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Effect of Methylglyoxal Modification and Phosphorylation on the Chaperone and Anti-Apoptotic Properties of Heat Shock Protein 27

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Abstract Heat shock protein 27 (Hsp27) is a stress-inducible protein in cells that functions as a molecular chaperone and also as an anti-apoptotic protein. Methylglyoxal (MGO) is a reactive dicarbonyl compound produced from cellular glycolytic intermediates that reacts non-enzymatically with proteins to form products such as argpyrimidine. We found considerable amount of Hsp27 in phosphorylated form (pHsp27) in human cataractous lenses. pHsp27 was the major argpyrimidine-modified protein in brunescent cataractous lenses. Modification by MGO enhanced the chaperone function of both pHsp27 and native Hsp27, but the effect on Hsp27 was at least three-times greater than on pHsp27. Phosphorylation of Hsp27 abolished its chaperone function. Transfer of Hsp27 using a cationic lipid inhibited staurosporine (SP)-induced apoptotic cell death by 53% in a human lens epithelial cell line (HLE B-3). MGO-modified Hsp27 had an even greater effect (62% inhibition). SP-induced reactive oxygen species in HLE-B3 cells was significantly lower in cells transferred with MGO-modified Hsp27 when compared to native Hsp27. In vitro incubation experiments showed that MGO-modified Hsp27 reduced the activity of caspase-9, and MGO-modified pHsp27 reduced activities of both caspase-9 and caspase-3. Based on these results, we propose that Hsp27 becomes a better anti-apoptotic protein after modification by MGO, which may be due to multiple mechanisms that include enhancement of chaperone function, reduction in oxidative stress, and inhibition of activity of caspases. Our results suggest that MGO modification and phosphorylation of Hsp27 may have important consequences for lens transparency and cataract development. J. Cell. Biochem. 99: 279–291, 2006.

Key words: heat shock protein 27; methylglyoxal; apoptosis; chaperone; lens epithelial cells

Heat shock protein 27 (Hsp27) belongs to the family of small heat shock proteins. It is a stress-inducible protein that functions as a molecular chaperone and an anti-apoptotic protein in cells. The anti-apoptotic action of Hsp27 has been ascribed to its interaction with proteins directly or indirectly involved in apoptosis. Possible mechanisms include inhibition of mitochondrial release of cytochrome c [Paul et al., 2002], inhibition of cytochrome c-dependent activation

Abbreviations used: Hsp27, heat shock protein 27; pHsp27, phosphorylated Hsp27; MGO, methylglyoxal; MODIC, N^6-(2-{[(4*S*)-4-ammonio-5-oxido-5-oxopentyl]amino}-5-methyl-3,5-dihydro-4*H*-imidazol-4-ylidene)-L-lysinate; MOLD, 6-{1-[(5*S*)-5-ammonio-6-oxido-6-oxohexyl]-4-methylimidazolium-3-yl}-L-norleucinate; MAPKAP kinase-2, mitogen activated protein kinase kinase-2; SP, staurosporine; WS, water soluble proteins; WI, water insoluble proteins; CS, citrate synthase; BLEC, bovine lens epithelial cells; ROS, reactive oxygen species; AMC, 7-amino-4-methylcoumarin; AFC, 7-amino-4-trifluoromethylcoumarin; CM-H_2DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; CNBr, cyanogen bromide; TUNEL, terminal dUTP nick-end labeling; FADD, Fas-associated death domain; Bid, BH3-interacting domain death agonist; DD, death domain; DED, death effector domain; DISC, death-inducing signaling Complex; Apaf-1, apoptosis-activating-factor-1.

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of procaspase-9 [Garrido et al., 1999] direct interactions with cytochrome c and procaspase-3 [Bruey et al., 2000; Concannon et al., 2001] and interaction with actin and inhibition of its polymerization [Schneider et al., 1998]. Hsp27 undergoes phosphorylation in response to stress, which results in deaggregation of its usual polymeric state [Kato et al., 1994]. Phosphorylation mediated by MAPKAP kinase-2 [Rogalla et al., 1999] is necessary for some anti-apoptotic functions of Hsp27 [Charette et al., 2000; Benn et al., 2002], although phosphorylation drastically reduces its chaperone function [Rogalla et al., 1999].

Alpha-crystallin is the major small heat shock protein in the human lens. Compared to α-crystallin, Hsp27 is found in much smaller amounts in the lens. Hsp27 and α-crystallin share significant sequence homology [Singh et al., 1996] and can interact with each other through subunit exchange [Boelens et al., 1998]. The significance of such an interaction is not understood, although in the case of one mutant αB-crystallin (R120G), association with Hsp27 is thought to reduce its translocation into cellular inclusion bodies [Ito et al., 2003].

Human lens proteins are long-lived and thus undergo numerous physico-chemical changes during aging and cataract formation. Several mechanisms are involved in such changes, one of which is the Maillard reaction of protein amino groups on lysine and arginine residues with carbonyl compounds. Methylglyoxal (MGO), a dicarbonyl compound is a byproduct in the glycolytic pathway. It is a major Maillard reaction initiator in tissue and cellular proteins [Nagaraj et al., 1996; Karachalias et al., 2003], including proteins of the lens. MGO concentrations in the lens are significantly higher than in plasma [Thornalley, 1993]. Several specific MGO-derived Maillard reaction products are found in the lens, including argpyrimidine, hydroimidazolones, N^6-(2-{[(4*S*)-4-ammonio-5-oxido-5-oxopentyl]amino}-5-methyl-3,5-dihydro-4*H*-imidazol-4-ylidene)-L-lysinate (MODIC) and 6-{1-[(5*S*)-5-ammonio-6-oxido-6-oxohexyl]-4-methylimidazolium-3-yl}-L-norleucinate (MOLD) [Padayatti et al., 2001b; Biemel et al., 2002; Ahmed et al., 2003].

Hsp27 is particularly vulnerable to MGO modification. In kidney mesangial cells, Hsp27 is the primary target of MGO modification under hyperglycemic conditions [Padival et al., 2003]. Sakamoto et al. [2002] found that MGO modifies Hsp27 at the C-terminal R188 in 293T and HT1080 cells. We have previously reported that MGO modification enhanced the chaperone function of Hsp27 [Nagaraj et al., 2003]. These observations prompted us to undertake a detailed study of how MGO modification and/or phosphorylation influences the chaperone and anti-apoptotic properties of Hsp27 and its interaction with α-crystallin in lens epithelial cells.

MATERIALS AND METHODS

Bovine serum albumin (BSA), dithiothreitol (DTT), horseradish peroxidase-linked anti-mouse IgG, 3,3′,5,5′-tetramethylbenzidine and MGO were obtained from Sigma Chemical Co. (St. Louis, MO). MGO was purified by distillation under low pressure and quantified as described by Chaplen et al. [1996]. Human lenses were obtained from the Cleveland Eye Bank (Cleveland, OH) and from the National Disease Research Interchange (Philadelphia, PA). Human recombinant Hsp27, antibodies to human Hsp27 and α-crystallin were obtained from Stressgen Biotechnologies Corp. (Victoria, BC, Canada). Hsp27 monoclonal antibody, pHsp27 (Ser82) polyclonal antibody, and horseradish peroxidase-linked anti-rabbit IgG were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Bovine α-crystallin was purified from calf lenses as described previously [Chellan and Nagaraj, 2001]. Recombinant human αA- and αB-crystallins were a kind gift from Dr. J. Mark Petrash, University of Washington, St. Louis, MO.

Preparation of Human Lens Proteins

Each lens was homogenized in a motor-driven homogenizer in 2.0 ml of 50 mM Tris-HCl buffer (pH 7.4) containing 0.9% NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol and 1 mM PMSF. The homogenized samples were centrifuged at 20,000*g* for 30 min at 4°C to yield a water-soluble (WS) supernatant and a water-insoluble (WI) pellet. The WS fraction was dialyzed for 24 h against the extraction buffer.

