Journal of Cellular Biochemistry 99:1085–1095 (2006)

Proteasome Inhibition Induces Differential Heat Shock Protein Response but not Unfolded Protein Response in HepG2 Cells

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Abstract Liver, a central organ responsible for the metabolism of carbohydrates, proteins, and lipoproteins, is exposed to various kinds of physiological, pathological, and environmental stresses. We hypothesized that blockage of proteasome degradation pathway induces heat shock protein (HSP) response and unfolded protein response in the liver cells. In this study, we have characterized cellular responses to proteasome inhibition in HepG2 cells, a well-differentiated human hepatoma cells. We found that proteasome inhibition induced differential response among cytosolic HSPs, that is, increased expression of HSP70, but no change in HSP40, HSC70, and HSP90. However, proteasome inhibition did not induce typical unfolded protein response as indicated by absence of stimulation of GRP78 and GRP94 proteins. Upon proteasome inhibition, inclusion bodies were accumulated, and ubiquitin-conjugated proteins appeared in insoluble fraction, together with HSP40, HSP70, HSC70, and HSP90. After proteasome inhibition, misfolded proteins were increased in the cytosol and in the ER compartment as evaluated by examining ubiquitin-conjugated proteins. However, essentially all ER-associated ubiquitin-conjugated proteins were located on the surface of the ER, which explains why proteasome inhibition does not induce unfolded protein response. In conclusion, proteasome inhibition induces differential HSP response, but not unfolded protein response in HepG2 cells. Our study also suggests that HSPs play important roles in directing proteasomal degradation and protein aggregate formation. J. Cell. Biochem. 99: 1085–1095, 2006.

Key words: apoB; uibiquitin; proteasome; hepatocytes

Protein degradation is as essential to the cell as protein synthesis. There are two major intracellular machineries for protein degradation, that is, lysosome and proteasome. Lysosome deals primarily with extracellular proteins endocytosized, whereas proteasome primarily degrades endogenous proteins. The cytoplasmic 26 S proteasome is the structure basis for proteasome-mediated degradation of proteins. It contains a barrel-shaped proteolytic core complex (20 S proteasome, which catalyzes protein degradation), capped at one or both ends by 19 S regulatory complexes (which recognize ubiquitinated proteins) [Orlowski and Wilk, 2000; Glickman and Ciechanover, 2002]. The proteasome is implicated in a number of important biological functions, such as mitosis, cellular differentiation, signal transduction, modulation of immune and inflammatory

Abbreviation used: ALLN, *N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal; apo, apolipoprotein; ER, endoplasmic reticulum; FBS, fetal bovine serum; GRP, glucose regulated protein; HSP, heat shock protein; NEM, *N*-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; PDI, protein disulfide isomerase; RER, rough ER; SER, smooth ER.

Grant sponsor: American Heart Association National Center; Grant sponsor: American Liver Foundation; Grant sponsor: Shanghai Education Commission; Grant number: E03007; Grant sponsor: US National Institutes of Health; Grant number: HL51586.

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Received 12 January 2006; Accepted 29 April 2006

DOI 10.1002/jcb.20996

responses, transcriptional activation, DNA repair, chromosome maintenance, and apoptosis, etc [Kim and Arvan, 1998; Ciechanover et al., 2000a,b; Ciechanover and Schwartz, 2002]. The disruption or corruption of this process can have deleterious effects on cell growth and viability and lead to a variety of human diseases.

Proteasome-mediated protein degradation pathway involves two successive steps. The first step is the covalent conjugation of polyubiquitin chain to the target proteins (ubiquitination), which provides exquisite selectivity and precise regulation. It requires the activities of E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzyme), and often E3 (ubiquitin ligase). Polyubiquitinated proteins are then recognized and degraded by proteasome. Recent studies also demonstrate that some proteins can be degraded by proteasome via ubiquitin-independent pathway [Benaroudj et al., 2001; Liao et al., 2003; Orlowski and Wilk, 2003]. Blockage of proteasome degradation pathway leads to an accumulation in the cells of misfolded proteins otherwise targeted for proteasomal degradation. The accumulation of misfolded proteins is believed to be linked to several diseases such as ageing-related neurodegeneration and systemic amyloidosis [Ciechanover and Schwartz, 2002; Bossy-Wetzel et al., 2004; de Vrij et al., 2004; Ross and Pickart, 2004; Snyder and Wolozin, 2004; Song and Jung, 2004]. On the other hand, proteasome has been the target for cancer therapy [Kisselev and Goldberg, 2001; Voorhees et al., 2003; Adams, 2004], based on the fact that the rate of protein translation and degradation is higher in cancer cells than that in normal cells.

Molecular chaperones are involved in the folding, assembly, and degradation of proteins and therefore play an essential role in preventing the intracellular accumulation of aggregated, misfolded, or damaged proteins. They represent a basic defense mechanism employed by cells to protect cells against various injurious conditions. In the cytosol, heat shock or other harsh conditions stimulate a group of heat shock proteins (e.g., HSP70) [Feder and Hofmann, 1999]. In the endoplasmic reticulum (ER), cells respond to an accumulation of unfolded proteins in the ER by increasing ER resident proteins, glucose regulated protein (GRP)78 and GRP94, namely unfolded protein response [Schroder and Kaufman, 2005].

