Retinal Ganglion Cell Protection with Geranylgeranylacetone, a Heat Shock Protein Inducer, in a Rat Glaucoma Model

Yoko Ishii, Jacky M. K. Kwong, and Joseph Caprioli

**Purpose.** To study the effects of geranylgeranylacetone (GGA) on the expression of inducible (HSP72) and constitutive (HSC70) heat shock proteins (HSPs) on retinal ganglion cells (RGCs) in a rat model of glaucoma.

**Methods.** Adult Wistar rats were given intraperitoneal injections of GGA at 200 mg/kg daily. Western blot analysis and immunohistochemical staining for HSP72 and HSC70 were performed after 1, 3, and 7 days of treatment with GGA. After 7 days of GGA pretreatment, intraocular pressure (IOP) was elevated unilaterally by repeated trabecular argon laser photocoagulation 5 days after intracameral injection of India ink. After the first laser photocoagulation, GGA was administered twice a week. RGC survival was evaluated after 5 weeks of elevated IOP. Immunohistochemistry and TdT-mediated dUTP nick end labeling (TUNEL) were performed after 1 week of elevated IOP. Quercetin, an inhibitor of HSP expression, was also administered to a separate group.

**Results.** There was increased expression of HSP72 in RGCs at 3 and 7 days after administration of GGA, but HSC70 was unchanged. After 5 weeks of elevated IOP, there was a 27% ± 6% loss of RGCs. The administration of GGA significantly reduced the loss of RGCs, lessered optic nerve damage, decreased the number of TUNEL-positive cells in the RGC layer, and increased HSP72. Quercetin abolished these protective effects.

**Conclusions.** These results demonstrate that systemic administration of GGA protects RGCs from ischemic damage in a rat model and suggest a novel pathway for neuroprotection in patients with glaucoma. (Invest Ophthalmol Vis Sci. 2003;44:1982-1992) DOI:10.1167/iovs.02-0912

The stress response is a highly conserved mechanism of gene regulation in response to a wide variety of physiological challenges.1-3 The response is characterized by the induction of specific cellular proteins with protective functions. The synthesis of heat shock proteins (HSPs) is rapidly increased in response to many forms of metabolic stress. They function as molecular chaperones to prevent protein aggregation and facilitate refolding of dysfunctional proteins, critical to the survival of all organisms.4-6

The HSP70 family, classified according to molecular mass (70 kDa), comprises several members, such as the inducible (HSP72), the constitutive (HSC70), the mitochondrial (HSP60), and the endoplasmic reticular (GRP78) forms. These proteins function under normal, developmental, and stressful conditions.7 The induction of HSP72 in the mammalian central nervous system by hyperthermia has been associated with the neuronal tolerance to ischemic insults8,9 and neuroprotective effects against light-induced injury in the rat retina.10 Intracellular expression of HSP72 has been demonstrated to protect cerebral neurons against heat shock,11 oxidative stress, apoptotic stimuli,12 excitotoxic insults,13,14 and ischemia-like conditions.15 Neurons of transgenic mice expressing HSP7216 or those of rats injected with the herpes virus containing HSP72 genes17 also have been shown to be resistant to ischemia and seizures, which suggests that HSP72 is essential for neuroprotection.

Geranylgeranylacetone (GGA), an acyclic polyisoprenoid developed and used clinically in Japan, is a unique antiulcer drug that protects gastric mucosa without affecting gastric acid or pepsin secretion.18 Its cytoprotective effects have been found to correlate with the expression of HSPs in gastric mucosal cells induced by the systemic administration of GGA.19-23 GGA induces the expression of proteins in the HSP60, -70, and -90 families in gastric mucosal cells in vivo and in vitro by activating heat shock factor (HSF)-1, the transcription factor for HSPs.19 GGA induces HSPs in numerous tissues including rat small intestine,24 liver,25 lung,26 kidney,24,27 and heart.24,29 In addition, GGA has been proposed to have potential therapeutic benefits for treatment and prevention of ischemia-reperfusion injury, trauma, inflammation, infection, stress ulcers, and organ transplantation.30 The effects of GGA in neuronal tissue have not yet been investigated.

