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Proteasome Inhibitors Activate the Transcription Factors C/EBP-β and δ in Human Intestinal Epithelial Cells

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In recent studies, induction of the heat shock response by hyperthermia upregulated the expression and DNA binding activity of the transcription factor C/EBP. This is an important observation because it may at least in part explain why the heat shock response upregulates IL-6 production in the intestinal mucosa and in the enterocyte. A novel method to induce the heat shock response is proteasome inhibition. The influence of this treatment on the expression and DNA binding activity of C/EBP is not known. We treated cultured Caco-2 cells, a human intestinal epithelial cell line, with one of the proteasome inhibitors, MG-132 or lactacystin, and measured C/EBP-β and δ DNA binding activity by electrophoretic mobility shift assay and supershift analysis. In addition, nuclear levels of C/EBP-β and δ protein were determined by Western blot analysis. Treatment of the cells with the proteasome inhibitors resulted in increased cellular levels of heat shock protein 72, consistent with induction of the heat shock response. Treatment also resulted in increased DNA binding activity and nuclear protein levels of C/EBP-β and δ. The effects of the proteasome inhibitors on C/EBP were inhibited by treating the cells with quercetin, a substance known to block the heat shock response. The results suggest that proteasome inhibition activates the transcription factors C/EBP-β and δ in human intestinal epithelial cells and that this response, at least in part, is caused by induction of the heat shock response. The observations are important because they provide support for a novel method to influence gene activation in the enterocyte.

It is now well established that the intestinal mucosa and enterocyte are active participants in the metabolic and inflammatory responses to severe injury and sepsis (1). For example, the production of several acute phase proteins and cytokines, including IL-6, is increased in gut mucosa during sepsis and endotoxemia (2–4) and in cultured enterocytes after exposure to inflammatory stimuli (5, 6). Studies from our and other laboratories have provided evidence that acute phase protein and cytokine production in the enterocyte is regulated at the transcriptional level during inflammation and that activation of several transcription factors, including NF-κB, AP-1 and C/EBP, is essential for the inflammatory response in the enterocyte (7–11).

In recent studies we found that induction of the heat shock response downregulated NF-κB activity and provided protective effects in the intestinal mucosa of endotoxemic mice (12, 13). Interestingly, mucosal and enterocyte IL-6 production was augmented by the heat shock response, despite inhibited NF-κB DNA binding activity (13, 14). These observations suggest that other transcription factor(s) may be activated and become important for the regulation of the IL-6 gene after induction of the heat shock response. Consistent with that concept, we recently found evidence that C/EBP activity was increased in enterocytes after induction of the heat shock response (15), suggesting that this transcription factor may be involved in the upregulation of IL-6 production and perhaps in the protection of the mucosa after induction of the heat shock response.

In most of the studies described above, the heat shock response was induced by hyperthermia. A novel method to induce the heat shock response is treatment with proteasome inhibitors as described initially by Zhou *et al.* in hepatocytes (16). In recent studies in our laboratory, treatment of cultured Caco-2 cells, a human intestinal epithelial cell line, with one of the proteasome inhibitors MG-132 or lactacystin, induced the heat shock response as evidenced by activation of heat shock factor-1 (HSF-1) and increased cellular levels of heat shock protein 72 (17). In the same experiments, the proteasome inhibitors blocked NF-κB activation

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and increased IL-6 production. In contrast, the influence of treatment with proteasome inhibitors on C/EBP activity in the enterocyte is not known.

C/EBP is a family of transcription factors consisting of at least six members, C/EBP-α through ϵ and CHOP 10 (18, 19). Among the different members of the C/EBP family, the C/EBP-β and δ isoforms are particularly important for the inflammatory response (10). C/EBP regulates the transcription of the IL-6 gene (20) as well as several other cytokines and acute phase proteins (10). In recent studies we found evidence that C/EBP-β and δ regulate IL-6 production in stimulated enterocytes (Hungness *et al.*, unpublished observations).

In the present study, we tested the hypothesis that proteasome inhibitors upregulate the DNA binding activity of C/EBP-β and δ and that this response at least in part reflects the induction of the heat shock response. We found that treatment of cultured Caco-2 cells with MG-132 or lactacystin increased C/EBP-β and δ activity and that this effect of the proteasome inhibitors was blocked by quercetin, a substance known to inhibit the induction of the heat shock response (21).

