Heat shock protein concentration and clarity of porcine lenses incubated at elevated temperatures

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Purpose: To quantify the concentration of heat shock proteins in lenses in lens organ culture at elevated temperatures, and to examine the relation between elevated temperature and lens clarity.

Methods: Pig lenses obtained from a local abattoir were dissected aseptically and incubated in medium M199 without serum for 4 days to stabilize, and lenses with protein leakage of less than 10 mg/l were obtained for heat shock exposure. Heat shock was performed by incubation for 1 h in M199 without serum at various temperatures ranging from 37 °C to 55 °C. After incubation for 24 h, cataract blurring of the images was assessed using Scantox™ and Scion Image analysis of the lens photographs. Lens homogenates were subsequently analyzed for Hsp70 and Hsp27 with western blotting.

Results: The degree of cataract blurring of the images increased with increasing temperature, but the two functional measures provided different results. Focal length inconsistency, as assessed with the back vertex distance standard error of the mean (BVD SEM); the variability in focal lengths measured at 20 equally spaced locations across the lens, Scantox™, increased nearly linearly with the heat treatment temperature. In contrast, decreased clarity, evident by a fuzzy image with lower contrast, was not markedly altered as the temperature rose until a threshold of approximately 47.5 °C. The inducible isoform of the Hsp70 family (Hsp70) of heat shock proteins was increased at all temperatures above the control except those above 50 °C. Changes in Hsp27 were less clear as the protein content increased only at the incubation temperatures of 39 °C and 48.5 °C.

Conclusions: The porcine lens demonstrates subtle changes in the variability of the focal length, and the variability increases as the incubation temperature rises. In contrast, lens clarity is relatively stable at temperatures up to 47.5 °C, above which dramatic changes, indicative of the formation of cataracts, occur. The lens content of Hsp70 was elevated in lenses exposed to heat shock only up to 50 °C. These data suggest that in a stressful environment, Hsp70 may be associated with protection against loss of clarity. In addition, the functional measures BVD SEM and clarity assess different qualities of the lens, with the former likely more sensitive to subtle changes in the protein structure.

The formation of cataracts results in partial or complete cloudiness of the crystalline lens of the eye, interfering with vision. Cataracts in humans and cataracts in various animal models have been studied for many decades and are formed in response to various agents and environmental stresses. The pioneering work of Sasaki suggested that either increased exposure to ultraviolet (UV) light, or elevated environmental temperature, or both might be causative factors in the formation of cataracts [1-3]. For example, infrared irradiation experienced by glassblowers [4-7], ironworkers [8,9], and bakery workers [10] has been implicated as a potential factor in the development of cataracts [11]. More recent studies by Truscott’s group [12] have suggested a role for heat induction of presbyopia associated with the increased stiffness of porcine lenses exposed to elevated temperatures. Work by Truscott’s group associated presbyopia with incorporation of the small heat shock protein, α-crystallin, into large molecular weight aggregates in the lens nucleus.

The heat shock response is a conserved response that is protective against various environmental insults, including elevated temperature, mitochondrial dysfunction, oxidative stress, and protein denaturation [13,14]. De Jong et al. [15] previously investigated the heat shock response of the cultured rat lens and found that synthesis of the inducible isof orm of heat shock protein 70 (Hsp70) started between 30 and 60 min after the heat shock, peaked after 3 h, and stopped after 8 h. Bagchi et al. [16] found Hsps in the epithelium, cortex, and nucleus of adult and embryonic chicken lenses, suggesting that Hsp40, Hsp70, and Hsc70 can interact with proteins in the deep cortex and the nucleus and protect them from heat-induced denaturation. Although Hsp27 is also found in high quantity in lenses [17,18] and is involved in maintaining α-crystallin solubility [19], the mRNA of Hsp27 was not altered in response to contusion of the eye or whole body heating to 40.5–41.5 °C for 8 min in rats [18]. The possible role of other types of stress in cataract development was...
investigated by Sivak and West-Mays [20-22] using an explant model to suggest that Hsp70 may be involved in protecting against the formation of subcapsular cataracts.