Glaucoma, one of the world’s leading causes of blindness, is characterized by progressive optic nerve damage with selective loss of retinal ganglion cells (RGCs).31-33 It has been postulated that apoptosis, a highly regulated process of cell death, is the final common pathway for RGC death in glaucoma.34-35 Although the exact mechanism of injury to RGCs in glaucoma is not yet known, studies suggest that the induced expression of HSP72 enhances RGC survival in harmful conditions36 and ameliorates glaucomatous damage in a rat model.37 We investigate in the current study whether HSP72 is induced in rat RGCs with systemic administration of GGA; whether the induction of HSP72 by GGA enhances RGC survival and prevents axonal injury in the optic nerve of a rat glaucoma model; and whether apoptosis-like cell death in RGCs is inhibited by GGA.

**Methods.** The procedures used in this study were approved by the Animal Research Committee of the University of California, Los Angeles, and the Research Committee of the University of California, Los Angeles, and the Glaucoma Research Foundation.

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GGA, intraperitoneal injection of GGA; Q, intraperitoneal injection of quercetin.

General Scheme

Three experiments are summarized as follows, with details provided in the subsequent sections. Experiment 1 was performed after systemic administration of GGA to evaluate the expression of HSP72 and HSC70 in RGCs by Western blot analysis and immunohistochemistry. For Western blot analysis, 12 rats were equally divided into 6 groups. Three groups of animals were given daily intraperitoneal injections of GGA (200 mg/kg) and were killed after 1, 3, or 7 days of administration of GGA. Three control groups were as follows: intraperitoneal injection daily for 7 days of (group 1) saline-vehicle or of (group 2) GGA with 4 mg/kg of quercetin (Sigma, St. Louis, MO) and (group 3) no treatment. The enriched RGC fraction was harvested from two retinas of each group and used for Western blot analysis. The same experiment for isolation of RGCs and Western blot analysis was repeated with the other two retinas from each group. For immunohistochemical staining for HSP72 and HSC70, six rats were given GGA and another six rats were given saline systemically for 7 days.

The number of animals used in experiments 2 and 3 are listed in Table 1. Experiment 2 was performed to investigate whether the induction of HSP72 by GGA enhances RGC survival and protects optic nerve axons in a rat glaucoma model. After pretreatment with GGA (200 mg/kg daily) for 7 days, trabecular laser photoocoagulation was performed on one eye of each rat (intracamerat injection of India ink was performed 5 days before photoocoagulation), whereas the contralateral eye remained untreated. GGA was then given twice a week at the same dose until death. Sustained elevation of intraocular pressure (IOP) was maintained by performing trabecular laser photoocoagulation 3 weeks after the first photoocoagulation. To elucidate the role of IOP in the neuroprotective effects of GGA, systemic administration of quercetin at 4 mg/kg was given in the same manner as GGA. Administrations of saline-vehicle, GGA, or GGA with 4 mg/kg of quercetin without trabecular laser photoocoagulation were included as controls. IOP and body weight were measured once a week. After 5 weeks of elevated IOP, the number of RGCs retrogradely labeled with dextran tetramethylrhodamine (DTMR; 3000 molecular weight, anionic, lysine fixable; Molecular Probes, Eugene, OR) was counted (n = 53). The grading of optic nerve injury and the counting of cresyl-violet-stained cells in the retinal ganglion cell layer (RGCL) was also performed (n = 54).

Experiment 3 was performed to investigate the inhibition of apoptosis with GGA administration after 1 week of elevated IOP (n = 50). TdT-mediated biotin-dUTP nick end labeling (TUNEL) and immunohistochemical analysis for HSP72 and HSC70 were performed.

Administration of GGA

GGA was a gift from Esai Co., Ltd. (Tokyo, Japan). A solution of 80 mg/mL GGA was prepared in saline (Balanced Salt Solution; Alcon Laboratories, Inc., Fort Worth, TX) and emulsified for 1 hour in an ultrasonic generator (Branson Ultrasonic Corp., Danbury, CT) immediately before administration. Intraperitoneal injections of GGA were given at a dose of 200 mg/kg. Saline vehicle was prepared and administered in the same fashion in vehicle-treated control groups.

