Glutamate-Induced Glutamine Synthetase Expression in Retinal Müller Cells after Short-term Ocular Hypertension in the Rat

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Purpose. To determine the effect of intraocular pressure (IOP) elevation on glutamate-induced expression of glutamine synthetase (GS) in retinal Müller cells of rat eyes.

Methods. Six groups of three rats each were studied. Group I was a normal control group. In Group II, one eye received an intravitreal glutamate injection (75 nmoles) while the contralateral eye served as control. In Groups III and IV, IOP was raised in one eye by episcleral vein cauterization. Moderately elevated IOP was maintained for 1 day in Group III or 1 week in Group IV (35 ± 1.9—45 ± 5.2 mm Hg). An additional two groups of rats received bilateral intravitreal glutamate injections (75 nmoles) immediately (Group V), or 6 days (Group VI), after induction of IOP elevation in one eye. One day after glutamate injection the rats in all groups were killed, and the eyes enucleated and fixed. Retinas from left and right eyes of each animal were embedded together in LR White resin (Ted Pella, Redding, CA). Sections were processed for GS immunolabeling with antibodies to GS by two-stage immunogold labeling with silver enhancement. Images of labeled retinas from the two eyes were captured under identical light microscopic conditions and the GS immunoreactivity in Müller cells was compared between the left and right retinas in the same section by image analysis. An additional five rats were included in Group II and the retinas were analyzed by Western blot analysis to confirm immunohistochemical findings.

Results. Similar to the finding in the control group (Group I), the GS immunoreactivity of the left and right eyes of Group III and IV remained unchanged even though the right eyes in the two groups had elevation of IOP lasting for 24 hours and 1 week, respectively. However, GS levels were significantly increased by 40 ± 5.7% in normotensive eyes 24 hours after intravitreal injection of glutamate (Group II). The rise in GS immunoreactivity was abolished in eyes with acute IOP elevation (Group V). In contrast, when the eyes were exposed to high IOP for 1 week (Group VI), the glutamate-induced increase in GS immunoreactivity was restored.

Conclusions. Elevated levels of vitreal glutamate can increase the expression of GS in retinal Müller cells. This increase was blocked if IOP was acutely elevated for 24 hours but was restored if IOP remained elevated for 1 week. This finding suggests that moderate elevation of IOP causes only short-term functional changes of glutamate metabolism (amidation) by retinal Müller cells. However, it is not known to what extent endogenous extracellular glutamate can regulate GS expression in normal eyes or in eyes with glaucoma. (*Invest Ophthalmol Vis Sci.* 2004;45:3107–3112) DOI:10.1167/iovs.03-0948

The role of retinal Müller cells in the pathophysiology of glaucoma is not fully understood. The response of Müller cells to a variety of stresses on the retina, such as high intraocular pressure (IOP), may be deleterious[1,2] as well as protective[3] for retinal neurons.

This study is focused on glutamine synthetase (GS), an important enzyme located mainly in retinal Müller cells, that catalyzes the amidation of glutamate to glutamine and is a component of the major mechanism for the clearance of extracellular glutamate.[4–8] A prolonged elevation of extracellular glutamate, possibly caused by chronic ocular hypertension, has been previously hypothesized to cause retinal ganglion cell (RGC) death.[9] The hypothesis is based on reports of the presence of increased levels of glutamate in the vitreous of some patients with glaucoma and in some animal models of the disease.[9–11] There is also evidence that casts doubt on these findings that glutamate is elevated in glaucoma to the extent that it causes RGC death.[12,13] Elevated vitreal glutamate levels would imply that the glutamate–glutamine cycle, which involves several enzymes and transporters and is the major pathway for clearance of extracellular glutamate, may be malfunctioning in a retina stressed by pressure. However, previous work showed that GS expression, as determined by immunohistochemistry, appears to be unaffected in chronic (1 to 10 months) ocular hypertensive glaucomatous monkey eyes (Shen F, et al. *IOVS* 1997;38:ARVO Abstract 2806). The absence of upregulation of the GS level in the glaucomatous monkey eye can be due either to the fact that in the pressure-stressed retina the level of extracellular glutamate is not high enough to upregulate GS or that upregulation is inhibited by high IOP.

