RETINAL GANGLION CELL PROTECTION WITH GERANYLGERANYLACETONE, A HEAT SHOCK PROTEIN INDUCER, IN A RAT GLAUCOMA MODEL

BY Joseph Caprioli MD, Yoko Ishii MD PhD, AND Jacky M. K. Kwong PhD

ABSTRACT

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, 200 mg/kg daily. Western blot analysis and immunohistochemical staining for HSP72 and HSC70 were performed after 1, 3, and 7 days of GGA administration. 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 given twice a week. RGC survival was evaluated after 5 weeks of IOP elevation. 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 GGA administration, but HSC70 was unchanged. After 5 weeks of IOP elevation, there was 27% ± 6% loss of RGCs. The administration of GGA significantly reduced the loss of RGCs, lessened optic nerve damage, decreased the number of TUNEL-positive cells in the RGC layer, and increased HSP72. Quercetin administration abolished these protective effects.

Conclusions: These results demonstrate that systemic administration of GGA protects RGCs from glaucomatous damage in a rat model and suggest a novel pathway for neuroprotection for patients with glaucoma.


INTRODUCTION

The stress response is a highly conserved mechanism of gene regulation in response to a wide variety of physiological challenges. 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. Geranylgeranylacetone (GGA), an acyclic polyisoprenoid developed and used clinically in Japan, is a unique anti-ulcer drug that protects gastric mucosa without affecting gastric acid or pepsin secretion. Its cytoprotective effects have been correlated with the expression of proteins in heat shock protein 60, 70, and 90 (HSC70), the mitochondrial (GRP75), and the endoplasmic reticular (GRP78) forms. These proteins function under normal, developmental, and stressful conditions. The induction of HSP72 in the mammalian central nervous system by hyperthermia has been associated with the neuronal tolerance to ischemic insults and neuroprotective effects against light-induced injury in the rat retina. Intracellular expression of HSP72 has been demonstrated to protect cerebral neurons against heat shock, oxidative stress, apoptotic stimuli, excitotoxic insults, and ischemia-like conditions. Neurons of transgenic mice expressing HSP72 or those of rats injected with the herpes virus containing HSP72 genes also have been shown to be resistant to ischemia and seizures, which suggests that HSP72 is important for neuroprotection.
families in gastric mucosal cells in vivo and in vitro by activating heat shock factor-1 (HSF1), the transcription factor for HSPs. GGA induces HSPs in numerous tissues, including rat small intestine, liver, lung, kidney, and heart. Additionally, 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. 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). It has been postulated that apoptosis, a highly regulated process of cell death, is the final common pathway for RGC death in glaucoma. 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 conditions and ameliorates glaucomatous damage in a rat model. We investigate here (1) whether HSP72 is induced in rat RGCs with systemic administration of GGA; (2) whether the induction of HSP72 by GGA enhances RGC survival and prevents axonal injury in the optic nerve of a rat glaucoma model; and (3) 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 complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male Wistar rats weighing 250 to 300 g were housed with standard chow and water provided ad libitum. The animal room was lit with fluorescent lights (330 lux) turned on at 6 AM and off at 6 PM, and was maintained at 21ºC.

GENERAL SCHEME

Three experiments are summarized here, and details are provided in the subsequent sections. Experiment 1 was performed to evaluate the expression of HSP72 and HSC70 in RGCs after systemic administration of GGA with Western blot analysis and immunohistochemistry. For Western blotting, 12 rats were equally divided into six groups. Three groups of animals were given intraperitoneal injections of GGA, 200 mg/kg daily, and were euthanized after 1, 3, or 7 days of administration of GGA. Three control groups were (1) intraperitoneally administered saline vehicle daily for 7 days; (2) intraperitoneally administered GGA with 4 mg/kg of quercetin (Sigma, St Louis, Mo) daily for 7 days; and (3) untreated. 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 blotting was repeated with the other two retinas from each group. For immunohistochemical staining for HSP72 and HSC70, six rats were administered GGA and another six rats were given saline systemically for 7 days.

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 photocoagulation was performed on one eye of each rat (intracameral injection of india ink was performed 5 days before photocoagulation), while the contralateral eye remained untreated. GGA was then given twice a week at the same dose until euthanasia. Sustained elevation of intraocular pressure (IOP) was maintained by performing trabecular laser photocoagulation 3 weeks after the first photocoagulation.