Isolation of RGCs

A previously described method was modified to partially purify RGCs from other retinal cells in rat retinas. Briefly, two dissected rat retinas from each subgroup were washed in 2.5 mL of calcium- and magnesium-free phosphate-buffered saline (PBS) at pH 7.4 and incubated in 1.25 mL of PBS containing 0.5 mg/mL trypsin and 0.01% DNase for 15 minutes at 37°C. This was followed by washing the retinas twice in 2.5 mL of minimal essential medium (MEM) containing 10% (vol/vol) fetal bovine serum. The retinas were subsequently washed in 2.5 mL of MEM twice and dissociated in 3 mL of MEM. The cell suspension was then mixed with 1.5 mL of 30% metrizamide (ICN Biomedicals, Inc., Aurora, OH) in MEM to a final concentration of 10% metrizamide. This mixture was then overlaid with 5% metrizamide in MEM, and the gradient was centrifuged at 4500 rpm (HB-4; Sorvall Instruments, Newtown, CT) for 25 minutes at 4°C. The cells in the 5% to 10% interface were collected and washed in 25 mL of cold MEM. The washed cells were pelleted by centrifugation at 250g for 10 minutes (model 5000C centrifuge; Juan, Winchester, VA). The cells were then resuspended in 400 μL of MEM buffer, and the protein concentration in the cell suspension was measured with a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL).

Immunoblot

Western blot analysis was performed according to the procedure described by Towbin et al. Aliquots of 20 μg of protein from enriched RGCs were separated on a 12% SDS-polyacrylamide minigel (Bio-Rad, Hercules, CA) and transferred to the membrane (Immobilon-P; Millipore Corp., Bedford, MA). The membrane was blocked by incubation in 0.1% Tween-20 in 100 mM Tris-buffered saline containing 10% nonfat dried milk for 1 hour. The membranes were incubated with mouse monoclonal antibody against HSP72 (1:1000; StressGen Biotechnologies Corp., Victoria, British Columbia, Canada) or with rat monoclonal antibody against HSC70 (1:1000; StressGen) overnight and then biotinylated rabbit anti-mouse secondary antibody (1:500; Amersham Biosciences, Piscataway, NJ) or biotinylated goat anti-rat secondary antibody (1:500; Amersham Biosciences) for 1 hour. This was followed by incubation with streptavidin-conjugated horseradish peroxidase (1: 2000; Amersham Biosciences) for 40 minutes. The immunoreactive bands were detected by chemiluminescence with an enhanced chemiluminescence Western blot reagent (Amersham Biosciences).

Immunohistochemistry

Animals were deeply anesthetized with intramuscular injections of 0.8 mL/kg of a cocktail of 5 mL ketamine (100 mg/mL), 2.5 mL xylazine (20 mg/mL), 1.0 mL acepromazine (10 mg/mL), and 1.5 mL normal saline. They were then transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. The enucleated eyeballs were immersed in fixative for 1 hour, bisected, and postfixed overnight. The eyes were embedded in paraffin and sectioned at a 4-μm thickness along the vertical meridian through the optic nerve head. After deparaffinization and hydration, a species-specific avidin-biotin complex (ABC) kit (Vectorstain; Vector Laboratories, Inc., Burlingame, CA) was chosen to match the species of primary antibody and the manufacturer’s procedures were followed. The tissue sections were incubated with blocking serum solution in PBS for 1 hour. This was followed by incubation with primary antibody at 4°C overnight. The antibodies were mouse monoclonal antibody against HSP72 (1:500; StressGen Biotechnologies Corp.), goat polyclonal antibody against HSP72 (1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or rat monoclonal antibody against HSC70 (1:200; StressGen Biotechnologies Corp.). Antigen-antibody complexes were detected by an avidin-biotin-peroxidase technique (Vectorstain ABC Kit; Vector Laboratories). Diaminobenzidine

### Table 1. Sample Size in Experiments 2 and 3

<table>
<thead>
<tr>
<th>Group</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>24</td>
<td>8</td>
</tr>
<tr>
<td>Laser + Vehicle</td>
<td>24</td>
<td>9</td>
</tr>
<tr>
<td>Laser + GGA</td>
<td>23</td>
<td>13</td>
</tr>
<tr>
<td>Laser + GGA + Q</td>
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<td>13</td>
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<tr>
<td>GGA + Q</td>
<td>6</td>
<td>7</td>
</tr>
</tbody>
</table>

## References

- [1983] RGC Protection by Geranylgeranylacetone
(DAB) was used to produce a brown color in the target tissue, and the sections were permanently mounted. As a negative control, alternate retinal sections were incubated with blocking solution by replacing the primary antibody or with anti-rabbit secondary antibody by replacing the original secondary antibody.

Immunohistochemical staining was analyzed quantitatively with a computer-assisted image-processing unit (Image-Pro Plus software; Media Cybernetics, Silver Spring, MD) and the “count–measure” function. Images of immunostained sections were captured with a digital camera (Coolsnap; RS Photometrics, Tucson, AZ) attached to the microscope (Axioplan; Carl Zeiss, Oberkochen, Germany) at 630× magnification under oil immersion. The system was calibrated according to the supplier’s manual before the analysis. For each digital image, all individual cells in the RGCL were marked by a masked examiner and the optical density of each cell was measured. The relative intensities of cells in the RGCL were measured and averaged (± SEM) to yield a single value representing one retina.

