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Hsp70 in bovine lenses during temperature stress

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Purpose: To determine the effects of heat shock treatment on cold cataract formation in bovine lenses.
Methods: A laser scanning system (ScanTox™) was used to analyze the optical quality of bovine lenses during a cooling and warming cycle. Cycloheximide, a compound that prevents new protein synthesis was used to inhibit inducible heat shock protein 70 (Hsp70) production during heat stress. Cycloheximide was also used to verify that the induction of Hsp70 takes place in lenses during heat stress. Western blots determined the relative accumulation of Hsp70 in urea soluble lens fractions. Lenses from animals approximately 2 years of age (n=60) were used in this experiment.
Results: The decrease in relative light transmittance during cold cataract varies for each group of lenses, with the greatest decrease appearing in the heat shock group in culture medium and the least in the control groups and the heat shock group with cycloheximide. The primary result is that heat shock lenses were most affected by the cold cataract (57% decrease in intensity of refracted beam), and that this effect is prevented by cycloheximide. Western blot results show an increase of Hsp70 with heat shock in the urea soluble lens fractions.
Conclusions: The results show that heat shock treatment increased both light scattering and the presence of Hsp70. Also cycloheximde prevented both the heat shock effect and the expression of Hsp70 in bovine lenses.

The cold cataract phenomenon, as demonstrated in mammalian lenses, involves the completely reversible opacification of the lens nucleus [1,2]. The cataract is caused by lowering lens temperature to well below body temperature. For example, cold cataract formation occurs when the temperature falls below 17 °C for bovine lenses [3]. It is reversed by a return to body temperatures. The fact that it is completely reversible and that the focal length profile of the lens (spherical aberration) is identical before and after, indicates that cold cataract is a result of supramolecular change [4]. It has been shown that the precipitation of γ-crystallin in the lens is correlated with the formation of cold cataract [5-9]. The precipitation of γ-crystallin at low temperatures is simply due to a sharp decrease in solubility as it approaches its isoelectric point [5]. It is also suggested that cold cataract formation is due to phase-separation associated with the γ-crystallins, a protein-rich phase and a protein-poor phase coexist at and below the critical temperature (T_c) and these cause light scattering [1,6]. The opacity developed is due to localized changes in refractive index within the lens [2,10].

While the mechanism for formation and recovery of cold cataract is still not well understood, the chaperone actions of certain proteins within the lens may be involved. Molecular chaperones maintain the native structure of all proteins. Chaperones aid in the refolding of denatured proteins and prevent protein aggregation [11]. Hsp70 is a major family of heat shock proteins which serve as molecular chaperones to assist cell recovery after stress by preventing the misfolding and aggregation of many kinds of cellular proteins [12,13]. Heat shock of *Xenopus laevis* embryos results in increased accumulation of Hsp70 mRNA in the lens placode [13]. Embryonic and adult chicken lenses also demonstrate the expression of inducible Hsp70 [11,14]. Recent studies show that Hsp70 plays a protective role in the visual system, as in retinal cell protection in rats under elevated intraocular pressure and in the increased expression of Hsp70 in bovine lenses after UV-A irradiation [12,15]. Further investigations of the role of Hsp70 in the lens could clarify the mechanism by which the lens deals with thermal stress.

This research examines the optical effects of cold cataract formation of the intact bovine lens using an in vitro whole lens culture approach in conjunction with a specially designed laser scanning instrument. This research also examines whether heat shock treatment of lenses prior to cold cataract induction enhances the ability of the lens to cope with thermal stress. The possible role of heat shock proteins was investigated by determining whether variations in the concentration of inducible Hsp70 in the lens can be related to thermal stress and cold cataract formation.

METHODS

Bovine lens culture: Fresh adult bovine eyes approximately 2 years of age were purchased from a local abattoir (Better Beef Ltd., Guelph, Ontario). Eyes were dissected under sterile conditions and lenses were carefully excised on the same day, as described in previous work [16]. The lenses were then placed in a two-chambered cell made of borosilicate glass and silicon rubber with an aluminum base and immersed in culture medium. The culture medium consisted of M199 (Sigma, M3769) with Earl's salts, and the addition of L-glutamine (0.1 g/l), sodium bicarbonate (2.2 g/l), Hepes (5.92 g/l), penicillin/streptomycin (100 units/ml) and 3% dialyzed fetal bovine serum. The lenses were incubated at 37 °C in an atmosphere of

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4.0% CO_2 in air. Lenses were incubated for 24 or 48 h before any treatment to ensure that they were not damaged during the dissection.

