Upregulation of Heat Shock Protein Expression by Proteasome Inhibition: An Antiapoptotic Mechanism in the Lens

Niranjan Awasthi¹ and B. J. Wagner¹,²

PURPOSE. Studies have shown that proteasome inhibition protects lens epithelial cells (LECs) against interferon (IFN)-γ-induced apoptosis. The present study was conducted to test the hypothesis that proteasome inhibition can protect lens cells against apoptosis by upregulating heat shock protein (HSP) expression.

METHODS. Murine lens epithelial αTN4-1 cells were treated with combinations of 100 U/mL IFN-γ, 10 μM MG132 (proteasome inhibitor), and 100 μM quercetin (HSP inhibitor). mRNA and protein expression were observed by RT-PCR and Western blot analysis, respectively. Caspase activities were measured by using cleavage of colorimetric substrate. Apoptosis was measured by phase-contrast microscopy and flow cytometry.

RESULTS. At the mRNA level, the proteasome inhibitor, MG132, caused a >10-fold increase in HSP27 and a small increase (1.2- to 1.6-fold) in αB-crystallin but no change in HSP70 or -90. At the protein level, a more than twofold increase in HSP27 and -90, a marked increase in HSP70, but no significant change in αB-crystallin, was observed. Downregulation of αA-crystallin by MG132 was observed at both the mRNA and protein levels. MG132 caused no significant change in heat shock factor (HSF)-1, but a more than twofold increase in HSF2 and -4 protein expression. MG132 prevented the IFN-γ-induced increase in caspase-1, -6, and -8 activities. Quercetin decreased MG132-induced expression of HSP27, -70, and -90 by more than 70%, and heat shock factors HSF2 and -4 by more than 65%. Quercetin pretreatment significantly reversed the decrease in caspase-1, -6, and -8 activities and the antiapoptotic effect of MG132 on IFN-γ-treated LECs.

CONCLUSIONS. The antiapoptotic effect of proteasome inhibition of IFN-γ-induced apoptosis in LECs correlates with increased expression of HSPs and inhibition of caspase activities. Inhibition of HSP expression restores caspase activities and abolishes the antiapoptotic effect of proteasome inhibition, implicating HSPs as mediators of the protective effect of proteasome inhibition. (Invest Ophthalmol Vis Sci. 2005;46:2082–2091) DOI:10.1167 iovs.05-0002

Apoptosis, or programmed cell death, is an essential process for normal development, homeostasis, and maintenance of multicellular organisms.¹-³ It has also been implicated in certain pathologic conditions.⁴ Apoptosis can be triggered by a variety of stimuli, including cytokines, hormones, viruses, and toxic insults.

The proteasome is a 700-kDa multicatalytic cytoplasmic and nuclear complex that is responsible for most nonlysosomal protein degradation. It plays a primary role in protein turnover, antigen processing, cell cycle regulation, apoptosis, and other regulatory pathways.⁵-⁷ Studies have shown that the proteasome takes part in apoptosis, based on its capacity to degrade or process certain apoptosis-related regulatory proteins.⁸ The proteasome may have anti- or proapoptotic action, depending on cell type, proliferating activity of cells, type of apoptotic stimuli, availability of growth factors and conditions of treatment.⁹-¹⁰ Proteasome inhibitors have been demonstrated to cause complex effects on programmed cell death, and while inhibition is usually proapoptotic,¹¹-¹³ under certain conditions, it prevents apoptosis triggered by harmful stimuli.¹⁴-¹⁰ In addition, recent publications indicate that proteasome inhibition causes upregulation of heat shock protein (HSP) expression.¹¹-¹³