To elucidate the role of HSP expression in the neuroprotective effects of GGA, systemic administration of quercetin at 4 mg/kg was done in the same manner as GGA. Administrations of saline vehicle, GGA, or GGA with 4 mg/kg of quercetin without trabecular laser photocoagulation were included as controls. IOP and body weight were measured once a week. After 5 weeks of IOP elevation, the number of retrogradely labeled RGCs with dextran tetramethylrhodamine (DTMR) 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) were also performed (n = 54).

Experiment 3 was performed to investigate the inhibition of apoptosis with GGA administration after 1 week of IOP elevation (n = 56). 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

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GGA, intraperitoneal geranylgeranylacetone injection; Q, intraperitoneal injection of quercetin.
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salt solution; Alcon Laboratories, Inc, Fort Worth, Tex) and emulsified for 1 hour in an ultrasonic generator (Branson Ultrasonics Corp, Danbury, Conn) immediately before administration. Intrapitoneal 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% deoxyribonuclease 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, Ohio) in MEM to a final concentration of 10% metrizamide. This mixture was then overlayed with 5% metrizamide in MEM, and the gradient was centrifuged at 4,500 rpm (HB-4; Sorvall Instruments, Newtown, Conn) for 25 minutes at 4°C. The cells in the 5% to 10% interface were collected and washed in 2.5 mL of minimal essential medium (MEM) containing 10% (vol/vol) fetal bovine serum. The retinas were pelleted by centrifugation at 250g for 10 minutes (3000C centrifuge, Jouan, Inc, Winchester, Va). The cells were then resuspended in 400 mL of MEM buffer, and the protein concentration in the cell suspension was measured with a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, Ill).

IMMUNOBLOT
Western blot analysis was performed according to the procedure described by Towbin and associates. Aliquots of 20 pg of protein from enriched RGCs were separated on a 12% SDS-polyacrylamide minigel (Bio-Rad, Hercules, Calif) and transferred to the membrane (Immobilon-P; Millipore Corporation, Bedford, Mass). 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 (1:1,000; StressGen Biotechnologies Corp, Victoria, British Columbia, Canada) or with rat monoclonal antibody against HSC70 (1:1,000; StressGen). Antigen-antibody complexes were detected by an avidin-biotin-peroxidase technique ( Vectastain ABC Kit; Vector Laboratories). Diaminobenzidine (DAB) was used to produce a brown color in the target tissue, and the slides were permanently mounted. As a negative control, alternate retinal section was 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, Victoria, British Columbia, Canada), goat polyclonal antibody against HSP72 (1:1,000; Santa Cruz Biotechnology, Inc, Santa Cruz, Calif), or rat monoclonal antibody against HSC70 (1:200; StressGen). Antigen-antibody complexes were detected by an avidin-biotin-peroxidase technique ( Vectastain ABC Kit; Vector Laboratories). Diaminobenzidine (DAB) was used to produce a brown color in the target tissue, and the slides were permanently mounted. As a negative control, alternate retinal section was incubated with blocking solution by replacing the primary antibody or with anti-rabbit secondary antibody by replacing the original secondary antibody.