**Rat Glaucoma Model**

Rats were anesthetized with intramuscular injections of 0.8 mL/kg of the anesthetic cocktail described earlier. A previously published procedure was modified to produce chronic moderately elevated IOP unilaterally, with the untreated contralateral eye serving as the comparative control.49 Animals were injected intracamerally with 10 μL of 35% India ink (BD Biosciences, Cockeysville, MD) in 0.01 M PBS after removing a similar volume of aqueous. At the end of the procedure, tobramycin 0.3% ophthalmic ointment (Tobrex; Alcon, Fort Worth, TX) was applied topically. Five days after intracameral injection of India ink, a dark band at the limbus was noted due to the aggregation of carbon particles in the trabecular meshwork.50 After anesthesia, approximately 200 laser burns were delivered ab externo to the pigmented trabecular band at laser settings of 200-μm diameter, 200-nmW power, and 0.2-second duration. Three weeks after the first laser treatment, another trabecular laser photoagulation was performed without further injection of ink.

**Measurements of IOP**

Dark-phase IOP measurements were monitored once a week with a portable tonometer (Tonopen XL; Mentor O&O, Norwell, MA) and were performed 1 hour after lights off.51 All IOP measurements were performed with animals in the awake state.52 After topical instillation of proparacaine hydrochloride 0.5% (Alcaine; Alcon, Fort Worth, TX), the tonometer was gently and briefly applied to the cornea and IOP readings were recorded. Five consecutive readings were taken. The IOP data collected in this study represented as uncorrected tonometer units. The readings generated by a very light touch or excessive force were ignored. Three readings were obtained by eliminating the minimum and maximum measurements and were averaged.

**Evaluation of RGC Density**

Rats were killed after 5 weeks of elevated IOP to evaluate the number of DTRM-labeled cells, which were considered as surviving RGCs.54 At 48 hours before death, retrograde labeling was performed in anesthetized animals. The optic nerve was exposed through a lateral conjunctival incision and the optic nerve sheath was incised with a needle knife 2 mm longitudinally starting 3 mm behind the eye. A cross section of the optic nerve was made with the needle knife through the opening of the optic nerve sheath, with care not to damage the adjacent blood supply. DTRM crystals were applied to the proximal cut surface of the optic nerve to label RGCs by fast retrograde axonal transport. After death and exsiccation of the eyes, the retinas were dissected and flattened with four radial cuts (superotemporal, inferotemporal, superonasal, and inferonasal). They were placed vitreous side up on glass slides, dried in the dark at room temperature overnight, and mounted. The retinas were examined with a fluorescence microscope (Axioplan; Carl Zeiss) equipped with a filter that permits visualization of rhodamine fluorescence (excitation filter BP 546, barrier filter LP590; Carl Zeiss). The counting of RGCs was conducted by two examiners in a masked fashion. Three areas per retinal quadrant (superior, temporal, inferior, and nasal) at 1, 2, and 3 mm from the optic disc were analyzed, yielding 12 separate retinal areas for RGC counting. Each rectangular area measured 0.475 × 0.562 mm, and the total counted area corresponded to approximately 3.1% of each total retinal area. Data are expressed as number of RGCs per square millimeter.

**Grading of Optic Nerve Injury and Cell Counting in the RGCL**

To examine the effect on RGC axons, optic nerve injury was evaluated with an established method.41 After 5 weeks of elevated IOP, deeply anesthetized animals were perfused with a solution of 4% paraformaldehyde and 1% glutaraldehyde. Optic nerve segments 1 mm behind the globe were dissected, washed, postfixed with 5% glutaraldehyde, dehydrated, and embedded. One-micrometer-thick sections were cut and stained with 1% toluidine blue. Optic nerve cross sections were examined under light microscopy and assessed by three independent masked observers. A graded scale of optic nerve injury ranging from 1 (normal) to 5 (total degeneration) was used. Data obtained from three observers were averaged and presented as the mean ± SEM.

