α - and β -Crystallins Modulate the Head Group Order of Human Lens Membranes during Aging

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PURPOSE. To examine the physical properties of human lens cell membranes as a function of age.

METHODS. The environment of the phospholipid head groups in fiber cell membranes from human lenses, aged 22 to 83 years, was assessed with Laurdan and two-photon confocal microscopy. The effect of mild thermal stress on head group order was studied with lens pairs in which one intact lens was incubated at 50°C. Dihydrosphingomyelin vesicles were preloaded with Laurdan, α -, β -, or γ -crystallin was added, and surface fluidity was determined.

RESULTS. The membrane head group environment became more fluid with age as indicated by increased water penetration. Furthermore, these changes could be replicated simply by exposing intact human lenses to mild thermal stress; conditions which decreased the concentration of soluble α - and β -crystallins. Vesicle binding experiments showed that α - and β -, but not γ -, crystallins markedly affected head group order.

CONCLUSIONS. The physical properties of cell membranes in the lens nucleus change substantially with age, and α - and β -crystallins may modulate this effect. β -Crystallins may therefore play a role in lens cells, and cells of other tissues, apart from being simple structural proteins. Age-dependent loss of these crystallins may affect membrane integrity and contribute to the dysfunction of lenses in older people. (*Invest Ophthalmol Vis Sci.* 2010;51:5162–5167) DOI:10.1167/iovs.09-4947

The state of membrane lipids is important for membrane organization, for cellular processes such as signaling, fusion, and endocytosis, and for the activities of membrane-bound enzymes.^{1–4} Cellular membranes are complex structures containing lipid domains, of which lipid rafts are the best known. Lipid rafts are enriched with cholesterol and sphingolipid, which results in these domains being highly ordered compared with the surrounding membrane. The different bio-

physical environment may affect cell communication and receptor trafficking.^{5,6}

When cells are exposed to elevated temperatures, non-bilayer structures can be induced, leading to membrane defects and changes in permeability.⁷ It is, therefore, crucial for normal cell function that membrane integrity be preserved during such thermal stress. One mechanism appears to involve the synthesis of small heat shock proteins (sHsps) that have a pronounced stabilizing effect on model membranes.⁸ For example, sHsps such as α -crystallin and HSP 17 interact with membranes by way of the polar head groups of the phospholipids, which also strongly affect the hydrophobic core of the membrane.

The sHsp α -crystallin is the most abundant structural protein in the human lens⁹ and is also found in brain, heart, and liver.¹⁰ It is composed of two subunits, α A- and α B-crystallin. The biological role of α -crystallin in the lens is not yet fully established, but it is thought to bind to lens proteins as they denature over time, thereby helping to ensure long-term lens transparency. This is particularly important in the lens because there is no protein turnover¹¹ and proteins, once synthesized, are thus present for the lifetime of the individual. In addition to this chaperone role, α -crystallin binds to phospholipid vesicles¹² and to lens membrane preparations,^{13,14} though the precise mechanism of interaction and any functional significance have not yet been characterized. Two other classes of structural proteins are present in the lens: β -crystallins and γ -crystallins. In humans, the major β -crystallin polypeptides are β A1/A3-, β A4-, β B1-, β B2-, and β B3-crystallin.¹⁵

In other systems, it has been reported that aging is accompanied by a decrease in cell membrane fluidity.^{16,17} The objective of the current work was to examine the properties of fiber cell membranes in human lenses of different ages, and the factors that influence it.

METHODS

Ethics Statement

Human tissue was handled in accordance with the tenets of the Declaration of Helsinki. The work was approved by the human research ethics committee of Sydney University (Ethics #7292).

Tissue Sectioning

Twenty-three pairs of human lenses, ranging in age from 22 to 83 years, were obtained from the Lions NSW Eye Bank. The time between death of the donor and removal of the lenses ranged from 3 to 14 hours. One of each pair of lenses was mounted in OCT compound (Tissue-Tek; ProSciTech, Kirwan, Australia) on prechilled chucks and then sliced in a cryostat (Leica, Wetzlar, Germany) at -25°C . Ten-micrometer equatorial sections were cut and thaw-mounted onto gelatinized glass slides and then dried at room temperature (1 hour).

