Neutrophil Elastase

DOI: 10.1002/anie.201109226

Spatially Resolved Monitoring of Neutrophil Elastase Activity with Ratiometric Fluorescent Reporters**

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Neutrophil elastase (NE), a serine protease mainly secreted by neutrophils under physiological and pathophysiological conditions, has important protective functions including remodeling of the extracellular matrix and host defense against bacterial infections.[1,2] However, in diseases characterized by chronic neutrophilic inflammation such as cigarette-smoke-induced chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) lung disease, excessive NE activity has been implicated in proteolytic damage of the airways and lung parenchyma causing bronchiectasis and emphysema, ultimately leading to respiratory failure and death.[3–7] Indeed, mice that lack NE are partially resistant to cigarette-smoke-induced lung damage.[3] A critical role of the protease/antiprotease balance is also highlighted in patients who lack the major endogenous inhibitor of NE, α1-antitrypsin (A1AT); they develop early-onset emphysema,[8] indicating that proper regulation of NE activity is of major significance for global lung homeostasis. In neutrophils NE is stored in its active form in azurophilic granules and released upon stimulation.[1] Since local enzyme activity in the immediate vicinity of the cell membrane is required for migration of neutrophils to sites of inflammation, it was hypothesized that membrane-associated NE might be responsible for tissue transmigration, whereas released NE is inhibited in the airway lumen to protect the lung from uncontrolled proteolytic damage.[6] In this context, it appears to be crucial to understand the activity distribution and function of both cell-surface-associated NE and soluble NE.

So far, studies on the role of NE in chronic lung disease have focused on the soluble form of NE,[4,5,9] generally measured using the chromophoric substrate NMeOSuc-AAPV-pNA or its fluorescent variant, which in humans displays good selectivity over the structurally related proteinase 3 (PR3).[10,11] However, this specificity is mostly lost for the mouse variants of the enzymes.[12] Of note, the detection limit of assays using this substrate is in the nanomolar range, so that NE activity in biological samples such as bronchoalveolar lavage (BAL) is often too dilute to be detected.[4] Moreover, several reports demonstrated the presence of a membrane-bound fraction of NE,[13,14] likely based on electrostatic interactions with the positively charged enzyme surface,[15] and the relative roles of membrane-associated versus soluble NE activity have to our knowledge not been studied.

For reliable monitoring of protease activity in a complex matrix at the level of intact cells and tissues from mouse models and patient specimens, high specificity and sensitivity of the detection probe are mandatory. We therefore selected a longer peptide sequence fitting the modeled binding pockets of mouse and human NE in both directions from the substrate cleavage site.[16] The primary goal was the improved detection of mouse NE activity to permit the use of mouse models. As was previously successfully applied for the monitoring of matrix metalloproteinase 12 (MMP12) activity on cell membranes,[17] the new NE reporter was also lipidated. Measurements on cell surfaces not only provide local activity information, but also the signal accumulates and therefore a much lower reporter concentration is required, which is important for future clinical applications.

We synthesized two specific small-molecule ratiometric NE monitoring reporters based on energy transfer: NEmo-1 to detect activity of the soluble enzyme and the lipidated variant NEmo-2 for insertion into the plasma membrane. We chose the peptide substrate sequence QPMA*VV*QSVPQ with a specific cleavage site between the valine residues accepted by mouse and human NE.[16] Both reporters were tested with recombinant mouse and purified human NE, NEmo-1 in buffer and NEmo-2 on liposomes and in cell culture. To demonstrate the applicability to biological samples, NEmo-1 and NEmo-2 were used to measure NE activity in lung fluid and on neutrophils, respectively, from a mouse model of acute neutrophilic lung inflammation.

The complete synthesis of both reporters was performed by standard 9-fluorenylmethoxycarbonyl(Fmoc)-based solid-