**Corresponding loss of cells from the RGCL was evaluated by counting cells in the RGCL in cresyl violet–stained retinas.** After collecting the optic nerves, enucleated eyeballs were postfixed in 10% neutral buffered formalin for 1 hour and washed in 0.1 M phosphate buffer (pH 7.4). The retinas were dissected and flattened on a slide, vitreous side up. Four radial cuts were made in the peripheral retinas. The specimens were dried overnight, stained with 1% cresyl violet, dehydrated, and covered with coverslips. Morphologically distinguishable glial and vascular endothelial cells were not counted. Cells with cytoplasm rich in Nissl substance and with irregular outlines were counted as neurons.55 The number of neurons in the RGCL in regions 1 mm (posterior), 2 mm (midperipheral), and 3 mm (peripheral) from the center of the optic nerve head were taken with an eyepiece reticle of a microscope at 400× magnification. Cell counting was performed by two investigators in a masked fashion and the results averaged. Results from the four quadrants (superior, temporal, inferior, and nasal) of each retina were averaged to arrive at one value (mean ± SEM).

**TUNEL Analysis**

Four-micrometer-thick paraffin-embedded sections along the vertical meridian of the optic nerve head were collected, and a minimum of six retinal sections (8 μm apart) per eyeball was used for counting the number of TUNEL-positive cells in the RGCL. Only full-length and undamaged retinal sections without oblique orientation were used. The procedures described in the kit (ApopTag Peroxidase In Situ Apoptosis Detection Kit; Intergen Co., Purchase, NY) were followed and diaminobenzidine (Sigma) was used as a color substrate. Counting was performed by two masked investigators with light microscopy and averaged.

**Statistical Analysis**

The data are expressed as the mean ± SEM. Mean data among groups were compared with one-way ANOVA, and data between groups were compared with the unpaired Student’s t-test. Statistical significance was declared for P < 0.05. Two-tailed tests were used for all comparisons.

**Results**

**Experiment 1: Induction of HSP72 in RGCs after Administration of GGA**

The immunoblots of proteins in the enriched RGC fraction from the rat retinas after systemic administration of GGA (200
There was no detectable difference among them. The expression of HSP72 in RGCs from GGA-treated rats was inhibited after administration of GGA with quercetin (4 mg/kg daily) for 7 days. Immunohistochemical staining for HSP72 showed mild immunoreactivity in RGCL cells of vehicle-treated retina (B) and an increased immunoreactivity in RGCL cells of retina treated with GGA for 7 days (C, arrowheads). Immunohistochemical staining for HSC70 showed strong immunoreactivity in RGCL cells of vehicle- (D) and GGA-treated retinas (E). Q, quercetin.

mg/kg daily) were probed with an antibody against HSP72 (Fig. 1A, top) that specifically recognizes the inducible form of HSPs and an antibody against HSC70 (Fig. 1A, bottom) that corresponds to the constitutive form. There was weak immunoreactivity for HSP72 in RGCs from the vehicle-treated retinas and normal untreated control retinas. One day after administration of GGA, a mild increase in immunoreactivity of HSP72 was noted in the RGCS. A strong increase in immunoreactivity was detected in RGCs treated with GGA for 3 and 7 days. The expression of HSP72 in RGCs from GGA-treated rats was inhibited by coadministration of quercetin (4 mg/kg). However, there was strong immunoreactivity against HSC70 in RGCs of the retinas from control groups and GGA-treated groups, but there was no detectable difference among them.

To localize the immunoreactivity of inducible and constitutive forms of HSPs in RGCs, immunohistochemical staining for HSP72 and HSC70 was performed on retinal sections after 7 days of GGA or vehicle treatment. Increased immunoreactivity of HSP72 was detected in most cells in the RGCL after treatment with GGA (Fig. 1C) when compared with vehicle-treated rat retinas (Fig. 1B). No detectable change in immunoreactivity of HSP72 was detected in other retinal layers (data not shown).

Similar to Western blot analysis, no observable difference in HSC70 expression was noted in the cells in the RGCL (Fig. 1E) or in other retinal layers of GGA-treated rats (data not shown), compared with vehicle-treated rats (Fig. 1D).

**Experiment 2: Protection of RGCs by Administration of GGA**

The baseline IOP in the awake rats was 15.0 ± 0.6 mm Hg, as measured by tonometer (Fig. 2; n = 53). Increased IOP was sustained for 5 weeks, with a maximum of 25.6 ± 1.0 mm Hg at 4 weeks. The relative increase of IOP at 5 weeks compared with the contralateral eyes was 66% (P = 0.001). In the GGA group, the increase of IOP at 5 weeks compared with contralateral control eyes was 82% with a maximum of 27.6 ± 1.2 mm Hg. In the group in which quercetin was coadministered with GGA, there was a 59% increase of IOP with a maximum of 25.0 ± 1.7 mm Hg compared with the contralateral eye. There were no statistically significant differences between the course of IOP in the groups that received vehicle, GGA, or GGA and quercetin.

