Cholesterol-derived bile acids enhance the chaperone activity of α -crystallins

Shuhua Song · Jack J. N. Liang · Michael L. Mulhern ·
Christian J. Madson · Toshimichi Shinohara

Received: 10 December 2010 / Revised: 9 February 2011 / Accepted: 10 February 2011 / Published online: 6 March 2011
© Cell Stress Society International 2011

Abstract Human lens membranes contain the highest cholesterol concentration of any known biological membranes, but it significantly decreases with age. Oxygenation of cholesterol generates numerous forms of oxysterols (bile acids). We previously showed that two forms of the bile acid components—ursodeoxycholic acid (UDCA) and tauroursodeoxycholic acid (TUDCA)—suppressed lens epithelial cell death and alleviated cataract formation in galactosemic rat lenses. We investigated whether these compounds also suppress the thermal aggregation of human lens crystallins. Total water-soluble (WS) proteins were prepared from human lenses, and recombinant human crystallins (α A-, α B-, β B2-, and γ C-crystallin) were generated by a prokaryotic expression system and purified by liquid chromatography. The light scattering of proteins in the presence or absence of UDCA or TUDCA was measured using a spectrofluorometer set at Ex/Em=400/400 nm. Protein blot analysis was conducted for detection of α -crystallins in the human lens WS proteins. High concentrations of UDCA and TUDCA significantly suppressed thermal aggregation of total lens WS proteins, which contained a low level of α A-/ α B-crystallin. Spectroscopic analysis with each recombinant human lens crystallin indicated that the bile acids did not suppress the thermal aggregation of γ C-, β B2-, α A-, or α B-crystallin. Combi-

nation of α -crystallin and bile acid (either UDCA or TUDCA) suppressed thermal aggregation of each individual crystallin as well as a non-crystallin protein, insulin. These results suggest that UDCA or TUDCA protects the chaperone activity of α -crystallin. It is believed that these two naturally occurring intermediate waste products in the lens enhance the chaperone activity of α -crystallin. This finding may lead to the development of UDCA and TUDCA as anticataract agents.

Keywords Chaperone activity · Lens crystallin · Lens water-soluble protein · Tauroursodeoxycholic acid · Thermal protein aggregation · Ursodeoxycholic acid

Introduction

The unfolded protein response (UPR) is an evolutionary conserved response to perturbation of normal endoplasmic reticulum (ER) physiology (ER stress). Many cataractogenic stressors also induce UPR, and prolonged ER stress induces the UPR in lens epithelial cells (LECs; Ikesugi et al. 2006). The UPR is also induced in galactose-fed rat lenses (Mulhern et al. 2006; Mulhern et al. 2007). Under these stresses, lens crystallins are modified and undergo structural changes and aggregation, but aggregation may be prevented by molecular chaperones, such as α -crystallins. In addition, pantethine and glutathione, products of common metabolic pathways, have been shown to modulate the chaperone-like effects on the anti-aggregation activity of α -crystallin (Clark and Huang 1996). Recent reports showed that some bile salts (metabolic products of cholesterol) have similar capability in stabilizing protein conformations and suppress the UPR activation (Ellis and Pinheiro 2002; Welch and Brown 1996; Perlmutter 2002). These small

S. Song · J. J. N. Liang
Ophthalmic Research/Surgery, Brigham and Women's Hospital,
Harvard Medical School,
Boston, MA, USA

M. L. Mulhern · C. J. Madson · T. Shinohara (✉)
Department of Ophthalmology and Visual Sciences,
University of Nebraska Medical Center,
Omaha, NE 68198-5840, USA
e-mail: tshinohara@unmc.edu

substances, which help to stabilize proteins in their natural conformation, were termed “chemical chaperones” (Welch and Brown 1996). Bile acids or oxysterols, typically represented by UDCA or taurine-conjugated UDCA (TUDCA; Fig. 1), are oxidative products of cholesterol (Bernstein et al. 2005; Hanukoglu 2006). In human lenses, cholesterol content decreases with aging, presumably due to oxidation (Li et al. 1985; Zelenka 1984), which generates numerous forms of oxysterols at relatively low levels (Girao et al. 2003; Girao et al. 1998). Previously, we have also shown that TUDCA suppressed LEC death and alleviated cataract formation in galactose-fed rat lenses (Mulhern et al. 2006; Mulhern et al. 2007), but we do not understand the underlying mechanism. We hypothesize that these bile acids may protect α -crystallin from aggregation or protect the chaperone activity of α -crystallin. To test our hypothesis, we investigated whether bile acids, either UDCA or TUDCA, suppress the protein aggregation induced by thermal aggregation of lens crystallins and whether these bile acids protect the chaperone activity of α -crystallin.