The porcine lens is approximately the same size as the human lens, with porcine lens crystallins sharing antigenic similarity with human crystallins [23]. The pig is also similar to the human in diurnal activity and omnivorous diet. Therefore, it was reasoned that the porcine lens may represent a good model for evaluating the effect of stress on the formation of cataracts. To test this, we subjected cultured porcine lenses to increasing levels of heat stress and evaluated the functional and Hsp70 and Hsp27 response 24 h later. We hypothesized that the heat shock would be accompanied by increased expression of Hsp70 and Hsp27 and that this would protect the lens from functional deficit (as evidenced by the back vertex distance standard error of the mean [BVD SEM] and clarity).

METHODS

This study was approved by the University of Western Ontario Council on Animal Care and was performed in accordance with the guidelines of the Canadian Council on Animal Care. Pig lenses (from pigs 36–42 weeks of age) from a local abattoir were dissected aseptically and incubated in medium M199 without serum for 4 days to stabilize. Those with protein leakage of less than 10 mg/l were taken for heat shock treatment as leakage at greater rates has been found to be indicative of lens damage [24]. Heat shock was performed by incubation for 1 h in M199 without serum at various temperatures ranging from 37 °C to 55 °C. M199 (pH 7.4) was made by adding 9.55 g Earle’s salts (Sigma Chemical, Mississauga, Canada) to 800 ml distilled water with 2.2 g sodium bicarbonate and 10 g L-glutamate. After the addition of 10 ml antimycotic antibiotic (10 mg streptomycin, 10,000 U/ml penicillin, and 25 µg amphotericin in 20 ml sterilized water), it was sterilized by filtration through a 0.22 µm filter. After the heat shock, the lenses were assessed for the formation of cataracts and then prepared for subsequent analysis. Frozen samples of approximately 60 mg were homogenized on ice (2–15 s bursts) in 19 volumes of 600 mm NaCl and 15 mm Tris- HCl, pH 7.5, using a Tekmar Ultra Turrax homogenizer (Tekmar, Cincinnati, OH). Homogenates and media were then heated for 3 min at 100 °C in boiling water. Samples were cooled to room temperature and spun at 20,000 × g for 20 s. Equal amounts of protein (60 µg/well) from each sample were loaded into the wells and separated according to their molecular weights by running the gel for 2 h at a constant 110 V in running buffer (25 mM Tris base, 0.5% SDS, 13% 2-mercaptoethanol, and bromophenol blue) and heated for 3 min at 100 °C in boiling water. Samples were run concurrently on each gel for accurate determination of Hsp70 and Hsp27. Proteins were transferred from the gels to nitrocellulose membranes (0.2 µm pore size; Bio-Rad Laboratories, Mississauga, Canada) according to the method described by Towbin et al. [30] using the Bio-Rad mini-protein II gel-transfer system. After transfer, nitrocellulose membranes were treated in a manner similar to that previously described [29,31], and western blots were probed for Hsp70 and Hsp27 (StressGenSPA-812 polyclonal; and SPA-803 polyclonal, respectively; Stressgen, Victoria, Canada). An alkaline phosphatase-conjugated secondary antibody (goat anti-rabbit immunoglobulin G alkaline phosphatase conjugate, Bio-Rad #170–65AP) was used to detect the antibody.

