Proteasome inhibitors induce heat shock response and increase IL-6 expression in human intestinal epithelial cells

TIMOTHY A. PRITTS,1,2 ERIC S. HUNGNESS,1 DAN D. HERSHKO,3 BRUCE W. ROBB,1,3 XIAOYAN SUN,1 GUANG-JU LUO,3 JOSEF E. FISCHER,1 HECTOR R. WONG,4 AND PER-OLOF HASSELGREN1,5

Departments of 1Surgery and 2Molecular and Cellular Physiology, University of Cincinnati, Cincinnati 45267-0558; 3Shriners Hospitals for Children, Cincinnati 45229-3095; 4Division of Critical Care, Children’s Hospital Medical Center, Cincinnati 45229; and 5Veterans Affairs Hospital, Cincinnati, Ohio 45220

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Pritts, Timothy A., Eric S. Hungness, Dan D. Hershko, Bruce W. Robb, Xiaoyan Sun, Guang-Ju Luo, Josef E. Fischer, Hector R. Wong, and Per-Olof Hasselgren. Proteasome inhibitors induce heat shock response and increase IL-6 expression in human intestinal epithelial cells. Am J Physiol Regulatory Integrative Comp Physiol 282: R1016–R1026, 2002; 10.1152/ajpregu.00492.2001.—In previous studies, the heat shock response, induced by hyperthermia or sodium arsenite, increased interleukin (IL)-6 production in intestinal mucosa and cultured human enterocytes. A novel way to induce the heat shock response, documented in other cell types, is treatment with proteasome inhibitors. It is not known if proteasome inhibition induces heat shock in enterocytes or influences IL-6 production. Here we tested the hypothesis that treatment of cultured Caco-2 cells, a human intestinal epithelial cell line, with proteasome inhibitors induces the heat shock response and stimulates IL-6 production. Treatment of Caco-2 cells with one of the proteasome inhibitors MG-132 or lactacystin activated the transcription factor heat shock factors (HSF)-1 and -2 and upregulated cellular levels of the 72-kDa heat shock protein HSP-72. The same treatment resulted in increased gene and protein expression of IL-6, a response that was blocked by quercetin. Additional experiments revealed that the IL-6 gene promoter contains a HSF-responsive element and that the IL-6 gene may be regulated by the heat shock response. The present results suggest that proteasome inhibition induces heat shock response and IL-6 production in enterocytes and that IL-6 may be a heat shock-responsive gene, at least under certain circumstances. The observations are important concerning the multiple biological roles of IL-6, both locally in the gut mucosa and systemically, and considering recent proposals in the literature to use proteasome inhibitors in the clinical setting to induce the heat shock response.

intestine; mucosa; enterocyte; stress response; cytokine

STUDIES DURING THE LAST DECADE have provided increasing evidence that the enterocyte and intestinal mucosa are active participants in the inflammatory response to sepsis, endotoxemia, and severe injury (42). For example, mucosal production of certain acute phase proteins (28, 29, 47, 50) and cytokines (13, 25, 27, 49) is increased in these conditions, and some of these substances may influence mucosal integrity and regulate intestinal permeability (15, 51).

Among cytokines produced in the intestinal mucosa during sepsis and endotoxemia, interleukin (IL)-6 is particularly important because of its multiple significant biological effects (32). Thus mucosal IL-6 may regulate IgA production in Peyer’s patch B cells, thereby influencing intestinal immune function (5). In addition, IL-6 is one of the strongest regulators of acute phase protein synthesis, both in hepatocytes (4) and enterocytes (28), and may influence enterocyte acute phase protein synthesis through a paracrine or autocrine mechanism or may participate in the regulation of hepatocyte acute phase protein synthesis after reaching the liver through the portal vein. Although commonly considered a proinflammatory cytokine (37), there is also evidence that IL-6 has important anti-inflammatory properties and may exert protective effects in various tissues (3, 41, 52). It is obvious, then, that methods to modulate IL-6 production in intestinal mucosa and in stimulated enterocytes may have important clinical implications.

In recent studies from our laboratory, mucosal production of IL-6 was increased in response to sepsis and endotoxemia (27, 49), and cultured enterocytes produced IL-6 after treatment with endotoxin (26) or IL-1β (35). In other experiments, we found that induction of the heat shock response resulted in augmented IL-6 production in mucosa of endotoxemic mice (48) and in IL-1β-stimulated cultured human enterocytes (33). In those studies, the heat shock response was induced by hyperthermia or treatment with sodium arsenite (33, 48). A novel way to induce the heat shock response is treatment with proteasome inhibitors as described initially by Zhou et al. (54). Treatment of...
cultured HepG2 cells with various proteasome inhibitors, including MG-132 and lactacycin, resulted in activation of the transcription factor heat shock protein 72 (HSP-72) (54). In other studies, Bush et al. (6) found that proteasome inhibition induced the heat shock response in cultured canine kidney cells and protected the cells from the noxious effects of high temperature (thermotolerance). From such and similar observations it was proposed that proteasome inhibitors may be used to induce the heat shock response in the clinical setting.

