Protection of Retinal Ganglion Cells from Ischemia-Reperfusion Injury by Electrically Applied Hsp27

Akiko Yokoyama,[1] *Toshiyuki Oshitari,*[1] *Hisanari Negishi,*[1] *Mari Dezawa,*[2] *Atsushi Mizota,*[1] *and Emiko Adachi-Usami*[1]

Purpose. To determine whether the Hsp27 protein can rescue retinal ganglion cells (RGCs) of rats from ischemia–reperfusion injury.

Methods. Retinal ischemia was induced in rats by clamping the ophthalmic artery within the dural sheath of the optic nerve. Immediately after removing the clamp and beginning the reperfusion, Hsp27 protein solution was injected into the vitreous, and electroporation was applied. To determine whether Hsp27 entered the RGCs, anti-Hsp27 immunohistochemistry was performed. The retinal damage was evaluated by counting the number of RGCs retrogradely labeled by 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine percholorate (diI) injected into the superior colliculus, and also by comparing the ratio of TUNEL-positive to all RGCs in the RGC layer.

Results. Electroporation successfully delivered Hsp27 protein into RGCs. In the Hsp27 electroinjected group, the number of RGCs 7 days after ischemia–reperfusion was significantly higher than in the control groups. The ratio of TUNEL-positive cells to all RGCs was lower in the group electroinjected with Hsp27 protein.

Conclusions. Electroporation of Hsp27 protein into RGCs increased the resistance of the RGCs to the apoptosis induced by ischemia–reperfusion injury. (*Invest Ophthalmol Vis Sci.* 2001; 42:3283–3286)

Exposure of cells to a mild heat challenge or other types of metabolic stress greatly enhances cell survival during a subsequent, and what would otherwise be a lethal, stress. This protective effect, referred to as acquired tolerance, is correlated with the expression of the heat shock proteins (Hsps).[1–4] For example, hyperthermic pretreatment of rats induced Hsp70 and resulted in a marked decrease in photoreceptor degeneration after exposure to bright light compared with rats pretreated with a normothermic environment.[5] Wagstaff et al.[6] prepared disabled herpes simplex virus–based vectors that were able to produce a high level of Hsp expression in infected neuronal cells without a damaging effect. Among the Hsps, Hsp70, Hsp56, and Hsp27, only Hsp27 can protect dorsal root ganglion neurons from the apoptosis induced by nerve growth factor withdrawal and the ND7 neuronal cell line from retinoic acid–induced apoptosis.[6]

The purpose of this study was to determine whether electroinjection of this novel neuroprotective factor, Hsp27, into retinal ganglion cells (RGCs) after ischemia–reperfusion injury, can protect the RGCs from apoptosis. We show that electroporation delivered Hsp27 to RGCs and increased the resistance of the RGCs to apoptosis in this ischemia–reperfusion injury model.

Materials and Methods

All animals used were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Nine-week-old male Wistar (200–250 g) rats were housed three per cage in a temperature-controlled environment and had free access to food and water.

Induction of Ischemia

Under deep anesthesia (intraperitoneal injection of a mixture of 500 mg urethane per kilogram body weight, 11 mg/kg ketamine, and 14 mg/kg xylazine), the left optic nerve was exposed by making an incision in the upper conjunctival fornix and detaching the superior rectus muscle. The eyeball was retracted, and the dural sheath surrounding the optic nerve was gently cut with microscissors to separate the central retinal artery from the nerve. The central retinal artery with the surrounding tissue was clamped with a cerebral aneurysm surgery clip. After 60 minutes, the clip was removed, and reperfusion of the retinal vessels was begun. Absolute cessation and reperfusion of blood flow in the retinal vessels was confirmed by ophthalmoscopy. Only those eyes in which reperfusion was confirmed within 5 minutes of clip removal were used. During these procedures, the body temperature of the rats was maintained at 37°C.

Hsp27 Electroinjection

Immediately after the reperfusion, 5 μl Hsp27 protein solution, containing 6.5 μg protein (StressGen Biotechnologies Corp., Victoria, British Columbia, Canada), was injected into the vitreous with a 31-gauge needle. After the injection, the eye was grasped with a forceps-type disc electrode (Forceps-Type electrode 449–10PRG; Meiwa Shoji, Tokyo, Japan). The disc on the surface of the cornea was attached to the cathode and the disc on the sclera to the anode (Fig. 1). Then, square-wave pulses of 99 msec duration were administered as follows: Five electric pulses with electric field strength of 12 V/cm were given twice with a 5-minute interval between administrations, as described in our previous study.[7] The electro square porator (T820; BTX, San Diego, CA) generated electric pulses at a rate of 1 Hz. The electrical resistance of the tissue was monitored with a graphic pulse analyzer (Optimizer 500; BTX). The mean resistance of the eyeball was 1.69 ± 0.52 kΩ.