To evaluate these possibilities by a timed study of IOP elevation on the GS level in retinal Müller cells, the earlier experiments done in monkeys (Shen F, et al. *IOVS* 1997;38: ARVO Abstract 2806) were extended to a rat glaucoma model and shorter periods of IOP elevation. The specific objective was to determine whether GS expression in Müller cells could be induced to upregulate in vivo by elevated extracellular glutamate, and if so, whether this induction is affected by acute (1 day) or more prolonged (1 week) elevation of IOP.

Methods

All animal care procedures and experimental protocols were approved by the Institutional Animal Care and Use Committee at the Mount Sinai

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School of Medicine and were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Ocular Hypertension and Glutamate Injection

Six groups, each of three Wistar rats (except Group II, $n = 8$), were used in this study. The first group (Group I) comprised of normal rats and was used to establish baseline information that the GS level in contralateral eyes of each animal is equivalent as determined by the quantitative immunohistochemical method used. To determine GS induction by a high concentration of vitreal glutamate in vivo, the right eye of each rat in the second group (Group II) received an intravitreal injection of glutamate (75 nmols; 1.5 μL of a 50 mM solution in 0.1M phosphate buffer, pH 7.4) 24 hours before enucleation. The contralateral left eyes received intravitreal injections of vehicle only and served as control eyes. In the third (Group III) and fourth (Group IV) groups of animals, elevation of IOP in the right eyes was created by episcleral vein cauterization. The contralateral left eyes were sham-operated. Elevated IOP (35 ± 1.9—45 ± 5.2 mm Hg) was maintained for 1 day (Group III) or 1 week (Group IV) before euthanasia to determine the effect of acute (1 day) and more prolonged (1 week) high IOP on GS level. To test if the induction of GS by glutamate could be affected by high IOP, another two groups of animals received intravitreal glutamate injections (75 nmoles) immediately (Group V) or 6 days (Group VI) after induction of IOP elevation in the right eyes. The contralateral left eyes were sham-operated at the same time as the right eyes for IOP elevation, and received the same dose of glutamate intravitreally 24 hours before euthanasia. The injected concentration of glutamate used in this study was based on preliminary experiments (data not shown) over the injected concentration range 5–50 mM glutamate which showed that the highest dose (75 nmoles per eye) gave a consistent elevation of GS in Müller cells of sufficient magnitude for reliable measurement.

Intravitreal injections were performed under acepromazine/ketamine/xylazine (1 + 4.5 + 9 mg/mL, 1–3 mL/kg body weight) anesthesia with a beveled 33-gauge needle connected to a microsyringe (Hamilton, Reno, NV) after the pupil was dilated with 1% cyclopentolate (Cyclogyl; Alcon, Fort Worth, TX). The needle tip was visualized at all times during the procedure through an ophthalmic surgical microscope to avoid retinal damage. Delivery of the solution was performed into the posterior vitreous over a period of 3 minutes to avoid reflux. Some animals (Groups V and VI) received bilateral glutamate injections because one eye was used for testing the effect of high IOP on glutamate metabolism with the contralateral eye serving as normal IOP control. No noticeable change of behavior was found after vitreal glutamate injection. To minimize the effects on the animals' well-being, the time of retina exposure to elevated glutamate was limited to 24 hours. Preliminary results (data not shown) had shown no morphologic retinal changes up to 48 hours after glutamate injection.

IOP was elevated immediately by cauterizing three episcleral veins[14] plus 270° of limbal veins,[15] under the same anesthesia as above. IOP was measured with the Tonopen-XL (Mentor, Norwell, MA) before, immediately after the surgical procedure, and just before euthanasia. All IOP measurements were performed shortly after ketamine/acepromazine/xylazine anesthesia. Table 1 is a summary of the experimental conditions applied to the left and right eyes of each group and the mean IOPs in the experimental and control eyes immediately after surgery and before euthanasia.