Although induction of the heat shock response by proteasome inhibitors has been reported in certain cell types (6, 19, 24, 54), it is not known if treatment with proteasome inhibitors results in induction of the heat shock response in the enterocyte. In addition, the influence of proteasome inhibitors on enterocyte IL-6 production has not been reported. This is particularly significant because the IL-6 gene is regulated at least in part by nuclear factor (NF)-κB (46), and inhibition of the proteasome blocks NF-κB activation secondary to inhibited degradation of inhibitory κB (IκB) (17). Thus the effect of proteasome inhibition on enterocyte IL-6 production is difficult to predict because, on one hand, inhibited NF-κB activity may reduce IL-6 production and, on the other hand, the heat shock response may increase the expression of IL-6.

The purpose of the present study was to test the hypothesis that treatment of cultured human enterocytes with proteasome inhibitors induces the heat shock response and that this response augments IL-6 production in IL-1β-stimulated cells. In addition, we examined the effect of proteasome inhibitors on NF-κB activation in IL-1β-treated enterocytes. We found that treatment of the enterocytes with MG-132 or lactacycin induced the heat shock response and that this response was associated with increased IL-6 production in IL-1β-stimulated cells. The same experimental conditions resulted in reduced NF-κB activity, suggesting that in enterocytes expressing the heat shock response, other transcription factors become important for the regulation of the IL-6 gene.

MATERIALS AND METHODS

Materials. Caco-2 cells were from American Type Culture Collection (Rockville, MD). DMEM, nonessential amino acids, low-endotoxin fetal bovine serum (FBS), l-glutamine, penicillin, streptomycin, and TRIZOL were purchased from Gibco-BRL (Grand Island, NY). Human recombinant IL-1β was purchased from Endogen (Woburn, MA). MG-132 (carbobenzoxy-l-leucyl-l-leucyl-l-leucinal), lactacycin, and quercetin were obtained from Calbiochem (La Jolla, CA). All other chemicals, unless stated otherwise, were from Sigma (St. Louis, MO).

Cell culture. Caco-2 cells, a human colon adenocarcinoma cell line that displays enterocyte-like features in culture (38), were grown at 37°C in 5% CO2 in DMEM supplemented with 10% FBS, nonessential amino acids, 6 mM glutamine, 10 mM HEPES, 10 mg/ml apotransferrin, 1 mM pyruvate, 24 mM NaHCO3, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells, between passages 5 and 25, were seeded at a density of 100,000 cells/cm2 onto 10-cm2 tissue culture plates for determination of NF-κB DNA binding activity, IκB-α protein levels, IL-6 mRNA levels, IL-6 gene transcription, and HSF DNA binding activity (Falcon-Becton Dickinson, Franklin Lakes, NJ). Six-well tissue culture plates were used for the determination of IL-6, IL-8, and HSP-72 levels and 96-well plates for determination of cell viability. Cells were grown for 72 h to 90% confluence before use.

Experimental conditions. Before experiments, cells were washed three times with serum-free DMEM and then pretreated with serum-free medium containing one of the proteasome inhibitors MG-132 (10 μM) or lactacycin (20 μM). In some experiments, cells were pretreated with quercetin (100 μM). The concentrations of these substances were based on previous studies in which they induced and blocked the heat shock response, respectively (6, 23, 31, 54). Because MG-132 and lactacycin were solubilized in DMSO, control cells were incubated in corresponding concentrations of DMSO. The concentration of DMSO in the culture medium did not exceed 0.75% (vol/vol). We have previously shown that IL-1β-induced NF-κB DNA binding activity, IκB-α degradation, and IL-6 production were not altered by DMSO concentrations up to 2% (vol/vol) (29).

After preincubation for 1 h with MG-132, lactacycin, quercetin, or DMSO, IL-1β (0.5 ng/ml) was added to the culture medium. Treatment of cultured enterocytes with this concentration of IL-1β resulted in maximal IL-6 production (35) and rapid IκB-α degradation and NF-κB activation in recent studies from our and other laboratories (16, 34). Cells were harvested after 30 min or 2 h for determination of IκB-α protein levels and NF-κB and HSF DNA binding activity, after 4 h for determination of IL-6 mRNA levels and gene transcription, and after 24 h for determination of IL-6 and IL-8 protein production and HSP-72 levels. All experiments were performed at least three times to ensure reproducibility.

