Proteasome inhibitors induce intracellular protein aggregation and cell death by an oxygen-dependent mechanism

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Abstract Clone 9 liver cells incubated under aerobic conditions with the proteasome inhibitors lactacystin, *clastro* lactacystin β-lactone, and tri-leucine vinyl sulfone, in the absence of an obvious oxidative challenge, underwent oxidative protein modifications, such as loss of solubility, formation of aggregates (predominantly by disulfide bridges), and increased carbonyl formation, similar to those seen with hydrogen peroxide treatment. These alterations were accompanied by modification of cell morphology and loss of cell viability. Remarkably, almost all of these modifications were prevented when cell incubation with proteasome inhibitors was performed under a 3% oxygen atmosphere instead of the 21% oxygen routinely used in cell culture experiments. Our results suggest an oxygen-dependent mechanism for the protein oxidation, protein aggregation, cellular dysfunction, and apoptosis induced by proteasome inhibitors.

Key words: Proteasome; Proteasome inhibitor; Protein oxidation; Proteolysis; Oxidative stress; Free radical; Lactacystin; Tri-leucine vinyl sulfone

1. Introduction

Inhibition of proteasome activity has been associated with apoptotic responses in various cell types [1,2]. It is known that the induction of apoptosis by proteasome inhibition occurs following an initial accumulation of short-lived proteins such as p53, p27, and pro-apoptotic Bcl-2 family members [2]. The mechanism by which apoptosis is triggered by decreased proteasomal activity, however, remains speculative. We have reported that proteasome inhibition in rat liver epithelial cells modifies glutathione metabolism, and causes intense protein *S*-glutathionylation [3]. Thus, alteration of cellular redox status and oxidative protein modifications should also be considered during proteasome inhibition. Our goal in the present study was to explore oxidative alterations to the pool of intracellular proteins after cell incubation with proteasome inhibitors, since accumulation of modified proteins may disrupt cellular function either by loss of catalytic and structural integrity, or by disruption of regulatory pathways [4].

We selected rat liver epithelial cells, and lactacystin, its derivative *clastro* lactacystin β-lactone and tri-leucine vinyl sulfone (NLVS) as proteasome inhibitors. Our criteria for the selection of the proteasome inhibitors included their ability to act as irreversible inhibitors of the proteasome [5], their previous utilization in functional studies of proteasomal activity, and their different structures. *Clastro* lactacystin β-lactone, the active product of lactacystin hydrolysis, is considered a more powerful inhibitor than is lactacystin [6,7]. NLVS, different from other structurally related proteasome inhibitors, was the first peptide found to exert irreversible and selective proteasome inhibition [8,9]. To compare damage promoted by proteasome inhibitors with that induced by oxidative stress, we used cell treatment with H_2O_2 as a common and biologically relevant form of oxidative challenge.

2. Materials and methods

2.1. Materials

Lactacystin, *clastro* lactacystin β-lactone, and NLVS were purchased from Calbiochem. The fluorogenic substrate succinyl-Leu-Leu-Val-Tyr-MCA (s-LLVY-MCA) was purchased from Sigma. [^{35}S]Methionine/cysteine and NaB[$^3H_4$] were purchased from NEN™ Life Science Products. Cell culture media, fetal bovine serum, and phosphate-buffered saline solution were purchased from Gibco. Bradford protein assay reagents were purchased from Bio-Rad.

2.2. Cells and cell culture

Clone 9 liver cells (normal rat liver epithelium) were obtained from American Type Culture Collection (ATCC CRL 1439). The cells were cultured in Ham's F-12K medium, supplemented with 10% fetal bovine serum. Samples of 2.5×10^6 cells were plated and grown for 4 days before treatment. Cells were grown in a model 3130 Forma Scientific incubator with 5% carbon dioxide, and either 21% or 3% oxygen; all gases were automatically regulated. All media were pre-equilibrated with the appropriate gas mixtures before application to cells.