The body weights of rats in the vehicle, GGA, and GGA with quercetin groups were monitored (Table 2). From the first day of saline injection (1 week before the first trabecular laser photocoagulation) to death (5 weeks after the first laser photocoagulation), there was a 38% increase in body weight in the vehicle-treated rats, 27% in the GGA group, and 38% in the GGA with quercetin group. The difference in gain in body weight among these groups was not statistically significant.

Retrograde labeling with DTMR was performed on optic nerves 2 days before death, to label surviving RGCs by retrograde axoplasmic transport (Figs. 3A–F). The DTMR-labeled RGCs were counted to evaluate the effect of administration of GGA (Fig. 3G). There was a statistically significant difference between the densities of DTMR-labeled RGCs among the six groups (P = 0.001, ANOVA). The density of DTMR-labeled RGCs for vehicle-treated control was 1230 ± 51 cells/mm². After 5 weeks of elevated IOP, the density of DTMR-labeled RGCs decreased to 904 ± 71 cells/mm² (Fig. 3B), which corresponded to a 27% ± 6% reduction when compared with the contralateral eyes (P = 0.0003). GGA preserved 57% more DTMR-labeled cells (1044 ± 36 cells/mm², Fig. 3C) than did vehicle. The preservation of RGCs by GGA in retinas with elevated IOP was partial (P = 0.003 when compared with the vehicle-treated control). Coadministration of quercetin abolished the protective effect of GGA in the retinas with elevated IOP (Fig. 3D; P = 0.002), which showed a density of 756 ± 88 cells/mm². The density of DTMR-labeled RGCs in the GGA-treated contralateral control (Fig. 3E) and the GGA and quercetin-treated contralateral control (Fig. 3F) was 1077 ± 48 and 1255 ± 51 cells/mm², respectively. There was no statistically significant difference between the densities of DTMR-labeled RGCs in the GGA- and the vehicle-treated control groups (P = 0.08) and between the GGA+quercetin- and the vehicle-treated control groups (P = 0.1).

Axonal injury in the optic nerve was demonstrated by light microscopy (Figs. 4A, 4B) and graded from 1 (no nerve injury) to 5 (severe nerve injury). A normal optic nerve with a grade of 1 is shown in Figure 4A, and an optic nerve with a grade 2 injury is shown in Figure 4B. There was significant damage to the optic nerve after 5 weeks of sustained IOP, with a grade of 1.64 ± 0.10 compared with the contralateral control (1.15 ± 0.02, P = 0.001), indicating mild to moderate injury. The optic nerve injury was significantly ameliorated by the administration of GGA, with a grade of 1.33 ± 0.05 (P = 0.026). The GGA-treated contralateral control eyes showed no statistically significant optic nerve injury (1.11 ± 0.02).
Cresyl violet staining and cell counting revealed a significant reduction of cells in the RGCL (2193 ± 75 cells/mm²) after 5 weeks of elevated IOP when compared with the contralateral control (2620 ± 78 cells/mm²; \(P = 0.001\); Fig. 4D). Administration of GGA inhibited the loss of cells in the RGCL with elevated IOP (2697 ± 70 cells/mm², \(P = 0.001\)) and had no significant effect on the number of cells in the RGCL of GGA-treated contralateral control retinas (2644 ± 59 cells/mm²).

**Experiment 3: Inhibition of Cell Death by GGA**

TUNEL staining was performed to label dying cells (Fig. 5B is shown as representative) in retinas with elevated IOP. The number of TUNEL-positive cells in the RGCL was counted and compared, to evaluate the effect of GGA (Fig. 5C). After 1 week of elevated IOP, the number of TUNEL-positive cells in the RGCL was 1.24 ± 0.29 per retinal section and was significantly higher than the control groups treated with vehicle (\(P = 0.026\)), GGA (\(P = 0.008\)), and GGA with quercetin (\(P = 0.017\)). The administration of GGA significantly reduced the number of TUNEL-positive cells to 0.53 ± 0.11 per retinal section (\(P = 0.02\)), corresponding to a 57% inhibition of cell death after 1 week of elevated IOP. The number of TUNEL-positive cells of quercetin-treated retinas with elevated IOP and GGA administration was 1.37 ± 0.31 per retinal section, similar to the vehicle-treated retinas with elevated IOP.