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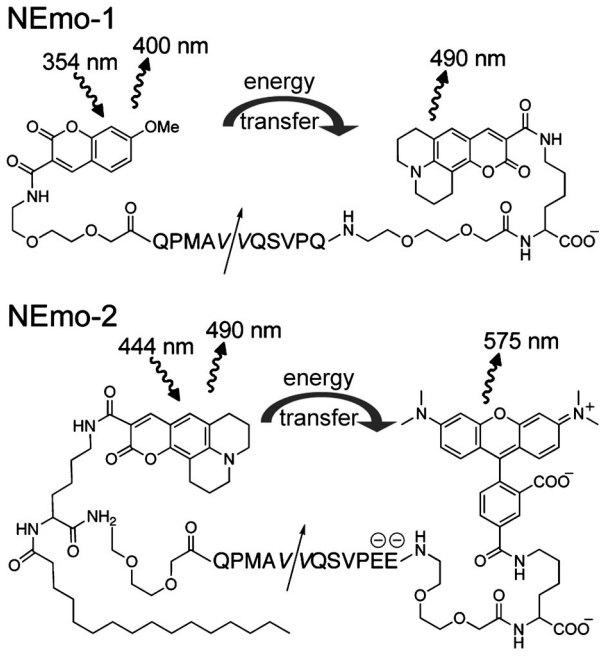
[**] We are grateful for excellent technical support by Stephanie Hirtz, Jolanthe Schatterny, and Heike Stichnoth as well as technical support by the EMBL ALMF core facility. C.S. acknowledges funding from LIVIMODE, a collaborative FP7 project of the EU. M.A.M. acknowledges funding from the Deutsche Forschungsgemeinschaft (MA 2081/4-1 and MA 2081/3-2). S.G. is a fellow of the Molecular Medicine Partnership Unit (MMPU) funded by EMBL and the University of Heidelberg.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201109226.

phase peptide synthesis. For the soluble variant NEmo-1, the substrate sequence QPMA*VV*QSVPQ was flanked by polyethyleneglcol (PEG) linkers to increase solubility and to introduce spacing elements. Lysine residues on the N and C termini were functionalized with methoxycoumarin and coumarin343, respectively, to build a fluorescence resonance energy transfer (FRET) pair. Coumarin dyes were selected based on our experience with the MMP12 reporter LaRee5,[17] because they showed less tendency to form nonfluorescing complexes. For NEmo-2, intended for imaging of NE activity on the surface of intact cells, the red-shifted fluorophore pair coumarin343 and TAMRA was chosen to avoid cell damage by high-energy light and to allow excitation by the 405 nm laser available in most standard fluorescence microscopes. An additional lysine on the N terminus was introduced to attach palmitoic acid used as a lipid anchor. To prevent diffusion of the intact reporter through the plasma membrane of the cell or its C-terminal part after cleavage, negative charges were included in the substrate sequence by replacing the C-terminal glutamine by two glutamic acid residues (Scheme 1). Choosing two fluorophores, instead of a fluorophore and a nonfluorescent quencher, allows for ratiometric readout of reporter cleavage. For the lipidated reporter NEmo-2, cleavage is also observable by following the release of the acceptor-coupled part from the cell surface by direct acceptor excitation.

To decrease the probability of unspecific reporter cleavage by other than the target protease, we attempted to optimize the reporter by shortening the NE substrate sequence to the P4-P4′[18] peptide-recognition sequence.



Scheme 1. Soluble (NEmo-1) and membrane-targeting lipidated (NEmo-2) neutrophil elastase monitoring fluorescent reporters prepared by solid-phase synthesis. The NE-specific peptidic target sequence is given by amino acids with single-letter codes. Two negative charges were introduced in NEmo-2 to prevent internalization from the cell membrane. Arrows indicate the cleavage site.

However, this reporter exhibited reduced performance. The same was observed for a reporter lacking the short PEG linkers. Both results hint towards the requirement of sufficient spacing between the bulky fluorophores and the active center of NE to allow for proper fitting of the recognition sequence. NEmo-1 showed a very high level of donor quenching when intact. Enzyme cleavage of the probe led to a strong increase in donor fluorescence and a small decrease in weak acceptor fluorescence, indicating that the acceptor fluorophore in the ratiometric reporter functions dominantly as a quencher. Probe cleavage was calculated from ratiometric change of the emission maxima over time (Figure 1 a,b).

NEmo-1 detected mouse and human NE activity in the subnanomolar range (Table 1); the detection limit is similar to that of the standard fluorescent NE substrate NMeOSuc-AAPV-AMC (Figure S1 in the Supporting Information). However, because of their ratiometric properties, NEmo-1 and NEmo-2 produce a signal independent of the probe concentration and other environmental factors in the experiment. NEmo-1 showed roughly 35-fold specificity for human NE over the structurally related proteinase 3 (PR3) (Figure S2 b) similar to what was demonstrated for the mouse enzymes by Kalupov et al.[16] In addition, a satisfactory