Phosphorylation of Hsp27 by MAPKAP Kinase-2 In Vitro

Phosphorylation of Hsp27 was performed according to a previously described procedure [Rogalla et al., 1999]. Briefly, 200 pmol of

recombinant Hsp27 was incubated at 30°C for 3 h with 60 mU of MAPKAP kinase-2 (Upstate Biotechnology, Lake Placid, NY) and 10 nmol of ATP in a reaction mixture (50 μl) containing 50 mM glycerol-phosphate (pH7.4), 0.1 mM EDTA, and 4 mM magnesium acetate. Phosphorylation was confirmed by Western blotting using a polyclonal antibody against pHsp27.

Enzyme-Linked Immunosorbent Assay (ELISA)

Aliquots (100 μl corresponding to 10 μg protein) of the WS fraction from lens homogenates were dispensed into 96-well microtiter plates and incubated for 20 h at 4°C. Unbound proteins were removed by washing with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBS-Tween). BSA (1%) in PBS-Tween was added (200 μl) to each well, and incubation was continued for 30 min at 37°C. The wells were then washed once with PBS-Tween. One hundred microliters aliquots of appropriately diluted antibody were added to each well, and incubation was continued for 2 h at 37°C. After discarding the supernatant and washing three times with PBS-Tween, 100 μl of secondary antibody was added. The secondary antibody was either goat anti-mouse or rabbit antibody conjugated to horseradish peroxidase in PBS-Tween. After incubation for 1 h at 37°C, the supernatant was discarded, and the plates were washed three times with PBS-Tween. The reaction was developed by addition of 50 μl of 3,3′,5,5′-tetramethylbenzidine in 0.05 M citrate-phosphate buffer (pH 5.0) and 0.04% hydrogen peroxide to each well. The reaction was terminated by addition of 2 M sulfuric acid, and absorbance at 450 nm was measured in a microplate reader.

Two-Dimensional Gel Electrophoresis

Isoelectric focusing for two-dimensional gel electrophoresis was performed with a PROTEAN IEF system from Bio-Rad Laboratories (Hercules, CA) according to the manufacturer's instructions. Aliquots of human lens proteins (100 μg) were suspended in 185 μl of rehydration buffer that contained 8 M urea, 2% (w/v) CHAPS, 50 mM dithiothreitol (electrophoresis grade), and 0.2% Bio-Lyte carrier ampholytes, pH 5–8. The protein suspension was loaded onto an IPG strip (11-cm, pH 5–8). Isoelectric focusing was carried out for 35,000 V-h. The samples were first equilibrated in 375 mM Tris-buffer (pH 8.8) containing 6 M urea, 30% glycerol, 2% SDS, and 2% DTT and then in the same Tris-urea and glycerol buffer with added 2.5% iodoacetamide. The strips were applied to a vertical 12.5% SDS gel without a stacking gel, and electrophoresis was carried at 200 V. Samples were electrophoretically transferred (100 V, 45 min) to a nitrocellulose membrane. The membrane was fixed in 10% methanol containing 7% acetic acid and developed with SYPRO Ruby protein membrane stain (Bio-Rad). Proteins were imaged using VersaDoc imaging system (Bio-Rad). The membrane was then used for Western blot analysis.

Western Blotting

Proteins from human lenses were separated by SDS–PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 5% non-fat dry milk in PBS-Tween for 1 h followed by incubation at 4°C for 16 h with a monoclonal antibody against Hsp27 (1:1,000 dilution) or a polyclonal antibody against pHsp27 (1:1,000 dilution). The membrane was then washed and incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-mouse antibody (for monoclonals) or horseradish peroxidase-conjugated anti-rabbit antibody (for polyclonal) in PBS-Tween. This incubation was followed by three 10-min washes in PBS-Tween. Protein bands were developed with Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

Immunoprecipitation

Human lens proteins (200 μg) were incubated for 16 h at 4°C with 4 μg of antibody specific to Hsp27 or pHsp27, and were captured by 75 μl gel suspension of protein G-sepharose (Pharmacia Biotech, Uppsala, Sweden) at 4°C for 4 h. After centrifugation the supernatant fraction were subject to direct ELISA using anti-argpyrimidine antibody. The immunoprecipitates were subsequently washed three times with PBS, boiled with the SDS–PAGE sample buffer for 5 min and subjected to Western blot analysis as described above except for using the anti-argpyrimidine antibody (1:9,000 dilution).

Chaperone Assay

Chaperone activity of Hsp27 was assayed in 96-microwell plates using a microplate reader (Model 190, Molecular Devices, Sunnyvale, CA). The total reaction volume was 250 μl.

Citrate synthase (7.5 μg protein in 0.1 M HEPES buffer, pH 7.4) was incubated at 43°C with or without 7.0 μg of Hsp27 or pHsp27, and light scattering was monitored at 360 nm. The efficacy of the chaperone function of Hsp27 was assessed by taking into consideration percent inhibition of protein aggregation at the end of the assay and considering the aggregation of protein alone as 100%.

Effect of MGO Modification of Hsp27 on the Binding to α-Crystallin

Bovine lens α-crystallin was chemically conjugated to CNBr-activated sepharose (Amersham Biosciences, Piscataway, NJ) and then nonspecific binding was blocked with 2% bovine serum albumin in PBS for 30 min at 4°C. One microgram of Hsp27 (MGO modified or unmodified) was incubated at 37°C for 24 h with 20 μg of bovine lens α-crystallin conjugated to CNBr-activated sepharose in 0.1 M phosphate buffer (pH 7.4). The precipitated pellets were washed three times with PBS and subjected to SDS–PAGE followed by immunoblotting with an anti-Hsp27 monoclonal antibody.

Cell Culture

Immortalized human lens epithelial cells (HLE B-3 cells) [Andley et al., 1994] were provided by Dr. Usha Andley, Washington University, St. Louis, MO, were maintained in serum-free minimal essential medium eagle (MEM, Mediatech, Inc., Herndon, VA) with 20% (v/v) fetal bovine serum (FBS), 50 μg/ml gentamicin, and 2 mM L-glutamine. The medium was changed twice a week. Cells between passages 8 and 21 were used for all experiments. Bovine lens epithelial cells (BLEC) were isolated according to the method of Andersson et al. [2000]. The medium was changed twice a week. Generally, these cells were used for experiments between the third to eighth passage.

Treatment of Hsp27 With BioPORTER

MGO-modified or control Hsp27 was treated with BioPORTER per the manufacturer's instructions (Gene Therapy System, Inc., San Diego, CA). Briefly, the BioPORTER reagent was suspended in methanol and 15 μl aliquots were air dried in 0.5 ml Eppendorf tubes. Hundred microliters of PBS containing 7.5 μg protein was added to each tube, thoroughly mixed by vortexing for 15 s, and then incubated at room temperature for 5 min.

Effect of Transfer of Hsp27 on Staurosporine (SP)-Induced Apoptosis in Lens Epithelial Cells

HLE B-3 cells that were 80%–90% confluent or BLEC that were 70%–90% confluent were incubated in 12-well culture dishes for 3.5 h at 37°C/5% CO_2 in 500 μl of serum free-MEM or serum free-MEM containing BioPORTER complexed with 7.5 μg MGO-modified or control Hsp27. After incubation, the cells were washed with PBS and then incubated for 3 h in serum-free-MEM with or without 1 μM SP (Kamiya Biomedical company, Seattle, WA).

DNA Fragmentation Detected by TUNEL (TdT-Mediated dUTP Nick End Labeling)

After incubation, DNA fragmentation was detected with an in situ Cell Death detection kit (Roche Diagnostics, Co., Indianapolis, IN). The cells were washed twice in PBS and fixed with 4% paraformaldehyde in PBS for 1 h at room temperature. All samples were rinsed with PBS and permeabilized on ice in a solution of 0.1% Triton-X100 and 0.1% sodium citrate for 5 min. After washing to remove the detergent 50 μl of TUNEL reaction mixture was added to each sample, and incubation was continued in the dark in a humidified atmosphere for 90 min at 37°C. The samples were analyzed by viewing under a fluorescence microscope.

