Protective Effect of *Heat Shock Proteins 70.1* and *70.3* on Retinal Photic Injury after Systemic Hyperthermia

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Purpose: This study aimed to determine the relationship between the heat shock protein 70 from *hsps70.1* and *70.3* on retinal photic injury after systemic hyperthermia.
Methods: Eight-week-old female C57BL/6 mice were kept at a constant temperature of 41~42℃ for 25~30 minutes. After dark-adaptation for 8 hours, intense light of 11000 lux was maintained for 6 hours. Histology and immunohistochemistry for the inducible heat shock protein 70 (hsp70), the constitutive heat shock protein 70 (hsc70), and western blot analysis, reverse transcriptase-polymerase chain reaction for *hsp70.1* and *hsp70.3* were performed just before photic injury and after 1, 4, 7, and 14 days.
Results: Light-induced retinal degeneration was prevented by thermotolerance. After hyperthermia, hsp70 was densely expressed in the inner segment of the photoreceptor layer on the photic injury. Hsp70 expression increased for 4 days after photic injury and slowly decreased thereafter. mRNA from *hsp70.3* was induced earlier than that of *hsp70.1*.
Conclusions: Retinal photic injury was prevented by hyperthermia-induced hsp70. Hsp70 from *hsp70.3* may be a rapid and short-lived responder, and that from *hsp70.1* is a slower and more sustained responder. Hsp70 from *hsp70.3* may be an initial retinal chaperone while hsp70 from *hsp70.1* may be a sustained chaperone. *Korean Journal of Ophthalmology 19(2):116-121, 2005*

Key Words: *Hsp70.1*, *Hsp70.3*, Hyperthermia, Retinal photic injury

Heat shock proteins (hsps) play a major role in protecting stressed cells.[1,2] They have a wide range of functions, which include protecting against external stress and injury and helping to regulate metabolism during normal development, differentiation, and growth.[3-7] In addition, cells acquire thermotolerance after heat stress through transcriptional activation of hsp genes.[8-10] Usually, hsp accumulation and the development of thermotolerance culminate 5~10 hours after the priming thermal stress.[11-13]

On the basis of molecular weight, hsps are classified into families of hsp90 (90-kilodalton, kd), hsp70 (70-kd), hsp60 (60-kd), and small hsps (25- to 30-kd). The hsp70 family consists of several proteins including inducible 70-kd hsps (hsp70) and constitutive 70-kd hsps (hsc70), which have a distinct distribution in the retina. These proteins are thought to be molecular chaperons that fold or unfold newly synthesized proteins during translation and transport through organelle membranes.[14,15] Although our previous results show that hsp70 plays an important role in retinal development and photic injury,[15-17] functions of hsp70 in ocular tissues are still unclear.[13-17]

Hsp70 in mice is known to originate from *hsp70.1*, *70.2*, or *70.3*.[18-21] In mice, the end products of each gene, hsp70, are highly homologous. This motivates the question of why different forms exist. Our previous results suggested that hsp70s from *hsp70.1* and *hsp70.3* play different roles.[16,17] The main difference may be the timing of action. The data suggested that hsp70 from *hsp70.1* may be a delayed responder to retinal light stress.[16,17]

This study aimed to determine the relationship between the hsp70s from *hsp70.1* and *70.3* on retinal light stress. To maximize hsp70 production, retinal light stress was maintained for 8 hours after the onset of hyperthermia, when thermotolerance culminates. The expression pattern of hsp70 from *hsp70.1* or *hsp70.3* was evaluated with the time sequence.

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Materials and Methods

1. Hyperthermia

The animals were handled in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. Eight-week-old female C57BL/6 mice were kept in a 12-hour cycle of 40 lux light and darkness between 22℃ and 24℃, for two weeks before the experiment. Each mouse was then kept at a constant temperature of 41-42℃. Body temperature was monitored intermittently with a rectal thermistor probe and reached 41-42℃ for 25-30 minutes. After drying, the mice were allowed to recover in another darkened chamber at 30℃ for 2 hours to prevent reactive hypothermia. Subsequently, the mice were kept at the same condition as before the experiment.

2. Photic injury

After hyperthermia, the mice were dark-adapted for 8 hours. Intense diffuse, cool, white fluorescent light was then applied with an equidirectional intensity of 11000 lux for 6 hours while maintaining the temperature between 22℃ and 24℃ by constant air flow. Just before photic injury and 1, 4, 7, and 14 days after light stress, six mice were sacrificed at each time.

