Elastase activity on sputum neutrophils correlates with severity of lung disease in cystic fibrosis

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Online Supplementary Material

SUPPLEMENTARY METHODS

Sputum processing

Sputum samples were collected in a specimen container, stored on ice immediately after production and processed within two hours. Sputum was separated from saliva, 4 times the volume of 10% sputolysin solution (Calbiochem, Darmstadt, Germany) was added and samples were homogenized for 15 minutes on a rocker at room temperature. Subsequently, the same volume of PBS was added, samples were filtered through 100 µm and 40 µm cell strainers and centrifuged at 300 g and 4°C for 10 minutes [E1, E2]. Cell pellets were resuspended in PBS and cell counts were determined using trypan blue (Sigma, St Louis, MO, USA). One portion of the sputum supernatants was treated with protease inhibitors (Roche Diagnostics, Rotkreuz, Switzerland) and supernatants were stored at -80°C until analysis.

Measurements of surface-bound NE activity

For quantification of membrane-associated neutrophil elastase (NE) activity on sputum neutrophils [E1, E3], 20,000-30,000 sputum inflammatory cells were incubated with 400 nM of the lipidated FRET reporter NEmo-2 and 1:1000 DRAQ5 (BioStatus Limited, Shepshed, UK) in 100 µL PBS for either 10 or 30 minutes. To control for NE specific cleavage, one aliquot of sputum cells was pre-incubated with 100 µM of the NE inhibitor sivelestat (Tocris Bioscience, Bristol, UK) for 15 minutes at room temperature and immediately put on ice after adding of NEmo-2 to determine background FRET signals. At the different time points, 100 µL PBS were added, cytospins were prepared, fixed with ice-cold methanol for 10 minutes, mounted with Roti®-Histokitt (Carl Roth, Karlsruhe, Germany) and stored at 4°C. Images were acquired using a Leica SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany) equipped with an HC PL APO CS2 40x/1.3 oil objective. Nuclear staining (DRAQ5) and membrane-associated NE activity (NEmo-2) were recorded sequentially. DRAQ5 was excited with a 633 nm HeNe laser

and emission was sampled between 650-715 nm. The pinhole was set to 195.9 µm (3.0 AU). The donor of NEmo-2 was excited with a 405 nm diode. Donor emission was sampled between 470-510 nm and acceptor emission between 570-610 nm. The pinhole was set to 326.6 µm (5.0 AU). Image analysis was performed and surface-bound NE activity determined as previously described [E1]. In brief, surface-bound NE activity was determined from the ratio of donor to acceptor fluorescence (D/A ratio) after 10 minutes of incubation with NEmo-2 and corrected for background signal by subtraction of the D/A ratios obtained in samples pretreated with the NE inhibitor sivelestat. Measurements were accepted when the D/A ratio of samples preincubated with sivelestat was ≤1.7 and the D/A ratio after 10 minutes of incubation did not exceed the D/A ratio after 30 minutes for more than 12.5%.

Measurements of free NE activity

Cell-free sputum supernatant was diluted in NE assay buffer (100 mM TRIS, 500 mM NaCl, pH 7.5). All kinetic assays were performed at 37°C using a fluorescence plate reader (Enspire 2300, PerkinElmer, Waltham, MA, USA). As negative control, sputum supernatants were pre-incubated with 100 µM Sivelestat (Tocris Bioscience, Bristol, UK) for 15 minutes at room temperature. Measurements of free NE activity were performed using the soluble FRET reporter NEmo-1 [E3] and chromogenic substrate MeO-Suc-AAPV-pNA (Sigma, St Louis, MO, USA) [E4, E5]. NEmo-1 detects free NE activity in the subnanomolar range with a detection limit ~0.02 nM for human NE and is ~35-fold more specific for NE over the structurally related proteinase 3 and ~230-fold more specific for NE over Cathepsin G. Further, NEmo-1 has high specificity for NE over a series of matrix metalloproteinases (MMPs) expressed in the lung [E3]. The chromogenic substrate MeO-Suc-AAPV-pNA detects free NE activity in the nanomolar range with a detection limit ~20 nM for human NE [E4, E5]. The peptide sequence of this substrate is selectively hydrolyzed by human NE, but not by Cathepsin G and Proteinase 3 [E3, E6, E7]. For quantification of free NE activity with NEmo-1, 40 µL of diluted sputum supernatant (CF diluted

1:400, control 1:1.) were added in polystyrene 96 well half area assay plates (Corning Inc., Acton, MA, USA). The reaction was initiated by adding 10 µL NEmo-1 (5 µM) and reporter cleavage was recorded over time using the following settings: λexc = 354 nm, λem(donor) = 400 nm and λem(acceptor) = 490 nm [E1]. For measurements of free NE activity by the chromogenic substrate MeO-Suc-AAPV-pNA (Sigma, St Louis, MO, USA), 75 µL diluted sputum supernatant (CF diluted 1:10, control 1:1) were added to a 96 well microplate (Greiner bio-one, Kremsmuenster, Austria). The reaction was started by adding 75 µL of substrate (1.2 mM) and absorbance was immediately recorded at 410 nm. A standard curve from known concentrations of purified human NE (Calbiochem, San Diego, CA, USA) was included in each assay [E4, E8]. Concentrations of active NE were estimated using the "R" package nCal [E9] for NEmo-1 and Enspire 2300 software (PerkinElmer, Waltham, MA, USA) for MeO-Suc-AAPV-pNA. In the present study, the lower and upper limits of detection for these assays were 0.008 µg/mL to 100 µg/mL for NEmo-1 and 0.5 µg/mL to 80 µg/mL for MeO-Suc-AAPV-pNA [E3, E4].