Fluorescence-Activated Cell Sorter (FACS) Analysis for FITC-Annexin V Binding

After treatment of HLE B-3 cells as described above, they were detached from the culture plates by treating with trypsin in the cultured medium. The cells were then centrifuged, washed with 1.0 ml cold Hank's balanced salt solution (HBSS) containing 2% BSA, resuspended in 100 μl of Annexin V and incubated for 15 min at room temperature. Binding buffer (400 μl) was added, and the cell populations were analyzed by flow cytometry (FACS, Becton Dickinson Immunocytometry Systems, Braintree, MA). The number of apoptotic cells was expressed as a percent of the total cells in each sample.

Intracellular Reactive Oxygen Species (ROS)

HLE B-3 cells in MEM containing 20% FBS were cultured in six-well plates. When cells reached 80% confluence, the adherent cells were transferred into new plates containing one of the following: BioPORTER alone, BioPORTER

with 15 μg of Hsp27, and BioPORTER with 15 μg of MGO modified Hsp27 in serum-free MEM. The cells were incubated for 4 h at 37°C with shaking. Then the cells were washed and 1 μM SP in MEM (serum free) was added. Incubation was continued for 3 h at 37°C. After the final incubation, adherent cells (approximately 1×10^5 cells) were loaded with 10 μM 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA, added in 1.0 ml HBSS) by incubation with shaking for 45 min at 37°C. The samples were centrifuged at 200*g* for 5 min at room temperature, the supernatant was discarded, and 800 μl of HBSS was added to the cell pellet. Fluorescence of this final cell suspension was measured at 530 nm (excitation-480 nm) with a microplate fluorescence reader.

Assay of Caspases 3, 8, and 9

Caspase-3 was assayed as described by Lopez-Hernandez et al. [2003] with slight modifications. Human recombinant caspase-3 (20 μg, approximately 200 U, Calbiochem, EMD Biosciences, Inc., San Diego, CA) was incubated at 37°C in 20 mM HEPES buffer (pH 7.4) containing 100 mM NaCl, 5 mM DTT, and 50 μM acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC, Calbiochem, EMD Biosciences, Inc., San Diego, CA) in the presence or absence of 750 ng of native and MGO-modified Hsp27 or pHsp27. The fluorescent product was measured at 505 nm (excitation at 400 nm). Human recombinant caspase-8 and caspase-9 (780 ng) (Calbiochem, EMD Biosciences, Inc., San Diego, CA) were incubated with 750 ng of native or MGO-modified Hsp27, MGO-modified or unmodified pHsp27 at 37°C in 100 mM HEPES buffer (pH 7.4) containing 10% glycerol, 0.5 mM EDTA, 1 mM DTT, and either 50 μM acetyl-Ile-Glu-Thr-Asp-7-amino-4-methylcoumarin (Ac-IETD-AMC) for caspase-8 or 20 μM acetyl-Leu-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin (Ac-LEHD-AFC) for caspase-9 (Calbiochem, EMD Biosciences, Inc., San Diego, CA) [Garrido et al., 1999]. The activity of caspase-8 was estimated by measuring fluorescence at 460 nm following excitation at 355 nm; for caspase-9 excitation was at 400 nm and emission was measured at 505 nm. Native and MGO-modified Hsp27 or pHsp27 were incubated with fluorogenic substrates to provide negative controls.

Statistics

The results are presented as mean ± SD. Statistical analyses were made by Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Our previous studies with rat mesangial cells indicated that argpyrimidine forms preferentially on Hsp27 [Padival et al., 2003]. We noted the immunoreactivity of a ~27 kDa protein in human lenses identified by an antibody to argpyrimidine [Padayatti et al., 2001b]. Assuming that this protein was probably Hsp27, we used 2-D electrophoresis and Western blotting to confirm its identity. Since Hsp27and α-crystallin share significant sequence homology, there was a possibility that antibodies against Hsp27 and pHsp27 might cross react with α-crystallin. Accordingly, we tested the specificity of our antibodies and found that the Hsp27 monoclonal antibody reacted with both Hsp27 and pHsp27, but the pHsp27 polyclonal antibody reacted only with pHsp27. Neither antibody reacted with human recombinant αA- or αB-crystallins (data not shown).

Two-dimensional electrophoresis on a pH strip that focuses proteins between pH 5.0 and 8.0 revealed a number of lens proteins with molecular weights between 20 and 30 kDa. Figure 1A shows the distribution of proteins in the WS fraction from the lens of a young donor (27 years old). When we used the monoclonal antibody for Hsp27 in 2-D Western blots, we found that the antibody reacted with several proteins of molecular weight between 24 and 27 kDa (Fig. 1B). Most proteins that reacted with antibody to Hsp27 reacted with an antibody to pHsp27 (Fig. 1C). In a lens from an older donor (76 years old), immunoreactivity for Hsp27 and pHsp27 (Fig. 1E,F) was more diffuse than that of the young lens. Results obtained with a highly pigmented cataractous lens from a 66-year-old donor were difficult to interpret. Although, proteins between 20 and 30 kDa were clearly visible, higher molecular weight proteins were poorly separated and appeared as streaks (Fig. 1G). The antibody against Hsp27 failed to show immunoreactivity, and the antibody against pHsp27 showed weak immunoreactivity (Fig. 1H). Taken together, these results suggest that in human lenses Hsp27

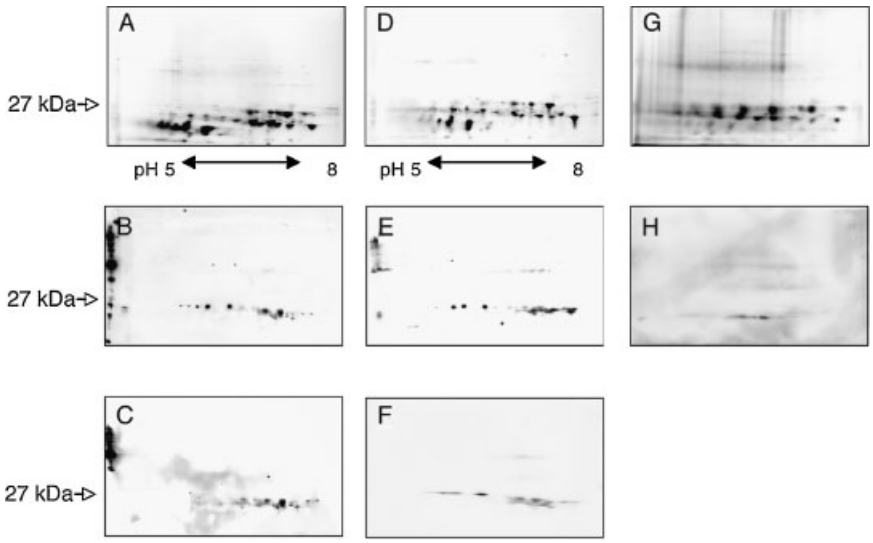

Fig. 1. Two-dimensional (2-D) electrophoresis and Western blot analysis of WS from human lens proteins. WS fraction from a young donor (27 years,) shown in **Panels A–C**; from aged donor (76 years) shown in **Panels D–F**; from a highly pigmented cataractous lens (66-year-old donor) shown in **Panels G** and **H**. Proteins stained with SYPRO Ruby shown in panels A, D, and G. Proteins were immunoblotted with antibodies specific for Hsp27 (1:1,000 dilution)(B and E) or pHsp27 (1:1,000 dilution) (C, F, and H).

occurs in multiple forms, possibly phosphorylated at serine residues.

To confirm the effect of age and cataract on the content and phosphorylation of Hsp27, we used direct ELISAs with antibodies specific for Hsp27 and pHsp27. At least four whole lenses each from young, middle aged, and aged donors as well as cataractous lenses were processed, and their WS fractions tested. As shown in Figure 2, Hsp27 and pHsp27 did not change significantly between young and aged lenses. However, the content of both Hsp27 and pHsp27 were significantly greater in brunescent cataractous lenses ($P < 0.001$). Furthermore, our data suggest that a significant portion of Hsp27 in brunescent cataractous lenses is phosphorylated. After immunoprecitation of WS fractions of brunescent cataractous lenses with anti-pHsp27 polyclonal antibody, the supernatant fraction displayed very little immunoreactivity for Hsp27 (Fig. 3). These data suggest that both the synthesis and phosphorylation of Hsp27 are elevated in brunescent cataractous lenses.

