Induction of Heat Shock Protein 72 Protects Retinal Ganglion Cells in a Rat Glaucoma Model

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PURPOSE. To investigate whether heat shock protein (Hsp) 72 is induced in retinal ganglion cells (RGCs) in experimental rat glaucoma and whether the induction of Hsp72 by heat stress or zinc (Zn2+) administration can increase survival of RGCs in the model.

METHODS. Intraocular pressure (IOP) was elevated unilaterally in Wistar rats with argon laser irradiation of the trabecular meshwork 5 days after intracameral injection of India ink. Immunohistochemical staining for Hsp72 was performed. The rats with elevated IOP were treated with heat stress once a week (six rats) or intraperitoneal injection of zinc (10 mg/kg) every two weeks (six rats). Untreated rats with elevated IOP served as a control group (six rats). Quercetin, an inhibitor of Hsp expression was injected in the rats with heat stress (six rats) and zinc injection (seven rats). Subsequent to 4 weeks of IOP elevation, RGCs were counted.

RESULTS. The IOP increase compared with the contralateral eyes was 48% ± 4% throughout the study period. Hsp72 was detected only in the eyes with elevated IOP at 1 and 2 days and was weakly detected at 1 week of IOP elevation. A single administration of zinc strongly induced Hsp72 in RGCs of rats with elevated IOP for 2 weeks. Treatment with heat stress or zinc in rats with elevated IOP increased RGC survival after 4 weeks of IOP elevation, compared with the untreated control group (P = 0.004, n = 6). Quercetin reversed the positive effect of heat stress or zinc injection on RGC survival.

CONCLUSIONS. These results demonstrate the possibility of a novel therapeutic approach to glaucoma through an enhanced induction of the endogenous heat shock response. (Invest Ophthalmol Vis Sci. 2001;42:1522–1530)

All organisms, from bacteria to humans, are known to respond to physiological or environmental stress by increasing the levels of a group of proteins referred to as heat shock or stress proteins. Heat shock proteins (Hsps), induced in cells by hyperthermia or other types of metabolic stress, enhance cell survival under conditions of further severe stress. In the mammalian central nervous system (CNS), the production of Hsps increases neuronal tolerance to ischemic insults.1,2 A protective role for Hsps, induced by hyperthermia, has also been demonstrated in the rat retina, against light-induced retinal damage.3

Among the various families of Hsps classified according to molecular weight, the Hsp70 family, which is expressed during preconditioning stress, is known to be essential for neuroprotective effects.4–7 Recently, neurons of transgenic mice overexpressing Hsp728 or those of rats injected with the herpes virus containing Hsp72 genes9 have been shown to be more resistant to ischemia and seizures. A neuroprotective role of Hsp72 against ischemic and excitotoxic cell death in cultured retinal ganglion cells (RGCs) has been demonstrated.10

Zinc, an essential cofactor for many enzymes, robustly induces Hsp72 in HeLa cells11 and rat astrocytes12 in vitro and in lung, liver, and kidney cells of the rat13 and the pig14 in vivo. However, there is no published evidence that zinc induces Hsp72 in neurons, especially in RGCs. Zinc is neuroprotective when subcutaneously injected twice at 24 and 48 hours before transient global ischemia in the gerbil.15

Glaucoma, one of the world’s leading causes of blindness, is characterized by progressive optic nerve damage with selective loss of RGCs.16–18 Although we still do not know the exact mechanism of injury of RGC damage in glaucoma, there is a strong possibility that any stressful insult to RGCs in glaucoma induces Hsp72 and that this stress protein is related to an endogenous neuroprotective mechanism. We investigated whether Hsp72 is induced in RGCs in an experimental rat glaucoma model with chronic intraocular pressure (IOP) elevation, whether systemically administered zinc induces Hsp72 in rat RGCs, and whether the induction of Hsp72 by heat stress or zinc enhances RGC survival in this model.

MATERIALS AND METHODS

General Scheme

Three stages of experiments are summarized in this section, and the technical details are given in the sections that follow. The first stage of experimentation was performed to investigate whether Hsc70 (constitutive form) and Hsp72 (inducible form) are induced in RGCs by IOP elevation in a rat glaucoma model. Eighteen Wistar rats were used. IOP was elevated in the right eye of each rat, as described later. Contralateral eyes were used as controls. Immunohistochemical staining for Hsc70 and Hsp72 was performed for 12 rats at 1 and 2 days and at 1, 2, 3, and 8 weeks of IOP elevation. Two eyes of a normal rat were enucleated at 24 hours after heat stress and served as a positive control. At 8 weeks, RGCs were counted for the right eye of four rats from the wholemounted retina, prepared 24 hours after retrograde labeling by dextran tetramethylrhodamine (DTMR) from the retrobulbar optic nerve. Another six eyes of six normal rats were labeled and counted to determine normal density of labeled cells.