GS Immunohistochemistry

Animals in all groups (I–VI) were euthanatized by anesthetic overdose and the eyes were immediately enucleated. The anterior segments were removed and the remaining posterior segments were fixed in 4% phosphate-buffered paraformaldehyde at 4°C overnight. Retinal pieces dissected from corresponding quadrants of the left and right eyes of each animal were dehydrated in a graded series of methanol/water (50–100%) and then infiltrated and embedded together in LR White resin (Ted Pella; Redding, CA). One-micron thick sections containing both retinas were cut. Sections were collected on silanized slides and processed for immunolabeling with antibody against GS using the two-stage immunogold labeling procedure and silver enhancement as described previously.[16,17] Briefly, the sections were etched with a saturated solution of sodium-periodate for 10 minutes at room temperature and then rinsed with 0.1 N HCl and distilled water sequentially. Nonspecific binding was blocked by incubating the sections in 5% normal goat serum. Sections were then exposed to monoclonal GS antibody (Transduction Laboratories, Lexington, KY; 1:1000, 4°C overnight). Sections were then treated with goat anti-mouse IgG conjugated to 1 nm gold particles (Amersham Life Sciences, Arlington Heights, IL; 1:50, 2 hours at room temperature). Immunolabeling on sections was visualized by applying the silver enhancement reagent (silver enhancement kit; Amersham Life Sciences) and examined under a light microscope (X40). Six pairs of nonadjacent wrinkle-free sections from each animal were selected for image analysis.

Vibratome (The Vibratome Company, St. Louis, MO) sections (40 μm) from corresponding quadrants of left and right retinas in Group II rats were made. Free-floating sections were labeled with the same GS antibody as described above at 4°C overnight. They were then treated with secondary antibody conjugated to indocarbocyanine (Cy3; Jackson ImmunoResearch Laboratory, West Grove, PA; 1:200, 2 hours at room temperature). Immunofluorescence-labeled sections were then examined by confocal microscopy (Leica Microsystems, Heidelberg GmbH, Germany).

Image Analysis

Digital images of silver-immunolabeled retinas were captured in gray-scale with a Polaroid digital camera mounted on a Zeiss Axioskop

Table 1. Treatment and Mean IOP (mm Hg) for Rats in Groups I to VI

Group	Eye	Treatment	Mean IOP Immediately after Surgery (mm Hg ± SD)	Mean IOP Before Euthanasia (mm Hg ± SD)
I	Left	None	—	18.5 ± 1.4
	Right	None	—	19.5 ± 2.2
II	Left	Vehicle* injection	—	18.6 ± 1.8
	Right	1 day after glutamate injection	—	18.5 ± 0.2
III	Left	None	18.7 ± 1.2	22.1 ± 3.7
	Right	1 day of high IOP	44.2 ± 5.3	41.4 ± 6.1
IV	Left	None	18.5 ± 3.5	20.7 ± 2.1
	Right	1 week of high IOP	43.1 ± 5.2	37.0 ± 1.7
V	Left	1 day after glutamate injection	19.1 ± 2.9	21.0 ± 1.4
	Right	1 day of high IOP + 1 day after glutamate injection	45.3 ± 3.3	43.5 ± 5.2
VI	Left	1 day after glutamate injection	20.7 ± 3.8	20.7 ± 2.9
	Right	1 week of high IOP + 1 day after glutamate injection	40.9 ± 2.1	35.0 ± 1.9

* The volume of vehicle injected is the same as the volume of glutamate injected into the contralateral eyes.

