

Proteasomes in lungs from organ donors and patients with end-stage pulmonary diseases

Short title: Proteasomes in normal and diseased lungs

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Summary

Proteasomes appear to be involved in the pathophysiology of various acute and chronic lung diseases. Information on the human lung proteasome in health and disease, however, is sparse. Therefore, we studied whether end-stage pulmonary diseases are associated with alterations in lung 20S/26S proteasome content, activity and 20S subunit composition. Biopsies were obtained from donor lungs (n=7) and explanted lungs from patients undergoing lung transplantation because of end stage chronic obstructive pulmonary disease (COPD; n=7), idiopathic pulmonary fibrosis (IPF, n=7) and pulmonary sarcoidosis (n=5). 20S/26S proteasomes in lung extracts were quantified by ELISA, chymotrypsin-like proteasome peptidase activities measured and 20S proteasome β subunits analyzed by Western blot. As compared with donor lungs, proteasome content was increased in IPF and sarcoidosis, but not in COPD. The relative distribution of free 20S and 26S proteasomes was similar; 20S proteasome was predominant in all extracts. Proteasome peptidase activities in donor and diseased lungs were indistinguishable. All extracts contained a mixed composition of inducible 20S β immuno-subunits and their constitutive counterparts; a disease associated distribution could not be identified. A higher content of lung proteasomes in IPF and pulmonary sarcoidosis may contribute to the pathophysiology of human fibrotic lung diseases.

Key words: 20S proteasome, 26S proteasome, chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis, pulmonary sarcoidosis

Introduction:

Proteasomes are important proteolytic machineries in all eukaryotic cells and among the major proteolytic systems that contribute to protein quality control (Orlowski 1990; Baumeister *et al.* 1998; Hershko and Ciechanover 1998; Groll and Huber 2004) . Proteasomes consist of a cylinder-shaped protein complex (20S core particle, 20S) which can be capped at each end by a 19S regulator complex when ATP/Mg²⁺ is present and is then termed 26S proteasome (26S). The 20S core particle is composed of four stacked rings. Each ring consists of seven α - and β -type subunits ($\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$). In eukaryotic 20S core particles, the proteolytic active sites are located in the subunits β_1 , β_2 and β_5 of the inner rings. These subunits can be replaced by inducible subunits β_{1i} , β_{2i} and β_{5i} . As two of these subunits are encoded within the MHC class II region, they are referred to as immuno-(i)-subunits (Rock and Goldberg 1999; Groll and Huber 2004; Gomes *et al.* 2006; Gallastegui and Groll 2010; Guillaume *et al.* 2010; Sijts and Kloetzel 2011). Whereas proteins that have been covalently modified with ubiquitin are substrates for ATP/Mg²⁺ dependent degradation by the 26S proteasome, the 20S core particle alone contributes to the removal of misfolded and damaged proteins, independent of ATP/Mg²⁺ or ubiquitylation (Eytan *et al.* 1993; Orlowski and Wilk 2003).

Several lines of evidence suggest that proteasomes are involved in the pathophysiology of various acute and chronic lung diseases. Besides their involvement in the pathophysiology of lung cancer and pulmonary inflammation, proteasomal dysfunction is thought to contribute to proteostasis imbalance in chronic obstructive lung disease (COPD) and idiopathic pulmonary fibrosis (IPF) (Bunn 2004; Malhotra *et al.* 2009; Weiss *et al.* 2010; Bodas *et al.* 2012; Zhao *et al.* 2012).

Curative treatment strategies for COPD, IPF or pulmonary sarcoidosis are currently not available and lung transplantation is the only treatment option for many patients with advanced disease. Thus, a better understanding of the regulation of the proteasome in normal and diseased lungs is essential to assess its role in pulmonary pathophysiology and as a possible drug target. Information on the human lung proteasome in health and disease, however, is sparse. Therefore, it was the aim of this pilot study to determine whether end-stage pulmonary diseases are associated with alterations in lung 20S/26S proteasome content, activity and 20S subunit composition.

