Extracellular proteasome in the human alveolar space: a new housekeeping enzyme?

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Sixt SU, Beiderlinden M, Jennissen HP, Peters J. Extracellular proteasome in the human alveolar space: a new housekeeping enzyme? Am J Physiol Lung Cell Mol Physiol 292: L1280–L1288, 2007. First published January 12, 2007; doi:10.1152/ajplung.00140.2006.—We hypothesized that 20S proteasome is present and functional in the extracellular alveolar space in humans. Proteasomal activity was measured in bronchoalveolar lavage (BAL) supernatant from eight humans using specific proteasomal fluorogenic substrates and 125I-albumin with and without specific proteasome inhibitors. Furthermore, gel filtration, Western blot technique, and mass spectrometry were applied for proteasome characterization. All proteasomal fluorogenic substrates were hydrolyzed by BAL supernatant, with hydrolysis inhibited by epoxomicin (P = 0.024) and other proteasome inhibitors as well. E64, a lysosomal inhibitor, did not inhibit enzyme activity. The majority of proteolytic activity was detected in BAL supernatant rather than in the cell pellet. No correlation was found between proteasomal hydrolysis in BAL supernatant and lactate dehydrogenase activity, the total cell count in the cell pellet, and the fraction of avital cells in the cell pellet, ruling out cell lysis as a major source of proteasomal activity. Gel filtration revealed hydrolyzing activity in the supernatant at 660 kDa and proteasome core proteins after analysis by ESI-QqTOF mass spectrometry. Furthermore, Western blots using a polyclonal antibody against proteasomal α/β-subunits detected proteasomal proteins in the typical 20- to 30-kDa range in BAL supernatant. Incubation of BAL supernatant with 125I-albumin showed a high mean cleavage rate (101.8 μg/ml × h lavage ± 46 SD) that was inhibited by epoxomicin (P = 0.013) and was ATP and ubiquitin independent. We identified for the first time extracellular, biologically active, ATP- and ubiquitin-independent 20S proteasome in the human alveolar space, with a high albumin cleavage rate. Possibly, the proteasome assists in maintenance of a low intra-alveolar oncotic pressure and/or alveolar protein degradation.

albumin; bronchoalveolar lavage; circulating proteasome; fluorogenic peptides; alveolar protein degradation; lung proteins

The ubiquitin/proteasome system is a major pathway for selective intracellular non-lysosomal protein degradation in eukaryotic cells and plays an important role in numerous processes (3, 13, 53). The 20S proteasome is a multicatalytic proteinase complex with a cylinder-shaped structure arranged as four axially stacked heptameric rings composed of seven α-subunits (outer rings) and seven β-subunits (inner rings) (36), respectively. Its catalytic sites are exclusively associated with the β-subunits (40, 56) and are ATP and ubiquitin independent. The caspase-like activity of the β1-subunit cleaves after acidic residues, the trypsin-like activity of the β2-subunit cleaves after basic residues, and the chymotrypsin-like activity of the β5-subunit cleaves after hydrophobic residues. Together, these three enzyme activities allow the proteasome to cleave many different substrates into diverse products. Within the cell, the 20S proteasome is associated with large ATP- and ubiquitin-dependent 19S regulatory cap-like complexes, together yielding a 26S complex. It is now well accepted that proteasomes are localized in both cell cytoplasm and nucleus (4, 50).

Whether proteasomes exist in the extracellular space under physiological conditions, however, has not been assessed. Vaithilingam et al. (61) described an extracellular proteasome-like structure with a molecular mass of 1,000 kDa released from cultured C6 astrocytoma cells that was able to degrade proteasomal fluorogenic substrates. This suggests that cells may secrete biologically active proteasome-like structures. However, investigations in vivo are missing, in particular, with regard to internal body surfaces.

Accordingly, we tested the hypothesis that biologically active proteasome is present physiologically in the extracellular alveolar space of humans and digests albumin, one of the predominant intra-alveolar proteins.