Laser scanning instrument: A laser scanning system (ScanTox™) was used to analyze the optical quality of the lenses. The laser scanner consists of a collimated laser source (on an X-Y table) that projects a beam onto a mirror mounted at 45 °C on a carriage assembly. The reflected beam then goes up through the scanner table surface and through the lens under examination. There are two digital cameras that capture images of the light beam after it is transmitted through the lens. The image information collected by the digital cameras is transferred to a computer, and the refracted direction for each of the beam positions is recorded with respect to the optical center, the point at which the slope of the laser beam approaches zero. Each scan involved 22 laser positions across an 11 mm diameter with a step size of 0.5 mm. Thus there were eleven measurements on each side of the optical center of each bovine lens tested. The results are given in relative scatter (intensity) of light for each scanning beam position across the lens from the level of pixel excitation for each refracted beam.

Induction of cold cataract: To induce the formation of cold cataract and the recovery process, a modified borosilicate glass two-chambered cell with two glass extensions, an inlet and an outlet was used. A closed circuit of circulating culture medium was established by attaching four meters of clear flexible tubing (1/4 inch ID x 5/16 inch OD) on each glass extension and connecting the inlet and outlet tubing to the corresponding ports on a small peristaltic variable-flow pump. The tubing was coiled and immersed inside a digitally controlled water bath. This allowed the culture medium to be circulated at temperatures controlled by the water bath. A digital thermometer (accuracy ±1 °C) was placed inside the glass chamber to monitor the temperature of the fluid surrounding the lens. The thermometer was removed each time an optical scan was made and then replaced.

The temperature cycle inside the chamber started at 37 °C, slowly cooled to 4 °C and then warmed back up to 37 °C. One cooling and warming cycle took approximately 2 h. Figure 1 shows the appearance of a cold cataract in an adult (2 years) bovine lens.

Treatment groups: The lenses (total n=60) were scanned initially at 37 °C and then divided into a control group and heat shock group. The control lenses were transferred into the modified glass chamber and subjected to one cooling and warming cycle. Scans were made periodically at different temperatures. The lenses in the heat shock group were subjected to 1 h of heat stress at 42 °C, a level sufficient to induce the heat shock response [17-19]. The lenses were then removed and stabilized at 37 °C for 4 h, after which they were transferred to the circulating chamber and examined under the same conditions as the control lenses described previously. Two control lenses and two treated (heat shock) lenses were tested per day.

A repeated measures experiment was performed on a group of bovine lenses (n=6). Each lens was subjected to three consecutive cooling and warming cycles (control, heat shock treatment, and recovery). Transmittance decreased during cooling and recovered when warmed. This experiment showed that there is no temporal effect (repeated-measures analysis of variance; $p>0.05$) on mean transmittance measurements for the same lens in that repeated cooling and warming cycles do not affect the results.

Cycloheximide experiment: Cycloheximide ($C_{15}H_{23}NO_4$, Sigma, St. Louis, MO), a compound that prevents new protein synthesis [20], was used to inhibit Hsp70 production during heat stress [21]. Cycloheximide was also used to verify that the induction of Hsp70 takes place in lenses during heat stress. This part of the experiment included five groups of