We performed several immuno-precipitation experiments to establish the extent of argpyrimidine modification of Hsp27 in human lenses. WS proteins from young, aged, and brunescent cataractous lenses were immunoprecipitated with either Hsp27 or pHsp27 antibody. First, we measured the effect of aging and cataract on argpyrimidine content. After centrifugation, the supernatant fractions were subjected to direct ELISA using monoclonal antibody against argpyrimidine, a modification of arginine by MGO. This monoclonal antibody is specific for argpyrimidine and it requires 4,6-dimethyl-5-hydroxyl moiety on argpyrimidine for reaction [Padayatti et al., 2001a]. Fifteen micrograms of WS protein was applied to coat each well. Our assays showed no difference in the argpyrimidine content between young and aged lenses. In contrast, the argpyrimidine content was significantly elevated in brunescent cataractous lenses ($P < 0.001$) (Fig. 4A,B). Immunoprecipitation experiments with Hsp27 or pHsp27 antibody revealed an almost complete loss of argpyrimidine immunoreactivity in brunescent cataractous lenses (Fig. 4A,B, closed bar). We also tested for argpyrimidine in immunoprecipitated pellets (by Hsp27 or pHsp27 antibody) with Western blotting. As expected, the pellets from brunescent cataractous lenses displayed stronger immunoreactivity for argpyrimidine compared to young and aged lenses (Fig. 4C). These results indicate that phosphorylated and MGO-modified Hsp27 abounds in brunescent cataractous lenses.

We then examined the effect of phosphorylation/MGO modification on the chaperone function of Hsp27 using citrate synthase (CS) as the target protein. Native Hsp27 inhibited CS

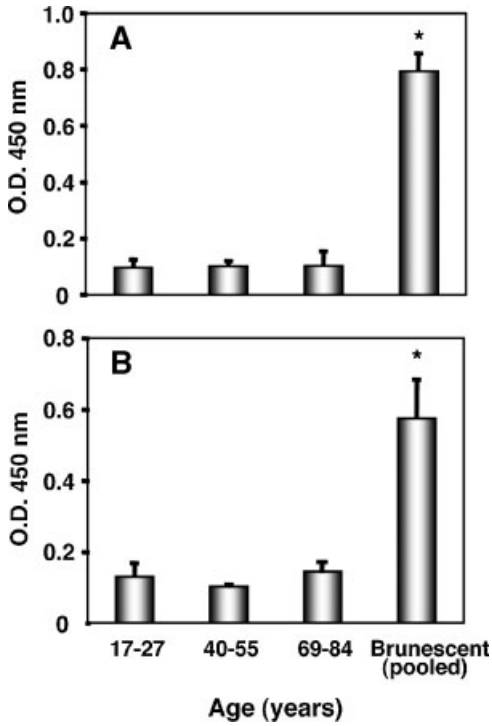

Fig. 2. Hsp27 and pHsp27 in WS fraction from human lens proteins. Hsp27 and pHsp27 were measured with direct ELISAs. Each well was coated with 10 μg protein from individual lenses. After blocking for non-specific binding, the proteins were incubated with indicated antibodies followed by secondary antibodies and substrate. **A**: Hsp27; **B**: pHsp27. At least four lenses were used for each experiment. Brunescent lens proteins (from donors aged 66 to 85 years) were pooled from five lenses. Data shown are mean ± SD $*P < 0.001$ between brunescent and 17–27 years old lenses.

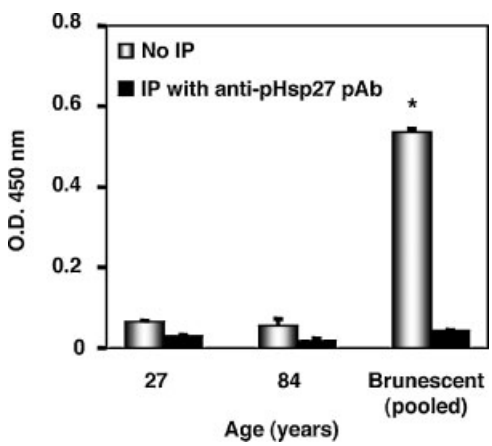

Fig. 3. Phosphorylation of Hsp27. WS fraction was immunoprecipitated (IP) with anti-pHsp27 polyclonal antibody, and supernatant fractions were applied to coat individual wells in the reaction plate. After blocking, the samples were incubated with anti-Hsp27 monoclonal antibody followed by secondary antibody and substrate. Data shown are mean ± SD $*P < 0.001$ between brunescent and the 27 years old lens.

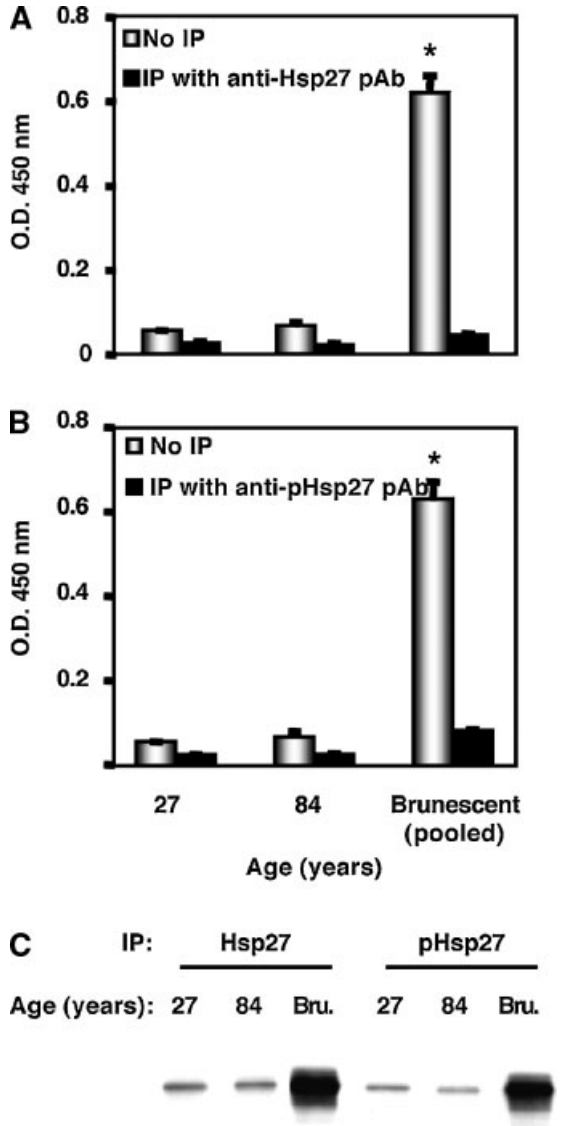

Fig. 4. Argpyrimidine immunoprecipitated with specific antibodies. WS fraction was immunoprecipitated by anti-Hsp27 and anti-pHsp27 antibodies. After centrifugation, supernatant fractions were analyzed by ELISA using a monoclonal antibody for argpyrimidine (1:9,000 dilution) (**A** and **B**). Fifteen micograms of protein was applied to coat each well. The immunoprecipitate pellet of argprimidine-Hsp27 and -pHsp27 were washed three times with PBS and then analyzed by SDS-PAGE followed by Western blotting with anti-argpyrimidine monoclonal antibody (**C**). Data shown are mean ± SD $*P < 0.001$ between brunescent and the 27 year old lens.

aggregation by ~25%. MGO-modification enhanced the chaperone function by ~63%. Phosphorylation resulted in an almost complete loss of the chaperone function of Hsp27 (~7% inhibition of CS aggregation) (Fig. 5). MGO-modification of pHsp27 improved the chaperone function, by 61%, but the overall effect on