METHODS

Eight Caucasian subjects (5 men, age 65 yr ± 4 SD) were included after approval of the local Ethics Committee and informed written consent. All subjects were free of lung, cardiac, infectious, and allergic disease and had no history of chemotherapy or radiation therapy.

Bronchoalveolar lavage (BAL) was performed during anesthesia preceding surgery [abdominal hernia repair (2), removal of cerebral tumor (4), thyroid resection (1), partial liver resection (1)] with a bronchoscope wedged in a right upper lobe segmental bronchus (2, 6). Recovered volume (81 ml ± 20, i.e., 51–63% of instilled volume) was filtered through gauze (34) to remove any visible particulate material and centrifuged (500 g, 10 min, 5°C). The supernatant was immediately frozen using liquid nitrogen and stored at −80°C. Cell counts were made in the pellet by counting an aliquot in a Neubauer chamber (34). Cell viability was determined by the Trypan blue dye exclusion technique (1). For differentiation of cells, smears were air-dried and stained according to May-Grunwald-Giemsa (6). The remaining cell pellet was frozen in liquid nitrogen and stored at −80°C. After cell lysis, the pellet was ultracentrifuged (30,000 g, 30 min), and its upper portion was used for further analysis (cell pellet lysate).

Hydrolyzing activities. Hydrolysis of proteasome-specific substrates was assayed with the fluorogenic substrates Suc-LLVY-AMC, Bz-Val-Phe-AMC, and Suc-FMK (17).

Suc-LLVY-AMC (3-carboxy-propiony-Leu-Leu-Val-Tyr-7-amido-4-methylcumarin) is a fluorogenic substrate for the chymotrypsin-like activity of the proteasome (17).

Bz-Val-Phe-AMC (benzoyl-Val-Gly-Arg-7-amido-4-methylcumarin) (5) is a substrate for the trypsin-like activity of the 20S proteasome.
Suc-LLE-AMC (3-carboxy-propionyl-Leu-Leu-Glu-7-amido-4-methylcoumarin) (48) is a fluorogenic substrate for the caspase-like (peptidylglutamyl-peptide hydrolyzing) activity of the proteasome. The final concentration in the assay buffer system was: 100 μM fluorogenic substrate, 50 mM Tris·HCl, pH 8.0, 1 mM dithioerythritol (DTE), and 5 mM MgCl₂. After incubation at 37°C for 60 min in a water bath, the reaction was inhibited irreversibly at specified time intervals by ethanol (20 min, 0°C). Release of 7-amido-4-methylcoumarin (AMC) was detected by spectrophotometry (RF-10A Xl, Shimadzu, Duisburg, Germany) with an excitation (λex) of 380 nm and an emission (λem) of 460 nm. The final protein concentration for the cell pellet assay was 2 mg/ml. Since protein concentration in the BAL supernatant was so low that a final protein concentration of 2 mg/ml could not be achieved, 104 μl of BAL supernatant were taken into each assay for all subjects. Samples from all subjects were measured separately in duplicate. Duplicate measurements were averaged for each single subject, and these eight values were used for statistical analysis.

**Inhibition of proteasome activity.** To assess potential inhibition of hydrolysis, 10 μM epoxomicin (43) (Calbiochem, San Diego, CA) was added to the incubation mixture in separate assays to samples from all eight subjects. For determination of EC₅₀ for epoxomicin inhibition of hydrolyzing activity in BAL supernatant, increasing concentrations of epoxomicin from 0.0001 to 15 μM were used. Epoxomicin is a novel, highly specific epoxy-ketone tetrapeptide proteasome inhibitor. It is cell permeable, potent, selective, and irreversible (43). It is the most potent and selective proteasome inhibitor currently available. Epoxomicin covalently binds to the LMP7, X, MECL1, and Z catalytic subunits of the proteasome. It inhibits all three enzyme activities of the proteasome (43).

