Expression of Hsp70 and Hsp27 in lens epithelial cells in contused eye of rat modulated by thermotolerance or quercetin

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Purpose: To investigate the dynamic expression of heat shock protein (Hsp)70 and Hsp27 in lens epithelial cells (LECs) of contused eyes and the effects of heat shock and quercetin.

Methods: Rat eyes were randomized into three groups: contusion, heat shock, and quercetin. Eye contusions were induced by dropping a 20 g steel ball from a height of 20 cm for 100 times. Heat shock was induced by raising the core body temperature to 40.5-41.5 °C for 8 min, 2-3 h before eye contusion. Quercetin, an inhibitor of Hsp expression, was given via gavage (100 mg/kg) 2-3 h before eye contusion. Experimental eyes were enucleated at 0, 1, 3, 6, and 24 h following each group treatment for the detection of Hsp70 and Hsp27 expression in LECs using RT-PCR. Lens sections were examined by immunohistochemistry with antibodies to Hsp70.

Results: Basic expression of Hsp70 and Hsp27 was observed in LECs. Expression of Hsp70 in LECs was increased after contusion in a time-dependent manner. Preconditioning hyperthermia (45 °C, 8 min) resulted in a significant increase of Hsp70 expression compared to the base level, and enhanced the expression in each subgroup in contrast to the corresponding subgroup in the contusion group. However, Hsp70 levels in the 1-6 h subgroup in the quercetin group was significantly lower. There was no notable change of expression of Hsp27 between each group. Immunostaining of Hsp70 was prominent in LECs of contusion eyes and heat shock eyes compared with those in normal eyes. Immunostaining of Hsp70 was faint in LECs of quercetin eyes.

Conclusions: Increased expression of Hsp70 in LECs of contused eyes may play a protective role against degeneration of lens protein. Thermal preconditioning possibly protects against lens injury by increasing the expression of Hsp70. Endogenous protective mechanisms may be important in eye contusion.

The lens is an indispensable part of the ocular optical system and an important intermediate of refraction. Thus, the maintenance of its transparency plays an important role in keeping normal optic function. It has been established that lens transparency is maintained by the supramolecular organization of its constituent proteins [1]. Preservation of this organization is maintained by the fidelity of native proteins. Quaternary protein structure depends on proper folding of newly synthesized polypeptides, and adequate protection from environmental and pathophysiological stress [2]. One possible protective mechanism suggested is the heat-shock response, which involves the transcriptional activation of a small set of specific genes and a subsequent synthesis of the “heat shock proteins” (Hsps) [3].

It has been shown that in vivo and in organ cultures, several mammalian tissues respond to heat shock or other forms of stress by the synthesis of one or more Hsps [4,5]. Regarded as molecular chaperones, many of these Hsps can not only prevent the accumulation of unfolded or aggregated polypeptides, but they can also promote the rapid degradation of such abnormal proteins. In addition, Hsps are protective proteins that aid in maintaining cell homeostasis under environmental stress [6-8], and this effect can be regulated by many factors. For instance, stressors including heat shock, ischemia, oxidative stress, pH extremes, nutrient limitation, osmotic variation, hypoxia, chemotherapeutic agents and noxious chemicals, anoxia, mechanical trauma, ethanol, glucose deprivation, and heavy metals can rapidly activate the expression of the genes for Hsps and subsequently synthesize the inducible forms of Hsps. Conversely, quercetin, a bioflavonoid from gingko biloba, regarded as an inhibitor of heat shock factor protein 1 (HSF1) [9,10], can block stress-inducible Hsps induction at the transcriptional levels.

Previous data have demonstrated that Hsp70, a major member of the Hsps family, is expressed in the lens under unstressed conditions, suggesting normal microenvironment of the lens is stressful and Hsp70 is continuously needed [11-13]. Further studies found that inducible Hsp70, Hsp40, and Hsp27 are expressed in the lens epithelium cells (LECs) under heat shock, oxidative stress, and osmotic stress [4,5,14], suggesting that certain Hsps may play a part in protecting LECs against a variety of insults that can damage or denature cell proteins. However, whether blunt trauma (a mechanical stress to the eye) could induce Hsp7s synthesis in lens has not been determined. Therefore, we performed this study to determine the dynamic expression of Hsp70 and Hsp27 in LECs of rats in vivo based on the contusion cataract model [15]. To test the...
modification on the expression of Hsp70 and Hsp27 in LECs, we used heat shock pretreatment to raise rat core body temperature and administered quercetin, an inhibitor of Hsps, via gavage prior to inducing eye contusion. Our results show there are basic expressions of Hsp70 and Hsp27 in LECs of rats. We find that contusion of eyes increases the expression of Hsp70 in rat LECs in vivo at both gene and protein levels, and this effect of contusion on the induction of Hsp70 synthesis can be modulated. Preconditioning hyperthermia can enhance the effect of contusion on Hsp70 induction, while quercetin can attenuate the size of the inducible response. However, our study also demonstrates Hsp27 levels remains unchanged after contusion insults.