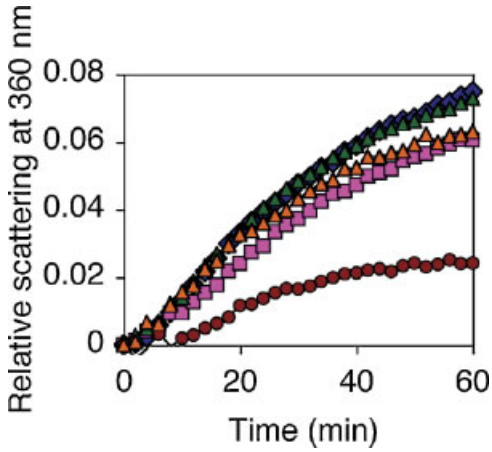

Fig. 5. Effect of phosphorylation and MGO-modification on Hsp27chaperone function Hsp27 and pHsp27 were incubated with MGO at a molar ratio of 1:10 (arginine content of protein: MGO) in phosphate buffer (pH 7.4) at 37°C for 5 days. The chaperone assay used CS as the target protein. ◆, control; ■, Hsp27; ●, MGO-modified Hsp27; ▲, pHsp27; ▲, MGO-modified pHsp27. Data shown are mean of three independent measurements. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

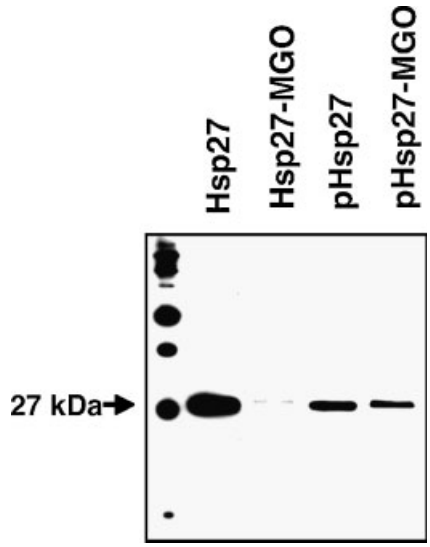

Fig. 6. Effect of MGO modification on interaction of Hsp27 with α-crystallin. Bovine α-crystallin conjugated CNBr-activated Sepharose was incubated with Hsp27, pHsp27, MGO-modified Hsp27, and MGO-modified pHsp27. The precipitated pellets were washed with PBS, separated by SDS–PAGE and immunoblotted with anti-Hsp27 monoclonal antibody.

the inhibition of CS aggregation was close to 21%, which was approximately three times less than that observed with MGO-modified Hsp27.

We also determined the effect of MGO modification of Hsp27 on the binding to α-crystallin. Bovine alpha-crystallin was chemically coupled to CNBr-activated Sepharose. Hsp27 and pHsp27 were modified with 0.1 mM MGO, and both MGO-modified and unmodified proteins were then incubated with the gel. After thoroughly washing three times with PBS, the precipitated pellet was subjected to SDS–PAGE followed by Western blot analysis with anti-Hsp27 antibody. The binding of Hsp27 to α-crystallin was clearly evident in Western blots (Fig. 6). Phosphorylation of Hsp27 considerably reduced this binding. Whereas MGO modification of Hsp27 completely abolished the binding, a similar modification of pHsp27 only reduced its binding to α-crystallin. These results suggest that MGO modification and phosphorylation of Hsp27 prevent/inhibit its interaction with α-crystallin.

To investigate the effect of MGO-modification on the anti-apoptotic property of Hsp27, we compared the protective effect of Hsp27 and MGO-modified Hsp27 on SP-induced apoptosis in HLE B-3 cells and BLE cells. Hsp27 and MGO-modified Hsp27 were introduced into HLE B-3 cells with the BioPORTER reagent, which can fuse directly with the plasma membrane and deliver the captured protein into cells by endocytosis; endosomes release the captured protein into the cytoplasm. The BioPORTER reagent is a cationic lipid mixture that forms complex with proteins and peptides. The complex formed is non-covalent and it therefore does not interfere with the biological activity of the protein. This technique is rapid and relatively uncomplicated. In HLE B-3 cells, apoptosis was assayed by FITC-Annexin V binding (by FACS). Figure 7A shows that SP treatment results in an increase in FITC-Annexin V positive cells. The introduction of Hsp27 reduced apoptotic cells by 53%, and MGO-modified Hsp27 further reduced it to 62% ($P < 0.001$) compared to SP treatment alone. We also analyzed the protective effects of MGO-modification of Hsp27 on apoptosis in BLEC, because BLEC are primary cells, while HLE B-3 cells are transformed cells. The transfer of Hsp27 into the cells decreased SP-induced apoptosis of BLEC by 54% (Fig. 7B). MGO-modified Hsp27 further reduced apoptosis by 62%. We wanted to determine if Hsp27 influenced SP-induced ROS formation in HLE B-3 cells. Figure 7C shows that Hsp27 significantly

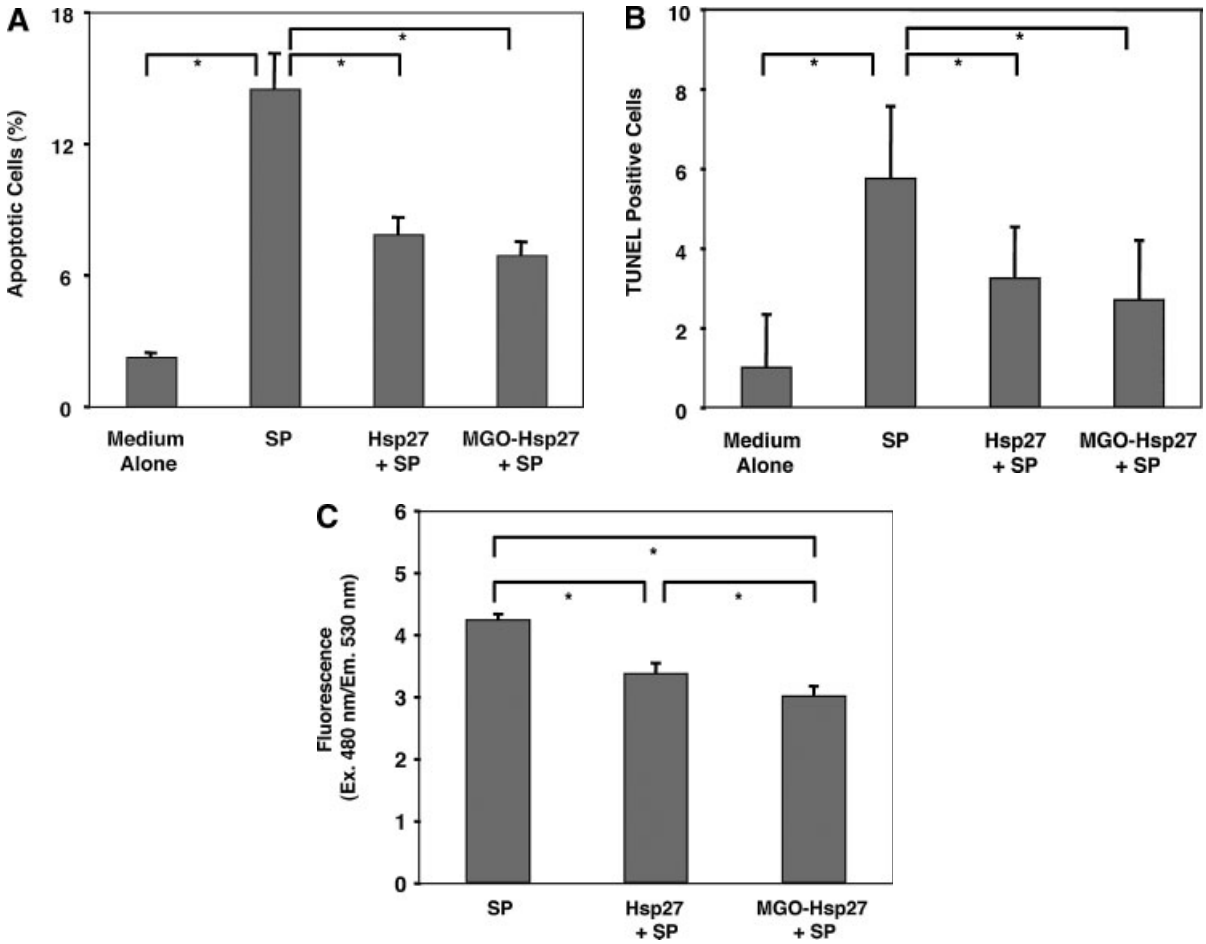

Fig. 7. Effect of MGO modification of Hsp27 on SP-induced apoptosis. Hsp27 or MGO-Hsp27 was introduced into HLE B-3 cells or BLEC with BioPORTER. After 3.5 h incubation, apoptosis was induced by treating the cells with SP for 3 h, and the extent of apoptosis in HLE B-3 was assayed by FACS (FITC-Annexin V binding) (**A**). Early apoptotic BLEC were quantified by TUNEL staining (**B**). Inhibition of SP-induced intracellular ROS formation in HLE B-3 cells by Hsp27 (**C**). ROS was assessed by incubating cells with CM-H_2DCFDA followed by measurement of fluorescence at 530 nm (excitation-480 nm) in a microplate fluorescence reader. Data shown are mean ± SD of three independent measurements. $^*P < 0.001$.

inhibited SP-induced production of ROS in the HLE B-3 cells ($P < 0.001$). Inhibition was on the order of 21%. Remarkably, MGO-modified Hsp27 inhibited the formation of ROS more aggressively, on the order of 30%. These data suggest that MGO-modified Hsp27 provides stronger protection against ROS formation in lens epithelial cells than the native form.

