Online Data Supplement

Proteolytic cleavage of elafin by 20S proteasome may contribute to inflammation in acute lung injury

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Supplementary Materials and Methods

Materials

Recombinant human elafin was purchased from Proteo Biotech AG (Kiel, Germany). Goat anti-human elafin antibody, biotinylated anti-human elafin antibody, anti-human SLPI mAb and biotinylated anti-SLPI antibodies were purchased from R&D systems (Abingdon, Oxon, UK). 20S proteasome α6 subunit mAb, 20S proteasome core subunit rabbit polyclonal antibody, human 20S proteasome and *N*-(methoxysuccinyl)-Ala-Ala-Pro-Val-7-amino-4-methylcoumarin (AAPV-AMC) were purchased from Enzo Life Sciences (Exeter, UK). Horseradish peroxidase (HRP)-conjugated streptavidin was obtained from Biolegend (San Francisco, CA, USA). Pefabloc, α1-antitrypsin (AAT), *N*-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), leupeptin, pepstatin, *N*-(methoxysuccinyl)-Ala-Ala-Pro-Val-chloromethyl ketone (AAPV-CMK), and N-Succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (LLVY-AMC) were purchased from Sigma-Aldrich (Dorset, England). E64, GM6001 and phosphoramidon were purchased from Merck Biosciences (Darmstadt, Germany). Antipain, chymostatin and complete protease inhibitor cocktail were purchased from Roche Applied Science (Germany). Human NE was obtained Elastin Products Company,

Inc. (Owensville, MO, USA). All other reagents were of analytical grade and purchased from Sigma-Aldrich unless otherwise stated.

Determination of elafin levels in BALF

Elafin levels in BALF were determined by ELISA as previously described with some minor modifications (E1). Greiner® high binding 96 well plates were coated with goat anti-human elafin antibody in Voller's buffer overnight at 4°C. The plate was washed three times with PBS 0.05% Tween 20 (PBST) and blocked in 1% BSA/PBST for 1 hr at room temperature. After washing 3 times in PBST, elafin standards and ALI BALF samples were added to the plate for 2 hr at room temperature. The plate was then washed and biotinylated anti-human elafin antibody was added to the plate for 2 hr. After washing, the plate was incubated with HRP-conjugated streptavidin for 20 min and washed with PBST. Peroxidase activity was measured by the addition of ABTS and reading the absorbance at 405 nm on a microtitre plate reader (Synergy HT using Gen5™ software, BioTek UK).

Determination of SLPI levels in BALF

SLPI levels in BALF were determined by ELISA as previously described (E2). Briefly, Greiner® high binding 96 well plates were coated with rabbit anti-human SLPI in Voller's buffer overnight at 4°C. The plate was washed three times with PBST and blocked in 1% BSA/PBST for 1 hr at room temperature. After washing, SLPI standards and SLPI samples were added for 2 hr at room temperature. The plate was then washed and biotinylated anti-human SLPI antibody was added to the plate for 2 hr. After washing, the plates were incubated with HRP-conjugated streptavidin for 20 min and peroxidase activity was measured by reading the A405 after the addition of ABTS as described above.

Determination of 20S proteasome concentrations in BALF

20S proteasome levels were measured by ELISA as described previously (E3). Briefly, Greiner® high binding 96 well plates were coated with 20S proteasome α6 subunit mAb (BML-PW8100) in Voller's buffer overnight at 4°C. The plates were washed with PBST, samples and 20S proteasome standards (1250 – 19.5 ng/ml) added and plates incubated overnight at 4°C. The plate was then washed and 20S proteasome core subunit rabbit pAb added in 1% BSA/PBST for 2 hr at room temperature. Anti-rabbit HRP (Fisher Scientific UK) was added for 20 min and peroxidase activity was measured by reading the A405 after the addition of ABTS as described above.

Western blot analysis of recombinant elafin incubated with ALI BALF

Recombinant human elafin (40 ng) and recombinant human SLPI (100ng) were incubated with 10 µl ALI BALF in TBS in a final volume of 20 µl for 24 hr at 37°C. In some experiments, ALI BALF was pre-incubated for 1 hr at 37°C with the following protease inhibitors prior to the addition of elafin: 100 µM chymostatin, 10 µM pepstatin, 50 ng antipain, 0.1 mM E64, 100 µM leupeptin, 10 µM GM6001, 1X complete protease inhibitor cocktail, 100 µM phosphoramdion, 5 mM pefabloc, 0.1 mM MeOSuc-AAPV-CMK, 1 mM TLCK, 1 µg SLPI, 1 µg AAT, 1µg anti-thrombin. In other experiments, elafin and SLPI were incubated with 20S proteasome for 10 min - 24 hr at 37°C. All incubations were stopped by adding non-reducing sample treatment buffer containing and boiled for 5 min. Samples were separated by Tricine SDS-PAGE on a 17.5% polyacrylamide gel and blotted onto 0.2 µm nitrocellulose membrane. The membrane was blocked for 1 hr at room temperature in 3% BSA/PBST. Elafin and SLPI were detected by using a biotinylated anti-elafin antibody (1:250, overnight at 4°C) or biotinylated SLPI antibody (1:500, overnight at 4^{o}C) following by HRP-conjugated

streptavidin (20 min at room temperature). Elafin was visualized by chemiluminescence (GE Healthcare UK, Buckinghamshire) and analysed using the Syngene G:Box and GeneSnap software (SynGene UK, Cambridge).