METHODS

Animal handling and euthanasia: Animals were handled under a license from Shanghai Laboratory Animal Center, Chinese Academy of Sciences. Rats were kept under standard lighting conditions with a 12:12 h light/dark cycle at room temperatures maintained between 21-24 °C. All experiments and procedures were carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Seventy-two male Sprague-Dawley rats weighing 50-100 g were randomized into three groups (n=24 each): contusion, heat shock (thermal preconditioning 2-3 h prior to trauma), and quercetin groups (100 mg/kg of quercetin given orally 2-3 h prior to trauma). Preoperative examinations were performed under anesthesia with 10% chloral hydrate by intraperitoneal injection (3 mg/kg). We performed slit lamp (Haag-streit 900, Bern, Switzerland) biomicroscopic examinations before preconditioning to exclude any other defects such as corneal abnormality or cataract. Each group was further randomized into six subgroups: C (control group), 0 h, 1 h, 3 h, 6 h, and 24 h groups (according to the time that eyes were enucleated at 0, 1, 3, 6, and 24 h following trauma).

Animal protocols: Rats were anesthetized with 10% chloral hydrate by intraperitoneal injection (3 mg/kg). Blunt trauma was induced by dropping a 20 g steel ball from 20 cm height 100 times on the eyes of anesthetized rats. Heat shock (hyperthermia) was induced by increasing the core body temperature of animals to 40.5-41.5 °C by partial immersion in a water bath (45 °C) [16] and remaining at 40.5-41.5 °C for 8 min, and allowing to recover at room temperature for 2-3 h before contusion. Rats anesthetized in the quercetin group received quercetin (100 mg/kg; Sigma Chemical Co., St. Louis, MO) via gavage 2-3 h before eye contusion. Then, the rats were killed by CO₂ inhalation, and the eyes or appropriate ocular

Figure 1. Expression of Hsp70 mRNA in rat lens epithelial cells. In the contusion group (A), Hsp70 is expressed normally but increased progressively by 1 h after eye trauma, peaked at 3 h, and decreased progressively by 24 h. In the heat shock group (B), Hsp70 was expressed higher after heat shock, increased progressively by 1 h, peaked at 3 h, and decreasing at 24 h but still remained higher than the control subgroup. In the quercetin group (C), Hsp70 also increased at 1 h and peaked at 3 h, then decreased progressively until 24 h, but the densitometry value of each subgroup was lower than the control group. D: Comparison of three groups shows the heat shock group was the highest and the quercetin group was the lowest. M indicates the molecular weight marker. The lanes are identified as control (C) or by the duration until enucleation for the subgroup within each group.
tissues were removed immediately. The dissection room was maintained between 21-24 °C during euthanization. The lenses were removed from the eyes, and anterior capsule with LECs were dissected and stored immediately in liquid nitrogen.

**Reverse transcript-polymerase chain reaction analysis:** After 0, 1, 3, 6, and 24 h of contusion, experimental rats were sacrificed and total RNA was isolated from lenses using Trizol Reagen (Invitrogen, Carlsbad, CA). cDNA was synthesized and subjected to RT-PCR for detecting Hsp70 and Hsp27 mRNAs using a One-Tube Expand RT-PCR System Kit (Roche Biochemicals, Basel, Switzerland). The following primer pairs were used: 5’ Hsp70 cDNA primer (GenBank L16764), 5’-AGG GAG GAG TCC GCC GAG G; 3’ Hsp70 cDNA primer, 5’-AGG TCG AAG ATG AGC ACG TTT (amplifying a 238 bp fragment); 5’ Hsp27 cDNA primer (GenBank M86389), 5’-TCA GCC AAG ACC ATG ACC G; 3’ Hsp27 cDNA primer, 5’-CGT GTA TTT CCG GGT GAA GC (amplifying a 300 bp fragment); 5’ α-actin cDNA primer (GenBank NM_031144), 5’-GCA TTG TTC CGG GTG GAA GC (amplifying a 280 bp fragment, used as the internal standard). Amplification was performed in a thermal cycler using the following conditions: 30 cycles of 94 °C for 30 s, 56 °C for 20 s, 72 °C for 30 s, and 72 °C for 30 s. PCR products were analyzed by electrophoresis in 0.8% agarose gels. The intensities of the amplified cDNA fragments were estimated using a video-densitometer (Kodak2000, Japan).