Materials and methods

Preparation of lens crystallins

The human lens water-soluble (WS) protein was prepared from lenses collected from a 55-year-old patient (NDRI, Philadelphia, PA). The lenses were homogenized, and the WS protein was separated by centrifugation at $10,000\times g$ for 10 min and then dialyzed against 50 mM phosphate buffer, pH 7.6, overnight at 4°C.

Fig. 1 Chemical structures of UDCA and TUDCA

Recombinant human crystallins (α A-, α B-, β B2-, and γ C-crystallin) were generated by a prokaryotic expression system and purified by liquid chromatography as described previously (Sun et al. 1997; Fu and Liang 2002). Insulin was purchased from Sigma-Aldrich, St. Louis, MO.

Thermal aggregation

Protein samples (~ 0.1 mg/ml) were heated to between 60°C and 65°C; protein aggregation was measured with increasing light scattering (aggregation) in the presence or absence of a small amount of chemical chaperone compound (1–10 mM of either UDCA or TUDCA) using a spectrofluorometer set at Ex/Em=400/400 nm (Fu and Liang 2002).

Protein blot analysis

The WS proteins and crystallins (10–20 mg/ml) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred onto nitrocellulose filters, followed by blocking for 1 h in a buffer containing 5% nonfat milk. The primary polyclonal antibody specific to human α B-crystallin (Sun et al. 1997) was applied overnight at 4°C, and the alkaline phosphatase (AP)-conjugated secondary antibody was added and incubated at room temperature for 1 h. The filters were incubated with AP color development reagents.

Materials

TUDCA (Tokyo Chemical Industry Co. Ltd (TCI); Toshima-Ku, Tokyo, Japan) and UDCA (Sigma Chemicals, Atlanta, GA, USA) were purchased from commercial vendors.

Results

UDCA and TUDCA were protected against thermal aggregation of human WS proteins

WS protein samples (0.17 mg/ml) were used throughout the experiments, and light scattering was measured with a spectrofluorometer at 60°C. Major components of the WS proteins are crystallins and comprise 16 different structural proteins. Increased light scattering suggested aggregation of WS proteins. Initially, scattering intensity remained at a minimum value for 5–8 min but increased steeply over the next 10–30 min, finally reaching a plateau at 60 min. In the presence of TUDCA (1.8 mM or 3.6 mM; Fig. 2a) or UDCA (2.4 mM or 4.8 mM; Fig. 2b), light scattering intensities were significantly suppressed. These results suggest that both TUDCA and UDCA are potent suppressors of thermal aggregation of lens WS proteins.

Fig. 2 Suppression of thermal aggregation of human water-soluble protein (WS) at 60°C by **a** TUDCA and **b** UDCA. Increased light scattering or aggregation of the mixtures of WS proteins (0.17 mg/ml) and either TUDCA or UDCA at two different concentrations was detected using a spectrofluorometer set with Ex/Em at 400/400 nm. WS (0.17 mg/ml) alone; WS+TUDCA (1.8 mM) or WS+UDCA (2.4 mM), and WS+TUDCA (3.6 mM) or WS+UDCA (4.8 mM)

Our SDS-PAGE and western blot clearly indicate the presence of α B-crystallin in human WS proteins prepared from 55-year-old human lenses (Fig. 3), but we did not determine exactly how much α -crystallin is present in our WS preparation. It has been reported that α -crystallin decreases with age (McFall-Ngai et al. 1985), and young human lenses contain about 28% α A/ α B-crystallin (Lampi et al. 1997). Therefore, it is reasonable to suggest that our WS preparations contain some levels of α A/ α B-crystallin, the majority is β - and γ -crystallins.

UDCA and TUDCA did not suppress thermal aggregation of each individual recombinant crystallin

We speculated that UDCA and TUDCA also affect thermal aggregation of recombinant β B2-crystallin and γ C-crystallins. When β B2-crystallin (0.11 mg/ml) was mixed with either UDCA (2.1 mM) or TUDCA (2.4 mM) at 60°C,

Fig. 3 Protein blot analysis of human lens WS protein. An antibody specific to human α B-crystallin was used. Lane 1 markers from top 107, 94, 52, 37, 28, and 18 kDa, lane 2 α B-crystallin, lane 3 calf lens WS proteins, and lane 4 human WS protein

increased light scattering or aggregation was observed for UDCA over 60 min (Fig. 4a). The results suggest that UDCA, but not TUDCA, significantly stimulated aggregation of β B2-crystallin.