Materials and Methods:

Lung biopsies: This study was approved by the Institutional Review Board and informed consent was obtained for the use of the lung biopsies. Lung biopsies were obtained from discarded donor lungs that fulfilled transplantation criteria (n = 7, age: 56 ± 27 years, 3/1/3 male/female/unknown) and from explanted lungs from patients undergoing single or double lung transplantation because of end stage COPD (n = 7, age: 64 ± 6 years, 6/1 male/female), IPF (n=7, age: 67 ± 5 years, 5/2 male/female) or sarcoidosis (n = 5, 1/4 male/female, age: 51 ± 4 years). Sixteen of the transplanted patients were of Caucasian origin, one patient with sarcoidosis was of African-American origin and one patient with IPF was of Hispanic origin. The diagnosis was confirmed by histology in all transplanted patients. Biopsies were snap frozen in liquid nitrogen immediately after resection and stored at - 70°C until further processing.

Tissue processing: Snap frozen tissues were homogenized in 1/10 phosphate

buffered saline, 2 mM ATP, 5 mM MgCl₂, pH 7.4 (1:5 weight/volume), centrifuged (16,600g, 4°C, 30 min) and supernatants (= extracts) aliquoted, as described (Majetschak *et al.* 2008a; Geng *et al.* 2009). All measurements in tissue extracts were standardized to total protein content (Lowry *et al.* 1951) and are reported per mg of protein.

Proteasome enzyme linked immunosorbent assays (ELISA): The concentration of total 20S proteasomes (free 20S core particles and 20S core particles within the 26S proteasome complex) and 26S proteasomes in lung extracts was quantified as described in detail previously (Majetschak and Sorell 2008). For the calculation of the molar concentration of 20S core particles, a molecular mass of 700 kDa was used. For the 26S proteasome complex, a molecular mass of 1.7 MDa was assumed to account for a mixture of 20S core complexes that are capped with either one or two 19S regulator complexes. The concentration of free 20S proteasomes was calculated as concentration of total 20S proteasomes minus the concentration of 26S proteasome complexes.

Proteasome peptidase activity assay. Proteasome peptidase activities (=total peptidase activity minus activity in the presence of epoxomicin) in lung extracts were measured employing the fluorogenic peptide substrate *N*-Suc-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (chymotryptic-like, CT-L, Biomol), as described (Majetschak *et al.* 2008a; Geng *et al.* 2009; Baker *et al.* 2010). Reaction mixtures contained 1 mmol/L DTE, 2 mmol/L ATP, 5 mmol/L MgCl₂, 10 mmol/L Tris/HCl, pH 8.0, 200 μmol/L peptide

substrate (0 - 400 $\mu\text{mol/L}$ peptide substrate for the measurement of the substrate dependency) and 0.5 mg/mL tissue extract. All enzyme assays were performed immediately after preparation of the lung extracts to prevent from proteasome inactivation by freeze-thawing.

Western blots: Western blotting with anti-proteasome core (anti- $\alpha 5,7, \beta 1,5,5i,7$), β subunit specific antibodies ($\beta 1-7$, $\beta 1i$, $\beta 2i$, $\beta 5i$) and densitometric quantification of the chemiluminescence signals was performed as described (Majetschak *et al.* 2008a; Geng *et al.* 2009). All antibodies were obtained from Enzo. Chemiluminescence signals were detected with a Chemidoc imaging system and analyzed using the Quantity One gel analyses software (BioRad). Human erythrocyte 20S proteasome (Boston Biochem) was used as a standard.

Analytical gel filtration: Analytical gel filtration was performed on a Superose 6 - column (GE Healthcare) in 25mmol/L Tris/HCl, pH 7.5, 150 mmol/L NaCl, 2 mmol/L ATP, 5 mmol/L Mg^{2+} at 5°C using the DuoFlow-chromatography system (Bio-Rad). The flow rate was 0.25 ml/min and fractions of 0.5 ml were collected. The column was calibrated using proteins of known molecular mass (thyroglobulin (670kDa), bovine γ -globulin (158kDa), chicken ovalbumin (44kDa), equine myoglobin (17kDa) (all from Bio-Rad).

Statistics. Data are described as mean \pm SD. One-way analysis of variance with Dunnett's multiple comparison test to control for multiple testing were used to compare

differences between groups. Statistical analyses and non-linear regression analyses were calculated with the GraphPad-Prism program (GraphPad-Software). A two-tailed $p < 0.05$ was considered significant.