**Histology:** The eyes from normal animals and animals 24 h after contusion, 24 h after heat shock and trauma, and 24 h after quercetin via gavage and trauma were dissected, fixed with 10% buffered formaldehyde at room temperature overnight, immersed in a series of graded isopropanol solutions, with 10% buffered formaldehyde at room temperature overnight after quercetin via gavage and trauma were dissected, fixed, rinsed several times with PBS, and then embedded in paraffin, m thick paraffin sections were cut along the vertical meridians and mounted onto microscope slides. Only sections including the lens were used for immunohistochemistry.

**Immunohistochemical analysis:** Sections were deparaffinized, rehydrated, and pretreated with 0.3% hydrogen peroxide in PBS to decrease endogenous peroxidase activity. Sections were incubated with an anti-Hsp70 monoclonal antibody made from inducible Hsp70 (1:200, Maixin Bio, Fuzhou, China) at room temperature for 45 min, and the primary antibodies were localized by immunoperoxidase using a commercial kit (ElivisionTM Kit, Maixin Bio). Sections were rinsed with PBS then incubated with appropriated biotinylated secondary antibodies for 30 min at room temperature. Sections were rinsed again with PBS before being exposed to horseradish peroxidase labeled streptavidin for 30 min. After several washes with distilled water, bound antibody was visualized with a DAB-nickel detection system. Sections were counterstained with hematoxylin and eosin and coverslipped with a mounting medium. Slides were examined and documented with a microscope (Olympus, Tokyo, Japan) equipped with bright-field illumination. Images were recorded by means of a digital camera (1000-Olympus DP11, Tokyo, Japan) attached to the microscope. The stained sections were examined by two observers (Yan QH and Li P). Qualitative evaluation of the immunostaining (negative, faint, or increased immunostaining) in the LECs was independently recorded by these two observers. Judgment of increased immunostaining was made only when both observers independently agreed.

**Statistical analysis:** All results were expressed as mean±standard deviation. Independent sample t-test and one way ANOVA analysis (SPSS 13.0 statistical software) were used to assess the data. A p<0.05 was considered to be statistically significant.

**RESULTS**

Reverse transcript-polymerase chain reaction analysis: Amplification of Hsp70 mRNA from rat LECs produced the predicted 238 bp signal before eye contusion in all three groups (Figure 1). Hyperthermal preconditioning significantly elevated the baseline value of Hsp70 mRNA (1.83±0.30 compared to 1.11±0.08, p<0.05; Figure 1B). In all three groups, Hsp70 mRNA increased 1 h after contusion, peaked after 3 h, descended gradually after 6 h, and then decreased to base level again at 24 h (Figure 1D). Densitometry values of Hsp70 of the contusion, heat shock, and quercetin groups are summarized in Table 1. Hyperthermal preconditioning significantly enhanced the expression in each subgroup in contrast to the corresponding subgroup of contusion group. The expression of Hsp70 mRNA in the heat shock group also decreased 24 h after contusion, but it was still higher than that of the control group. Intake of quercetin did not significantly decrease the baseline expression of Hsp70 mRNA, but significantly decreased the expression of Hsp70 in the 1-6 h subgroups compared to the corresponding contusion subgroups. There was no significant difference in the expression level of Hsp70 mRNA at 24 h in the quercetin group when compared to that of the control group. While a 300 bp PCR product of Hsp27 mRNA was detected in each subgroup in all three groups (Figure 2), there was no significant difference among them (Table 2).

