Proteasome Inhibition Leads to a Heat-shock Response, Induction of Endoplasmic Reticulum Chaperones, and Thermotolerance*

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The accumulation of misfolded proteins in the cytosol leads to increased expression of heat-shock proteins, while accumulation of such proteins in the endoplasmic reticulum (ER) stimulates the expression of many ER resident proteins, most of which function as molecular chaperones. Recently, inhibitors of the proteasome have been identified that can block the rapid degradation of abnormal cytosolic and ER-associated proteins. We therefore tested whether these agents, by causing the accumulation of abnormal proteins, might stimulate the expression of cytosolic heat-shock proteins and/or ER molecular chaperones and thereby induce thermotolerance. Exposure of Madin-Darby canine kidney cells to various proteasome inhibitors, including the peptide aldehydes (MG132, MG115, N-acetyl-leucyl-leucyl-norleucinal) and lactacystin, inhibited the degradation of short-lived proteins and increased markedly the levels of mRNAs encoding cytosolic heat-shock proteins (Hsp70, polyubiquitin) and ER chaperones (BiP, Grp94, Erp72), as shown by Northern blot analysis. However, inhibitors of cysteine proteases (E64), serine proteases (leupeptin), or metalloproteases (1,10-phenanthroline) had no effect on the levels of these mRNAs. The relative efficacies of the peptide aldehyde inhibitors in inducing these mRNAs correlated with their potencies against the proteasome. Furthermore, reduction of the aldehyde group of MG132 decreased its inhibitory effect on proteolysis and largely prevented the induction of these mRNAs. Although treatment with the proteasome inhibitors caused rapid increases in mRNA levels (as early as 2 h after treatment with MG132), the inhibitors did not detectably affect total protein synthesis, total protein secretion, ER morphology, or the retention of ER-luminal proteins, even after 18 h of treatment. Together, the findings suggest that inhibition of proteasome function induces heat-shock proteins and ER chaperones due to the accumulation of sufficient amounts of abnormal proteins and/or the inhibition of degradation of a key regulatory factor (e.g. heat-shock factor). Since expression of heat-shock proteins can protect cells from thermal injury, we tested whether the proteasome inhibitors might also confer thermotolerance. Treatment of cells with MG132 for as little as 2 h, markedly increased the survival of cells subjected to high temperatures (up to 46 °C). Thus, these agents may have applications in protecting against cell injury.

The cellular “heat-shock response,” manifested by increased expression of heat-shock proteins, represents a basic defense mechanism employed by cells to protect themselves against high temperature and various other injurious conditions (1, 2). Most of the major heat-shock proteins function as molecular chaperones involved in the folding, assembly, and/or degradation of proteins and therefore appear to prevent the accumulation of aggregated, misfolded, or damaged proteins in the affected cell (1, 3, 4). In the cytosol, heat-shock or other harsh conditions cause increased transcription and translation of a group of chaperones (e.g. Hsp70) and polyubiquitin (a cofactor in intracellular protein degradation). In the endoplasmic reticulum (ER),1 harsh growth conditions (e.g. glucose deprivation) induce the increased production of ER molecular chaperones, i.e. the glucose-regulated proteins (e.g. Grp78/BiP, Grp94, and Erp72) (2–5). It is now well established in bacterial (6) and animal (1, 7) cells that the signal for the induction of heat-shock proteins is the accumulation of abnormal proteins in the cytosol. Similarly, conditions that perturb folding or glycosylation of surface or secreted proteins cause a buildup of misfolded proteins in the ER, which appears to be a common signal, at least indirectly, for the increased expression of the ER chaperones (8, 9).

Abnormal proteins in the cytosol and nucleus are degraded primarily through the ubiquitin-proteasome pathway (10–14). The proteasome is the major neutral proteolytic apparatus in the cell and is an essential component of the ATP-dependent degradative pathway (10, 15, 16). Recent studies with inhibitors in lymphoblasts suggest that the proteasome is responsible not only for the degradation of many rapidly turned-over proteins, but also for the bulk of other proteins (17). The degradation of misfolded proteins transiting the ER is less well understood, although at least in the case of one transmembrane protein, the cystic fibrosis transmembrane conductance regulator, the proteasome appears to be involved (18, 19).

