Proteasome inhibition induces TNFR1 shedding from human airway epithelial (NCI-H292) cells

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Proteasome inhibition induces TNFR1 shedding from human airway epithelial cells. For example, TACE (TNF-α protease inhibitor 2) attenuated clasto-lactacystin β-lactone-mediated TNFR1 shedding, which is consistent with its ability to inhibit the zinc metalloprotease-catalyzed cleavage of TNFR1 ectodomains. Clasto-lactacystin β-lactone also induced soluble TNFR1 (sTNFR1) release from the A549 pulmonary epithelial cell line, as well as from primary cultures of human small airway epithelial cells and human umbilical vein endothelial cells. Furthermore, sTNFR1 release induced by clasto-lactacystin β-lactone was not a consequence of apoptosis or the extracellular release of TNFR1 exosome-like vesicles. The clasto-lactacystin β-lactone-induced increase in TNFR1 shedding was associated with reductions in cell surface receptors and intracytoplasmic TNFR1 stores that were primarily localized to vesicular structures. As expected, the broad-spectrum zinc metalloprotease inhibitor TNF-α protease inhibitor 2 (TAPI-2) attenuated clasto-lactacystin β-lactone-mediated TNFR1 shedding, which is consistent with its ability to inhibit the zinc metalloprotease-catalyzed cleavage of TNFR1 ectodomains. TAPI-2 also reduced TNFR1 on the cell surface and attenuated the clasto-lactacystin β-lactone-induced reduction of intracytoplasmic TNFR1 vesicles. This suggests that TNFR1 shedding induced by clasto-lactacystin β-lactone involves the zinc metalloprotease-dependent trafficking of intracytoplasmic TNFR1 vesicles to the cell surface. Together, these data are consistent with the conclusion that proteasomal activity negatively regulates TNFR1 shedding from human airway epithelial cells, thus identifying previously unrecognized roles for the proteasome and zinc metalloproteases in modulating the generation of sTNFR1.

TUMOR NECROSIS FACTOR (TNF) is a key mediator of inflammation, immunity, and apoptosis (4, 8). Although TNF can signal through two receptors, TNFR1 (TNFRSF1A, CD120a, p55 TNFR) and TNFR2 (TNFRSF1B, CD120b, p75 TNFR), the majority of TNF-mediated biological events in nonlymphoid cells are mediated via TNFR1 signaling (43). Furthermore, TNFR1 plays an important role in the regulation of lung inflammation. For example, Staphylococcus aureus protein A induces airway epithelial cell inflammatory responses and neutrophil recruitment by binding to and activating TNFR1 signaling (15). TNFR1 signaling in airway smooth muscle cells mediates the TNF-induced secretion of IL-6 and regulated upon activation, normally T-expressed, and presumably secreted and activates the p38 and p42/44 MAPK pathways (2). Hydrogen peroxide signaling through TNFR1 and TNF receptor-associated factor 2 (TRAF2) in lung fibroblasts selectively activates c-Jun NH2-terminal kinase and prevents IκB and NF-κB signaling, thereby creating a signaling imbalance that favors apoptosis (33). Transgenic mouse models have also implicated TNFR1 in the pathogenesis of lung disease. In a murine model of hemorrhage-induced acute lung injury, TNFR1 knockout mice have reduced lung neutrophil accumulation and microvascular leak, consistent with a role for TNFR1 signaling in disease pathogenesis (40). TNFR1 knockout mice are unable to resolve the pulmonary inflammatory response to Rhodococcus equi infection as a consequence of impaired apoptosis at the site of infection (21). In a murine model of Pseudomonas aeruginosa pneumonia, TNFR1 knockout mice display augmented lung inflammation and bacterial clearance, suggesting that TNFR1 attenuates lung inflammation in response to bacterial infection (39). Elevated plasma levels of soluble TNFR1 (sTNFR1) and TNFR2 may also serve as biomarkers for morbidity and mortality in patients with acute lung injury (34). In addition, utilization of a low tidal volume ventilation strategy has been associated with reduced plasma sTNFR1 levels, which may be indicative of attenuated alveolar epithelial injury (34).

sTNFRs function as TNF-binding proteins that modulate TNF bioactivity. TNFR1 can be released from cells to the extracellular compartment by two distinct mechanisms. Proteolytic cleavage of the TNFR1 extracellular domain generates soluble TNF-binding proteins that bind and inhibit TNF bioactivity (12, 29, 31, 37, 38). The major TNFR1 cleavage site is located in the spacer region adjacent to the transmembrane domain between Asn-172 and Val-173, with a minor site between Lys-174 and Gly-175 (7, 37, 44). TNF-α converting enzyme (TACE) ADAM 17, a member of the metalloprotease-disintegrin (ADAM) family of zinc metalloproteases, has been proposed as a TNFR1 sheddase, based on the finding that TACE-deficient cells have lower ratios of shed to cell surface TNFR1 than cells reconstituted with TACE (36). The receptor sheddase(s) responsible for TNFR1 cleavage, however, remains incompletely defined (43).

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The second mechanism by which TNFR1 can be released from cells to the extracellular compartment is via the generation of exosome-like vesicles. Exosomes are small membrane-enclosed vesicles, 30–200 nm in diameter, that correspond to the internal vesicles of endolysosome-related multivesicular bodies and are released from the cell via exocytic fusion with the plasma membrane (11, 16, 35, 41). Human vascular endothelial cells constitutively release a full-length 55-kDa TNFR1 within the membranes of exosome-like vesicles (17). These human umbilical vein endothelial cell (HUVEC)-derived TNFR1 exosome-like vesicles bind TNF but do not appear to have intrinsic signaling capabilities, based on the presence of silencer of death domains and the absence of TNFR-associ-
ated death domain, receptor-interacting protein (RIP), and TRAF2 (17).