**Immunohistochemical study:** Maximal expressions of Hsp70 after preconditioning were observed and localized in the nucleus or cytoplasm around the nucleus. The degree of Hsp70 expression exhibited the increased level after preconditioning. In the control group (normal eyes) Hsp70 was not expressed in the lens epithelium, but it was expressed strongly in both contusion and heat shock eyes. Quercetin eyes dem-

**Table 1. Densitometry value of Hsp70 of contusion, heat shock, and quercetin groups**

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Contusion</th>
<th>Heat shock</th>
<th>Quercetin</th>
<th>F value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>1.11±0.08</td>
<td>1.83±0.30</td>
<td>1.11±0.06</td>
<td>21.50</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>1.06±0.04</td>
<td>2.06±0.16</td>
<td>1.16±0.04</td>
<td>141.26</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>4.39±0.12*</td>
<td>5.85±0.26*</td>
<td>3.20±0.13</td>
<td>39.37</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>7.72±0.27*</td>
<td>8.95±0.62*</td>
<td>3.05±0.41</td>
<td>135.85</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>5.67±0.15*</td>
<td>6.88±0.36*</td>
<td>3.00±0.18</td>
<td>273.22</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>1.32±0.54</td>
<td>3.14±0.54</td>
<td>1.19±0.05</td>
<td>45.25</td>
<td>0</td>
</tr>
</tbody>
</table>

Subgroups are identified as controls (C) or by the duration until enucleation (h) for the subgroup. The asterisks denote statistically significant differences between a subgroup and the control subgroup (p<0.05; n=4; mean±SD).
onstrated faint expression of Hsp70. These results conformed to those of RT-PCR analysis and are presented in Figure 3.

DISCUSSION

The heat shock response is one possible protective mechanism in maintaining normal microenvironment in the lens. Our results confirm the separate observations of previous studies, suggesting that Hsp70 and Hsp27 are expressed constitutively in the lens. The lens is a closed system with limited capability to repair or regenerate itself [17]. However, the eye chamber is exposed constantly to aqueous humor containing reactive oxygen species (ROS) generated by light-catalyzed reactions [18]. These free radicals may cause increased oxidation in tissues of the anterior eye segment and finally lead to oxidative damage. Therefore, such constitutive expression of Hsp70 and Hsp27 in the lens may be due to continual oxidative stress.

Researchers found the presence of a nuclear matrix protein of Hsps with significant sequence similarity to heat shock transcription factor which upregulates synthesis of Hsps. Many of these proteins act as molecular chaperones, suggesting sustained heat shock protein synthesis in the mouse lens [19]. Their study further demonstrated that Hsp27 was expressed in all regions of the lens, and Hsp70 was found in the epithelium cells and superficial cortical fiber cells. A recent study found that Hsp70 knockout mouse models showed more lens opacity than normal mice [16]. All of these findings suggest that the normal microenvironment of the lens is stressful and Hsps, such as Hsp27 and Hsp70, are continuously needed. Moreover, the main finding of the present study is that eye contusion can induce Hsp70 synthesis in LECs of rats in vivo in a time-dependent manner. In mammalian cells, Hsps are commonly classified according to their molecular masses, for example, Hsp90, Hsp70, Hsp60, and Hsp20 to Hsp30 KDa (small Hsp). Most Hsps are constitutive proteins, and only part of them can be induced by stress. For Hsp70, both constitutive and inducible forms exist. Hsp70 can be induced in a wide variety of cells such as rat islet cells, cardiomyocytes or cultured hepatocytes in response to many stressors, and then play an important role in keeping normal levels of protein synthesis and a higher survival rate of cells under environmental stress [20-22]. Thus, the induction of Hsp70 also suggests a potential protective mechanism of the lens against eye contusion. Furthermore, the finding that Hsp70 peaked at 3 h after eye contusion may suggest that its expression conforms to eye traumatic process. In addition, our study also finds that preconditioning hyperthermia and quercetin treatment can modulate the effect of this mechanical stress. Many previous studies confirmed that thermotolerance, which can be induced by a short exposure to a nonlethal heat treatment, could enhance Hsp70 induction and its effect of protecting cells against heat

![Figure 2](http://www.molvis.org/molvis/v12/a51/)

