Pressure-Induced Syneretic Response in Rhesus Monkey Lenses

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PURPOSE. To investigate the effect of pressure on the freezable and nonfreezable water content of the lens.

METHODS. Excised rhesus monkey lenses in tissue culture media were subjected to three different hydrostatic pressures (2 atm, 1 atm, and 0.03 atm) for 24 hours. Then while still under the experimental pressure, the vessels were cooled in dry ice-acetone until the lenses were frozen. While the lenses were kept frozen, nuclear and cortical samples were dissected, enclosed in a sample pan, and weighed. Differential scanning calorimetry (DSC) measurements were performed between −30°C and 30°C. Total water content of each lens sample was obtained by thermogravimetric analysis at 105°C. The nonfreezable water content was obtained by subtracting the freezable water content calculated from the DSC data from the total water content.

RESULTS. The total water content of the lenses did not change significantly as a function of pressure applied. This was true both for cortical and for nuclear sections. The freezable water content increased as the pressure decreased both in cortex and nucleus. Similarly, the freezable water/nonfreezable water ratio also decreased with increasing pressure.

CONCLUSIONS. External hydrostatic pressure would generate an influx of water into the lens. To alleviate this diluting tendency and to prevent turbidity as a result of dilution, the lens must effect an osmotic pressure change equivalent to the applied pressure. Change in the osmotic pressure is caused by changing the activity of the water (i.e., converting free water to bound water). This is a reversible and energetically the least expensive response. The release of bound water from the hydration layers of macromolecules and its conversion to free water in condensed systems are known as syneresis. In the lens decreasing pressures induce syneresis as demonstrated by the increase in freezable water content and the freezable water/nonfreezable water ratio. Such a response may be operative also in accommodating lenses. *(Invest Ophthalmol Vis Sci.* 1999;40:1285-1288)

Syneresis is a process in which bound water is released from the hydration layer of biopolymers and becomes free water. The physical process itself has a number of potential consequences. In the eye an irreversible syneretic process accounts for the liquid pocket formation in the aging vitreous.[1] The consequence of this is a change in the viscoelastic properties of the vitreous, occasionally resulting in detached retinas in older persons. A similar irreversible syneretic process is involved in cataract formation.[2] In aging and cataractous lenses irreversible syneresis has an optical consequence because the refractive index of bound water is different from that of the free water. In the lens, syneresis causes a change in the refractive index of the environment surrounding the protein aggregates. It has been shown[3-4] that syneresis contributes to the turbidity of the lens by increasing the amplitude of refractive index fluctuations. In human lenses[5-7] and in various animal models of cataract[8-12] it has been shown that syneresis may account for 30% to 85% of the total turbidity.

It has recently been predicted on theoretical grounds that a reversible syneretic process could be a possible operative response to hydrostatic pressure changes.[13] The consequence of such syneresis is a change in the colligative properties. When water is released from its bound state and becomes free water, the effective concentration, the activity of water increases. This is, in essence, a diluting effect. A hydrostatic pressure, even though it is nondirectional, has a vectorial component that would drive water molecules across the lens capsule and fiber cell membranes into the cytoplasm. This would upset the osmotic balance. An immediate response that would counter this stress is an inverse syneretic process. This means that the excess water activity on pressure/stress could be relieved by changing the free water to bound water. Preliminary experiments with bovine lenses have shown that this is indeed the case in vitro. The ratio of free water to bound water decreased with increasing pressure and increased when the pressure was reduced.[13] The purpose of the present study was to look more closely at the possible role of syneresis in stress responses in the lens.

MATERIALS AND METHODS

Rhesus monkey eyes were obtained from animals euthanatized in the FDA vaccine testing program. Twenty-six eyes were enucleated shortly after death. The lenses were dissected as previously described[14] and were immersed in the prepared medium. The medium used was Leibowitz's L-15 with glutamine but no phenol red, adjusted to pH 7.5. Antibiotics (penicillin and streptomycin) were added, and the osmolarity of the medium was adjusted to 300 ± 2 milliosmoles.