Results:

Proteasome content

The content of total 20S proteasomes in donor lung extracts was 4.1 ± 0.9 $\mu\text{g}/\text{mg}$. As compared with the total 20S content, the amount of 26S proteasome complexes in donor lung extracts was 4-fold lower (0.96 ± 0.23 $\mu\text{g}/\text{mg}$). The molar ratio between free 20S core particles and 26S complexes was 10 ± 3 . As compared with donor lungs (molar 20S content: 5.4 ± 1.3 pmol/mg), the content of free 20S proteasomes per mg of protein was similar in extracts from patients with COPD (6.2 ± 1.3 pmol/mg) and significantly increased in lung extracts from patients with IPF (11.1 ± 2.2 pmol/mg) and sarcoidosis (9.8 ± 3.4 pmol/mg ; Fig. 1A). Whereas quantification of the 26S content showed a similar tendency, these differences did not reach statistical significance (Fig. 1B). The molar ratios between 20S and 26S proteasomes in lung extracts were similar in all groups (molar 20S/26S ratio: donor lungs - 10.0 ± 3.1 ; IPF - 9.3 ± 2.9 ; sarcoidosis - 11.8 ± 6.0 ; COPD - 7.7 ± 3.9 ; $p > 0.05$; Fig. 1C). To confirm the relative distribution of 20S and 26S proteasomes, we then performed gel filtration experiments with donor lung extracts (Fig. 1D). Consistent with the molar 20S/26S ratio that we determined by ELISA, 90% of 20S proteasomes in donor lung extracts eluted in fractions that corresponded to a molecular mass of 700 kDa (fractions #27-33), whereas only 10% of 20S proteasomes were detectable at elution positions corresponding to a molecular mass of >1 MDa (fractions #23-26).

Proteasome peptidase activity

Next, we determined the time progression and substrate dependency of the proteasome peptidase activity in the lung extracts. We observed linear enzyme kinetics over 40 min in extracts from donor and diseased lungs (Fig. 2A). The substrate dependency of the proteasome peptidase activity for the chymotrypsin-like peptide substrate showed Michaelis-Menten kinetics. The Michaelis constant (K_m) was similar in all extracts (K_m for Suc-LLVY: donor lungs - $126 \pm 17 \mu\text{M}$, IPF - $134 \pm 30 \mu\text{M}$, Sarc - $247 \pm 138 \mu\text{M}$, COPD - $218 \pm 148 \mu\text{M}$; r^2 : 0.83-0.99; $p > 0.05$; Fig. 2B).

As expected based on the quantification of the proteasome content in the lung extracts, chymotrypsin-like proteasome peptidase activity was significantly increased in lung extracts from IPF patients when the enzyme activity was expressed per mg of total extract protein (Fig. 2C). There were no differences among the lung extracts, however, when the specific proteasome peptidase activity was expressed per mol of 20S proteasome, which contains the catalytically active sites (Fig. 2D).

To further characterize the enzymatic properties of the lung proteasome, we tested the sensitivity of the proteasome peptidase activity in lung extracts to the specific proteasome inhibitor epoxomicin (Meng *et al.* 1999). We detected similar inhibition kinetics in extracts from donor lungs and diseased lungs (LogIC_{50} : 6.3 - 6.6, $p > 0.05$; Fig. 2E).

Proteasome subunit composition

To assess the subunit composition of donor and diseased lungs, we first analyzed lung extracts by Western blotting utilizing an antibody against multiple α and β 20S subunits (Fig. 3). Purified human erythrocyte 20S proteasome was used for

comparison because it does not contain inducible immuno-subunits (Claverol *et al.* 2002; Chen *et al.* 2009). In contrast to erythrocyte proteasomes in which two bands were detectable, this antibody recognized four major bands in all lung extracts (Fig. 3A/B), suggesting differences in the subunit composition between erythrocyte and lung 20S proteasomes. We then analyzed the expression of constitutive and inducible 20S proteasome β subunits by Western blotting with subunit specific antibodies (Fig. 4). Donor lung extracts contained all constitutive (β 1-7) and immuno (β 1i, β 2i, β 5i) subunits (Fig. 4A-D). All immuno-subunits and their constitutive counterparts were also detectable in extracts from diseased lungs, whereas immuno-subunits were not detectable in human erythrocyte 20S proteasomes (Fig. 4A-E). When membranes with lung extracts that showed obvious differences in the band intensities for the subunits β 1, β 2 or β 5 were re-probed for the corresponding inducible immuno-subunits, we observed an inverse relationship of the band intensities, and vice versa (Fig. 4E). As shown in Fig. 4E for lung extracts from patients with COPD, the chemiluminescence signals for the bands corresponding to the immuno-subunits and their corresponding counterparts in donor lung extracts and extracts from diseased lungs varied considerably and did not provide evidence for a disease associated pattern by Western blotting.