2.3. Incubation of clone 9 liver cells with proteasome inhibitors and treatment with hydrogen peroxide

On the fourth day after seeding, cells were incubated with proteasome inhibitors (1–10 μM with 1×10^5 cells/cm^2) in culture medium, or treated with H_2O_2 (200–400 μM with 3×10^6 cells/cm^2) for 30 min at 37°C in phosphate-buffered saline (pH 7.4). After H_2O_2 treatment, cells were washed three times and incubated in culture medium for 24 h.

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Abbreviations: GSH, reduced glutathione; NLVS, tri-leucine vinyl sulfone; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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2.4. Cellular viability

Variation in the number of viable cells following incubation with proteasome inhibitors, and after treatment with hydrogen peroxide, was determined with the CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega), according to the manufacturer's protocol. The cells were seeded at a density of 10 000/well/100 μl (96-well plate, Corning) and incubated in the presence of proteasome inhibitors or treated with H_2O_2 4 h after plating. Cell viability was determined every 4 h.

2.5. Proteolysis determination

Proteolysis ratios were determined in cell extracts (100–200 μg protein) by hydrolysis of the fluorogenic substrate s-LLVY-MCA as previously described [3].

2.6. [^{35}S]Methionine/cysteine protein labeling

Cells were incubated with [^{35}S]methionine/cysteine, in methionine/cysteine-free minimal essential Eagle's medium, for 16 h at 37°C. After incubation, the labeling mixture was removed and the cells were washed three times with phosphate-buffered saline. This was followed by a 2 h incubation in the methionine/cysteine-free medium, and a further washing procedure.

2.7. Preparation of cell extracts and protein samples

Cell extracts were prepared by sonication of cell pellets. Detergent-soluble cell proteins were obtained by resuspension of cell pellets in a detergent-buffered solution consisting of: 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS), in 10 mM Tris–HCl/1 mM EDTA, pH 8 [10]. After cell lysis (15 min at 4°C followed by vigorous stirring), samples were centrifuged at 13 000×*g* for 10 min. The supernatant contained detergent-soluble proteins, whereas the pellet contained detergent-insoluble proteins. Quantification of the detergent-insoluble protein fraction was achieved using a [^{35}S]methionine/cysteine protein labeling procedure before treatments. The detergent-insoluble pellet, after three washes, was dissolved in 0.1 N NaOH and subjected to scintillation counting. Detergent-insoluble proteins were solubilized, when specified, in a detergent-buffered solution consisting of: 4% Triton X-100, 2% sodium deoxycholate, and 0.4% SDS, in 10 mM Tris–HCl/1 mM EDTA pH 8 [10]. Protein concentration was determined using Bradford reagents.

2.8. Polyacrylamide gel electrophoresis (PAGE)

Protein preparations, ^{35}S-labeled or unlabeled proteins, were electrophoresed by SDS–PAGE according to Bollag et al. [11].

2.9. Determination of protein carbonyl derivatives

Protein carbonyls were quantified as described in [12] by ^{3}H-labeling carbonyl groups during reduction with NaB[$^{3}H_4$]. A Western blotting immunoassay was performed as described in [13]. The antibodies were: rabbit anti-dinitrophenyl IgG (monoclonal, Sigma) and horseradish peroxidase-conjugated AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch). Blotting identification was performed with the chemiluminescence kit ECL Western Blotting Detection (Amersham).

3. Results

We initially undertook this investigation to explore the hypothesis that the proteasome plays an important role in overall cell defenses against oxidants by catalyzing the selective removal of oxidatively modified proteins [14–19]. We started by testing for an intracellular accumulation of oxidatively modified proteins, after exposure of cells to hydrogen peroxide during inhibition of proteasomal activity. During the experiments, however, we observed that cells incubated in the presence of proteasome inhibitors alone accumulated oxidized proteins to a similar extent as did cells solely pretreated with