MATERIALS AND METHODS

Cell culture. Caco-2 cells (American Type Culture Collection, Rockville, MD) were grown at 37°C in 5% CO_2 in DMEM supplemented with 10% fetal bovine serum (FBS), nonessential amino acids, 6 mM glutamine, 10 mM Hepes, 10 μg/ml apotransferrin, 1 mM pyruvate, 24 mM $NaHCO_3$, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Gibco-BRL, Grand Island, NY). Cells, between passages 5 and 25, were seeded at a density of 100,000 cells/cm^2 onto 10-cm tissue culture plates (Falcon-Becton–Dickinson, Franklin Lakes, NJ). Cells were grown for 72 h to 90% confluence before use.

Experimental conditions. Before experiments, Caco-2 cells were washed three times with serum-free DMEM. Cells were then treated for 4 h with serum-free medium containing one of the proteasome inhibitors MG-132 (10 μM) or lactacystin (20 μM) (both from Calbiochem, La Jolla, CA). The concentrations of MG-132 and lactacystin used here were based on previous reports in which they inhibited proteasome activity and induced the heat shock response in other cell types (16, 22, 23). Because MG-132 and lactacystin were solubilized in dimethyl sulfoxide (DMSO), control cells were incubated in corresponding concentrations of DMSO. The concentration of DMSO in the culture medium did not exceed 0.75% (v/v). In some experiments, cells were pretreated with 100 μM quercetin (Sigma Chemical Co., St. Louis, MO) for 1 h prior to addition of MG-132 or DMSO. This concentration of quercetin effectively inhibited induction of the heat shock response by blocking the activation of heat shock factor-1 (HSF-1) in other studies (21).

Preparation of nuclear fraction. After the different treatments, the culture medium was aspirated and the cells were harvested in 1 ml of ice-cold phosphate-buffered saline (PBS, pH 7.4). The samples were centrifuged for 5 min at 3000*g*. The cells were weighed and resuspended in one packed-cell volume of lysis buffer [10 mM Hepes, pH 7.9; 10 mM KCl; 1.5 mM $MgCl_2$; 0.1 mM ethylenediaminetetraacetic acid (EDTA); 1 mM 1,4-dithiothreitol (DTT); 0.25 vol% Nonidet P-40; 0.1 mM phenylmethylsulfonyl fluoride (PMSF); protease inhibitor cocktail; and phosphatase inhibitor cocktail 1; all from Sigma]. After incubation for 5 min on ice, the nuclear pellets were isolated by centrifugation (3000 g for 5 min). The nuclear pellets were then resuspended in one packed-cell volume of extract buffer [20 mM Hepes, pH 7.9; 420 mM NaCl; 0.1 mM EDTA; 1.5 mM $MgCl_2$; 25% (v/v) glycerol; 1 mM DTT; 0.5 mM PMSF; protease inhibitor cocktail; and phosphatase inhibitor cocktail 1], and incubated for 15 min. Samples were centrifuged at 16,000*g* for 20 min and the supernatants were saved as the nuclear extracts. Protein concentrations were determined using a modified Bradford assay (Bio-Rad Laboratories, Hercules, CA).

Electrophoretic mobility shift assay (EMSA). C/EBP gel shift oligonucleotide (5′-TGC AGA TTG CGC AAT CTG CA-3′) (Santa Cruz Biotechnology, Santa Cruz, CA) was end labeled with [γ-^{32}P]ATP using polynucleotide kinase T4 (Gibco-BRL). End-labeled probe was purified from unincorporated [γ-^{32}P]ATP using a purification column (Bio-Rad Laboratories) and recovered in Tris–EDTA buffer (TE), pH 7.4. Nuclear proteins (15 μg) were incubated in buffer containing 12% glycerol (v/v), 12 mM Hepes, pH 7.9, 4 mM Tris–HCl, pH 7.9, 1 mM EDTA, 1 mM DTT, 25 mM KCl, 5 mM $MgCl_2$, 0.04 μg/μl poly [d(I–C)] (Boehringer Mannheim, Indianapolis, IN), and TE, pH 7.4. Labeled probe was added and the samples were incubated for 30 min at room temperature. For supershift reactions, 2 μl of antibodies to C/EBP-β or δ (Santa Cruz Biotechnology) was added 30 min after the addition of the radiolabeled probe. Samples were then subjected to electrophoretic separation on a nondenaturing 5% polyacrylamide gel at 100 V using Tris–borate–EDTA buffer (0.45 M Tris–borate, 1 mM EDTA, pH 8.3). Blots were dried overnight and analyzed by exposure on PhosphorImager screens (Molecular Dynamics, Sunnyvale, CA).