Gel electrophoresis and western blotting: Protein concentrations were determined using Lowry et al.’s technique [28] with bovine serum albumin (BSA) as the standard. Polyacrylamide gel electrophoresis (PAGE) was performed as described previously [29]. Briefly, lens homogenates (diluted 19:1) were mixed with an equal volume of sample buffer (0.5 M Tris base, 13% glycerol, 0.05% sodium dodecyl sulfate (SDS), 13% 2-mercaptoethanol, and bromophenol blue) and heated for 3 min at 100 °C in boiling water. Samples were run concurrently on each gel for accurate determination of Hsp70 and Hsp27. Proteins were transferred from the gels to nitrocellulose membranes (0.2 µm pore size; Bio-Rad Laboratories, Mississauga, Canada) according to the method described by Towbin et al. [30] using the Bio-Rad mini-protein II gel-transfer system. After transfer, nitrocellulose membranes were treated in a manner similar to that previously described [29,31], and western blots were probed for Hsp70 and Hsp27 (StressGenSPA-812 polyclonal; and SPA-803 polyclonal, respectively; Stressgen, Victoria, Canada). An alkaline phosphatase-conjugated secondary antibody (goat anti-rabbit immunoglobulin G alkaline phosphatase conjugate, Bio-Rad #170–65AP) was used to detect the antibody.

Functional measures: Scantox™ (purchased from J. Sivak, School of Optometry, University of Waterloo, Canada) and Scion Image analysis (Scion Corporation, Frederick, MD) of the photographs of the lenses were used to estimate the blurring of the images of the cataracts [26,27]. The Scantox™ uses a laser scanning system to send a beam of light through the lens (20 measures from edge to edge) and calculates the standard error of the mean of all back vertex distance measurements. The greater the variability in the back vertex distance, the greater the degradation of the image quality (see [26] for a full description of the method). Additionally, photographs of the lenses in tissue culture medium in culture dishes sitting on a sheet of graph paper (black and white and calibrated in 1 mm increments) were taken (Figure 1). Using the Scion image program, the average gray value, divided by the number of pixels of the grid within the total area of the lens, was divided by the average of the same measure of two to three adjacent areas (the same diameter) outside the lens. Increased cataract resulting in increased fuzziness of the image caused decreased clarity (the image contrast ratio) as the cataract progressed. To correct for initial differences in the lenses, the Scion image grade was normalized to the initial ratio found at the beginning of incubation for each lens.
reacted in a carbonate buffer (100 mm Na₂CO₃, 1 mm MgCl₂, pH 9.8) containing 3% (w/v) p-nitro blue tetrazolium chloride p-toluidine salt in 70% N,N-diethylformamide and 15% (w/v) 5-bromo-4-chloro-3-indolyl phosphate in 100% N,N-diethylformamide to visualize the protein band. After the blots were dried and scanned, Scion Image blot analysis software (NIH, Bethesda, MD) [27] was used for densitometric quantification. Data were corrected relative to the pooled soleus standard. To reduce any differences due to gel to gel variability, the gels were loaded with samples that had been incubated at different temperatures, and the densitometric readings of individual bands were normalized to the standard on each gel. Group means of the normalized bands were used for statistical analysis.

Statistical analysis was performed using IBM SPSS for Windows version 21.0 (Armonk, NY). To examine statistical differences in Hsp expression between the heat incubations of the lenses, a one-way ANOVA was performed with Tukey’s post hoc testing. Data are presented as the mean and standard error of the mean (SEM) of each group. Best fit curves for relations between the variables were accomplished using the curve fitting module in SPSS and segmental linear regression in GraphPad Prism (San Diego, CA). A p value of less than 0.05 was considered statistically significant, and the best fit model and r² were reported.

RESULTS

Twenty-four hours after 1 h incubation at elevated temperature, the Scantox™ measure of sharpness of focus (the BVD SEM) increased as a linear function of the incubation temperature (r² = 0.869, p = 0.001; Figure 2). These changes coincided with a loss of clarity (reduced image contrast ratio) as the incubation temperature was raised (Figure 1 and Figure 3). The relation between the change in clarity and temperature was not linear but was fitted using a segmental linear model (r² = 0.841) with an apparent threshold above 47.5 °C (Figure
3) where clarity suddenly declined. Hsp70 expression was increased relative to the control temperature of 37 °C at all incubation temperatures except 55 °C and peaked at 48.5 °C (Figure 4). In contrast, Hsp27 content was largely unaltered with the exception of increases at 39 °C and 48.5 °C (Figure 5). The two independent optical measurements of the cataract focusing ability of cataractous lenses, Scantox™ BVD SEM and clarity (the contrast ratio) by Scion imaging also exhibited a nonlinear relation (r^2 = 0.768, Figure 6) suggesting that their temperature dependence is not the same and that BVD SEM, a measure of sharpness of focus, is more resistant to temperature increases at higher temperatures than is clarity.