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. Then they were 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-mm thickness along the vertical meridian through the optic nerve head. After deparaffinization and hydration, a species-specific Vectastain ABC kit (Vector Laboratories, Inc, Burlingame, Calif) 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, Victoria, British Columbia, Canada), goat polyclonal antibody against HSP72 (1:1,000; Santa Cruz Biotechnology, Inc, Santa Cruz, Calif), or rat monoclonal antibody against HSC70 (1:200; StressGen). Antigen-antibody complexes were detected by an avidin-biotin-peroxidase technique ( Vectastain ABC Kit; Vector Laboratories). Diaminobenzidine (DAB) was used to produce a brown color in the target tissue, and the slides were permanently mounted. As a negative control, alternate retinal section was incubated with blocking solution by replacing the primary antibody or with anti-rabbit secondary antibody by replacing the original secondary antibody.
RAT GLAUCOMA MODEL
Rats were anesthetized with intramuscular injections of 0.8 mL/kg of the anesthetic cocktail already described. A previously published procedure was modified to produce chronic moderately elevated IOP unilaterally, while the untreated contralateral eye served as the comparative control. Animals were injected intracamerally with 10 µL of 35% india ink (Becton Dickinson, Cockeysville, Md) in 0.01 M PBS after removal of a similar volume of aqueous. At the end of the procedure, Tobrex ophthalmic ointment (tobramycin 0.3%; Alcon, Fort Worth, Tex) was applied topically. Five days after intracameral injection of india ink, a dark band at the limbus, caused by the aggregation of carbon particles in the trabecular meshwork, was noted. After anesthesia was obtained, approximately 200 laser burns were delivered ab externo to the pigmented trabecular band at laser settings of 200 µm diameter, 200 mW power, and 0.2 seconds duration. Three weeks after the first laser treatment, another trabecular laser photocoagulation 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, Mass) and were performed 1 hour after lights off. All IOP measurements were performed with animals in the awake state. After topical instillation of Alcaine (proparacaine hydrochloride 0.5%; Alcon, Fort Worth, Tex), 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 Tonopen 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 euthanized after 5 weeks of IOP elevation to evaluate the number of DTMR (3,000 molecular weight, anionic, lysine fixable; Molecular Probes, Eugene, Ore) labeled cells, which were considered as surviving RGCs. At 48 hours before euthanasia, retrograde labeling was performed 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 mm × 0.362 mm, and the total counted area corresponded to approximately 3.1% of each total retinal area. Data are expressed as number of RGCs per mm².

GRADING OF OPTIC NERVE INJURY AND CELL COUNTING IN THE RETINAL GANGLION CELL LAYER
To examine the effect on RGC axons, optic nerve injury was evaluated with an established method. After 5 weeks of IOP elevation, 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-µm-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 mean ± SEM.

Corresponding loss of cells from the RGCL was evaluated by counting cells in the RGCL in cresyl violet–stained retinas. After the optic nerves were collected, 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 flat mounted on a slide, vitreal 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 cells and vascular endothelial cells were not counted. Cells with cytoplasm rich in Nissl substance and with irregular outlines were counted as neurons. The numbers 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 400x magnification. The counting was performed by two
investigators in a masked fashion and averaged. Results from the four quadrants (superior, temporal, inferior, and nasal) of each retina were averaged to give one value (mean ± SEM).

TUNNEL ANALYSIS
Four-µm-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 ApopTag Peroxidase In Situ Apoptosis Detection Kit (Intergen Co, Purchase, NY) were followed, and diaminobenzidine (Sigma, St Louis, Mo) was used as a color substrate. Counting was performed by two masked investigators with light microscopy, and the counts were averaged.

STATISTICAL ANALYSIS
The data are expressed as mean ± SEM. Mean values among groups were compared with one-way analysis of variance (ANOVA), and values between groups were compared with the unpaired Student's t test. Statistical significance was declared for P < .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 mg/kg daily) were probed with antibody against HSP72 (Figure 1A, upper panel) that specifically recognized the inducible form of HSPs as well as antibody against HSC70 (Figure 1A, lower panel) that corresponded to the constitutive form. There was a weak immunoreactivity against HSP72 in RGCs from the vehicle-treated rat retinas (lane 1) and normal untreated control rat retinas (lane 2). One day after administration of GGA, a mild increase in immunoreactivity of HSP72 was noted in RGCs (lane 3). A strong increase in immunoreactivity was detected in RGCs given GGA for 3 and 7 days (lanes 4 and 5, respectively). The expression of HSP72 in RGCs from GGA-treated rats was inhibited by coadministration of quercetin (4 mg/kg; lane 6). However, there was strong immunoreactivity against HSC70 in RGCs of the retinas from control groups (lanes 1, 2, and 6) and GGA-treated groups (lanes 3 through 5), but there was no detectable difference among them.

To localize the immunoreactivity of inducible and constitutive forms of HSPs in RGCs, immunohistochemi-
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 Tonopen (Figure 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 = .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 IOP course of 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 II). From the first day of saline injection (1 week before the
first trabecular laser photocoagulation) to euthanasia (5 weeks after the first laser photocoagulation), the percentage increase of body weight was 38% in vehicle-treated rats, 27% in the GGA group, and 38% in the GGA with quercetin group. The gain in body weight among these groups showed no statistically significant difference.

