DETECTION AND POSSIBLE ROLE OF PROTEASOMES IN THE BRONCHOALVEOLAR SPACE OF THE INJURED LUNG

Short title: **Proteasomes in the alveolar space of the injured lung**

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Summary:

Recent observations suggest the presence of 20S proteasomes (20S) in the lung epithelial lining fluid. However, the physiological relevance of 20S in the alveolar space and possible contribution to disease processes are unknown. Thus, we evaluated whether extracellular proteasomes could have a pathophysiological role in the injured lung using a rat model of lung contusion (LC). Bronchoalveolar lavage fluids (BALF) were obtained at various time points for up to 168h after LC or sham procedure. Enzyme activities, ELISA and western blots indicated enzymatically active 20S, the 19S subunit Rpt5 and ubiquitin in BALF. 20S and ubiquitin increased significantly after LC, peaked at 24h and normalized within 168h. Mg^{2+}/ATP-dependent peptidase activities were detectable 6-24h after LC. BALF after LC also contained ubiquitin-protein-ligase activity. Addition of Mg^{2+}/ATP to BALF after LC led to significant proteolysis and could be prevented with epoxomicin and EDTA. These data suggest for the first time that the Mg^{2+}/ATP-dependent 26S proteasome complex exists outside the cell, is released into the lung epithelial lining fluid after LC and contributes to the proteolysis of the bulk of protein in the alveolar space of the injured lung. We infer that proteasome complexes may have a pathophysiological role during lung edema clearance.

Key words:

26S proteasome, 20S core particle, 19S regulator complex, ubiquitin, ubiquitin-protein ligase

Introduction:

The ubiquitin-proteasome-pathway (UPP) is the principal non-lysosomal proteolytic system in all eukaryotic cells. In this pathway, ubiquitin-protein ligase systems catalyze the covalent ligation of ubiquitin to intracellular proteins (Hershko and Ciechanover 1998). The ubiquitylated protein is then destined for degradation by the multicatalytic 26S proteasome (26S). The 26S is formed from a cylinder-shaped multimeric protein complex referred to as the 20S proteasome core particle (20S) and singly or doubly capped by a regulatory component termed the 19S regulator complex (19S), which confers Mg^{2+}/ATP-dependency and ubiquitylated substrate specificity. While Mg^{2+}/ATP is required for assembly and function of the 26S complex, the 20S alone is involved in the degradation of misfolded and damaged proteins, independent of Mg^{2+}/ATP or ubiquitylation (Hershko and Ciechanover 1998, Baumeister *et al.* 1998).

Some of the protein components of the UPP have also been identified in extracellular fluids, such as serum, cerebrospinal, epididymal or bronchoalveolar fluids (BALF) (Wada *et al.* 1993, Magi *et al.* 2005, Majetschak *et al.* 2003 and 2005a, Zoeger *et al.* 2006, Bai *et al.* 2007, Baska *et al.* 2007, Sixt *et al.* 2007). While previous data suggest a pathophysiological role of free extracellular ubiquitin during inflammation (Kieffer *et al.* 2003, Majetschak *et al.* 2003 and 2005b), the functional relevance of extracellular proteasomes, their natural protein substrates and possible contribution to disease processes are unknown.

As compared with healthy volunteers, circulating levels of ubiquitin and 20S are significantly elevated in severely injured patients (Majetschak *et al.* 2003, Roth *et al.* 2005), but their possible alterations and function in the alveolar space of the injured lung have not been studied. Thus, using a well described model of lung contusion (LC) in rats, we evaluated whether increased release of UPP components into the alveolar space occurs after trauma and whether those components could play a pathophysiological role in the injured lung.

Methods:

Animal protocol. All procedures were performed according to NIH Guidelines for the Use of Laboratory Animals and approved by the IACUC. LC was induced using a blast wave model, as described in detail previously (Jaffin *et al.* 1987, Knöferl *et al.* 2003). In brief, anesthetized (3% servoflurane (Abbott, Wiesbaden, Germany), 97% oxygen, flow 0.8 L/min) and spontaneously breathing male Sprague Dawley rats (n=50, 250-275 g, Charles River, Sulzfeld, Germany) were placed on an acrylic glass plate in supine position. A single blast wave (exposure distance: 2 cm, peak pressure: 0.73 bar, duration: 3.4 ms) centered on the thorax (n=25) was delivered using a blast wave generator (Jaffin *et al.* 1987). With this non-lethal model a bilateral LC is induced without additional injury to abdominal organs or bony structures of the chest (Knöferl *et al.* 2003). Sham animals (n=25) underwent the same procedures but no blast wave was delivered. Animals were allowed to recover from anesthesia and then brought back to their cages with free access to food and water. To collect BALF, each five sham and LC animals were sacrificed by exsanguination (aortal puncture) under general anesthesia after 6h, 12h, 24h, 48h and 168h. The trachea was cannulated and lungs were lavaged twice with 5 mL of ice cold PBS. The recovery was 3-4 mL per lavage. BALF were pooled, centrifuged (16,000×g, 4°C, 2 min) and supernatants stored at -80°C until analyses.

Ubiquitin ELISA. Ubiquitin concentrations were quantified with a competitive ELISA, as described in detail previously (Majetschak *et al.* 2005a). Addition of purified hemoglobin to the assay did not affect measurements. The lower detection limit was 11 ng/mL.

20S ELISA. 20S were quantified by ELISA, as described (Majetschak *et al.* 2008). The correlation coefficients for each standard curve were 0.98–1. The lower detection limit was 15 ng/mL.