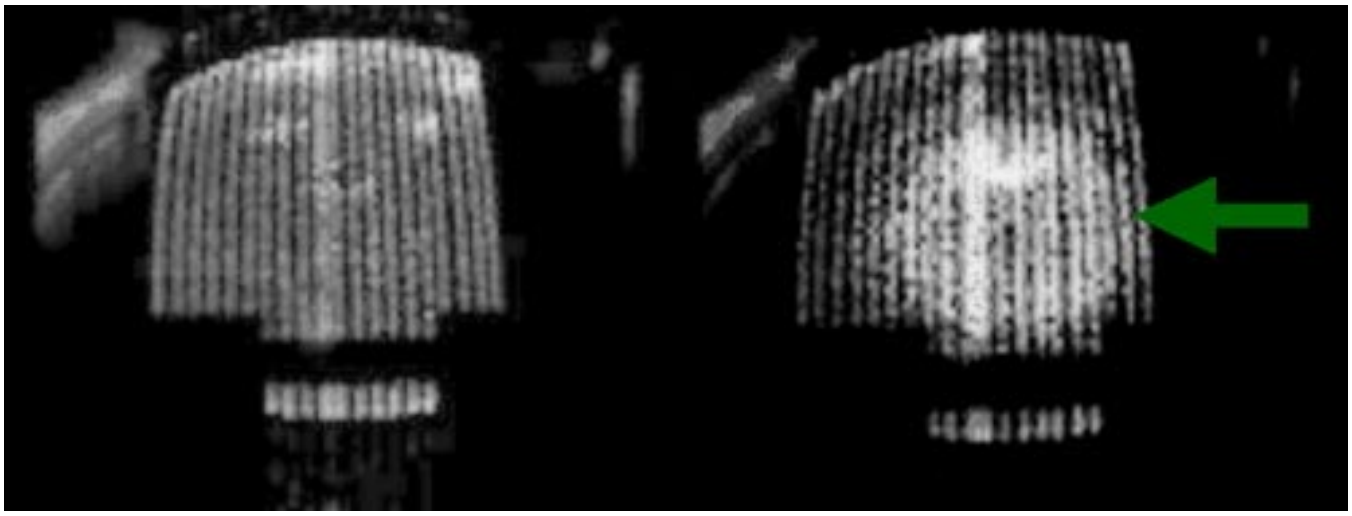

Figure 1. Cold cataract in a bovine lens. The above images are optical scans of a bovine lens (2 years of age) in a glass chamber. The left image represents a lens before and after cold cataract. The right image shows a lens during cold cataract, the arrow points to the nuclear opacity.

lenses: 1. Control (n=14); 2. Control with 0.0001% ethanol (n=10); 3. Control with cycloheximide (cycloheximide was dissolved in 0.0001% ethanol and the final concentration in culture medium was 1 μM, n=10); 4. Heat shock with culture medium (n=14); and 5. Heat shock with 1 μM cycloheximide (n=12). A control group with ethanol was used to ensure that the optical changes in the cycloheximide groups (control and heat shock) were due to the effects of cycloheximide and not due to the effects of ethanol (used to prepare the cycloheximide solution). The cycloheximide solution was added 1 h prior to any treatment. The control group with ethanol was exposed to the ethanol for 1 h prior to any treatment. The other control lenses remained in fresh culture medium before experimentation. The heat shock group with cycloheximide was then incubated at 42 °C for 1 h and then stabilized at 37 °C for 4 h. After the 4 h of stabilization, the lens was transferred into the circulating chamber (culture medium without cycloheximide) and optically scanned for one cooling and warming cycle. The three control groups were subjected to the same procedure as the control lenses of the previous section.

Detection of Hsp70: After optical analysis, lenses were decapsulated and the cortex of 2 year old lenses (where n=10 for the heat shock group and n=4 for each of the control, control ethanol, control cycloheximide, and heat shock cycloheximide groups) were collected and used to detect inducible Hsp70. The cortex was homogenized in 100 mM Tris buffer (pH 7.5) and centrifuged at 4 °C (12,000x g, for 30 min). The supernatant (water-soluble lens fraction) was removed and the pellet was resuspended in 1 ml of 5 M urea solution and centrifuged [15]. The urea-soluble fraction was diluted in Lammeli's sample buffer (1:1) with 5% β-mercaptoethanol. The mixtures were then heated for 8 min, cooled on ice and then stored at -20 °C until ready to be used for western blots. The Bicinchoninic Acid (BCA) Assay [22], with bovine serum albumin (BSA) as the standard, was used to determine the protein concentration in the tissue samples.

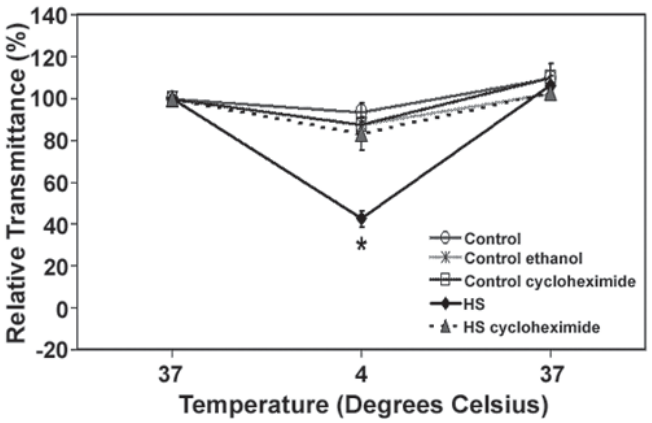

Figure 2. Normalized relative light transmittance of heat shock and cycloheximide experiment (2 year old bovine lenses). Relative transmittance (%) on the Y axis versus the temperature points (°C) on the X axis (pre-cold cataract, cold cataract, and post-cold cataract). The values and bars represent normalized mean and standard error of the mean, respectively. There was both a significant temperature and treatment effect (repeated-measures analysis; $p<0.05$). The heat shock group (HS, with culture, marked with a star) was significantly different (unpaired student-t test; $p<0.05$) from the control groups (control with culture medium, control ethanol, and control cycloheximide). HS with cycloheximide was not significantly different from the control groups (control with culture medium, control ethanol, and control cycloheximide).