In addition, we also studied (n = 8) the effects of other specific inhibitors (Calbiochem) of proteasomal activity such as proteasome inhibitor II (49) (concentrations of 0.001–70 μM), MG-132 (39) (concentrations of 0.01–70 μM), and thyrreopetin A (45) (concentrations of 0.01–100 μM). Proteasome inhibitor II (48) is a substrate-related inhibitor (Z-LLE-CHO) and inhibits the chymotryptic-like and trypic-like activity. The inhibitor is cell permeable. Proteasome inhibitor IV (63) also mediates substrate-related inhibition and is a weak inhibitor of the chymotryptic-like activity. It also inhibits the caspase-like activity and weakly inhibits the trypic-like activity. MG-132 (39) is a potent and selective reversible proteasome inhibitor mediating substrate-related inhibition (Z-Leu-Leu-CHO). It inhibits the chymotryptic-like activity and the trypic-like activity. The caspase-like activity is not inhibited by MG-132. It also inhibits cathepsins and calpains (cysteine proteases). Thyrreopetin A (45) is cell permeable and mediates competitive and reversible inhibition. It inhibits the chymotryptic-like and the trypic-like activity of the proteasome.

To exclude effects on enzyme activity by lysosomal enzymes, we also studied the effect of the lysosomal inhibitor E64 (500 μM), E64 [trans-epoxysuccinyl-l-leucylamido-(4-guanidino)butane] (28) is an potent, irreversible, cell-permeable, and highly selective cysteine protease inhibitor, and E64 does not inhibit serine proteases (except trypsin) like the cysteine protease inhibitors, leupeptin, and antipain. The trans-epoxysuccinyl group (active moiety) of E64 irreversibly binds to an active thiol group of many cysteine proteases such as papain, actinidase, and cathepsins B, H, and L (58) to form a thioether linkage. The mechanism of inhibition of some cysteine proteases including cathepsins B and L, and of trypsin, have been reported (58).

We used fluorogenic substrate cleavage related to total protein [picokatal (pkt)/mg] to describe the specific enzyme activity of extracellular proteasome. We also calculated the volume-related enzyme activity of the BAL (pkt/ml) to derive total enzyme activity of the BAL recovery expressed as pkt (pkt₄₀₀AL = pkt/ml × ml BAL recovery). Thus volume-related activity is expressed as U/ml (= katal/ml), and specific activity is expressed as U/mg (= katal/mg = mol/mol × s).

**125I-labeling of BSA.** Twenty-five micrograms of BSA were iodinated (62), mixed with 2.5 mg BSA, acetylated (67), and dialyzed against 20 mM Tris·HCl, pH 7.8. It was then precipitated by 5% wt/vol TCA. The pellet was resuspended in 300 μl of 1.0 M NaOH, diluted to a total volume of 6 ml in millipore water, heated to 60°C for 15 min, and stored at −80°C (125I-BSA stock solution). Methylated 125I-BSA was prepared according to Hershko et al. (29).

**Proteolysis of 125I-BSA.** ATP-dependent proteolysis was measured as previously described (14, 67), and the assay was modified (25) for the measurements in BAL supernatant from eight individuals in separate assays. Proteolysis was measured in the presence and absence of 10 mM EDTA and 10 μM epoxomicin, respectively. Incubation mixture 1 (total vol 0.1 ml) for ATP/Mg²⁺-dependent activity (+ ATP) had the following final concentrations: 10 μg/ml pure 125I-BSA and 50 μl sample of BAL supernatant, 50 mM Tris·HCl, pH 8.0, 1 mM DTE, 5 mM ATP, and 5 mM Mg²⁺. Mixtures with buffer instead of sample were incubated as controls. For measurement of ATP/Mg²⁺-independent proteolytic activity (mixture 2), 10 mM EDTA (−ATP) was added to mixture 1. For the incubation mixture −ATP/+epoxomicin (proteasome-independent activity), 10 μM (final concentration) epoxomicin (mixture 3) was added to mixture 2. After incubation at 37°C for 120 min, 0.125 ml 10% wt/vol TCA and 0.025 ml unlabeled BSA (30 mg/ml) were added to the mixtures and placed on ice (0°C) for 20 min. After centrifugation (Eppendorf 5415; Eppendorf, Hamburg, Germany) for 2 min at 14,000 g, the TCA-soluble radioactivity in the supernatant was measured in a gamma counter (Gammasizt BF 5300; Berthold, Cologne, Germany). Activity was expressed as U/ml as follows