Nuclear and cytoplasmic extracts. Nuclear and cytoplasmic fractions were prepared as previously described (34). All steps were carried out on ice. Cells were harvested by scraping into ice-cold phosphate-buffered saline, pH 7.4, and were pelleted by centrifugation at 3,800 g for 5 min. Cells were then suspended in 1 packed-cell volume of lysis buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1.5 mM MgCl2, 0.2% (vol/vol) Nonidet P-40, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 100 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 1.5 μM pepstatin A, 1.4 μM transeposoxyucciyl-l-leucylamidol, 4 μM bestatin, 2.2 μM leupeptin, 0.08 μM apritin, 0.0045 μM microxycytin LR, 0.46 μM cantharidin, and 0.2 μM (–) bromotetramisole. After incubation on ice for 5 min with intermittent vortexing, the nuclear pellet was isolated by centrifugation at 3,800 g for 5 min. The supernatant was removed and saved as the cytoplasmic fraction. The pellet was resuspended in 1 cell volume of extract buffer containing 20 mM HEPES, pH 7.9, 420 mM NaCl, 0.1 mM EDTA, 1.5 mM MgCl2, 25% (vol/vol) Nonidet P-40, 1 mM DTT, 0.5 mM PMSF, 100 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 1.5 μM pepstatin A, 1.4 μM transeposoxyucciyl-l-leucylamidol, 4 μM bestatin, 2.2 μM leupeptin, 0.08 μM apritin, 0.0045 μM microxycytin LR, 0.46 μM cantharidin, and 0.2 μM (–) bromotetramisole. After incubation on ice for 15 min with intermittent vortexing, the nuclear pellet was isolated by centrifugation at 16,000 g for 20 min. Protein concentrations of nuclear and cytoplasmic extracts were determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as a standard.
Electrophoretic mobility shift assays. Electrophoretic mobility shift assays (EMSAs) were performed as previously described in detail (39). Aliquots of the nuclear fractions (7.5 μg protein) were incubated in buffer containing 12.5% glycerol (vol/vol), 12 mM HEPES, pH 7.9, 4 mM Tris- HCl, pH 7.9, 1 mM EDTA, 1 mM DTT, 25 mM KCl, 5 mM MgCl₂, 0.04 μg/μl poly(dIdC) (Boehringer Mannheim, Indianapolis, IN), and Tris-EDTA buffer, pH 7.4. NF-κB gel shift oligonucleotide 5'-AGT TGA GGC GAC TTT CCC AGC G-3' was purchased from Santa Cruz Laboratories (Santa Cruz, CA). Oligonucleotides corresponding to the known heat shock-responsive element (HRE) 5'-GCC TCG AAT GTT CGC GAA GTT TCG-3' (11) and the potential HREs pHRE1 (5'-AGC ACG AAC GAA AGA GAA GCT A TA CCT TCT CAC GAG CTG-3'), pHRE2 (5'-AAA AAG AAA GTA AAG GAG GAG TGC TGG TTC TGC TTC TAG C-3'), and pHRE3 (5'-CAG AGG AAA CTC AGT TCA GAA CAT CT-3') were synthesized by the Univ. of Cincinnati DNA Core Facility. Conformational standards were annealed using a DNA thermocycler (Perkins-Elmer, Branchburg, NJ).

Probes were end-labeled with [γ-32P]ATP using polynucleotide kinase T4 (GIBCO BRL). End-labeled probe was purified from unincorporated [γ-32P]ATP using a purification column (Bio-Rad Laboratories) and recovered in Tris-EDTA buffer, pH 7.4. Labeled probe was added to nuclear extracts, and the samples were incubated for 30 min on ice. Where indicated in results, an excess (20 ng) of unlabelled NF-κB, HRE, or pHRE2 DNA was added for competition reactions. For supershift analysis, 2 μl of antibody to HSF-1 (Stressgen Biotechnologies, Victoria, British Columbia, Canada) or to HSF-2 (kindly provided by Dr. R. I. Morimoto, Northwestern Univ., Chicago, IL) were added 30 min before addition of the radiolabeled probe. Samples were then subjected to electrophoretic separation on a nondenaturing 5% polyacrylamide gel at 30 mA using Tris borate EDTA buffer (0.45 M Tris-hydrochloride, 0.03 M borate, 0.001 M EDTA, pH 8.3). Blots were dried at 80°C for 1 h and analyzed by exposure to PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA).

Western blot analysis. Aliquots of cytoplasmic fractions containing 25 μg of protein were boiled in equal volumes of loading buffer (125 mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate, 20% glycerol, and 10% 2-mercaptoethanol) for 3 min and then separated by electrophoresis on an 8–16% Tris-glycine gradient gel (Novex, San Diego, CA). A protein ladder (See-Blue Standard, Novex) was included as a molecular weight marker. The proteins were transferred to nitrocellulose membranes (Xcell II Blot Module; Novex). Equal loading of proteins was confirmed by staining with Ponceau S (Bio-Rad Laboratories). The membranes were blocked with 5% nonfat dried milk in Tris-buffered saline (TBS), pH 7.6, containing 0.05% Tween-20 (TTBS), for 30 min and then incubated with a polyclonal rabbit anti-mouse antibody to IκBα (Santa Cruz Laboratories) or a polyclonal antibody to HSP-72 (Stressgen Biotechnologies) for 1 h. After being washed twice in TTBS, the blots were incubated with a peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 20 min. The blots were washed in TTBS for 5 min three times and then in TBS for 5 min, incubated in enhanced chemiluminescence reagents (ECL, Amersham Life Sciences, Buckingham, UK), and exposed on radiographic film (X-omat AR; Eastman-Kodak, Rochester, NY).