Peptidase assays. Peptidase activities were measured employing the fluorogenic peptide substrates *N*-Suc-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (chymotryptic-like, CT-L) and Bz-Val-Gly-Arg-7-amino-4-methylcoumarin (tryptic-like, T-L; both from Biomol), as described

(Majetschak *et al.* 2008). Reaction mixtures contained 1 mM DTE, 2 mM ATP, 5 mM $MgCl_2$, 10 mM Tris/HCl, pH 8.0, 100 μM peptide substrate and 20 μL BALF. Mixtures were incubated for 60 min at 37ºC. Ethanol (2:1 v/v) was added, mixtures placed on ice for 10 min and centrifuged (16000 g, 5ºC, 6 min). Supernatants were transferred into microplates (Corning, Acton, MA) and free 7-amino-4-methylcoumarin cleaved from the substrates measured in a fluorescence reader (FLX8000, Bio-Tek, Woburn, MA, $\lambda_{excitation/emission}$=360/455nm) against standard curves of 7-amino-4-methylcoumarin (Sigma). To differentiate the proteasome from other peptidase activities, the Mg^{2+}/ATP-dependent and epoxomicin-sensitive proportions were determined by addition of 10 mM EDTA and 7 μM epoxomicin (Biomol) to the mixtures (Eytan *et al.* 1993, Meng *et al.* 1999). Proteasome peptidase activities were determined as mol of 7-amino-4-methylcoumarin cleaved per h and mL.

Western blots. Immunoblotting to ubiquitin and proteasome subunits was performed as described previously (Majetschak et al. 2003, 2005a and 2008). In brief, BALF proteins were separated by SDS-PAGE (4-15% gels, Bio-Rad, Hercules, Ca), transferred to nitrocellulose membranes (Hybond-ECL, Amersham Biosciences) and probed with anti-ubiquitin (1:800, Sigma, St. Louis, MO) and a HRP-labeled secondary antibody (1:10000, Amersham Biosciences). Visualization and quantification of immunoreactive proteins were performed with chemiluminescence detection (Super-Signal, Pierce, Rockford, IL) using the ImageMaster system with the ImageQuant analysis software (Amersham Biosciences). For immunoblotting to proteasome subunits anti-20S "core" which has been shown to react with the 20S subunits α5, α7, β1, β5, β5i, β7 (1:2000; Biomol, Plymouth Meeting, PA), anti-20S subunit β3 and anti-19S ATPase subunit Rpt5 (both 1:1000; Biomol) were used in combination with corresponding secondary HRP-labeled anti-mouse (Pierce) and anti-rabbit (Amersham), accordingly.

Ubiquitylation assay. Ubiquitin protein ligase activity was assessed as incorporation of $ubiquitin_b$ into the sum of BALF proteins, as described (Majetschak *et al.* 2008). Incubation mixtures contained 1 mM DTE, 0 or 2 mM ATP, 0 or 5 mM $MgCl_2$, 10 mM Tris/HCl, 10 μg/mL $ubiquitin_b$

and 25 μL BALF at pH 8. Mixtures were incubated for 60 min at 37°C. After incubation, Laemmli sample buffer (50%v/v) was added and the mixtures were boiled for 5 min, and then separated by SDS-PAGE. Immunoblotting was performed using a goat anti-biotin antibody conjugated to horseradish peroxidase (Sigma), as described.

ATP assay. ATP concentrations were determined with recombinant firefly luciferase and its substrate D-luciferin using a commercially available assay kit (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. The lower detection limit was 15 nM.

Detection of hemolysis. Oxyhemoglobin and bilirubin levels were estimated spectrophotometrically according to (Cruickshank 2001, Majetschak *et al.* 2005a). For the detection of oxyhemoglobin absorption (A) was measured at 414 nm. Bilirubin absorption was measured at 453 nm and 578 nm. Bilirubin concentration (mM) was calculated as (A 453 nm–A 578 nm) x 23, where 578 nm is the wavelength at which oxyhemoglobin has identical absorbance to its absorbance at 453 nm, and 23 is the extinction coefficient for bilirubin at 453 nm (Cruickshank 2001).

Other methods. For trichloroacetic acid (TCA) precipitation of proteins, samples were mixed 1:1 (volume/volume) with 10% TCA, placed on ice for 30 min and centrifuged (16,000 g, 20 min). The pellet was resuspended in 0.1N NaOH, 1% SDS. Protein was measured using a commercially available assay kit (Bio-Rad). SDS-gels were stained with Coomassie blue (Bio-Rad) and analyzed using the ImageMaster system and ImageQuant analysis software. After images were captured, pixel densities of each lane were plotted against the Rf ((distance of protein migration)/(distance of tracking dye migration)) value. Spline curves were calculated, normalized and plotted. For each gel the corresponding molecular masses were calculated using the Rf-values of protein standards (Precision Plus, Bio-Rad).

Statistics: Data are described as the mean±SEM. Paired and independent samples t-test and Spearman correlation coefficients (r_s) were calculated with the SPSS-program (Chicago, IL). Spline curves were calculated with the GraphPad-Prism-program (GraphPad-Software, San Diego, CA). A

two-tailed $p<0.05$ was considered significant.