Immunohistochemistry

To confirm the entry of Hsp27 into RGCs, we first applied dextran rhodamine B (Molecular Probes, Eugene, OR) to the right superior

From the [1]Department of Ophthalmology and Visual Science, Graduate School of Medicine, Chiba University, Japan; and [2]Department of Anatomy, Yokohama City University School of Medicine, Japan.

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Corresponding author: Akiko Yokoyama, Department of Ophthalmology and Visual Science, Graduate School of Medicine, Chiba University, Inohana 1-8-1, Chuo-ku, Chiba City, Chiba 260-8670, Japan. yokoyama@ophthalm.m.chiba-u.ac.jp

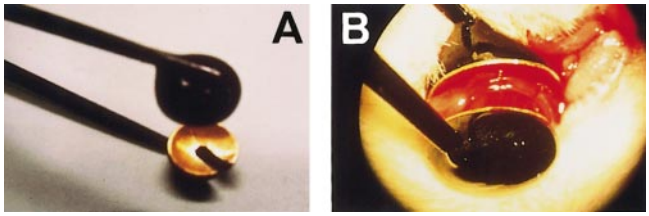

FIGURE 1. (**A**) Forceps-type electrode. (**B**) Eye is grasped by the forceps-type electrode to administer the electrical pulses. The disc with the slit is used on the optic nerve side of the eye, and the other disc is placed on the cornea.

colliculus for retrograde labeling of the RGCs of the left eye. Five days later, 60 minutes of ischemia was induced, and Hsp27 was injected into the left vitreous after the 60 minutes. The pulses were administered immediately in five rats (the Hsp27 electroinjection group). For the controls, five rats were injected with Hsp27 intravitreally without electroporation after the ischemia (Hsp27 without-pulses group), and five rats underwent only 60 minutes of ischemia–reperfusion (ischemia group).

All rats were killed by an intracardiac perfusion of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) under deep ether anesthesia. The retina of left eyes was removed from the eyecup, postfixed in the same fixative for 3 hours, and mounted in optimal cutting temperature (OCT) compound (Tissue Tek; Miles, Elkhart, IN). Frozen sections of 10-μm thickness were cut with a cryostat and mounted on MAS-coated microslide glass (Superfrost; Matsunami, Tokyo, Japan). These sections were stained with rabbit polyclonal antibody raised against recombinant human Hsp27 (1:200; StressGen Biotechnologies Corp.) as the primary antibody and FITC-conjugated goat antibody raised against rabbit IgG (1:1000; Antibodies Incorporated, Davis, CA) as the secondary antibody. The sections were then examined and photographed with a confocal laser microscope (Radians 2000; Bio-Rad, Hertfordshire, UK). The number of anti-Hsp27–positive RGCs was counted by two masked observers in 50 retinal sections for each of the three groups of animals. In all, 100 fields for each group of rats were counted. For controls, the primary antibodies were omitted in the staining of the sections.

Count of Living RGCs 7 Days after Ischemia

To count the number of living RGCs, we applied 7 μl of the lipophilic tracer, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine percholorate (diI; Molecular Probes), on the right superior colliculus 10 days before ischemia for retrograde labeling of the RGCs in the left eye. The diI suspension was prepared by placing 15 mg diI in 1 ml *N*,*N*-dimethylformamide.[8]

DiI labeling was performed in 31 rats. Seven animals were used to determine the level of diI labeling in the normal untreated retinas. These animals were perfused with 4% paraformaldehyde in 0.1 M PBS while under deep ether anesthesia 10 days after the diI application (intact group).

Ten days after diI application, the other 24 animals underwent 60 minutes of retinal ischemia-reperfusion. In seven of these rats, Hsp27 was injected and electric pulses were administered as described (Hsp27 electroinjection group), and in five other animals, Hsp27 was injected without electric pulses (Hsp27 without-pulses group). In five animals, PBS was injected into the vitreous and electric pulses were administered (pulses group), and seven animals served as control subjects and had only ischemia (ischemia group).