We have been studying the biogenesis process of apolipoprotein B (apoB) in the liver. As demonstrated in the liver cell cultures, there is significant proportion of the newly translated apoB that is degraded intracellularly before its secretion. The ubiquitin-proteasome pathway is the major mechanism responsible for this intracellular degradation of apoB [Chan et al., 2000; Fisher and Ginsberg, 2002]. Misfolded apoB is accumulated in the cells treated with proteasome inhibitors, as evident by accumulation of ubiquitinated apoB [Fisher et al., 1997; Liao et al., 1998, 2003; Liao and Chan, 2000, 2001]. Little information is available regarding hepatic response to the accumulation of unfolded (or misfolded) proteins. We reasoned that blockage of proteasome degradation pathway induces HSP response and unfolded protein response in the liver cells. In this study, we have characterized cellular responses to proteasome inhibition in HepG2 cells, a well-differentiated human hepatoma cells [Dashti and Wolfbauer, 1987]. We found that proteasome inhibition induces differential response among cytosolic HSPs, but surprisingly it does not induce ER unfolded protein response. We further demonstrated that essentially all ER-associated ubiquitin-conjugated proteins were located on the surface of the ER, which explains why proteasome inhibition does not induce unfolded protein response.

EXPERIMENTAL PROCEDURE

Materials

Nitrocellulose membrane was from Schleicher & Schuell. *N*-ethylmaleimide (NEM), *N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal (ALLN) was from Sigma. Lactacystin was from Calbiochem. Mouse monoclonal antibody against ubiquitin, goat polyclonal antibodies against human apoB was from Chemicon. Antibodies against protein disulfide isomerase (PDI), GRP78 & GRP94, and HSPs were from Stressgen Biotechnologies Corp. or Santa Cruz Biotechnology, Inc., Tris-glycine gradient gels were from Invitrogen.

Cell Culture

HepG2 cells and 3T3-L1 cells were from American Type Culture Collection and were maintained at 37°C in an atmosphere with 5% CO_2 and in RPMI 1640 (for HepG2 cells) or DMEM medium (for 3T3-L1 cells) containing

10% fetal bovine serum (FBS) (HyClone), penicillin (100 U/ml), and streptomycin (100 μg/ml) (GibcoBRL).

Differentiation of 3T3-L1 Preadipocytes into Adipocytes and Fat Staining

3T3-L1 cells were grown and maintained in DMEM containing 10% FBS in a humidified atmosphere 10% CO_2. For inducing differentiation, 2 days post-confluence, the cells were exposed to the medium DMEM containing 10% FBS and 0.4 μg/ml dexamethasone, 0.5 mmol/L isobutylmethylxanthine, and 5 μg/ml insulin as described [Imamura et al., 2001; Ishibashi et al., 2001]. Four days after the induction, the media was changed and the cells were maintained with DMEM containing 10% FBS. For fat staining, the cells were washed with PBS and fixed for 10 min with 10% formaldehyde and stained with 0.5% Oil Red O for 1 h at room temperature. Cells were washed with 70% methanol three times followed by rinsing with water.

Treatment of the Cell Culture With Proteasome Inhibitors and Tunicamycin

HepG2 cells and 3T3-L1 cells were grown to 75% confluence. Proteasome inhibitors and tunicamycin were dissolved in DMSO and added to cell culture at concentration of 100 μM ALLN, 10 μM lactacystin, or tunicamycin (5 μg/ml) for 17 h incubation with the exception indicated. The control cells were treated with equal amount of DMSO only.

Immunoblot Analysis

Western blot analysis was performed on the cell lysate as described previously [Liao et al., 1998, 1999; Liao and Chan, 2000, 2001]. In brief, 17 h after the treatment with proteasome inhibitors, the cells were washed with cold PBS and lysed in 2% sodium cholate in HEPES-buffered saline (50 mM HEPES, pH 7.4, 200 mM NaCl) containing 1 mM PMSF, 0.1 mM ALLN, 5 mM NEM, and complete protease inhibitors (Boehringer Mannheim). Soluble fraction and insoluble material in the cell lysate was separated by centrifugation at 10,000*g* for 15 min. Insoluble material in the pellets were resuspended in appropriate volume of 1% SDS containing the inhibitors described above and sonicated for 20 s with a microtip sonicator. The samples were then boiled for 5 min in SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer. The proteins were separated by SDS–PAGE, transferred overnight onto nitrocellulose membranes, probed with indicated primary antibodies followed with appropriate horseradish peroxidase-conjugated secondary antibody and detected by enhanced chemiluminescence (ECL kit, Amersham).

Electron Microscopy

Cells were grown to 75% confluence and treated 17 h with ALLN or lactacystin as described above. Cells were washed twice in phosphate buffer (100 mM phosphate buffer, pH 7.5), scraped, and collected by centrifugation for 10 min at 10,000*g*. The pellet was washed twice with PBS and then fixed for 90 min with 1.5% glutaraldehyde in 0.1 M sodium cacodylate pH 7.4. Cells were washed three times with sodium cacodylate and post-fixed in OsO_4 in 0.1 M sodium cacodylate pH 7.4 for 60 min on ice. After washing three times with sodium cacodylate 0.1 M, cells were subjected to a series of graded ethanol dehydration (30, 50, 70, 90, 95, 100%) followed by 1 h incubation in 1:1 100% ethanol/Polybed resin (Polysciences, Inc.). After two changes of fresh 100% resin, the cell pellets were transfected to gelatin molds and polymerized in fresh resin overnight at 60°C.