3. Tissue preparation for histology and immunohistochemistry

Both eyes from each animal were removed and fixed by immersion in Carnoy's solution for 2 hours at room temperature. Each eye was then dehydrated in a graded ethanol series and embedded in paraffin using standard techniques. The paraffin-embedded eyes were sectioned at 4 μm and mounted on slides coated with bovine serum albumin for hematoxylin and eosin staining, or 0.5% Elmer's glue for the immunohistochemistry. Only the sagittal sections parallel to the superior-inferior axis of the eye, including the optic nerve, were collected.

4. Histology & immunohistochemistry

The sections were deparaffinized, rehydrated and stained with hematoxylin and eosin according to normal histologic procedures. For immunohistochemistry, slide-mounted sections were deparaffinized, rehydrated, and treated with 0.3% H_2O_2 to eliminate any endogenous peroxidase activity. The sections were treated with 10% normal rabbit serum to block the nonspecific antigenic sites and then incubated overnight at 4℃ with anti-hsp70 (5 μg/ml) and anti-hsc70 antibodies (0.25 μg/ml). The next day, the sections were incubated with biotinylated anti-mouse IgG antibodies (1 μg/ml), followed by a reaction with peroxidase-conjugated streptavidin (1 μg/ml). The sections were rinsed, dehydrated, cleared in xylene, and then mounted in Permount. The bound antibodies were visualized using the DAB-nickel detection system (Vector Laboratories).

5. Western blot analysis

The dissected retinal tissue was homogenized in a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer (2% SDS, 20% glycerol, 5% β-mercapto-ethanol, 0.01% bromophenol blue, 10 mM Tris-HCL [pH 8.0], 1 mM phenylmethylsulfonyl fluoride and 1 mM EDTA) and incubated for 3 minutes at 95℃. Aliquots of 2 μg were then separated on an 8% SDS-polyacrylamide Minigel (Bio-Rad). Samples of recombinant human hsp70 (>90% hsp70; SPP755; StressGen Biotechnologies) were included as controls. The proteins in the gel were transferred electrophoretically to nitrocellulose (Hoefer Mighty Small Transphor System) and the filter was split into sections. After blocking with 5% blotto (5% nonfat milk in 10mM phosphate-buffered saline [pH 7.4], 0.1% Tween-20 [PBS-T]) for 1 hour, the membranes were incubated for another hour in 5% blotto containing either of the hsp70 (1 μg/ml; SPA-815; StressGen) primary antibodies, followed by incubation for 45 minutes in 5% blotto containing either the peroxidase-conjugated goat anti-rat (1 μg/ml; Kirkegaard & Perry Laboratories, KPL) or goat anti-mouse (1 μg/ml; KPL) secondary antibodies, where appropriate. The immune complexes were visualized using an ECL detection system (Amersham).

6. Reverse transcriptase-polymerase chain reaction (RT-PCR)

The *hsp70.1* and *hsp70.3* mRNA was detected in the RNA extracts from the retinal tissues of two eyeballs from the same mouse using TRIzol reagent and Rnase-free Dnase I (Life Technologies, Gaithersburg, MD, USA). Duplicate cDNAs were reverse transcribed from two sets of these RNAs using random hexamer primers from an RNA PCR kit (Perkin-Elmer Corp., Norwalk, CT, USA). The negative-control cDNAs were produced from the extracted RNAs in the absence of reverse transcriptase. The PCR products were generated using primers corresponding to the 3'-untranslated regions of *hsp70.1* (upper primer 5'-TGCTTGGGCACCGAT TACTGTCAAGG-3' and lower primer 5'-GGCAGCTAGA-CTATATGTCTTCCCAGGCTACTG-3') and *hsp70.3* (upper primer 5'-AGATATGTGG-CCTTGAGGACTGTCATTATTT C-3' and lower primer 5'-CTGGGGCAGTGCTGAA-TTGAA GAATATA-3') identical to a previous report.[21] Each PCR utilized one-eighth of the volume of any one batch of cDNA, amplified for 40 cycles (94℃, 1 min; 53℃, 2 min; 72℃, 30 sec) at pH 8.3 and with 1.5 mM $MgCl_2$. The *hsp70.1* amplicon is 285 bp and the *hsp70.3* amplicon is 220 bp. The control RT-PCRs were run using primers for hsc70 mRNA (positive control) and hsc70t mRNA (negative control).