HSPs are ubiquitous and highly conserved proteins, with expression induced by a variety of physiological and environmental stresses. According to molecular weight, HSPs are classified into four major families: HSP90, -70, and -60 and the small HSPs, such as HSP10, HSP27, αA-crystallin, and αB-crystallin.¹⁴ HSP70, HSP27, αA-crystallin, and αB-crystallin are reported to be antiapoptotic, whereas HSP60 and -10 are proapoptotic. The role of HSP90 in controlling apoptosis is ambiguous and depends on the apoptotic stimulus, but the effect is usually antiapoptotic.¹⁴ Recent publications have indicated that the antiapoptotic action of HSPs is due to an inhibitory effect on caspase activation.¹⁵-¹⁷

Caspases are cysteine proteases that selectively cleave key apoptotic proteins at aspartate residues, thereby switching their function to promote cell death.¹⁸ Caspases are synthesized as proenzymes that are activated by trans- or autoproteolytic cleavage at aspartate residues. Among the 14 caspases identified so far, some act as initiators of apoptosis (caspase-8, -9, and -10), some act as downstream executioners (caspase-3, -6, and -7) and others act as inflammatory agents (caspase-1 and -11).¹⁹ Although caspase-1 is known to play a major role in inflammation, it also has been shown to be involved in apoptosis, including that mediated by IFN-γ and TNF.²⁰-²⁵

The lens epithelium is a single layer of cuboidal cells at the anterior surface of the lens that is important for maintaining metabolic homeostasis and transparency.²⁴ Under normal conditions, lens epithelial cells (LECs) have a long lifespan. Elevated apoptosis of LECs has been reported to be associated with cataractogenesis.²⁵-²⁷ We have reported the induction of lens epithelial cell apoptosis by IFN-γ, which is prevented by proteasome inhibition.²⁰ The present study implicated HSPs as mediators, by proteasome inhibition, of the rescue of lens epithelial cells from IFN-γ–induced apoptosis.

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MATERIALS AND METHODS

Cell Culture and Treatment

The murine lens epithelial cell line, αTN4-1,26 (kindly provided by Paul Russell, National Eye Institute, Bethesda, MD), which responds to IFN-γ treatment by upregulating immunoproteasome subunits similar to IFN-γ-expressing transgenic mice,26 was grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 2 mM glutamine, and gentamicin (100 μg/mL) at 37°C in humidified 5% CO2. Cells were grown in 25 cm² flasks to 90% to 95% confluence and treated with 100 U/mL murine recombinant IFN-γ (Cell Sciences, Canton, MA), either alone or in combination with 10 μM proteasome inhibitor (MG132 or clasto-lactacystin M proteasome inhibitor cocktail tablet (Roche Diagnostics GmbH, Mannheim, Germany) per 10 mL of lysis buffer. After centrifugation for 15 minutes at 13,000 rpm, supernatant protein concentrations were measured by bicinchoninic acid (BCA) assay.33 Proteins were separated on 15% Tris-Cl SDS-polyacrylamide gels (10 μg lysate protein for HSP27, αβ-crystallin, HSP70, and HSF2; 25 μg for HSP90 and HSF1; and 50 μg for α-crystallin and HSF4) and transferred to nitrocellulose membranes. The membranes were blocked overnight at 4°C in TBS-T (10 mM Tris-HCl, [pH 7.6], 150 mM NaCl, 0.05% Tween-20) containing 5% nonfat milk powder, and incubated for 1 hour at room temperature with the corresponding antibody, with the following antibody dilutions: goat polyclonal anti-HSP27 (1:1000); rabbit polyclonal anti-α-crystallin (1:2000), rabbit polyclonal anti-αβ-crystallin, HSP70, and HSF2 (1:1000); rabbit polyclonal anti-α-crystallin (1:2000), rabbit polyclonal anti-αβ-crystallin, anti-HSP70, anti-HSF1, and anti-HSF4 (1:1000); rabbit polyclonal anti-HSP90 (1:15,000); rat monoclonal anti-HSF2 (1:1000); and mouse monoclonal anti-α-tubulin (1:2000). These blots were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hour at room temperature, and specific bands were detected using the enhanced chemiluminescence reagent (ECL; Perkin Elmer Life Sciences, Boston, MA) on autoradiographic film. Protein bands were quantitated by densitometry, and protein loading was normalized with α-tubulin. For quantitation of protein levels, the amount of protein loaded on the gel was optimized, and multiple exposures were performed to ensure that the signals were within the linear response range of the film. Anti-HSF4 antibody was a generous gift from Akiri Nakai (Yamaguchi University School of Medicine, Japan). Anti-HSP27 and HSF1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-α-crystallin antibody was purchased from Sigma-Aldrich. All other antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-α-tubulin antibody was purchased from Sigma-Aldrich. All other antibodies were purchased from Stressgen (Victoria, British Columbia, Canada).