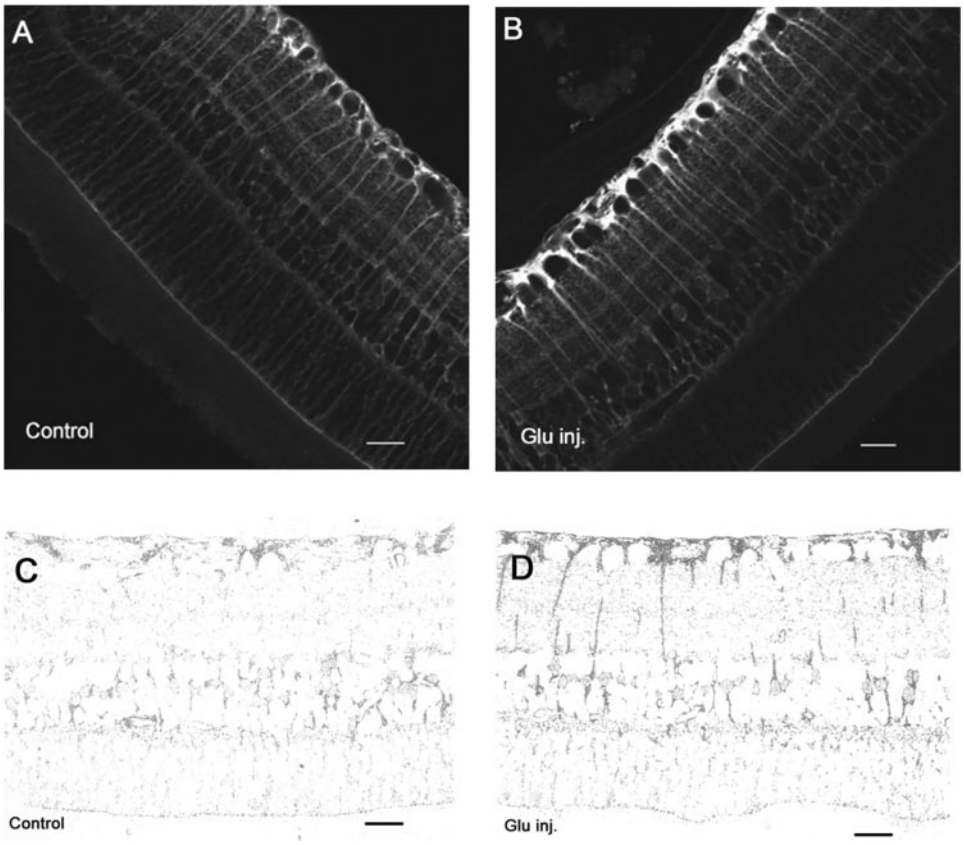

FIGURE 1. GS immunostained sections of Group II rat eyes given a glutamate injection in the right eyes (**B**, **D**; Glu inj.) and buffer injection in the left eyes (**A**, **C**; Control). Note the overall increase in GS immunostaining in the glutamate-injected eyes (**B**, **D**). The increase is more noticeable in the end-feet regions of Müller cells in both fluorescent confocal imaging (**B**) and silver immunostaining (**D**) when the left and right eyes are compared. Scale bars: (**A**, **B**) 20 μm; (**C**, **D**) 15 μm.

microscope (Zeiss, Thornwood, NY) as described previously.[17] Exposure conditions were kept the same for both of the side-by-side paired retinas in each section. Pixel intensity (0 to 255) of immunolabeled Müller cell end-feet was determined using the Image Tool, v2.0, image analysis software (UTHSCSA; ImageTool, San Antonio, TX). Intensity measurements were only performed at the Müller cell end-feet as they are close to the vitreous and possess relatively large unramified areas within which to measure intensity. Average labeling intensity (immunoreactivity) was determined from twenty measurements taken from all Müller cell end-feet in each image analyzed. Immunoreactivity of GS in Müller cell end-feet was compared between left and right retinas in six nonadjacent wrinkle-free sections from each animal in each group. Background intensity (determined as grayscale intensity over adjacent areas of the sections without tissue) was subtracted from the intensity measured within Müller cell end-feet. Percent difference and normalized GS immunoreactivity between the contralateral retinas in each animal group were plotted. In Figure 7, the data are normalized by setting the immunoreactivity of the left eyes at 100% for comparison with the right eyes. Student's *t*-test was used for statistic analysis.