Quantitative analysis of immunoreactive intensity of HSP72 (Fig. 6A) and HSC70 (Fig. 6B) in the RGCL was performed 1 week after trabecular laser photocoagulation. The expression of HSP72 immunoreactivity was significantly different among the groups (\(P = 0.001\), ANOVA). A significantly increased expression of HSP72 was induced by elevated IOP (\(P = 0.01\)). HSP72 expression in retinas with elevated IOP increased further after administration of GGA (\(P = < 0.001\) when compared with vehicle control), but this increase was not statistically significant when compared with the retinas with elevated IOP alone. Expression of HSP72 in GGA-treated retinas with elevated IOP was significantly reduced by the coadministration of quercetin (\(P = 0.002\)). Systemic administration of GGA alone caused an increased expression of HSP72 in the RGCL when

**TABLE 2. Time Course of Body Weight in Experiment 2**

<table>
<thead>
<tr>
<th>Group</th>
<th>-1 Week</th>
<th>0 Week</th>
<th>1 Week</th>
<th>2 Week</th>
<th>3 Week</th>
<th>4 Week</th>
<th>5 Week</th>
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<tr>
<td>Vehicle</td>
<td>346 ± 7</td>
<td>376 ± 8</td>
<td>405 ± 10</td>
<td>432 ± 11</td>
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<tr>
<td>GGA</td>
<td>371 ± 6</td>
<td>379 ± 8</td>
<td>400 ± 9</td>
<td>424 ± 9</td>
<td>448 ± 11</td>
<td>454 ± 12</td>
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<tr>
<td>GGA + Q</td>
<td>322 ± 4</td>
<td>342 ± 7</td>
<td>374 ± 8</td>
<td>398 ± 9</td>
<td>423 ± 10</td>
<td>439 ± 11</td>
<td>445 ± 12</td>
</tr>
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</table>

Data are expressed as the mean ± SEM. GGA, intraperitoneal GGA injection; Q, intraperitoneal quercetin injection. (\(P = 0.07\); ANOVA).

FIGURE 2. Course of IOP in each group during experiment 2. There was a significant increase of IOP in all groups with trabecular laser photocoagulation (\(P = 0.001\)) when compared with groups without photocoagulation. Administration of GGA, vehicle, or GGA + quercetin did not cause a significant change in IOP. Laser, trabecular laser photocoagulation after intracameral ink injection; GGA, GGA injection; Q, quercetin injection. Data are expressed as the mean ± SEM.
compared with the vehicle-alone control (\( P = 0.005 \)), but this increase was abolished by coadministration of quercetin. In contrast, there was no statistically significant difference in the expression of HSC70 in RGCL among all the groups.

**DISCUSSION**

The preinduction of HSP72 in RGCs enhances the survival of RGCs under hypoxic, excitotoxic, and glaucomatous stress. In this study, HSP72 expression was induced in rat RGCs by systemic administration of GGA, a heat shock inducer. We further demonstrated the protective effects of GGA on RGC survival in a rat glaucoma model. The protection appears to relate to the inhibition of apoptosis-like cell death and the induction of HSP72 in RGCs, but does not appear to be related to HSC70. The neuroprotective effect of HSP72 induction was blocked by the coadministration of quercetin, an inhibitor of HSP expression. This is the first report to demonstrate that GGA induces HSP72 in retinal neurons, and provides evidence of a neuroprotective effect for RGCs in a rat glaucoma model.

GGA was developed in Japan and is used clinically for the treatment of gastric ulcers and gastritis. In gastric mucosal cells, GGA promotes biosynthesis of glycolipid intermediates in microsomes and improves metabolism of mucous glycoprotein. Its major pharmacologic effects include promotion of biosynthesis of gastric mucus, protection of the gastric mucosa, and increased gastric blood flow. Many studies have shown that the administration of GGA protects, in vivo and in vitro, rat and guinea pig gastric mucosa against various stressors. Takahashi et al. reported that pretreatment with GGA fails to prevent ethanol-induced damage in cultured rabbit gastric mucosal cells, suggesting species-specific vulnerability. It has also been suggested that GGA may exert its cytoprotective action through an increase of prostaglandin E2, maintenance of nitric oxide synthase activity, or induction of HSPs. There are several published reports that show GGA to be cytoprotective in other organs, such as lung, heart, liver, and kidney. Our results indicate that the neuroprotective effects of GGA may be related to the expression of HSP72, because coadministration with quercetin inhibited HSP and blocked the protection of RGCs induced by GGA in a rat glaucoma model. To confirm the role of HSP72 in the protection of RGCs, further study to inhibit the production of HSP72 with specific antisense oligonucleotides in vivo should be considered.