Our group (25) has previously demonstrated that both phorbol ester and interleukin-1β induce sTNFR1 release from human vascular endothelial cells. In this study, we hypothesized that the proteasome might regulate the release of sTNFR1 into the extracellular compartment. An important role for the proteasome has recently been identified in regulating levels of TNFR1, which undergoes TNF-dependent internalization and proteasomal degradation to attenuate excessive proinflammatory signaling (24). Using NCI-H292 cells as a model human airway epithelial cell line system, we demonstrate that the specific proteasome inhibitor, clasto-lactacystin β-lactone, induces the proteolytic cleavage and shedding of TNFR1 ectodomains into the extracellular compartment, which is associated with a reduction in cell surface receptors and intracytoplasmic TNFR1 stores that were primarily localized to vesicular structures. This identifies an unexpected function for the proteasome, as proteasomal inhibition has been shown to induce a blockade of ligand-mediated receptor endocytosis, with resultant increases in receptor expression at the plasma membrane (28, 42, 46). Furthermore, we show that zinc metalloprotease activity mediates the trafficking of vesicle-associated intracellular receptors and the release of sTNFR1 into the extracellular space. These data provide new insights into the role of the proteasome and zinc metalloproteases in the generation of sTNFRs.

METHODS

Cells and reagents. The NCI-H292 human pulmonary mucoepidermoid carcinoma cell line and the A549 human pulmonary adenocarcinoma cell line were purchased from the American Type Culture Collection (Manassas, VA). NCI-H292 cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum under 5% CO2 at 37°C. A549 cells were grown in Ham's F12K with 2 mM-glutamine and 1.5 g/l sodium bicarbonate supplemented with 10% fetal bovine serum. Normal human small airway epithelial cells and HUVEC were grown in small airway epithelial growth medium and endothelial growth medium, respectively (Cambrex, East Rutherford, NJ). Clasto-
lactacystin β-lactone, MG-132, Z-VAD-FMK, and Boc-D-FMK were purchased from Peptides International (Louisville, KY).

Quantification of sTNFR1 by ELISA. NCI-H292 cells were grown to confluence in six-well plates; incubated with vehicle (DMSO), clasto-lactacystin β-lactone, or MG-132 for 1, 2, or 4 h; and washed twice with ice-cold PBS. Cells were collected by scraping and disrupted by sonication (2×10 s) in 50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% Triton X-100, and 1% n-octyl β-D-glucopyranoside with Complete protease inhibitor (Roche). Protein concentrations were determined with the BCA protein determination kit (Pierce). We separated proteins (40 μg) via SDS-PAGE using 4–12% Bis-Tris Nupage gels (Invitrogen), electroblotted to nitrocellulose membranes, and incubated with the murine H-5 IgG2a monoclonal antibody (2 μg/ml) that reacts with the extracellular domain of human TNFR1 or ubiquitin (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were stripped (Re-blot Western blot recycling kit, Chemicon, Temecula, CA) and reacted with antibodies directed against TACE, RIP, TRAF2, or β-tubulin (2 μg/ml) (Santa Cruz Biotechnology). The antibody against poly-ADP ribose polymerase (PARP) was from BD Transduction Laboratories (Palo Alto, CA). The rabbit polyclonal antibody aminopeptidase regulator of TNFR1 shedding (ARTS-1) antibody was utilized at a dilution of 1:1000. Cells and reagents.

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lactacystin β-lactone, MG-132, Z-VAD-FMK, and Boc-D-FMK were purchased from Calbiochem (San Diego, CA). DMSO, staurosporine, and 1.5 g/l sodium bicarbonate supplemented with 10% fetal bovine serum.

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were grown to confluence in 175-cm² flasks and incubated with RPMI 1640 containing 2% FBS that had been pre-exosomes via centrifugation at 175,000 g for 16 h. Cell supernatants were collected and cleared of cellular debris by sequential centrifugation at 1,500 g for 10 min and 10,000 g for 30 min × 2, as well as by filtration through a 0.22-μm filter. The 10,000-g supernatant was then centrifuged at 175,000 g for 2 h, and pellet and supernatant fractions were analyzed by immunoblotting.

Quantification of sTNFR1 by immunoblotting. To assess clasto-lactacystin β-lactone-induced sTNFR1 release, NCI-H292 cells were grown in six-well plates, washed three times with RPMI-1640, and incubated with medium, vehicle (DMSO), clasto-lactacystin β-lactone (25 μM), or TAPI-2 (50 μM) for 2 h in 750 μl of RPMI 1640 without serum. Supernatants were collected and cleared of cellular debris by sequential centrifugation at 1,500 g for 5 min and twice at 10,000 g for 30 min. Total proteins in the 10,000-g supernatants were precipitated with 20% TCA and analyzed by SDS-PAGE and immunodetection as described above.