**DISCUSSION AND CONCLUSIONS**

Humans often live and work in environments that may predispose them to the formation of cataracts [2,9,10,32]. In the present study, the porcine lens was selected as a model for lenses exposed to stress, specifically heat stress. The results suggest that the lens is resistant to heat stress as far as functional changes are observed up to temperatures near 50 °C. Our data suggest that the heat shock response as exemplified by changes in Hsp70 may partially explain the observed protection. Nonetheless, protection was most evident regarding lens clarity whereas sharpness of focus as
assessed with BVD SEM showed a more gradual deterioration as the temperature or stress increased.

Damage to lenses resulting in cataract appears to be multifactorial in its etiology [33]. Heat is a damaging agent that can potentially lead to several changes, including protein denaturation, free radical formation, mitochondrial dysfunction, and disruption of signaling pathways [13]. These insults are typical of those that lead to protein cross-linking, clumping, and ultimately decreased lens clarity [34]. The α-crystallins, which are major components in the lens, are chaperones that help keep protein aggregates soluble and maintain lens clarity [35]. They are part of a class of
heat-shock proteins that respond to stress with increased synthesis in other tissues. One purpose of this response is to hold and refold denaturing proteins to retain function or to ensure orderly disposal of proteins that are beyond repair [35]. Generally, α-crystallins alter their structure as the temperature increases to “hold” denatured proteins that are folded later by other chaperones, most notably Hsp70, in an ATP-dependent fashion [36]. Hsp27 also interacts directly with α-crystallins, thus enhancing their stability [19]. Additionally, Hsp27 and Hsp70 may inhibit apoptosis directly via inhibiting release of apoptotic agents from the mitochondria and inhibition of caspase activity and indirectly by influencing the cell death signaling pathways [37]. In this fashion, Hsps may limit protein damage, aggregation, and the eventual formation of cataracts in lenses exposed to elevated temperatures.

In the present study, the lenses demonstrated a loss in functional capacity 24 h after the heat shock as a function of increasing temperature. In the case of the BVD SEM (Figure 2), which is a marker of the lens’s ability to focus light at a specific focal point, the increasing variability (SEM) suggests increasing damage to the lens as the temperature rises. Although this effect is slightly greater per the temperature gradation at higher temperatures, the increasing SEM is nearly linear with the increase in the incubation temperature. This could be due to several causes, including changes in the protein concentration in the lens due to leakage or due to conformational changes in the proteins that are in the lens. The most abundant crystallin proteins are known to change their conformation as the temperature rises in order for them to more readily interact with their client proteins, prevent frank clumping, and potentially retain clarity in the lens [38]. This could presumably impact the ability of the lens to maintain a sharp focal point. In contrast, the measure of clarity (Figure 3) showed a curvilinear response with a marked decrease in clarity (increased fuzziness) at temperatures above 47.5 °C. In fact, the clarity was relatively unchanged at incubation temperatures ranging from 37 °C to 47.5 °C, suggesting that major changes in the fuzziness of the lens are protected over a large range of temperatures until a threshold is reached at which significant formation of cataracts occurs.