Results:

Protein content averaged 1.3 ± 0.16 mg/mL in BALF from control animals and did not show significant alterations during the observation period. Protein concentrations in BALF were significantly elevated between 6h-48h after LC (average: 4.6 ± 0.5 mg/mL) and returned to control values after 168h (0.8 ± 0.2 mg/mL; Fig.1A). There was noticeable hemolysis in BALF after LC. Oxyhemoglobin and bilirubin levels increased with peak levels at 24-48h and normalized within 168h (Fig.1B/C). Ubiquitin was detectable in 40% of BALF after sham procedure and in all BALF after LC. 20S protein was detectable in 83% and 90% of BALF from controls and injured animals, respectively. Compared with BALF from control animals at corresponding time points, 20S and ubiquitin concentrations increased 5-10-*fold* in BALF after LC (Fig. 1 D/E). 20S and ubiquitin peaked at 24h after LC (20S: 2397 ± 71 ng/mL; ubiquitin: 469 ± 124 ng/mL) and returned to control levels at 168h (20S: 42 ± 30 ng/mL; ubiquitin: 31 ± 6 ng/mL). Western blots confirmed these findings (Fig.1F) and showed that the signals for ubiquitin (lanes 1 and 2), 20S "core" subunits (lanes 3 and 4) and 20S subunit β3 (lanes 5 and 6) were stronger in BALF 24h after LC than after sham procedure. BALF probed also positive for the 19S ATPase subunit Rpt5, with stronger signals in BALF after LC (lanes 7 and 8).

After sham procedure, epoxomicin sensitive chymotryptic-like activities were detectable in 96% and epoxomicin sensitive tryptic-like activities in 28% of all specimens, and in 100% and 60% after LC, respectively. Changes in epoxomicin sensitive chymotryptic- and tryptic-like peptidase activities paralleled changes in 20S protein content (Fig. 2A/B). Compared with sham controls, epoxomicin sensitive chymotryptic-like activities were 22-*fold* and tryptic-like activities 79-*fold* elevated in BALF obtained 24h after LC. Ubiquitin and 20S concentrations as well as chymotryptic- and tryptic-like proteasome activities correlated significantly positive with oxyhemoglobin and

bilirubin levels (Tab.1).

Since the immunoblotting experiments suggested that 19S could also be present in BALF, we tested for ATP/Mg^{2+}-dependence of the peptidase activities to discriminate the ATP/Mg^{2+} dependent 26S complex. ATP-dependent activity was not detectable in BALF after sham procedure. In contrast, peptidase activities in BALF that was obtained between 6-24h after CT were higher when assayed in the presence of ATP/Mg^{2+} than in the presence of ATP/Mg^{2+} and EDTA (Fig. 3A/C). The ATP-activity ratio (activities in the presence of ATP/Mg^{2+}/activity in the presence of ATP/Mg^{2+} and EDTA) of the individual samples reached 2.6 ± 0.1 (chymotryptic-like, Fig. 3B) and 2.8 ± 0.3 (tryptic-like, Fig. 3D) at 12h after LC and returned to ATP independency (ATP ratio≈1) at later time points.

We further explored the possibility that ubiquitin protein ligase activity is detectable in BALF after LC. As shown in Fig. 4A, incubation of BALF with $ubiquitin_b$ led to newly formed $ubiquitin_b$ protein conjugates (Fig. 4A, arrows a/b) and ubiquitylation was enhanced in the presence of ATP/Mg^{2+} (pixel density: arrow a: lane 1 - 342, lane 2 - 1988, lane 3 – 2791; arrow b: lane 1 – 554, lane 2 – 606, lane 3 – 1214).

ATP was not detectable in any of the BALF (not shown). The TCA precipitated protein content remained constant when BALF obtained 12-48h after LC were incubated for 15h at 37ºC and decreased by 46 ± 3% ($p<0.001$) when specimens were supplemented with ATP/Mg^{2+} (Fig. 4B). The average BALF protein concentration in these specimens was 5.8 mg/mL and the degradation rate in the presence of ATP/Mg^{2+} 125 ± 19 μg/mL/h. ATP/Mg^{2+} induced proteolysis could be inhibited by EDTA and epoxomicin. Supplementation of BALF with ATP/Mg^{2+} and ubiquitin did not increase ATP/Mg^{2+} induced protein degradation.

To obtain initial information on the natural substrates of the ATP/Mg^{2+} dependent proteolytic activity, BALF were incubated as described before, equal protein amounts were separated by SDS-PAGE and the molecular mass profiles were analyzed. Fig. 4C shows a typical Coomassie stained

SDS-gel and Fig. 4D the average molecular mass profiles from three independent experiments. The protein pattern shifted towards the lower molecular mass range after incubation without exogenous ATP/Mg^{2+} (Fig. 4C, lanes 2 and 3). Compared with BALF that were incubated without ATP/Mg^{2+} (Fig. 4D, black line), addition of ATP/Mg^{2+} led to a decrease of proteins in the range of 37-100 kDa and an increase in low molecular mass proteins (≤ 10 kDa; Fig. 4C, lane 4; Fig. 4D, red line). This could be prevented with epoxomicin (Fig. 4C, lane 6; Fig. 4D, green line). Addition of EDTA (Fig. 4C, lane 5; Fig. 4D, blue line) also prevented ATP/Mg^{2+} induced changes. As compared with BALF incubated with and without Mg^{2+}/ATP, it resulted in a protein pattern that resembled the protein pattern in BALF pre-incubation more closely.

Discussion:

In the present study we show for the first time that UPP components are released into the bronchoalveolar space of the injured lung and provide initial experimental evidence for a functional role of extracellular proteasome complexes. First, we detected significantly increased concentrations of ubiquitin and 20S in BALF after LC. Second, BALF probed positive for the 19S ATPase subunit Rpt5. Third, ATP/Mg^{2+}-dependent and epoxomicin sensitive peptidase activities were present in BALF after LC. Fourth, addition of ATP/Mg^{2+} to BALF obtained after LC induced proteolysis of endogenous BALF proteins. Fifths, ATP/Mg^{2+} induced proteolysis in BALF could be inhibited by EDTA and by the specific proteasome inhibitor epoxomicin.