20S proteasome and NE activity assay

20S proteasome activity was measured using the substrate LLVY-AMC. BALF was diluted in 20S buffer (50 mM Tris HCl, 1 mM DTT, 5 mM $MgCl_2$, pH 8.0) to a final volume of 50 µl. Experiments were performed ± 100 µM epoxomicin to correct for non-proteasomal activity. The samples were incubated with substrate (65 µM) and fluorescence (substrate turnover) was determined by excitation at 360 nm and emission at 460 nm in a 96 well microplate reader (Synergy HT using Gen5™ software, BioTek UK). NE activity was measured using the substrate AAPV-AMC. BALF was diluted in 0.1 M Hepes, 0.5 M NaCl, pH 7.5 to a final volume of 50 µl. The samples were incubated with substrate (50 µM) and fluorescence (substrate turnover) was determined as described above. The rate of substrate hydrolysis was monitored at 37°C over time and results were expressed as the change (Δ) in relative fluorescence units (ΔRFU).

Evaluation of the anti-elastase activity of 20S proteasome-cleaved elafin

To evaluate the antiprotease activity of 20S proteasome-cleaved elafin, we tested the ability of cleaved elafin to inhibit one of its target proteases, NE, using the substrate MeOSuc-AAPV-AMC. 20S proteasome (1 µg) was incubated with elafin (100 ng) in HEPES/NaCl buffer for 1 hr. Control reactions included elafin pre-treated with epoxomicin for 1 hr before addition of 20S proteasome, elafin incubated with NE and NE alone. Samples were then incubated with NE (500 ng) in HEPES/NaCl buffer for 30 minutes. After this time, the NE

substrate AAPV-AMC (50 μM) was added to the samples and and substrate hydrolysis was monitored at 37°C over time by excitation at 350 nm and emission at 460 nm in a 96 well microplate reader (Synergy HT using Gen5™ software, BioTek UK). Results were expressed as relative fluorescence units (RFU).

HPLC mass spectrometry

Cleavage of elafin by 20S proteasome was assessed by incubating recombinant elafin (500 ng) with recombinant 20S proteasome (5 μg) in 20S buffer for 0, 15, 30 and 60 min. Reactions were stopped by addition of guanidine hydrochloride. Samples were lyophilized until analysis when they were reconstituted in H_2O and treated with 10 mM DTT for 1 hr at $37^{O}C$. Samples were then analyzed by reverse phase HPLC coupled to electrospray mass spectrometry as previously described (E1, E2).

Statistical analysis

All data were analyzed with GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). Descriptive results of continuous variables were expressed as mean (±SEM) or median (IQR) depending on the normality of their distribution. Means were compared by unpaired t-test or Mann Whitney test for comparison between two groups. For comparison between three or more groups, data were analyzed by means of one-way analysis of variance (ANOVA) or Kruskal-Wallis test using Dunn's multiple comparison test when significance was indicated (at the $P < 0.05$ level). Statistical significance is presented as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ in all figures.

Supplement References

E1. Guyot N, Butler MW, McNally P, Weldon S, Greene CM, Levine RL, O'Neill SJ, Taggart CC, McElvaney NG. Elafin, an elastase-specific inhibitor, is cleaved by its cognate enzyme neutrophil elastase in sputum from individuals with cystic fibrosis. *J Biol Chem* 2008;283(47):32377-32385.

E2. Weldon S, McNally P, McElvaney NG, Elborn JS, McAuley DF, Wartelle J, Belaaouaj A, Levine RL, Taggart CC. Decreased levels of secretory leucoprotease inhibitor in the Pseudomonas-infected cystic fibrosis lung are due to neutrophil elastase degradation. *J Immunol* 2009;183(12):8148-8156.

E3. Sixt SU, Adamzik M, Spyrka D, Saul B, Hakenbeck J, Wohlschlaeger J, Costabel U, Kloss A, Giesebrecht J, Dahlmann B, Peters J. Alveolar extracellular 20S proteasome in patients with acute respiratory distress syndrome. *Am J Respir Crit Care Med* 2009;179(12):1098-1106.

ALI Patient Information

The aetiology of the patient's recruited was as follows:

Pneumonia	12
Aspiration	7
Sepsis	4
Trauma	4
Burns	2
Pancreatitis	1
Other	7