Recently a variety of reversible (17) and irreversible (20) inhibitors of the 20 S proteasome have been identified that can enter mammalian cells and inhibit degradation of proteins by the ubiquitin-proteasome pathway. One group of such inhibitors are peptide aldehydes (e.g. carboxyanilide-leucinyl-leucinyl-leucinal (MG132)) which reversibly bind to active sites and

1The abbreviations used are: ER, endoplasmic reticulum; MDCK, Madin-Darby canine kidney; BiP, immunoglobulin-binding protein; Grp94, 94-kDa glucose-regulated protein; Erp72, 72-kDa endoplasmic reticulum protein; Hsp, heat-shock protein; aLLN, N-acetyl-leucyl-leucyl-norleucinal; MG132, carboxyanilide-leucinyl-leucinyl-leucinal; MG115, carboxyanilide-leucinyl-leucinal-norvaline.

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inhibit cleavage of hydrophobic or acidic substrates (17). A more specific inhibitor is the naturally occurring bacterial compound, lactacystin, which covalently modifies threonine residues in the proteasome's active site and does not seem to affect any other known protease (20). Such agents can inhibit protein degradation (17–19) and major histocompatibility class I antigen presentation (17) in a variety of mammalian cells and have been widely used to probe the physiological function of the ubiquitin-proteasome pathway.

Since the blockage of protein breakdown in the cell (e.g. by inhibition of proteasomes) should lead to an accumulation in cells of proteins otherwise targeted for degradation, it seemed likely that these agents might also signal the induction of the cytosolic and/or ER chaperones. To test this possibility, we examined the ability of several types of protease inhibitors to increase the levels of mRNA encoding the cytosolic heat-shock proteins (i.e. Hsp70 and polyubiquitin) and/or the ER chaperones (i.e. BiP, Grp94, and ERP72). In addition to specific inhibitors of the proteasome, we also studied the effects of an inhibitor of cysteine proteases (such as lysosomal cathepsins B, H, and L and calpains) and a metal chelating agent that inhibits metalloproteases. These other inhibitors have also been reported to affect the degradation of certain ER proteins (21–24). However, in our studies, only those agents which have been reported to inhibit the activity of the proteasome (17, 20) were able to consistently stimulate expression of mRNAs for heat-shock proteins and ER chaperones. Furthermore, proteasome inhibition was found to protect cells from subsequent thermal injury, a finding that may have broad implications, not only for the interpretation of experimental studies using these inhibitors, but possibly for therapeutic use of these highly specific inhibitors.

EXPERIMENTAL PROCEDURES

Reagents and Chemicals—DNA probes were kindly provided by the following investigators: ERp72 and Grp94 by Michael Green (St. Louis University); BiP, Grp78, and BiP by Mary-Jane Gething (Melbourne University, Australia). The cDNA probe for Hsp70 was from ATCC (Rockville, MD). Carboxyamidomethylated and carbobenxoxyl-leucinyl-leucinyl-leucinal (MG132) and carbobenzoxyl-leucinyl-leucinyl-norvaline (MG115) were kindly provided by ProScript (whatman), washed with ice-cold 5% trichloroacetic acid, ice-cold absolute acetone, and leupeptin were from Sigma. Antibodies to antibody was from Affinity Bioreagents (Nashane Station, NJ), and fluorescent isothiocyanate-labeled secondary antibodies were from Jackson Immunologicals (West Grove, PA).

Treatment Conditions and Northern Blot Analysis—Confluent monolayers of Madin-Darby canine kidney (MDCK) cells were incubated for up to 18 h in the absence or presence of either tunicamycin (a potent inducer of ER stress proteins) or protease inhibitors in Dulbecco's modified Eagle's medium (DME) supplemented with 5% fetal calf serum (FCS). Total RNA was isolated from the cells by phenol-chloroform extraction, electrophoresed on 1% formamide/formaldehyde-agarose gels, and transferred to nitrocellulose as described previously (5, 25). The nitrocellulose blots were then hybridized with random primed 32P-labeled cDNA overnight at 42 °C, washed with SSC, and exposed to autoradiographic film (26).

Protein Degradation Assay—To examine the degradation of short-lived proteins, MDCK cells were incubated at 37 °C for 1 h in tyrosine-free DMEM supplemented with 5% FCS to which 5 μCi/ml [3H]tyrosine (Whatman), washed in DPBS and chased for 1 h in normal DMEM, 5% FCS (104 μCi/ml tyrosine-2Na2H2O; 20 μCi chloroquine was added for the final 30 min of this chase). After this chase, the medium was replaced with fresh unlabeled DMEM, 5% FCS containing inhibitors and 20 μCi chloroquine and chased for an additional 2 h at 37 °C. Determination of total acid-soluble radioactivity was performed at the end of this chase as described above.