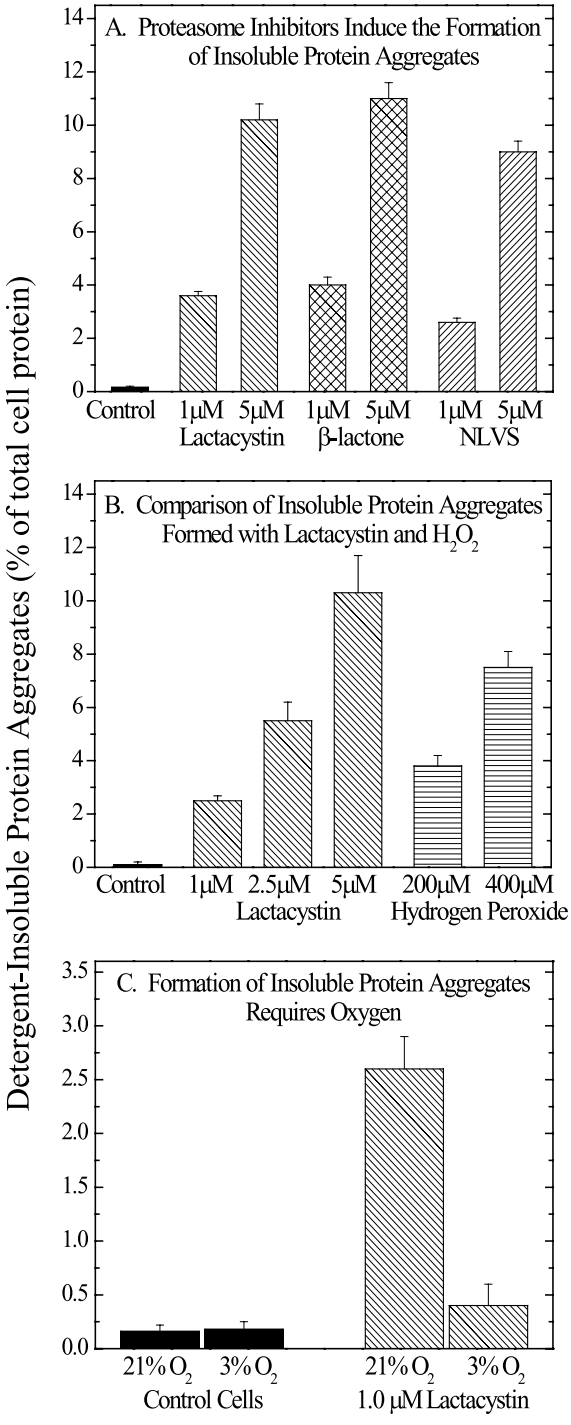

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Fig. 1. Formation of detergent-insoluble oxidized protein aggregates in clone 9 liver cells treated with proteasome inhibitors or hydrogen peroxide. A cellular protein pellet was obtained as described in Section 2. A: Cells (at 1×10^5 cells/cm^2) were incubated for 24 h with proteasome inhibitors at the concentrations indicated. B: Treatment with H_2O_2 was performed for 30 min (at 3×10^6 cells/cm^2) followed by a further 24 h incubation. C: The cells (at 1×10^5 cells/cm^2) were maintained for 24 h under either a 3% or a 21% oxygen atmosphere, in the presence or absence of 1 μM lactacystin. In A the formation of protein aggregates after treatment with proteasome inhibitors is expressed as a percentage of ^{35}S counts in the aggregates compared to ^{35}S counts per mg of total cell protein. B shows a comparison of lactacystin and H_2O_2 as inducers of protein aggregates. C shows the production of protein aggregates as a function of ambient oxygen concentration. In B and C ^{35}S counts of detergent-insoluble pellets were compared to control samples set as 1.00. Results shown are mean ± S.D. of four independent experiments.