The second stage of experiments was performed to investigate whether zinc or heat stress induces Hsp72 in rat RGCs. The divalent cation zinc (Zn2+), 10 mg/kg was injected intraperitoneally into four normal rats and four with elevated IOP in the form of zinc sulfate (24.6 mg/kg). Two control rats were injected intraperitoneally with saline. Immunohistochemical staining for Hsp72 was performed simultaneously with staining for Thy-1 at 1 and 2 days and at 1 and 2 weeks.
after injection, as described later. Thy-1 reacts specifically with RGCs.23

For Western blot analysis of Hsp72 in RGCs, two rats were injected intraperitoneally with zinc sulfate and two were treated with heat stress. An additional two rats were injected intraperitoneally with 400 mg/kg of quercetin (Sigma Chemical Co., St. Louis, MO), an inhibitor of Hsp expression20–22 followed by heat stress 6 hours later, and two normal rats served as a control. RGCs were isolated from four retinas in each subgroup and Western blot analysis was performed with the methods described later.

The third stage of experiments was performed to investigate whether the induction of Hsp72 by heat stress or zinc would increase the survival of RGCs in the rat glaucoma model. Thirty-one rats were divided into five groups (six rats each of groups I–IV; seven rats in group V). Intraocular pressure was elevated in all five groups over a span of 4 weeks, as will be described. Group I served as a control group with elevated IOP but without any treatment. The rats in group II were treated with heat stress every week, as described later. The rats in group III were intraperitoneally injected with zinc sulfate every 2 weeks. The rats in group IV were intraperitoneally injected with 400 mg/kg of quercetin 6 hours before heat stress every week, as will be described. The rats in group V were intraperitoneally injected with quercetin 6 hours before injection of zinc sulfate every 2 weeks. At 4 weeks of IOP elevation and treatment, RGCs were counted from wholemounted retinas, prepared 24 hours after retrograde labeling by dextran tetrathylrhodamine (DTMR) applied to the retrobulbar optic nerve, as described later.

Rat Glaucoma Model

Male Wistar rats, weighing 250 to 300 g, were used for all three stages of the experiments. They were housed in a standard animal room lit with fluorescent lights (350 lux), which were automatically turned on at 6 AM and off at 6 PM. Room temperature was maintained at 21°C. All experiments complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Rats were anesthetized by intramuscular injection of 0.8 ml/kg of a cocktail solution 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. The right eye of each animal was injected intracamerally with 10 μl of 55% India ink (Becton Dickinson Co., Cockeysville, MD) diluted in BSS (Alcon Laboratories, Inc., Fort Worth, TX), after the same amount of aqueous was drained. After injection of India ink, carbon particles aggregated in the trabecular meshwork and formed a black pigmented band along the limbus. Five days after India ink injection, ab externo argon laser photocoagulation to the pigmented trabecular meshwork was performed.23 Seventy to 90 laser burns were delivered around the pigmented trabecular band at laser settings of 200 μm, 260 to 300 mW, and 0.2 seconds.

IOP Measurements

IOP was measured between 10 AM and 2 PM with a tonometer (Tono-Pen XL; Mentor O&O, Norwell, MA) 5 minutes after intramuscular injection of 0.6 ml/kg of cocktail solution (stage 1 experiment) or 8 mg/kg of acepromazine (stage 3 experiment), and 30 seconds after topical instillation of 0.5% proparacaine hydrochloride. Five readings with firm contact of the tonometer tip with the cornea were recorded in each eye. Any readings that occurred just before the tip contacted the cornea (contact with the tear film meniscus) or just after the tip was removed from the cornea were ignored because of their unreliability.24 The average of these readings was recorded as the IOP for that measurement.

