Proteasome Inhibitors Activate Stress Kinases and Induce Hsp72

DIVERSE EFFECTS ON APOPTOSIS*

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Inhibition of the major cytosolic protease, proteasome, has been reported to induce programmed cell death in several cell lines, while with other lines, similar inhibition blocked apoptosis triggered by a variety of harmful treatments. To elucidate the mechanism of pro- and antiapoptotic action of proteasome inhibitors, their effects on U937 lymphoid and 293 kidney human tumor cells were tested. Treatment with peptide aldehyde MG132 and other proteasome inhibitors led to a steady increase in activity of c-Jun N-terminal kinase, JNK1, which is known to initiate the apoptotic program in response to certain stresses. Dose dependence of MG132-induced JNK activation was parallel with that of apoptosis. Furthermore, inhibition of the JNK signaling pathway strongly suppressed MG132-induced apoptosis. These data indicate that JNK is critical for the cell death caused by proteasome inhibitors. An antiapoptotic action of proteasome inhibitors could be revealed by a short incubation of cells with MG132 followed by its withdrawal. Under these conditions, the major heat shock protein Hsp72 accumulated in cells and caused suppression of JNK activation in response to certain stresses. Accordingly, pretreatment with MG132 reduced JNK-dependent apoptosis caused by heat shock or ethanol, but it was unable to block JNK-independent apoptosis induced by TNFα. Therefore, proteasome inhibitors activate JNK, which initiates an apoptotic program, and simultaneously they induce Hsp72, which suppresses JNK-dependent apoptosis. A balance between these two effects might define the fate of cells exposed to the inhibitors.

Mammalian cells respond to various stressful conditions by activation of stress kinase signaling cascades. Activation of a stress-kinase, c-Jun N-terminal kinase (JNK),1 by strong oxidants, UV irradiation, and some other stressful conditions proceeds through a signal transduction pathway that involves small GTP-binding proteins and a cascade of kinases (1, 2).

other components of the kinase cascade inhibits programmed cell death in response to heat shock, UV irradiation, oxidative stress (5), and certain other inducers (3, 4, 6). However, there are certain types of apoptosis (e.g. Fas- or TNF-induced) that are JNK-independent (7, 8).

Many stressful conditions also induce heat shock proteins, including Hsp72, which increases a cell’s tolerance to stresses. Therefore, the cell’s decision to die or to survive is determined by the fine balance of these two systems. In cells affected by stresses, Hsp72 binds to damaged and misfolded polypeptides and can either facilitate their repair or target nonreparable polypeptides for degradation by the ubiquitin- and proteasome-dependent pathway (see Refs. 9 and 10 for review). Our recent findings demonstrate, however, that the cell-protective action of Hsp72 may be unrelated to its activity in protein refolding or degradation. In fact, Hsp72 interferes with the activation of stress kinase JNK, and thus prevents apoptotic signaling during certain stresses (11). This suppression of JNK appears to be responsible for Hsp72-mediated protection of cells from apoptosis induced by heat shock, ethanol, and some other stresses (11).2

Recently, inhibition of major cellular protease, proteasome, was shown to activate a cell death program (12–18). In fact, incubation of cells with potent proteasome inhibitors, namely peptide aldehydes and lactocystin, either facilitated apoptosis caused by TNF or Fas-ligand (14) or was alone sufficient to induce apoptosis (13, 15–18). Since many important regulatory proteins, e.g. p53, are the substrates of proteasome, it was suggested that inhibition of the breakdown of these proteins could result in their accumulation, thereby leading to apoptosis (16, 17). On the contrary, other laboratories reported that treatment with inhibitors of proteasome-dependent protein breakdown protected cells from apoptosis triggered by growth factor withdrawal, ionizing irradiation, exposure to glucocorticoids, or phorbol ester (19, 20). Such protection was observed with quiescent cells, while induction of apoptosis by proteasome inhibitors was demonstrated mainly with proliferating cultures. This led to the idea that inhibition of protein degradation has much more severe effects on cells progressing through the cell cycle. However, there are some examples where exposure of quiescent cells to inhibitors also activated apoptosis (16, 20).