Retrograde labeling with DTMR was performed on optic nerves 2 days before euthanasia to label surviving RGCs by retrograde axoplasmic transport (Figure 3A through F). The DTMR-labeled RGCs were counted to evaluate the effect of administration of GGA (Figure 3G). There was a statistically significant difference between the densities of DTMR-labeled RGCs among the six groups ($P = .001$, ANOVA). The density of DTMR-labeled RGCs for vehicle-treated control was $1,230 \pm 51$ cells/mm$^2$. After 5 weeks of IOP elevation, the density of DTMR-labeled RGCs dropped to $904 \pm 71$ cells/mm$^2$ (Figure 3B), which corresponded to a $27\% \pm 6\%$ reduction when compared to the contralateral eyes ($P = .0003$). Administration of GGA preserved $57\%$ more DTMR-labeled cells ($1,044 \pm 36$ cells/mm$^2$, Figure 3C) compared with vehicle. The preservation of RGCs by administration of GGA in retinas with IOP elevation was partial ($P = .003$ when compared with vehicle-treated controls). Coadministration of quercetin abolished the protective effect of GGA in the retinas with IOP elevation (Figure 3D; $P = .002$), which showed a density of $756 \pm 88$ cells/mm$^2$. The density of DTMR-labeled RGCs in GGA-treated contralateral controls (Figure 3E) and GGA and quercetin-treated contralateral controls (Figure 3F) was $1,077 \pm 48$ cells/mm$^2$ and $1,235 \pm 51$ cells/mm$^2$, respectively. There was no statistical significance between the densities of DTMR-labeled RGCs in GGA-treated controls and vehicle-treated controls ($P = .1$).

Axonal injury in the optic nerve was demonstrated by light microscopy (Figure 4A and B) 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, while an optic nerve with a grade 2 injury is shown in Figure 4B. There was significant damage to the optic nerve after 5

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

*Data are expressed as mean ± SEM ($P = .07$, ANOVA).

**TABLE II: TIME COURSE OF BODY WEIGHT IN EXPERIMENT 2**

**FIGURE 4**

Representative micrographs illustrate optic nerve cross section for 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 IOP elevation 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 IOP elevation when compared with vehicle- or GGA-treated controls ($^*P < .05$). This axonal damage and reduction of cells in the RGCL was inhibited by administration of GGA ($^†P < .05$). GGA, GGA injection; Q, quercetin injection. Data are expressed as mean ± SEM.
weeks of sustained IOP, with a grade of 1.64 ± 0.10 compared with contralateral controls (1.13 ± 0.02, \( P = .001 \)), indicating mild to moderate injury. The optic nerve injury was significantly ameliorated by the administration of GGA (1.33 ± 0.05, \( P = .026 \)) compared with elevated IOP retina with administration of GGA. 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 (2,193 ± 75 cells/mm² corresponding to 16% loss) in eyes after 5 weeks of elevated IOP when compared with contralateral eyes (2,620 ± 78 cells/mm²; \( P = .001 \)), as shown in Figure 4D. Administration of GGA inhibited the loss of cells in the RGCL with IOP elevation (2,697 ± 70 cells/mm², \( P = .001 \)) and had no significant effect on the number of cells in the RGCL of GGA-treated contralateral control retinas (2,644 ± 59 cells/mm²).

**EXPERIMENT 3: INHIBITION OF CELL DEATH BY GGA**

TUNEL staining was performed to label dying cells (Figure 5B is shown as representative) in retinas with elevated IOP. The TUNEL-positive cells in the RGCL were counted and compared to evaluate the effect of GGA (Figure 5C). After 1 week of IOP elevation, the number of TUNEL-positive cells in the RGCL was 1.24 ± 0.29 per retinal section and was statistically significantly higher than in the control groups treated with vehicle (1.11 ± 0.02), GGA (1.02 ± 0.08), or GGA with quercetin (1.04 ± 0.08). The administration of GGA inhibited the loss of TUNEL-positive cells to 0.53 ± 0.11 per retinal section (\( P = .02 \)), corresponding to a 57% inhibition of cell death after 1 week of IOP elevation. The number of TUNEL-positive cells of quercetin-treated retinas with IOP elevation and GGA administration was 1.37 ± 0.31 per retinal section, similar to the vehicle-
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treated retinas with IOP elevation.