Western Blot Analysis

Eyes from five additional animals in Group II were used for Western blot analysis to confirm the immunohistochemical findings of GS upregulation induced by the increase of vitreal glutamate. Retinal homogenates were prepared and equal amounts of proteins (100 μg) were loaded and electrophoresed in a 10% SDS-polyacrylamide gel and then transferred to nitrocellulose membranes. Nonspecific binding was blocked in a solution containing 5% dry milk and 0.1% Tween-20 in Tris-buffered saline for 2 hours. Membranes were then incubated with GS primary antibody (Transduction Laboratories, Lexington, KY; 1:2000). Detection was performed using a horseradish peroxidase chemiluminescence system (HRP-ECL; Amersham Pharmacia Biotech, Piscataway, NJ). Experiments were performed in triplicate for each retinal sample.

RESULTS

Short-term elevation of IOP up to 1 week was created in the right eye of each animal by cauterizing episcleral and limbus veins. IOPs were significantly higher than those in the noncauterized contralateral eyes ($P < 0.001$). This increase was maintained for up to 1 week without significant drop off (Table 1).

GS immunoreactivity was primarily localized to retinal Müller cells and labeling intensity was similar between left and right eyes in normal rats (Group I). GS immunoreactivity significantly increased 24 hours after the intravitreal glutamate injection (Group II). This increase was more noticeable in the Müller cell end-feet regions, as observed by both fluorescent confocal (Figs. 1A, 1B) and silver immunostaining imaging (Figs. 1C, 1D), and supported by Western blot analysis as an increase in total amount of retinal GS (Fig. 2). GS immunoreactivity in Müller cell end-feet was 40 $\pm$ 5.7% higher ($P < 0.001$) in retinas exposed to glutamate (Fig. 3). Retinas exposed only to high IOP for periods of up to 1 week (Groups III

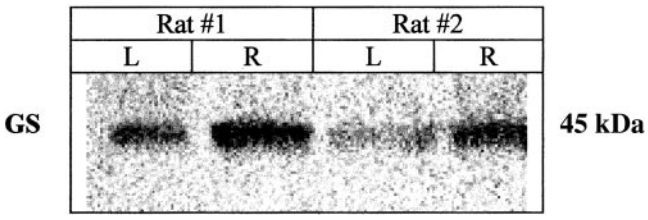

FIGURE 2. Representative Western blot analysis of GS expression in left (L) and right (R) retinas from two out of the five Group II rats in which the right eyes received glutamate injection and the left eyes were buffer-injected controls. 100 μg protein loaded into each lane. Note the significant increase of GS in the right eyes confirming immunohistochemical findings.

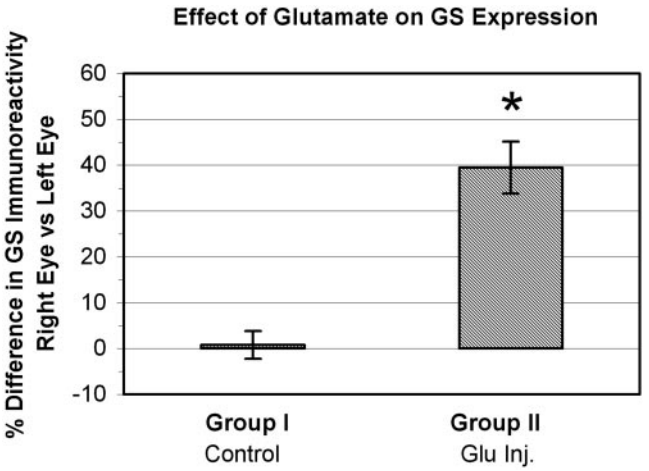

FIGURE 3. The mean percent difference (± SEM) of GS silver-enhanced immunoreactivity between the left and right eyes of Groups I and II rats showing a significant increase in the glutamate injected Group II compared with normal control Group I (*$P < 0.001$).