For sequential centrifugation experiments to assess clasto-lactacystin β-lactone-induced sTNFR1 release, NCI-H292 cells were grown in six-well plates and treated with clasto-lactacystin β-lactone for 2 h in 750 μl of RPMI 1640 without serum. For sequential centrifugation experiments to assess constitutive TNFR1 release, NCI-H292 cells were washed twice with serum-free medium before treatment with 25 μM clasto-lactacystin β-lactone for 2 h or in medium containing 2% exosome-depleted FBS for 24 h as described above for the sequential centrifugation experiments. Supernatants were cleared of cellular material by sequential centrifugation at 1,500 g for 10 min and 10,000 g for 30 min × 2, followed by filtration through a 0.22-μm filter. Protein concentrations were quantified, and a volume containing 1 mg of protein was overlaid on a continuous sucrose gradient (0.2 to 2.0 M in 20 mM Tris, pH 8.0) and centrifuged for 16 h. Fractions (0.5 ml) were collected from the bottom of the gradient, and proteins were quantified. Samples of proteins (60 μg) were precipitated with 20% TCA and analyzed by proteasome inhibition induces sTNFR1 release from pulmonary epithelial cell lines and primary cultures of normal airway epithelial and vascular endothelial cells. A: NCI-H292 cells were incubated for 2 h with the indicated concentrations of clasto-lactacystin β-lactone, and concentrations of sTNFR1 present in culture supernatants were determined by ELISA (n = 6). *P < 0.001, single-factor ANOVA. B: NCI-H292 cells were incubated for 2 h with medium (control), vehicle (DMSO), or 25 μM clasto-lactacystin β-lactone (β-lactone). Supramaximal stimuli (10 mM N-acetyl-l-leucyl-l-leucyl-l-norvaline (AHLN), 20 mM cycloheximide, and 50 μM chloroquine, and concentrations of sTNFR1 present in culture supernatants were determined by ELISA (n = 6). *P < 0.001 vs. DMSO-treated cells, two-tailed Student’s t-test with a Bonferroni correction for multiple comparisons. D–F: A549 cells (D; n = 5), normal human small airway epithelial cells (E; SAEC, n = 6), and human umbilical vein endothelial cells (F; HUVEC, n = 6) were treated for 2 h with control medium, vehicle (DMSO), or 25 μM clasto-lactacystin β-lactone, and concentrations of sTNFR1 present in culture supernatants were determined by ELISA. *P < 0.005 vs. DMSO-treated cells, two-tailed Student’s t-test.

Fig. 2. Proteasome inhibition induces sTNFR1 release from pulmonary epithelial cell lines and primary cultures of normal airway epithelial and vascular endothelial cells. A: NCI-H292 cells were incubated for 2 h with the indicated concentrations of clasto-lactacystin β-lactone, and concentrations of sTNFR1 present in culture supernatants were determined by ELISA (n = 6). *P < 0.001, single-factor ANOVA. B: NCI-H292 cells were incubated for 2 h with medium (control), vehicle (DMSO), or 25 μM clasto-lactacystin β-lactone (β-lactone). Supramaximal stimuli (10 mM N-acetyl-l-leucyl-l-leucyl-l-norvaline (AHLN), 20 mM cycloheximide, and 50 μM chloroquine, and concentrations of sTNFR1 present in culture supernatants were determined by ELISA (n = 6). *P < 0.001 vs. DMSO-treated cells, two-tailed Student’s t-test with a Bonferroni correction for multiple comparisons. D–F: A549 cells (D; n = 5), normal human small airway epithelial cells (E; SAEC, n = 6), and human umbilical vein endothelial cells (F; HUVEC, n = 6) were treated for 2 h with control medium, vehicle (DMSO), or 25 μM clasto-lactacystin β-lactone, and concentrations of sTNFR1 present in culture supernatants were determined by ELISA. *P < 0.005 vs. DMSO-treated cells, two-tailed Student’s t-test.
immunoblotting. A Palm Abbe digital refractometer (Misco) was utilized to measure the density of each fraction.

Quantification of cell surface TNFR1. NCI-H292 cells grown to confluence in six-well plates were treated with medium, vehicle (DMSO), clasto-lactacystin β-lactone, or TAPI-2 for 2 h, washed twice in ice-cold PBS, and harvested by incubation for 10 min with Versene (HBSS containing 0.02% EDTA without calcium, magnesium, or trypsin) and reacted with fluorescein-conjugated antibodies against TNFR1 or an IgG1 isotype control (R&D Systems). We analyzed cells by flow cytometry using an EPICS XL-MCL flow cytometer (Beckman-Coulter, Miami, FL). Dead cells were excluded by propidium iodide uptake, and 10,000 events were recorded for each sample. Results are presented as mean fluorescence intensity corrected for background fluorescence, as determined with the IgG1 isotype control.

Immunofluorescence confocal laser scanning microscopy. NCI-H292 cells grown on collagen I-coated, two-well slides (Becton-Dickinson, Bedford, MA) were incubated with vehicle (DMSO), clasto-lactacystin β-lactone (25 μM), or TAPI-2 (50 μM) for 2 h. Cells were fixed in 4% paraformaldehyde for 15 min, washed three times in PBS, blocked with 10% donkey serum in PBS, and incubated overnight at 4°C with the H-5 murine anti-TNFR1 monoclonal antibody, diluted 1:40 in 1% donkey serum. The goat polyclonal anti-TACE antibody was purchased from Santa Cruz Biotechnology and utilized at a concentration of 0.2 μg/ml. Cells were washed three times in PBS containing 0.1% bovine serum albumin and incubated for 1 h with Alexa Fluor 488 donkey anti-mouse or Alexa Fluor 568 donkey anti-goat secondary antibodies (Molecular Probes, Eugene, OR). Cells were mounted utilizing Vectashield mounting medium with 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) and visualized utilizing a Leica SP laser scanning confocal microscope (Leica, Heidelberg, Germany). For comparative studies, the settings of gain, laser power, and acquisition times were identical to permit valid comparisons of data from different experiments. The fluorescence intensity of individual cells was quantified using Meta morph software (Universal Imaging, Downingtown, PA) and reported as arbitrary fluorescence units.

Statistical analyses. Statistical analyses were performed using a two-tailed Student’s t-test or single-factor ANOVA. A P value of <0.05 was considered significant. A Bonferroni correction was utilized to determine statistical significance when multiple comparisons were made.