Quantitative analysis of immunoreactive intensity of HSP72 (Figure 6A) and HSC70 (Figure 6B) in the RGCL was performed 1 week after trabecular laser photocoagulation. The expression of HSP72 immunoreactivity was statistically significantly different among the groups \((P = .001, ANOVA)\). There was a statistically significantly increased expression of HSP72 induced by IOP elevation \((P = .01)\). HSP72 expression in retinas with IOP elevation apparently further increased after GGA administration \((P = <.001\) when compared with vehicle control), but this increase was not statistically significant when compared with the retinas with IOP elevation alone. HSP72 expression in retinas with IOP elevation and GGA administration was significantly reduced by the coadministration of quercetin with the retinas with IOP elevation \((P = .002)\). Systemic administration of GGA alone caused an increased expression of HSP72 in the RGCL when compared with vehicle-alone controls \((P = .005)\), 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.

CONCLUSIONS

The preinduction of HSP72 in RGCs enhances the survival of RGCs under hypoxic, excitotoxic, and glaucomatous stress. In this study, we demonstrated that HSP72 expression is 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 it 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 and associates reported that pretreatment with GGA failed 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 \(E\), maintenance of nitric oxide synthase activity, or induction of HSPs. There are a number of published reports that show a cytoprotective role of GGA in other organs, such as lung, heart, liver, and kidney. Our results indicate that the neuroprotective effects of GGA may be related to HSP72 expression, since coadministration with quercetin inhibited HSP expression and blocked the protection of RGCs induced by GGA in a rat glaucoma model. To confirm the role of HSP72 expression 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 expression of HSP72 in neuronal cells of the adult rat retina. The bioavailability of GGA \((125 \text{ mg/kg})\) given by intravenous injection peaks at 6 hours after administration and can be detected for up to 42 days. We administered GGA intraperitonically 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, which 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 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 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 require further investigation.

The rat model of glaucoma used here 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 IOP elevation. 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 glaucoma patients with progressive optic nerve head degeneration by enhancing endogenous self-defense mechanisms. Beere and Green
proposed that HSP72 is an anti-apoptotic chaperone protein that may interfere with multiple stages of the apoptotic pathway.\textsuperscript{11,12} These stages include suppression of c-Jun N-terminal kinase (JNK) activation,\textsuperscript{13,14} prevention of cytochrome-c release,\textsuperscript{15,16} disruption of apoptosome formation,\textsuperscript{17,18} inhibition of apoptotic protease activating factor 1 (Apaf-1) oligomerization,\textsuperscript{19} and suppression of procaspase recruitment.\textsuperscript{20,21} More recently, Ikeyama and associates\textsuperscript{22} 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 proteases 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 toward protecting rescuing neurons in glaucoma. There is evidence that shows the involvement of HSPs in the eyes of glaucomatous humans\textsuperscript{23} and in experimental animal models of glaucoma.\textsuperscript{24} A nontoxic, therapeutic treatment to enhance endogenous protection is lacking. 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 are 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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Retinal Ganglion Cell Protection With Geranylgeranylacetone, A Heat Shock Protein Inducer, In a Rat Glaucoma Model


DISCUSSION

Dr Douglas E. Gaasterland. In a unique approach to protection against glaucoma damage of retinal ganglion cell and optic nerve, Dr Caprioli and co-workers have previously shown that induction of heat shock proteins (HSFs) in the eye is effective. They now report inducing intracellular HSPs with geranylgeranylacetone (GGA), a systemic medication commercially available in Japan to treat gastric ulcers, and they convincingly demonstrate protection from glaucoma damage in a rat glaucoma model.