Similar results were obtained for γ C-crystallin (0.062 mg/ml) with either TUDCA (1.8 mM) or UDCA (2.4 mM; Fig. 4b); UDCA, but not TUDCA, significantly promoted the thermal aggregation of γ C-crystallin. These results clearly demonstrated that both UDCA and TUDCA cannot suppress the thermal aggregation of β B2- and γ C-crystallins.

We next sought to determine whether UDCA and TUDCA suppress the thermal aggregation of α A- and α B-crystallins. When α B-crystallin (0.14 mg/ml) was mixed with either TUDCA (1.8 mM) or UDCA (1.4 mM), increased light scattering was measured at 65°C (Fig. 5a). Neither UDCA nor TUDCA suppressed the thermal aggregation of α B-crystallin.

We also tested the other α -crystallin (α A-crystallin), which is very thermally stable and cannot be unfolded and aggregated at 60°C (Sun and Liang 1998). In fact, we found that α A-crystallin is not aggregated even at 80°C, but α A/ α B-crystallin at 1:1 ratio can be induced to aggregate at 80°C. The effect of UDCA (4.2 mM) and TUDCA (4.2 mM) on the thermal aggregation of α A/ α B-crystallin (both 0.08 mg/ml) showed that UDCA significantly suppressed protein aggregation, but TUDCA significantly promoted protein aggregation (Fig. 5b). These results suggest that neither UDCA nor TUDCA suppresses protein aggregation of homo-oligomer α B-crystallin, but UDCA can significantly suppress aggregation of the hetero-oligomer α A/ α B-crystallin.

UDCA and TUDCA protect chaperone activity of α -crystallin

Next, we investigated whether the chaperone activity of α -crystallin is protected by UDCA or TUDCA. In the control

Fig. 4 Effects of thermal aggregation of human recombinant β B2- and γ C-crystallin by either TUDCA or UDCA: **a** β B2-crystallin (0.11 mg/ml) was mixed with either TUDCA (2.4 mM) or UDCA (2.1 mM). **b** γ C-crystallin (0.0031 mM) was mixed with either UDCA (2.4 mM) or TUDCA (1.8 mM)

study, we mixed γ C-crystallin (0.062 mg/ml) with increasing amounts of α A-crystallin (0.00, 0.02, or 0.04 mg/ml). As expected, α A-crystallin suppressed γ C-crystallin aggregation, and the extent of suppression increased with the amount of α A-crystallin (Fig. 6a). In a subsequent study, we extended these findings to human WS proteins: (WS+ α A-crystallin+UDCA) and (WS+ α A-crystallin+TUDCA). WS protein aggregation with both (α A-crystallin+UDCA) and (α A-crystallin+TUDCA) was much more strongly suppressed than with α A-crystallin alone (Fig. 6b). The effect of these chemical chaperones on the crystallin activity is at this moment still miniscule, and no quantification is provided in this study.

To confirm that TUDCA protects the chaperone activity of α -crystallin, we studied aggregation of a non-crystallin protein, insulin. We studied the following systems: (insulin+TUDCA), (insulin+ α A/ α B-crystallin), and (insulin+ α A/ α B-

Fig. 5 Effects of thermal aggregation of human recombinant α B- and α A/ α B-crystallin by either TUDCA or UDCA: **a** α B-crystallin (0.14 mg/ml) was mixed with either TUDCA (1.6 mM) or UDCA (1.4 mM), and the solutions were heated at 65°C. **b** α A/ α B-crystallin (0.08 mg/ml each) was mixed with either UDCA (3.4 mM) or TUDCA (4.2 mM), and the solutions were heated at 80°C

crystallin+TUDCA). They all suppressed insulin aggregation, but suppression was greater for (α A/ α B-crystallin+TUDCA) than with α A/ α B-crystallin alone (Fig. 7). These results suggest that the chaperone activity of α -crystallin was protected by TUDCA.