In two rats in each group in the third stage of experiments, dark-phase IOP measurements were performed 2 hours after the room lights were turned off. Dark-phase measurements were made with a long-wavelength 16-W bulb (Bright Laboratory Jr. CPM, Inc., Dallas, TX) that emits the far-red range of the visible light spectrum and does not affect circadian rhythms.25,26

Hyperthermia (Heat Stress)

All rats in group II and IV of the third stage of experiments, four rats from the second stage, and one rat from the first stage (see general scheme described earlier) were treated with heat stress as follows: Each rat was anesthetized with an intramuscular injection (8 ml/kg) of the same cocktail solution described in the creation of the rat glaucoma model. Five minutes after being anesthetized, each rat was placed in an 18 × 8 × 7-cm hollow, unlidded, buoyant, aluminum receptacle. Rat and receptacle were then immediately placed in a water bath at a constant temperature of 42°C. Increasing body temperatures were continuously monitored with a rectal thermometer until rectal temperatures were kept between 40°C and 42°C for 15 minutes. Usually, it took 25 to 30 minutes for rats to achieve a rectal temperature of 40°C. The rats were then removed from the water bath and allowed to recover from the anesthesia. A similar treatment had been shown previously to robustly induce Hsp72 in the retina.3

Evaluation of RGC Density

For analyses of RGC density, four rats were killed at 8 weeks of IOP elevation, 24 hours after retrograde labeling. Retrograde labeling was performed with the animals under anesthesia with 0.8 ml/kg of the cocktail solution. The right optic nerve was incised with a needle knife 2 mm longitudinally at least 5 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. DTMR (3000 molecular weight, anionic, lysine fixable, Molecular Probes, Eugene, OR) crystals were applied to the proximal cut surface of the optic nerve to label RGCs by fast axonal diffusion.27–29 The same procedure was performed for the right optic nerve of six normal rats that served as a control group. The rats were perfused first with saline and then with 4% paraformaldehyde in phosphate-buffered saline (PBS), and the eyes were then enucleated. The retinas were dissected, flattened with four radial cuts (the deepest in the superior pole and the others in the inferior, temporal, and nasal poles), fixed for an additional 30 minutes, and mounted with the vitreal side up on glass slides. The retinas were examined with a fluorescence microscope (Axioplan; Carl Zeiss, Oberkochen, Germany) equipped with an ultraviolet filter that permits visualization of rhodamine fluorescence (excitation filter BP 546, barrier filter LP590; Carl Zeiss).

Labeled RGCs were counted from printed fluorescence micrographs of 12 standard areas of each retina. Each rectangular area measured 0.34 × 0.22 mm², and there were three areas in each retinal quadrant (superotemporal, inferotemporal, superonasal, and inferonasal) at 1, 2, and 3 mm, respectively, from the optic disc. The number of labeled cells in the 12 photographs was divided by the area of the region and pooled to calculate mean densities of labeled neurons per square millimeter for each retina. RGC counts were conducted by two investigators in a masked fashion and averaged. The identity of the retinas that led to the micrographs was unknown to the counters until cell counts from different groups were completed.

Immunohistochemistry

At 1 and 2 days and at 1, 2, 3, and 8 weeks after IOP elevation, the rats were perfused with 4% paraformaldehyde in PBS after deep anesthesia with the cocktail solution. The eyes were dissected and then immersed in the same cocktail solution described in the creation of the rat glaucoma model. Five minutes after being anesthetized, each rat was placed in an 18 × 8 × 7-cm hollow, unlidded, buoyant, aluminum receptacle. Rat and receptacle were then immediately placed in a water bath at a constant temperature of 42°C. Increasing body temperatures were continuously monitored with a rectal thermometer until rectal temperatures were kept between 40°C and 42°C for 15 minutes. Usually, it took 25 to 30 minutes for rats to achieve a rectal temperature of 40°C. The rats were then removed from the water bath and allowed to recover from the anesthesia. A similar treatment had been shown previously to robustly induce Hsp72 in the retina.3
isolation of RGCs

RGCs were partially purified from other cells in the rat retinas by a modification of a method previously described.29,30 Retinas were isolated from normal rats and 24 hours after intraperitoneal zinc injection, were incubated with blocking solution without primary antibody. Retinal tissue enucleated 24 hours after heat stress was used as a positive control.