RT-PCR Analysis

Total RNA was isolated from cells using RNAzol (Tel-Test, Friendswood, TX) and reverse transcribed at 42°C for 60 minutes, followed by heat denaturation at 95°C for 5 minutes and cooling at 4°C for 5 minutes. The reaction mixture (40 μL) included 1 μg of total RNA, 2.5 μM oligo (dT)16, 40 U RNase inhibitor, and 100 U MuLv reverse transcriptase (Applied Biosystems, Inc. [ABI], Foster City, CA). The mixture (50 μL) included 5 μL from the reverse transcription reaction, 0.3 μM each sense and antisense primer, and 1.25 U DNA polymerase (AmpliTaq; ABI). The PCR was performed with an initial denaturation for 2 minutes at 95°C, followed by 25 or 30 amplification cycles, each comprising 1 minute at 94°C, 30 seconds at 55°C, and 1 minute at 72°C, followed by 7 minutes at 72°C and cooling to 4°C. The PCR products were separated by 2% agarose gel electrophoresis and visualized with ethidium bromide. The bands were quantitated by densitometry and normalized with β-actin. Product formation for indicated genes was linear at 25 and 30 PCR cycles. The sequences of mouse-specific primers, corresponding reference or GenBank accession numbers, and expected product sizes are shown in Table 1 (GenBank is available at http://www.ncbi.nlm.nih.gov/GenBank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD).

Western Blot Analysis

The cells were washed in PBS and lysed in buffer containing 25 mM HEPES (pH 7.5), 0.3 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.05% TritonX-100, 20 mM β-glycerophosphate, 1 mM orthovanadate, 0.5 mM dithiothreitol (DTT), and one protease inhibitor cocktail tablet (Roche Diagnostics GmbH, Mannheim, Germany) per 10 mL of lysis buffer. After centrifugation for 15 minutes at 13,000 rpm, supernatant protein concentrations were measured by bicinchoninic acid (BCA) assay.33 Proteins were separated on 15% Tris-Cl SDS-polyacrylamide gels (10 μg lysate protein for HSP27, αβ-crystallin, HSP70, and HSF2; 25 μg for HSP90 and HSF1; and 50 μg for α-crystallin and HSF4) and transferred to nitrocellulose membranes. The membranes were blocked overnight at 4°C in TBS-T (10 mM Tris-HCl, [pH 7.6], 150 mM NaCl, 0.05% Tween-20) containing 5% nonfat milk powder, and incubated for 1 hour at room temperature with the corresponding antibody, with the following antibody dilutions: goat polyclonal anti-HSP27 (1:1000); rabbit polyclonal anti-α-crystallin (1:2000), rabbit polyclonal anti-αβ-crystallin, anti-HSP70, anti-HSF1, and anti-HSF4 (1:1000); rabbit polyclonal anti-HSP90 (1:15,000); rat monoclonal anti-HSF2 (1:1000); and mouse monoclonal anti-α-tubulin (1:2000). These blots were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hour at room temperature, and specific bands were detected using the enhanced chemiluminescence reagent (ECL; Perkin Elmer Life Sciences, Boston, MA) on autoradiographic film. Protein bands were quantitated by densitometry, and protein loading was normalized with α-tubulin. For quantitation of protein levels, the amount of protein loaded on the gel was optimized, and multiple exposures were performed to ensure that the signals were within the linear response range of the film. Anti-HSF4 antibody was a generous gift from Akiri Nakai (Yamaguchi University School of Medicine, Japan). Anti-HSP27 and HSF1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-α-tubulin antibody was purchased from Sigma-Aldrich. All other antibodies were purchased from Stressgen (Victoria, British Columbia, Canada).