Determination of IL-6 and IL-8 protein. IL-6 and IL-8 protein levels were determined in cell culture media by ELISA using commercially available kits (Endogen). The lower limit of detection as described by the manufacturer was <1 pg/ml for both assays.

RNase protection assay. Total RNA was isolated and extracted from cell monolayers by the acid guanidinium thiocyanate-phenol-chloroform method using a commercially available reagent as previously described (7). RNA concentration was determined spectrophotometrically, and purity was verified by electrophoresis on a 1.0% agarose/formaldehyde gel with subsequent visualization by ethidium bromide staining. cDNA fragments for IL-6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized by RT-PCR using an RT-PCR kit (Perkin-Elmer, Branchburg, NJ) according to the manufacturer’s instructions. Sequences of the PCR primers were as follows: human IL-6, forward PCR primer 5'-ATG AAC TCC TTC ACC AGC GC-3' and reverse PCR primer 5'-G AAG AGC CCT CAG GCT GGA CTG-3' (628 bp) (14); human GAPDH, forward PCR primer 5'-ACA TCG TCT AGA CAC CAT G-3' and reverse PCR primer 5'-GAA GCC GCT GAC GGT CTT-3' (710 bp) (44). After purification, cDNA fragments were subcloned into pGEM3. Probes were synthesized using bacteriophage polynucleotide kinase and [32P]UTP (DuPont, Boston, MA) according to the manufacturer’s instructions. RNase protection assays (RPA) were performed utilizing the RPA II kit (Ambion, Austin, TX). Following electrophoresis of the RNase-treated samples on 5% polyacrylamide/urea gels, the gels were exposed on PhosphorImager screens and then quantified. The signals for IL-6 mRNA were normalized to GAPDH mRNA bands on the same gel.

Nuclear run-on assay. Cells were spun down, washed once with ice-cold phosphate-buffered saline (pH 7.4), and incubated on ice for 5 min. The nuclei were centrifuged at 500 g, resuspended in storage buffer [50 mM Tris-HCl, pH 8.3, 5 mM MgCl₂, 0.1 mM EDTA/NaOH, pH 8.0, 40% (vol/vol) glycerine], aliquoted, frozen in liquid nitrogen, and stored at −80°C. Aliquots of nuclei were thawed on ice and incubated for 12 min at room temperature with or without 4 μl of a-amanita (0.1 mg/ml). Nuclei were then mixed with 100 μl of reaction buffer [500 mM KCl, 5 mM MgCl₂, 0.5 mM each of ATP, UTP, and GTP, 100 μCi [α-32P]CTP; and 10 mM Tris-HCl, pH 8.0, with or without 1.2% (wt/vol) sarcosyl] and incubated for 15 min at 28°C. Forty units of RNase-free DNase I (Roche Diagnostics) were added, and the incubation was continued for 12 min at room temperature. The DNAase I treatment was repeated if sarcosyl was part of the reaction buffer. After isolation of nuclear transcripts by Sephadex G-50 column filtration, radiolabeled RNA was hybridized to nylon membrane-immobilized cDNA restriction fragments for IL-6 and GAPDH at 65°C for 36 h in 5 ml of Church buffer [0.5 M sodium phosphate, pH 7.1, 7% (vol/vol) SDS, 0.1 mM EDTA/NaOH, pH 8.0]. Membranes were washed with 2× standard saline citrate twice at room temperature, allowed to air dry, mounted onto filter paper, exposed on PhosphorImager screens, and quantitated by densitometry. For analysis, IL-6 mRNA transcriptional rate was normalized to GAPDH transcription rates.

Determination of cell viability. Cell viability was determined by measuring mitochondrial respiration, assessed by the mitochondria-dependent reduction of 3-(4,5 dimethyl-thiazol-2-yl)-2,5 diphenyltetrazolium bromide to formazan as described previously (40). Cell viability was not influenced by any of the experimental conditions in the present study (data not shown).

Statistical analysis. Where appropriate, results were expressed as means ± SE. ANOVA followed by Student-Newman-Keuls test was used for statistical analysis. To allow direct comparison between experiments and account for vari-
ation between different groups of cultured cells, some results were expressed as percentage of IL-1β-induced IL-6 production.