Effects of the inhibition of proteasome-dependent proteolysis on apoptosis could provide new insights into the mechanisms triggering programmed cell death. Furthermore, information about the nature of the inhibitor’s cytotoxicity could also be helpful in drug development, since some of the proteasome

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1 The abbreviations used are: JNK, c-Jun N-terminal kinase; TNF, tumor necrosis factor; PARP, poly(ADP-ribose) polymerase; aLLN, N-acetyl-leucinyl-leucinyl-norleucinal.

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inhibitors are currently in use as lead compounds for drug design. In this work, we have studied effects of proteasome inhibitors on the activation of JNK and the role of this kinase in cytotoxicity of such inhibitors. We also investigated the role of induction of Hsp72 in the antiprotective effects of the proteasome inhibitors.

EXPERIMENTAL PROCEDURES

Materials and Cell Culture—U937 human lymphoid tumor cells were grown in RPMI 1640 medium with 10% fetal bovine serum and were used for experiments while in the midlog phase (3–7 \times 10^5 cells/ml). 293 human cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and were used in transient transfection experiments while at 40% of confluence. MG132 was purchased from Biomol Research Laboratory, Inc. N-Acetyl-leucinyl-leucinyl-norleucinal (aLLN) was purchased from Sigma. MG132 analogs were synthesized and purified by high pressure liquid chromatography. Anti-hsp72 antibody (SPA810) was from StressGene Biotechnologies Corp. (Canada), and anti-p53 antibody (PAb1801) was from Santa Cruz Biotechnology, Inc.

JNK and p38 Assay—JNK activity was assayed using GST-c-Jun protein as a substrate after immunoprecipitation with anti-JNK1 antibodies (sc-474, Santa Cruz Biotechnology) or anti-HA antibody for transfected JNK (Berkeley Antibody Company, Richmond, CA). Briefly, cells were lysed in a buffer containing 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2 mM EDTA, 1% Triton X-100, 25 mM \( \gamma \)-glycerophosphate, 10 mM NaF, 1 mM Na3VO4, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride and a 25 \mu g/ml concentration each of aprotinin, pepstatin, and leupeptin). After immunoprecipitation with anti-JNK1 or anti-HA antibody and protein A-Sepharose for 2 h at 4 °C and washing, a kinase reaction was carried out in a buffer containing 25 mM HEPES, 10 mM MgCl2, 2 mM dithiothreitol, 25 mM \( \gamma \)-glycerophosphate, 2 mM Na3VO4, and 25 \mu Ci of [\( \gamma \]-\( ^{32} \)P]ATP at 37 °C for 10 min. Then the samples were subjected to SDS-polyacrylamide gel electrophoresis followed by transfer to a nitrocellulose membrane and autoradiography. This membrane was later used for immunoblot with anti-JNK1 antibody to ensure that equal amounts of the kinase were immunoprecipitated. p38 kinase activity was determined by Western blot with polyclonal antibody (New England Biolabs) specifically recognizing the phosphorylated (active) form of p38. Later the same blot was treated with anti-p38 antibody (New England Biolabs) to ensure equal loading of the kinase on different lanes.

Apoptosis Assays—Poly(ADP-ribose) polymerase (PARP) degradation was followed by Western blotting with anti-PARP monoclonal antibodies (C2–10; G. Poirier, Montreal, Canada). Apoptosis of 293 cells was detected by cleavage of a truncated form of U1 70-kDa protein d12 (21). Cells were transfected by calcium phosphate method with a plasmid encoding d12 tagged with the T7 epitope alone or together with a dominant-negative mutant form of SEK1 (K/R) or c-Jun (TAM67). After 48 h, cells were either treated with 10 \mu M MG132 or subjected to heat shock at 45 °C for 15 min. After an additional 24 h, cells were lysed, and cleavage of d12 was detected by Western blot with anti-T7 epitope antibody.