The lens content of the inducible isoform of the 70 kDa family of heat shock proteins, Hsp70, increased in response to heat shock at incubation temperatures as low as 37 °C, and elevated levels were maintained at all incubation temperatures up to 50 °C, above which the lens content of Hsp70 declined. The temperature range in which increased Hsp70 content was observed corresponds closely with the range over which obvious formation of cataracts was prevented (limited change in clarity) despite apparent functional changes in the lens as indicated by progressive increases in the BVD SEM. Although it is unclear whether increases in Hsp70 were important in buffering the lens against changes in clarity, there are several roles played by this protein that could account for such protection. Although the α-crystallins are critical in maintaining the protein of the lens in a soluble state, Hsp70 in conjunction with cochaperones is critical in helping refold such proteins to their original conformation [35]. This is an ATP-dependent process, and importantly, Hsp70 has also been implicated in protecting the mitochondria during heat shock and maintaining their function under stress [14,39]. A previous study showed that mitochondrial poisoning results in increased calcium staining, suggesting that mitochondria are a major calcium storage site in the lens [40]. Srivastava’s group has shown that elevation of calcium causes isolated fiber cells to form globules [41,42], similar to those we previously demonstrated with scanning microscopy.
in human and model cataracts [43]. In the diabetic ex vivo model, initiation of apoptosis, activation of endogenously present calpain, proteolysis of fodrin, blebbing of cell cytoplasm, and loss of clarity may all be initiated by increased calcium release as a result of mitochondrial damage [44].

The elevation of lens Hsp70 at temperatures up to 48.5 °C, followed by a progressive decrease after exposure to higher temperature treatments, is also consistent with cellular changes being involved in the clarity observed. Nevertheless, the heat-induced aggregation of α-crystallin, which shows a critical temperature of 45 °C [45], could also be involved in increased light scattering that would be responsible for the cloudiness of the images of the lenses used for Scion imaging to determine the contrast of the image. Heat-induced aggregation of α-crystallin has been associated with the loss of lens flexibility leading to the onset of presbyopia in a model using a heat-exposed pig lens [12].

Although a full data set was not obtained, the release of Hsp70 into the media surrounding the lens was measured at some temperatures, and in vivo, this could have a protective function for other ocular tissues. External Hsp70 has been shown to protect neural tissue cells by interaction with toll-like receptors (TLRs) and stimulation of anti-inflammatory action [46], as well as stimulation of cytokine production in human oxidized low density lipoprotein (LDL)–treated macrophages [47]. Thus, there could be some protection of tissues in contact with the aqueous humor, including the trabecular meshwork, and via Schlemm’s canal, the superficial vasculature [48] if the release of Hsp70 were to occur in vivo.

It is more difficult to ascribe a role for Hsp27 in the functional changes observed in the heat-shocked lenses. It is unclear why changes in protein content occurred only at 39 °C and 48.5 °C (Figure 5). However, previous research has indicated that when whole animals are heated to 40.5–41.5 °C for 8 min, there is no change in the levels of the Hsp27 mRNA transcripts [18]. Further, it is known that Hsp27 is highly phosphorylated in the lens [49], and changes in Hsp27 phosphorylation status may influence whether Hsp27 acts more in a chaperone capacity or in the prevention of apoptosis. Greater phosphorylation of Hsp27 reduces the chaperone function and increases antiapoptotic capacity [50] and could possibly play a role in the prevention of the formation of cataracts although this remains to be determined.

In conclusion, the present study used a pig lens to model the response to the stress of heating regarding functional outcomes. The results suggest that the porcine lens, which is a good representation of a human lens, demonstrates subtle changes in the variability of the focal length with increasing variability as the incubation temperature increases. In contrast, lens clarity is stable at temperatures up to 47.5 °C. As expected, the lens content of Hsp70 was elevated in the lenses exposed to heat shock, reaching a threshold at 50 °C. Above that threshold, the lens content of Hsp70 declines. In contrast, Hsp27 shows a limited increase in expression over the range of temperatures assessed. Thus, the Hsp70 content of heat-shocked lenses may be associated with the finding that obvious formation of cataracts does not occur until higher temperatures. The role of Hsp27 remains to be clarified. These data also suggest that the functional measures BVD SEM and clarity assess different qualities of the lens with the former being perhaps more sensitive to subtle changes in the protein structure.

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