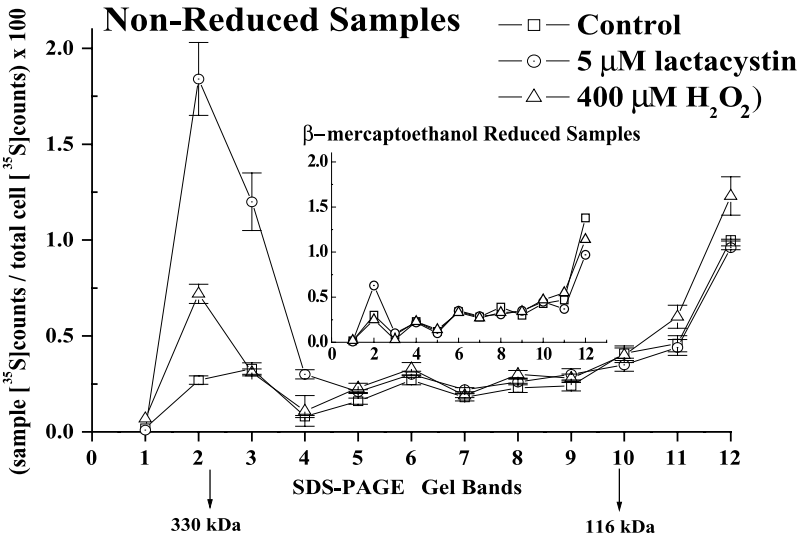

Fig. 2. Disulfide protein cross-linking in clone 9 liver cells treated with lactacystin or hydrogen peroxide. Cell proteins were metabolically labeled with [^{35}S]methionine/cysteine and incubated for 24 h in the presence of proteasome inhibitors, or were exposed to H_2O_2 treatment (as in Fig. 1). Cellular detergent-soluble protein samples were applied to SDS–PAGE (50 μg/well). Stacking and resolving gels were 3% and 5% polyacrylamide, respectively. After Coomassie blue staining, the gels were dried and divided in vertical slices of 0.4 cm width. The slices were cut in 12 equal pieces (indicated as 'Gel bands' on the *x*-axis) and maintained overnight in liquid scintillation cocktail before counting. Results are expressed as a percentage ^{35}S counts per band compared to ^{35}S counts/mg total cell protein. The results shown are mean ± S.D. of four independent experiments. Inset: Values obtained from samples following reduction with 0.04% β-mercaptoethanol.

hydrogen peroxide. This observation motivated both a previous publication [3] and the present experiments.

In this study proteolysis was tested in cell extracts, obtained after cell incubation with proteasome inhibitors, by hydrolysis of the fluorogenic substrate s-LLVY-MCA. The effects of the inhibitors were dose-dependent, but were quite similar for all three agents tested. In cells incubated for 24 h with 1–5 μM lactacystin, *clastro* lactacystin β-lactone, or NLVS we found 40 ± 10% to 90 ± 5% inhibition of proteolysis, in comparison with control samples.

Cell viability followed over a 2–24 h period was dependent on the concentration of proteasome inhibitor, or H_2O_2 utilized. After 24 h, cell viability was 30% with 1 μM lactacystin, 28% with 5 μM NLVS, and 59% with 400 μM H_2O_2, of control cell values. However, contrary to cells incubated with proteasome inhibitors, the growth of H_2O_2-treated cells was almost completely recovered 48 h later. Cells treated with both a proteasome inhibitor and H_2O_2 failed to grow, and did not recover at any point.

Since oxidatively modified proteins can form large aggregates due to covalent cross-linking and consequent loss of solubility [10,17,20–27], we looked for the formation of insoluble aggregates after treatment with H_2O_2 and incubation with proteasome inhibitors, by measuring the formation of detergent-insoluble protein fractions. Newly synthesized cell proteins were metabolically labeled with [^{35}S]methionine/cysteine before cell treatment, and the total protein content of treated cells was recovered after 24 h in a detergent-buffered solution. Labeled protein that could not be solubilized in detergent was recovered as the detergent-insoluble protein pellet, after centrifugation (see Section 2). The amount of material in the detergent-insoluble protein pellet was increased at least 13-fold in samples from cells treated with 1 μM NLVS compared to the insoluble protein pool of control cells, and as much as a 60-fold increase was seen in samples obtained from cells incubated with 5 μM lactacystin (Fig. 1A). The loss of protein solubility was dependent on the dose of proteasome inhibitor and did not differ significantly between samples obtained from cells incubated with any of the three inhibitors tested (Fig. 1A). Loss of protein solubility after cell incubation with 5 μM lactacystin was similar to that obtained after treatment with 200 or 400 μM hydrogen peroxide (Fig. 1B). The loss of protein solubility after cell incubation with proteasome inhibitors was a relatively early event, since the formation of a detergent-insoluble protein pellet was observed after only 4 h of incubation in the presence of 2.5 μM lactacystin: 30% higher than control values.