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Laurdan Labeling

Three serial sections from the middle of each lens were used. Tissue slices were covered with 40 μ L of 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan; Invitrogen, Mulgrave, Australia, 50 μ M in PBS), incubated at 25°C in the dark for 30 minutes, and washed three times with PBS. Each section was fixed for 20 minutes with 4% paraformaldehyde (40 μ L) and washed three times with PBS. A comparison of fixed with unfixed sections showed that paraformaldehyde did not affect the fluorescence readings. After drying at room temperature, the slides were mounted in 20 μ L medium (Mowiol; Calbiochem, Schwalbach, Germany), covered with a coverslip, and stored at room temperature overnight in the dark and then at 4°C until analysis.

Microscopy

Images were obtained with a confocal microscope (TCS SP5; Leica, Gladesville, Australia). Laurdan was excited with a two-photon laser, and intensity images were recorded simultaneously in the emission range of 400 to 460 nm and 470 to 530 nm for the two channels, respectively. The relative sensitivity of the two channels was determined with 0.5 μ M Laurdan in dimethyl sulfoxide (DMSO) for each experiment, and the G-factor was calculated as previously described.¹⁸ To extend the total field of view, a number of overlapping images ($775 \times 775 \mu\text{m}^2$, 512×512 pixels) were acquired such that the whole length of the section was covered.

Image Analysis

The pairs of Laurdan intensity images were converted to generalized polarization (GP) images with ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>) using the relationship:

$$\text{GP} = \frac{I_{(400-460)} - I_{(470-530)}}{I_{(400-460)} + I_{(470-530)}},$$

where I = signal intensity.

Final GP images were pseudocolored in a graphic editing program (Photoshop; Adobe, Mountain View, CA). To construct a montage of adjacent images in the x - y plane, a series of overlapping images from the same lens were stitched together using the Mosaic J plugin of ImageJ.

GP values at 0, 0.75, 2, 3, 3.5, 4, and 4.5 mm from the central point were obtained from the reconstructed GP images. For each lens, at the specified distances from the center, three readings for each of three sections were used to yield an average GP value. GP values were corrected using the G factor obtained for Laurdan in DMSO.

Cholesterol Analysis

Contralateral lenses of those used for Laurdan staining were dissected into four regions using three trephines, as described.¹⁹ The ends (~ 1 mm) of the 8-mm diameter billet were removed before using the 6-mm and 4.5-mm trephines. Tissues were homogenized in 400 μ L methanol/ CHCl_3 (1:2 vol/vol) containing 0.01% butylated hydroxytoluene and were extracted overnight. D6 cholesterol was added, and cholesterol was quantified by direct-insertion electron ionization mass spectrometry (QP5050 GC-MS; Shimadzu, Sydney, Australia), as previously described.²⁰

Heating of Intact Lenses

Three pairs of lenses aged 30, 41, and 63 years were used. One lens from each pair was incubated at 50°C for 20 hours, and the other was kept at -80°C . Lenses were sectioned and then incubated with Laurdan, as described. After sectioning, a 4.5-mm trephine was used to obtain nuclear samples from the remaining tissue. These were homogenized, and extracts were centrifuged at 25,000g for 20 minutes (4°C). Homogenization was repeated four times. Gel filtration HPLC (Shi-

madzu, Sydney, Australia) was used to determine the content of soluble α -crystallin, β -crystallin, and HMW protein.²¹

Heating Lens Tissues

A time course experiment was performed using dissected lens regions from one pair of 38-year-old lenses. One lens was sliced, and sections from the middle of the lens were collected for incubation at 50°C for 0 hour, 4 hours, 8 hours, 16 hours, or 24 hours and subsequent Laurdan staining, as described. The other lens was dissected using the 6-mm trephine. The ends (~ 1 mm) of the 6-mm billet were removed, and the nucleus was divided into five equal parts. These nuclear portions were incubated as described for the lens slices. Tissues were extracted as described to determine the soluble content of α -crystallin, β -crystallin, and HMW protein by gel filtration HPLC.