Discussion

In the present pilot study, we detected that proteasome content in lung extracts from patients with IPF and pulmonary sarcoidosis was increased, as compared to that in lung extracts from organ donors and patients with COPD. The relative distribution between 20S and 26S proteasomes was similar and proteasome peptidase activities

were indistinguishable in donor and diseased lungs. All lung extracts contained a mixed composition of inducible 20S β immuno-subunits and their constitutive counterparts. Disease associated alterations in 20S proteasome subunit distribution, however, could not be identified.

The amount of 20S proteasomes that we determined in donor lungs is in agreement with the cellular 20S content that has been estimated based on Western blot analyses in human embryonic pulmonary epithelial cells (L132; $6.9 \pm 2.8 \mu\text{g}/\text{mg}$) (Brooks *et al.* 2000). A several fold excess of 20S over 26S proteasomes has been reported in rat liver and L132 cells previously (Brooks *et al.* 2000). Along with our finding that the molar 20S/26S proteasome ratio is approximately 10 in human lung extracts, these data suggest that the free 20S is predominant in solid organs. Our findings suggest that IPF and, to a lesser extent, sarcoidosis are associated with increased expression of lung proteasomes in which the physiological equilibrium between 20S and 26S proteasomes is maintained. It should be noted, however, that quantification of proteasome content per mg of protein likely underestimates proteasome content in lungs from patients with IPF and sarcoidosis, because in both diseases the fibrotic remodeling will lead to an increased protein content of the homogenates.

Whereas the human lung proteasome in patients with IPF and sarcoidosis has not been studied previously, reduced lung proteasome peptidase activity per mg of protein has been described in patients with COPD (Malhotra *et al.* 2009). In this study, however, proteasome peptidase activity was tested after sodium dodecyl sulfate (SDS) activation of a proteasome fraction that was enriched using a ubiquitin-like (UbL) domain linked to affinity matrix beads (Malhotra *et al.* 2009). Although this observation

may suggest changes in substrate recognition by the proteasome (Tanaka *et al.* 1989; Dahlmann *et al.* 1993), information on the enzymatic activity of proteasomes without SDS activation has not been provided (Malhotra *et al.* 2009). Thus, these findings are not contradictory with our enzyme activity measurements in minimally manipulated lung extracts.

Human liver, colon, small intestine and kidney have been shown to contain 20S proteasomes with a mixed composition of standard and inducible immuno-subunits (Baldovino *et al.* 2006; Visekruna *et al.* 2009; Guillaume *et al.* 2010). Furthermore, changes in the subunit composition of 20S proteasomes under pathological conditions have been described in animal models and in patients (Barton *et al.* 2002; Visekruna *et al.* 2009; Zheng *et al.* 2012). Our findings demonstrate for the first time that all immuno-subunits and their constitutive counterparts are also detectable in extracts from donor and diseased lungs. Our Western blot experiments further suggest that both inducible immuno-subunits and their constitutive counterparts form the 20S core particle in lung extracts, which is in agreement with recent observations in lung tumor cell lines (Guillaume *et al.* 2010). Furthermore, the observation that multiple antibodies reacted with more than one band is consistent with the existence of multiple isoforms of most of the 20S proteasome subunits (Claverol *et al.* 2002; Chen *et al.* 2009).

The heterogeneity of the subunit distribution and the large variability among individual specimens that we detected could be explained by the exposure to inhaled particles and microbes throughout a person's lifetime (Nelson *et al.* 2000; Kwak *et al.* 2007; Kremer *et al.* 2010), which is specific to the individual and may have masked disease associated alterations.