RESULTS

Proteasome inhibition induces sTNFR1 release. Experiments were performed to assess whether proteasome inhibition modulates the release of sTNFR1 into the extracellular compartment. The NCI-H292 pulmonary epithelial cell line was utilized as a model system, and proteasome-mediated proteolysis was inhibited utilizing the specific proteasome inhibitor clasto-lactacystin β-lactone, as well as the peptide aldehyde proteasome inhibitor MG-132, which can inhibit both proteasomal and lysosomal enzymes (27). Confluent NCI-H292 cells were incubated with 25 μM clasto-lactacystin β-lactone, MG-132, or vehicle (DMSO) for 1, 2, or 4 h, and the amount of TNFR1 released into culture supernatants was quantified by ELISA. As shown in Fig. 1, at each time point, clasto-lactacystin β-lactone or MG-132 significantly increased sTNFR1 release into culture supernatants compared with cells treated with vehicle alone. Release of sTNFR1 was dependent on clasto-lactacystin β-lactone concentration (Fig. 2A). Furthermore, Western blots of total cell lysate proteins from clasto-lactacystin β-lactone-treated cells showed more polyubiquitinated proteins than cells incubated with medium alone or vehicle (Fig. 2B), which is consistent with the inhibition of proteasome-mediated proteolysis. Experiments were also performed to assess whether inhibition of lysosomal enzyme activity contributed to sTNFR1 release from NCI-H292 cells. As shown in Fig. 2C, sTNFR1 release was enhanced by treatment with the proteasome-specific inhibitor clasto-lactacystin β-lactone but not with the lysosomal enzyme inhibitors ammonium chloride (NH₄Cl) or chloroquine. These data are consistent with the conclusion that clasto-lactacystin β-lactone-mediated sTNFR1 release is not mediated by lysosomal enzyme activity. Also, differences in the absolute amount of sTNFR1 released between experiments are consistent with the biological variability of culture conditions performed on different days. Within each experimental set, however, equal numbers of cells were utilized and treated in an identical fashion so that comparisons could be made to appropriate controls.

Because NCI-H292 cells are derived from a human pulmonary mucoepidermoid carcinoma, we investigated whether the ability of proteasome inhibition to induce sTNFR1 release was specific for this cell line. As shown in Fig. 2, D, E, and F, 25 μM clasto-lactacystin β-lactone significantly increased sTNFR1 release from the A549 lung adenocarcinoma epithelial cell line, as well as from normal human small airway epithelial cells and HUVEC. Thus proteasome inhibition induced sTNFR1 release from both human lung epithelial carcinoma-derived cell lines and primary cultures of normal human airway and vascular endothelial cells. In subsequent experiments, the
NCI-H292 cell line was utilized as a model human airway epithelial cell system.

Proteasome inhibition decreases cell-associated TNFR1. Because proteasome inhibition increased the constitutive release of TNFR1 into culture supernatants, we reasoned that this effect should be associated with decreases in the quantity of cell-associated TNFR1. Alternatively, if TNFR1 is constitutively targeted for proteasomal degradation, then proteasome inhibition should be associated with an increased quantity of cell-associated TNFR1. To assess the effect of proteasome inhibition on cell-associated TNFR1 levels, NCI-H292 cells were treated with 25 μM clasto-lactacystin β-lactone or media for 1, 2, or 4 h, and immunoblots were performed on total cellular proteins. As shown in Fig. 3A, treatment with clasto-lactacystin β-lactone selectively decreased total cellular TNFR1 protein, which was maximal at 2 h and not associated with changes in the quantity of RIP or TRAF2. Similarly, proteasome inhibition did not significantly alter the quantity or maturation of TACE or ARTS-1, a type 2 integral membrane protein that binds TNFR1 and promotes its extracellular release (9, 36). Because the reduction of cell-associated TNFR1 in response to clasto-lactacystin β-lactone was maximal at 2 h, all subsequent experiments were conducted at this time point. The clasto-lactacystin β-lactone-mediated reduction in total cellular TNFR1 stores is shown in Fig. 4A. NCI-H292 cells were grown on collagen I-coated slides and treated for 2 h with 25 μM clasto-lactacystin β-lactone or vehicle. A: immunofluorescence confocal laser scanning microscopy was performed to quantify the clasto-lactacystin β-lactone-induced decrease in intracytoplasmic TNFR1 vesicles, which were identified by reaction with the H-5 murine anti-TNFR1 monoclonal antibody and an Alexa Fluor 488 donkey anti-mouse secondary antibody (green). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (blue). No reaction was detected with the IgG2b isotype control antibody (data not shown). B: immunofluorescence confocal laser scanning microscopy was performed to evaluate TNFR1 colocalization with TACE. TACE was identified by reaction with a goat polyclonal anti-TACE antibody and an Alexa Fluor 568 donkey anti-goat secondary antibody.

Proteasome inhibition does not induce poly-ADP ribose polymerase (PARP) cleavage. NCI-H292 cells were treated with 25 μM clasto-lactacystin β-lactone, vehicle (DMSO), or 1 μM staurosporine (S) for 8 h. Cells were lysed in sample loading buffer containing 6 M urea and 6% β-mercaptoethanol, and equivalent volumes (25 μl) were separated by SDS-PAGE, transferred to nitrocellulose, and reacted with an antibody against PARP. C: staurosporine induces PARP cleavage. NCI-H292 cells were treated with 25 μM clasto-lactacystin β-lactone, vehicle (DMSO), or 1 μM staurosporine (S) for 8 h. Cells were lysed in sample loading buffer containing 6 M urea and 6% β-mercaptoethanol, and equivalent volumes (25 μl) were separated by SDS-PAGE, transferred to nitrocellulose, and reacted with an antibody against PARP.
lar TNFR1 levels began to recover after 4 h despite persistent sTNFR1 release (Fig. 1), which is consistent with new TNFR1 production.

To confirm that proteasome inhibition selectively decreases the quantity of cell-associated TNFR1, NCI-H292 cells were treated with 25 μM clasto-lactacystin β-lactone, MG-132, or vehicle (DMSO) for 2 h and immunoblots were performed on total cellular proteins. As shown in Fig. 3B, total cellular TNFR1 was lower after treatment with either inhibitor for 2 h compared with cells receiving medium alone or vehicle, demonstrating that this was not a nonspecific effect of the DMSO diluent. Neither inhibitor had a significant effect on TACE maturation or on the quantity of cellular TACE, RIP, TRAF2, or ARTS-1.