Inactivation of MG132—Since it is the aldehyde group of the peptide aldehyde inhibitors that is thought to interact with the proteasome and thus interfere with its ability to degrade proteins (34, 35), inactivation of this aldehyde group should ameliorate the effects of these inhibitors. Inactivation of the aldehyde group was accomplished by reduction of this group to an alcohol. DPBS with or without MG132 (final concentration 0.7 mM) was treated with 0.1 mM sodium borohydride for 1 h at room temperature. The reaction was then stopped by acidification of the samples to pH 5 with glacial acetic acid to inactivate the sodium borohydride. The entire solution was then refrigerated for at least 1 h to ensure complete inactivation of the sodium borohydride. Confluent monolayers of MDCK cells were then incubated in DMEM, 5% FCS in the absence or presence of tunicamycin, MG132, borohydride-reduced MG132, or an equivalent volume of borohydride-treated DPBS. After 18 h of incubation, total RNA was collected, and Northern blot analysis was performed as described above.

Protein Synthesis and Secretion—Confluent monolayers of MDCK cells were treated with inhibitors as described above. A time course of incorporation of 35S-labeled methionine into cellular and secreted proteins was performed. Cells were pulsed with 35S for 30 min in methionine-free DMEM, 5% FCS following either 0, 3, 6, 9, 12, or 15 h of growth in normal DMEM, 5% FCS (30 μCi/ml methionine) containing protease inhibitors. The cells were then chased in nonradioactive normal DMEM, 5% FCS (30 μCi/ml methionine) containing protease inhibitors for an additional 2.5 h. Cells and media were collected separately and labeled proteins were precipitated with 10% trichloroacetic acid. Acid-precipitated proteins were collected on glass microfiber filters (Whatman), washed with ice-cold 5% trichloroacetic acid, ice-cold absolute acetone, and the total acid-precipitable radioactivity (representing the total incorporation of 35S-labeled methionine into cellular or secreted proteins) was measured in a scintillation counter.

Immunofluorescence—Confluent monolayers of MDCK cells growing on coverslips were treated with the inhibitors for 12–18 h. They were then fixed by plunging into ~80 °C methanol and processed for indirect immunofluorescence as described previously (27).

Immunoblot Protein Analysis—Following 12–18 h of growth in the presence of protease inhibitors, the medium was collected, trichloroacetic acid-precipitated material was solubilized in SDS-polyacrylamide gel electrophoresis sample buffer. Equivalent amounts of the conditioned medium were electrophoresed on a 10% SDS-polyacrylamide gel. Western blots and probing of nitrocellulose membranes with primary antisera were performed as described previously (27–29). Immunoblots were developed using the ECL chemiluminescent system (Amersham Corp.) with horseradish peroxidase-conjugated secondary antisera.

Thermotolerance—Confluent monolayers of MDCK cells were incubated at 37 °C for 2 h in the absence or presence of the inhibitors. Following this “pretreatment,” cells were washed three times (15 min each wash) in fresh media to remove the inhibitors. After the final wash, the cells were incubated at 37 °C in fresh medium for an additional 3 h. The culture media was then changed a final time, and the cells were incubated in a separate CO2 incubator maintained at 46 °C for an additional 4–5 h. The cells were then rinsed in PBS and exposed for 5 min to 0.1% trypsin blue in PBS (supplemented with physiological levels of Ca2+ and Mg2+), and the percentage of nonviable cells was determined.