RESULTS

Treatment of the cultured Caco-2 cells with one of the proteasome inhibitors MG-132 or lactacystin resulted in stimulated HSF DNA binding activity, determined by EMSA, and increased HSP-72 levels, determined by Western blotting, consistent with induction of the heat shock response (Fig. 1). The activation of HSF was noted after treatment with proteasome inhibitor at both time points studied here (30 min and 2 h). Stimulation of the cells with IL-1β alone did not influence HSF activity or HSP-72 levels.

Addition of an excess amount of unlabeled HRE oligonucleotide to the EMSA reaction, but not an excess amount of a nonspecific (NF-κB) oligonucleotide, resulted in deletion of the HSF band, confirming the specificity of the EMSA (Fig. 2A). Addition of an antibody to HSF-1 resulted in a supershifted band and almost completely deleted the HSF band (Fig. 2B), indicating that HSF-1 was involved in the induction of the heat shock response by proteasome inhibitors. When an antibody against HSF-2 was added, the HSF band decreased in intensity, suggesting that HSF-2 as well was involved in the induction of the heat shock response by proteasome inhibition. It should be noted, however, that no clear supershifted band was seen when the HSF-2 antibody was used. More studies will be needed to better define the exact role of HSF-2 in the heat shock response induced by proteasome inhibitors under the present experimental conditions. Treatment of the cells with quercetin reduced, but did not completely abolish, HSF DNA binding activity and the HSF-1 supershifted band (Fig. 2B).

We next examined the effect of MG-132 and lactacystin on IL-6 production in cultured Caco-2 cells.

**Fig. 1.** A: heat shock factor (HSF) DNA binding activity determined by electrophoretic mobility shift assay (EMSA; top) and cellular heat shock protein (HSP)-72 protein levels determined by Western blotting (bottom) in Caco-2 cells after treatment with 10 μM MG-132 (MG), 20 μM lactacystin (Lacta), or 0.5 ng/ml interleukin (IL)-1β (IL-1). Cells were pretreated for 1 h with MG-132 or lactacystin followed by treatment with IL-1β for 4 h (for EMSA) or 24 h (for HSP-72). Control cells were treated with vehicle (Veh). NS, nonspecific binding. B: HSF DNA binding activity in Caco-2 cells after treatments identical to those in A, except that the length of IL-1β treatment was 30 min.

**Fig. 2.** A: competition reactions and supershift analysis of HSF EMSA. Cells in all lanes except the first (Veh) were treated with 10 μM MG-132 for 4 h; +HRE, addition of excess nonradiolabeled heat shock-responsive element (HRE) oligonucleotide; +NF-κB, addition of excess nonradiolabeled nuclear factor (NF)-κB oligonucleotide. B: HSF DNA binding activity in Caco-2 cells treated with quercetin (Q; 100 μM) and/or MG-132 (10 μM) for 4 h. Antibody to HSF-1 (anti-HSF-1) or HSF-2 (anti-HSF-2) was added to the reaction as indicated above the gel. SS, supershift.
Treatment of the cells with the proteasome inhibitors resulted in increased IL-6 production with the most pronounced increase noted after treatment with MG-132 (Fig. 3A). Confirming previous results from this laboratory (35), treatment of the cells with IL-1β also increased IL-6 production. When IL-1β was added to cells that were treated with MG-132 or lactacystin, a substantial augmentation of the IL-1β-induced IL-6 production was noted. To test if the effects of the proteasome inhibitors reflected a generalized effect on cytokine production, we measured the production of an additional cytokine, i.e., IL-8. In contrast to IL-6, IL-8 production was inhibited by MG-132 in IL-1β-stimulated cells and was not affected by MG-132 alone (Fig. 3B).

To examine the transcriptional regulation of IL-6 by proteasome inhibition, we measured IL-6 mRNA levels by RPA and IL-6 gene transcription rates by nuclear run-on assay in Caco-2 cells treated with IL-1β or MG-132. Results from those experiments showed that IL-6 mRNA levels were increased by treatment with IL-1β and MG-132 and that the combined treatment with IL-1β and MG-132 resulted in an additive, rather than a synergistic, effect on IL-6 mRNA levels (Fig. 4). Both IL-1β and MG-132 increased IL-6 gene transcription as determined by nuclear run-on assay with no further increase noted when IL-1β and MG-132 were added together (Fig. 5). The changes in gene transcription determined by nuclear run-on assay were less pronounced than the changes in mRNA levels, suggesting that the increased mRNA levels seen after treatment of the cells with MG-132 and IL-1β reflected both increased gene transcription and increased mRNA stability. It should also be noted that the effects of MG-132 on IL-6 gene expression were less pronounced than the effects on IL-6 protein levels (compare with results in Fig. 3A), indicating that the increased expression of
IL-6 protein after treatment with proteasome inhibitors did not reflect increased IL-6 gene transcription only.