To determine if direct action on caspases could account for the greater inhibition of apoptosis by MGO-modified Hsp27, we measured caspase activity with and without the modified proteins. Human recombinant caspase-3, 8, and 9 were incubated with unmodified and MGO-modified Hsp27 and pHsp27 at 37°C for 0, 1, and 24 h. Enzyme activities were determined by cleavage of specified fluorogenic substrates. Reaction mixtures of unmodified and MGO-modified Hsp27 and pHsp27 with fluorogenic substrates in the absence of caspase were negative controls. Caspase-3 activity was significantly decreased ($P < 0.005$) in the presence of MGO-modified pHsp27 compared to control (Fig. 8A). After 24 h incubation, caspase-3 was almost completely inactivated in the presence of MGO-modified pHsp27 ($P < 0.005$), but was inactivated by 70%–75% in the presence of other types of Hsp27. All types of Hsp27 examined increased the activity of caspase-8, but MGO-modified Hsp27 was the most effective (Fig. 8B, $P < 0.005$). Caspase-9 activity was unaffected by unmodified Hsp27 up to 1 h,

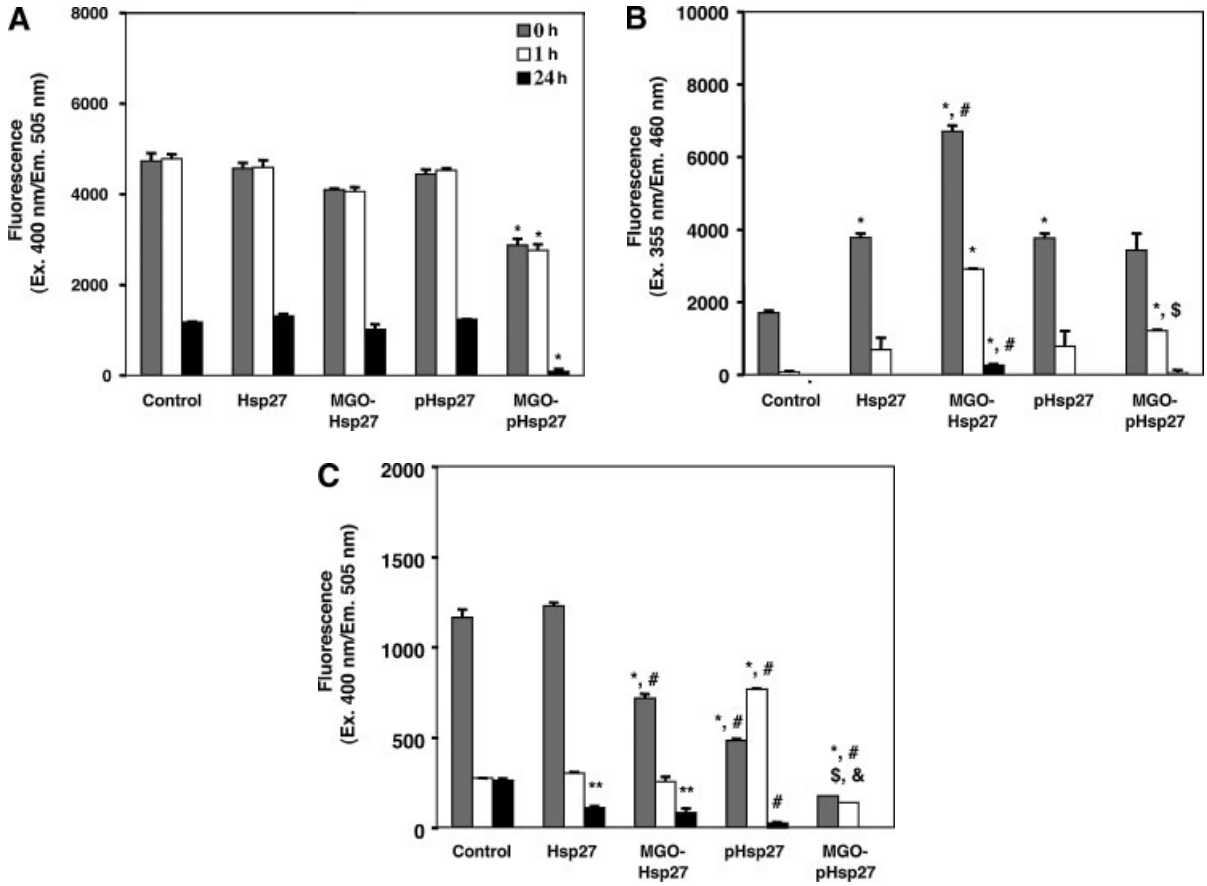

Fig. 8. Effect of Hsp27 on activities of caspase-3, 8, and 9. Human recombinant caspases were incubated with unmodified or MGO-modified Hap27 and pHsp27 at 37°C for 0, 1, and 24 h. Caspase-3 (**A**), caspase-8 (**B**) and caspase-9 (**C**) activities were determined by cleavage of the fluorogenic substrates Ac-DEVD-AFC, Ac-IETD-AMC, and Ac-LEHD-AFC, respectively. The cleavage product AFC was measured at 505 nm (excitation at 400 nm), and release of AMC was measured at 460 nm (excitation at 355 nm). Data shown are mean ± SD of three independent measurements. $^{*}P < 0.005$, statistical difference from Control; $^{**}P < 0.05$, statistical difference from Control; $^{\#}P < 0.005$, statistical difference from Hsp27; $^{\$}P < 0.001$, indicates statistical difference from MGO-Hsp27; $^{\&}P < 0.001$, statistical difference from pHsp27.

but was inhibited to 46% after 24 h (Fig. 8C). MGO-modified Hsp27 suppressed caspase-9 activity by 38.5%, 18%, and 65.1% after 0, 1, and 24 h, respectively. MGO-modified pHsp27 was the most efficacious for inhibiting caspase-9 activity; significant inhibitions ($P < 0.005$) was observed at all three time points of incubation and the inhibition was total after 24 h.

DISCUSSION

We found that phosphorylated Hsp27 is the major argpyrimidine-modified protein in human lenses and that brunescent cataracts have more pHsp27 than normal lenses. Phosphorylation of Hsp27 is a general stress response necessary to prevent actin filament disruption [Huot et al., 1996] and dissociation of Hsp27 from Akt during inhibition of apoptosis [Rane et al., 2003]. Hsp27 phosphorylation is also involved in cell migration [Hedges et al., 1999]. These events may not be relevant for terminally differentiated lens fiber cells that lack organelles, but they might affect homeostasis and survival of lens epithelial cells, and cells in other tissues. Whether MGO-modification of pHsp27 compromises such functions remains to be investigated.

Our finding that increased pHsp27 corresponds to higher argpyrimidine immunoreactivity in brunescent cataractous lenses suggests that an increase in environmental or metabolic stress (e.g., oxidative or other) promotes phosphorylation of Hsp27 along with increased formation of MGO. We do not yet know whether increased oxidative stress, as is well documented in cataracts [Varma et al., 1984; Bando and Obazawa, 1990; Bhat et al., 1991], actually

increases MGO production, but we believe it to be a reasonable possibility. The facts that ascorbate oxidation increases in cataracts [Lohmann et al., 1986; Nagaraj et al., 1991; Linetsky et al., 1999] and ascorbate is a precursor for argpyrimidine support this assumption. Other investigators also noted MGO production in response to increased oxidative stress in cells [Abordo et al., 1999].