Vesicle Experiments

Dihydrosphingomyelin 12:0 (*N*-lauryl-D-erythro-sphinganylphosphorylcholine; Avanti Polar Lipids, Alabaster, AL) (15 μ L, 10 mg/mL in chloroform) was vortexed for 2 minutes and sonicated for 2 minutes (Ultrasonic Cleaner; Ultrasonics, Sydney, Australia), and then the chloroform was dried under nitrogen. The lipid film was reconstituted in 300 mM sucrose (1.5 mL) and heated in a water bath at 60°C for 36 hours. This is a modification of the method of Nicolini et al.²² One hour before imaging, Laurdan was added to the samples such that the final concentration of Laurdan was 5 μ M. After they had been purified by gel filtration HPLC, as described,²¹ and lyophilized, α -, β -, and γ -crystallins were dissolved separately (5.0 mg/mL) in 10 mM *N*-[tris(hydroxymethyl) methyl]-2-aminoethanesulfonic acid buffer (pH 7.1). Vesicles (180 μ L, 0.1 mg/mL) were incubated for 30 minutes with the proteins (20 μ L) at 25°C. Samples were measured in a fluorometer (SpectraMax M2; Molecular Devices, Sydney, Australia) at an excitation wavelength of 400 nm. A ratio of emission intensities from 410 to 460 nm and 470 to 530 nm was used to calculate GP values.

RESULTS

The fluorescent membrane dye Laurdan shifts its peak emission from approximately 500 nm in fluid membranes to approximately 440 nm in ordered membranes.^{18,23} A normalized ratio of the two emission regions, GP, can therefore be used as an index of membrane fluidity that is independent of membrane ruffles and dye concentration. Theoretical GP values range from -1 (most fluid) to $+1$ (most ordered).

It should be noted that the Laurdan fluorescence emission ratio is a measure of water penetration into the membrane and thus more accurately reflects the environment of the phospholipid head groups at the membrane surface. Representative GP images of selected human lens sections (Fig. 1A) are pseudocolored so that ordered membranes are yellow to red and more fluid membranes are green. As can readily be seen, there were pronounced changes in the properties of the membranes in the centers of the lenses as a function of donor age. The surfaces of cell membranes in the centers of older lenses appear more fluid than the centers of young lenses and the edges of the same lenses.

To quantify this relationship, mean GP values at specific distances from the lens center were measured (Fig. 1B). GP values in the outer parts of the lens varied little as a function of lens age. This was unsurprising because this part of the lens has been synthesized most recently, and the environment in all lenses should be similar.

In the young lenses (20–40 years), GP values across the sections remained constant as a function of distance from the lens center. By contrast, in older lenses, a different, but consistent, pattern was observed in which GP values dropped from 3.5 mm to 2 mm. Then, within the inner 2-mm radius, values were relatively constant (Fig. 1B). In addition, in the

FIGURE 1. The effect of age on membrane GP values across the diameter of the lens. (A) Representative pseudocolored GP images of lens sections across the age range. A number of confocal images ($775 \times 775 \mu\text{m}^2$, 512×512 pixels) were acquired, and the edges overlapped to enable the whole length of the lens section to be covered. (B) Membrane properties as a function of lens age. GP values (mean $\pm$ SEM) at 0, 0.75, 2, 3, 3.5, 4, and 4.5 mm from the geometric center of the lenses were calculated from the reconstructed GP images. Lenses: 20 to 40 years, $n = 5$; 40 to 50 years, $n = 4$; 50 to 60 years, $n = 5$; 60 to 70 years, $n = 5$; older than 70 years, $n = 4$.

central part of the lens, there was a progressive decrease in GP values as a function of age. These changes are summarized in Figure 2.