RESULTS

Inhibitors of proteasome function can block the degradation of most short- and long-lived proteins (17), and therefore treatment with these agents is likely to cause the accumulation of such proteins not only in the cytosol, but possibly also in the ER, since protein degradation by this pathway has been reported to function in the quality control of some ER-associated proteins (18, 19). To test whether treatment of cells with proteasome inhibitors may cause the induction of the Hsps and ER chaperones, we examined the ability of several inhibitors of the proteasome to increase the content of the mRNAs for the cytoplasmic Hsps, Hsp70 and polyubiquitin,
and/or ER chaperones, BiP, ERP72, and Grp94 (2, 5, 28–30), in MDCK cells, a widely studied polarized epithelial cell line. When cells were incubated for 12–16 h with the peptide aldehyde proteasome inhibitor, MG132, the mRNA levels of both the cytosolic Hsps and the ER chaperones increased several-fold (Fig. 1). Another aldehyde proteasome inhibitor, aLLN (at concentrations greater than 10 μM), was also able to induce consistent increases in both groups of mRNAs (Fig. 2A). In the case of MG132 (10 μM), the most potent inhibitor of the proteasome, the levels of BiP and Grp94 mRNA were roughly comparable with those achieved with tunicamycin (10 μg/ml) treatment (Fig. 1), one of the most potent inducers of ER chaperones described (31–33). Two other protease inhibitors, which have been reported to retard the degradation of certain proteins transiting the ER, including the specific inhibitor of cysteine proteases, E64 (22), and the metalloprotease inhibitor, 1,10-phenanthroline (23, 24), were found to have little or no effect on these mRNAs (Fig. 1). In addition, leupeptin, a potent inhibitor of certain serine proteases and many thiol proteases (e.g., the lysosomal cathepsins and calpain), which blocks most lysosomal proteolysis, but does not affect degradation of short-lived or abnormal proteins, also had no effect on these mRNA levels (Fig. 1). Thus, only those agents that inhibited the function of the proteasome (17) were found to increase mRNAs for cytosolic heat-shock proteins or ER chaperones (Figs. 1 and 2A), both of which are generally believed to be induced by the accumulation of abnormally folded protein (1, 3, 4, 6–9). To confirm that these effects were due to a reduction in proteolysis, the rate of breakdown of short- and long-lived proteins was measured in control and treated cells. Although measurement of protein degradation following treatment of cells with either MG132 or aLLN showed that both MG132 and aLLN were capable of blocking the degradation of proteins, MG132 (particularly at the concentrations used to induce the chaperones (< 10 μM)) was a more potent inhibitor of the degradation of short-lived proteins (Fig. 3A). Treatment of cells with as little as 1 μM MG132 reduced the degradation of short-lived proteins up to 60–70% of control, while this level of inhibition was achieved only after treatment with 30 μM aLLN. Moreover, MG132 was found to inhibit the degradation of long-lived proteins with much greater efficacy than aLLN at all concentrations tested (Fig. 3B). Furthermore, even a concentration of 30 μM aLLN was only able to achieve roughly half the inhibition of long-lived protein degradation as 1 μM MG132 (Fig. 3B). By contrast, borohydride-reduced MG132 (see below) was much less effective in blocking protein degradation (<30% inhibition of proteolysis) (Fig. 3C). Moreover, as expected, tunicamycin, an inhibitor of N-linked glycosylation, which also induces BiP expression, had no effect of total proteolysis (data not shown).

To determine whether the observed increases in the mRNAs for the Hsps and ER chaperones were in fact due to the inhibition of the proteasome and not the result of nonspecific effects, cells were treated with an inactive form of MG132 incapable of inhibiting proteolytic activity of the proteasome. The aldehyde group of peptide aldehydes, such as MG132, forms a transition state hemiacetyl complex with the threonine active site of the proteasome and interferes with its ability to degrade proteins (34, 35). Thus, if this aldehyde group were altered (e.g., reduced to an alcohol), the ability of MG132 to inhibit the proteasome should largely be lost. We therefore inactivated MG132 by reducing its aldehyde group with sodium borohydride. Incubation of MDCK cells with the reduced MG132 had no effect on the mRNA levels for Hsp70, polyubiquitin, or the ER chaperones, even after 16 h of incubation (Fig. 2B). Together with the finding that the borohydride-reduced MG132 was ineffective in blocking protein degradation (see above; Fig. 3), these experiments indicate that the increases in the message levels for the Hsps and ER chaperones observed with active peptide aldehyde inhibitors are most likely due to inhibition of the ubiquitin-proteasome proteolytic pathway and the accumulation of undegraded proteins and/or inhibition of a key regulatory factor (e.g., heat-shock factor).

Additional support for this hypothesis was obtained when cells were treated with the less potent peptide aldehyde, carboxenzoxy-leucinyl-leucinyl-norvaline (MG115), or the irreversible natural product, lactacystin. MG115 (like MG132) is a hydrophobic peptide aldehyde that also inhibits the proteasome (17). Lactacystin, which reacts with the proteasome active site threonine on the β subunit, appears to be totally ineffective for the proteasome (20). Northern blot analysis revealed that treatment of cells with either of these inhibitors of the proteasome (both at 10 μM) caused increases in the mRNA levels for both Hsp70 and BiP (Fig. 4A). The similar results obtained upon treatment with MG115 (as well as the other peptide aldehyde inhibitors, MG132 and aLLN) and lactacystin, which inhibit the proteasome by very different mechanisms, argues that the mRNA increases directly result from specific inhibition of the proteasome (probably due to an accu-
MDCK cells were labeled for 18 h with [3H]tyrosine, washed, and chased for 2 h in fresh medium with or without 1, 10, or 30 μM MG132 for short-lived proteins, MDCK cells were labeled for 1 h with [3H]tyrosine, washed and chased for 2 h in fresh complete medium. The cells were then chased for 1 h in fresh complete medium. The cells were then chased for 1 h in fresh complete medium with or without 1, 10, or 30 μM MG132 or 1, 10, or 30 μM aLLN. B, for long-lived proteins, MDCK cells were labeled for 18 h with [3H]tyrosine, washed, and chased for 1 h in fresh complete medium. The cells were then chased for an additional 2 h in complete medium with or without 1, 10, or 30 μM MG132 or 1, 10, or 30 μM aLLN. C, reduction of the aldehyde group of MG132 attenuates the inhibitory effects of MG132 on protein degradation.