**Table 2. Densitometry values of Hsp27 of contusion, heat shock, and quercetin groups**

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Contusion</th>
<th>Heat shock</th>
<th>Quercetin</th>
</tr>
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<tbody>
<tr>
<td>C</td>
<td>0.298 ± 0.021</td>
<td>0.290 ± 0.012</td>
<td>0.295 ± 0.017</td>
</tr>
<tr>
<td>0</td>
<td>0.278 ± 0.013</td>
<td>0.288 ± 0.017</td>
<td>0.280 ± 0.016</td>
</tr>
<tr>
<td>1</td>
<td>0.300 ± 0.026</td>
<td>0.290 ± 0.022</td>
<td>0.288 ± 0.017</td>
</tr>
<tr>
<td>3</td>
<td>0.295 ± 0.024</td>
<td>0.293 ± 0.015</td>
<td>0.293 ± 0.015</td>
</tr>
<tr>
<td>6</td>
<td>0.268 ± 0.010</td>
<td>0.270 ± 0.008</td>
<td>0.300 ± 0.014</td>
</tr>
<tr>
<td>24</td>
<td>0.303 ± 0.021</td>
<td>0.298 ± 0.017</td>
<td>0.295 ± 0.006</td>
</tr>
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</table>

Subgroups are identified as controls (C) or by the duration until enucleation (h) for the subgroup. There were no statistically significant differences among groups or subgroups (n=4; mean±SD).
or other stresses [23,24]. When undergoing thermal (45 °C, 15 min) stimulation, mesangial cells increase Hsp70 expression to protect themselves against subsequent oxidative injury [25]. Heat-shock treatment also could increase light scattering and the expression of Hsp70 in bovine lenses [13]. Another study demonstrated that heating, which caused overexpression of Hsp70 in vivo was found in LECs of rats as a result of preconditioning hyperthermia (45 °C, 8 min) regardless of whether eye contusion was present, suggesting that LECs might execute thermotolerance and become resistant to eye contusion. Conversely, quercetin, an inhibitor against Hsp synthesis, also decreased the expression of Hsp70 in LECs of contusion eyes. Previous data showed that quercetin was able to inhibit Hsp70 synthesis at the transcriptional level due to the loss of HSF phosphorylation or HSF-HSE binding activity [26]. Also, quercetin blocks Hsp induction and the protective effect of glutamine and DON (6-diazo-5-oxo-L-norleucine), a

Figure 3. Micrographs showing the distribution of Hsp70 immunoreactivity in rat lens epithelium. Immunostaining of normal eyes revealed Hsp70 was negative in lens epithelial cells (A) and the fiber cells of the lens (B) and is highlighted by arrows. Hsp70 reactivity in contusion eyes indicated it was localized in the nucleus or cytoplasm around the nucleus, and exhibited increased immunostaining in the lens epithelial cells (C) and the fiber cells of the lens (D) and is highlighted by arrows. Hsp70 is expressed strongly in lens epithelial cells in the heat shock eye (E), and faintly in lens epithelial cells in the quercetin eye (F). Magnification was 400x.
nonmetabolizable analog of glutamine [27]. Since it is commonly accepted that quercetin inhibits the syntheses of Hsps at the transcriptional level, we propose that the induction of Hsp70 possibly occurs at the cell transcriptional level.

In summary, our study clearly demonstrated that there was basic expression of Hsp70 and Hsp27 in LECs of rat in vivo. Contusion of eyes induced increasing expression of Hsp70 in LECs in a time-dependent manner, and preconditioning hyperthermia and quercetin administration can modulate this inducible effect. Together, basal expression and stress-induced induction of heat shock proteins could play an important role in cellular protection against physiologic or environmental stress. Further studies are required to determine the mechanism of how inducible Hsp70, even if only located in superficial cortical lens regions, helps maintain lens transparency during eyes contusion or thermal stress.

REFERENCES

4. Bagchi M, Katar M, Maisel H. Effect of exogenous stress on the basic expression of Hsp70 and Hsp27 in LECs of rat in vivo. Contusion of eyes induced increasing expression of Hsp70 in LECs in a time-dependent manner, and preconditioning hyperthermia and quercetin administration can modulate this inducible effect. Together, basal expression and stress-induced induction of heat shock proteins could play an important role in cellular protection against physiologic or environmental stress. Further studies are required to determine the mechanism of how inducible Hsp70, even if only located in superficial cortical lens regions, helps maintain lens transparency during eyes contusion or thermal stress.

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