The ELISA measurements, western blots and peptidase activity measurements confirmed the presence of ubiquitin and enzymatically active proteasomes in BALF (Magi *et al.* 2005, Bai *et al.* 2007, Sixt *et al.* 2007) and showed consistently that their concentrations in BALF are significantly increased after LC. As expected, BALF protein content was also significantly elevated after LC, indicating that the blast wave injury induced relevant post-traumatic capillary leakage. The finding that ubiquitin and 20S levels did not parallel total BALF protein content after CT argues against their

origin from the systemic circulation. Their positive correlations with parameters of hemolysis point towards erythrocytes as one cellular origin after LC.

It has been shown previously that the 19S regulator complex can be dissociated into lid- and base- sub-complexes and intact 19S lid sub-complexes have been isolated from erythrocytes, thus suggesting that pre-assembled 19S sub-complexes exist in a free form (Glickman *et al.* 1998, Henke *et al.* 1999, Braun *et al.* 1999). Furthermore, it was shown that 19S and 20S can be released from 26S proteasomes by ATP depletion and that re-addition of ATP leads to the reformation of 26S proteasomes (Eytan *et al.* 1993, Peters *et al.* 1994). These data along with the findings from the present study suggest that 26S complexes re-assemble in BALF obtained after LC if ATP/Mg^{2+} is present.

Although the 19S ATPase subunit Rpt5 was also detectable in BALF after sham procedure, we were unable to demonstrate ATP/Mg^{2+} dependent peptidase activities in these BALF specimens. Similarly, Sixt et al. (2007) could also not detect ATP/Mg^{2+}- dependent proteolysis of chemically modified and denatured bovine serum albumin in BALF from patients without lung pathologies. There are several possible explanations for these observations. Besides the possibility that ATP/Mg^{2+}-dependent enzyme activities in BALF from patients without lung pathologies (Sixt *et al.* 2007) or in BALF obtained after sham procedure in the present study were simply below the detection limit of the assays, artificial test substrates may not accurately reflect the enzymatic properties of proteasome complexes for natural protein substrates. On the other hand, the volume of the lung epithelial lining fluid is extremely small when compared with the volume of the BALF, the recovery of its chemical components may vary largely, and ectonucleotidases and their soluble forms are widely distributed (Stephens *et al.* 1996, Zimmermann 2000). Thus, it was not surprising that ATP concentrations in BALF were below the detection limit. As a consequence, 19S and 20S would be released from 26S complexes in BALF (Eytan *et al.* 1993) and it is conceivable that re-assembly of 26S complexes requires additional protein factors which may not reach sufficient concentrations in BALF from sham

controls, such as Hsp90 or Ecm29 (Imai *et al.* 2003, Gorbea *et al.* 2004). The finding that Hsp90 has already been identified in BALF strengthens this hypothesis (Sixt *et al.* 2007).

Nevertheless, it is well established that physiologically relevant concentrations of ATP are released into the extracellular space under various conditions, including tissue damage, low oxygen tension or inflammation (Kerkweg and de Groot 2005, Idzko *et al.* 2007, Sprague *et al.* 2007). Thus, the results of the present study imply the presence of ATP/Mg^{2+} dependent 26S proteasomes in the alveolar space of the injured lung.

Incubation of BALF in the absence of ATP/Mg^{2+} led to a shift of the molecular mass distribution of BALF proteins towards the lower molecular mass range while protein amounts after TCA precipitation remained constant. This finding is consistent with spontaneous and non-proteasomal breakdown of proteins into larger fragments without processing of oligopeptides, which are known to be produced by the proteasome (Greenberg and Shipe 1978, Hershko and Ciechanover 1998). The detection of an ATP/Mg^{2+}-dependent decrease in TCA precipitated BALF protein content, the proteolytic cleavage rate for proteins with molecular masses between 37-100 kDa and the findings that EDTA and epoxomicin consistently abolished ATP/Mg^{2+} induced proteolysis in BALF quantitatively and prevented the ATP/Mg^{2+} induced shift of BALF proteins towards the lower molecular mass range, strongly suggest the presence and a functional role of 26S complexes in the alveolar space of the injured lung. In addition, it should be noted that the protein pattern in BALF after incubation with 2 mM ATP, 5 mM Mg^{2+}, 10 mM EDTA was more similar to its protein pattern pre-incubation than the resulting protein pattern after incubation without addition of ATP, Mg^{2+} and EDTA. This observation could point towards neutralization of the effects of divalent metal ions on protein structure and thermal stability by EDTA (Sissi et al. 2005).

Along with the detection of ubiquitin protein ligase activity in BALF after LC the findings of the resent study open up the possibility that the entire UPP could be involved in processing of proteins in the alveolar space. The observation that addition of ubiquitin did not increase the ATP/Mg^{2+}-

dependent proteolytic activity is not contradictive since the concentrations of endogenous ubiquitin in the incubation mixtures were at least 4-*fold* above the *K*m for ubiquitin protein ligase systems (Majetschak *et al.* 1998).

Taken together, the present study provides further evidence for the novel concept that the UPP exists in the extracellular space (Baska *et al.* 2007). Our data suggest for the first time that 26S proteasome complexes are present outside the cell and contribute significantly to the degradation of the bulk of endogenous proteins in the alveolar space of the injured lung. Based on these findings, we conclude a pathophysiologial role of proteasomes during lung edema clearance. Nevertheless, the clinical relevance of these findings remains to be determined since proteasome release into the alveolar space could also be harmful if proteins such as surfactant proteins are among its natural substrates.

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Table 1:

Correlation of 20S and ubiquitin levels and proteasome peptidase activities with oxyhemoglobin and bilirubin levels in BALF.

r_s(p)	**Oxyhemoglobin** (OD 414 nm)	**Bilirubin** (mM)
20S proteasome (ng/mL)	0.668(<0.001)	0.680(<0.001)
Ubiquitin (ng/mL)	0.813(<0.001)	0.368(0.017)
CT-L (pmol/h/mL)	0.768(<0.001)	0.413(0.006)
T-L (pmol/h/mL)	0.634(<0.001)	0.384(0.012)

r_s: Speaman correlation coefficient. p: level of statistical significance. CT-L: Chymotryptic-like proteasome peptidase activity (epoxomicin sensitive proportion). T-L: Tryptic-like proteasome peptidase activity (epoxomicin sensitive proportion).