After deep anesthesia with the cocktail solution, all rats from stage 2 were perfused with 4% paraformaldehyde in PBS. Eyes were dissected and then immersed in the same fixative for 1 hour. The cornea and lens were then removed and the rest of the eye immersed in 30% sucrose until it sank to the bottom of the sucrose solution. The eyes were then frozen in optimal cutting temperature (OCT) media (Tissue-Tek; Sakura Finetechical Co., Ltd., Tokyo, Japan) and sectioned at a 14-μm thickness along the vertical meridian, through the optic nerve head. Tissue sections were then incubated with blocking solution containing 10% FCS, 2% donkey serum, and 0.1% Triton X-100 in PBS for 1 hour. This was followed by incubation with mouse monoclonal antibodies to Hsp72 (StressGen Biotechnologies) 1:50 and goat polyclonal antibody to Thy-1 (Research Diagnostics, Inc., Flanders, NJ) 1:400 for 1 hour and donkey anti-mouse IgG conjugated with FITC (Research Diagnostics, Inc.) 1:100 for 1 hour at room temperature and donkey anti-goat IgG conjugated with rhodamine Red-X (Research Diagnostics, Inc.) 1:400 for 1 hour at room temperature. This double immunofluorescent staining was also performed in stage 1 experiments to confirm that the cells expressing Hsp72 are RGCs. The immunofluorescence from all staining was observed with a fluorescence microscope.

FIGURE 1. Percentage of increase in IOP compared with contralateral control eyes. The IOP increase was highest at 1 week of laser application and stabilized thereafter through 8 weeks. The average IOP increase compared with the contralateral eyes was approximately 50% throughout the study period.

RESULTS

Stage 1: Characterization of Hsp72 Induction with Elevated IOP

The baseline IOP measured at 5 minutes after cocktail anesthesia was 11.4 ± 1.1 mm Hg in the right eye and 11.5 ± 1.2 mm Hg in the left eye. The increases in IOP compared with contralateral control eyes were 54% ± 23%, 42% ± 16%, 47% ± 27%, 49% ± 16%, and 47% ± 13% at 1, 2, 3, 4, and 8 weeks, respectively, after laser irradiation (Fig. 1). The IOP of the right eye was highest at 1 week (17.7 ± 2.3 mm Hg) and stabilized thereafter to 8 weeks. The final IOP at 8 weeks was 17.2 ± 1.0 mm Hg in the right eye and 11.8 ± 1.0 mm Hg in the left eye. The average IOP increase was 48% ± 4% of contralateral control eyes. The RGC densities at 1, 2, and 3 mm from the center of the optic nerve head were 1230, 1937, and 314, respectively, for the rats with elevated IOP and 2075 ± 273, 1937 ± 265, and 1502 ± 288 cells/mm², respectively, for the normal rats. The density of labeled cells was significantly decreased at all locations compared with control eyes at 8 weeks of IOP elevation (P = 0.011 for each, Mann–Whitney test; Fig. 2): these were 41%, 41%, and 51% reductions at locations of 1, 2, and 3 mm, respectively, from the center of the optic nerve head compared with control eyes.
The immunohistochemical reaction for Hsp72, the inducible form, was strongest in the RGC layer of eyes with elevated IOP at 1 day, whereas control eyes showed no staining. Double immunofluorescent staining for Hsp72 and Thy-1 confirmed that these cells were RGCs. Superimposed image for both Hsp72 and Thy-1 is shown in (E). Hsp72 immunoreactivity became weaker at 1 week after IOP elevation. A constitutive form, Hsc70, stained RGCs of both control and glaucomatous eyes at 2 weeks of IOP elevation. This pattern of Hsc70 staining was detected throughout the whole period of the study. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; IS/OS, photoreceptor inner and outer segment layer. Scale bar, 50 μm.

Stage 2: Induction of Hsp72 by Zinc Injection or Heat Stress

The normal control eye without zinc treatment showed negative staining for Hsp72 (Fig. 4A). Intraperitoneally injected zinc...
induced Hsp72 production in RGCs of normal rat strongly until 2 weeks (Figs. 4B–E). The rats with elevated IOP treated with zinc also showed strong immunoreactivity for Hsp72 in RGCs for 2 weeks (Figs. 4F, 4G).

Western blot analysis with anti-Hsp72 monoclonal antibody was performed to investigate the expression of Hsp72 in RGCs of rats that were treated with heat stress or intraperitoneally injected with zinc. Thy-1 immunoreactivity was enriched in the partially purified RGC fraction (lane 1) and was detected in the whole retina extract (lane 3), but was not detected in the ganglion cell–poor fraction collected from the metrizamide gradient purification (lane 2). A strong increase in the immunoreactivity of Hsp72 was noted in RGCs of zinc-injected rats (lane 3) and a moderate increase in heat-stressed rats compared with the normal control rats (lane 1). The expression of Hsp72 in RGCs of heat-stressed rats was inhibited when these rats were pretreated with quercetin (400 mg/kg) before heat stress (lane 4).