They created an experimental, sub-acute, secondary glaucoma in the eyes of laboratory rats by first injecting India ink into the anterior chamber then, several days later when the trabecular meshwork was stained, applying laser energy from outside the eye. Coagulation and subsequent healing of the outflow pathways caused sustained elevation of intraocular pressure into the high 20's during the following weeks. The experiments were done after 5 weeks, at which point, as found in some cases of human glaucoma, there was widespread, early damage of the retinal ganglion cells and optic nerve axons in control eyes. The investigators had previously shown that inducing heat shock proteins was protective against this damage. In this work they convincingly show that systemic GGA induces production of HSP72 in retinal ganglion cells, and that this is associated with enhanced survival of retinal ganglion cells in laboratory rat eyes with the experimental glaucoma. Treatment prevents axonal injury and inhibits apoptosis-like retinal ganglion cell death. In short, they have shown systemic GGA provides neuroprotection in laboratory rat experimental glaucoma.

During the Glaucoma 2002 Subspecialty Day meeting of the American Academy of Ophthalmology, Dr Stuart J. McKinnon presented a talk entitled “Paradigms for Neuroprotection.” It provided a review of methods under investigation as approaches to protect the optic nerve in glaucoma. He listed eight general strategies, as follows: 1) Inhibit apoptosis; 2) prevent tumor necrosis factor receptor (TNFR) activation; 3) stabilize calcium homeostasis; 4) block glutamate excitotoxicity; 5) block nitric oxide toxicity; 6) inhibit amyloid-β production; 7) modulate expression of heat-shock proteins; and 8) supply neurotrophins or modify intracellular signaling pathways.

His review of methods to modulate expression of heat-shock proteins centered on ways to reduce intrinsic levels of antibodies to these proteins. The work of Dr Caprioli is the first to identify a potentially clinically useful method to induce production of these protective “chaperones” in the retina.

We can add two strategies to the list of Dr McKinnon—first, improving blood flow to the optic nerve, and second intraocular pressure (IOP) reduction. These may not be mutually exclusive.

We have learned from glaucoma clinical trials that therapeutically reduced IOP is protective. The Advanced Glaucoma Intervention Study (AGIS) has shown a “dose response” relation of amount of IOP reduction and rate of visual field defect progression. However, the study has found among those patients with the lowest levels of IOP some whose field defects worsened. The Early Manifest Glaucoma Trial (EMGT) has shown that disease progression is less frequent and delayed after a 25% IOP reduction in newly diagnosed glaucoma. The Ocular Hypertension Treatment Study (OHTS) has shown that the occurrence of glaucoma damage is reduced by about half at 5 years after a 20% reduction of IOP in patients with ocular hypertension. In each study, protection from reduction of IOP is imperfect—some eyes progress in spite of substantially lower IOP.

In the Results section of the manuscript the authors note that the “preservation of RGCs by administration of GGA in retinas with IOP elevation was partial....” This raises several questions: 1) How much protection might we expect from geranylgeranylacetone (GGA), or other heat shock protein inducers, in human glaucoma? Can such an approach be completely protective? 2) This study is in subacute, experimental glaucoma; will it work in primary open angle glaucoma? 3) Is it safe? 4) Intraperitoneal dosing seems impractical for human glaucoma; how might human glaucoma dosing be carried out? Can GGA get to the eye after oral dosing or topical administration?

REFERENCES

Dr Joseph Caprioli. How much will it protect? Will it be complete or partial? While I don’t know the answer to these questions, if it works in human glaucoma, I suspect the protection would be partial. One of the things I like about this approach in that is that it’s not mechanistically specific. That is to say, the protection of cells in this manner would theoretically work in the setting of a wide variety of insults. Dr Spaeth pointed out this morning that, like macular degeneration, primary open angle glaucoma is probably a multitude of diseases. There are many different mechanisms of damage, all having in common a final common pathway of optic nerve “rot” that we identify as glaucoma. I’m hopeful that, regardless of the mechanisms of damage, this sort of kicking up of the endogenous protective mechanism may work for a wide variety of glaucomas.

Is it safe? We know this drug is used clinically and has passed the Japanese equivalent of the FDA. There’s been a lot of work with respect to its safety and efficacy, and also with respect to penetration of body organs. It does penetrate the CNS when given orally. When we first started to test teprenone, we gave it orally to rats, and it did cause a substantial increase of HSP-72 in retinal ganglion cells. We did not take it further to show that oral administration protected ganglion cells in the glaucoma model, but that would be a good next step.