Figure Legends:

Figure 1: BALF concentrations of 20S proteasome core particles (20S) and ubiquitin are increased in BALF after lung contusion (LC). Measurements in BALF (n = 5 per group and time point). Data are mean ± SEM. Open bars: Sham. Grey bars: LC. *:$p<0.05$ vs. sham. **A.** Protein (mg/mL). **B.** Oxyhemoglobin (OD 414 nm). **C.** Bilirubin (mM). **D.** 20S (ng/mL). **E.** Ubiquitin (ng/mL). **F.** Western blotting to ubiquitin (lanes 1/2), 20S "core" subunits α5,7,β1,5,5i,7 (lanes 3 and 4), 20S subunit β3 (lanes 5 and 6) and 19S subunit Rpt5 (lanes 7 and 8). Each lane contains 20µl of BALF. Lanes 1, 3, 5 and 7: Sham, 24h. Lanes 2, 4, 6 and 8: LC, 24h. Left: Migration positions of molecular mass standards.

Figure 2: Proteasome peptidase activities are increased in BALF after lung contusion (LC). Chymotryptic-like (CT-L; **A.**) and tryptic-like (T-L; **B.**) proteasome peptidase activities in BALF (epoxomicin sensitive proportions; n=5 per group and time point). Open bars: Sham; Grey bars: LC. Data are mean ± SEM. *:$p<0.05$ vs. sham.

Figure 3: ATP/Mg^{2+} dependent peptidase activities are detectable in BALF after lung contusion (LC). **A.** and **C**: Chymotryptic-like (CT-L; **A.**) and tryptic-like (**C.**) peptidase activities measured with (ATP/Mg^{2+} +, EDTA -) and without ATP/Mg^{2+} (ATP/Mg^{2+} +, EDTA +). N = 5 per group and time point. Open bars: Sham. Grey bars: LC. Data are mean ± SEM. *:$p<0.05$ vs. measurements in the absence of ATP/Mg^{2+}. **B.** and **D**: ATP-activity ratios (activities in the presence of ATP/Mg^{2+}/activity in the presence of ATP/Mg^{2+} and EDTA) of the chymotryptic-like (**B.**) and tryptic-like (**D.**) peptidase activities measured in the individual BALF after LC.

Figure 4: **A.** Detection of ubiquitin protein ligase activity in BALF 24h after lung contusion (LC). Left: Migration position of molecular mass standards. Lane 1: Incubation 0 min, no ATP/Mg^{2+}. Lane 2: Incubation 60 min, no ATP/Mg^{2+}. Lane 3: Incubation 60 min, with 2 mM ATP/ 5 mM Mg^{2+}. The arrows mark newly formed $ubiquitin_b$-protein conjugates. **B.** ATP/Mg^{2+} supplementation of BALF obtained after LC induces proteolysis of natural BALF proteins that can be inhibited by the specific proteasome inhibitor epoxomicin. TCA-precipitated protein content in BALF incubated for 15h at 37ºC with or without 2 mM ATP/ 5 mM Mg^{2+} (ATP/Mg^{2+}), ubiquitin (1 μg/mL), epoxomicin (7 μM) and EDTA (10 mM). Protein content is expressed as %pre-incubation. BALF were obtained 12-48h after CT. Data are mean ± SEM. *:$p<0.05$ vs. pre-incubation. The number of experiments (n) and incubation conditions are shown. **C.** Typical Coomassie stained SDS-gel with BALF obtained 24h after LC incubated as described in B. Lanes 2-6 contain 20 μg of protein. Lanes 1-2 and 3-7 were run on separate gels. Lanes 1/7: Protein standards. Lane 2: BALF, no incubation. Lanes 3-6: same BALF as in lane 2 after 15h of incubation at 37ºC with or without 2 mM ATP/ 5 mM Mg^{2+} (ATP/Mg^{2+}), epoxomicin (7 μM) and EDTA (10 mM). **D.** Molecular mass profiles of BALF proteins incubated as described in B/C. The lines represent mean pixel densities from three independent experiments. The abscissa shows Rf values and corresponding molecular masses.