To investigate the basis for these age-related changes, we first quantified cholesterol in regions of the individual lenses (Fig. 3) because cholesterol content is an important factor governing the fluidity of membranes.²⁴ In the lenses used in this study, no clear pattern was observed either across individual lenses or in the central lens regions as a function of age, suggesting that changes in the levels of this major membrane constituent are not responsible for the changes in GP values that were observed.

In previous studies, heating of intact lenses at 50°C has been used to mimic changes that occur to human lenses in the eye, where the temperature is approximately 35°C , over several decades. In such studies, lens stiffness increased substantially after incubation at 50°C .²¹

To determine whether incubation at this temperature also affected the properties of lens membranes, lens pairs were used with one lens of the pair incubated for 20 hours at 50°C while the other was stored at -80°C . Subsequent Laurdan labeling and imaging at room temperature were performed, as described, for each lens. Figure 4A shows the GP values obtained from heated and unheated contralateral lenses. Heating of intact lenses resulted in significant decreases in lens GP values. Interestingly, the patterns obtained depended on the age of the lenses; however, the data were consistent in that the

FIGURE 2. Effect of age on membrane GP values in the lens center and outer region. Age dependence of GP values in the very center of the lens with those 3.5 mm from the lens center. 3.5 mm: $y = -0.0001x + 0.4786$; $R^2 = 0.0129$. Center: $y = -0.0028x + 0.5453$; $R^2 = 0.8004$.

GP value in the nucleus of the heated lens was significantly lower than that of the unheated control. Of particular interest, the GP values in the outer lens regions were largely unaffected by heating. Similar GP changes were obtained by heating lens slices at 50°C (data not shown).

From previous experiments we had documented significant decreases in the content of α -crystallin and other soluble proteins when lenses were exposed to similar thermal stress.²¹ To confirm that this was also observed in the current incubations, nuclear regions from heated and control lenses were extracted and examined by gel filtration HPLC to determine the content of soluble crystallins. HMW protein is formed in the lens,²⁵ and in model systems,²⁶ when α -crystallin binds to denatured proteins. Once α -crystallin is depleted, proteins become insoluble.²⁷ In the intact lenses, heating led to a decrease in the content of soluble proteins (Fig. 4B). In the 63-year-old lens pair, no soluble α -crystallin was present initially because, as has been documented previously,^{21,28,29} it had presumably been used in chaperoning other proteins as they denatured over this time span. The results of this experiment suggested that pro-

FIGURE 3. Cholesterol content in lens regions as a function of age. Cholesterol concentration (mean $\pm$ SEM) was determined using electron ionization mass spectrometry.

FIGURE 4. The influence of mild thermal stress on (A) membrane GP values in intact human lenses. Three lens pairs (30 years, 41 years, and 63 years) were used with one lens of each pair incubated at 50°C (20 hours) and the other not heated. (B) Soluble proteins. The nuclear regions of three lens pairs were extracted, and the soluble proteins were separated by gel filtration HPLC with absorbance monitored at 280 nm. (A, B) Lenses were sectioned and treated with Laurdan (mean $\pm$ SD).

teins such as α -crystallin, and possibly other soluble crystallins, could be involved in modulating membrane properties.

To determine whether membrane properties changed in parallel with the concentration of crystallins, a lens nucleus was divided into five equal parts and then heated at 50°C for different periods of time. Over time, the HMW form of α -crystallin and the β -crystallins decreased in the soluble fraction, and there was a corresponding decrease in membrane GP values (Fig. 5). Examination of the insoluble, membrane-containing fraction revealed that there was a corresponding increase over time in crystallin content (data not shown). This is consistent with the conversion of soluble proteins into insoluble aggregates in response to thermal stress.

To confirm that such fluorescence changes were due to a direct effect on the lipids in membranes, vesicle experiments were undertaken. Vesicles were prepared using the major human lens phospholipid, dihydrosphingomyelin (DHSM). DHSM comprises of half the total phospholipids of human fiber cells.²⁰ As shown (Supplementary Fig. S1, <http://www.iovs.org/cgi/content/full/51/10/5162/DC1>), α - and β -crystallins markedly affected GP values in a concentration-dependent manner. In contrast, γ -crystallin had no effect.