Measurement of Caspase Activities

Caspase-1, -6, and -8-like activities were measured colorimetrically, using the substrates Ac-YVAD-pNA, Ac-VEID-pNA, and Ac-IETD-pNA, respectively (Biomol Research Laboratories, Plymouth Meeting, PA). Cells were washed three times with PBS and dissolved in lysis buffer containing 50 mM HEPES (pH 7.4), 0.1% CHAPS (3-[3-cholamidopropyl]dimethylammonio-2-hydroxy-1-propanesulfonate), 5 mM DTT, and 0.1 mM EDTA. The samples were centrifuged at 13,000 rpm for 20 minutes at 4°C, and supernatant protein concentrations were measured by BCA assay. Protein lysate (40 μg) was mixed with the corresponding substrate (400 μM), diluted to 100 μL with assay buffer containing 50 mM HEPES (pH 7.4), 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA, and 10% glycerol and incubated at 37°C. The optical

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer Sequence (5’–3’)</th>
<th>Reference or GenBank Accession Number</th>
<th>Expected Product Size (bp)</th>
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<td>HSP70</td>
<td>Forward-AATTGGCTGTTATGAAGATG</td>
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<tr>
<td>β-Actin</td>
<td>Forward-TTGAGCTTACAGGAGACGG</td>
<td>Reference 30</td>
<td>243</td>
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</table>
density at 405 nm was measured with a microplate reader (model 450; Bio-Rad, Hercules, CA).

**Phase-Contrast Microscopy**

αTN4-1 cells were treated with IFN-γ, MG132, and quercetin, either alone or in combination and photographed under a phase-contrast microscope (Eclipse TE 300; Nikon, Melville, NY).

**Flow Cytometric Analysis**

Apoptosis was quantitated by annexin V-FITC and propidium iodide (PI) staining. The annexin V-positive and PI-negative cells were considered to be early apoptotic cells. After incubation, as indicated in the figure legend (see Fig. 8), cells were washed with PBS and harvested using Accutase (Innovative Cell Technologies Inc., San Diego, CA). Cell pellets were washed and resuspended in 1 mL of binding buffer (Caltage Laboratories, Burlingame, CA). The 100 μL cell suspension was stained with 5 μL annexin V-FITC (Caltage Laboratories) and 2.5 μL PI (Sigma-Aldrich) from 50 μg/mL stock. After incubation for 10 minutes at room temperature in the dark, 400 μL binding buffer was added and samples were immediately analyzed by flow cytometry (FACScalibur System; BD Biosciences, Franklin Lakes, NJ).

**Statistical Analysis**

Statistical significance was analyzed by the two-tailed Student’s t-test. Values of $P < 0.05$ were considered to be statistically significant.
RESULTS

Proteasome Inhibition and HSP mRNA Expression

To investigate the effect of proteasome inhibition on expression of HSP mRNA, RT-PCR assays were performed on total RNA extracted from cells treated with IFN-γ, MG132, and IFN-γ+MG132, after 1, 4, 8, and 12 hours of incubation. A more than 10-fold increase in HSP27, a small but significant (1.2- to 1.6-fold) increase in αB-crystallin mRNA, but no significant change in expression of HSP70 or -90 mRNA, was observed in MG132- and IFN-γ+MG132-treated cells, compared with control and IFN-γ-treated cells, after 12 hours of incubation (Fig. 1). Data at early incubation times (1, 4, and 8 hours) showed increased HSP27 mRNA after 4 and 8 hours of incubation; a small but significant increase in αB-crystallin mRNA expression at 1, 4, and 8 hours of incubation; but no significant change in HSP70 and -90 mRNA expression in cells at any time after incubation in MG132 or IFN-γ+MG132 (data not shown). The mRNA expression of β-actin, a housekeeping gene, was used as an internal control, and its expression was similar in all samples. Similar results were obtained when clasto-lactacystin β-lactone was used as a proteasome inhibitor (data not shown).