Next we incubated cells in the presence of 1 μM lactacystin, either under an atmosphere of only 3% oxygen, or in the regular 21% oxygen atmosphere (room air) routinely used by almost all investigators for cell culture studies. After 24 h of incubation the cells were assayed for loss of protein solubility. We found that at 3% O_2 the lactacystin-induced decrease in protein solubility was essentially blocked in comparison with cells grown under the usual 21% O_2 atmosphere (Fig. 1C). This result indicates that formation of an insoluble protein pool, induced by proteasome inhibitors, is dependent on oxygen.

Detergent-insoluble pellets obtained from cells 24 h after treatment with 400 μM H_2O_2 or with 2.5 μM lactacystin were solubilized and protein carbonyl derivatives were measured by reaction with NaB[$^3H_4$]. The amount of carbonyl protein derivatives formed was relatively similar in samples from H_2O_2-treated cells (480 ± 15 ^{3}H counts × 10^3/mg protein) and lactacystin-treated cells (400 ± 21 ^{3}H counts × 10^3/mg protein) but both were five- to six-fold higher than in extracts from control cells (83 ± 14 ^{3}H counts × 10^3/mg protein). In actuality our control values are probably an overestimation, since control cells subjected to the same procedure did not yield a large enough detergent-insoluble protein pellet to be assayed, hence carbonyl derivatives were measured in total cell protein.

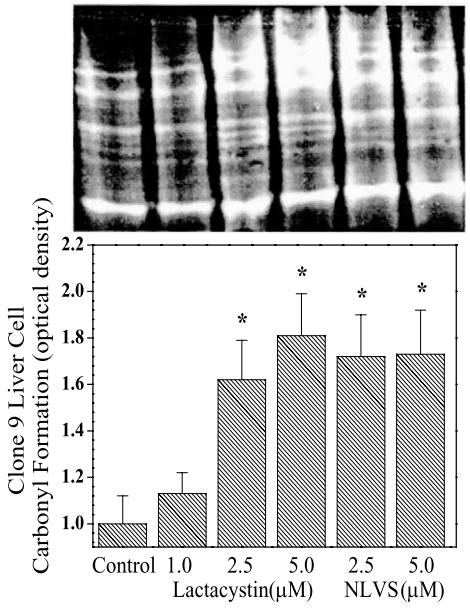

Fig. 3. Protein carbonyl formation induced by proteasome inhibitors in clone 9 liver cells. Cells were lysed in a detergent-buffered solution 24 h after incubation (as in Fig. 1) with inhibitors at the indicated concentrations. After derivatization with dinitrophenylhydrazine, the lysates (5 μg protein/well) were applied to 8% SDS–PAGE. The carbonyl immunoassay was performed according to Shacter et al. [13]. The blot shown in the upper panel is representative of all experiments. In the lower panel gels from three independent experiments were scanned, and the optical density of each experimental sample was expressed relative to control values, arbitrarily set as 1.00. Each histogram represents mean ± S.D. of three independent experiments. Significant differences (*$P < 0.001$ compared to control samples) are indicated.