Immunofluorescence confocal laser scanning microscopy experiments were performed to investigate further the effect of proteasome inhibition on cell-associated TNFR1. TNFR1 is reported to be localized to the Golgi apparatus in human vascular endothelial cells (6, 20, 45). As shown in Fig. 4A, TNFR1 in NCI-H292 cells was predominantly detected in a diffuse punctate pattern, consistent with its localization to intracytoplasmic vesicles. After treatment with clasto-lactacystin β-lactone for 2 h, there was a marked reduction of intracellular TNFR1 vesicles, consistent with transfer of cell-associated TNFR1 to the extracellular space. The fluorescence intensities of cells treated with vehicle- or clasto-lactacystin β-lactone were measured to quantify further the effect of proteasome inhibition on cell-associated TNFR1. As shown in Fig. 4A, the mean fluorescence intensity of clasto-lactacystin β-lactone-treated cells was significantly less than that of vehicle-treated cells (20.5 ± 0.8, n = 15 cells, vs. 43.8 ± 2.0, n = 16 cells, arbitrary fluorescence units; P < 0.001, two-tailed Student’s t-test). Together, these data are consistent with the conclusion that proteasome inhibition induced a redistribution of TNFR1 from intracytoplasmic vesicles to the extracellular space.

Experiments were also performed to determine whether TNFR1 and TACE were colocalized in NCI-H292 cells. As shown in Fig. 4B, both TNFR1 and TACE were present in intracytoplasmic vesicles, which were minimally colocalized in cells treated with vehicle or clasto-lactacystin β-lactone. Because the quantity and processing of TACE, as determined by Western blotting, were not affected by proteasome inhibition, these data suggest that TACE may not regulate the clasto-lactacystin β-lactone-induced increases in sTNFR1 release from NCI-H292 cells.

*Increased sTNFR1 release in response to proteasome inhibition is not a consequence of apoptosis. Because proteasome inhibitors are known to induce apoptosis in a time- and concentration-dependent fashion, experiments were performed to assess whether a pro-apoptotic effect was responsible for increasing sTNFR1 release. As shown in Fig. 5A, neither the irreversible, broad-spectrum caspase inhibitor Z-VAD-FMK nor Boc-d-FMK prevented the clasto-lactacystin β-lactone-induced increases in sTNFR1 release into culture supernatants at 2 h. Similarly, neither Z-VAD-FMK nor Boc-d-FMK altered sTNFR1 release.

Additional experiments were performed to assess whether proteasome inhibition induces the extracellular release of TNFR1 via a proapoptotic mechanism. Cleavage of PARP into a 85-kDa product is an early marker of apoptosis. As shown in Fig. 5B, treatment with 25 μM clasto-lactacystin β-lactone for 4 h was not associated with cleavage of the 116-kDa PARP into a 85-kDa product, whereas total cellular TNFR1 levels were markedly decreased. As shown in Fig. 5C, the 85-kDa PARP cleavage product was detected in total cellular lysates after treatment with 1 μM staurosporine for 8 h but not in cells treated with medium alone, vehicle (DMSO), or clasto-lactacystin β-lactone. The 85-kDa PARP cleavage product was not detected after treatment with 1 μM staurosporine for 2 or 4 h (data not shown). Together, these experiments support the conclusion that the increased sTNFR1 release during the first 2 h of proteasome inhibition is not a consequence of apoptosis.

Proteasome inhibition induces the release of proteolytically cleaved TNFR1 ectodomains into the extracellular compartment. Experiments were conducted to assess whether the TNFR1 released into the extracellular compartment represented the proteolytically cleaved ectodomain or full-length TNFR1 that is associated with exosome-like vesicles. We have previously demonstrated that both the proteolytically cleaved TNFR1 ectodomain and exosome-associated TNFR1 can be detected with the H-5 murine monoclonal antibody that reacts...
with the human TNFR1 extracellular domain (17). NCI-H292 cells grown to confluence in six-well plates were treated with 25 μM clasto-lactacystin β-lactone or vehicle (DMSO) for 2 h in serum-free medium. Culture supernatants were collected, and debris was removed by sequential centrifugation. As shown in Fig. 6A, a 34-kDa TNFR1 ectodomain form was present in supernatants of cells treated with clasto-lactacystin β-lactone but not from cells treated with medium alone or vehicle. This finding is consistent with the initial descriptions of sTNFR1, which was reported to have a size range from 27 to 33 kDa (12, 13, 31, 38). Furthermore, the release of sTNFR1 into the extracellular compartment was associated with a 51% decrease in total cellular TNFR1 content compared with cells treated with vehicle, consistent with a redistribution of TNFR1 to the extracellular space as a consequence of proteolytic cleavage and ectodomain shedding (Fig. 6, B and C).

To characterize the 34-kDa TNFR1 that was released into NCI-H292 cell culture supernatants following treatment with 25 μM clasto-lactacystin β-lactone for 2 h in serum-free media, culture supernatants were collected and debris was removed by sequential centrifugation. As shown in Fig. 7A, the 34-kDa TNFR1 in the 10,000-g supernatant was recovered almost entirely in the supernatant fraction (S2) after centrifugation at 175,000 g, consistent with a soluble, cleaved TNFR1 ectodomain. Supernatants from NCI-H292 cells that were incubated for 24 h in control medium containing 2% exosome-depleted FBS were likewise fractionated by sequential centrifugation to characterize the TNFR1 released into the extracellular compartment under basal conditions. As shown in Fig. 7B, a 48-kDa TNFR1 was present in the 10,000-g supernatant (S1) and largely sedimented in the 175,000-g pellet (P2), consistent with its presence in a membrane-associated form, such as an exosome-like vesicle.