Subcellular Organelle Fraction

Total microsomes were prepared as described [Liao et al., 1998; Liao and Chan, 2000] with the exception that 10,000g supernatant (3 ml) was layered on 1 ml of 2 M sucrose cushion (2 M sucrose, 10 mM HEPES, pH 7.4) for centrifugation at 100,000*g* for 90 min at 4°C. The resulting microsomes were collected. Subfraction of the total microsomes into rough ER (RER), smooth ER (SER), and Golgi complex were prepared according to Banerjee and Redman [1984] as described previously [Liao et al., 2003]. The total microsomes were adjusted to density 1.16 g/ml with an appropriate volume of 2 M sucrose. The total microsome fraction (3 ml) was layered on a 2 M sucrose (3 ml) and layered successively with 1.1 M sucrose (3 ml), 0.6 M sucrose (1.5 ml), and 0.25 M sucrose (1 ml) and centrifuged at 100,000*g* for 18 h at 4°C. Golgi, SER, and SER banded at 0.6 M/1.1 M, 1.1 M/1.38M, and 1.38 M/2 M sucrose interfaces, respectively, were collected. Golgi, SER, and RER samples were from our previous study and the relative purity of the fractions was examined by determining ER markers (PDI and BiP) and Golgi marker

(membrin) [Liao et al., 2003]. The samples were digested with trypsin [Liao et al., 1998] and then mixed with with SDS–PAGE loading buffer and denatured by boiling at 100°C for 5 min in the presence of 5% 2-mercaptoethanol for immunoblot analysis.

RESULTS

Differential Response of Heat Shock Proteins and ER Proteins to Proteasome Inhibition

It is known that proteasome inhibition blocks protein degradation leading to the accumulation of ubiquitin-conjugated proteins in the cells. We then confirmed these findings in HepG2 cells. As shown in Figure 1, treatment of HepG2 cells with proteasome inhibitors, ALLN or lactacystin, induced marked accumulation of ubiquitin-conjugated proteins (Fig. 1A, compare lanes 2 and 3 vs. lane 1). ApoB, a prominent liver secretory protein degraded by ubiquitin proteasome pathway, was increased markedly (~four fold) by proteasome inhibitors (Fig. 1B, compare lanes 2 and 3 vs. lane 1). In contrast, albumin (a typical liver secretory protein which does not undergo ubiquitin/proteasome degradation) and γ-tubulin did not increase upon proteasome inhibition (Fig. 1B, compare lanes 2 and 3 vs. lane 1). Our results thus indicate that under our experiment conditions, both ALLN and lactacystin blocked proteasome-mediated degradation pathway, leading to the accumulation of unfolded (misfolded) proteins in the cells as evident by presence of ubiquitin-conjugated proteins. Under such optional conditions, we determined whether proteasome inhibition induces cytosolic HSP response and ER unfolded protein response.

Among the cytosolic HSPs determined, proteasome inhibitors stimulated HSP70 expression (~three fold), whereas they did not stimulate heat shock cognate, HSC70. Two other inducible HSPs, HSP40 and HSP90, were also not altered upon proteasome inhibition (Fig. 1C, compare lanes 2 and 3 vs. lane 1). Furthermore, proteasome inhibition did not stimulate expression of ER proteins, GRP78 and GRP94 (Fig. 1D, compare lanes 2 and 3 vs. lane 1), the marker proteins for unfolded protein response. Another ER protein, PDI, was also not altered by proteasome inhibition. Our results thus indicate that proteasome inhibition induces differential response of cytosolic HSPs but not ER unfolded protein response in HepG2 cells.

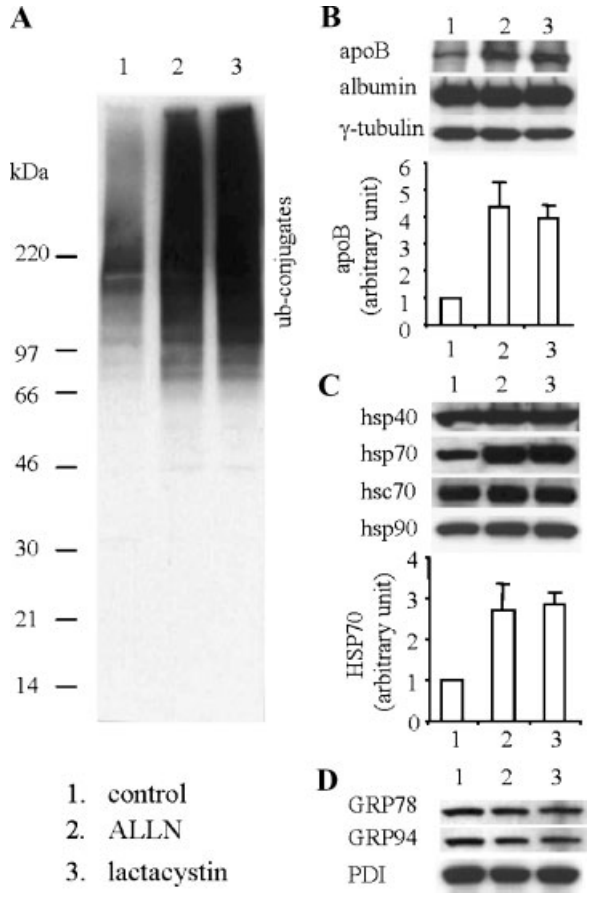


Fig. 1. Proteasome inhibitors induce differential cytosolic heat shock protein (HSP) response but not ER unfolded protein response in HepG2 cells. HepG2 cells were treated with proteasome inhibitor, ALLN (100 μM) or lactacystin (10 μM) for 17 h. The control cells were treated with vehicle only. After the treatment, the cells were lysed for immunoblot analyses for detecting ubiquitin-conjugated proteins (**panel A**), apoB, albumin, and γ-tubulin (**panel B**), cytosolic HSPs (**panel C**) and ER proteins (**panel D**) using antibodies as indicated. The experiments were repeated twice, showing similar results. The signal intensity of apoB and HSP70 were quantitated using NIH Image software and presented as the mean ± SEM from three independent experiments.