Proteasome Inhibition and Cellular Stress Response

![Fig. 3. Proteasome inhibitors block degradation of short-lived and long-lived proteins in MDCK cells.](image)

- **A** shows the effects of tunicamycin, MG132, sodium borohydride-reduced MG132, or aLLN on the degradation of short- and long-lived proteins in MDCK cells. A, for short-lived proteins, MDCK cells were labeled for 1 h with [3H]tyrosine, washed and chased for 2 h in fresh medium with or without 1, 10, or 30 μM MG132 or 1, 10, or 30 μM aLLN. B, for long-lived proteins, MDCK cells were labeled for 18 h with [3H]tyrosine, washed, and chased for 1 h in fresh complete medium. The cells were then chased for an additional 2 h in complete medium with or without 1, 10, or 30 μM MG132 or 1, 10, or 30 μM aLLN. C, reduction of the aldehyde group of MG132 attenuates the inhibitory effects of MG132 on protein degradation.

Additional inhibitors of the proteasome also induce the ER molecular chaperones. Northern blot analysis of total RNA from MDCK cells grown for 12–16 h in the absence (control lanes 1 and 5) or presence of either 10 μg/ml tunicamycin (positive control lanes 2 and 6), 10 μM MG132 (lanes 3 and 7), 10 μM MG115 (lane 4), or 10 μM lactacystin (lane 8) as described under “Experimental Procedures.” Blots were probed with 32P-labeled probe for BiP and Hsp70. Ethidium bromide staining of the 28 S rRNA was used to indicate the equivalence of the load.

**Fig. 4.** Additional inhibitors of the proteasome also induce the ER molecular chaperones. Northern blot analysis of total RNA from MDCK cells grown for up to 6 h in the absence (control lane A) or presence of either 10 μg/ml tunicamycin (positive control lane B), 10 μM MG132 (lane C), 10 μM aLLN (lane D), or 10 μM lactacystin (lane E). Total RNA was collected 1, 2, 3, and 6 h after incubation with the agents (as indicated on the left), and Northern analysis was performed using the 32P-labeled probe for BiP or the 32P-labeled probe for Hsp70.

**Fig. 5.** Hsp70 and BiP are induced rapidly upon incubation with proteasome inhibitors. Northern blot analysis of total RNA from MDCK cells grown for 12–16 h in the absence (control lane A) or presence of either 10 μg/ml tunicamycin (positive control lane B), 10 μM MG132 (lane C), 10 μM aLLN (lane D), or 10 μM lactacystin (lane E). Total RNA was collected 1, 2, 3, and 6 h after incubation with the agents (as indicated on the left), and Northern analysis was performed using the 32P-labeled probe for BiP or the 32P-labeled probe for Hsp70.

Increases were seen after 12 h (Figs. 1 and 2A).

To test if the induction of Hsps and ER chaperones could also conceivably be a consequence of cell toxicity, we studied what effect the proteasome inhibitors had on the overall ability of cells to synthesize and secrete proteins. Fig. 6A shows that treatment with the protease inhibitors MG132, lactacystin, or aLLN caused no inhibition of total protein synthesis in MDCK cells, even after 18 h of growth in medium containing the inhibitors (Fig. 6A). Likewise, the total amount of protein secreted by MDCK cells remained unaffected following treatment with the inhibitors (Fig. 6B). Thus, the observed increases in the mRNA encoding the ER chaperones are not likely to be due to an accumulation of proteins in the ER resulting from a generalized blockage of the secretory pathway. While it is possible that the secretion of specific proteins may be affected by these inhibitors, the profile of secreted proteins analyzed by standard SDS-polyacrylamide gel electrophoresis from 35S-labeled cells showed no obvious differences (data not shown).