Results

1. Histology

Hyperthermia-induced thermotolerance prevented retinal degeneration by photic injury. When photic injury occurred without hyperthermia, diffuse disarrangement and deterioration in the outer nuclear layer (ONL) was observed after 14 days. However, retinal photic injury after systemic hyperthermia caused no definite disarrangement in the retina (Fig. 1).

2. Immunohistochemistry

Hsp70 immunoreactivity was detected in some retinal layers (ONL, outer limiting membrane, and photoreceptors) with differing intensities. Hsp70 expression was most prominent in the inner segment of the photoreceptors. Hsp70 immunoreactivity in the inner segment increased for 4 days after photic injury and slightly decreased thereafter (Fig. 2). Hsc70 immunoreactivity was detected in all retinal layers. Hsc70 immunoreactivity was almost constant exhibiting little temporal and spatial variation (Fig. 2).

3. Western blot analysis

Hsp70 level was enhanced even before light stress, which originated from hsp-induction through systemic hyperthermia. The production of hsp70 was detected at each time sequence. Although the change with time was subtle, hsp70 increased for 4 days after photic injury, and then slowly decreased (Fig. 3).

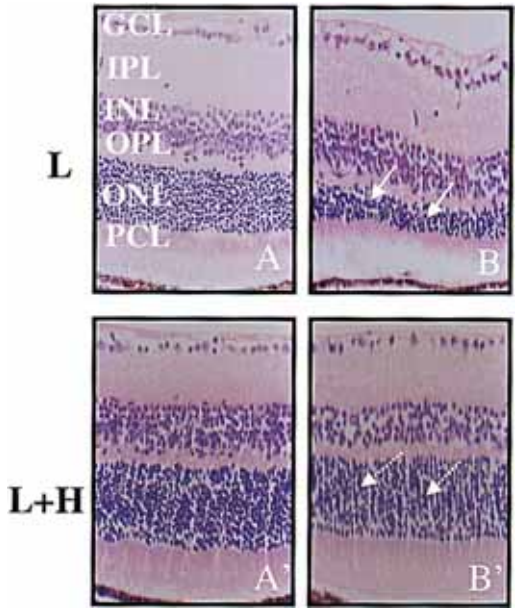

Fig. 1. Histology of the retina after photic injury with or without hyperthermia. On photic injury without previous hyperthermia, diffuse disarrangements in the ONL and photoreceptor cell layer was observed (white arrows in B). However, photic injury after hyperthermia made no definite change in retina structure (white arrows with dotted line in B'). Hematoxylin & Eosin staining, magnification ×400 (A, A': before photic injury; B, B': 14 days after photic injury, L; light stress only, L+H; light stress after systemic hyperthermia)