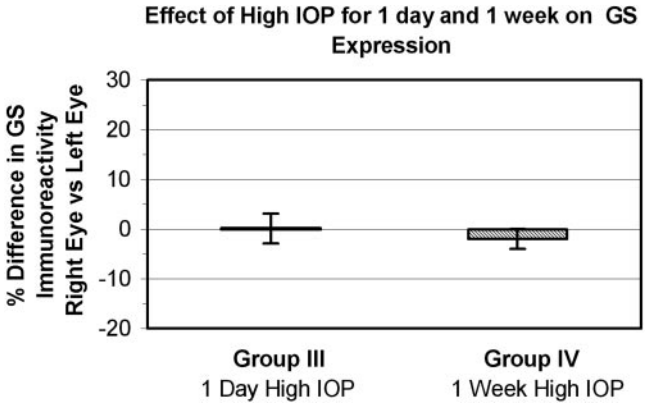

FIGURE 5. The mean percent difference (± SEM) of GS silver-enhanced immunoreactivity between the left and right eyes of Groups III and IV. No significant difference was found between the two eyes in Group III and Group IV rats which had high IOP for 1 day and 1 week, respectively, in the right eye.

and IV) did not show a noticeable increase in GS immunoreactivity (Fig. 4). The result from quantitative image analysis of GS labeling showed no significant difference between the control eyes and eyes with 1 day (Group III; $P = 0.88$) or 1 week (Group IV; $P = 0.42$) of high IOP alone (Fig. 5). However, in the eyes with 1 day elevation of IOP plus glutamate injection (Group V), the labeling intensity of GS in Müller cells was significantly less than that in the contralateral eyes, which had normal ocular tension and received glutamate injection alone (Fig. 6). A 42 ± 2.7% decrease was found by quantitative image analysis in Group V (Fig. 7), indicating concurrent IOP elevation with glutamate intravitreal injection for 1 day significantly inhibited the glutamate response to increase GS immunoreactivity in Müller cell end-feet ($P < 0.001$). However, this effect of high IOP was not sustained. By 1 week of high IOP (Group VI), the Müller cell GS immunoreactivity response to intravitreal glutamate was restored to levels similar to that in eyes receiving glutamate only ($P = 0.2$; Fig. 7).

DISCUSSION

GS is the primary glial enzyme involved in the clearance of extracellular glutamate.[4–8] GS immunoreactivity in the retina localizes mainly in Müller cells and has in the past been used as a specific label for these cells.[18] The present work indicates that GS immunoreactivity can be induced to increase in Müller cell end-feet in vivo in response to an elevated level of vitreal glutamate. This induction has been previously observed in in vitro systems.[19] Cultured rat astrocytes upregulate GS activity after exposure to glutamate (4.11 mM),[20] whereas GS mRNA is increased over baseline level when glutamate is added to either pure astrocytic or astrocytic/neuronal co-cultures.[21] Conversely, GS levels decline when a major source of glutamate is lost, such as during loss of photoreceptors due to inherited degeneration,[22,23] light damage,[24] and retinal detachment.[25] On the basis of these findings it has been proposed that regulation of this enzyme may be normally dependent on extracellular glutamate levels.[19]

In the present study, quantitative immunohistochemistry was used to determine changes of the amount of GS in retinal Müller cells. Although quantitative data from traditional immunohistochemical techniques should be used with caution, silver immunostaining is more reliable and has been used for quantitation by several independent investigators.[17,26,27] To further reduce variability introduced by tissue processing, the tissues from both the treated and control eyes were embedded together in one plastic block and both tissues cut and processed simultaneously in every step of the experiment. This approach allowed paired comparisons. To further validate this approach some of the results were confirmed by Western blot analysis. The significant advantage of immunohistochemistry over protein level measurement lies in the fact that it provides information on the localization and pattern of immunolabeling.