**Stage 3: Increased Survival of RGCs by Enhanced Induction of Hsp72**

The baseline IOP measured after the anesthesia with acemprazine injection and topical proparacaine application was 19.3 ± 0.8 mm Hg in group I, 18.7 ± 1.3 mm Hg in group II, 19.1 ± 1.4 mm Hg in group III, 19.6 ± 0.8 mm Hg in group IV, and 19.2 ± 0.8 mm Hg in group V. These IOPs were not statistically different among the five groups (P = 0.389, Kruskal-Wallis test). The final IOP at 4 weeks measured with the same anesthetics was 30.7 ± 0.9 mm Hg in group I, 31.6 ± 1.2 mm Hg in group II, 31.3 ± 0.8 mm Hg in group III, 30.9 ± 0.8 mm Hg in group IV, and 30.2 ± 1.2 mm Hg in group V. The follow-up IOPs for 4 weeks were not statistically different among the five groups (P > 0.05, Kruskal-Wallis test). The IOPs increased approximately 50% over baseline IOP in each group. The dark-phase IOP measured at 2 hours after lights off also increased approximately 50% over baseline dark-phase IOP and 50% over light-phase IOPs (Fig. 6).

The average RGC densities were 890 ± 137 cells/mm² for group I (a control group with IOP elevation), 1318 ± 133 cells/mm² for group II (heat stress), 1594 ± 273 cells/mm² for group III (zinc injection), 1069 ± 190 cells/mm² for group IV (quercetin injection and heat), and 1308 ± 161 cells/mm² for group V (quercetin and zinc injection) (Table 1). There were significant differences in the density of labeled cells among the five groups (P = 0.001, Kruskal-Wallis test). When the RGC densities of treated groups (groups II and III) were compared with untreated control group I, both treated groups had significantly higher RGC densities (P = 0.004 between groups I and II, P = 0.004 between groups I and III, Mann–Whitney test) than the control group (Fig. 7). Even though there was a tendency for the density of labeled cells of group III to be higher than that of group II, there was no statistically significant difference in the density of labeled cells between groups II and III (P = 0.109). Group IV with quercetin injection and heat stress showed a significantly lower density of labeled cells than in group II with heat stress (P = 0.037). Group V with quercetin and zinc injection showed significantly lower density of labeled cells than did group III with zinc injection (P = 0.046). Group IV with quercetin injection and heat stress did not show a significant difference in the density of labeled cells compared with the control group I (P = 0.078), whereas group V with quercetin and zinc injection showed significantly higher density of labeled cells than the control group I (P = 0.004).

**Discussion**

It is well known that Hsp72 is the major form of Hsp induced in brain injuries, such as ischemia2,3,5 and seizures,4 and that Hsp72 induced by preconditioning stress has a neuroprotective effect.1,2 Few studies have been performed on Hsp72 induction in RGCs. In this study we demonstrated that Hsp72 is induced in RGCs in a rat glaucoma model with the strongest immunoreactivity at 1 day after IOP elevation. We further induced Hsp72 in RGCs by heat stress or systemic administration of the divalent cation zinc and showed increased survival of RGCs in the groups treated with heat stress or zinc, compared with the control group. The effect of heat stress or zinc treatment was reversed by injection of quercetin, an inhibitor of Hsp expression. This is the first report that demonstrates the neuroprotective effect of Hsp72 on RGCs in an animal glaucoma model.

Although many papers have been published about the neuroprotective role of Hsp72, the exact mechanism of

**FIGURE 5.** (A) Western blot analysis for Thy-1 in control rats. Thy-1 immunoreactivity was noted in the whole retina extract (lane 3) and was enriched in the partially purified RGC fraction (lane 1), but was barely detectable in the ganglion cell–poor fraction collected from the metrizamide gradient purification (lane 2). A total of 15 μg of protein was applied in each lane. (B) Western blot analysis for Hsp72 in RGCs of normal rats (lane 1) and 24 hours after heat stress (lane 2), zinc injection (lane 3), and quercetin injection and heat stress (lane 4). A total of 30 μg of proteins was applied on the blot for each lane. A strong increase in heat-stressed rats (lane 2) compared with the control rats (lane 1). The expression of Hsp72 in RGCs of heat-stressed rats was inhibited when these rats were pretreated with quercetin (400 mg/kg) before heat stress (lane 4).