Rate zonal centrifugation through continuous sucrose gradients was performed to characterize further the TNFR1 present in cell culture supernatants from NCI-H292 cells. As shown in Fig. 7C, the 34-kDa sTNFR1 in medium from cells treated with 25 μM clasto-lactacystin β-lactone for 2 h sedimented to a peak density of 1.05–1.06 g/ml. In contrast, supernatants from NCI-H292 cells incubated with exosome-depleted medium for 24 h contained a 48-kDa TNFR1 that sedimented to a peak density of 1.11–1.13 g/ml, as is typical of exosome-like vesicles (35, 41). Furthermore, this density is similar to that of exosome-associated TNFR1 released from human vascular endothelial cells, which also sediments to a peak density of 1.1 g/ml (17). Together, the finding of a 34-kDa TNFR1 in supernatants from cells treated with clasto-lactacystin β-lactone, which preferentially localizes to the supernatant fraction fol-

![Fig. 7. Characterization of TNFR1 in cell culture supernatants. A: clasto-lactacystin β-lactone-induced release of sTNFR1. NCI-H292 cells were grown to confluence in 6-well plates and treated with 25 μM clasto-lactacystin β-lactone for 2 h in serum-free media. Culture supernatants were collected, and debris was removed by sequential centrifugation at 1,500 g for 10 min and twice at 10,000 g for 30 min, followed by filtration through a 0.22-μm filter. The 10,000-g supernatant (S1) was then subjected to high-speed centrifugation at 175,000 g for 2 h, and the resulting pellet (P2) and supernatant (S2) were collected. Total proteins from the 10,000-g (S1) and 175,000-g (S2) supernatants were precipitated with 20% TCA and suspended in NuPAGE sample buffer. Total proteins in the 175,000 g pellet (P2), as well as 40 μg of total cellular proteins from unstimulated NCI-H292 cells (T), were also suspended in sample buffer and analyzed by immunoblotting with the H-5 murine monoclonal antibody, which reacts with the TNFR1 extracellular domain. B: characterization of constitutive TNFR1 release from NCI-H292 cells. NCI-H292 cells were grown to confluence in 175-cm² flasks in exosome-depleted medium for 24 h, and TNFR1 present in culture supernatants and cell lysates were prepared as described above. Proteins (19 μg) from the 10,000-g supernatant and 175,000-g pellet and supernatant were utilized for immunoblotting with the H-5 anti-TNFR1 antibody. Total cellular proteins (40 μg) from unstimulated NCI-H292 cells were again included for comparison. C: rate of zonal centrifugation through continuous sucrose gradients. Supernatants from NCI-H292 cells incubated in medium with 2% exosome-depleted FBS for 24 h (control) or with 25 μM clasto-lactacystin β-lactone for 2 h in serum-free medium were collected and cleared of cellular material by sequential centrifugation at 1,500 g for 10 min and 10,000 g for 30 min × 2, followed by filtration through a 0.22-μm filter. A volume containing 1 mg of protein was overlaid on a continuous sucrose gradient (0.2–2.0 M in 20 mM Tris, pH 8.0) and centrifuged at 175,000 g for 16 h. Fractions (0.5 ml) were collected from the bottom of the gradient, and proteins were quantified. Samples of proteins (60 μg) were precipitated with 20% TCA and analyzed by immunoblotting with the H-5 murine anti-TNFR1 monoclonal antibody. Specific gravities of individual fractions are shown at bottom.](http://ajplung.physiology.org/)}
TAPI-2 on clasto-lactacystin
Thus experiments were performed to assess the effect of inhibitors, such as TAPI-2, on clasto-lactacystin-induced shedding of sTNFR1, as we have also detected the 34-kDa sTNFR1 in supernatants from cells incubated in exosome-free medium for 24 h.

TAPI-2 inhibits clasto-lactacystin β-lactone-mediated sTNFR1 release. Hydroxamic-acid based zinc metalloprotease inhibitors, such as TAPI-2, can block sTNFR1 release (9, 45). Thus experiments were performed to assess the effect of TAPI-2 on clasto-lactacystin β-lactone-induced TNFR1 shedding to the extracellular compartment. As demonstrated by immunoblots of TCA-precipitated proteins from culture supernatants (Fig. 8, A–C), incubation with 50 μM TAPI-2 for 2 h inhibited the clasto-lactacystin β-lactone-induced shedding of the 34-kDa sTNFR1 into culture supernatants. TAPI-2 also prevented the clasto-lactacystin β-lactone-induced decrease in total cellular TNFR1 content (Fig. 8, A–C).

To characterize further the role of zinc metalloproteases in the release of sTNFR1, immunofluorescence confocal laser scanning microscopy experiments were performed to quantify intracellular receptor content. As shown in Fig. 8D, treatment with 25 μM clasto-lactacystin β-lactone for 2 h was associated with a marked reduction in the quantity of intracellular TNFR1 vesicles compared with cells treated with vehicle alone. The clasto-lactacystin β-lactone-induced reduction in intracellular TNFR1 vesicles was prevented by TAPI-2, as the quantity of TNFR1 vesicles in cells treated with clasto-lactacystin β-lactone plus TAPI-2 was significantly greater than that in cells treated with clasto-lactacystin β-lactone alone.

The effect of zinc metalloprotease inhibition on NCI-H292 cell surface TNFR1 levels was assessed by flow cytometry (Fig. 8E). As expected, cells treated with 25 μM clasto-lactacystin β-lactone demonstrated a significantly lower mean surface TNFR1 expression. Data from triplicate experiments, corrected for the isotype control, are reported as means ± SE. *P < 0.05 vs. DMSO-treated cells, two-tailed Student’s t-test with a Bonferroni correction for multiple comparisons. **P < 0.01 clasto-lactacystin β-lactone (n = 6) vs. clasto-lactacystin β-lactone plus TAPI-2 (n = 15), two-tailed Student’s t-test with a Bonferroni correction for multiple comparisons.