Protein Degradation Assays—U937 cells were washed once with PBS, resuspended in RPMI 1640 without leucine and with dialyzed fetal bovine serum, and incubated for 40 min. Proteins were then labeled for 1 h with 10 \mu Ci/ml of [14C]leucine. Cells were washed once and chased in regular RPMI 1640 media with 0.2 mg/ml unlabeled leucine, 150 mM cycloheximide (to inhibit protein synthesis and reincorporation of a labeled leucine), and 20 mM chloroquine (to inhibit lysosomal protein degradation) in the presence of different concentrations of MG132 (preincubation of cells with MG132 for 3 h did not change the level of inhibition of degradation). At the end of the chase period, 10% trichloroacetic acid was added, and samples were left for 30 min on ice. Then samples were spun down in Eppendorf centrifuge for 5 min, and the radioactivity in the supernatant was measured. A percentage of degradation was calculated as a ratio of trichloroacetic acid-soluble and total radioactivity in a sample. To assay for inhibition of I-\alpha-B degradation by MG132, cells were incubated for 45 min with different concentrations of the inhibitor, and then TNF\( \alpha \) (5 ng/ml) was added. Samples were collected 10 min later and subjected to polyacrylamide gel electrophoresis followed by Western blot with anti-I-\alpha-B antibody (sc-847, Santa Cruz Biotechnology) and quantitated with a laser scanner.

RESULTS

Proapoptotic Effects of the Proteasome Inhibitors—To dissect the mechanisms of induction of apoptosis by proteasome inhibitors, we tested their effects on activation of JNK, a stress kinase required for initiation of the apoptotic program in response to a variety of stressful conditions. Effects of a very potent proteasome inhibitor, benzyloxy carbonyl-leucinyl-leucinyl-norleucinyl-leucinyl (MG132), on the U937 cell line were studied (Fig. 1A). The degree of apoptosis was followed by the cleavage of PARP and the appearance of an 86-kDa fragment of PARP, which is the hallmark of this process. A fraction of cleaved PARP correlated with a fraction of cells that underwent shrinkage and nuclear condensation (not shown). The first signs of apoptosis were seen at 4–5 h of incubation with 50 \mu M of MG132, and after 7 h of incubation, more than 50% of cells underwent apoptosis. Prolonged exposure to the inhibitor (20 h) caused programmed cell death in almost 100% of the cell population. Similar results (100% apoptosis) were observed upon titration of MG132 down to 1.5 \mu M (Fig. 2A). Therefore, dose dependence of apoptosis on MG132 covered a narrow range of concentrations between 0.2 and 1.5 \mu M.

Apoptosis caused by MG132 closely correlated with activation of JNK. JNK1 activity steadily and strongly increased during incubation with the inhibitor (Fig. 1B) and preceded PARP degradation (Fig. 1A). In addition, the dose dependence of apoptosis was almost parallel with that of activation of JNK (Fig. 2). In fact, incubation of the cells with 1.5 \mu M MG132 activated JNK1 to the maximal level. This saturation plateau...
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Fig. 2. Dose dependence of MG132-mediated induction of apoptosis and activation of JNK. A, dose dependence of MG132-induced PARP cleavage. PARP cleavage was measured after a 16-h incubation with the indicated concentrations of MG132. B, dose dependence of JNK activation by MG132. Samples from the experiment showed in panel A were taken after a 4.5-h incubation with the indicated concentrations of MG132 for measurement of JNK activity. C, quantitation of data from panels A and B by laser scanner. The dose dependence of JNK activation strongly correlates with the dose dependence of PARP cleavage.

Critical role of JNK in MG132-induced apoptosis, we studied if a dominant-negative mutant form of c-Jun, an immediate downstream component of JNK cascade, suppresses such apoptosis. Cotransfection of c-Jun dominant-negative mutant (TAM67) together with d12 strongly suppressed d12 cleavage induced by MG132 (Fig. 3C). Therefore, inhibition of JNK signaling pathway either immediately upstream or immediately downstream of JNK suppressed MG132-induced apoptosis. Taken together, these data strongly indicate that MG132-induced apoptotic pathway involves SEK1, JNK, and c-Jun.