To test if there is a causative relationship between the heat shock response and augmented IL-6 production after treatment with proteasome inhibitor, we treated cells with quercetin, a flavonoid compound that has been shown in previous studies to suppress the heat shock response by downregulating HSF-1 (26) and decreasing HSF-1 activity (23). Treatment of the Caco-2 cells with quercetin before addition of MG-132 reduced the increase in HSP-72 levels, consistent with inhibition of the heat shock response (Fig. 6, top). Quercetin also resulted in a substantial reduction of IL-6 production in cells treated with MG-132 or a combination of IL-1β and MG-132 (Fig. 6, bottom). These results suggest that the increased IL-6 production induced by the proteasome inhibition is, at least in part, dependent on induction of the heat shock response. This interpretation of the data needs to be done with caution, however, because quercetin is not specific in its inhibitory effects on the heat shock response but may cause other cellular effects as well, including modulation of various kinases and antioxidative effects (10, 12).

In addition to induction of the heat shock response, treatment with proteasome inhibitors may prevent the activation of the transcription factor NF-κB by blocking the degradation of IkB, at least in some cell types (17). This effect of proteasome inhibitors is particularly pertinent for the present experiments because the IL-6 gene is at least in part regulated by NF-κB (46). To test whether proteasome inhibitors block NF-κB activation in enterocytes, IkB-α levels and NF-κB DNA binding activity were determined in cultured Caco-2 cells treated with IL-1β or MG-132. Similar to previous reports from this laboratory, stimulation of the cells with IL-1β resulted in reduced levels of IkB-α and increased NF-κB DNA binding activity, and these effects were seen after both 30-min and 2-h IL-1β treatment (Fig. 7, A and B, respectively). These effects of IL-1β were inhibited by both MG-132 and lactacystin with the most complete inhibition noted for MG-132. Interestingly, quercetin did not influence the response of NF-κB to IL-1β or MG-132 (Fig. 7C). Taken together with the results shown in Fig. 3A, the data suggest that treatment of the Caco-2 cells with proteasome inhibitors increases IL-6 production despite inhibited NF-κB activation. Thus it is possible that other transcription factors regulate the IL-6 gene in cells after induction of the heat shock response. In addition to NF-κB, the IL-6 gene is regulated by CCAAT/enhancer binding protein (C/EBP), activator protein (AP)-1, and cAMP response element binding protein (CREB) (46).

The role of the different transcription factors in the regulation of the IL-6 gene varies with the treatment and condition of the cell, and previous studies suggest that the IL-6 gene is not always regulated by NF-κB (45).

Because in the present study, induction of the heat shock response with proteasome inhibitors was associated with a strong upregulation of HSF DNA binding activity (see Fig. 1), we speculated that the IL-6 gene may be regulated by HSF as well. Because it is not known if the IL-6 promoter contains binding sites for HSF, we examined the published sequence of the 5′-flanking region of the human IL-6 gene (53) for pHREs. HREs typically consist of a series of repeating pentameric motifs, the most common being nGAGn and nCTCn, where “n” represents less conserved nucleotides (2). The sequences of HREs vary, however, and they may contain other motifs as well, the most common of which are nGAGn and nCTCn. Examination of the 5′-flanking region of the human IL-6 gene revealed three segments of the promoter that contained sequences at least partially consistent with naturally occurring HREs (Fig. 8). We called these segments pHRE1, pHRE2, and pHRE3.

To determine if the pHREs bind protein after induction of the heat shock response with proteasome inhibitor, we prepared oligonucleotide probes corresponding to a known HRE (the same probe that was used in the experiments shown in Figs. 1 and 2) and to each of the pHREs shown in Fig. 8. When cells were treated with MG-132, EMSA revealed a DNA/protein complex with the known HRE probe (similar to the results in Figs. 1

![Fig. 6. HSP-72 protein levels determined by Western blotting (top) and IL-6 production in Caco-2 cells (bottom) after treatment with 10 μM MG-132 and 0.5 ng/ml IL-1β. Cells were pretreated with MG-132 for 1 h followed by treatment with IL-1β for 24 h. Additional cells were preincubated with 100 μM quercetin (+QCTN) for 1 h before addition of MG-132 or MG-132 + IL-1β; n = 6 for each condition. *P < 0.05 vs. all other groups; **P < 0.05 vs. Veh, IL-1, MG-132, and MG-132 + IL-1β group without quercetin by ANOVA.](http://ajpregu.physiology.org/article/fig/6)

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and 2) and with the pHRE2 probe (Fig. 9A). The binding of protein to the pHRE2 probe was reduced in cells treated with quercetin (Fig. 9B), providing support (but not proof) for the concept that a HSF binds to the pHRE2 after treatment with MG-132. The protein/DNA complex seen when the pHRE2 probe was used for EMSA was obliterated by an excess amount of unlabeled pHRE2 oligonucleotide but not by an excess of nonspecific (NF-κB) probe, providing evidence for the specificity of the EMSA (Fig. 10). A supershift was seen after addition of an antibody to HSF-1 (Fig. 10). Taken together, the results shown in Figs. 9 and 10 suggest that the pHRE2 segment from the IL-6 promoter binds HSF-1 in Caco-2 cells treated with MG-132, consistent with the concept that the IL-6 gene may be a HSF-responsive gene, at least under the present experimental conditions.