Western blotting. Aliquots of the nuclear fractions containing 50 μg of protein were boiled in equal volumes of loading buffer [125 mM Tris–HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 20% glycerol, and 10% 2-mercaptoethanol] for 3 min and separated by electrophoresis on an 8–16% Tris–glycine gradient gel (Invitrogen, Carlsbad, CA). See-Blue Standard (Invitrogen) was included as a molecular weight marker. The proteins were then transferred to nitrocellulose membranes using the Novex Xcell II blot module (Invitrogen). The membranes were blocked with 5% nonfat dried milk in Tris-buffered saline, pH 7.6 (TBS) containing 0.05% Tween 20 (TTBS) for 30 min and incubated with antibodies to C/EBP-β or δ (Santa Cruz Biotechnology) or HSP-70 (Stressgen Biotechnology Corp.). After washing twice with TTBS, the blots were incubated with a peroxidase-conjugated IgG secondary antibody for 60 min. The blots were washed in TTBS for 5 min three times, incubated in enhanced chemiluminescence reagents (ECL, Amersham–Pharmacia Biotech, Uppsala, Sweden), and exposed on radiographic film (Eastman Kodak Co., Rochester, NY).

Experiments were performed at least three times to ensure reproducibility of results.

RESULTS

Treatment of cultured Caco-2 cells with MG-132 or lactacystin resulted in increased C/EBP DNA binding activity (Fig. 1). Supershift analysis of the EMSAs suggested that both C/EBP-β and δ were activated by the proteasome inhibitors. Among the two proteasome inhibitors used here, MG-132 is the more potent drug (24). In subsequent experiments in the present study we used MG-132 to achieve proteasome inhibition.

To test the potential role of the heat shock response for the effect of the proteasome inhibitors, cellular levels of hsp-72 were measured. Treatment of the cells with MG-132 resulted in increased hsp-72 levels (Fig. 2), consistent with previous results in Caco-2 cells (17)

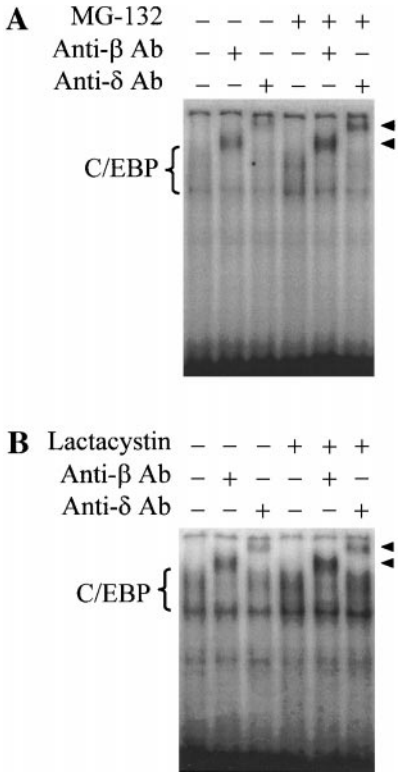

FIG. 1. Effect of MG-132 (A) and lactacystin (B) on C/EBP DNA binding activity in cultured Caco-2 cells. Cells were treated with 10 μM MG-132 or 20 μM lactacystin for 4 h, after which nuclear fractions were prepared and EMSA was performed. Supershift analysis was performed by adding anti-C/EBP-β or anti-C/EBP-δ antibodies to the reaction as indicated above the gels. Supershifted bands are indicated by arrowheads.