One possibility that proteasome inhibition did not induce unfolded protein response in HepG2 cells might be due to defective unfolded protein response signal transducing machinery in the cells. We thus performed separate experiments to demonstrate whether the unfolded protein response signal transduction pathway in HepG2 cells is intact. The unfolded protein response has well been documented in the cells treated with agents that disrupt the

protein folding in the ER, such as tunicamycin, which blocks an enzyme essential for the *N*-glycosylation [Elbein, 1987]. We treated HepG2 cells with tunicamycin. In parallel, some cells were treated with ALLN or lactacystin. Our results showed again that proteasome inhibitors, ALLN and lactacystin, did not stimulate expression of GRP78 and GRP94 (Fig. 2, compare lanes 3 and 4 vs. lane 2), whereas tunicamycin stimulated marked expression of GRP78 and GRP94 (~2.8-fold) (Fig. 2, compare lane 1 vs. lane 2). Neither tunicamycin nor proteasome inhibitors stimulated PDI expression. Therefore, HepG2 cells have intact unfolded protein response signal transduction machinery.

The finding that proteasome inhibition did not induce ER unfolded protein response prompted us to explore if this is unique for HepG2 cells, we therefore examined this issue in 3T3-L1 cells, a cell line readily committed to differentiation into adipocytes upon exposing the cells to the differentiation mix (insulin/dexametheson/isobutylmethylxan thine) [Green and Meuth, 1974; Green and Kehinde, 1975, 1976]. As shown in Figure 3A, essentially all cells became adipocytes 8 days post-differentiation as demonstrated by massive accumulation of intracellular lipid droplets. We then treated 3T3-L1 preadipocytes and adipocytes with proteasome inhibitors and determined the ER unfolded protein response. Treatment with proteasome inhibitors, ALLN or lactacystin, induced marked accumulation of ubiquitin-conjugated proteins in 3T3-L1 preadipocytes (Fig. 3B, compare lanes 2 and 3 vs. lane 1) and in 3T3-L1 adipocytes (Fig. 3D, compare lanes 2 and 3 vs. lane 1). However, proteasome inhibition did not stimulate expression of GRP78 and GRP94 in 3T3-L1 preadipocytes (Fig. 3C, compare lanes 2 and 3 vs. lane 1) and in adipocytes (Fig. 3E, compare lanes 2 and 3 vs. lane 1).

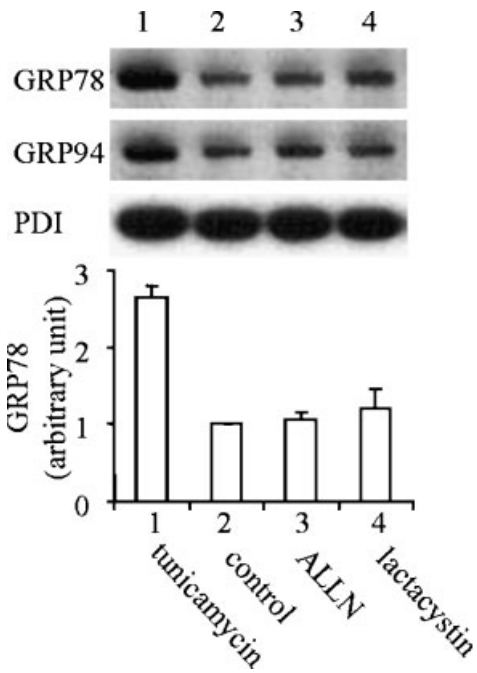


Fig. 2. Tunicamycin induces ER unfolded protein response in HepG2 cells. HepG2 cells were treated with tunicamycin (5 μg/ml), or proteasome inhibitors (ALLN (100 μM) or lactacystin (10 μM)) for 17 h. The control cells were treated with vehicle only. After the treatment, the cells were lysed for immunoblot analyses for detecting ER proteins using antibodies as indicated. The experiments were repeated twice, showing similar results. The signal intensity of GRP78 was quantitated using NIH Image software and presented as the mean ± SEM from three independent experiments.

Proteasome Inhibition Induces Inclusion Bodies in HepG2 Cells

In the previous study [Liao et al., 2003], by immunofluorescence staining, we found that the accumulated apoB in HepG2 cells treated with proteasome inhibitors showed a broad distribution which was colocalized with ER marker, but not with centrosomes where misfolded proteins can accumulate as "aggresomes." Thus, apoB in the cells treated with the proteasome inhibitors is associated with the ER rather than with aggresome. Aggresome is usually observed in the cells that overexpress exogenous mutant proteins [Johnston et al., 1998; Garcia-Mata et al., 1999; Wigley et al., 1999; Kopito, 2000]. It might be possible that proteasome inhibition does not induce aggresome formation in HepG2 cells. In the current study, we addressed this issue by electron microscopy. At the ultrastructural level, a number of electron-dense particles surrounding a clearly visible centriole and in proximity to the nucleus in lactacystin- or ALLN-treated cells (Fig. 4, #3, and #4), but not in untreated or DSMO-treated cells (Fig. 4, #1, and #2). This finding is similar to that described by Johnston et al. [1998]. These data indicate that proteasome inhibition induces inclusion bodies in HepG2 cells, some of which are within aggresomes in their centrosomal localization.