Western blots were performed in triplicate, as in A; a representative blot is shown. Nitrocellulose membranes were stripped and reacted with anti-β-tubulin antibodies, as a control for protein loading. TNFR1 values normalized to those of β-tubulin, to control for protein loading, are presented as means of corrected densitometry units. *P = 0.002, clasto-lactacystin β-lactone vs. clasto-lactacystin β-lactone plus TAPI-2 (n = 3), two-tailed Student’s t-test. TAPI-2; βT, clasto-lactacystin β-lactone plus TAPI-2. E: effect of TAPI-2 on clasto-lactacystin β-lactone-induced decreases in intracytoplasmic TNFR1 vesicles. NCI-H292 cells were grown on collagen I-coated slides and incubated for 2 h with DMSO (vehicle), 25 μM clasto-lactacystin β-lactone, 50 μM TAPI-2, or clasto-lactacystin β-lactone plus TAPI-2 (βT). Intracytoplasmic TNFR1 vesicles were identified by reaction with the H-5 murine anti-TNFR1 monoclonal antibody and an Alexa Fluor 488 donkey anti-mouse secondary antibody (green). Nuclei were stained with DAPI (blue). Fluorescence intensity of individual cells is reported as means of arbitrary fluorescence units (MFI) ± SE. *P < 0.001 clasto-lactacystin β-lactone (n = 6) vs. DMSO-treated cells (n = 12), two-tailed Student’s t-test with a Bonferroni correction for multiple comparisons. **P < 0.01 clasto-lactacystin β-lactone (n = 6) vs. clasto-lactacystin β-lactone plus TAPI-2 (n = 15), two-tailed Student’s t-test with a Bonferroni correction for multiple comparisons. E: effect of zinc metalloprotease inhibition on cell-surface TNFR1 expression. NCI-H292 cells were incubated with vehicle, 25 μM clasto-lactacystin β-lactone, 50 μM TAPI-2, or clasto-lactacystin β-lactone plus TAPI-2 for 2 h, and flow cytometry was performed to quantify the MFI of cell surface TNFR1 expression. Data from triplicate experiments, corrected for the isotype control, are reported as means ± SE. Cell surface TNFR1 expression was decreased in cells treated with clasto-lactacystin β-lactone or TAPI-2 compared with vehicle (*P < 0.05 vs. DMSO-treated cells, two-tailed Student’s t-test with a Bonferroni correction for multiple comparisons). There was no difference in cell surface TNFR1 expression between cells treated with clasto-lactacystin β-lactone compared with cells treated with clasto-lactacystin β-lactone plus TAPI-2 [P = not significant (NS), n = 3, two-tailed Student’s t-test].
fluorescence intensity of cell surface TNFR1 than cells treated with vehicle alone. Interestingly, treatment with TAPI-2 alone also resulted in lower levels of cell surface TNFR1 expression than that of cells treated with vehicle alone. If the increases in sTNFR1 released in response to proteasome inhibition were a consequence of increased proteolytic cleavage of cell surface receptors, an increase in cell surface TNFR1 might be expected to result from inhibition of zinc metalloproteases. In contrast, cells treated with TAPI-2 plus clasto-lactacystin β-lactone for 2 h had the same mean fluorescence intensity of cell surface TNFR1 compared with cells treated with clasto-lactacystin β-lactone alone. Together, these data suggest that TAPI-2 inhibited trafficking of vesicle-associated TNFR1 to the cell surface, as well as clasto-lactacystin β-lactone-mediated proteolytic cleavage of TNFR1.

DISCUSSION

The ubiquitin-proteasome pathway plays a central role in regulating cellular activities by modulating the abundance of key proteins (5). Furthermore, the proteasome may regulate the quantity of cell surface receptors (46). For example, proteasome inhibitors impair internalization of the interleukin-2/interleukin-2 receptor complex (IL-2/IL-2R), as well as its subsequent lysosomal degradation, possibly by regulating trafficking to lysosomes (46). Specific proteasome inhibitors also block internalization of cell surface growth hormone receptors (GHR), although ubiquitination of the GHR is not required for its proteasome-dependent internalization (42). Similarly, proteasome inhibition significantly increases cell surface levels of the low-density lipoprotein receptor-related protein, as a consequence of impaired trafficking to multivesicular bodies (28).

Here, we hypothesized that a proteasomal pathway might also modulate the release of TNFR1 into the extracellular compartment. We utilized the specific proteasome inhibitor clasto-lactacystin β-lactone, as well as the peptide aldehyde proteasome inhibitor MG-132, to investigate the role of the proteasome in extracellular TNFR1 generation (14, 23). Clasto-lactacystin β-lactone, the active form of the Streptomyces product lactacystin, binds specifically and covalently to catalytic subunits of the 20S proteasome and inhibits peptide hydrolysis by the 26S complex without altering lysosomal protein degradation or the activity of other known proteases, making it a useful reagent for investigating proteasome-mediated events (14, 32).

Using a model airway epithelial cell system, we demonstrate that proteasome inhibition induces the shedding of a 34-kDa protein, consistent with proteolytic cleavage of the TNFR1 extracellular domain by a zinc metalloprotease receptor sheddase. Furthermore, clasto-lactacystin β-lactone-induced TNFR1 shedding was associated with a marked reduction in total cellular TNFR1 protein levels, as determined by immunoblotting and confocal microscopy, as well as diminished cell surface TNFR1 protein. These data suggest that the proteasome regulates TNFR1 via a different mechanism than has been described for IL-2/IL-2R, GHR, and lipoprotein receptor-related protein, where proteasome inhibition blocks receptor internalization, with a resulting increase in cell surface expression. In contrast, proteasome inhibition was associated with decreased cell surface TNFR1 and mobilization of the intracellular TNFR1 vesicles. Therefore, these data are consistent with the conclusion that proteasome inhibition targeted intracellular vesicle-associated TNFR1 for proteolytic cleavage and receptor shedding into the extracellular space (Fig. 9). Furthermore, this is consistent with the prior description of an intracellular TNFR1 reservoir located in the Golgi apparatus in human vascular endothelial cells, which can be mobilized and shed in response to histamine stimulation (20, 45). In NCI-H292 cells, however, intracellular TNFR1 appears to be diffusely localized to intracytoplasmic vesicles, which represents the predominant storage pool.