Three pressure conditions were applied. A pressure of 0.03 atm was attained by placing the lenses in the media in a vacuum chamber that was evacuated using a vacuum pump and sealed by a stopcock. The pressure therefore was the vapor pressure of the aqueous medium at 37°C. Two atmosphere pressure was obtained by connecting the pressure chamber to a nitrogen cylinder and maintaining the pressure with control valves. A third group of lenses was incubated at normal atmospheric pressure. All incubations were kept at 37°C by placing the vessels inside a tissue culture incubator. After 24 hours' exposure the vessels containing the lenses, but still under the experimental pressure, were immersed in a

TABLE 1. Mean Values of Hydration Parameters of Rhesus Monkey Lenses under Different Experimental Conditions

Sample	TW%	FW%	NFW%TW	FW/NFW
2 atm cortex	75.16 ± 2.77	51.42 ± 3.45	28.06 ± 1.04	2.16 ± 0.24
2 atm nucleus	65.68 ± 0.91	45.45 ± 1.03	30.83 ± 1.04	2.25 ± 0.11
1 atm cortex	73.23 ± 1.80	54.50 ± 2.00	25.72 ± 1.32	2.91 ± 0.19
1 atm nucleus	63.28 ± 1.03	47.45 ± 1.42	25.02 ± 1.77	3.00 ± 0.26
0.03 atm cortex	72.69 ± 1.70	58.89 ± 1.97	19.06 ± 1.46	4.26 ± 0.13
0.03 atm nucleus	64.60 ± 1.97	49.93 ± 2.19	23.43 ± 2.43	3.45 ± 0.32

Values are means ± SD; sample size: 9 lenses at 2 atm, 10 lenses at 1 atm and 7 lenses at 0.03 atm. TW%, total water percent of lens; FW%, freezable water percent of lens; NFW%TW, nonfreezable water percent of total water.

−70°C dry ice-acetone bath until the lenses were frozen. Subsequently, the vessels were transported into a cold room and opened. The frozen lenses were removed from the vessels and were placed immediately in a petri dish resting on dry ice. The lenses were dissected into cortical and nuclear sections (6–15 mg) and positioned in the center of preweighed cold pans, sealed immediately, reweighed, and quickly packed into labeled containers and stored at −70°C.

The pans containing the samples were used to perform differential scanning calorimetry (DSC) to obtain freezable water content. The dry ice-cooled pans were placed into the apparatus and heated to −30°C at which point they were equilibrated for 20 minutes. From this point the pans were heated (3°C/min) to 30°C. The amount of heat needed to effect the change between the two temperatures was recorded against a blank (a pan holding no sample). The heat of melting, recorded as Joules per gram of sample, was converted to freezable water per gram of sample using calibration curves. The calibration curve was obtained using seven samples of triple distilled water of different masses; for each of these, the heat of melting was obtained.[7,9] The pans were reweighed on an analytical balance and placed in the thermogravimetric apparatus (TGA) in which their weights were established on a Cahn electrical balance. The pans were punctured with a special needle in three places, taking care not to touch the samples residing in the center of the pans. The pans were placed again on the Cahn electrobalance and the apparatus closed. The samples were heated to 105°C and held there for 20 minutes. During this period a constant weight was established. The loss in weight recorded by the instrument was taken as total water of the sample. As a double-check, the dried pans were reweighed on an analytical balance, and the weight loss was obtained also in this manner. The nonfreezable water content was obtained as the difference between total and freezable water content.

RESULTS

The first important result concerns the total water content given as a percentage of the sample. It is clear that in monkey lenses (Table 1) there are no statistically significant differences between the three experimental conditions used. This is true for both the cortex and the nucleus. At the same time the cortex-to-nucleus gradient was maintained under the different experimental conditions.

The overall effect of changing the hydrostatic pressure is evident in the free water-to-bound water ratio, that is freezable water-to-nonfreezable water ratio (FW/NFW). The nonfreezable water content was calculated as the difference between the total water (TGA) and freezable water (DSC) content. As the hydrostatic pressure increases the freezable water-to-nonfreezable water ratio decreases. This is a statistically significant trend at the 95% confidence level. The increase in this ratio is evident both in the cortex and in the nucleus of monkey lenses (Table 1).

The dominant individual hydration parameter in the FW/NFW ratio seems to be the freezable water. In the cortex and in the nucleus, the freezable water, presented as percentage of the lens, decreases with increasing pressure. Freezable water content is directly obtained from DSC measurements, and it does not involve the total water content. However, because the total water content of the lenses does not change significantly with pressure, it is obvious that the nonfreezable water (as a percentage of the lens) is increasing with increasing pressure.