**DISCUSSION**

The results in the present study suggest that treatment with proteasome inhibitors induces the heat shock response and upregulates basal and IL-1β-stimulated IL-6 production in human enterocytes. Because the proteasome inhibitors blocked NF-κB activation, the results indicate that transcription factors other than NF-κB become important for the regulation of the IL-6 gene in enterocytes expressing the heat shock response. When one considers the multiple important biological roles of IL-6, both locally in the mucosa and systemically (3–5, 32, 37, 41, 52), this means that modulating enterocyte and mucosal IL-6 production may have important clinical implications. Methods to...
increase IL-6 production may be particularly important in light of recent reports suggesting that IL-6 is an anti-inflammatory cytokine that can provide cell protection in different tissues and under various conditions (3, 4, 5, 41, 52).

Although induction of the heat shock response by proteasome inhibition has been reported in a number of different cell types (6, 19, 24, 54), the relationship between proteasome inhibition, expression of heat shock proteins, and cell protection is not universal. For example, in recent studies, inhibition of proteasome activity in neurons caused cell death and increased vulnerability to oxidative injury (24). In addition, the noxious effects of proteasome inhibition were counteracted by heat shock proteins in the same cells. Thus it is important to establish the effect of proteasome inhibition and its relationship to the heat shock response as well as other cellular functions in individual cell types. The importance of examining the effects of proteasome inhibition in enterocytes is further illustrated by the fact that proteasome inhibitors have been proposed as therapeutic agents in various disease states (6, 22), including chronic colitis (8). The safety of using proteasome inhibitors in the clinical treatment of patients has been established in recent studies in which the treatment was administered to patients with multiple myeloma, chronic lymphocytic leukemia, and different solid tumors (9).

In the present experiments, the induction of the heat shock response was monitored by determining HSF DNA binding activity and HSP-72 levels. Although supershift analysis of the HSF EMSA suggested that HSF-1 was involved in the induction of the heat shock response, the results do not rule out the possibility that other members of the HSF transcription factor family were activated as well. Indeed, our results suggest that HSF-2 was activated by MG-132 although those results were less clear than the results for HSF-1. Previous studies suggest that multiple HSFs can be activated by proteasome inhibitors, at least in some cell types (19).

Cellular levels of HSP-72 (the inducible form of HSP-70) were measured here because this protein was expressed in most previous studies in which proteasome inhibitors induced the heat shock response (6, 19, 24, 54). In some cell types, proteasome inhibition upregulated the expression of multiple heat shock proteins (6, 19, 24), whereas in other cell types, including human HepG2 cells, treatment with proteasome inhibitors selectively increased HSP-72 levels without affecting cellular levels of HSP-25, HSP-27, HSP-60, HSP-86, HSP-90, HSP-104, and Bip (54). The influence of proteasome inhibition on heat shock proteins other than HSP-72 in the enterocyte remains to be determined.

Although the mechanisms by which proteasome inhibition induces the heat shock response are not fully understood, accumulation of abnormal proteins secondary to inhibition of their degradation by the proteasome may at least in part explain the heat shock response (6). In that model, HSP-70 that is normally bound to HSF-1 is diverted to the abnormal proteins as...
chaperones. This reduces the inhibition of HSF-1 normally exerted by HSP-70, allowing for the activation of HSF with transactivation of the HSP-70 gene and increased production of the heat shock protein (1). An additional mechanism that has been proposed in heat shock response induced by proteasome inhibition is reduced degradation of a short-lived protein that is a positive regulator of heat shock protein transcription (54). Support for the role of a short-lived protein was provided in experiments showing that the proteasome inhibitor-induced heat shock response was blocked by cycloheximide (54). Interestingly, in the same study, evidence was found that hyperthermia-induced heat shock response is not dependent on de novo protein synthesis, supporting the concept that mechanisms of heat shock response induction may be different, depending on the stimulus. Finally, treatment of cells with proteasome inhibitors resulted in hyperphosphorylation, trimerization, and increased DNA binding activity of HSF-1, and it was suggested that inhibited degradation of a short-lived kinase targeting HSF-1 and/or cofactors for the kinase may be a mechanism of the heat shock response after inhibition of the proteasome (20).