Proteasome inhibitor-induced TNFR1 shedding was further characterized using the broad-spectrum, hydroxamic acid-based, zinc metallopeptase inhibitor TAPI-2. As expected, TAPI-2 decreased the proteasome inhibitor-induced release of the 34-kDa sTNFR1 into the extracellular compartment, consistent with an inhibition of zinc metalloprotease-dependent cleavage of the TNFR1 ectodomain. Because proteasome inhibition blocks internalization of IL-2/IL-2R, GHR, and LPR, with a resulting increase in cell surface receptor expression, we assessed whether a similar phenomenon occurred with TNFR1, as an increase in cell surface expression could provide additional receptors for proteolytic cleavage and ectodomain shedding. If this hypothesis was correct, then one would expect a clasto-lactacystin β-lactone-induced increase in cell surface TNFR1 with zinc metallopeptase inhibition, as receptor shedding activity would be blocked. Surprisingly, we found that clasto-lactacystin β-lactone reduced cell surface TNFR1 ex-
pression despite zinc metalloprotease inhibition with TAPI-2. Furthermore, TAPI-2 treatment attenuated the reduction of intracytoplasmic vesicle-associated TNFR1 that was seen with proteasome inhibition. Together, these data show that zinc metalloprotease-dependent trafficking of intracytoplasmic TNFR1 vesicles to the cell surface is important in the mechanism by which release of sTNFR1 is regulated. Cell surface TNFR1 levels were also decreased by treatment with TAPI-2 alone, which is further evidence consistent with zinc metalloprotease-dependent trafficking of intracytoplasmic TNFR1 vesicles.

Although we have shown that proteasome inhibition induces TNFR1 shedding from NCI-H292 human airway epithelial cells, further studies will be required to determine the mechanism underlying this effect. Western blots of NCI-H292 cell lysates did not demonstrate an accumulation of modified forms of TNFR1 following proteasomal inhibition, which suggests that TNFR1 is neither monoubiquitinated nor polyubiquitinated under basal conditions. Our data are consistent with a recent report that also found that TNFR1 is not constitutively ubiquitinated (24). Together, these findings suggest that levels of a protein other than TNFR1 may be altered by proteasome inhibition and thereby increase constitutive TNFR1 shedding. Our data suggest that this protein is neither TACE nor ARTS-1, as we did not demonstrate an accumulation of modified forms following proteasome inhibition.

Because proteasome inhibition has been associated with a time- and concentration-dependent induction of apoptosis in several cell types, the role of programmed cell death in TNFR1 shedding was examined (10, 19, 22). Apoptosis can result in the generation of microvesicles or microparticles, which are formed by plasma membrane vesiculation or blebbing (3, 18). Apoptosis-inducing agents, such as staurosporine, can also induce TNFR1 shedding (26). In NCI-H292 cells, pan-caspase inhibitors did not alter the quantity of TNFR1 released into the extracellular compartment and proteasome inhibition did not induce PARP cleavage over 4 h. In contrast, PARP cleavage was demonstrated after 8 h of staurosporine treatment. Together, these data suggest that the mechanism by which proteasome inhibitors induce TNFR1 shedding from NCI-H292 cells does not involve induction of programmed cell death or the release of apoptotic microvesicles.

We have also characterized the inducible 34-kDa sTNFR1 isoform that is released in response to proteasome inhibition as the proteolytically cleaved TNFR1 ectodomain. This is based on the recovery of a 34-kDa sTNFR1 form in the supernatant fraction after high-speed centrifugation at 175,000 g and on its sedimentation to a specific density of 1.05-1.06 g/ml in a continuous sucrose gradient, which is below that of the exosome-associated 48-kDa TNFR1. In contrast, under basal conditions, NCI-H292 cells release a 48-kDa TNFR1 isoform, which can be pelleted from the medium by high-speed centrifugation at 175,000 g and sedimented to a specific density of 1.1-1.13 g/ml. This behavior is most consistent with exosome-associated TNFR1, as exosomes characteristically are sedimented by high-speed centrifugation and equilibrate on sucrose gradients at densities ranging from 1.08 to 1.22 g/ml, as is typical of lipid vesicles (35, 41). Thus these data are consistent with the conclusion that inducible TNFR1 shedding in response to proteasome inhibition results from proteolytic cleavage of receptor ectodomains.

In summary, we have demonstrated that proteasome inhibition induces the proteolytic cleavage and shedding of sTNFR1 ectodomains from human pulmonary epithelial cells, which is associated with a reduction of intracytoplasmic TNFR1 vesicles and cell surface receptors (Fig. 9). Furthermore, the mechanism of TNFR1 shedding may involve the zinc metalloprotease-dependent trafficking of intracytoplasmic TNFR1 vesicles to the cell surface. These findings identify new roles for the proteasome and zinc metalloproteases in regulating the proteolytic cleavage and shedding of the TNFR1 extracellular domain. In addition, the ability of proteasome inhibition to induce the proteolytic cleavage and shedding of sTNFR1 ectodomains may have physiological relevance in the setting of oxidative stress, which has been associated with impaired proteasome function (30). The enhanced release of sTNFR1 ectodomains may also be important for patients receiving proteasome inhibitors as therapeutic agents for neoplastic disease (1).

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GRANTS

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