Proteasome Inhibition and HSP Expression

We also looked at HSP expression at the protein level by Western blot analysis in IFN-γ-, MG132-, and IFN-
MG132-treated cells. Again, we saw increases in HSP expression, but the pattern was different. A marked increase in HSP70 and a more than twofold increase in HSP27 and -90 were observed in MG132- and IFN-γ-treated cells, compared with control or IFN-γ-treated cells (Fig. 2). Although we saw a small and significant change in αB-crystallin mRNA, no significant change in its expression was observed at the protein level (Fig. 2). The expression of α-tubulin, a housekeeping protein, was used as an internal control, and its expression was similar in all samples. Similar results were obtained when clasto-lactacystin β-lactone was used as the proteasome inhibitor (data not shown).

Effect of Proteasome Inhibition on αA-Crystallin Expression

We also examined the effect of proteasome inhibition on expression of αA-crystallin, a member of the small HSP family that is more specifically expressed in the lens. In our study, a marked decrease in αA-crystallin mRNA expression was observed after 12 hours of incubation in MG132- and IFN-γ+MG132-treated cells, compared with control or IFN-γ-treated cells (Fig. 1). Data at early incubation times showed no significant change in αA-crystallin mRNA expression after 1 hour of incubation, but a marked decrease in expression after 4 and 8 hours of incubation in MG132- and IFN-γ+MG132-
triplicates, in one of three independent experiments with similar results were obtained when clasto-lactacystin expression was also observed at the protein level after 12 caspase substrates in a microtiter plate. The plate was read in a controls remained the same in all the samples.

Our previous studies showed increased activity of caspase-1 and -32. Caspases are widely involved in the execution of apoptosis.32 IFN-γ treatment caused a 2.0- to 3.4-fold increase in HSF2 and -4 protein expression but no change in HSF1 protein (Fig. 3). In addition, proteasome inhibition did not cause any significant change in LEDGF mRNA expression, compared with control or IFN-γ treatment alone (data not shown). β–Actin and α–tubulin controls remained the same in all the samples.

Effect of Proteasome Inhibition on HSF Expression

To investigate the mechanism of elevated synthesis of HSPs by proteasome inhibition, we measured the expression of heat shock transcription factors, HSF1, -2, and -4 and lens epitheli-um derived growth factor (LEDGF). Proteasome inhibition increases HSP expression, which blocks caspase cleavage. In addition, specific caspase-inhibitor–treated cell lysates were used as a control for nonspecific cleavage of caspase substrates by cell extracts.

We have also verified that quercetin inhibited apoptosis in nonapoptotic cells and that the proteasome inhibitors MG132 and clasto-lactacystin β-lactone had no effect on commercially available caspase-1, -6, and -8 enzymes (data not shown).

Effect of Quercetin on the Antiapoptotic Activity of Proteasome Inhibitor

To establish a direct correlation between MG132-induced HSPs and its antiapoptotic effect on IFN-γ- induced apoptosis, cells were pretreated with quercetin, a natural flavonoid that inhibits the stress-induced synthesis of HSPs and then were treated with MG132 and/or IFN-γ. We observed that quercetin pre-treatment significantly reversed the induction of caspase-1, -6, and -8 activities caused by MG132 (Fig. 6).

We used MG132, a peptide aldehyde inhibitor of the cata-lytic subunits of the proteasome. MG132 is highly potent, but inhibition of calpain and cathepsin B has been reported.33 Therefore, we also used clasto-lactacystin β-lactone, which is a more specific inhibitor of proteasome.34 Although in one study it was shown to inhibit the human platelet A-like protease.35 MG132 and clasto-lactacystin β-lactone gave similar results in untreated, MG132-treated cells, indicating the antiapoptotic effect of MG132 on IFN-γ-induced apoptosis.20 The morphology of the cells treated with quercetin alone was similar to that of the untreated control. However, a significant number of cells with apoptotic morphology were present in samples pretreated with quercetin and then cotreated with IFN-γ+MG132 (Fig. 7).