Whereas we were unable to differentiate a disease specific pattern of the lung 20S proteasome subunit composition, we cannot exclude that quantification of the subunit composition with more accurate and precise methods, such as subunit specific ELISAs, may be able to detect statistically significant differences between proteasomes from donor and diseased lungs. Our finding that the specific proteasome peptidase activity was similar in all extracts, however, argues against this possibility, because such differences would be expected to alter the specific enzyme activity (Eleuteri *et al.* 1997; Guillaume *et al.* 2010; Zu *et al.* 2010; Rodriguez *et al.* 2012; Zheng *et al.* 2012).

Limitations of this pilot study are the small sample size and the limited information about the donor and patient history. Therefore, we can currently not comment on the possible effects of other biological or behavioral factors, such as cigarette smoking or alpha1-antitrypsin mutations (Novoradovskaya *et al.* 1998; van Rijt *et al.* 2012). Furthermore, we have utilized crude lung extracts for enzyme activity measurements; thus, proteasomes from non-lung cells, such as erythrocytes, and extracellular proteasomes may have confounded our findings. As erythrocyte proteasomes, however, do not contain 20S immuno-subunits and the concentrations of extracellular proteasomes are far below intracellular concentrations, such confounding effects appear negligible (Claverol *et al.* 2002; Chen *et al.* 2009; Majetschak *et al.* 2008b, Albright *et al.* 2009).

Several lines of evidence suggest that the proteasome is involved in the pathophysiology of organ fibrosis and proteasome inhibition has been shown to inhibit TGF- β 1 induced gene expression in primary human lung fibroblasts from healthy individuals and patients with IPF (Fineschi *et al.* 2006; Mutlu *et al.* 2012). Moreover,

increases in proteasome peptidase activities have been detected in lung extracts several days after injury, which is known to lead to repair with fibrosis, and proteasome inhibition has been described to reduce lung fibrosis in some animal models (Matute-Bello *et al.* 2008; Manning *et al.* 2009; Mutlu *et al.* 2012). As IPF and end-stage pulmonary sarcoidosis are characterized by a fibrotic remodeling of the lung, our findings support the concept that the higher content of lung proteasomes contributes to the pathophysiology of human fibrotic lung diseases. Further exploration of the proteasome as a drug target for these currently incurable diseases appears justified.

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Figure Legends:

Figure 1: Proteasome content in human lung extracts. DL: donor lung. IPF: idiopathic pulmonary fibrosis. COPD: chronic obstructive lung disease. Sarc: sarcoidosis. RFU: relative fluorescence units. Boxes extend from the 25th to 75th percentile, the horizontal line and error bars show the median, minimum and maximum. *: p<0.05 vs. DL. **A.** 20S/mg; n=5-7/group. **B.** 26S/mg, n=5-7/group. **C.** molar 20S/26S ratio, n=5-7/group. **D.** A donor lung extract (0.5 ml) was separated on a Superose 6 column. The 20S concentrations in the eluted fractions were quantified by enzyme linked immunosorbent assay. The arrows show the elution positions of proteins with known molecular masses.

Figure 2: Proteasome peptidase activities in human lung extracts. DL: donor lung. IPF: idiopathic pulmonary fibrosis. COPD: chronic obstructive pulmonary disease. Sarc: sarcoidosis. RFU: relative fluorescence units. Boxes extend from the 25th to 75th percentile, the horizontal line and error bars show the median, minimum and maximum. *: p<0.05 vs. DL. **A.** Time progression of the chymotrypsin-like proteasome peptidase activity (CT-L). **B.** Substrate dependency of the CT-L. **C.** CT-L/mg, n=5-7/group. **D.** CT-L/mol 20S, n=5-7/group. **E.** Epoxomicin sensitivity of the CT-L in % (100% = no epoxomicin; 0% = 7 μ M epoxomicin), n=3/group, mean \pm SD.

Figure 3: 20S proteasome core subunits (α 5,7, β 1,5,5i,7) in human lung extracts. Western blots with anti-proteasome core (α 5,7, β 1,5,5i,7). Each lane contains 300 pmol of 20S proteasomes. The migration positions of molecular mass standards are

indicated. **A.** E: human erythrocyte 20S proteasome. DL: donor lung. IPF: idiopathic pulmonary fibrosis. **B.** DL: donor lung. COPD: chronic obstructive pulmonary disease. IPF: idiopathic pulmonary fibrosis. Sarc: sarcoidosis.