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\text{U/ml} = \frac{(\text{cpm}_{\text{assay}} - \text{cpm}_{\text{zero value}}) \times 100 \times \text{dilution of sample}}{\text{cpm}_{\text{cal}} \times \text{incubation time (min)}}
\]

\[
1 \text{ U/ml} = 600 \mu g \ 125I \text{albumin/ml per hour.}
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**SDS-PAGE.** SDS-PAGE was performed with Mini-Protein 3 Electrophoresis (Bio-Rad) with 15% gels according to Laemmli (38). Fifty micrograms of protein per lane were applied. The molecular weight standard was SeeBlue Pre-Stained Standard obtained from Invitrogen.

**Western blots.** To detect the presence of proteasomal proteins, samples (50 μg/lane) were subjected to SDS-PAGE and transferred to PVDF (Bio-Rad) under semidry conditions with the use of a Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). After blocking the PVDF membranes by incubation with TBS-Tween buffer (5% Tween 20, 150 mM NaCl, 20 mM Tris·HCl, pH 7.6) and Starting-Block blocking buffer (Pierce) for 24 h at 4°C, the membranes were incubated (dilution 1:1,000, 2 h, room temperature) with rabbit polyclonal antibody to 20S proteasome α-β-subunits (Biomol International; PW 8155). After being washed with TBS-Tween buffer (5% Tween 20, 150 mM NaCl, 20 mM Tris·HCl, pH 7.6), the membranes were incubated (1:10,000, 1 h, room temperature) with peroxidase-conjugated, affinity-isolated goat anti-rabbit IgG (Sigma Aldrich). After washing, the chemiluminescence method was employed to detect the peroxidase activity using an ECL kit (SuperSignal West Pico Chemiluminescence Substrate, Pierce).

**Gelfiltrations.** Gelfiltrations (6 separate runs from 6 individuals) were performed on a Superose 6PG column (internal diameter: 1 cm, length: 40-cm packed gel, Amersham Bioscience) in 20 mM Tris·HCl, 10% glycerol, 100 mM NaCl, pH 7.5 (44), at 5°C. The column had been previously calibrated using proteins of known molecular mass: thyreglobulin (668 kDa), ferritin (446 kDa), catalase (232 kDa), aldolase (158 kDa), IgG (160 kDa), BSA (67 kDa), ovalbumin (45 kDa), trypsin inhibitor (20.1 kDa), and cytochrome c (12.4 kDa). Molecular masses of the enzyme fractions were calculated by nonlinear regression based on the respective calibration function. **Lactate dehydrogenase activity.** As a marker of possible cell damage, BAL supernatants were analyzed for total (LDH₁-LDH₃)
lactate dehydrogenase (LDH) activity by a kinetic UV test (Diaglobal) using an optimized standard method (IFCC).

**Albumin and total protein concentration.** Albumin concentration in BAL supernatant in eight subjects was measured using an ELISA (Dade Behring, Marburg, Germany). Total protein in the BAL supernatant was determined after TCA precipitation (5%), washing, and resolubilization according to Lowry et al. (41) using an AutoAnalyzer (Technicon) employing BSA as a standard. Since albumin is the predominant protein (18) in the BAL, measured albumin concentrations were subtracted from the total protein. Hence, all data on hydrolyzing activity were referenced to albumin-depleted total protein.