In U937 cells, reduction of the amount of JNK by incubation of cells with an antisense oligonucleotide covering the 5′-translated region of JNK1 coding sequence (23) also suppressed MG132-induced apoptosis. This effect was modest (about 50%), since even after prolonged incubation of cells in the presence of the oligonucleotide, cellular content of JNK1 decreased only about 50% (not shown).

It is noteworthy that exposure of cells to MG132 also led to an activation of another stress-kinase p38 (Fig. 4). This kinase had been shown to participate in production of interleukin-6, activation of phospholipase A2, regulation of phosphorylation of Hsp27, and other processes (24, 25). The pattern of activation of p38 kinase by 1.5 μM MG132 was similar to that of JNK activation (Fig. 4A), but a high concentration of this proteasome inhibitor (50 μM) caused much faster activation of p38 (not shown). Although p38 was reported to be critical for cer-
SB203580 alone did not cause apoptosis). The extent of the inhibition did not change significantly after incubation of cells with a high concentration of MG132 (50 μM). Therefore, there was a possibility that the breakdown of distinct unstable proteins could selectively be inhibited by MG132 even at concentrations that do not affect the breakdown of the bulk of short lived proteins. Defining such unstable protein(s) that regulates JNK is an important goal for future research.

Antipapoptotic Effects of the Proteasome Inhibitors—Recent publications indicate that under certain conditions, inhibition of proteasome-dependent protein breakdown prevents apoptosis triggered by harmful stimuli (19, 20). We suggested that proteasome inhibition may cause both pro- and antipapoptotic effects, and depending on which effect dominates, cells either undergo apoptosis or become protected from apoptosis caused by another stress. As shown above, proapoptotic effects of the proteasome inhibitors are associated with the activation of the JNK pathway. What could be the nature of antipapoptotic action of the inhibitors? It was described previously that exposure to proteasome inhibitors induces the 70-kDa heat shock protein, Hsp72 (12, 29). Recently, we and others reported that Hsp72 accumulation confers resistance to apoptosis (11, 30–33). Therefore, we suggested that antipapoptotic effects of proteasome inhibitors could be mediated by accumulation of Hsp72. In our experiments, incubation of U937 cells with MG132 (Fig. 7A), lactacystin-β-lacton, NIP-L-VS, or aLLN (not shown) was found to dramatically increase the levels of Hsp72. Hsp40 and Hsp27 also appeared to be induced by incubation with MG132 (not shown). By contrast, treatment with even high concentrations of less active and inactive MG132 analogs, benzoyloxy carbonyl-leucyl-leucyl-leucyl-leucine, benzoyloxy carbonyl-leucyl-leucyl-norvaline, or benzoyloxy carbonyl-leucyl-leucyl- norvaline, did not lead to any significant induction of...
Hsp72. These data indicate that induction of Hsp72 was caused by inhibition of the proteasome-dependent protein breakdown.

To investigate whether the induction of Hsp72 by proteasome inhibitors leads to suppression of apoptosis caused by certain stresses, we first searched for the conditions under which antiapoptotic action of proteasome inhibitors could be separated from their proapoptotic effect. Incubation of cells with 1.5 \( \mu \text{M} \) MG132 for 4.5 h, followed by its withdrawal from the culture, activated JNK only temporally (Fig. 1C), which was insufficient to induce apoptosis (not shown). This treatment, on the other hand, was sufficient to cause strong accumulation of Hsp72 after several (4–16) hours of recovery (Fig. 7B). These conditions appeared to be suitable for revealing an antiapoptotic action of the proteasome inhibitor. Using heat shock as an apoptotic stimulus, we found that pretreatment with MG132 reduced the extent of cell death from about 40% to a negligible level (Fig. 8A). Thus, long incubation of U937 cells with the proteasome inhibitor caused prolonged activation of JNK leading to apoptosis. By contrast, short incubation of the same cells with this inhibitor caused temporal nontoxic activation of JNK (Fig. 1C) and induction of Hsp72, which protected cells from certain stresses.

What could be the mechanism of protection of cells from stresses after pretreatment with the proteasome inhibitors?