Discussion

Although the thermal aggregation of human lens WS proteins was suppressed by a high concentration of UDCA and TUDCA, these compounds did not suppress, but rather stimulated the thermal aggregation of each individual crystallin, including α A-, α B-, β B2, and γ C-crystallins. These results suggest that neither UDCA nor TUDCA suppresses the thermal aggregation of each individual crystallin. Interestingly, while UDCA significantly suppresses aggregation of α A/ α B (1/1)-crystallin, TUDCA

Fig. 6 Effects on aggregation of either γ C-crystallin by α A-crystallin or human WS by α A-crystallin and UDCA or TUDCA: **a** Control γ C-crystallin (0.0031 mM), γ C-crystallin+ α A-crystallin (0.02 mg/ml); γ C-crystallin+ α A-crystallin (0.04 mg/ml); or γ C-crystallin+ α A-crystallin (0.08 mg/ml). Light scattering intensity was measured at 60°C. **b** Human lens WS proteins (0.17 mg/ml); WS+ α A-crystallin (0.04 mg/ml); WS+ α A-crystallin + TUDCA (1.8 mM) or WS+ α A-crystallin+UDCA (2.4 mM). Light scattering intensity was measured at 60°C

shows significant stimulation, indicating that UDCA and TUDCA might alter protein conformation and thus affect chaperone activity. At present, we only observed that UDCA and TUDCA protected or affected the chaperone activity differently, but did not know the underlying mechanisms.

It has been reported that the molecular chaperones (α A/ α B) (3/1)-crystallin are not denatured in thermal treatment at or near 60–65°C for 60 min (Sun and Liang 1998). Therefore, molecular chaperones must be intact in our WS preparation, but the majority of other crystallins are thermally aggregated by treatment at 60°C or 65°C for 60 min (Fig. 4). This agrees with the reports that the hetero-oligomer of α A/ α B (3/1)-crystallin is the most stable

Fig. 7 Effects on DTT-induced insulin aggregation by TUDCA and a mixture of TUDCA and α -crystallin: Control, insulin (0.5 ml of 1 mg/ml)+DTT (0.2 ml of 2 mM); Insulin+DTT+TUDCA (3.9 mM); Insulin+DTT+ α A/ α B (3/1, 0.15 mg/ml); and Insulin+DTT+ α A/ α B (3/1, 0.15 mg/ml)+TUDCA (3.3 mM). Light scattering intensity was measured at 37°C

complex and has the highest chaperone activity (Sun and Liang 1998; McFall-Ngai et al. 1985). The observation of aggregation of the lens WS protein is because of low levels of α -crystallin; its chaperone activity is not sufficient to suppress the aggregation of other crystallins. We believe that the protein aggregation occurs only when chaperone concentration is not sufficiently high or partially lost chaperonic activity. Our results, however, indicate that addition of either UDCA or TUDCA to the WS proteins protected or preserved chaperone activity of α -crystallin and resulted in effective protection (Fig. 2), implying that the old lens may remain clear despite decreased α -crystallin because of protected chaperone activity by bile acids.

Oxysterols or bile acids are oxidation products of cholesterol; they are water-soluble, are released gradually from the lens, and may be important in the maintenance of the chaperone activity of α -crystallin. Furthermore, α -crystallin is attached to the plasma membrane of the lens fiber cells (Boyle and Takemoto 1996; Mulders et al. 1985; Liang and Li 1992), suggesting that the microenvironment of the α -crystallin may have a high concentration of bile acids. The age-related decrease of bile acids may thus play a role in age-related cataracts because the amount of bile acid is insufficient to protect the chaperone activity of α -crystallin. Interestingly, pantethine (a dimeric form of pantothenic acid or vitamin B5) is used as a dietary supplement in managing cholesterol levels; a high concentration of pantethine (10–20 mM) also enhances the chaperone-like activity of bovine α -crystallin (Clark and Huang 1996). We suspect that bile acids may have the similar mechanism as pantethine in modulating the chaperone activity of α -crystallin.

The protection of the chaperone activity of α -crystallin was also demonstrated by insulin aggregation assay. This observation is important because thermal aggregation is non-physiological; human lenses are seldom subjected to high temperatures. The fact that insulin was protected from aggregation at low temperatures further reinforces the important role of bile acids in anti-aggregation activity.

In conclusion, our results clearly show suppression of protein aggregation by bile acids through enhancement of α -crystallin chaperone activity; its biological significance is further supported by our recent findings that treatment of TUDCA suppressed the UPR and delayed LEC death in galactose-fed rat lenses (Mulhern et al. 2007), suggesting that bile acids may also enhance and retain the chaperone activity of Bip in the cholesterol-producing ER membrane (Ridsdale et al. 2006; Iddon et al. 2001). Bip is a chaperone protein in the ER of LECs that recognizes unfolded proteins and suppresses prolonged ER stress. Further study may shed further light on our speculations on the protective capability of bile acids against aggregation.

Acknowledgments The authors are grateful to Dr. J. Horwitz for his valuable suggestions regarding chemical chaperones. This work was supported in part by the RPB, funding from the Department of Ophthalmology, and NIH grants EY013968 (JL) and EY0180172 (TS).