Accumulation of undegraded proteins could conceivably lead to alterations in cellular and/or ER morphology. However, indirect immunofluorescence with antibodies against the ER chaperone, BiP, revealed that the morphology of the ER was not affected at this level of analysis, even after 12–16 h of treatment with any of the proteasome inhibitors (Fig. 7B). Moreover, the retention mechanism of ER luminal proteins, which has been reported to be affected by other perturbations, such as Ca2+ ionophores (36), remained intact. Thus, Western blot analysis of the media from cells treated with the proteasome inhibitors demonstrated that the cells did not nonspecifically release the ER resident luminal chaperones BiP and Grp94 (Fig. 7A). Together, these results suggest that inhibition of the proteasome has minimal (if any) effects on the processing of normal proteins, at least in MDCK cells, admittedly a “harpy” cell line. Since several highly sensitive measures of cellular and ER well-being appear unaffected, it is likely that the ob-
radiolabeled MDCK cells incubated in the absence or presence of either or aLLN on the secretion rate of MDCK cells. The growth medium from cystin (55). In addition, cells were also treated with tunicamycin (10 mM, 3 h, and the level of radiation was measured. Even after 18 h of incubation the protease inhibitors have little or no effect on the secretary rate of MDCK cells.

A graph showing effects of MG132, lactacystin, or aLLN on the rate of protein synthesis in MDCK cells. 3S-Labeled cells grown in the absence (control) or presence of the inhibitors (10 mM MG132, 15 mM aLLN, 10 mM lactacystin) were collected and solubilized every 3 h for 18 h, and the amount of radioactive protein was measured. Protein synthetic rate of MDCK cells is apparently unaffected, even after 18 h of incubation in the protease inhibitors. B, graph showing effects of MG132, lactacystin, or aLLN on the secretion rate of MDCK cells. The growth medium from radiolabeled MDCK cells incubated in the absence or presence of either 10 mM MG132, 10 mM lactacystin, or 15 mM aLLN was collected every 3 h, and the level of radiation was measured. Even after 18 h of incubation the protease inhibitors have little or no effect on the secretory rate of MDCK cells.

FIG. 6. Neither protein synthesis nor cellular secretion is affected by treatment with proteasome inhibitors. A, graph showing effects of MG132, lactacystin, or aLLN on the rate of protein synthesis in MDCK cells. 3S-Labeled cells grown in the absence (control) or presence of the inhibitors (10 mM MG132, 15 mM aLLN, 10 mM lactacystin) were collected and solubilized every 3 h for 18 h, and the amount of radioactive protein was measured. Protein synthetic rate of MDCK cells is apparently unaffected, even after 18 h of incubation in the protease inhibitors. B, graph showing effects of MG132, lactacystin, or aLLN on the secretion rate of MDCK cells. The growth medium from radiolabeled MDCK cells incubated in the absence or presence of either 10 mM MG132, 10 mM lactacystin, or 15 mM aLLN was collected every 3 h, and the level of radiation was measured. Even after 18 h of incubation the protease inhibitors have little or no effect on the secretory rate of MDCK cells.

Effects on Thermotolerance—The observed increases in mRNA expression induced by proteasome inhibition will very likely lead to increased cellular content of Hsp70 and ER stress proteins. Induction of the heat-shock proteins, especially Hsp70, such as occurs during exposure of cells to high temperature, protects cells against the lethal effects of subsequent exposure to very high temperature or other toxic insults (1, 37–41). To determine if the proteasome inhibitors induced this protective response, we investigated whether exposure of MDCK cells to these agents could protect them against high temperature (thermotolerance), as occurs upon induction of Hsps by other mechanisms. Incubation of MDCK cells at 37 °C for 2 h with 1 mM MG132 was found to dramatically increase cell survival after subsequent exposure to temperatures as high as 46 °C for 4 h (Fig. 8). Moreover, cellular survival was increased also upon exposure for 5 h at temperatures as high as 50 °C (data not shown). Treatment with 1 mM MG132 for this period appeared to maximize enhancement of thermotolerance of MDCK cells at higher temperatures. In contrast, however, treatment with 5 mM and 10 mM MG132 appeared to reduce cell survival at high temperature, while treatment with 0.1 mM and 0.01 mM MG132 had little or no effect on survival (data not shown). In addition, the survival rate of MDCK cells “pretreated” longer (>4 h) or with intermediate concentrations of MG132 (12.5 mM) was similar to that of control cells (data not shown). Thus, there appears to be a relatively narrow window for enhancement of thermotolerance by MG132 in MDCK cells, which, in our experience, are more resistant to environmental stress than many cell lines. Treatment of cells with other protease inhibitors, including aLLN, borohydride-reduced MG132, E64, and 1,10-phenanthroline, was found to have little if any effect on survival of cells at 43 °C for 4 h (Fig. 8). On the contrary, some of these nonproteasome inhibitors appeared to reduce cell survival at higher temperature (Fig. 8). The fact that treatment of cells with aLLN had no significant effect on the survival of cells at high temperatures is probably due to the much lower potency of this drug and the longer time required for any observable increase in the mRNAs for the Hsps (Fig. 5). The effects of lactacystin could not be examined in such experiments as it irreversibly binds to the proteasome and cannot be washed out from the cells. Thus, of all the inhibitors tested, only MG132 proteasome inhibition appears to confer thermotolerance to MDCK cells.