DISCUSSION

It has been hypothesized that ubiquitous sHsps may protect cells from thermal stress in two distinct ways.^{8,30} One involves a chaperone action by which sHsps bind to denaturing proteins²⁷ and in this way minimize insoluble protein content in the cell cytosol. The other is less well documented and involves interactions with membranes to stabilize the lipid bilayer structure.⁸ Until now, such a putative function has been inferred solely on the basis of model studies using synthetic and naturally occurring lipids.^{13,31}

In the experiments reported here, evidence has been obtained that supports a role for the sHsp α -crystallin in modulating membrane properties in a human tissue: the lens. Our data agree with those of Tang and Borchman,³² who showed that incubation of α -crystallin at 47°C caused it to undergo a conformational change that resulted in binding to the head group of sphingomyelin and enabled greater water penetration. In addition, our results suggest a similar role for the β -crystallins. At this stage it is unknown which of the β -crystallin subunits is chiefly responsible; this is under investigation. This finding implies that one or more β -crystallins could play a role in cells that is separate from the role of a structural protein, which is its other function in the lens.

We took advantage of the fact that exposure of intact lenses to mild thermal stress (50°C) results in changes to the content of soluble proteins within the lens cells. Under these conditions, soluble α -crystallin decreases,²¹ and high molecular weight (HMW) aggregates are formed. The HMW aggregates are composed mostly of α -crystallin in a noncovalent complex with other lens proteins that presumably have been denatured.³³ The flexible C-terminal regions of α -crystallin in the HMW form can readily be observed by nuclear magnetic resonance spectroscopy,³⁴ and it has been reported that it is more hydrophobic³⁵ than native α -crystallin and may, therefore, be more likely to bind to membranes.¹⁴ In the human lens, HMW aggregates increase in amount until about age 40 and then decrease as insoluble protein is formed.^{21,36} These data are consistent with HMW protein forming and then later becoming insoluble. Such a view is supported by model studies in which crystallins have been heated.²⁷

FIGURE 5. Effect of exposure time to mild thermal stress on lens membrane GP values and soluble protein content. Concentration of the HMW form of α -crystallin and β -crystallin in portions of a 38-year-old human lens core, as a function of time of incubation at 50°C, compared with GP values of sections from the contralateral lens heated at 50°C. In this lens nucleus, α -crystallin was present only in the HMW form. HMW: $y = -0.2264x + 6.3907$; $R^2 = 0.9287$. β -crystallins: $y = -0.8574x + 44.044$; $R^2 = 0.7369$. GP: $y = -0.0055x + 0.4316$; $R^2 = 0.8756$.

Under our experimental conditions, membrane GP values decreased significantly in the centers of lenses after exposure to mild thermal stress in a manner that mimicked the changes observed with age (Figs. 1B, 4A). Moreover, the magnitude of the decreases in GP values was comparable in the heated intact lenses and the older normal lenses. A lens temperature of 50°C is little different from that which can be observed under some environmental conditions. For example, the lens temperature in monkeys exposed to the midday sun at an ambient temperature of 49°C reached 41.5°C within 9 minutes.³⁷

The human lens, unlike the lenses of experimental animals, contains very few polyunsaturated fatty acids,²⁰ and, in separate experiments, we have not observed evidence of oxidation. It would also be expected that any potential oxidation arising from increased exposure to oxygen during the incubations would be mitigated by the presence of high concentrations of antioxidants, such as glutathione and ascorbate, in the intact lenses. Nevertheless, it cannot be ruled out as a possible contributor to the observed decrease in GP values after heating. Although it has not been reported previously, we also cannot eliminate the possibility that Laurdan may interfere with the head group structure of phospholipids or may interact directly with proteins associated with lens membranes.

On the basis of protein changes that could be reproduced simply by exposure of intact lenses to thermal stress, a hypothesis was formulated that long-term exposure to body temperature is a key determinant of lens changes in humans.²¹ The finding in this study that thermal stress can also reproduce age-related changes in the physical properties of cell membranes supports this proposal.