**FIGURE 6.** IOP change of each group during the period of the stage 3 experiment. The IOPs increased approximately 50% in each group, and they were not significantly different among the five groups (P > 0.05). Dotted line: dark-phase IOP measured at 2 hours after lights off. The dark-phase IOPs also increased approximately 50% over baseline dark-phase IOP and 50% over light-phase IOPs. Q, quercetin injection; HS, heat stress; Zn, zinc injection.
neuroprotection by this stress protein remains unknown. It may be explained by the chaperoning function of Hsp72 on proteins that are important for the survival of cells. Hsps participate in the folding and assembly of nascent and unfolded peptides, and they facilitate protein transport to specific subcellular compartments and disposal by degradation.35,36 Hsps are also involved in multiple stages of the apoptosis pathway and function to inhibit apoptosis.37,38 Hsp70 overexpression protects mitochondria from the deleterious effect of reactive oxygen species (ROS).39 Hsp70 inhibits apoptosis downstream of cytochrome c release and upstream of caspase-3 activation.40 Inhibition of stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) by Hsp70 has been reported.41

### Table 1. DTMR-Labeled RGC Densities

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<th>Group I (↑ IOP)</th>
<th>Group II (HS+ ↑ IOP)</th>
<th>Group III (Zn+ ↑ IOP)</th>
<th>Group IV (Q+HS+ ↑ IOP)</th>
<th>Group V (Q+Zn+ ↑ IOP)</th>
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Mean ± SD

| % Decrease compared with normal | 52% | 28% | 13% | 42% | 29% | 0% |

RGC densities are expressed in cells/mm². ↑ IOP, increased intraocular pressure; HS, heat stress; Zn, intraperitoneal zinc injection; Q, quercetin.

*P = 0.004, compared with group I, Mann-Whitney test.

†P = 0.037, compared with group II, Mann-Whitney test.

‡P = 0.046, compared with group III; P = 0.004 compared with group I, Mann-Whitney test.

**Figure 7.** RGCs labeled with DTMR staining of normal retina (A), control retina with IOP elevation (B; group I), retina with heat stress and IOP elevation (C; group II), retina with zinc injection and IOP elevation (D; group III), retina with quercetin injection, heat stress, and IOP elevation (E; group IV), and retina with quercetin and zinc injection and IOP elevation (F; group V) at 4 weeks after IOP elevation. The density of labeled cells in group II (C) and III (D) were significantly higher than in control group I (B). The density of labeled cells in groups IV (E) and V (F) were significantly lower than in groups II (C) and III (D), respectively. Scale bar, 50 μm.
Zinc is an essential element for normal growth and function in humans. It is present in highest concentration in the retina and choroid in the body and functions as an essential cofactor for more than 100 enzymes in the body. Ultrastructural studies have suggested that brain zinc is present within the synaptic vesicles of glutaminergic neurons. In the present study systemically administered zinc induced Hsp72 in RGCs of both normal and glaucomatous rat eyes. Although we do not know yet the exact mechanism of zinc in the induction of Hsp72, it has been proposed that the translocation of synaptic zinc to postsynaptic neuron induces Hsp72 induction in the rat hippocampus. Because excessive extracellular zinc and excessive influx of zinc into postsynaptic neurons may contribute to neuronal death after transient global ischemia or sustained seizures, systemic zinc administration in this experiment may have presented a preconditioning stress to RGCs and thus may have induced Hsp72.

Zinc is quite nontoxic compared with other transition metals. The dosage (10 mg/kg of zinc twice a week) in this experiment did not show any observable systemic side effects during the 4 weeks of the study period. It has been reported that rats receiving 16 mg/kg zinc orally every day for 32 weeks showed no striking pathologic alterations. In a human clinical study, zinc has been tried in macular degeneration with an oral dosage of 200 mg of zinc sulfate daily for 2 years with no significant adverse effects. Thus, any nontoxic pharmacologic agent that can increase Hsp72 in RGCs may have a beneficial effect in neuroprotection in a rat glaucoma model. Garanyleranalacetone, an antieucler drug developed in Japan, may be another candidate for a safe inducer of Hsp72. The drug protects gastric mucosal cells under conditions of stress through Hsp72 expression.