Measurements of cytokines and antiproteases

Concentrations of interleukin-8 (IL-8) were determined in cell-free sputum supernatant treated with protease inhibitors. Concentrations of myeloperoxidase (MPO), secretory leukocyte protease inhibitor (SLPI) and α1-antitrypsin-NE complexes (AAT-NE) were measured in untreated sputum supernatants [E4]. Cytokines and antiproteases were quantified using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA and Calbiochem, San Diego, CA, USA) according to the manufacturer's instructions.

SUPPLEMENTARY REFERENCES

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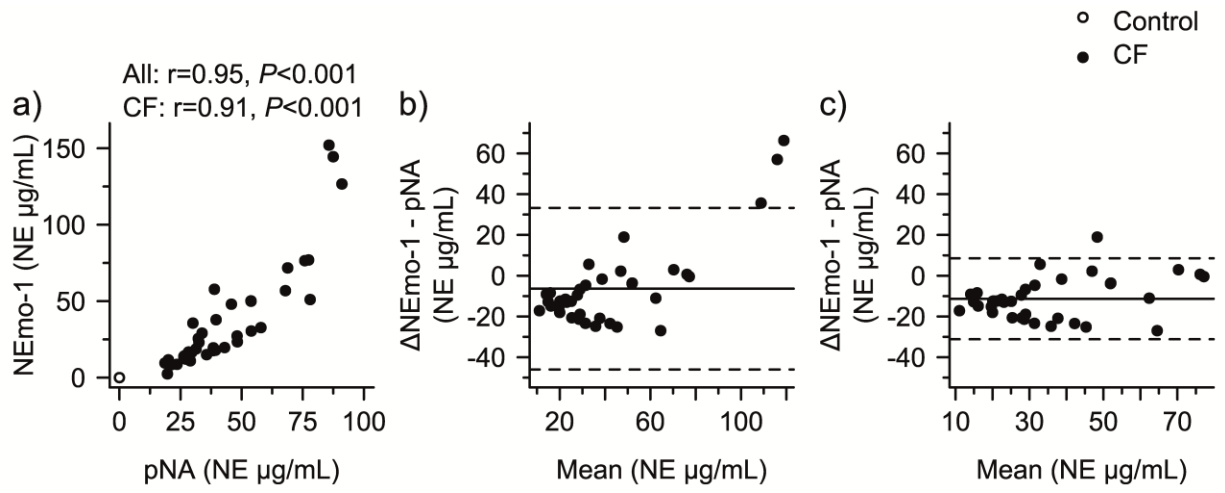
SUPPLEMENTARY TABLES

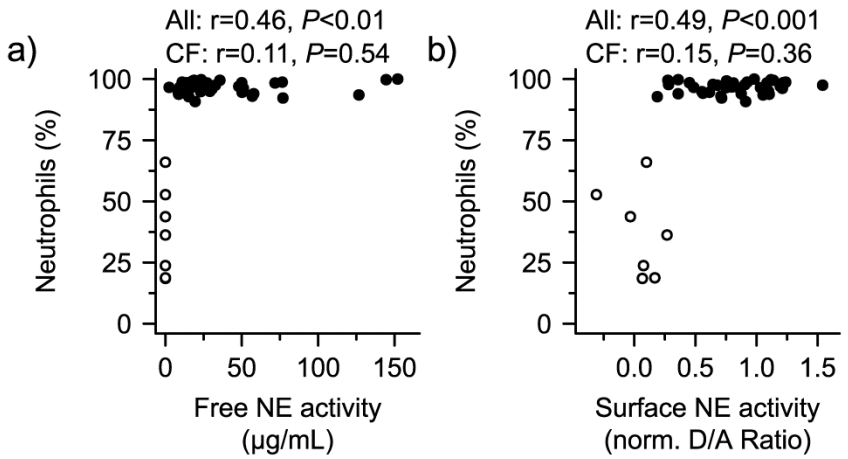
Supplementary table S1. CFTR genotypes and pancreatic status of patients with cystic fibrosis

Pancreatic insufficient		**Pancreatic sufficient**	
***CFTR* genotype**	**subjects (n)**	***CFTR* genotype**	**subjects (n)**
F508del/F508del	13	F508del/R117H	1
F508del/2789+5G->A	1	F508del/T1299I	1
F508del/3821delT	1	G542X/X	1
F508del/3849+10kbC->T	1	R553X/3849+10kbC->T	1
F508del/3905insT	1	W1282X/R347P	1
F508del/711+3A->G	1		
F508del/CFTR del 17 (2,5 Kb)	1		
F508del/E403D	1		
F508del/E60X	1		
F508del/G551D	2		
F508del/I507del	2		
F508del/M1101K	3		
F508del/N1303K	1		
F508del/Q220X	1		

SUPPLEMENTARY FIGURES



Supplementary figure S1. a) Correlation of free NE activity determined by the soluble NE FRET reporter NEmo-1 and the chromogenic substrate MeO-Suc-AAPV-pNA (pNA) in sputum from the entire study population (control and CF; all) and from patients with CF. b, c) Bland-Altman plots comparing free NE activity determined with NEmo-1 and MeO-Suc-AAPV-pNA for all patients with CF (b) and for values captured by the standard curve of the respective assay (≤ 80 µg/mL for pNA and ≤ 100 µg/mL for the NEmo-1 assay) (c). Solid lines represent the mean and dashed lines the mean ± 1.96 x standard deviation.



Supplementary figure S2. a, b) Relationship of free (a) and surface-bound (b) NE activity with percentage of neutrophils in sputum from the entire study population (control and CF; all) and from patients with CF.