**ESI-QqTOF-mass spectrometry.** To analyze proteins in the peak Suc-LLVY-AMC eluate activity of the BAL supernatant (fractions 22–26) (as described above) and the cell pellet lysate (fractions 21–24) gel spots in a molecular range (above 20 kDa) likely to contain proteasomal proteins, if present, were excised from the SDS-PAGE of one individual and subjected to electrospray ionization mass spectrometry measurements (44). This technique provides a plot of detector signal intensity (abundance of ions) vs. the mass-to-charge ratio of ions produced in the instrument (m/z). Such a plot is called a mass spectrum and is considered a “fingerprint” of a molecule, which may be identified by a positive comparison with a mass spectrum obtained from a known sample. Specifically, a sample is introduced into the gas phase and exposed to an ion source. Here, the substance (MH) is bombarded with electrons. Electrons interact in such a way as to produce positive ions of the substance, according to the equation \( MH + e^- \rightarrow MH^+ 2e^- \). The \( MH^+ \) ion has essentially the same mass as the original substance, so it is called a molecular ion. Enough energy is imparted to the substance during ionization that most molecules fragment, producing fragment ions with a smaller mass-to-charge ratio. The mass of the fragment ions and the abundance of ions at each mass varies widely among molecules and thus are unique for a particular molecule. Some molecules break apart so readily that no molecular ion remains. The fingerprint of the cracked molecules allows identification of (all) proteins present in the gel as long as they are listed in the database.

**Chemicals.** All chemicals were of highest available or analytical grade. Water was deionized, distilled, and passed through a Milli-Q System (Millipore) before use.

**Statistical analysis.** Data are described as medians (box and whisker plots) or means ± SD. Values of variables were compared using a Wilcoxon or paired sample t-test, as appropriate, using SPSS 11.0 (Chicago, IL). Differences were regarded as statistically significant with an α-error (P) of less than 0.05.

**RESULTS**

All three proteasomal-specific fluorogenic substrates (16, 59) were hydrolyzed in the BAL supernatant and in the cell pellet lysate (Fig. 1, A and B). Furthermore, all three hydrolytic activities in BAL supernatant were significantly inhibited by 10 μM epoxomicin (43) (Fig. 1, A and D), the most potent and selective inhibitor currently available, with an EC₅₀ of 210 nM, 1.1 μM, and 3.4 μM for hydrolysis of Suc-LLVY-AMC (Fig. 1D), BZ-VGR-AMC, and Suc-LLE-AMC, respectively. The EC₅₀ for the hydrolysis of Suc-LLVY-AMC, BZ-VGR-AMC, and Suc-LLE-AMC was 200 nM, 40.7 μM, and 2 μM for proteasome inhibitor II, 2.1 μM, 10.65 μM, and 3.12 μM for thyropeptin A, and 0.31 μM, 0, and 0 for MG-132, respectively. Incubation with the lysosomal inhibitor E64 (Fig. 1E) had no effect on the hydrolysis of fluorogenic substrates.

Of note, the majority (94–62%) of the total proteolytic activity was found in BAL supernatant (Fig. 1C) rather than in the cell pellet lysate. Cell lysis resulting from the centrifugation step itself can be excluded (Fig. 2D) as a major source of proteasome activity in BAL supernatant since only 0.1% of the hydrolyzing activity resulted from cell lysis, as shown by exemplary centrifugation of blood samples. Furthermore, all alveolar cells observed by direct microscopy and after staining with May-Grunwald-Giemsa and trypan blue had intact cell membranes. Also, there was no correlation between the enzyme activities in the BAL supernatant measured with proteasome-specific substrates and LDH activity (Fig. 2A), a marker of cell lysis, the cell count in the cell pellet (Fig. 2B), and the fraction of cells in the cell pellet taking up trypan blue (Fig. 2C).

Determination by gel filtration of molecular mass in the BAL supernatant yielded a molecular weight of 660 kDa, but one of 1,000 kDa in the cell pellet lysate (Fig. 3), consistent with the 20S proteasome and 26S proteasome, respectively. Similar results were obtained for BZ-VGR-AMC and Suc-LLE-AMC. The purification table (Fig. 3) illustrates that Suc-LLVY-AMC enzyme activity of the eluate peaks (fractions 22–26) of the BAL supernatant was purified 2.49-fold by gel filtration and represents 11.4% of protein. This indicates a yield of 249%, possibly due to the elimination of an inhibitor.

Western blots of BAL supernatant using the primary polyclonal antibody against α/β-proteasomal subunits showed typical proteasomal protein bands in the 20- to 30-kDa range in eight subjects (Fig. 4, A and B). Of note, the Western blot showed a different arrangement of proteasomal protein bands in BAL supernatant and cell pellet.