FIG. 7. Treatment of MDCK cells with proteasome inhibitors does not detectably alter ER morphology by immunofluorescence or cause release of ER molecular chaperones, BiP and Grp94. A, Western blot analysis of levels of BiP and Grp94 proteins in MDCK cells (Cells) and growth medium (Media) following 16–20 h of growth in the absence (Control) or presence proteasome inhibitors (10 mM MG132 (MG132), 15 mM aLLN (ALLN), 10 mM lactacystin (Lactacystin)). In addition, cells were also treated with tunicamycin (10 μg/ml) as a positive control. In all cases, little or no protein was detected in the medium. B, MDCK cells grown overnight in the absence (Control) or presence of proteasome inhibitors (10 mM MG132 (MG132), 15 mM aLLN (ALLN), 10 mM lactacystin (Lactacystin)) were fixed and processed for immunofluorescent localization of the ER chaperone BiP. Examination of cells revealed little apparent alteration in distribution of this ER protein following treatment with the inhibitors.
DISCUSSION

Potent inhibitors of the proteasome, including the peptide aldehydes MG132, MG115, as well as the highly specific inhibitor, lactacystin, represent novel tools to study the importance of this cellular degradative pathway. In this study, we examined the ability of these inhibitors, as well as aLLN (which was initially described as an inhibitor of calpain, but has since also been shown to inhibit the ubiquitin-proteasome degradation pathway (17)), to induce the Hsps and ER chaperones. Included in our analysis were other protease inhibitors (E64 and 1,10-phenanthroline) that have been suggested to interfere with the degradation of certain proteins in the ER (Refs. 21–24; reviewed in Ref. 42), as well as leupeptin (a potent inhibitor of lysosomal proteases).

We observed that inhibition of the proteasome with the peptide aldehydes, aLLN, and lactacystin resulted in increases in the mRNAs encoding both the cytoplasmic Hsps and the ER chaperones (Figs. 1, 2, and 4). Although peptide aldehyde proteasome inhibitors (MG132, MG115, and aLLN) can also inhibit calpains and lysosomal proteases in vitro, the higher potency of these agents in blocking proteasome-mediated degradation (Fig. 3; Ref. 17), together with the fact that other calpain inhibitors (such as E64) showed no effect on the mRNA levels of either Hsps or ER chaperones (Fig. 1) and the finding that lactacystin (the most selective proteasome inhibitor currently known (20)) also caused marked increases in mRNAs for both the Hsps and the ER chaperones (Fig. 4), indicates that these increases are the result of an effect of the inhibitors on the proteasome.

One surprising finding is the rapidity with which the transcription of the heat-shock proteins and ER chaperones rise upon addition of MG132. Under conditions where intracellular protein breakdown is inhibited by about 70% (Fig. 3), the mRNAs for Hsp70 and BiP clearly increased within 2 h (Fig. 5), presumably due to the accumulation of abnormal proteins or various short-lived, normal regulatory proteins. In Escherichia coli, inhibitors of the cell's major ATP-dependent proteases can also trigger the heat-shock response (6). These findings support the notion that saturation of the cell's proteolytic capacity can trigger the expression of the heat-shock genes, most of which encode either molecular chaperones or components of the proteolytic pathway (e.g. polyubiquitin). These proteins together can promote the refolding or the destruction of damaged polypeptides whose accumulation could be toxic. Molecular chaperones appear to be important in the cellular mechanism monitoring the buildup of such proteins. The accumulation of such unfolded proteins in E. coli leads to a stabilization of the normally short-lived, positive regulatory factor, \( \sigma^32 \), the specific subunit of RNA polymerase necessary for transcription of heat-shock genes. The rapid degradation of \( \sigma^32 \) requires, as cofactors, the bacterial molecular chaperone, DnaK (the Hsp70 homolog) and its cofactors, DnaJ and GrpE, as well as the ATP-dependent protease (43). Apparently, the accumulation of unfolded proteins saturates DnaK and DnaJ proteins, and thus prevents degradation of \( \sigma^32 \) and leads to increased transcription of Hsps. In eukaryotic cells, a distinct, but related, mechanism involving molecular chaperones seems to function in regulating heat-shock proteins (44). The heat-shock factor is normally associated with Hsp70, which inhibits the expression of heat-shock genes (45). The buildup of abnormal proteins saturates the chaperone and competitively blocks this inhibition of heat-shock factor by Hsp70, leading to expression of heat-shock proteins. The findings presented here would be consistent with such models and provide a new experimental system for its analysis.