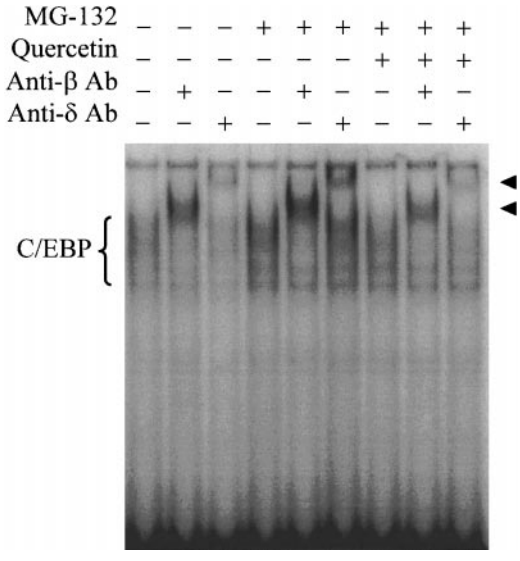

FIG. 3. Effect of MG-132 and quercetin on C/EBP DNA binding activity in cultured Caco-2 cells. Cells were treated with MG-132 (10 μM) for 4 h as indicated above the gels. Some cells underwent pretreatment with 100 μM quercetin for 1 h before treatment with MG-132. Supershift analysis was performed by adding antibodies to C/EBP-β or C/EBP-δ as indicated above the gels. Supershifted bands are indicated by arrowheads.

and other cell types as well (16, 22, 25). When cells were treated with quercetin, the MG-132-induced increase in hsp-72 levels was blocked.

We next determined the effect of quercetin on C/EBP activity in cells treated with MG-132. When cells were pretreated with quercetin, the activation of C/EBP-β and δ by MG-132 was blocked (Fig. 3), supporting the interpretation that activation of C/EBP by the proteasome inhibitor was associated with induction of the heat shock response.

Because activation of C/EBP at least in part reflects translocation from the cytoplasm to the nucleus of the various isoforms, we next determined nuclear levels of C/EBP-β and δ by Western blotting. Treatment of the cells with MG-132 resulted in increased nuclear levels of both isoforms and this effect of MG-132 was blocked by quercetin (Fig. 4).

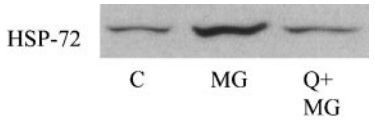

FIG. 2. Heat shock protein 72 levels in Caco-2 cells treated with 10 μM MG-132 for 4 h with or without pretreatment with 100 μM quercetin for 1 h. Control cells were cultured in the absence of MG-132 and quercetin. C, control; MG, MG-132; Q + MG, MG-132 + quercetin.

DISCUSSION

In the present study, treatment of cultured Caco-2 cells with one of the proteasome inhibitors MG-132 or lactacystin resulted in activation of C/EBP-β and δ. Because the same treatment resulted in increased cellular levels of hsp-72 and because the effects of the

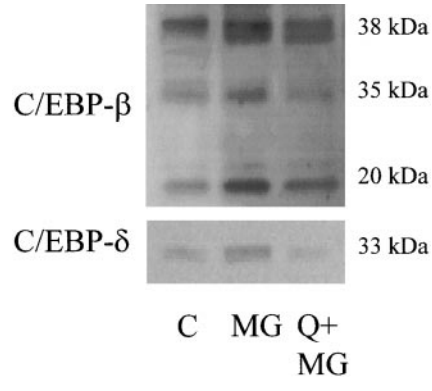

FIG. 4. C/EBP-β and δ protein levels in the nuclear fraction of Caco-2 cells treated with 10 μM MG-132 for 4 h or 10 μM MG-132 for 4 h preceded by 100 μM quercetin for 1 h. C, control; MG, MG-132; Q + MG, MG-132 + quercetin.

proteasome inhibitors on C/EBP binding activity was blocked with quercetin, the results were interpreted as indicating that C/EBP activation by the proteasome inhibitors at least in part was caused by the heat shock response.

The present results are in line with, but not identical to, previous reports of upregulated C/EBP activity after induction of the heat shock response by hyperthermia. Thus, in recent studies, subjecting cultured Caco-2 cells to hyperthermia resulted in the induction of the heat shock response and increased C/EBP DNA binding activity (15). In those experiments, the heat shock response was associated with activation of the β, but not the δ, isoform of C/EBP. In other studies, induction of the heat shock response by hyperthermia in mice resulted in increased C/EBP DNA binding activity in liver (26). Interestingly, in those studies as well, the hyperthermia-induced heat shock response upregulated the C/EBP-β but not δ isoform. Thus, it is possible that the influence of the heat shock response on the individual C/EBP isoforms depends upon the method by which the heat shock response is induced. This may be related to previous studies in which evidence was found that proteasome inhibitors and hyperthermia induce the heat shock response by different mechanisms (16).