We have previously observed that overproduction of Hsp72 blocks stress-induced apoptosis by suppression of JNK (11). In line with this data, MG132-induced protection of cells from heat shock also resulted from suppression of JNK. In fact, severe heat shock of pretreated cells led to much lesser activation of this kinase compared with activation of JNK under the same conditions in untreated cells (Fig. 8A). Thus, long incubation of U937 cells with MG132 reduced PARP cleavage during heat shock-induced apoptosis. U937 cells were incubated with 1.5 \( \mu \text{M} \) MG132 for 4.5 h. Then MG132 was removed, and after 13.5 h of recovery, cells were subjected to heat shock at 43 °C for 60 min. PARP processing was assayed 20 h later. C, control; HS, heat-shocked cells. B, pretreatment of cells with MG132 inhibits activation of JNK in response to the heat shock and TNFα. U937 cells were pretreated with MG132 as indicated in Fig. 7A and then exposed to either heat shock (43 °C for 60 min) or TNFα (5 ng/ml for 15 min), and JNK activity was assayed. C, pretreatment of cells with MG132 does not protect cell from TNFα-induced apoptosis. U937 cells were pretreated with MG132 as indicated in Fig. 7B and then exposed to TNFα (5 ng/ml for 15 min), and PARP cleavage was measured in 20 h. D, pretreatment of cells with MG132 or mild heat shock renders temporary suppression of JNK activation by subsequent incubation with this inhibitor. After either short incubation with 1.5 \( \mu \text{M} \) MG132 for 4.5 h or mild heat shock at 43 °C for 20 min, cells recovered for 16 h and then were incubated with MG132 for the indicated time periods or subjected to heat shock (HS; 43 °C for 60 min, as a control stress), and JNK1 activity was measured.
sequent long term incubation with MG132. In fact, this pretreatment caused strong suppression of JNK at early (up to 6 h) but not late (after 8 h) time points (Fig. 8D). Temporal suppression of JNK in the pretreated cells correlated with a delay in apoptosis; e.g. after 8-h incubation with the proteasome inhibitor, only 10% of PARP were cleaved in MG132-pretreated cells, while the extent of cleavage in control cells was 30%. However, after 18 h of incubation, almost all PARP was cleaved in both types of cells. The effect of MG132 pretreatment on activation of JNK and apoptosis caused by subsequent stress was similar to the effects of pretreatment with mild heat shock, i.e. mild heat shock also rendered transient suppression of JNK (Fig. 8D) and delayed apoptosis caused by MG132 (not shown). Probably, during long incubation with this inhibitor, a putative activator of JNK accumulates and reaches a level when protective effects of pretreatment with MG132 or mild heat shock become insufficient.

To further evaluate the suggestion that pretreatment with MG132 prevents apoptosis by suppression of JNK, we tested effects of such pretreatment on two other types of apoptosis: ethanol-induced apoptosis, which requires JNK, and TNF-induced apoptosis, which is able to mobilize a distinct, JNK-independent pathway (7, 8). MG132 strongly suppressed activation of JNK by both ethanol (not shown) and TNF (Fig. 8B), while effects on cell death were strikingly different. Similarly to its effects on heat shock-induced apoptosis, pretreatment with MG132 suppressed cell death caused by ethanol (not shown). By contrast, pretreatment with this inhibitor did not rescue cells from TNF-induced apoptosis and even potentiated it (Fig. 8C). Likewise, pretreatment of the cells with mild heat shock, which induced Hsp72, strongly reduced activation of JNK after exposure to TNF, but no protection from TNF-induced apoptosis was observed under these conditions (not shown). Therefore, pretreatment with proteasome inhibitors appears to protect cells only from JNK-dependent apoptosis.