We next looked for aggregates in the detergent-soluble protein fraction on SDS–PAGE, after cell incubation under similar experimental conditions (Fig. 2). Both the formation of high molecular weight protein bands (220–330 kDa) and their attenuation after chemical reduction with 0.04% β-mercaptoethanol was observed (Fig. 2). Greatest protein aggregation was found in the high molecular weight range (220–330 kDa, bands 1–4) in samples from cells incubated with 5 μM lactacystin (5.4% of total ^{35}S counts) or treated with 400 μM H_2O_2 (1.2% of total ^{35}S counts). In contrast, less than 0.7% of total protein was found in same gel bands from control samples. All high molecular weight protein bands (220–330 kDa) were profoundly attenuated when samples were reduced with β-mercaptoethanol (inset in Fig. 2). Protein concentration in the high molecular weight bands of lactacystin- and H_2O_2-treated samples after reduction with β-mercaptoethanol was close to that found in control samples subjected to the same procedure, ca. 0.6% of total protein. From these results one may infer that inter-protein disulfide bond formation was a major cause for the appearance of detergent-soluble protein aggregates. Even upon mild proteasome inhibition (40% inhibition after 24 h of cell incubation in the presence of 1 μM lactacystin) we observed formation of protein aggregates that could be partially dispersed by chemical reduction with β-mercaptoethanol (result not shown).

To further explore whether protein modifications are caused by oxidative mechanisms, protein carbonyl derivatives [4] were determined in samples from cells incubated with proteasome inhibitors. Immunoblotting of protein carbonyl groups evaluated in detergent-soluble protein samples from cells incubated with 1, 2.5, and 5 μM lactacystin or 2.5 and 5 μM NLVS showed increased carbonyl formation at all inhibitor concentrations (upper panel of Fig. 3). When averaged over three independent experiments, carbonyl formation was seen

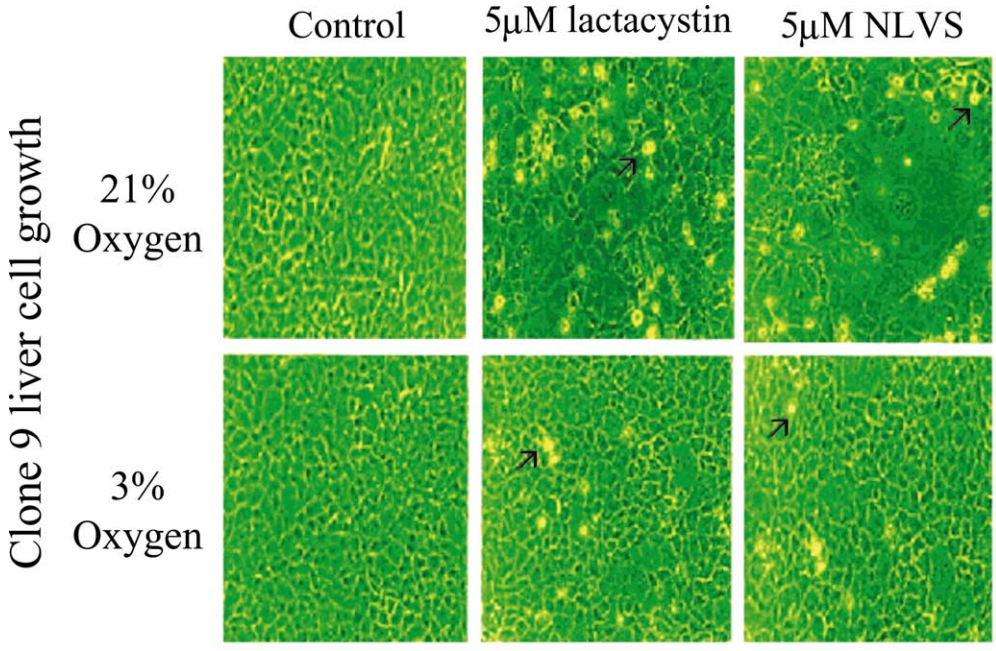

Fig. 4. Oxygen dependence of clone 9 cell death induced by proteasome inhibitors. Representative cell pictures of a light microscopic view (20× magnification) (Nikon Diaphot) 24 h after incubation (as in Fig. 1) with lactacystin or NLVS at the concentrations indicated. The top three panels all show results with cells grown under a 21% O_2 atmosphere. In contrast, the bottom three panels show cells grown under an atmosphere of only 3% O_2. Pictures were taken from three or four random fields in the culture plates.

to increase by 60–80% after 24 h of cell incubation in the presence of 2.5–5.0 μM lactacystin or NLVS (Fig. 3, lower panel).