The heat shock response has been shown in previous studies to provide protection against various noxious influences, at a cellular, tissue, and whole-organism level (27, 28). Understanding the effects of different methods to induce the heat shock response, therefore, has important clinical implications. Induction of the heat shock response by treatment with proteasome inhibitors was reported previously in a number of different cell types (16, 22, 25) and more recently in the enterocyte as well (17). The use of a drug, such as a proteasome inhibitor, to induce the heat shock response, may be more practical in the clinical setting than subjecting a person to hyperthermia. Indeed, proteasome inhibitors have been proposed as therapeutic agents in various disease states (22, 24), including chronic colitis (29).

Increased C/EBP activity may be a mechanism of the increased mucosal and enterocyte IL-6 production that we recently observed after induction of the heat shock response (13, 14). Although IL-6 is commonly considered a proinflammatory cytokine (30), there is evidence that IL-6 may have important anti-inflammatory and protective effects as well (31–33). It is possible that the biological role of IL-6 depends on tissue and cell type and local concentrations. The fact that the heat shock response was associated with increased mucosal IL-6 levels and maintained mucosal morphology in endotoxemic mice (13) supports the concept that high IL-6 levels in the gut after induction of the heat shock response may be beneficial. In more recent studies, we found that the heat shock response significantly blunted the increase in mucosal permeability in mice with endotoxemia (Wang and Hasselgren, unpublished observations). Thus, understanding the effects of the heat shock response on C/EBP, an important regulator of the IL-6 gene, has significant clinical implications. It will be important to determine the influence of the heat shock response in the enterocyte on other transcription factors involved in the regulation of the IL-6 gene, including AP-1 and CREB (20). Because mucosal NF-κB activity was downregulated by the heat shock response in endotoxemic mice (12), it is likely that the other transcription factors regulating the IL-6 gene are involved in the response to heat shock.

Quercetin was used in the present study to block the heat shock response. In previous studies, quercetin was found to be an effective and selective inhibitor of the heat shock response, mainly by blocking the activation of HSF-1 (21). It should be noted that quercetin is not completely specific in its actions. For example, quercetin is known to inhibit protein synthesis (34). In a recent study, however, evidence was found that the inhibition of HSF-1 activation did not reflect inhibited protein synthesis (21).

The present finding of increased hsp-72 levels after treatment with MG-132 and inhibition of this response by quercetin does not necessarily mean that the influence of the heat shock response on C/EBP was mediated by hsp-72. 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 (16, 17, 22, 25). Thus, in the present experiments, hsp-72 was used as a marker of the heat shock response and it is likely that other heat shock proteins were expressed as well (25). Additional studies are needed to define which heat shock protein(s) (if any) is responsible for the upregulation of C/EBP activity. Indeed, it is possible that none of the heat shock proteins is responsible for the activation of C/EBP but this effect of the heat shock may be mediated by a heat shock factor; in a recent study, evidence was found that the promoter of mouse C/EBP-β contains a heat shock responsive element (26).

The mechanisms by which proteasome inhibitors induce the heat shock response are not fully understood, but accumulation of abnormal proteins secondary to inhibition of their degradation by the proteasome may at least in part explain the heat shock response (22). According to 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-1 with transactivation of the hsp-70 gene and increased production of the heat shock protein. An additional mechanism that has been proposed in proteasome inhibitor-induced heat shock response is reduced degradation of a short-lived protein that is a positive reg-

ulator of heat shock protein transcription (16). Support for the role of a short-lived protein was found in experiments in which the proteasome inhibitor-induced heat shock response was blocked by cycloheximide (16). A third potential mechanism was found in a study in which 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 (35).

Although the present results and those in recent studies (15) are consistent with the concept that the increase in C/EBP activity after treatment with proteasome inhibitors reflected the heat shock response, other mechanism(s) may be possible. For example, a recent study suggests that the homolog of C/EBP-β in the sea slug *Aplysia californica*, ApC/EBP, is degraded by the proteasome (36). Proteasome-dependent degradation of C/EBP could explain why treatment with the proteasome inhibitors resulted in increased levels of the C/EBP isoforms in the present study but further experiments are needed to test that hypothesis. Regardless of the mechanism, the present finding of upregulated expression and activity of C/EBP after treatment with proteasome inhibitors is important because it provides support for a novel method to influence gene activation in the enterocyte.

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