-test, respectively), or as compared with those of normal control eyes (P = .0004 and P = .0465 by Fisher’s PLSD test, respectively). Thereafter, levels of glutamate in the vitreous of the operated eyes reduced to control levels (Figure 1A). Levels of glutamine in the vitreous of operated eyes did not differ significantly from those of the normal control or the sham-operated control eyes throughout the study (Figure 1B).

All retinas from the eyes subjected to ischemia showed vacuolation of the cytoplasm in the pigment epithelium. In the retinas from the eyes subjected to more than 45 minutes of reperfusion, the outer segments were fragmented, and the abnormalities were more marked toward the distal regions of the outer segments. Retinas from the eyes subjected to 45 minutes of reperfusion showed associated swelling of ganglion cell bodies. After 75 minutes of reperfusion, the nuclei of the ganglion cells and neurons in the inner nuclear layer were swollen, and less intensely stained with clumped chromatin. Edema in the inner and outer plexiform layers was also observed in the eyes subjected to 75 minutes of reperfusion. In addition, serous detachment of the retina and subretinal accumulation of exudative materials were observed in some portions of the retinas that were subjected to longer than 45 minutes of reperfusion (Figure 2).
Discussion

The roles of glutamate in ischemic damage of the retina have been intensively investigated. However, glutamate content in the retina during ischemia remains controversial. More recently, a microdialysis technique has demonstrated glutamate elevation in the extracellular space of the retina in ischemia-reperfusion models. Changes in the vitreous glutamate levels during ischemia-reperfusion in this study resembled those in the retinal extracellular space revealed by microdialysis techniques, although the latter showed that glutamate levels in the extracellular space of the retina increased five- to sevenfold during reperfusion. Ours showed a twofold elevation of glutamate in the rabbit vitreous after the ischemia-reperfusion, but there was no significant change in glutamine levels.

These changes in the vitreous glutamate levels may correspond to alterations in the glutamate metabolism of the retina. Acute ischemia induces depolarization of neurons and the synaptic release of glutamate. Also, glutamate uptake systems are impaired by reduced adenosine triphosphate (ATP) levels, and the activities of glutamine synthetase (GS)—an enzyme that converts glutamate to glutamine—decrease in the Müller cells. As a result, glutamate levels may be elevated in the extracellular retina, and, therefore, elevated levels of glutamate in the vitreous may result from diffusion of glutamate from the extracellular retina.

In the early phase of reperfusion, the glutamate level is still elevated in the vitreous of operated eyes. At this time, the glutamate accumulated in the extracellular space may cause prolonged neuronal depolarization. Because either prolonged depolarization or an increased availability of inorganic phosphate are known to stimulate the activation of phosphate-activated glutaminase (PAG), an enzyme that converts glutamine to glutamate, retinal glutamate pools may recover in association with blood supply; they would then provide glutamate for continuous release in this phase. Finally, glutamate levels decreased to control levels in the late phase of reperfusion. This may have resulted from the inhibition of PAG activity resulting from suppression by an elevated glutamate concentration. The normalization in glutamate level may be also attributed to the recovery of the glutamate uptake or degradation system. Increases in glutamate uptake or GS activities have been observed in the postischemic surviving neurons or glial cells.

We observed the elevation of glutamate levels in the rabbit vitreous during ischemia-reperfusion, which may reflect an involvement of glutamate neurotoxicity in the retina during ischemia-reperfusion. The accumulation of released glutamate in the vitreous may be toxic to retinal neurons; this is evidenced by histology of the retinas that showed swelling of ganglion cells, inner plexiform layer, and neurons in the inner nuclear layer of eyes subjected to a longer time of reperfusion in our study.

Figure 1. Levels of glutamate and glutamine in operated and sham-operated eyes. (A) Glutamate concentration in ischemic eyes (●) and in sham-operated control eyes (○) during ischemia-reperfusion. Closed circle at 0 minutes shows glutamate level in right normal controls and open circle at 0 minutes shows that in left normal controls. Data are expressed as mean ± SEM (n = 6). Significant differences from controls are indicated: *P < .05 (Fisher’s PLSD test), **P < .001 (Fisher’s PLSD test), #P < .05 (paired t-test), ##P < .01 (paired t-test). (B) Glutamine concentration in ischemic eyes (●) and in sham-operated control eyes (○) during ischemia-reperfusion. Closed circle at 0 minutes shows glutamine level in right normal controls and open circle at 0 minutes shows that of left normal controls. No significant difference was found between eyes subjected to ischemia-reperfusion or sham-operation, or among ischemic eyes and normal control eyes.
Our results support systemic administration of glutamate receptor antagonists to treat patients with ischemic damage to the retina.\textsuperscript{10} They also suggest some beneficial effect in removing the vitreous containing high amounts of glutamate and perfusing the vitreous cavity with glutamate receptor antagonists in patients with acute or chronic ischemic diseases of the retina.

Figure 2. Histological retinal samples from experimental animals. Retina from normal control eye (A), retina from eyes enucleated at end of ischemia (B), after 15 minutes (C), after 45 minutes (D), and after 75 minutes (E) of reperfusion. Retinas subjected to longer time of reperfusion showed prominent swelling of ganglion cells and neurons in inner nuclear layer. In retinas from eyes subjected to longer than 45 minutes of reperfusion, outer segments were fragmented and disorientated. Bar = 25 \mu m.

References
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