Western blots were performed under the same conditions as mentioned above, with 8% SDS-PAGE gels (35 μg of total protein loaded for each lane) and rabbit anti-human Hsp70 polyclonal (1:1000, Stressgen, Victoria, BC) as the primary antibody. Alkaline phosphatase detecting reagents (NBT 0.033% w/v and BCIP 0.017% w/v) were used to develop visible color bands. A semi-quantitative measurement of the relative intensity of each protein band was evaluated using the AlphaEase™ software (Alpha Innotech, San Leandro, CA) [23].

Statistical analysis: The repeated measures analysis of variance and the unpaired Student's t-test (SPSS™ 11.0 statistical software) were used to assess treatment and temperature effects on relative light transmittance. A p value <0.05 was considered to be significant.

Western blotting data (Hsp70 accumulations) were statistically analyzed using the unpaired Student's t-test, where a p value <0.05 was significant.

RESULTS

Relative light transmittance: Three critical temperature points were used to demonstrate the effect of the cold cataract; pre-cold cataract (37 °C), cold cataract (4 °C), and recovery or post-cold cataract (37 °C). Each lenses from each group of shows a significant decrease in transmittance at 4 °C, which recovers when the lenses are warmed to 37 °C.

Lenses in each treatment group developed cold cataracts. However, while the reduction of light transmittance varied from 7% to 17% (all control groups and heat shock group with cycloheximide) the differences were not significant (Figure 2). The primary result is that heat shock lenses were most affected by the cold cataract (57% decrease in intensity of refracted beam), and that this effect was prevented by cycloheximide.

Hsp70 detection: The lenses from the cycloheximide experiment described above were used for the detection of Hsp70. Figure 3 represents the western blot results. The top panel shows a representative western blot, while the lower panel with a histogram shows the density of the bands from the top panel expressed as a percent of the control. Lanes 1 to 4 in the top panel represents control, control cycloheximide, control ethanol, and heat shock cycloheximide, respectively (n=4 for each group). No bands were detected for lanes 1 to 4 while there is an obvious band for Hsp70 in lane 5 (heat shock with culture medium, n=10). The bar graph in Figure 3 shows that there were no significant differences ($p>0.05$) between control (100%), control cycloheximide (87%), control ethanol (146%), and heat shock cycloheximide (173%) lenses. Heat

shock treatment with culture medium shows the largest amount of Hsp70 (1,060%) and this amount is significantly different from all other treatment groups. This shows that treatment with cycloheximide prevented the expression of inducible Hsp70.

DISCUSSION

The ocular lens grows throughout life by continuous layering of new fiber cells on top of older lens fibers [24]. Thus, a minimal turnover rate of proteins occurs in the nuclear regions of the lens [24-26]. As the age of the lens increases, there is a decrease in the amount of γ-crystallin. The exception is γS-crystallin, which increases with age [10,25,27]. Hence, the cold cataract phenomenon is most apparent in young lenses (Figure 1). However, cold cataract formation in older lens extractions can still be induced at much lower temperatures [1,7]. This is also supported by a report that shows that temperature sensitivity to phase transition of the intact rat lenses decreases with age, correlating with a lower critical temperature (T_c) for cold cataract in older lenses [6].

The enhanced cold cataract reaction after heat shock could be explained by changes that may occur in the phase transition curve of γ-crystallin. It has been shown that the addition of polyethlylene glycol to γD-crystallin protein fractions shifts the transitional curve of γD-cyrstallin and increases the critical temperature [28]. Also, liquid-liquid phase experiments have demonstrated that separate γE-, γF-, γB-, γD-, and γS-crystallin fractions have different critical temperatures, with