Fig. 1

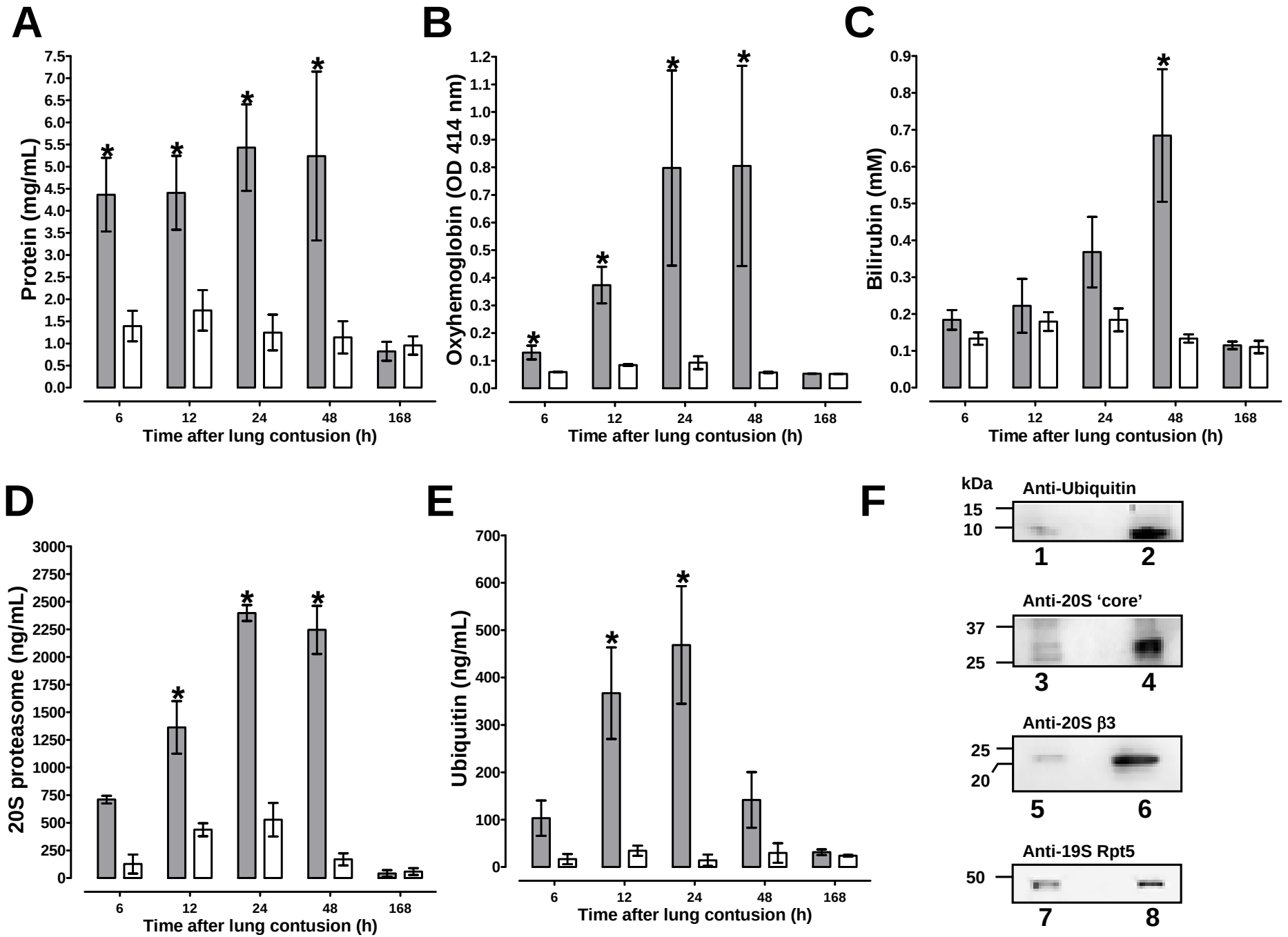

A
Protein (mg/mL)
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
6
12
24
48
168
Time after lung contusion (h)
B
Oxyhemoglobin (OD 414 nm)
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
6
12
24
48
168
Time after lung contusion (h)
C
Bilirubin (mM)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
6
12
24
48
168
Time after lung contusion (h)
D
20S proteasome (ng/mL)
3000
2750
2500
2250
2000
1750
1500
1250
1000
750
500
250
0
6
12
24
48
168
Time after lung contusion (h)
E
Ubiquitin (ng/mL)
700
600
500
400
300
200
100
0
6
12
24
48
168
Time after lung contusion (h)
F
kDa
Anti-Ubiquitin
15
10
1
2
Anti-20S 'core'
37
25
3
4
Anti-20S β3
25
20
5
6
Anti-19S Rpt5
50
7
8