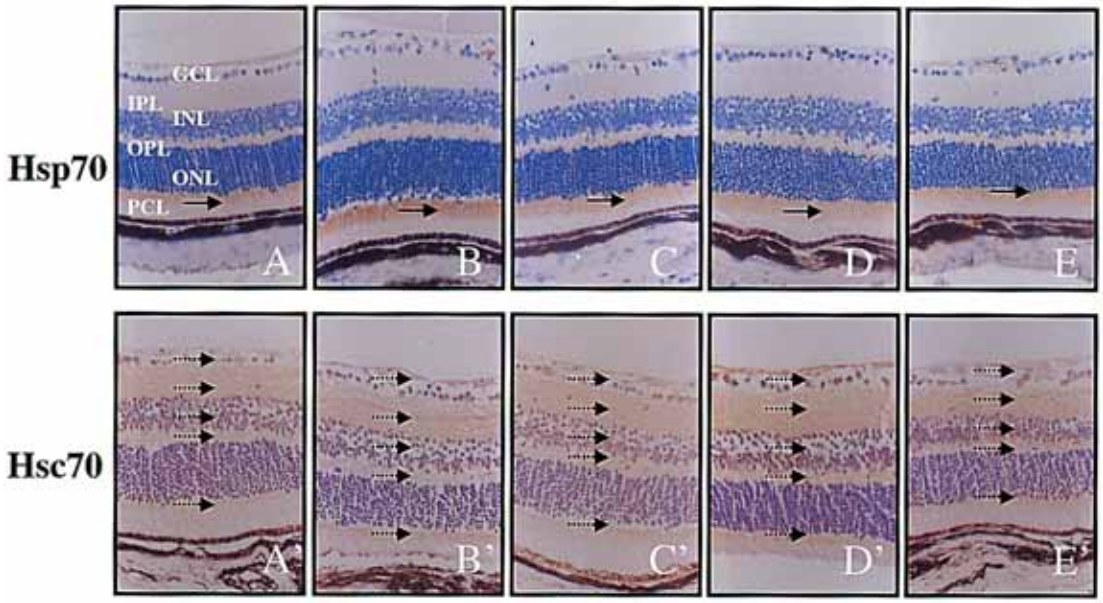

Fig. 2. Immunohistochemistry for hsp70 and hsc70 in the retina. Hsp70 expression was most prominent in the inner segment of photoreceptors (black arrows) for 4 days after photic injury following hyperthermia. Hsc70 expression was detected in most retinal layers (black arrows with dotted line), and nearly constant without temporal or spatial difference. Hematoxylin & Eosin staining, magnification × 400 (A, A': before photic injury; B, B': 1 day after photic injury; C, C': 4 days; D, D': 7 days; E, E': 14 days).

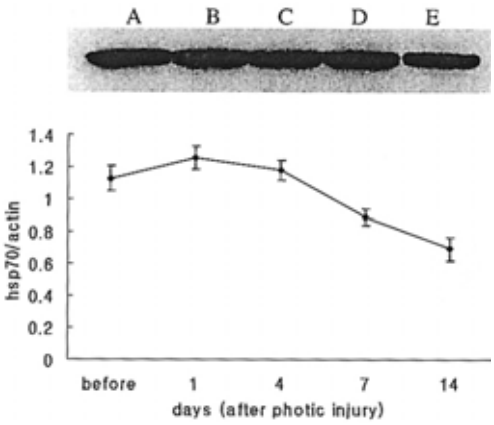

Fig. 3. Western blot analysis for hsp70. In A, enhanced hsp70 was due to hsp induction by systemic hyperthermia. The expression of hsp70 was sustained for 7 days. Although changes were subtle, the quantitative measurement showed that production of hsp70 increased for 4 days after photic injury and slowly decreased thereafter. (A: before photic injury; B, C, D and E: 1, 4, 7 and 14 days after photic injury, respectively.)

4. RT-PCR

Semiquantitative analysis of mRNA expression, RT-PCR, showed some interesting results. mRNAs of *hsp70.1* and *hsp70.3* were expressed more after light stress than before. mRNA from *hsp70.3* was induced earlier than that of *hsp70.1*. mRNA expression of *hsp70.3* reached a peak 1 day after photic injury, whereas *hsp70.1* expression peaked after 4 days (Fig. 4).

Discussion

Heat shock proteins are important for cellular protection. Substantial evidence has shown that hsps have a protective function against various noxious conditions as well as otherwise lethal heat stress.[1-5] Furthermore, hsps must not be overlooked for their clinical importance. They may act as immunodominant antigens of infecting organisms, immunoreactive agents, and powerful antineoplastic vaccines.[22,23] Recently, it was found that it is possible to lengthen the life span of an organism using hsps.[24-26] In ocular tissue, especially the retina, hsp70 is a major stress protein. Hsp70 in mice is composed of the end products of *hsp70.1*, *70.2*, and *70.3*[18-21] and has retinal protective effects against various stresses.[13-18] However, it is not known which gene plays the major role in retinal photic injury. The main question is whether or not overexpression of hsp70 can be a therapeutic method for either apoptotic or necrotic retinal disease processes. There have been some interesting reports showing the effect of hsp70 overexpression.[27,28] However, no study has been able to answer this question with certainty. There may be different results depending on which hsp70, (from *hsp70.1* or *70.3*), is overexpressed. Therefore, the first step