Ubiquitin-Conjugated Proteins Become Insoluble Together With Heat Shock Proteins Upon Proteasome Inhibition

Proteins that normally degraded by proteasome are delivered to the inclusion bodies if proteasome is inhibited. The ubiquitin-conjugated proteins are likely to be clustered together, becoming insoluble. To investigate this, we separated cell lysates into soluble and insoluble cellular fractions. In the control cells, ubiquitin-conjugated proteins were observed primarily in the soluble fraction, whereas little was detected in the insoluble fraction (Fig. 5A, compare lane 2 vs. lane 1). However, in the cells treated with either ALLN or lactacystin, the distribution of ubiquitin-conjugated proteins changed dramatically. The amount of ubiquitin-conjugated proteins in the insoluble fraction was even slightly more than that in the soluble fraction (Fig. 5A, compare lane 4 vs. lane 3 for ALLN treated cells; compare lane 6 vs. lane 5 for lactacystin-treated cells).

In the control cells, apoB as well as albumin were detected primarily in the soluble fraction with little in the insoluble fraction (Fig. 5B, compare lane 2 vs. lane 1). However, in the cells treated with either ALLN or lactacystin, significant amount of apoB appeared in insoluble fractions, whereas the distribution of albumin was not altered by proteasome inhibition (Fig. 5B, compare lane 4 vs. lane 3 for ALLN treated cells; compare lane 6 vs. lane 5 for lactacystin-treated cells).

As demonstrated above, proteasome inhibition induces differential response among HSPs, that is, stimulating the protein expression of HSP70 but no changes in HSP40, HSC70, and HSP90. Ubiquitin and HSP70 have been detected in the aggresome [Johnston et al., 1998; Garcia-Mata et al., 1999]. Thus, it is likely that HSPs are co-fractionated with ubiquitin-conjugated proteins in the insoluble fraction. Determination of their distribution in soluble and insoluble fractions revealed that all HSPs tested appeared in the insoluble fraction with varying degrees after proteasome inhibitor treatment (Fig. 5B). The most prominent one was HSP40 followed by HSC70 and HSP70, whereas HSP90

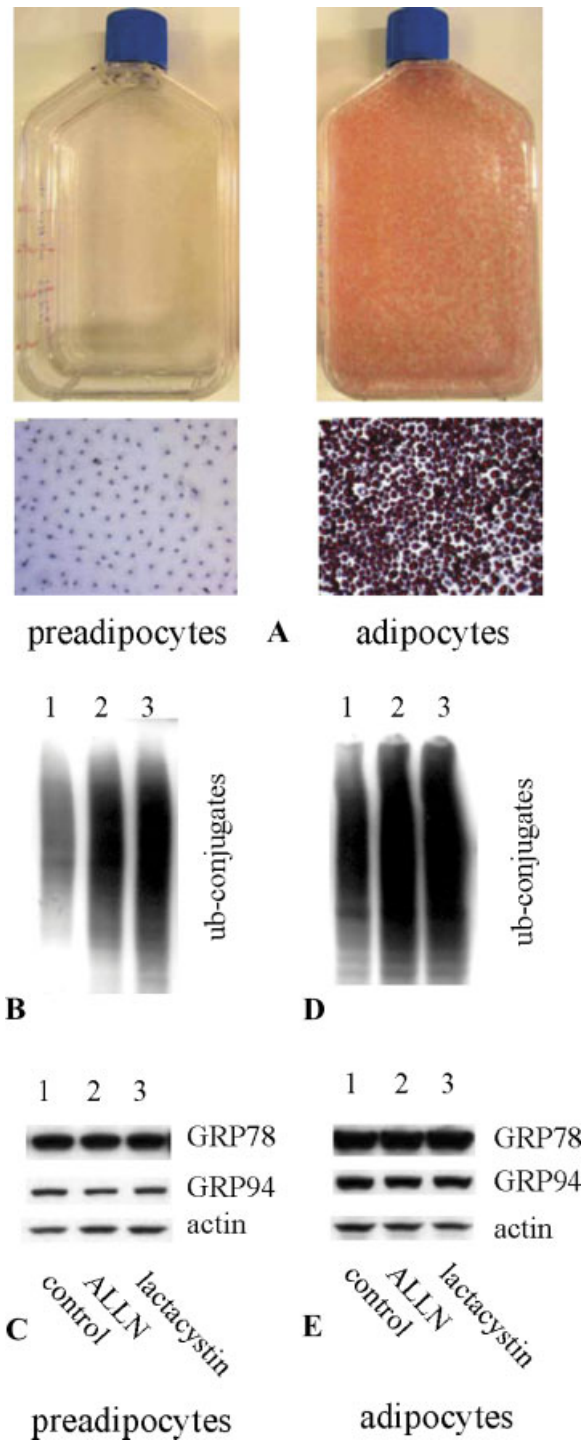
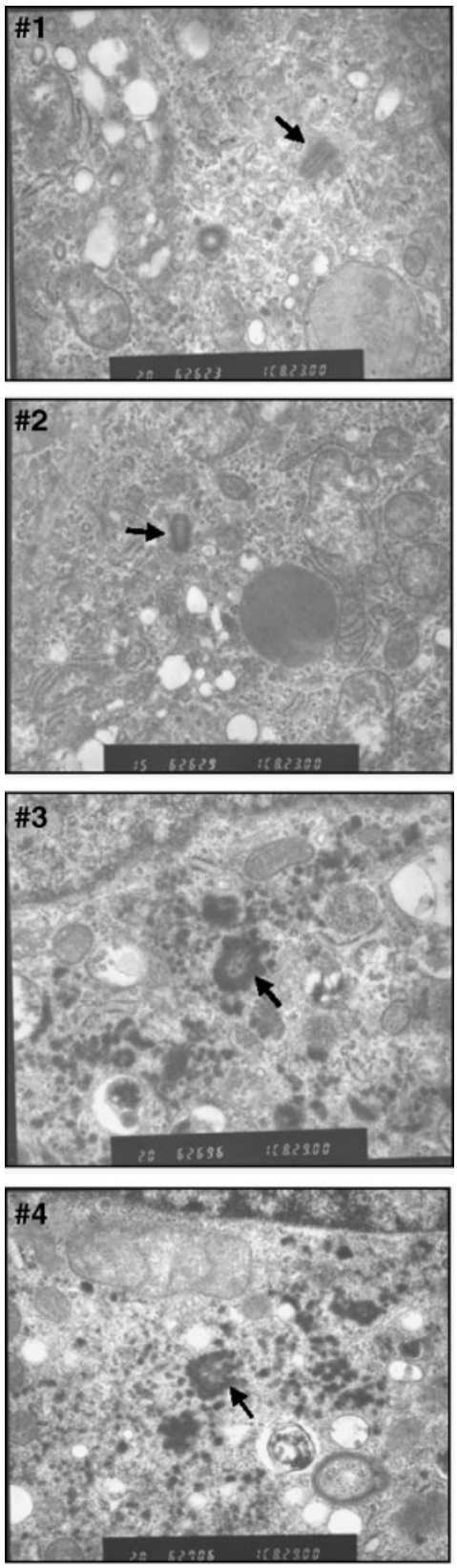