Although there was no statistically significant difference in the total density of labeled cells between the heat stressed and the zinc-treated group in this study, there was a trend in the zinc-treated group toward a higher density of labeled cells than in the heat stressed group. Further, the density of labeled cells in group V after inhibition of Hsp expression by quercetin in the zinc-treated rats was significantly higher than in control group I (Table 1). These findings may be explained as follows: First, the peak expression of Hsp72 is known to be at 18 hours after heat stress, whereas the effect of zinc lasts longer by at least 2 weeks, as shown in this study. The second explanation is that zinc itself may have a neuroprotective effect in addition to Hsp induction. It has been reported that zinc blocks the action of N-methyl-D-aspartate (NMDA) on cortical neurons.

In stage 1 experiments, IOP was measured after cocktail anesthesia (ketamine, xylazine, and acepromazine), whereas in stage 3 experiments IOP was measured after acepromazine injection only. That explains why the baseline and postlaser IOPs in stage 1 were lower than those in stage 3. It has been reported that with increasing duration of anesthesia, the measured IOP continues to decrease in a nonlinear fashion after cocktail anesthesia. This effect seems to be due to a muscle-relaxation effect of xylazine combined with suppressed sympathetic neural function and decreased aqueous production. When acepromazine only was used as in stage 3 experiments, the rats maintained normal muscle tone but were calm enough for the measurement of IOP. The level of baseline IOPs measured with acepromazine only is equivalent to that in a previous study in which IOP was measured without general anesthesia.

In this study we used the method of labeling from the retrobulbar optic nerve instead of labeling from the superior colliculus. Although we did not directly compare our method with the labeling method from the superior colliculus, the normal density of labeled cells measured from our method correlates well with previous studies using the superior colliculus. The advantage of retrobulbar labeling is that the method is simple and results can be acquired quickly—24 hours after labeling. Thus, there is less concern about a decrease in the intensity of the dye or fluorescent material due to metabolism or loss of fluorescence. Although the optic nerve is transected, the transection itself does not affect the results, because RGC death begins 5 days or more after the axotomy. There may be concern about the possibility that elevated IOP affects the active transport of dye to the RGCs. However, because 3000-molecular-weight DTMR is transported by fast axonal diffusion rather than active transport, the possibility of the blockade of axonal diffusion in response to elevated IOP is very low. Even though anything is possible, the RGC count was compared among the eyes with IOP elevation in the stage 3 experiment, and the IOP conditions were therefore the same for all groups and the statistical analysis was performed among those five groups (Table 1).

It cannot be ruled out that Hsp72 may partially restore the axonal transport yielding an artificially greater number of cells that are labeled. Thus, it may require additional conventional staining of wholemounts or regular histology to confirm the result of retrograde labeling. However, we should not miss the point that when the staining of wholemounts or regular histology is used, rather than retrograde labeling, displaced amacrine cells are another problem that may introduce an artifact.

To rule out any possibility that the laser treatment itself may be responsible for the expression of Hsp72, we previously had performed the laser treatment on the peripheral cornea anterior to the trabecular meshwork, not to elevate IOP (unpublished data). Those eyes did not show any Hsp72-positive staining in RGCs, which suggests the laser treatment itself at the peripheral cornea does not affect the expression of Hsp72 in RGCs.

In glaucoma treatment, patients often experience progression of disease, even after maximum reduction of IOP. Also, in patients with normal-tension glaucoma, we cannot entirely depend on IOP reduction, because substantial further reduction of IOP is often difficult. Because we know that the final common pathway of glaucoma is RGC death, an approach to protect RGCs by inducing an Hsp response—an endogenous neuroprotection strategy—can widen the field of glaucoma treatment. Recently, Hsp 60 and Hsp 27 immunoreactivities have been shown to be increased in human glaucomatous eyes compared with normal eyes, which may reflect a role of Hsps as a cellular defense mechanism in response to stress or injury in glaucoma. In this study, we identified a possible role for at least one Hsp, Hsp72, in RGC survival in eyes with elevated IOP. This study demonstrates the feasibility of a novel therapeutic approach to glaucoma through an enhanced Hsp response. However, further study with other models of optic nerve damage and functional evaluations of RGCs should be conducted to confirm the neuroprotective effect of stress proteins. So we should consider the possibility that other Hsps, not investigated in this study, may have some role in RGC survival.

References

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