DISCUSSION

It has been reported that proteasome inhibition causes upregulation of HSPs.8,11,12 Recent reports15–17 have indicated that some HSPs are antiapoptotic and directly inhibit caspase activation. Because our previous study showed that proteasome inhibition protects LECs from IFN-γ-induced apoptosis,20 in the present study, we tested the hypothesis that proteasome inhibition increases HSP expression, which blocks caspase activation and thus could prevent IFN-γ-induced apoptosis of LECs.

There has been a growing interest in the role of the ubiquitin-proteasome pathway in the regulation of gene expression. The ubiquitin-proteasome pathway is responsible for degra-dation of abnormal proteins. Proteasome inhibitors block intracellular protein breakdown; the resultant accumulation of abnormal proteins activates HSFs, which results in elevated synthesis of HSPs.8, 11–13, 36.
may cause accumulation of HSPs through reduction in degradation of these proteins. In our study, proteasome inhibition caused an increase in HSP27, -70, and -90 protein levels (Fig. 2). Although we saw a small but significant change in H9251 B-crystallin mRNA by proteasome inhibitor treatment, we did not observe any increase in its expression at the protein level (Fig. 2), perhaps because of its posttranslational modification by other proteases, such as calpain.37 A decrease in H9251 A-crystallin expression (Figs. 1, 2) may actually protect the cells under stress conditions as part of the antiapoptotic mechanism of proteasome inhibition. This observation is consistent with a previous report in which a decrease in αA-crystallin expression was observed in hydrogen-peroxide–resistant αTN4 cells, compared with normal cells.38 Because αA-crystallin has also been shown to protect cells from apoptosis,39,40 there is a possibility that the decrease in αA-crystallin expression is a proapoptotic effect of proteasome inhibition but the net effect of the proteasome inhibitor is antiapoptotic, due to the involvement of other factors.

The induction of heat shock gene expression by stress is mediated by HSFs, which bind to heat shock elements in the promoters of heat shock genes.41,42 HSFs have been shown to be affected during downregulation of the ubiquitin-proteasome pathway. The specific function of HSFs in regulation of heat shock gene expression in response to proteasome inhibition is not clear. Proteasome inhibition has been shown to activate

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**Figure 5.** Western blot analysis for expression of HSP27, -70, and -90 and HSF2 and -4 and α-tubulin protein after 12 hours' incubation with MG132, with and without 24-hour pretreatment with quercetin (100 μM). (A) Total cell lysate was prepared from αTN4-1 cells, proteins were separated by SDS-PAGE, and the gel was immunoprobed. Data are representative of at least two independent experiments. (B) The intensity of bands was quantitated by densitometry. For HSP27, HSP70, and HSF4, each data point represents the mean ± SD (n = 3). *P < 0.001, **P < 0.01 versus untreated control. For HSP90 and HSF2, each data point represents the average of two values (n = 2).
Regarding effector caspases (-3, -6, and -7), we have reported that caspase-3 (and by implication caspase-7, which has the same substrate specificity) is not involved in IFN-γ-induced apoptosis.20 We therefore looked at the activity of caspase-6, which was increased by IFN-γ treatment and blocked by MG132. The basal level of caspase-6 activity in control (untreated) cells was also blocked by proteasome inhibitor treatment (Fig. 4).

To establish a direct relationship between HSP induction, blockage of caspase activity, and the antiapoptotic effect of proteasome inhibition, we used quercetin to inhibit HSP induction. A bioflavonoid, quercetin has been widely studied, and numerous experiments have reported its biological, pharmacological, and medicinal properties.54–55 Quercetin can affect the stress response to injury by inhibiting the synthesis of HSPs.56–58 Intervention in apoptosis by quercetin has been reported in several cell types. In general, quercetin facilitates apoptosis of tumor cells, but not of normal cells, through depression of HSP70.59 However, quercetin may inhibit apoptosis in some nontumorigenic cells.60,61 In our study, quercetin pretreatment decreased the induction in HSP expression.