In addition to testing the hypothesis that treatment of enterocytes with proteasome inhibitors results in induction of the heat shock response, the present experiments were designed to determine the influence of proteasome inhibition on enterocyte IL-6 production. The results suggest that proteasome inhibition stimulates basal IL-6 production and, to an even greater extent, potentiates the effect of IL-1β on enterocyte IL-6 production. The present observations of reduced HSF activation and HSP-72 levels and inhibited IL-6 production in cells treated with quercetin are consistent with the concept that the increased IL-6 production was dependent on the heat shock response. However, because quercetin is not a specific HSF inhibitor, this interpretation needs to be made with caution. It is not known from the present results if the effect was caused by HSP-72 or some other heat shock protein(s). Although previous studies support a specific role of HSP-72 in some of the biological effects associated with the heat shock response, further experiments are needed to determine which specific heat shock protein(s) is responsible for increased IL-6 production in enterocytes treated with proteasome inhibitor.

In the present study, proteasome inhibition resulted in a four- to sixfold increase in IL-6 production in IL-1β-treated Caco-2 cells with a much smaller increase in IL-6 mRNA levels and gene transcription. These results suggest that the increased IL-6 protein levels were not only caused by transcriptional upregulation but by other mechanisms as well, e.g., increased translational efficiency of IL-6 mRNA and/or inhibited degradation of IL-6. In this context it is interesting to note that previous studies suggest that heat shock proteins may protect mRNAs from degradation (18, 21, 30, 43), and it may be speculated that a similar mechanism can protect IL-6 from degradation as well. Regardless of the mechanisms of increased IL-6 levels in Caco-2 cells treated with the proteasome inhibitors, reduced IL-6 levels in the same cells suggest that different mechanisms are involved in the regulation of individual cytokines by proteasome inhibition.

Results from the nuclear run-on assay suggest that the increased IL-6 mRNA levels were at least in part caused by increased gene transcription although other mechanisms may have also been involved. We reported previously that the heat shock response increased mRNA stability (30). This observation was confirmed in recent studies in which induction of the heat shock response resulted in HSP-70 sequestration of the AU-rich element (ARE)-binding protein AUF-1 (21). AUF-1 is a protein that promotes the degradation of short-lived mRNAs (such as cytokine mRNAs) containing AREs. Consequently, sequestration of AUF-1 by HSP-70 results in inhibited degradation of ARE mRNAs. The potential role of this mechanism in the upregulated IL-6 mRNA levels in our experiments remains to be determined.

Increased IL-6 production, despite downregulated NF-κB activity, as noticed here in cells expressing the heat shock response, may seem contradictory to previous reports demonstrating an important role of NF-κB in the regulation of the IL-6 promoter (46). It should be noted, however, that the IL-6 gene is under the regulation of multiple transcription factors (46), and there is evidence that the role of the different transcription factors varies with different stimuli and cell conditions. In a recent study, treatment of cultured fibroblasts with tumor necrosis factor (TNF)-α or staurosporin resulted in increased IL-6 production (45). When experiments were performed using plasmids with different promoter constructs, evidence was found that TNF-α-induced IL-6 production was regulated by NF-κB with no involvement of C/EBP, AP-1, or CREB, whereas staurosporin-induced IL-6 production was mediated by C/EBP, AP-1, and CREB with no involvement of NF-κB. Thus although NF-κB is an important regulator of IL-6 production in most cell types and conditions, IL-6 production can be regulated without NF-κB involvement under certain circumstances. The present results suggest that the increased IL-6 production in enterocytes expressing the heat shock response is not regulated by NF-κB. It should be noted that in previous studies, we found evidence that the IL-6 gene is under the regulation of NF-κB in cultured enterocytes that have not been subjected to induction of the heat shock response (36).

The reason why downregulation of NF-κB activity in enterocytes treated with proteasome inhibitors did not result in reduced IL-6 production may at least in part reflect increased activity of other IL-6-related transcription factor(s). In recent studies in this laboratory, treatment of cultured Caco-2 cells with MG-132 or lactacystin increased the expression and DNA binding activity of C/EBP-β and -δ (Hungness ES, Robb BW, Luo GJ, Pritts TA, Hershko DD, and Hasselgren PO, unpublished observations), supporting the concept that other transcription factors may become predominant in the regulation of the IL-6 gene after induction.
of the heat shock response. An interesting novel observation in the present study was the finding that the IL-6 promoter contains several HREs and that at least one of these regions of the promoter may bind HSF-1 in enterocytes expressing the heat shock response. Although further experiments are needed to better define the role of HSF-1 (and other HSFs) in the regulation of the IL-6 gene, the present results suggest that IL-6 may be a HSF-responsive gene.

In summary, the present results suggest that proteasome inhibition results in induction of the heat shock response and upregulated IL-6 production in human enterocytes. Because NF-xB activation was blocked by the same treatment, it is likely that other transcription factors become important for regulation of the IL-6 gene in enterocytes expressing the heat shock response, and it is possible that HSF-1 may be one of those transcription factors. The present results are important considering the multiple biological roles of IL-6, including anti-inflammatory and protective effects both locally in the gut mucosa and systemically, and considering recent proposals in the literature to use proteasome inhibitors in the clinical arena to induce the heat shock response.

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