Seven days after the ischemia, the animals were perfused with 4% paraformaldehyde in 0.1 M PBS under deep ether anesthesia. The retinas were taken for confocal laser microscopy (Radians 2000; Bio-Rad) within 6 hours, and the number of diI-labeled cells was counted by two masked observers. The counting was performed in 10 areas within a 1-mm radius of the optic disc because the current density from the electrodes was highest around the optic nerve.

TUNEL Staining

To demonstrate the effect of Hsp27 on preventing apoptosis in RGCs, five animals that underwent ischemia–reperfusion and had Hsp27 electroinjection and five animals with only ischemia were killed 24 hours after the beginning of reperfusion, because the peak of apoptosis occurred 24 hours after reperfusion.

The retina of left eyes was removed from the eyecup and frozen sections were made similar to the method of immunohistochemistry. TUNEL staining was performed with an in situ apoptosis detection kit (Trevigen, Gaithersburg, MD). Labeled nuclei were then detected by streptavidin-FITC. The sections were stained with the fluorescent dye propidium iodide to label all nuclei, and the sections were studied under a fluorescence microscope (Carl Zeiss, Jena, Germany). TdT was omitted during the staining procedure as a negative control. The numbers of TUNEL-positive cells among all the cells in the RGC layers were counted by the same two masked observers in 25 frozen sections for each of the two groups of animals.

Statistical Analysis

The Mann–Whitney test was used to compare the number of diI-labeled RGC in the three groups, and the ratio of TUNEL-positive cells to all cells in the RGC layer was determined in the three groups. In all cases, $P < 0.05$ was considered to be significant and $P < 0.01$ was considered to be highly significant.

RESULTS

In the immunohistochemical study, the RGCs in the three groups were identified by the dextran rhodamine label (Figs. 2A, 2B, 2C). The immunoreactivity of Hsp27 was observed only in the RGCs in the electroinjected retina (Fig. 2A). In the retinas of rats injected with Hsp27 protein without electric pulses (Fig. 2B), there were only two or three cells among the 100 cells examined that were positive for anti-Hsp27, and in the retinas that underwent only 60 minute of ischemia (Fig. 2C), none of the 100 cells examined was positive for anti-Hsp27.

The number of living RGCs labeled by diI 7 days after ischemia is shown in Figure 3. In the control retinas, the mean number of RGCs was 2795.0 ± 351.1 cells/mm^2 (mean ± SD),

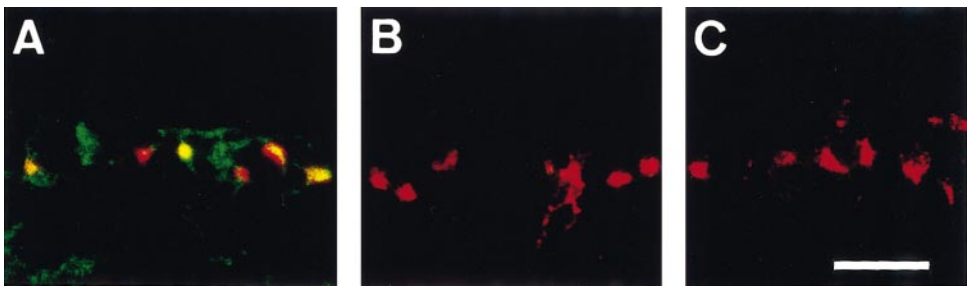

FIGURE 2. Photograph of RGC layer prelabeled by dextran rhodamine (*red*) and immunostained by anti Hsp27 antibody (*green*). (**A**) Hsp27 electroinjection group; (**B**) Hsp27 without-pulses group; (**C**) ischemia group. In (**B**) and (**C**), none of the RGCs is labeled by anti-Hsp27 antibody. Scale bar, 20 μm.

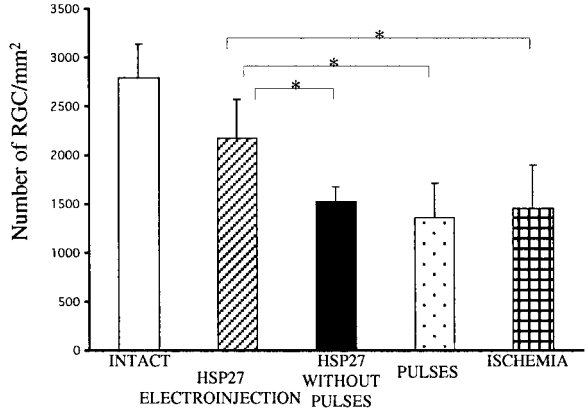

FIGURE 3. The number of living RGCs 7 days after ischemia. Number of RGCs in Hsp27 electroinjection group is significantly higher than in the ischemia group, the pulses group, and the Hsp27 without-pulses group. $^*P < 0.01$ highly significant.