Consistent with the observed antiapoptotic effect of HSPs by direct inhibition of caspase activation,15–17 we found that the proteasome inhibitor completely blocked the increase in caspase-1 and -8 activities caused by IFN-γ (Fig. 4). The proteasome has three activities, one of which, the peptidylglutamyl peptide-hydrolyzing (PGPH) activity, is described as caspaselike. Therefore, we performed a control experiment and demonstrated that purified proteasome had no detectable activity on specific caspase substrates in a microtiter plate. The plate was read in a microplate reader at 405 nm. Data, expressed as the mean ± SD of triplicates, are from one of two independent experiments with similar results. *P < 0.001, **P < 0.005 versus untreated control.

Recently, HSF4 has been shown to be important for normal development of the lens.46–48 We observed a more than two-fold increase in HSF2 and -4, but no change in HSF1 protein expression on proteasome inhibition (Fig. 3), suggesting the involvement of HSF2 and -4 in elevated synthesis of HSPs. HSF4 has two isoforms, HSF4a (56kDa) and -4b (62 kDa), and only HSF4b has the potential to activate transcription.69–70 Consistent with previous reports,48,49 we also detected only the b isoform of HSF4. In addition, in our study, low-mobility bands, suggestive of a phosphorylated form of HSFs, were not observed after proteasome inhibitor treatment (Fig. 3). Indeed, other reports have suggested that the increases in HSF2 concentration alone by proteasome inhibition is sufficient to upregulate HSP expression.51,52

Consistent with the observed antiapoptotic effect of HSPs by direct inhibition of caspase activation,15–17 we found that the proteasome inhibitor completely blocked the increase in caspase-1 and -8 activities caused by IFN-γ (Fig. 4). The proteasome has three activities, one of which, the peptidylglutamyl peptide-hydrolyzing (PGPH) activity, is described as caspaselike. Therefore, we performed a control experiment and demonstrated that purified proteasome had no detectable effect on caspase-1 and -8 substrates. This finding suggests that the increase in IFN-γ-induced caspase activity in our experiments is due to the increase in activity of caspaselike enzymes and not due to the proteasome’s effect on caspase substrates. This is consistent with the report53 that the proteasome has a very low caspase-1-like activity compared with its chymotrypsin-like activity. Also, we verified that the proteasome inhibitors MG132 and clasto-lactacystin β-lactone had no effect on commercially obtained caspase-1, -6, and -8 enzymes, suggesting that proteasome inhibitors do not directly inhibit caspase. These control experiments suggest that the blockage of caspase activities by proteasome inhibition is an indirect effect, such as we propose occurs through the involvement of HSPs.
Early apoptotic cells with high annexin-V and low PI staining were flow cytometry after staining with FITC-conjugated annexin-V and PI. In independent experiments performed in duplicate (authors also acknowledge the support of the late Joseph Fu for this and Harold I. Calvin for a critical reading of the manuscript. The casease-1, -6, and -8 activities caused by MG132. Of note, caused by MG132. It also significantly reversed the blockade of caspase-1, -6, and -8 activities caused by MG132. Of note, quercetin pretreatment significantly decreased the antiapoptotic activity of MG132, as observed by phase-contrast microscope and flow cytometry.

In summary, the results in our study indicate a causal linkage between proteasome inhibition, HSP induction, blockade of caspase activity, and prevention of apoptosis. Proteasome inhibitors are currently in clinical trials and are approved for treatment of cancer, based on the induction of apoptosis. Our studies, by contrast, provides novel findings that support the exploitation of the antiapoptotic response of lens epithelial cells to proteasome inhibition as a therapeutic tool for the prevention of cataract.

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