Figure 4: Constitutive and inducible 20S proteasome β subunits in human lung extracts. E: human erythrocyte 20S proteasome. DL: donor lung. IPF: idiopathic pulmonary fibrosis. COPD: chronic obstructive pulmonary disease. Sarc: sarcoidosis. Each lane contains 300 pmol of 20S proteasomes. **A.** Western blots with anti- β 1 and anti- β 1i. **B.** Western blots with anti- β 2 and anti- β 2i. **C.** Western blots with anti- β 5 and anti- β 5i. **D.** Western blots with anti- β 3,4,6,7. **E.** Top: Western blots with anti- β 1, anti- β 2i and anti- β 5. Bottom: Membranes were stripped and re-probed with antibodies against the corresponding inducible or constitutive counterparts, as indicated. Lanes 1 and 2 contain lung extracts from two different patients with COPD.

Figure 1

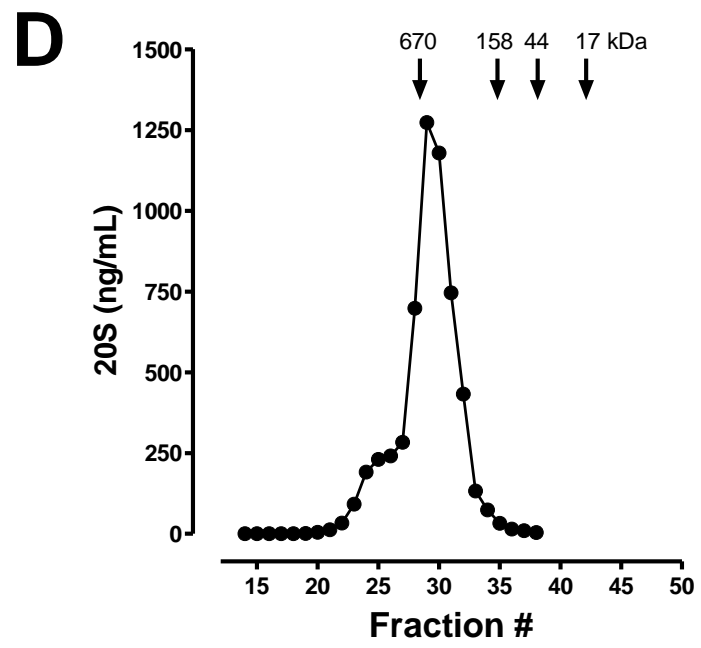
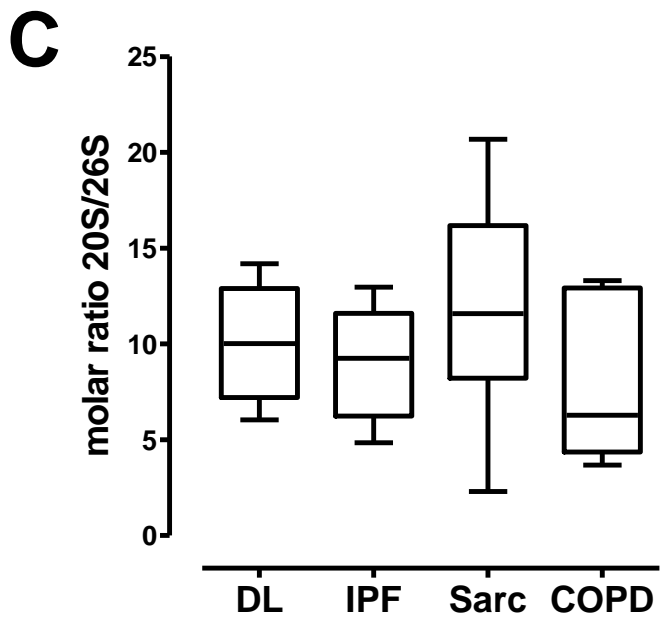
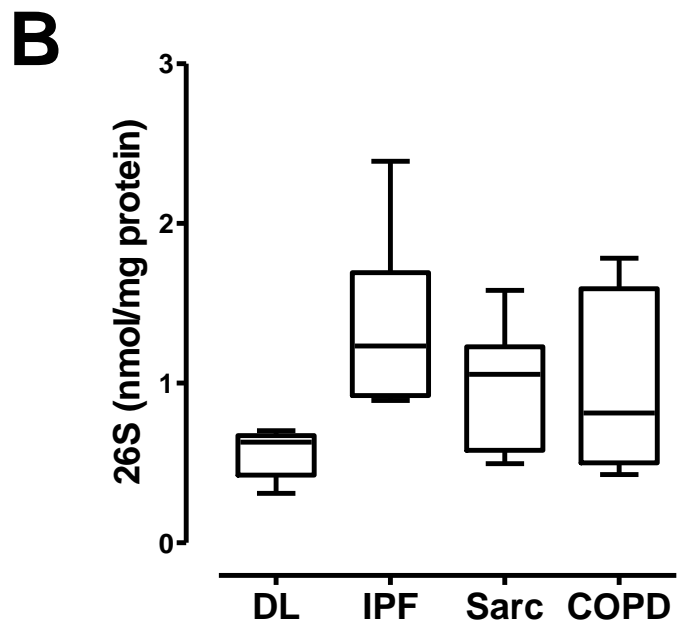
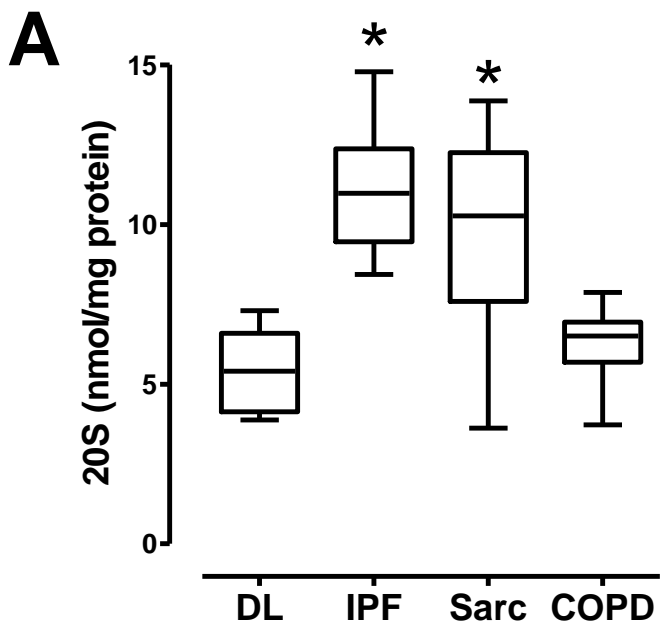