Fig. 3. Response of 3T3-L1 cells to proteasome inhibitors. 3T3-L1 preadipocytes were differentiated into adipocytes. Fat staining was done with Oil Red O for gross photography (**panel A**, upper) followed by nuclei staining with hemotoxylin for microphotography under microscope (panel A, lower, magnification: 100×). Preadipocytes and adipocytes were treated with proteasome inhibitor, ALLN (100 μM) or lactacystin (10 μM) for 17 h. The control cells were treated with vehicle only. After the treatment, the cells were lysed for immunoblot analyses for detecting total ubiquitin-conjugated proteins (**panels B** and **D**) and cytosolic HSPs and ER proteins (**panels C** and **E**) using antibodies as indicated. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]



was much less affected (compare lane 4 vs. lane 3 for ALLN treated cells; compare lane 6 vs. lane 5 for lactacystin-treated cells).

Ubiquitin-Conjugated Proteins Are Localized on the Cytosolic Surface of the ER

As demonstrated above, proteasome inhibition appeared not to induce ER unfolded protein response. This may be due to that there was no accumulation of misfolded proteins in the ER upon proteasome inhibition. We then further addressed this issue in the following experiments.

As shown in Figure 1A, misfolded proteins accumulated in response to proteasome inhibition as evaluated by measuring ubiquitin-conjugated proteins. This was due to the increase of ubiquitin-conjugated proteins in the cytosol and in the microsomes (data not shown). We determined the distribution of ubiquitin-conjugated proteins in the cytosol and microsomes and along the secretion pathway. First, we noted that ubiquitin-conjugated proteins were more abundant in the microsomes than in cytosol (Fig. 6A). By subfractioning the microsomes into RER, SER, and Golgi, it was found that ubiquitin-conjugated proteins were mainly located in the RER and SER whereas little ubiquitin-conjugated proteins were associated with Golgi (Fig. 6B). To investigate whether the ER-associated ubiquitin-conjugated proteins are located in the ER lumen or not, we treated the organelle fractions with trypsin, and then immunobloted with anti-ubiquitin antibody. Again, in the untreated fraction we detected ubiquitin conjugates associated the RER and SER fractions (Fig. 6C, lanes 1 and 3) but little ubiquitin-conjugated proteins in the Golgi fraction (Fig. 6C, lane 5). Trypsin treatment almost completely removed the ubiquitin conjugates in the RER (Fig. 6C, lane 2 vs. lane 1) and SER fractions (Fig. 6C, lane 4 vs. lane 3), whereas it had no effect on a lumen protein, albumin (Fig. 6D, lane 2 vs. lane 1; lane 4 vs. lane 3). These data indicate that ubiquitin-conjugated proteins are largely located on the cytosolic surface of the ER rather than in the ER lumen.

Fig. 4. Proteasome inhibitors induce inclusion bodies in HepG2 cells. HepG2 cells were treated with proteasome inhibitor, ALLN (#3), or lactacystin (#4) for 17 h. The control cells were treated with vehicle only (#2) or without treatment (#1). After the treatment, the cells were prepared for electron microscopy as described in Experimental Procedure. Arrows indicate the centriole. Magnification: 20,000×.