Conflict of interest There are no conflicts of interest among the authors of the manuscript.

References

- Bernstein H, Bernstein C, Payne CM, Dvorakova K, Garewal H (2005) Bile acids as carcinogens in human gastrointestinal cancers. *Mutat Res* 589(1):47–65
- Boyle DL, Takemoto L (1996) EM immunolocalization of α -crystallins: association with the plasma membrane from normal and cataractous human lenses. *Curr Eye Res* 15(5):577–582
- Clark JI, Huang QL (1996) Modulation of the chaperone-like activity of bovine α -crystallin. *Proc Natl Acad Sci USA* 93(26):15185–15189
- Ellis RJ, Pinheiro TJ (2002) Medicine: danger—misfolding proteins. *Nature* 416(6880):483–484
- Fu L, Liang JJ (2002) Conformational change and destabilization of cataract γ -crystallin T5P mutant. *FEBS Lett* 513(2–3):213–216
- Girao H, Mota MC, Ramalho J, Pereira P (1998) Cholesterol oxides accumulate in human cataracts. *Exp Eye Res* 66(5):645–652
- Girao H, Shang F, Pereira P (2003) 7-Ketocholesterol stimulates differentiation of lens epithelial cells. *Mol Vis* 9:497–501
- Hanukoglu I (2006) Antioxidant protective mechanisms against reactive oxygen species (ROS) generated by mitochondrial P450 systems in steroidogenic cells. *Drug Metab Rev* 38(1–2):171–196
- Iddon CR, Wilkinson J, Bennett AJ, Bennett J, Salter AM, Higgins JA (2001) A role for smooth endoplasmic reticulum membrane cholesterol ester in determining the intracellular location and regulation of sterol-regulatory-element-binding protein-2. *Biochem J* 358(Pt 2):415–422
- Ikesugi K, Yamamoto R, Mulhern ML, Shinohara T (2006) Role of the unfolded protein response (UPR) in cataract formation. *Exp Eye Res* 83(3):508–516
- Lampi KJ, Ma Z, Shih M, Shearer TR, Smith JB, Smith DL, David LL (1997) Sequence analysis of β A3, β B3, and β A4 crystallins completes the identification of the major proteins in young human lens. *J Biol Chem* 272(4):2268–2275
- Li LK, So L, Spector A (1985) Membrane cholesterol and phospholipid in consecutive concentric sections of human lenses. *J Lipid Res* 26(5):600–609
- Liang JJ, Li XY (1992) Spectroscopic studies on the interaction of calf lens membranes with crystallins. *Exp Eye Res* 54(5):719–724
- McFall-Ngai MJ, Ding LL, Takemoto LJ, Horwitz J (1985) Spatial and temporal mapping of the age-related changes in human lens crystallins. *Exp Eye Res* 41(6):745–758
- Mulders JW, Stokkermans J, Leunissen JA, Benedetti EL, Bloemendal H, de Jong WW (1985) Interaction of α -crystallin with lens plasma membranes. Affinity for MP26. *Eur J Biochem* 152(3):721–728
- Mulhern ML, Madson CJ, Danford A, Ikesugi K, Kador PF, Shinohara T (2006) The unfolded protein response in lens epithelial cells from galactosemic rat lenses. *Invest Ophthalmol Vis Sci* 47(9):3951–3959
- Mulhern ML, Madson CJ, Kador PF, Randazzo J, Shinohara T (2007) Cellular osmolytes reduce lens epithelial cell death and alleviate cataract formation in galactosemic rats. *Mol Vis* 13:1397–1405
- Perlmutter DH (2002) Chemical chaperones: a pharmacological strategy for disorders of protein folding and trafficking. *Pediatr Res* 52(6):832–836
- Ridsdale A, Denis M, Gougeon PY, Ngsee JK, Presley JF, Zha X (2006) Cholesterol is required for efficient endoplasmic reticulum-to-Golgi transport of secretory membrane proteins. *Mol Biol Cell* 17(4):1593–1605
- Sun TX, Liang JJ (1998) Intermolecular exchange and stabilization of recombinant human α A- and α B-crystallin. *J Biol Chem* 273(1):286–290
- Sun TX, Das BK, Liang JJ (1997) Conformational and functional differences between recombinant human lens α A- and α B-crystallin. *J Biol Chem* 272(10):6220–6225
- Welch WJ, Brown CR (1996) Influence of molecular and chemical chaperones on protein folding. *Cell Stress Chaperones* 1(2):109–115
- Zelenka PS (1984) Lens lipids. *Curr Eye Res* 3(11):1337–1359