However, it is instructive to look at the syneretic process also from the point of view of the total water content. If the nonfreezable water content is calculated as percentage of the total water, one finds that this hydration parameter is increasing with increasing pressure, albeit the effect in the monkey lens nucleus is less pronounced than in the cortex. Furthermore, the difference between 1 atm and 0.03 atm conditions in the nucleus is not statistically significant. This reflects the variability in the total water content, which did not show any statistically significant trend.

DISCUSSION

The hydration parameters examined in rhesus monkey lenses exposed to different hydrostatic pressures show that there is no significant change in total water content and that there is an increase in nonfreezable water and a decrease in freezable water content with increasing pressure (Table 1). Thus, the FN/NFW ratio (free water/bound water ratio) is decreasing with increasing pressure (Fig. 1). This confirms the hypothesis[13] that a syneretic response is operative in rhesus monkey lenses similar to that observed in bovine lenses. An important feature of these results is that the syneretic response is reversible. It is also a rapid response. We found that if lenses were kept under 2 atm pressure for 20 hours and the pressure was released to 1 atm 1 hour before dissection of the lenses, the
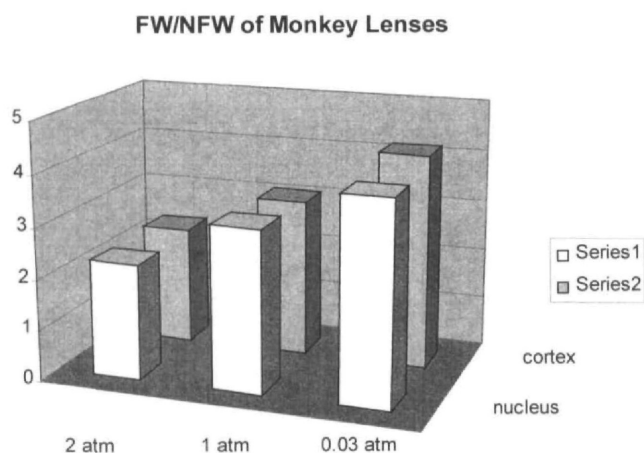
FW/NFW of Monkey Lenses

FIGURE 1. Freezable water/nonfreezable water ratio in rhesus monkey lenses as a function of hydrostatic pressure.

hydration behavior was the same as if the lenses were under 1 atm pressure throughout the incubation. Also, if the dissected lenses were not kept strictly below −20°C, the hydration behavior of the lenses subjected to 2 atm or 0.03 atm conditions were similar to those under 1 atm pressure. The reversible nature of this syneretic response is all the more important because under acute and very large hydrostatic pressures, irreversible changes do occur. This was demonstrated when fish lenses were exposed to 1000 atm pressure, when an irreversible turbidity was observed.[15] It is germaine to extend the inquiry to determine at what limit the reversibility can exist both in terms of hydration parameters and of turbidity. If syneretic response is a protective measure against osmotic imbalance-induced cataract formation, does the syneretic response fail when irreversible cataract develops? This question may be important in considering possible cataract hazards to human divers.

The reversible syneretic response of the lens to small changes in hydrostatic pressure may have physiological significicance in accommodation. Rhesus monkey lenses were chosen because they accommodate and because we wanted to examine the possibility that there is a similarity between pressure responses and responses during accommodation. In accommodation the equatorial diameter of the lens shrinks, the lens becomes thicker, and its surfaces, especially the anterior surface, become more sharply curved. Mathematical modeling[16,17] has shown that the zonules inserted into the lens capsule[18] apply stress that has both parallel (stretching) and perpendicular (compressive) components. These discrete stresses are transformed by the capsule into a uniform stress "approximately perpendicular to the lens surface."[19] Thus, during the unaccommodated state the stress normal to the lens surface (equivalent to hydrostatic pressure) would be at maximum and in the accommodated state at minimum. According to mathematical modeling, the pressure corresponding to the force of contraction of the ciliary muscles during accommodation is three orders of magnitude smaller than those used in this study.[20] Thus, the present study proves only the feasibility that a syneretic response may exist in accommodation. Nevertheless, to respond to the change in pressure associated with going from the unaccommodated to the accommodated state, syneresis is the fastest and energetically the least expensive