**DISCUSSION**

Recently, proteasome inhibitors were demonstrated to cause very complex effects on programmed cell death. With some exceptions, it appeared that dividing cells respond to the inhibitors by activation of apoptosis, while in nondividing cells such inhibitors showed antiapoptotic effects. Therefore, the common assumption was that this difference is attributed to differences in the cell types or in proliferating activity. Here we demonstrated that both pro- and antiapoptotic effects of proteasome inhibitors could be seen with the same cell line, U937, depending on the conditions of treatment (i.e. prolonged treatment resulted in apoptosis, while shorter exposure did not kill cells; it induced Hsp72 that caused protective effects). Therefore, we suggested that in cell types where JNK could not be highly activated by proteasome inhibitors, they do not manifest a proapoptotic effect (in fact, we observed that in the COS-7 cell line where MG132 does not activate JNK, it is unable to induce apoptosis). Then, accumulation of Hsp72 in response to proteasome inhibitors would render cells protected from certain types of apoptosis. In prior publications that reported antiapoptotic effects of proteasome inhibitors, these inhibitors were applied simultaneously with the apoptotic stimuli (19, 20). Probably MG132 is a poor activator of JNK in cell lines used in these experiments. Fast Hsp72 accumulation may also contribute to prevention of commitment of these cells to apoptosis.

Proteasome inhibitor-induced apoptosis was dependent on the stress kinase JNK, as with heat shock and certain other stresses. In fact, these inhibitors caused very strong activation of JNK, and dose dependence of such activation closely corre-
thermore, MG132 could not cause apoptosis in rapidly proliferating COS-7 cells (while activating expression of Hsp72), which suggests that correlation between the cell division and proapoptotic action of the proteasome inhibitors is not universal. It does not exclude, however, the possibility that certain rapidly proliferating cells are more sensitive to exposure to the proteasome inhibitors, because a protein regulator of JNK may accumulate in such cells more efficiently, for example, due to a faster synthesis.

What could be the mechanism of another cellular response to inhibition of proteasome synthesis of Hsp72? It is well established that accumulation of misfolded proteins induces Hsp72 (for a review, see Ref. 35). Therefore, we originally suggested that the primary signal for induction of Hsp72 by the proteasome inhibitors is accumulation of newly synthesized polypeptides, which for certain reasons cannot acquire a proper conformation. However, our data rule out this possibility, because MG132 was able to dramatically induce Hsp72 at concentrations that did not affect the breakdown of the bulk of short-lived proteins. Therefore, it appears that accumulation of a specific regulatory polypeptide(s) causes induction of Hsp72. Although we have not identified the putative short-lived protein inducer of Hsp72, probably it is different from the putative activator of JNK. In fact, although dose dependence of Hsp72 induction varies among cells, this effect of the proteasome inhibitors is specific to the apoptotic pathway that involves JNK activation. By contrast, JNK-independent apoptosis was expected not to be sensitive to preincubation of cells with MG132. Although TNFα is a strong activator of JNK, TNFα-induced apoptosis was reported to be independent of JNK (7, 8). Accordingly, we observed that cells pretreated with MG132 suppressed TNFα-activation of JNK but did not reduce TNFα-induced apoptosis (Fig. 8, B and C). Thus, the protective effects of the proteasome inhibitors indeed appeared to be limited to the JNK-dependent apoptotic pathway. Previously, it was reported that MG132 protects cells from apoptosis induced by the nerve growth factor withdrawal, γ-irradiation, and phorbol ester (19, 20). Although there are no data on the JNK-dependence of phorbol ester-induced apoptosis, it has been shown that JNK is essential for apoptosis induced by both nerve growth factor withdrawal and γ-irradiation (3, 4).

Therefore, the inhibition of proteasome activator of the apoptotic pathway dependent on the stress-kinase JNK and simultaneously induces synthesis of the protective protein Hsp72, which suppresses JNK. We suggest that the balance between these two activities could define whether pro- or anti-apoptotic action of the inhibitors would dominate.

The ability of the proteasome inhibitors to induce Hsp72 and to suppress JNK and p38 kinases could be very useful in the design of new drugs. In fact, JNK-mediated apoptosis appears to be involved in the pathology of myocardial ischemia (36), while p38 is critical for a variety of inflammatory responses (37). Therefore, MG132 could be used as a prototype of therapeutics against certain myocardial and inflammatory pathologies.

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