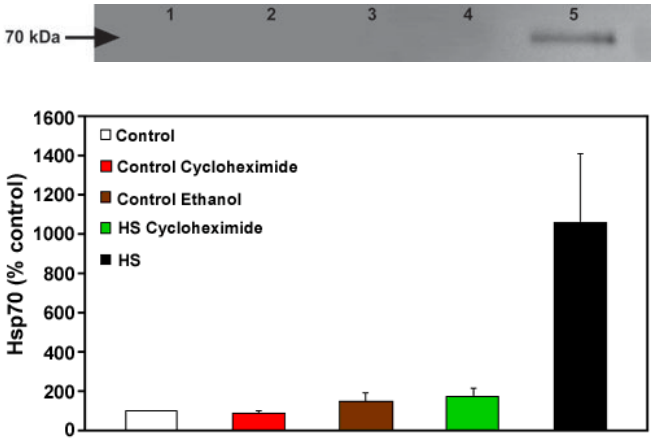

Figure 3. Hsp70 in 2 year old bovine lenses. The top panel shows a representative western blot using rabbit anti-Hsp70 polyclonal antibody. Heat shock cycloheximide (lane 4) and the control groups (culture medium, lane 1; cycloheximide, lane 2; and ethanol, lane 3) demonstrate no Hsp70 band. There was an obvious Hsp70 band for the heat shock group (lane 5). The bar graph (bottom panel) represents the density of the bands expressed as percent of control with culture medium. Values and bars represent the mean and standard error of the mean, respectively; n=10 for heat shock group, and n=4 for all other treatment groups. The heat shock (HS) group has significantly greater amounts of Hsp70 then all other treatment groups. Heat shock cycloheximide, control, control cyloheximide, and control ethanol were not significantly different (unpaired Student's t-test; p>0.05).

γS-crystallin having the lowest [29]. The addition of γS-crystallin, to other γ-crystallin fractions significantly lowers the T_c and suppresses the aggregation of γ-crystallin solutions [10].

Conformational changes of γ-crystallin during cold cataract has not been detected by using circular dichroic (CD) spectra and other spectroscopic methods [5,30]. The conformational stability of γ-crystallin provides a protective mechanism against various assaults on the lens nucleus [31]. The precipitate of γIV-crystallin (γA and γE) is thought to be the primary γ-crystallin protein related to cold cataract formation. Therefore, increased binding of γA- and γE- to α-crystallin with age decreases the amount of free γA- and γE-crystallins available to produce cold cataract [8,32]. Experiments have shown that acrylamide treatment of the lens protein fractions prevents cold cataract formation due to the cross-linking of acrylamide and γ-crystallins [7].

This research investigated the effects of heat shock treatment on the ability of the lens to cope with cold stress. Bovine lenses that were heat shocked in culture medium showed the most prominent light scatter, while treatment with cycloheximide lowered the heat shock effect (Figure 2). As mentioned in the methods, cycloheximide prevents synthesis of new proteins, including inducible Hsp70, as observed in this study. Western blot analysis of Hsp70 (Figure 3) confirmed that Hsp70 was only present in heat shock lenses in culture medium. Hence, heat shock treatment with cycloheximide did prevent the expression of Hsp70. The Hsp70 levels are higher in the water-insoluble fraction compared to the water-soluble fraction [15,33]. However, the location of Hsp70 within the lens is uncertain. As noted earlier, Hsp70 is found in bovine lens protein fractions, although its exact location was not specified and lens portions were not segregated [15]. Hsp70 is found only in the epithelium of human lenses. However, the results obtained from the same study also shown that Hsp70 was detected in all regions of a 24 h post-mortem human lens [33]. Hsp70 is present in all regions of the chicken lens and there were no significant changes in relative Hsp70 levels between embryonic and adult chicken lenses. Nor are chickens prone to age-related cataract [11]. Other experimental results have shown an absence of Hsp70 in the lens epithelium of a 76 year old noncataractous human lens, and thus human lenses show an age-related decline of Hsp70 [33]. Inducible Hsp70 is expressed under stress through out various tissues in the body [34]. However the ocular lens expresses Hsp70 in normal unstressed conditions, which suggests that the normal microenvironment of the lens is stressful and Hsp70 is continuously needed [33,34].

It is possible that increased Hsp70 binding to lens proteins causes the changes in phase transition and increases the critical temperature. Also, the larger aggregations formed increase the scattering effect at lower temperatures. We are not aware of data involving the phase separation analysis of the interaction between γ-crystallin and Hsp70. Future experiments investigating the interaction between Hsp70 and separate lens crystallins may reveal the changes occurring to the phase separation curves for specific lens regions. However, at this point one cannot assume that Hsp70 reacts with only α- or γ-crys-

tallin. The light scattering effect of the cold cataract is investigated with the whole lens and therefore protein aggregation and binding in either the cortical or nuclear layers could increase the overall scatter.

Cychloheximide prohibits Hsp70 induction therefore inhibiting the potential binding of Hsp70 to lens crystallins. Thus, the light scattering effects of treated lenses do not differ from the controls. Although the purpose of this experiment was to investigate the role of Hsp70 in the lens, cycloheximide could also have an effect on other lens proteins.

We conclude that heat shock treatment of lenses prior to cold cataract induction affected both light scattering properties and supramolecular organization of ocular lenses. While there is no evidence that links inducible Hsp70 to cold cataract formation, we suggest that the presence of Hsp70, even if only located in superficial cortical lens regions, helps maintain lens transparency during thermal stress.

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