mechanism.[13] Obviously a syneretic response is not the only mechanism by which an osmotic imbalance can be relieved. Other possible mechanisms include metabolic responses and transport responses. One metabolic response may be proteolysis. The breakdown of protein molecules into smaller peptides or amino acids may increase the osmolyte concentration. In this way the increase in water activity (concentration) induced by an applied hydrostatic pressure may be counteracted. Another metabolic response may be the synthesis of osmolytes such as betaine or sorbitol. A possible presumptive transport response to pressure stress may be activation of ATP-dependent gated channels to import ions in the lens. Such metabolic or transport responses require high energy derived from hydrolysis of ATP. In contrast, a syneretic response involves only 5 kJ/mole to 30 kJ/mole water to immobilize the water in the hydration layer by additional hydrogen bonding. This is at minimum a 10-fold more energy efficient process than the other mechanisms envisaged. Furthermore, the syneretic response is also much faster than metabolic or transport responses.[13] The model includes the assumption that a small amount of reversible exchange between bound water in the hydration layer of the lens proteins and the free water in the bulk can occur without adversely affecting protein conformation or viability.

The present experiments confirm that the application of small hydrostatic pressure on monkey lenses elicits a reversible syneretic response. Although this suggests that such a syneretic response may be operative during accommodation, the proof for such an effect must be sought with more refined techniques that are noninvasive and can be applied in vivo, as well.

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Molecular Identification and Immunolocalization of the Water Channel Protein Aquaporin 1 in CBCECs

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PURPOSE. Water channel proteins are important pathways for water movements across cell membranes, including those in the corneal endothelium that contribute to the fluid transport mechanism essential in maintaining corneal transparency. This study was conducted to identify and locate the water channel protein(s) in cultured bovine corneal endothelial cells (CBCECs).

METHODS. Poly(A)$^+$ RNA was isolated from CBCECs, and MMLV reverse transcriptase and random hexamer primers were used to generate a cDNA pool by reverse transcription-polymerase chain reaction (RT-PCR). Two specific degenerate primers were synthesized based on consensus sequences from the major intrinsic lens protein superfamily; a "touchdown" PCR protocol accommodated the degeneracy. Immunolocalization was performed by incubating sections of CBCECs with an antibody against human aquaporin 1 (AQP1). Cryo-sections (0.85 μm) of CBCECs were used for light microscopy, and 800-Å ultrathin cryosections were used for electron microscopy (EM).

RESULTS. A 372-bp fragment was isolated. Its encoded amino acid sequence was 100% identical with that of bovine AQP1 (AQP1_bovin). CBCECs reacted strongly with the anti-AQP1 antibody, and the labeling was selectively localized to the plasma membrane by light microscopy. Subcellular localization by EM revealed immunoreactivity with the inner leaflets of the plasma membrane.

CONCLUSIONS. The identity of the aquaporin, its abundance, and its membrane location suggest that it is a major pathway for fluid flow across endothelial cell membranes. This is consistent with transcellular endothelial fluid transport. (*Invest Ophthalmol Vis Sci.* 1999;40:1288-1292)

The corneal endothelium is essential in maintaining the normal level of dehydration and transparency of the cornea through its fluid pump. Yet, although fluid transport has been shown in many other epithelia including cultured corneal endothelial cells, the inner workings of the fluid pump remain unsolved. Some mechanisms presumably involved in it have been reviewed,[1] but cellular details such as the identity and location of the membrane transporters and channels involved and the signaling and regulatory pathways for them remain to be elucidated.

The discovery of the water channel protein, channel-forming integral membrane protein of 28 kDa (CHIP28), in human red blood cells[2] brought new impetus to studies in this field. The sequence of this protein turned out to be highly homologous to that of the major intrinsic lens protein (MIP), and to date, at least nine water channel isoforms (later renamed aquaporins, or AQP1-9) have been identified. The distribution of water channels is characteristic: For instance, within a nephron, all epithelia involved in fluid movement (and only those epithelia) have water channels.[3] This suggests that water channels are the major pathway for epithelial fluid transport and that such fluid movement is transcellular.

AQP1 has been located in rat corneal endothelium by in situ hybridization,[4] and we have reported the existence of mRNA encoding functional water channels in CBCECs, the expression of which is abolished by AQP1 antisense RNA.[5] From that study, we concluded that CBCECs express a CHIP28-type protein. In the present study, we identified a segment encoding nearly half of the coding region of AQP1 and localized it to the inner leaflet of the plasma membrane.

METHODS

Cell Culture and mRNA Preparation

Bovine eyes were obtained from a local abattoir. Corneas with a 2- to 3-mm-wide annulus of surrounding sclera were excised