and after 60 minutes of ischemia (ischemia group), the number was significantly reduced to 1458.2 ± 443.8 cells/mm^2. The number of living RGCs after an intravitreal injection of Hsp27 immediately after the 60 minutes of ischemia without electroporation (Hsp27 without-pulses group) and the group that received a vitreous injection of PBS and electroporation (pulses group) was 1525.5 ± 161.2 cells/mm^2 and 1365.1 ± 353.6 cells/mm^2, respectively. The mean number of RGCs under both of these conditions did not differ significantly from the number in eyes that underwent ischemia alone (1458.2 ± 443.8 cells/mm^2). However, the number of RGCs was significantly higher at 2185.2 ± 388.0 cells/mm^2 when Hsp27 was injected into the vitreous and pulses were administered immediately after 60 minutes of (Hsp27 electroinjection group).

The percentages of TUNEL-positive cells to all cells in the RGC layer 24 hours after ischemia are shown in Figure 4. The percentage in the Hsp27 electroinjection group was 17.0% ± 6.4% and that in the ischemia-alone group was 28.2% ± 10.3%. The lower percentage of apoptotic cells in the Hsp27 electroinjection group was highly significant ($P < 0.0001$).

DISCUSSION

We have shown that Hsp27 protein protected RGCs from ischemia–reperfusion injury but only when the protein entered the RGCs. Molecules of Hsp27 are large, and merely injecting the protein into the vitreous did not result in their entrance into the RGCs. The presence of the protein in the vitreous also did not rescue the RGCs from the ischemia–reperfusion injury. Although there are some reports that suggest that Hsps bind to the cell surface and internalize,[9–11] we could not find any RGCs clearly immunoreactive to anti-Hsp27 antibody in the Hsp27 without-pulses group. Earlier, we demonstrated that electrical pulses presented under the same conditions were successful in delivering a green fluorescent protein gene into RGCs, and the electric pulses themselves did not induce significant damage.[7] The same method was used in this study and the results demonstrated that electroporation will deliver Hsp27 into RGCs.

Hsp27 serves as a chaperone to stabilize the cytoskeleton,[12,13] and cytoskeletal breakdown is one of the key events in the initiation of apoptosis. Inhibition of protein synthesis during heat shock limits the accumulation of unfolded proteins that may damage eukaryotic cells. Chaperon Hsp27 is a heat shock–induced inhibitor of cellar protein synthesis, that binds elF4G and facilitates the dissociation of cap-binding initiation complexes known as elF4F.[14]

In human studies, elevated titers of serum antibodies to Hsp27 have been documented in some diseases such as cancer[15,16] and glaucoma.[17] The application of the antibody to Hsp27 exogenously facilitates apoptotic cell death in cultured retinal cells at concentrations similar to those found in patients with glaucoma,[17] and activates a proteolytic cascade, including caspase-8 and caspase-3 activation and the cleavage of poly-(ADP ribose) polymerase.[18] Hsp27 antibody enters neuronal cells in isolated human retinas by an endocytic mechanism, colocalizes with the actin cytoskeleton, and facilitates apoptotic cell death.[19] These results suggest that exogenous Hsp27 antibody may induce neuronal apoptosis by inactivating or attenuating the ability of native Hsp27 to stabilize actin cytoskeleton.

There was a good correlation between the number of anti-Hsp27-labeled RGCs in the Hsp27 electroinjection group (Fig. 2A) and the number of dil-labeled RGCs in the same group, and both numbers were significantly higher than in the ischemia group. Thus, we have demonstrated that electroinjected Hsp27 protected RGCs in a rat ischemia–reperfusion model for at least 7 days. We suggest that electroinjected Hsp27 inhibits RGC apoptosis by limiting the accumulation of unfolded proteins and stabilizes actin cytoskeleton.

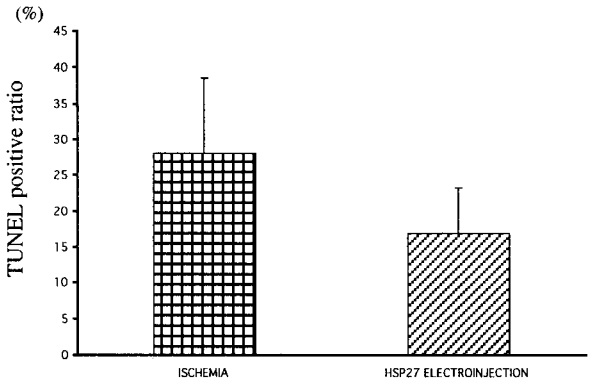

FIGURE 4. Ratio of TUNEL-positive cells to all cells in RGC layer 24 hours after ischemia. Hsp27 electroinjection group and ischemia group.