Fig. 2

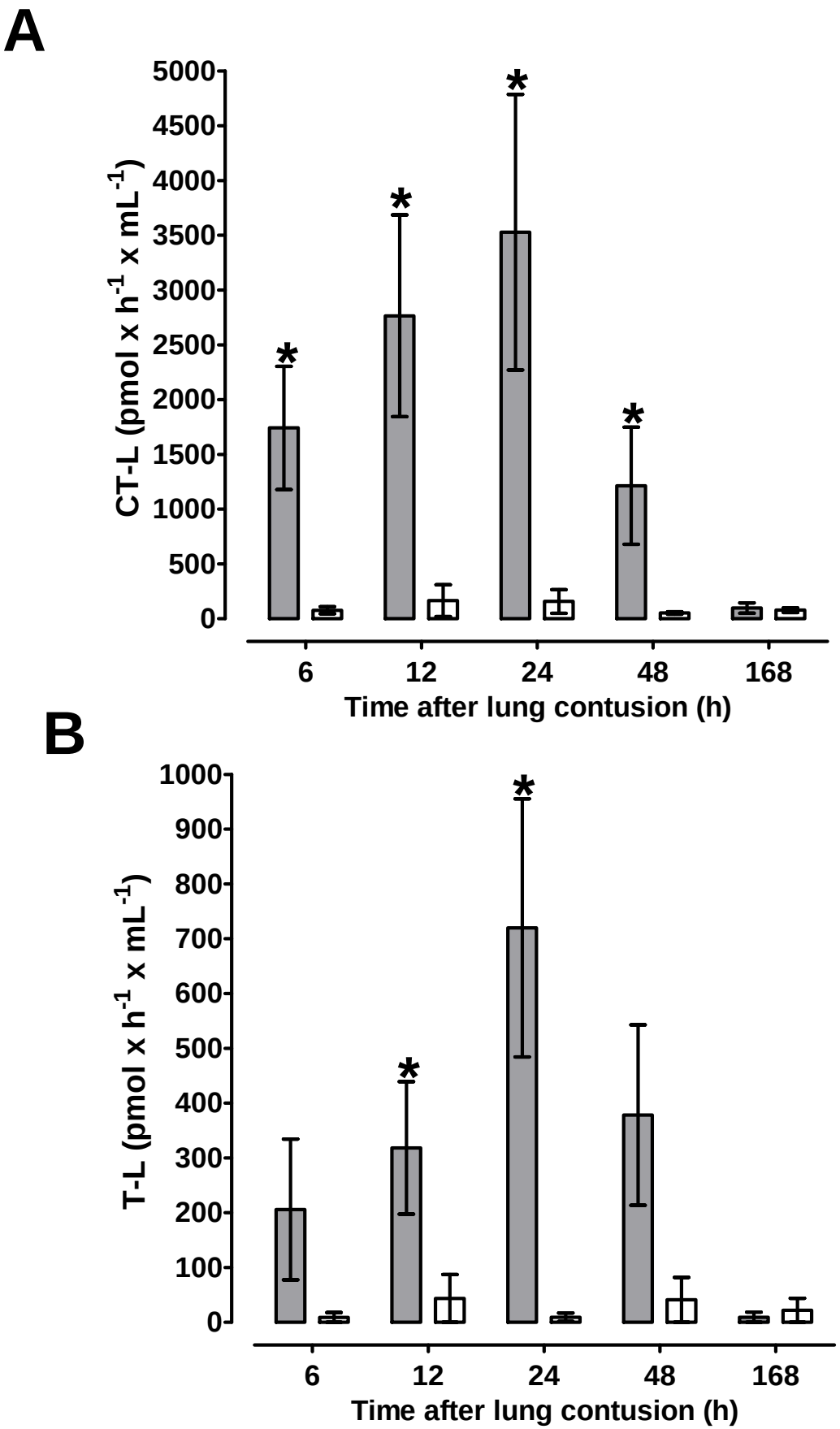

A
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
CT-L (pmol x h-1 x mL-1)
*
*
*
*
6
12
24
48
168
Time after lung contusion (h)
B
1000
900
800
700
600
500
400
300
200
100
0
T-L (pmol x h-1 x mL-1)
*
*
6
12
24
48
168
Time after lung contusion (h)