The immunioprecipitation and the ELISA results implied that most of the Hsp27 in brunescent cataractous lenses is phosphorylated. The non-reactivity of the pHsp27 antibody against Hsp27 rules out any contamination of Hsp27 in these experiments. We found that phosphorylation of Hsp27 nearly abolishes its chaperone function. MGO modification improved it slightly but was not as effective as native Hsp27. MGO modification would be thus unlikely to restore adequate chaperone function in brunescent cataracts. The combination of lost Hsp27 chaperone function by phosphorylation along with decreased ability to restore it by MGO modification may render Hsp27 dysfunctional in brunescent cataractous lenses.

We considered the possibility that the argpyrimidine antibody reacted with Hsp27 itself, but the following observations indicate otherwise. Sakamoto et al. [2002] measured incorporation of ^{14}C-MGO into proteins of cultured cells and showed that Hsp27 accumulates significant quantities of MGO. They also reported that substitution of R188 with another amino acid by point mutation caused a complete loss of argpyrimidine immunoreactivity. Their findings confirm that the antibody reaction is due to argpyrimidine modification in Hsp27. Just why Hsp27 in human lenses is targeted by MGO remains unclear. One possibility is that arginine residues on the surface of Hsp27 are exposed and readily available for reaction with MGO.

Our studies indicate that MGO-modification of Hsp27 diminishes its binding to α-crystallin. Several investigators noted an interaction of α-crystallin with Hsp27 [Fu and Liang, 2002; Fu and Liang, 2003]. One study reported interaction of Hsp27 amino acids 141 to 186 with α-crystallin [Liu and Welsh, 1999]. The consequence of such interactions is not known. Whether the chaperone function of these small

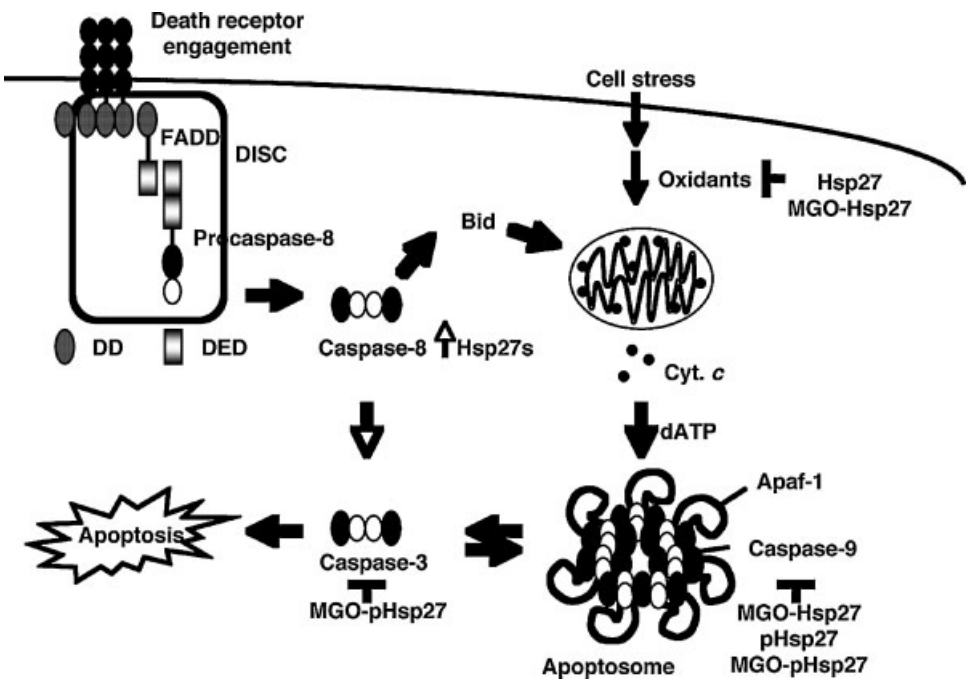

Fig. 9. Possible mechanisms of inhibition of apoptosis in lens epithelial cells by MGO-modified and native Hsp27. MGO-modified Hsp27 might inhibit apoptosis through multiple mechanisms, such as, inhibition of ROS formation, inhibition of caspase-9 and inhibition of and caspase-3. FADD, Fas-associated death domain; Bid, BH3-interacting domain death agonist; DD, death domain; DED, death effector domain; DISC, death-inducing signaling complex; Apaf-1, apoptosis-activating-factor-1.

heat shock proteins is enhanced by binding to α-crystallin remains unclear, but the fact that MGO-modification disrupts the interaction signals a potentially damaging effect in the lens.

Hsp27 protected lens epithelial cells from SP-induced apoptosis. MGO-modified Hsp27 displayed better protection than native Hsp27 against SP-induced apoptosis. Furthermore, our data indicate that MGO-modified Hsp27 inhibits oxidative stress in SP-treated HLE B-3 cells more effectively than native Hsp27. Whether this property confers greater inhibition of apoptosis remains unconfirmed, but it is likely a factor. Hsp27 can prevent apoptosis by regulation of activation of the PI3-K/Akt pathway [Rane et al., 2003] and inhibit cytochrome c-dependent activation of procaspase-9 [Garrido et al., 1999]. Because MGO-modified Hsp27 loses its ability to bind cytochrome c [Padival et al., 2003], we do not believe that this property is a mechanism underlying the inhibition of SP-induced apoptosis. However, we noted that MGO-modified Hsp27 was more effective than the native Hsp27 in lowering SP-induced production of ROS in lens epithelial cells. An increase in ROS levels can perturb the cell redox status, thereby damaging lipids, proteins, and DNA and eventually causing cell death. Taken together, our findings provide strong evidence that MGO-modified Hsp27 protects cells from apoptosis triggered by oxidative stress.

We noted marked inhibition of human recombinant caspase-9 in the presence of MGO-modified Hsp27; the MGO-modified phosphorylated form provided even greater inhibition. In addition, MGO-modified pHsp27 reduced the activity of caspase-3. These data suggest that the anti-apoptotic property of MGO-modified Hsp27 involves multiple mechanisms as shown in Figure 9. Possibly MGO-modified Hsp27 represses apoptosis not only through enhanced chaperone function but also by inhibition of specific caspases.

Our study indicates that MGO modification of Hsp27 has multiple effects. While it decreases its association with α-crystallin, it improved the chaperone and anti-apoptotic functions. The improvement in the anti-apoptotic function is likely to occur through inhibitions of ROS production, and activities of caspases. Our results indicate an interesting possibility that subtle modification of Hsp27 by MGO might enable lens epithelial cells to better cope with stresses that induce apoptosis during aging and in early cataractogenesis.

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REFERENCES

Abordo EA, Minhas HS, Thornalley PJ. 1999. Accumulation of alpha-oxoaldehydes during oxidative stress: A role in cytotoxicity. Biochem Pharmacol 58:641–648.

Ahmed N, Thornalley PJ, Dawczynski J, Franke S, Strobel J, Stein G, Haik GM. 2003. Methylglyoxal-derived hydroimidazolone advanced glycation end-products of human lens proteins. Invest Ophthalmol Vis Sci 44:5287–5292.

Andersson M, Sjostrand J, Petersen A, Honarvar AK, Karlsson JO. 2000. Caspase and proteasome activity during staurosporin-induced apoptosis in lens epithelial cells. Invest Ophthalmol Vis Sci 41:2623–2632.

Andley UP, Rhim JS, Chylack LT, Jr., Fleming TP. 1994. Propagation and immortalization of human lens epithelial cells in culture. Invest Ophthalmol Vis Sci 35:3094–3102.

Bando M, Obazawa H. 1990. Activities of ascorbate free radical reductase and H_2O_2-dependent NADH oxidation in senile cataractous human lenses. Exp Eye Res 50:779–786.