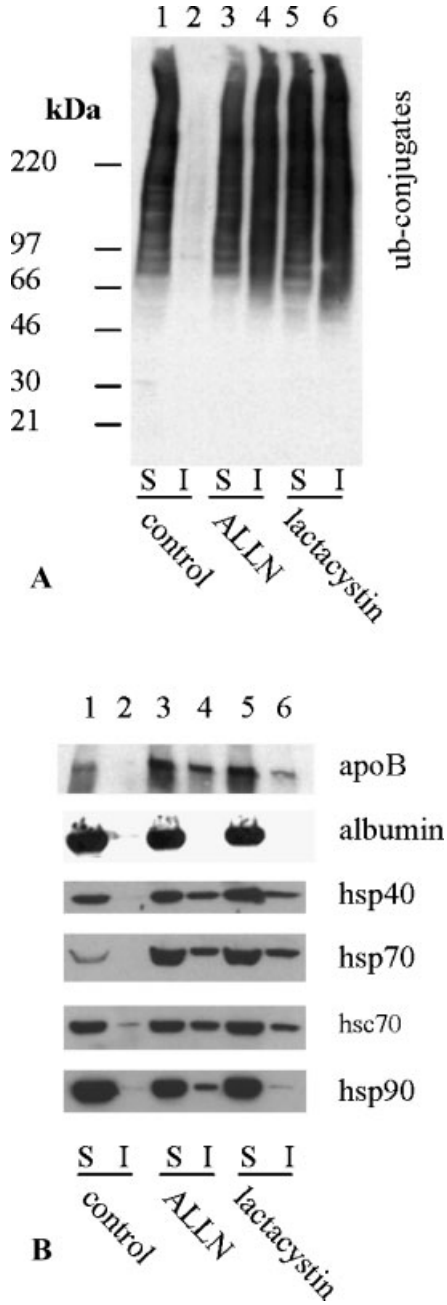


Fig. 5. Ubiquitin-conjugated proteins become insoluble upon proteasome inhibition. HepG2 cells were treated with proteasome inhibitor, ALLN (100 μM) or lactacystin (10 μM) for 17 h. The control cells were treated with vehicle only. After the treatment, the cells were lysed and the cell lysates were separated into soluble and insoluble fractions. Equivalent amounts of each fraction were used for immunoblot analyses for detecting ubiquitin-conjugated proteins (**panel A**) and for apoB, albumin, cytosolic HSPs, and ER proteins (**panel B**) using antibodies as indicated. Note that in the panel A, the control samples (both soluble and insoluble) were loaded threefold as ALLN- or lactacystin-treated ones. However, the ubiquitin-conjugated proteins were still barely detectable in insoluble fraction of the control cells. S, soluble fraction; I, insoluble fraction.

DISCUSSION

In this study, we have demonstrated that proteasome inhibition induces differential response of cytosolic HSPs and accumulation of inclusion bodies, but not typical ER unfolded protein response in HepG2 cells. We showed that proteasome inhibitors induce accumulation of ubiquitin-conjugated proteins in the cells. ApoB, a prominent liver secretory protein degraded by ubiquitin proteasome pathway, was increased upon proteasome inhibition, whereas albumin, a typical liver secretory protein which does not undergo ubiquitin/proteasome degradation, was not altered by such the manipulation. These findings are consistent with our previous studies [Liao et al., 1998, 2003; Liao and Chan, 2001].

As one would expect, proteasome inhibition stimulates the expression of inducible HSP70 but not its heat shock cognate, HSC70 in HepG2 cells. Two additional inducible HSPs tested, that is, HSP40 and HSP90, also do not respond to proteasome inhibitors. These data suggest the differential responses of cytosolic HSPs to the misfolded proteins upon proteasome inhibition in HepG2 cells.

Upon proteasome inhibition, misfolded proteins are accumulated as evidenced by increased amount of ubiquitinated proteins. The accumulated misfolded proteins appear to be refractory to proteolysis and to be delivered to inclusion bodies, some of which can be associated with aggresomes. We observed that the ubiquitinated proteins appear in insoluble fraction after the treatment of HepG2 cells with proteasome inhibitors. Among proteins detected in the insoluble fraction upon proteasome inhibition were included all HSPs tested, that is, HSP40, HSP70, HSC70, and HSP90. Existence of these HSPs in the insoluble fractions suggests the failure of HSPs in directing misfolded proteins for proteasomal degradation. By using electron microscopy, we found that in HepG2 cells treated with the proteasome inhibitors, a number of electron-dense particles were surrounding a clearly visible centriole and extending to nearby area close to the nucleus, a finding similar to that described by Johnston et al. [1998]. While it is unclear if sequestered proteins in cytoplasmic inclusions are indeed "aggresomes," some of them are within aggresomes in term of centrosomal localization. It is known that proteins in the