Fig. 3

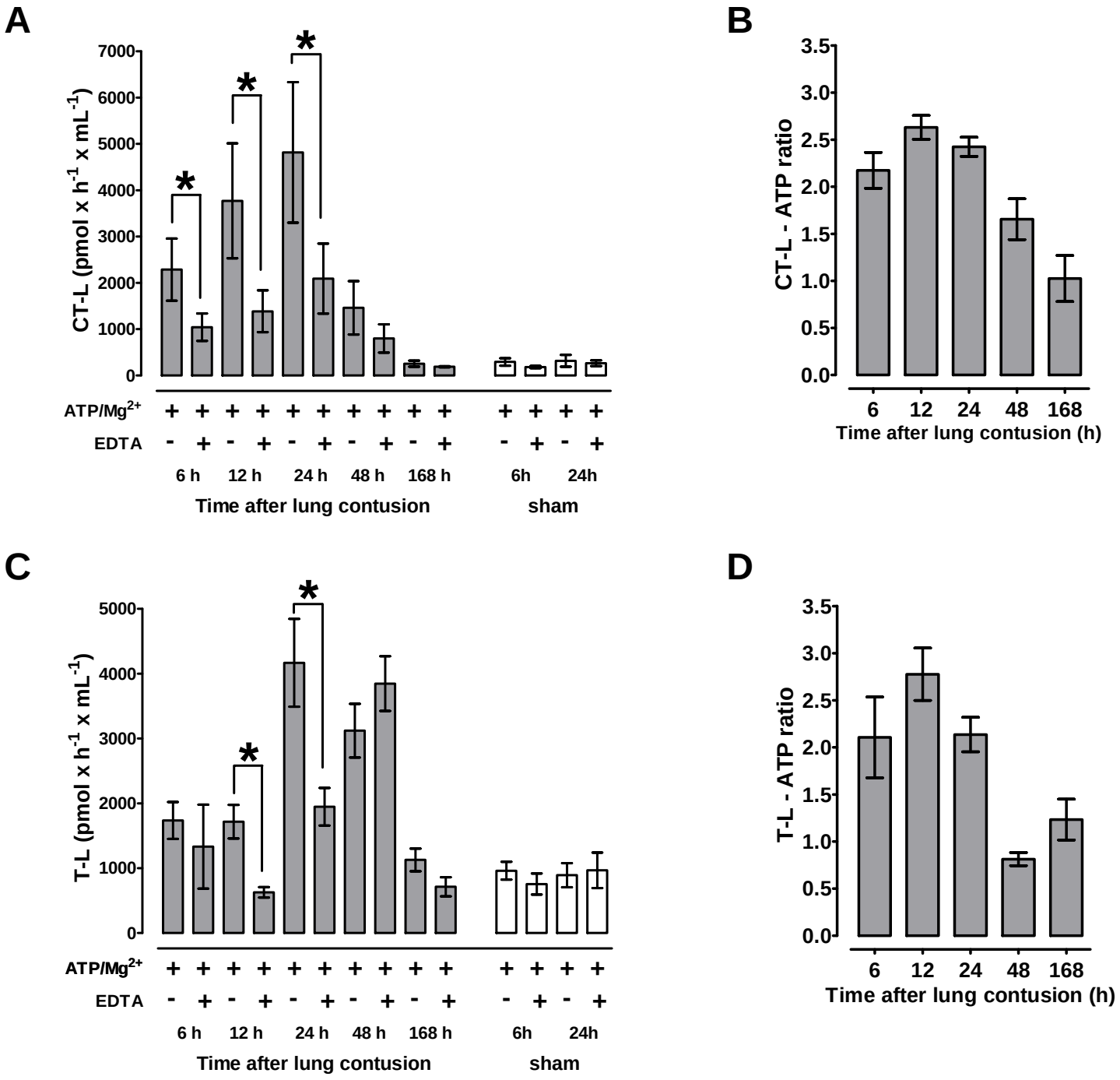
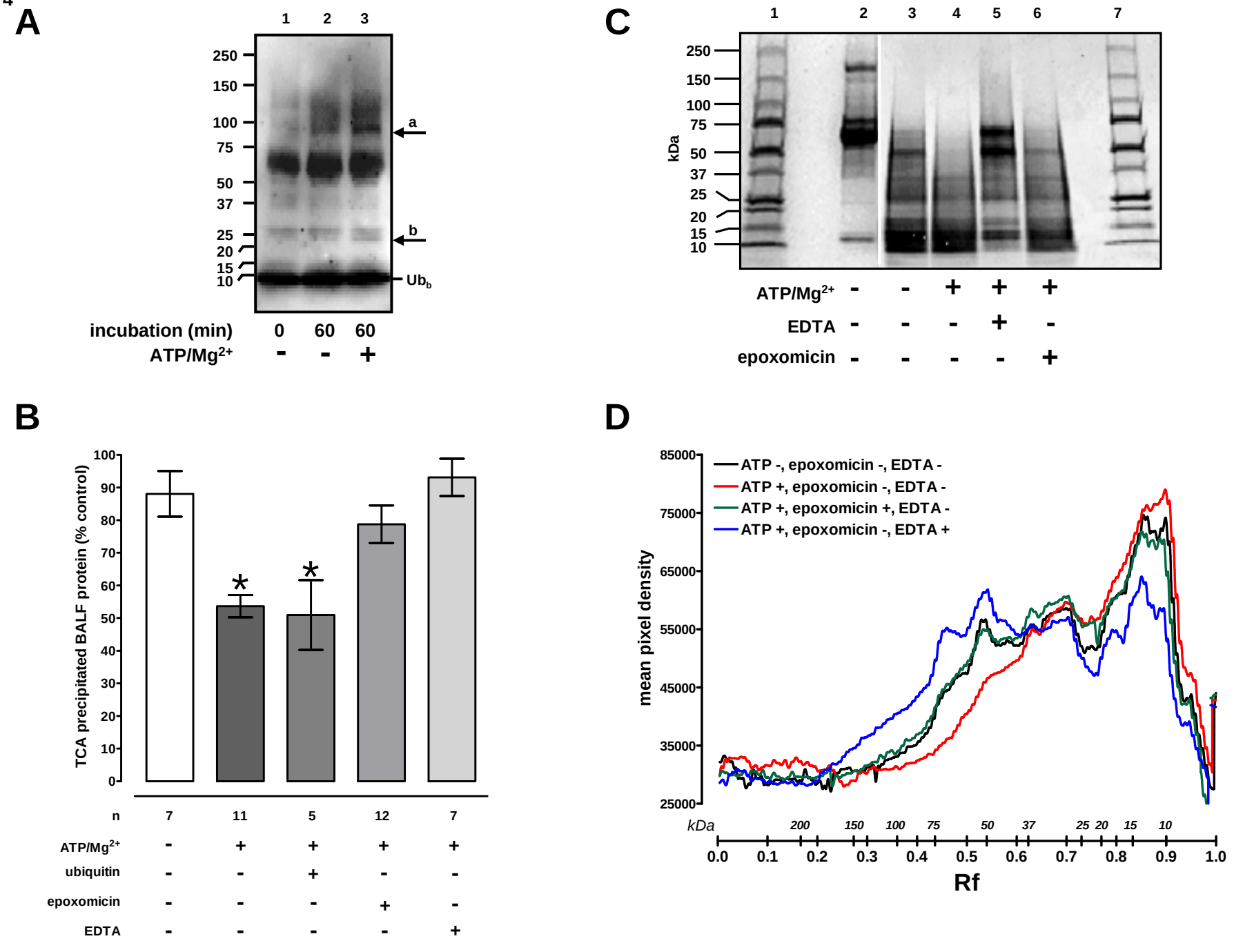

A
CT-L (pmol x h-1 x mL-1)
7000
6000
5000
4000
3000
2000
1000
0
*
*
*
ATP/Mg2+
EDTA
6 h
12 h
24 h
48 h
168 h
6h
24h
Time after lung contusion
sham
B
CT-L - ATP ratio
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
6
12
24
48
168
Time after lung contusion (h)
C
T-L (pmol x h-1 x mL-1)
5000
4000
3000
2000
1000
0
*
*
ATP/Mg2+
EDTA
6 h
12 h
24 h
48 h
168 h
6h
24h
Time after lung contusion
sham
D
T-L - ATP ratio
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
6
12
24
48
168
Time after lung contusion (h)

Fig. 4


A
1 2 3
250
150
100
75
50
37
25
20
15
10
a
b
Ub$_b$
incubation (min) 0 60 60
ATP/Mg^{2+} - - +
B
TCA precipitated BALF protein (% control)
100
90
80
70
60
50
40
30
20
10
0
*
*
n 7 11 5 12 7
ATP/Mg^{2+} - + + + +
ubiquitin - - + - -
epoxomicin - - - + -
EDTA - - - - +
C
1 2 3 4 5 6 7
kDa
250
150
100
75
50
37
25
20
15
10
ATP/Mg^{2+} - - + + +
EDTA - - - + -
epoxomicin - - - - +
D
mean pixel density
85000
75000
65000
55000
45000
35000
25000
ATP -, epoxomicin -, EDTA -
ATP +, epoxomicin -, EDTA -
ATP +, epoxomicin +, EDTA -
ATP +, epoxomicin -, EDTA +
kDa
200
150
100
75
50
37
25
20
15
10
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
Rf