Benn SC, Perrelet D, Kato AC, Scholz J, Decosterd I, Mannion RJ, Bakowska JC, Woolf CJ. 2002. Hsp27 upregulation and phosphorylation is required for injured sensory and motor neuron survival. Neuron 36:45–56.

Bhat KS, John A, Reddy PR, Reddy PS, Reddy VN. 1991. Effect of pigmentation on glutathione redox cycle antioxidant defense in whole as well as different regions of human cataractous lens. Exp Eye Res 52:715– 721.

Biemel KM, Friedl DA, Lederer MO. 2002. Identification and quantification of major maillard cross-links in human serum albumin and lens protein. Evidence for glucosepane as the dominant compound. J Biol Chem 277:24907–24915.

Boelens WC, Croes Y, de Ruwe M, de Reu L, de Jong WW. 1998. Negative charges in the C-terminal domain stabilize the alphaB-crystallin complex. J Biol Chem 273:28085–28090.

Bruey JM, Ducasse C, Bonniaud P, Ravagnan L, Susin SA, Diaz-Latoud C, Gurbuxani S, Arrigo AP, Kroemer G, Solary E, Garrido C. 2000. Hsp27 negatively regulates cell death by interacting with cytochrome c. Nat Cell Biol 2:645–652.

Chaplen FW, Fahl WE, Cameron DC. 1996. Method for determination of free intracellular and extracellular methylglyoxal in animal cells grown in culture. Anal Biochem 238:171–178.

Charette SJ, Lavoie JN, Lambert H, Landry J. 2000. Inhibition of Daxx-mediated apoptosis by heat shock protein 27. Mol Cell Biol 20:7602–7612.

Chellan P, Nagaraj RH. 2001. Early glycation products produce pentosidine cross-links on native proteins. novel

mechanism of pentosidine formation and propagation of glycation. J Biol Chem 276:3895–3903.

Concannon CG, Orrenius S, Samali A. 2001. Hsp27 inhibits cytochrome c-mediated caspase activation by sequestering both pro-caspase-3 and cytochrome c. Gene Expr 9:195–201.

Fu L, Liang JJ. 2002. Detection of protein-protein interactions among lens crystallins in a mammalian two-hybrid system assay. J Biol Chem 277:4255–4260.

Fu L, Liang JJ. 2003. Alteration of protein-protein interactions of congenital cataract crystallin mutants. Invest Ophthalmol Vis Sci 44:1155–1159.

Garrido C, Bruey JM, Fromentin A, Hammann A, Arrigo AP, Solary E. 1999. HSP27 inhibits cytochrome c-dependent activation of procaspase-9. FASEB J 13:2061–2070.

Hedges JC, Dechert MA, Yamboliev IA, Martin JL, Hickey E, Weber LA, Gerthoffer WT. 1999. A role for p38(MAPK)/HSP27 pathway in smooth muscle cell migration. J Biol Chem 274:24211–24219.

Huot J, Houle F, Spitz DR, Landry J. 1996. HSP27 phosphorylation-mediated resistance against actin fragmentation and cell death induced by oxidative stress. Cancer Res 56:273–279.

Ito H, Kamei K, Iwamoto I, Inaguma Y, Tsuzuki M, Kishikawa M, Shimada A, Hosokawa M, Kato K. 2003. Hsp27 suppresses the formation of inclusion bodies induced by expression of R120G alpha B-crystallin, a cause of desmin-related myopathy. Cell Mol Life Sci 60:1217–1223.

Karachalias N, Babaei-Jadidi R, Ahmed N, Thornalley PJ. 2003. Accumulation of fructosyl-lysine and advanced glycation end products in the kidney, retina and peripheral nerve of streptozotocin-induced diabetic rats. Biochem Soc Trans 31:1423–1425.

Kato K, Hasegawa K, Goto S, Inaguma Y. 1994. Dissociation as a result of phosphorylation of an aggregated form of the small stress protein, hsp27. J Biol Chem 269:11274–11278.

Linetsky M, James HL, Ortwerth BJ. 1999. Spontaneous generation of superoxide anion by human lens proteins and by calf lens proteins ascorbylated in vitro. Exp Eye Res 69:239–248.

Liu C, Welsh MJ. 1999. Identification of a site of Hsp27 binding with Hsp27 and alpha B-crystallin as indicated by the yeast two-hybrid system. Biochem Biophys Res Commun 255:256–261.

Lohmann W, Schmehl W, Strobel J. 1986. Nuclear cataract: Oxidative damage to the lens. Exp Eye Res 43:859–862.

Lopez-Hernandez FJ, Ortiz MA, Bayon Y, Piedrafita FJ. 2003. Z-FA-fmk inhibits effector caspases but not initiator caspases 8 and 10, and demonstrates that novel anticancer retinoid-related molecules induce apoptosis via the intrinsic pathway. Mol Cancer Ther 2:255–263.

Nagaraj RH, Sell DR, Prabhakaram M, Ortwerth BJ, Monnier VM. 1991. High correlation between pentosidine protein crosslinks and pigmentation implicates ascorbate oxidation in human lens senescence and cataractogenesis. Proc Natl Acad Sci USA 88:10257–10261.

Nagaraj RH, Shipanova IN, Faust FM. 1996. Protein cross-linking by the Maillard reaction. Isolation, characterization, and in vivo detection of a lysine-lysine cross-link derived from methylglyoxal. J Biol Chem 271:19338–19345.

Nagaraj RH, Oya-Ito T, Padayatti PS, Kumar R, Mehta S, West K, Levison B, Sun J, Crabb JW, Padival AK. 2003. Enhancement of chaperone function of alpha-crystallin by methylglyoxal modification. Biochemistry 42:10746–10755.

Padayatti PS, Jiang C, Glomb MA, Uchida K, Nagaraj RH. 2001a. High concentrations of glucose induce synthesis of argpyrimidine in retinal endothelial cells. Curr Eye Res 23:106–115.

Padayatti PS, Ng AS, Uchida K, Glomb MA, Nagaraj RH. 2001b. Argpyrimidine, a blue fluorophore in human lens proteins: High levels in brunescent cataractous lenses. Invest Ophthalmol Vis Sci 42:1299–1304.

Padival AK, Crabb JW, Nagaraj RH. 2003a. Methylglyoxal modifies heat shock protein 27 in glomerular mesangial cells. FEBS Lett 551:113–118.

Paul C, Manero F, Gonin S, Kretz-Remy C, Virot S, Arrigo AP. 2002. Hsp27 as a negative regulator of cytochrome C release. Mol Cell Biol 22:816–834.

Rane MJ, Pan Y, Singh S, Powell DW, Wu R, Cummins T, Chen Q, McLeish KR, Klein JB. 2003. Heat shock protein 27 controls apoptosis by regulating Akt activation. J Biol Chem 278:27828–27835.

Rogalla T, Ehrnsperger M, Preville X, Kotlyarov A, Lutsch G, Ducasse C, Paul C, Wieske M, Arrigo AP, Buchner J, Gaestel M. 1999. Regulation of Hsp27 oligomerization, chaperone function, and protective activity against oxidative stress/tumor necrosis factor alpha by phosphorylation. J Biol Chem 274:18947–18956.

Sakamoto H, Mashima T, Yamamoto K, Tsuruo T. 2002. Modulation of heat-shock protein 27 (Hsp27) anti-apoptotic activity by methylglyoxal modification. J Biol Chem 277:45770–45775.

Schneider GB, Hamano H, Cooper LF. 1998. In vivo evaluation of hsp27 as an inhibitor of actin polymerization: hsp27 limits actin stress fiber and focal adhesion formation after heat shock. J Cell Physiol 177:575–584.

Singh K, Zewge D, Groth-Vasselli B, Farnsworth PN. 1996. A comparison of structural relationships among alpha-crystallin, human Hsp27, gamma-crystallins and beta B2-crystallin. Int J Biol Macromol 19:227–233.

Thornalley PJ. 1993. The glyoxalase system in health and disease. Mol Aspects Med 14:287–371.

Varma SD, Chand D, Sharma YR, Kuck JF, Jr., Richards RD. 1984. Oxidative stress on lens and cataract formation: Role of light and oxygen. Curr Eye Res 3:35–57.