It should be noted that the Laurdan labeling and microscopy experiments were all performed at 25°C, and the acyl chains of human lens membrane lipids become more ordered at lower temperatures.³⁸ Recent work by Sane et al.³⁹ also indicates that the acyl component of porcine lens lipids undergoes a structural transition at physiological temperatures³⁹ even though porcine lenses have a different lipid composition from human lenses.²⁰ Therefore, with regard to the application of our results to the aged human eye, it is not possible to conclude that the measurements made at 25°C necessarily reflect the situation in the lens of the eye, which is typically maintained at 35°C. The high mole percentage of cholesterol in human lens membranes should, however, minimize any effect of temperature on phase transitions.

The lens is a unique system because there is no turnover of protein. Once crystallins and other proteins are synthesized, they are present for the life of the individual.¹¹ The lens grows continuously throughout life through the addition of newly differentiated cells to the lens that was present at birth. For the lens to remain transparent, it is clearly crucial in long-lived animals such as humans that processes are present within the lens to minimize structural damage to proteins and membranes that could result in opacification. A high content of α -crystallin has been hypothesized to be one way to minimize the effects of age-dependent changes to proteins that will occur inevitably over such a long time period.²⁷

On the basis of our data, it would appear that a decline in soluble α - and β -crystallins with age results in a marked decrease in GP values, consistent with a reduction in the head group order of lens nuclear membranes. The interaction of proteins with membranes, leading to alterations in fluidity, is not without precedent. Amyloid β -peptide 1–40 increased the fluidity of plasma membranes that were isolated from some brain regions.⁴⁰ By contrast, in experiments with model membranes, sHsps strongly stabilized the liquid crystalline state,⁸ a finding consistent with our results. The consequences of a more fluid membrane surface in the center of older lenses are unknown. It has been shown that the incidence of fused

membranes in the lens center increases with age.⁴¹ In older lenses, one result of this may be the formation of a macromolecular diffusion pathway⁴² in the center of the lens that allows cell-to-cell movement of large molecules such as proteins. Certainly ultrastructure data indicate that there may be more damage to the membranes in the nucleus of age-related cataract lenses,⁴³ and more convoluted membranes in older and cataractous lenses could affect head group order and, therefore, Laurdan measurements.

Given that the human lens stiffens with age⁴⁴ and that this is the probable basis for presbyopia,²¹ it seems unlikely on the basis of our data that this stiffness is due solely to changes in the fiber cell membranes because, on the basis of Laurdan fluorescence ratios, they become more fluid. Such a conclusion must be tempered by the fact that Laurdan measures primarily the surface environment, or head group order, of membranes. Data from other researchers indicate that hydrocarbon chain order increases with age in the lens nucleus and cortex, a phenomenon that may relate to the levels of saturation.^{45,46} It is important to recognize that hydrocarbon order can vary independently of head group dynamics, such as in sphingolipids.^{47,48} Although at this stage it is not possible to be definitive, it appears that alterations to cytosolic proteins are more likely to be involved in the age-related changes in lens stiffness.

Recent data from Raguz et al.⁴⁹ indicate that lipid bilayer membranes made of lipids extracted from lenses are completely in a liquid-ordered phase. Although the techniques used were different from those used here, this finding may suggest that in the lens, the properties of the cell membranes are markedly affected by the presence of other components, including extrinsic proteins.

There is a considerable body of data that demonstrate binding of α -crystallin to phospholipid vesicles and cell membrane preparations.^{12,13,31} It appears from our data that such interactions, particularly those involving α - and β -crystallin polypeptides, may be directly responsible for regulating membrane properties in the lens. Because α -crystallin is also present in heart, brain, and liver¹⁰ and β -crystallins have recently been reported in tissues such as the retina,⁵⁰ these polypeptides may also play a significant role in modulating the integrity of membranes in other tissues of the body. Future experiments will involve examining purified crystallins to determine which are of most importance and the mechanism of their interactions with membranes.

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