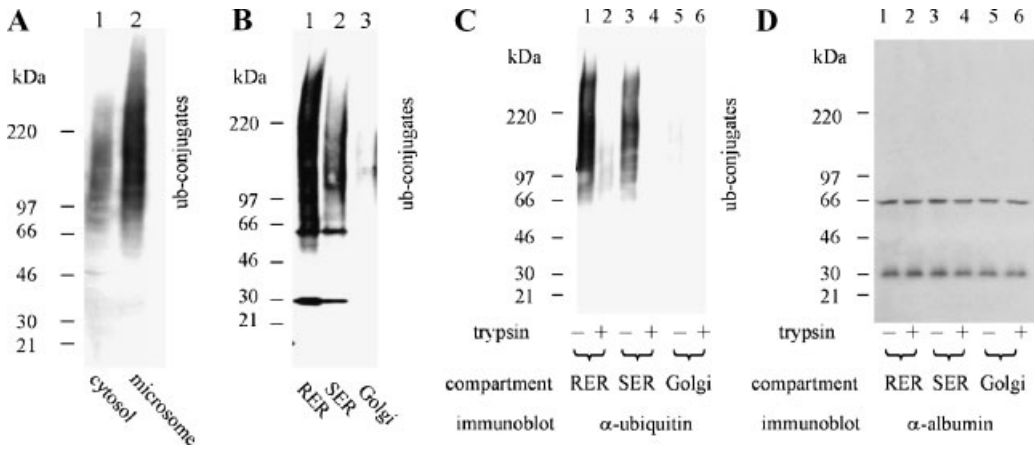


Fig. 6. Ubiquitin-conjugated proteins are localized on the cytosolic surface of the ER. The microsomes and cytosol were prepared from HepG2 cells treated with ALLN (100 μM) for 2 h. Equivalent amount of each compartment were fractionated by SDS–PAGE and immunobloted with anti-ubiquitin antibody (**panel A**). RER, SER, and Golgi complex were prepared from HepG2 cells treated with ALLN (100 μM) for 2 h. Equivalent amount of each compartment was either directly denatured, fractionated by SDS–PAGE and immunobloted with anti-ubiquitin antibody (**panel B**), or was treated with or without trypsin (200 μg/ml) for 20 min at room temperature. After incubation, soybean trypsin inhibitor (final concentration, 5 mg/ml) was added to stop digestion. The samples were then denatured, fractionated by SDS–PAGE, and then immunobloted with anti-ubiquitin antibody (**panel C**) or with anti-albumin antibody (**panel D**).

aggresomes are insoluble [Kopito, 2000]. It is currently believed that the formation of aggresome is an active process and requires dynein-dependent transport of aggregated molecules along microtubules [Kopito, 2000; Johnston et al., 2002].

In response to proteasome inhibition, apoB also appears in the insoluble fraction. Our previous study demonstrated that after proteasome inhibition, apoB is associated with the ER rather than aggresomes [Liao et al., 2003]. These data thus suggest that aggresome appears not to be the only source of insoluble proteins in the cells because little apoB is associated with aggresome, but significant amount of apoB (~10%) become insoluble after the treatment with proteasome inhibitors.

However, proteasome inhibition does not induce ER unfolded protein response in HepG2 cells as demonstrated by absence of stimulation of GRP78 and GRP94 proteins by proteasome inhibitors, which is somewhat surprising. Similar to what found in HepG2 cells, proteasome inhibition does not induce unfolded protein response in 3T3-L1 preadipocytes and adipocytes.

Apparently, the lack of typical ER unfolded protein response in HepG2 cells is not due to a defective ER sensing machinery in the cells, because tunicamycin elicits typical unfolded protein response in HepG2 cells. The reasons for this lack of a response to proteasome inhibition can be explained by the notion that upon proteasome inhibition, few misfolded proteins are accumulated in the ER lumen. Because proteasome is located in the cytoplasm, its inhibition could lead to the accumulation of its substrates in the fractionated cytosol. This explanation is rather intriguing, because we know that misfolded proteins are largely associated with the ER as evaluated by determination of ubiquitin-conjugated proteins. However, it should be noted that few misfolded proteins are in the ER lumen, because protease protection assay showed that essentially all ubiquitin-conjugated proteins are located on the cytoplasmic surface of the ER.

Our findings that proteasome inhibition induces differential HSP response and accumulation of cytoplasmic inclusion bodies, but not unfolded protein response in the liver cell cultures may have important implications. The liver is a central organ responsible for the metabolism of carbohydrates, proteins, and lipoproteins. The unique anatomic location of the liver constantly exposes it to various kinds of physiological, pathological, and environmental stresses. Inappropriate reactions to these stresses may underlie the pathological process of liver diseases such as fatty liver, cirrhosis, various liver injuries, and hepatitis, etc. Use of proteasome inhibitors as anti-cancer therapy

[Kisselev and Goldberg, 2001; Voorhees et al., 2003; Adams, 2004] is particularly of concern unless one can deliver the inhibitors only into the cancer cells. This is because proteasome inhibitors induce the accumulation of misfolded proteins which leads to formation of inclusion bodies in the cells that could eventually influence normal function of the cells. Future study in hepatocyte response to various stresses and injuries will provide novel information on pathological process of liver diseases and may also provide pharmaceutical targets for prevention and treatment of these diseases.

ACKNOWLEDGMENTS

We thank Jan Pferdekamper for 3T3-L1 cell culture and differentiation. This work was supported by grants from the American Heart Association National Center (Scientist Development Award, 0235286N, to W.L.), the American Liver Foundation (to W.L.), the Shanghai Education Commission (No. E03007, to X.L.), and the US National Institutes of Health (HL51586, to L.C.). L.C. is the Betty Rutherford Chair of the St. Luke's Episcopal Hospital and Baylor College of Medicine.

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