Cell morphology, visualized by optical microscopy, during cell incubation under a 21% oxygen atmosphere with 5 μM lactacystin or NLVS revealed profound modifications (Fig. 4, top three panels). Many cells can be seen to have detached from the surface (arrows in Fig. 4), or to have become enlarged, or ruptured (and clearly dead). When cells were incubated under a 3% oxygen atmosphere, however, these effects were almost completely blocked (Fig. 4, bottom three panels). These data, in agreement with other results shown above, indicate that oxidative processes underlie the protein modifications and cell death we now report during proteasome inhibition in mammalian cells.

4. Discussion

Proteasome inhibitors have been widely used in functional studies of proteasome-dependent protein turnover, and are considered important tools in the study of apoptosis. During the first semester of 2002, 46 articles were published on this issue, according to a search performed with Pubmed Tutorial system (US National Library of Medicine). Moreover, there is an important body of citations suggesting the possible utility of proteasome inhibitors in cancer therapy, based mainly on their pro-apoptotic activity (42 citations according to the same source and period of time). Our results show that during proteasome inhibition, without an obvious oxidative stress, the intracellular protein pool underwent modifications that resemble those observed after H_2O_2 treatment (utilized here for comparative purposes). Hydrogen peroxide levels utilized in the assays described above (200–400 μM H_2O_2 with 3×10^5 cells/cm^2; equivalent to a final concentration range of 66–133 μM) are related to mild oxidative damage when used in cultured cells [15–17]. The modifications reported here include complete insolubility even in detergents, aggregation even in detergents, carbonyl group formation, sulfhydryl oxidation, changes in cell morphology, and cell death. All of these modifications may be taken to suggest an oxidative mechanism underlying the effects of proteasome inhibition.

An increased pool of substrates for oxidation, since protein turnover is decreased when proteasome activity is inhibited, could be an explanation for increased oxidative protein damage during proteasome inhibition. However, 24 h protein turnover accounts for the degradation of no more than 5% of intracellular protein. Recently we reported intense protein *S*-glutathionylation in extracts of cells incubated with the same proteasome inhibitors utilized in the present study [3]. Protein *S*-glutathionylation was accompanied by increased levels of total glutathione, and of mRNA encoding the rate-limiting enzyme glutamate cysteine ligase (EC 6.3.2.2, formerly called γ-glutamylcysteine synthetase) in reduced glutathione (GSH) biosynthesis. Since in vitro experiments revealed that the 20S proteasome extracted from mammalian cells was ^{35}S-glutathionylated after incubation with the inhibitors [3], we concluded that proteasome inhibitors promote in vivo structural alterations in the 20S proteasome core, allowing its *S*-glutathionylation, and thereby altering intracellular GSH availability because of GSH diversion to protein *S*-glutathionylation. Increased GSH consumption may start by 20S proteasome core *S*-glutathionylation, thus decreasing intracellular reductive capacity, and consequently, increasing the size of the intracellular pool of oxidized proteins. We suggest that this mechanism may, at least partly, explain the results shown here, since intracellular protein damage during cell incubation with proteasome inhibitors is widespread and is similar to the damage observed during oxidative stress. Our results may also help to explain reports in the literature indicating that proteasome inhibition promotes oxidative damage to macromolecules such as proteins, lipids, and DNA [28] as well as alterations in glutathione metabolism [29].

Further experiments will be needed in order to truly understand the mechanisms of protein damage during proteasome inhibition. Of potentially greatest significance, however, is the new finding that the apoptotic cell death induced by proteasome inhibitors is largely dependent upon oxygen concentration. This result strongly suggests that proteasome inhibition causes apoptosis via increased oxidative stress. We suggest that the cellular oxidative protein damage here reported underlies the apoptotic phenomenon during proteasome inhibition. If this hypothesis can be verified it will have serious implications for the use of proteasome inhibitors in cancer therapy, as well as other medical applications.

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