**ESI-QqTOF** showed the presence of proteasome subunit α₃ (PSMA3), proteasome subunit α₄ (PSAM4), and proteasome subunit α₆ (PSMA6) (Table 1). Of note, peptides derived from 19S proteasomal caps (PSD3, PSD4, PSD11, PSD13) were exclusively found in the cell pellet lysate but not in the BAL supernatant. In addition, we found in BAL supernatant heat shock protein 90 (HSP 90), HSP70, and chaperonin containing TCP-1. Two of the protein spots could not be specified because signals could not be related to known proteins in the data base.

To test for proteasomal albumin degradation and for its ATP and ubiquitin dependency, BAL supernatants were incubated with 125I-albumin in subsequent experiments. As shown in Fig. 5, albumin degradation by BAL supernatant was present, was not ubiquitin or ATP dependent, and was inhibited (P = 0.013) by epoxomicin. Albumin degradation in BAL supernatant averaged 101.8 ± 46 μg/ml lavage/hour. The total protein concentration in the BAL supernatant ranged from 100 to 300 μg/ml, with an average albumin concentration of 41.5 ± 20 μg/ml.

**DISCUSSION**

These findings indicate that 20S proteasome is present physiologically in the extracellular alveolar space in humans, as shown by hydrolysis of specific proteasomal fluorogenic substrates in BAL supernatant, its inhibition by the highly specific proteasome inhibitor epoxomicin as well as by other specific proteasome inhibitors, absence of inhibition by lysosomal inhibitor E64, the presence of proteasomal proteins as revealed by Western blots and mass spectrometry, and a molecular mass of 660 kDa, consistent with 20S proteasome (66). Furthermore, 125I-albumin was degraded in an ATP- and ubiquitin-independent manner, and this was also inhibited by epoxomicin.
Fig. 1. A and B: hydrolysis of proteasome-specific fluorogenic substrates in bronchoalveolar lavage (BAL) supernatant and cell pellet lysate from 8 humans undergoing BAL. All measurements were performed in duplicate and averaged for each subject. Data from 8 individual values are shown as box and whisker plots with the median, the upper quartile, the lower quartile, and the 2 extreme values of the data set displayed. Suc-LLVY-AMC, BZ-VGR-AMC and Suc-LLE-AMC-hydrolyzing activity without (open bars) and with 10μM epoxomicin (hatched columns) in BAL supernatant (A) and BAL cell pellet lysate (B). Hydrolyzing activity was present for all substrates in BAL supernatant and was significantly inhibited by epoxomicin.

C: fraction of total hydrolyzing activity in BAL supernatant and cell pellet. Data from 8 subjects. The main fraction of total hydrolyzing activity is present in the BAL supernatant. D: EC50 for epoxomicin inhibition of hydrolyzing activity in BAL supernatant was 210 nM for Suc-LLVY-AMC. Data from 8 subjects (means ± SD). E: hydrolysis of proteasome-specific fluorogenic substrates in BAL supernatant from 6 subjects without (open bars) and with 500μM E64 (hatched columns), pkat, Picokatal.
indicating that the extracellular proteasome found in the alveolus is also biologically active and can degrade albumin.

This questions several fundamental aspects of currently favored models of physiological proteasome localization and function. First, our data demonstrate for the first time that 20S proteasome is not only present extracellularly in the lung but also that it is enzymatically intact and active in the human alveolar space. Second, the 20S proteasome found degrades albumin, physiologically present in the alveolar space, with a high cleavage rate. Although the proteasome is known to degrade albumin, a feature used to describe tissue-related proteasomal activity (32), the idea that albumin is a physiological substrate of the proteasome in the extracellular alveolar space has not been addressed.