Although GS expression and activity might be dependent on the concentration of extracellular glutamate, at the level of the Müller cells the dose–response for this cannot be estab-

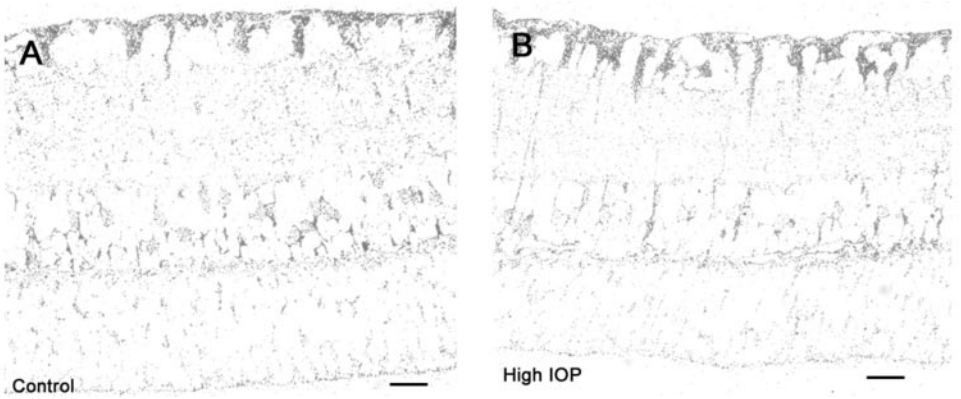

FIGURE 4. Representative GS immunostaining in Group III rats with high IOP in the right eyes for 1 day. No obvious difference in immunostaining is seen in the eye with high IOP (**B**), compared with the contralateral normotensive control eye (**A**). Scale bars: 15 μm.

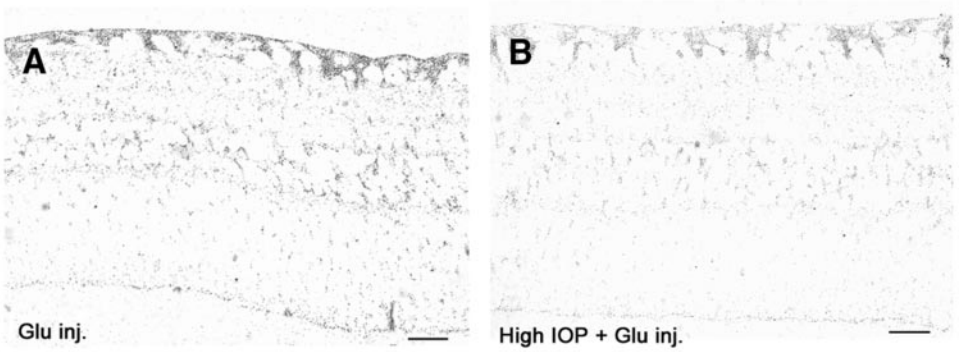

FIGURE 6. Representative GS immunostaining in Group V rat retinas with high IOP in right eyes for 1 day plus glutamate injection (Glu inj.) in both eyes. Significantly less GS immunostaining is observed in the eye with high IOP plus glutamate injection (**B**), when compared to the contralateral eye which received glutamate injection only (**A**). Scale bars: 15 μm.

lished in vivo. In the present study, relatively high doses of glutamate (75 nmoles) were injected into the vitreous cavity, and a significant change in GS immunoreactivity was observed within 24 hours. High concentrations of intravitreally injected glutamate seem to be necessary to observe an effect on GS, probably because the glutamate quickly spreads out from the point of injection into the whole vitreous, then diffuses into the retinal circulation and neural retina (through the inner limiting membrane) where it is rapidly transported and metabolized. Therefore the actual concentration of glutamate in the extracellular space of the neural retina cannot be determined, but is likely to be much lower than the concentration calculated by dividing the amount of injected glutamate by the volume of the vitreous.

In contrast to the effect of elevated glutamate, no obvious change was observed in GS immunoreactivity after exposure of the retina to high IOP for 1 day or for 1 week. This lack of change in GS expression is in agreement with the findings previously reported after longer times of IOP elevation in the monkey glaucoma model (Shen F, et al. *IOVS* 1997;38:ARVO Abstract 2806). GS expression is not affected in monkey eyes by IOP elevation from a few weeks to 10 months. Taken together, these results indicate that ocular pressure stress alone, both acute and chronic, does not affect GS expression levels in the retina.