Acknowledgments

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References

1. Caprioli J, Kitano S, Morgan JE. Hyperthermia and hypoxia increase tolerance of retinal ganglion cells to anoxia and excitotoxicity. *Invest Ophthalmol Vis Sci.* 1996;37:2376–2381.
2. Yang YL, Lu KT, Tsay HJ, Lin CH, Lin MT. Heat shock protein expression protects against death following exposure to heatstroke in rats. *Neurosci Lett.* 1998;252:9–12.
3. Sharp FR, Massa SM, Swanson RA. Heat-shock protein protection. *Trends Neurosci.* 1999;22:97–99.
4. Yenari MA, Fink SL, Sun GH, et al. Gene therapy with HSP72 is neuroprotective in rat models of stroke and epilepsy. *Ann Neurol.* 1998;44:584–591.
5. Berbe MF, Tytell M, Gower DJ, Welch WJ. Hyperthermia protects against light damage in the rat retina. *Science.* 1988;241:1817–1820.
6. Wagstaff MJD, Collaço-Moraes Y, Smith J, Belleroche JS, Coffin RS, Latchman DS. Protection of neuronal cells from apoptosis by Hsp27 delivered with a herpes simplex virus-based vector. *J Biol Chem.* 1999;274:5061–5069.

7. Dezawa M, Takano M, Negishi H, Mo X, Oshitari T, Sawada H. Gene transfer into retinal ganglion cells by in vivo electroporation: a new approach. *Micron.* 2002;33:1–6.
8. Vidal-Sanz M, Villegas-Perez MP, Bray GM, Aguayo AJ. Persistent retrograde labeling of adult rat retinal ganglion cells with the carbocyanine dye diI. *Exp Neurol.* 1988;102:92–101.
9. Hightower L, Guidon PT. Selective release from cultured mammalian cells of heat shock (stress) proteins that resemble glia axon transfer proteins. *J Cell Physiol.* 1989;138:257–266.
10. Grossfeld RM: Axon-glia exchange of macromolecules. *Ann New York Acad Sci.* 1991;633:318–330.
11. Guzhova IV, Arnholdt ACV, Darieva ZA, et al. Effects of exogenous stress protein 70 on the functional properties of human promonocytes through binding to cell surface and internalization. *Cell Stress Chaperones.* 1998;3:67–77.
12. Welch WJ, Suhan JP. Morphological study of the mammalian stress response: characterization of changes in cytoplasmic organelles, cytoskeleton, and nucleoli and appearance of intranuclear actin filaments in rat fibroblasts after heat-shock treatment. *J Cell Biol.* 1985; 101:1198–1211.
13. Lavoie JN, Lambert H, Hickey E, Weber LA, Landry J. Modulation of cellular thermoresistance and actin filament stability accompanies phosphorylation-induced changes in the oligomeric structure of heat shock protein 27. *Mol Cell Biol.* 1995;15:505–516.
14. Cuesta R, Laroia G, Schneider J. Chaperon Hsp27 inhibits translation during heat shock by binding elF4G and facilitating dissociation of cap-initiation complexes. *Genes Dev.* 2000;14: 1460–1470.
15. Conroy SE, Sasieni PD, Amin V, et al. Antibodies to heat-shock protein 27 are associated with improved survival in patients with breast cancer. *Br J Cancer.* 1998;77:1875–1879.
16. Jäättelä M. Escaping cell death: survival proteins in cancer. *Exp Cell Res.* 1999;248:30–43.
17. Tezel G, Seigel GM, Bax MB. Autoantibodies to small heat shock proteins in glaucoma. *Invest Ophthalmol Vis Sci.* 1998;39:2277–2287.
18. Tezel G, Wax MB. Inhibition of caspase activity in retinal ganglion cell apoptosis induced by various stimuli in vitro. *Invest Ophthalmol Vis Sci.* 1999;40:2660–2667.
19. Tezel G, Wax MB. The mechanism of hsp27 antibody-mediated apoptosis in retinal neuronal cells. *J Neurosci.* 2000;10:3552–3562.