A 26S proteasome-mediated degradation of albumin in the BAL supernatant can be excluded because of the strict ATP and ubiquitin independency found. In fact, only the 19S caps of the 26S proteasome require ATP and ubiquitin for protein degradation (13, 32). Remarkably, the albumin cleavage rate in BAL supernatant found in vitro by the extracellular proteasome is greater than albumin degradation formerly assumed to occur in the alveolar space (7, 22–24, 26, 42). Possibly, differences in radiolabeling techniques of albumin account for this difference. We used, for stable proteolysis, a chloramin T technique (62) with subsequent acetylation (67) and methylation of albumin (29) to allow more reliable and more stable measurements of proteasomal albumin degradation (29). In any case, 125I-albumin degradation by other catalytic enzymes of the extracellular alveolar space can be excluded because epoxomicin, a highly specific proteasome inhibitor, significantly inhibited albumin degradation in BAL supernatant.

Extracellular proteasome in the alveolar space by degrading albumin might be involved in the maintenance of a low intra-alveolar oncotic pressure. In fact, due to the high proteasome-dependent albumin cleavage rate observed, alveolar albumin load could increase 2.45-fold before degradation capacity would be exhausted. Considering that a BAL sample represents only 1.5–3% of the alveolar space (27) and that recovery of BAL fluid from the lung averaged 81 ml, albumin degradation capacity measured in BAL supernatant is substantial quantitatively. Of note, BAL of a lung segment is believed to represent 1 ml of epithelial lining fluid (64). Thus proteasome activity in the alveolar epithelial lining fluid in vivo might be many times greater than that measured in the BAL supernatant in our subjects.

Fig. 2. Correlation of Suc-LLVY-AMC hydrolyzing activity in BAL supernatant with markers of cell damage. There are no correlations between Suc-LLVY-AMC hydrolyzing activity in the BAL supernatant and lactate dehydrogenase (LDH) activity (8 subjects) in BAL supernatant (A), the cell count (8 subjects) in BAL cell pellet (B), and the fraction of cells in the cell pellet taking up trypan blue (C; 5 subjects). Centrifugation of blood (8 subjects) does not increase Suc-LLVY-AMC enzyme activity in the supernatant (D). Box and whisker plots are shown.
Since alveolar albumin concentration can increase in lung diseases (47), it would be of interest to assess alveolar proteasomal albumin degradation in lung disease. Inflammatory acute and chronic lung disease where alveolar protein concentrations can increase dramatically would be of particular interest. Furthermore, since the alveolar space is an external surface, second only to the gastrointestinal tract in dealing with environmental antigens, it is conceivable that extracellular proteasome in the lung, besides albumin, degrades other alveolar proteins as well. In this respect, HSPs (11) and chaperonins (51, 52) also found in the alveolar space may assist the 20S proteasome in unfolding and digesting target proteins in the absence of 19S caps and the intracellular ubiquitin system.

We used for BAL in humans the gold standard technique clinically available for alveolar sampling. We did not use the technique described by Ware et al. (65), inserting blindly a catheter into the airways via an endotracheal tube, since this method yields only a small recovery in healthy subjects with-
out pulmonary edema. Thus our sampling technique employed yields the most reliable alveolar sample possible in humans.

Traditionally, the proteasomal system is considered to have intracellular functions, and the extracellular role of proteasomes has not yet been explored in the human lung. However, there is increasing evidence that proteasomes can exert physiological roles in the extracellular space, too. Of interest, extracellular proteasomal activity has been suggested for ascidian fertilization and sperm penetration of the viteline coat (55). In addition, proteasome and proteasome subunits have been detected in sera of patients with autoimmune disease (21) and were described as “circulating proteasome” (19-21, 60). Furthermore, particles of proteasomal structure have been detected on the cell surface of lymphocytes (12), and Vaithilingam et al. (61) described an extracellular proteasome-like structure released from cultured C6 astrocytoma cells with a molecular mass of 1,000 kDa, able to degrade collagen IV, β-casein, and synthetic proteasomal peptide substrates. In support of our findings, this suggests that human cells may secrete biologically active proteasomal structures.