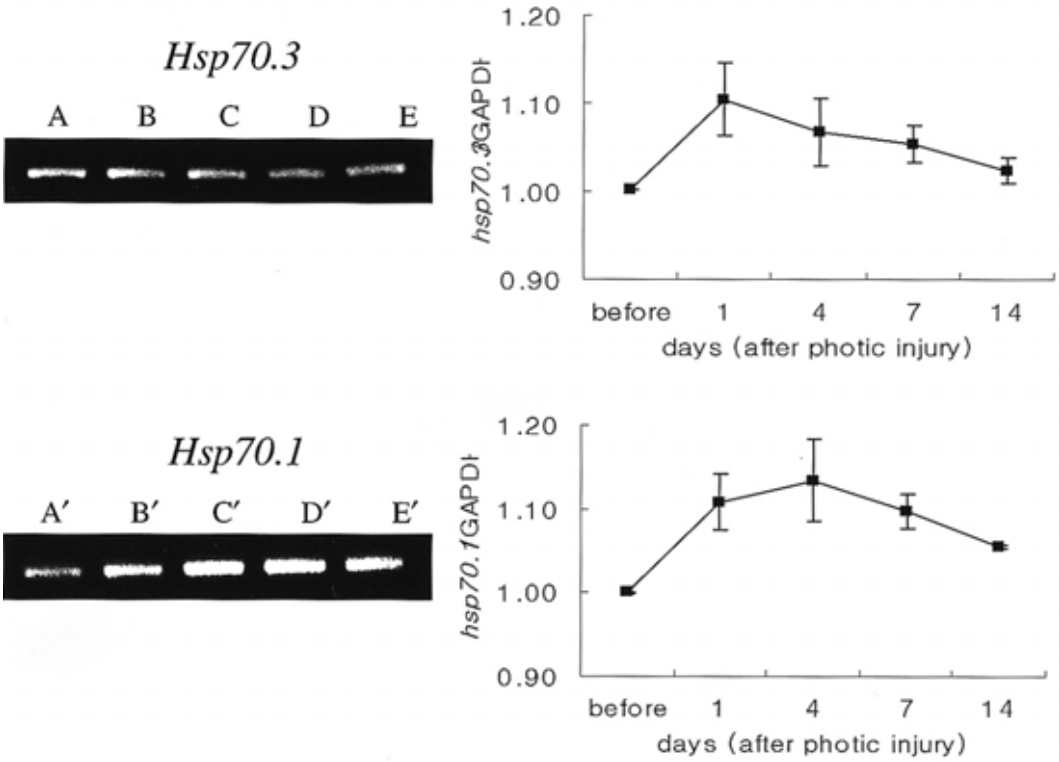

Fig. 4. Semiquantitative analysis of mRNA expression, RT-PCR, for *hsp70.1* and *hsp70.3*. mRNA expression of *hsp70.1* reached a peak 4 days after light stress and slowly decreased until day 14. The mRNA expression in hsp70.3 was most prominent at day 1 and slowly decreased thereafter. (A, A': before photic injury; B, B': 1 day after photic injury; C, C': 4 days; D, D': 7 days; E, E': 14 days)

to finding the answer is to accurately show the different functions of hsp70s from *hsp70.1* and *70.3*.

This study showed some interesting results regarding the retinal protective action of hsp70 that might be associated with hsp70 induction by systemic hyperthermia. Upon histologic examination, there was no definite disarrangement observed in retinal photic injury after systemic hyperthermia (Fig. 1). The hsp70 expression pattern in immunohistochemistry and western blotting supports the possibility of retinal protection by hsp70 against retinal deterioration after light stress (Figs. 2, 3).

The protein expression level cannot be predicted from the mRNA level.[29] However, mRNA expression is strong evidence of protein production. Because the hsp70s from *hsp70.1* and *hsp70.3* are highly homologous,[18-21] it is very difficult to distinguish between them at the protein level. Therefore, the production patterns of hsp70s from *hsp70.1* and *hsp70.3* were evaluated indirectly from the changes in mRNA expression. mRNA expression of *hsp70.1* reached a peak later than that of *hsp70.3*, 4 days after photic injury, and then slowly decreased. These facts suggest two hypotheses; first, that hsp70 from *hsp70.3* may provide initial protection against light stress, as it responds quickly, and second, that hsp70 from *hsp70.1* may have a delayed response.

Hsp70.1 and *70.3* both play major roles in protection against retinal photic injury. Specifically, hsp70 from *hsp70.1* may be a major protector, although it is a delayed responder. *Hsp70.3* may compensate for the delayed action of *hsp70.1*. Therefore, the expression level of the mRNA from *hsp70.3* increases immediately after light stress, and is expressed for a relatively short period of time.

There are some questions remaining: (1) Why does the mRNA from *hsp70.1* respond so much later? (2) If the hsp70s from *hsp70.1* and *hsp70.3* interact as complements, why is the mRNA from *hsp70.3* sustained for 14 days after light stress, without a compensational decrease? and (3) What is the role of hsp70 from *hsp70.1*, knowing that its inducement is delayed? Answers to these questions will be the focus of a future study ultimately aimed at treating apoptotic retinal disease.

This study showed that hsp70 has specific functions for retinal protection against various stresses, according to which gene they originate from. It is necessary to determine which gene would be most efficacious as a possible gene therapy for apoptotic or necrotic disease in the retina.

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