Although this mechanism can explain the increases in Hsp70 mRNA by proteasome inhibitors, the mechanism for increases in the mRNA encoding the ER chaperones remains unclear. It is possible that the ER chaperones are induced by the same signal as the Hsps. This is not without precedent, as it has been shown in yeast that KAR2 (the yeast BiP homolog) is up-regulated in response to heat-shock along with Hsp70 (46, 47). However, recent evidence suggests that the increases in ER chaperone mRNA we observed with proteasome inhibitors might be the result of a more direct effect on the ER. For example, the degradation of several membrane proteins have been found to require ubiquitination and the proteasome in yeast ER (48, 49). The degradation of the cystic fibrosis transmembrane conductance regulator is also blocked by the use of inhibitors of the proteasome, suggesting that the proteasome is also involved in the ER degradation of some mammalian membrane-associated proteins (18, 19). How the cytosolic proteasome may function in the degradation of proteins contained within an apparently inaccessible membrane-bound organelle such as the ER remains unclear and constitutes an important area for future study. However, the proteasome (or a proteasome-like structure) has been detected in the microsomal fractions of various cells, although it is believed to be in association with the cystosolic surface of the membrane (16, 50). Although the proteasome would appear to be in a strategic location to assist in the degradation of transmembrane ER proteins with extensive cytosolic domains, it remains unclear how the proteasome localized to the cytosolic face of the ER would affect the degradation of proteins contained entirely within the lumen of the ER, if indeed it does. However, if a coordinated degradative mechanism were to exist, such that the luminal, transmembrane, and cytosolic domains of transmembrane proteins were degraded simultaneously (as has been suggested (reviewed in Ref. 42)), it may be sufficient to inhibit the degradation of such transmembrane proteins alone to elicit an ER stress response. In addition, it is also possible that at least some proteins transiting the ER (transmembrane and secretory), which are targeted for degradation, are extruded from the ER into the cytosol where they are then degraded by the proteasome. Regardless of the mechanism involved, our data suggest that inhibition of the proteasome somehow results in a “backup” in the degradative pathway and accumulation of abnormal proteins (presumably within the ER) with consequent increases in the expression of ER luminal chaperones.

Treatment with MG132 was also found to impart thermotolerance to mammalian cells (Fig. 8), although the window of efficacy may be somewhat narrow, even for MDCK cells, a relatively hardy cell line. Similar findings have been obtained upon treatment of yeast with these proteasome inhibitors, which become resistant to several insults, including high temperature, anoxia, and alcohol following treatment with proteasome inhibitors. Together these findings suggest novel uses for the proteasome inhibitors in the protection of cells from injury. Previous studies have demonstrated that preinduction of the heat-shock proteins by mild stress can protect cells from a variety of subsequent, more severe stresses, including heat, H\(_2\)O\(_2\), anoxia, alcohol, heavy metals, and others (1, 37–41). Most likely, prior heat-shock induces the Hsps by causing the misfolding of cellular protein that is mild or reversible enough for the cell to withstand subsequent greater injury, presumably due to increased cytosolic chaperone synthesis and enhanced folding and/or degradative capacity. Nevertheless, heat-shock and many other stresses that induce cytosolic chaperones are likely to affect a wide variety of cellular functions, and finer tools are obviously necessary for use in therapeutic settings.

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The observed increases in the messages for cytosolic and ER stress response proteins following treatment with highly specific proteasome inhibitors may be due to the accumulation of abnormal protein targeted for degradation in these cellular compartments or alternatively to the inhibition of the degradation of a key factor responsible for the heat-shock response (e.g., heat-shock factor; see above) without any major effect on the processing of normal protein (Figs. 5–6). Since exposure to these inhibitors can induce thermotolerance, they may have investigative or perhaps therapeutic applications in protecting against cell injury.

REFERENCES