Figure 2

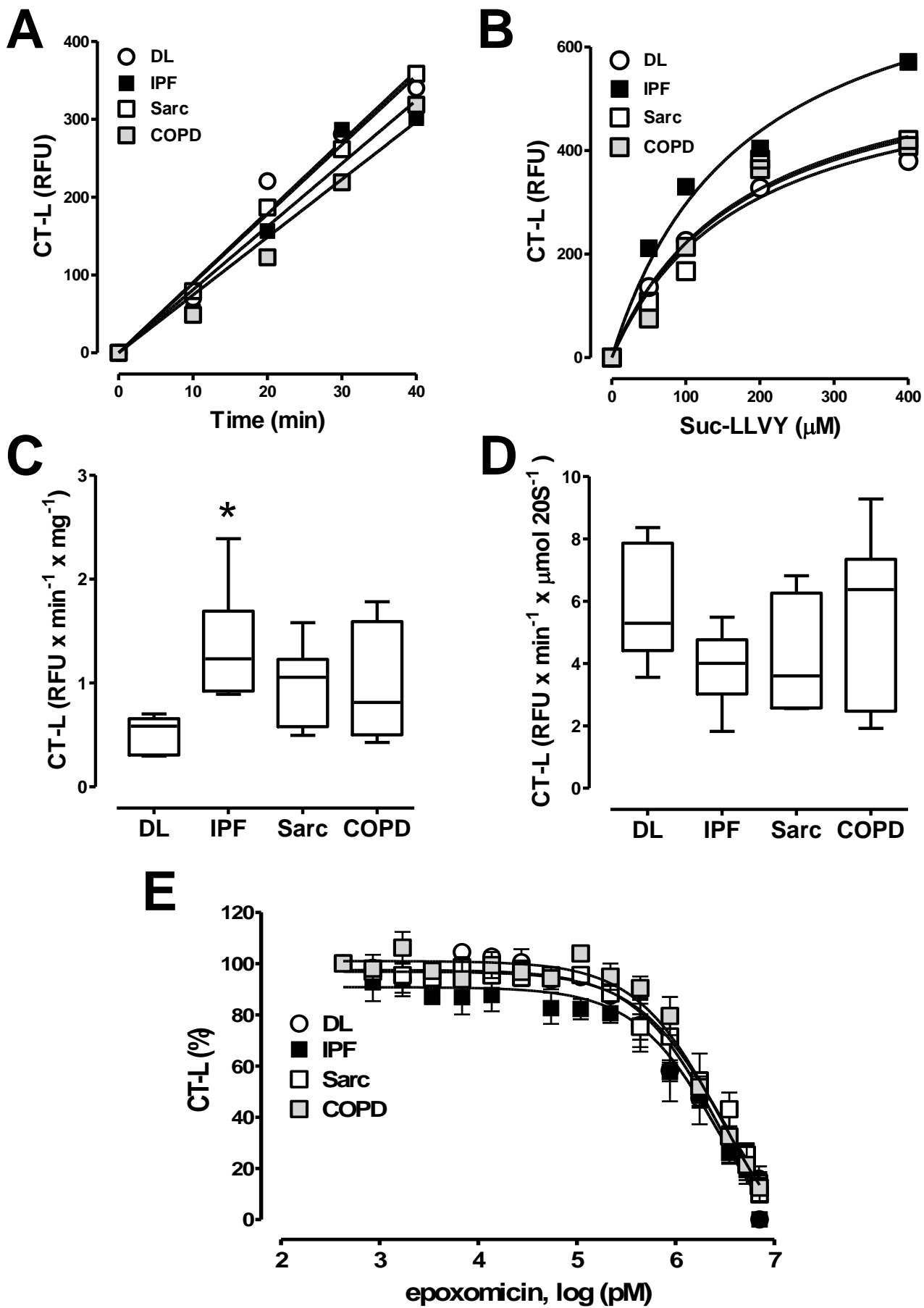


Figure 3

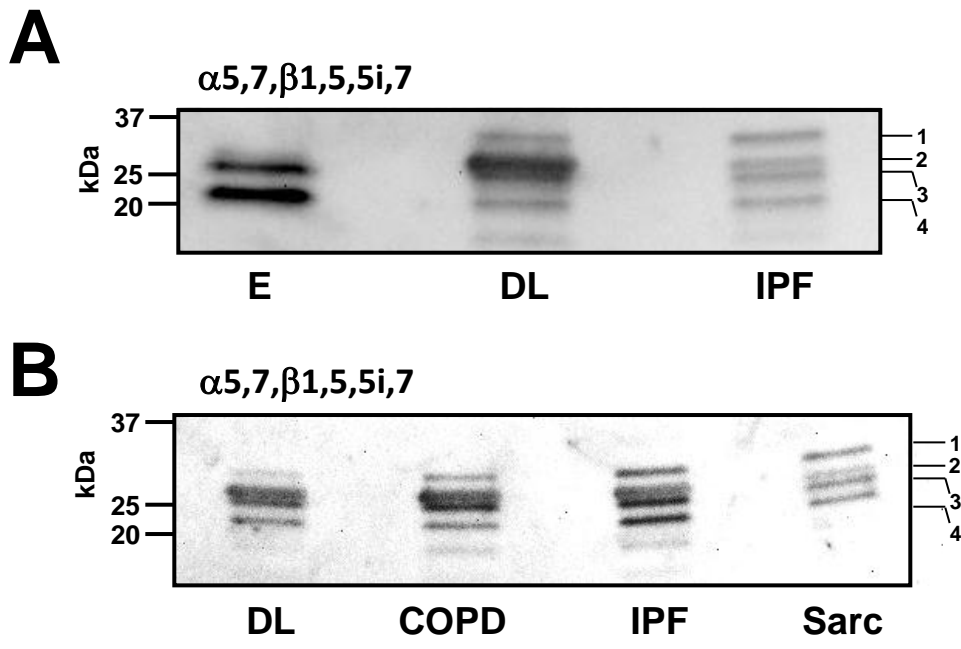


Figure 4