Proteins larger than the 20S proteasome, such as 2-macroglobuline (820 kDa) and immunoglobulin M (900 kDa), are found in the BAL, too (10, 18, 33). The dimension of the 20S proteasome is 15 \times 12 \text{ nm} (37). Williams (68) found vesicles in the alveolar epithelium with radii as large as 130 nm. Thus transport mechanisms of adequate size are present for expulsion of proteasome from the alveolar epithelium. Alternatively, alveolar macrophages could release proteasome core proteins, but this has not been assessed.

Furthermore, the potential cellular source of the extracellular proteasome is unknown. The fact that the majority of activity was found in BAL supernatant rather than BAL cell pellet does not imply that 20S proteasome is brought into the extracellular space exclusively by resident cells of the alveolar space. In fact, data from Zoeger et al. (69) show in the blood proteasomal molecules that do not derive from platelets, red blood cells, or other blood-borne cells, suggesting that endothelial cells or

| Table 1. Analysis by ESI-QqTOF-mass spectrometry of proteins contained in peak Suc-LLVY-AMC eluate activity (gel filtration fractions 22–26) of BAL supernatant from one individual |
|-----------------|-----------------|-----------------|
| **BAL Supernatant** | **Amino Acid Sequence** | **Protein** |
| **Peptide mass/charging/[M + H]^+** | **Amino Acid Sequence** | **Protein** |
| 609,35 | ++ | 1217,70 | AVENSSTAIGIR | proteasome subunit α 3 |
| 504,28 | ++ | 1007,56 | TTIFSPERG | proteasome subunit α 4 |
| 643,39 | ++ | 1285,78 | AINQGGLTSVAVR | proteasome subunit α 6 |

![Fig. 5. Albumin proteolysis in BAL supernatant. Data from 8 individuals are shown. Box and whisker plots are used. Albumin proteolysis was present in BAL supernatant and could be significantly inhibited by 10 \text{ uM} epoxomicin. Albumin degradation was ATP and ubiquitin independent.](http://ajplung.physiology.org/ by 10.220.33.6 on September 20, 2017)
other organs may secrete proteasome. Thus, extracellular alveolar proteasome could also derive from the blood stream.

In our study, we exclusively found 20S proteasome in the extracellular alveolar space, but not 26S proteasome, as indicated by gel filtration experiments and molecular weight determination, and the ATP/ubiquitin independence. The 20S proteasome is a smaller protein complex than the 26S proteasome. Possibly, it is easier for cells to transfer into the extracellular space a 660-kDa protein than a 1,000-kDa protein. Furthermore, intracellular protein degradation via the 20S proteasome system requires a very complex enzyme cascade (E1–E4) including ubiquitylation and deubiquitylation and the presence of 19S proteasomal caps. Teleologically, it is not plausible how the alveolar space would set up and maintain such a complex and regulated enzyme system outside the cell. Instead, HSPs including ubiquitylation and deubiquitylation and the presence of more, intracellular protein degradation via the 26S proteasome is a smaller protein complex than the 26S proteasome.

Possibly, it is easier for cells to transfer into the extracellular space a 660-kDa protein than a 1,000-kDa protein. Furthermore, our data showed protein degradation via the 20S proteasome independent of ATP and ubiquitin, without the presence of 19S caps required. This is consistent with the general principle that extracellular enzymes have to function without ATP because of its absence extracellularly.

Our findings also show that there is more extracellular alveolar machinery for protein degradation than previously known. Because of its ubiquitin independence, the extracellular proteasome described in this study may adopt a clearance function for proteins independent of the complex regulation described for the intracellular proteasome.

Furthermore, since proteasomal degradation is known to result in oligopeptides (13), the extracellular alveolar proteasome might be involved in extracellular antigen processing before antigens are presented to immune cells. However, it cannot be excluded that the alveolar proteasome besides protein degradation has other functions unrelated to protein digestion. In this context, the concept of moonlighting proteins, i.e., proteins that have completely different functions depending on their presence in different intracellular and extracellular compartments, is an interesting approach (8, 9, 15, 30, 31, 35, 46, 57).

In summary, biologically active 20S proteasome is physiologically present and functional in the extracellular human alveolar space and can degrade albumin. Its multifunctional character and substantial albumin cleavage rate suggest that it has a biological role in alveolar protein degradation.

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