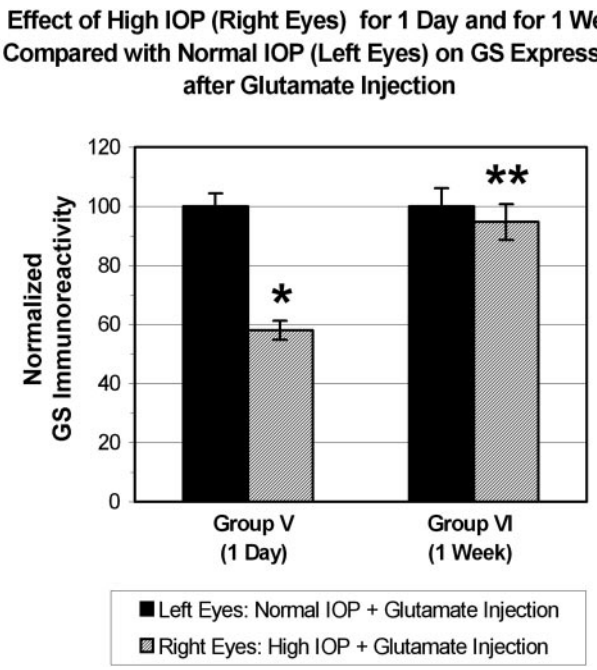

FIGURE 7. Normalized mean (± SEM) GS immunoreactivity of right and left eyes of animals in Groups V and VI. The data are normalized by setting the immunoreactivity of the left eyes at 100% for comparison of left and right eyes. GS immunoreactivity was approximately 40% lower (*$P < 0.001$) in the right eyes compared with that in the left eyes in Group V, in which the right eyes had high IOP for 1 day and both eyes received glutamate injection. A similar level of GS immunoreactivity was found in Group VI eyes, which had 1 week of high IOP in the right eyes plus glutamate injection in both eyes (**$P = 0.2$).

Glaucomatous damage has been proposed to result from an increase in vitreal and retinal glutamate levels. Although the issue remains controversial with various studies in support[9,11] or against[12–13] this theory, one would expect that if elevated IOP leads to glutamate elevation and if Müller cells respond normally, this would lead to increased GS expression. Since GS increase was not observed in the eyes with elevated IOP, there may be two possibilities. First, it could be that increased IOP cannot cause extracellular glutamate concentrations to become high enough to induce a GS increase. Second, it is possible that in a pressure-stressed retina, GS expression does not change from baseline levels despite a rise in extracellular glutamate, because such change is blocked by the effects of high IOP on Müller cells. The results in Group V animals that received intravitreal glutamate injection at the same time as acutely elevating IOP supports the second hypothesis. However, this inhibition effect was not sustained in rats if the IOP elevation was prolonged to 1 week. Glutamate-induced GS expression was completely restored by then (Fig. 7). The level of extracellular glutamate in the animals with high IOP was not known; however, it could be inferred that the extracellular glutamate level was not sufficient to induce GS elevation in the eyes with prolonged IOP elevation. This is in agreement with previous observations in glaucomatous monkey eyes that showed unchanged GS expression (Shen F, et al. *IOVS* 1997; 38:ARVO Abstract 2806) and vitreal glutamate levels.[12]

The variable effect of IOP from 1 day to 1 week on glutamate-induced GS suggests that Müller cells may malfunction when there are acute changes in their environment. However, it appears that longer-term exposure to a noxious stress (such as high IOP) may lead to Müller cell adaptation to the new condition, with a return of full functionality. Specifically, in regard to glutamate metabolism in the retina, the present results suggest that in primary open angle glaucoma (where IOP is chronically and moderately elevated), Müller cells are capable of responding to elevated glutamate levels equally well as in retinas not subjected to high IOP. However, in some forms of glaucoma (e.g., intermittent angle-closure glaucoma, pigmentary glaucoma, glaucomatocyclitic crisis)[28] in which acute episodic IOP elevation has been observed, Müller cells may not be able to remove increased extracellular glutamate efficiently by GS upregulation, and extracellular glutamate may reach toxic levels in these conditions.

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