Consistent with the results in other rat tissues, such as small intestine, liver, lung, kidney, and heart, we observed that the administration of GGA induces the expres-
Representative micrographs of optic nerve cross sections in the vehicle-treated control, with a grade of 1 (A) and degeneration in the optic nerve section of a laser-treated eye after 5 weeks of elevated IOP showing focal degenerating axons, with an injury grade of 2 (B). Optic nerve injury grading (C) and cell counting in the RGCL (D) showed significant axonal damage and reduction of cells in the RGCL after 5 weeks of elevated IOP when compared with vehicle- or GGA-treated controls (*P < 0.05). This axonal damage and reduction of cells in the RGCL was inhibited by administration of GGA (†P < 0.05). GGA, GGA injection; Q, quercetin injection. Data are expressed as the mean ± SEM.
sion of HSP72 in neuronal cells of the adult rat retina. The bioavailability of GGA (125 mg/kg) given by intravenous injection peaks at 6 hours after administration and can be detected for up to 42 days. We administered GGA intraperitoneally to the rats daily and demonstrated that there was an increased expression of HSP72 in RGCs after 3 days, with no observable side effects. The subsequent chronic administration of GGA (twice weekly) sustained the increased expression of HSP72, and appeared to be nontoxic. The mechanism of HSP induction by GGA is not clearly understood, but it is likely that GGA activates HSF1, a transcription factor that stimulates synthesis of mRNA for HSP72. HSF1 is present in the cytoplasm as an inactive monomer. When exposed to stressors, HSF1 undergoes oligomerization to a DNA-binding trimer and then phosphorylation, translocates into the nucleus, and binds to heat shock element (HSE). HSE is located upstream of the HSP72 gene and the binding causes synthesis of HSP72. One study has demonstrated cellular expression and activation of HSF1 in the central nervous system (CNS). HSP72 is believed to protect cerebral neurons against ischemia, seizure, stroke, and hyperthermia. The molecular mechanisms of HSP72 expression induced by GGA and the involvement of HSF1 in neurons are topics for further investigation.

The rat model of glaucoma used herein showed mild to moderate loss of RGCs in association with cell death and mild axonal damage in the optic nerve head after 5 weeks of moderate elevated IOP. The proportion of RGCs with elevated HSP72 induced by GGA has not yet been examined and the exact mechanism of HSP72 synthesis in RGCs remains unclear. However, the ameliorative effect of GGA on the loss of RGCs, optic nerve injury, and cell death in this model supports the notion that the use of GGA could be beneficial to patients with glaucoma who have progressive optic nerve head degeneration, by enhancing endogenous self-defense mechanisms. Becke and Green have proposed that HSP72 is an antiapoptotic chaperone protein that may interfere with multiple stages of the apoptotic pathway. These stages include suppression of c-Jun N-terminal kinase (JNK) activation, prevention of cytochrome c release, disruption of apoptosome formation, inhibition of apoptotic protease activating factor (ApaF)-1 oligomerization, and suppression of procaspase recruitment. More recently, Ikeyama et al. demonstrated that GGA induces a rapid accumulation of HSP72 mRNA and HSP72, suppresses hydrogen peroxide- and ethanol-induced phosphorylation of JNKs, and interferes with caspase-9 and subsequent activation of caspase-3-like pro-

![Figure 5](image-url)
teases in rat hepatocytes. Whether GGA inhibits apoptosis in this glaucoma model at multiple levels or whether there are preferential sites of action by HSP72 needs further evaluation.

The enhancement of endogenous self-defense mechanisms represents a novel avenue for rescuing neurons in glaucoma. There is evidence that shows the involvement of HSPs in the glaucomatous eyes of humans and in experimental animal models of glaucoma. A nontoxic, therapeutic treatment to enhance endogenous protection is not available. Our study confirms that the systemic administration of GGA induces the expression of HSP72 in RGCs and confers protection to RGCs by inhibiting cell death in a rat glaucoma model. How HSP72 regulates apoptosis and the roles of other members of the HSP family is not yet known. However, it appears likely that HSP72 plays an important role in promoting the survival of RGCs in neurodegenerative disease.

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**References**