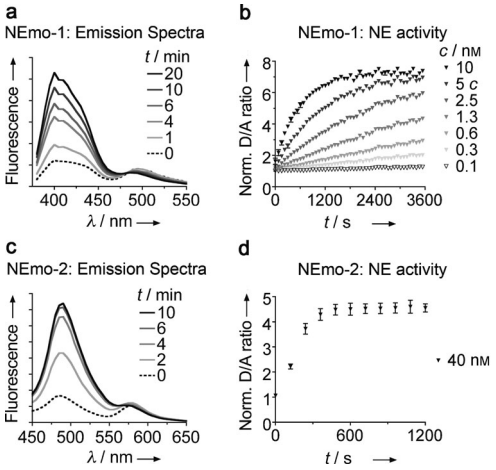


Figure 1. Cleavage of NE reporters in vitro. a) Change in fluorescence emission spectra of NEmo-1 after addition of NE. b) Dose-dependent NE activity detected by NEmo-1. Cleavage is shown by a change in the donor/acceptor (D/A) emission ratio (methoxycoumarin, $\lambda_{max}=400$ nm, coumarin343, $\lambda_{max}=490$ nm, normalized to control (no enzyme)). c) Change in the fluorescence emission spectra of NEmo-2 on liposomes after adding NE. d) Cleavage of NEmo-2 on liposomes plotted as normalized D/A emission ratio (coumarin343, $\lambda_{max}=490$ nm; TAMRA, $\lambda_{max}=575$ nm) in response to 40 nM NE over time.

Table 1: Performance of NEmo-1.

Initial D/A ratio	Maximal D/A ratio	$FRET_{eff}$[a]	Detection limit [nM] rmNE[b]	HNE[c]
1.0–1.2[d]	7.5–9.5[d]	0.86	≈0.1	≈0.02

[a] Effective fluorescence energy transfer. [b] Recombinant enzyme; activity dependent on activation by CathC, therefore, likely incomplete activation. [c] HNE = human NE. [b] Dependent on the buffer used.

specificity was found when a series of mouse and human matrix metalloproteinases relevant in lung inflammation (Figure S2c) were tested.

The performance of the lipidated probe NEmo-2 was tested on liposomes generated from a phosphatidyl choline/phosphatidyl serine (PC/PS) lipid mixture by extrusion (Figure 1c,d). Whereas NEmo-2 showed the expected response on liposomes, poor performance was observed in buffer, likely caused by the poor water solubility of TAMRA and/or aggregation effects induced by the fatty acid moiety (Figure S3).

To study the performance of NEmo-2 in the context of an intact cell model, cultured RAW macrophages (that do not express NE) were preincubated with the reporter, and donor fluorescence emission and the sensitized emission of the acceptor were imaged by fluorescence microscopy. NEmo-2 localized exclusively on the cell membrane. After addition of NE, a strong increase in the D/A emission ratio (about fourfold) was observed (Figure 2). In contrast to the MMP12 reporter LaRee1,[17] no internalization of the lipidated cleavage product was observed for up to four hours.

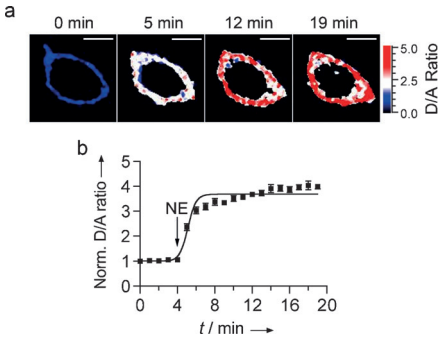


Figure 2. Cleavage of NEmo-2 on cultured RAW macrophages after 2 min incubation and subsequent washing. After 4 min, NE was added. a) Confocal D/A ratio images. Blue = low D/A ratio, intact NEmo-2; red = high D/A ratio, indicative of cleaved NEmo-2. b) D/A ratios were normalized to the starting value and plotted over time. Scale bar: 10 μm.

Next, we evaluated NEmo-1 and NEmo-2 in primary samples of a mouse model of acute neutrophilic airway inflammation. Wild-type (Wt) and NE-deficient ($NE^{-/-}$) mice were treated by intratracheal instillation of lipopolysaccharide (LPS) four hours before bronchoalveolar lavage (BAL) was performed.[19,20] BAL was separated into supernatant and the cell fraction, containing roughly 85% neutrophils (Figure S4). When we investigated the elastolytic activity present in the airway lumen by incubation of NEmo-1 with the BAL supernatant of Wt and $NE^{-/-}$ mice, no NE activity was observed. Instead, BAL supernatant could be used to inhibit the activity of purified NE, suggesting an excess of endogenous inhibitors present in BAL supernatant (Figure S5). A candidate antiprotease is A1AT, the major physiological inhibitor of NE in the lung, which inactivates NE by forming a stable complex.[21–23]

We then investigated the hypothesis that NE activity resides exclusively on the neutrophil surface and may hence be carried to the target substrates by the cell itself. To monitor cell-surface-associated NE activity, BAL neutrophils were incubated with NEmo-2 and spread on glass slides, and reporter cleavage was analyzed by fluorescence microscopy. A pronounced increase in D/A ratio was observed on the cell surface of neutrophils from Wt, but not from $NE^{-/-}$ mice indicating that the response was caused by NE-specific cleavage (Figure 3). Alternatively to spinning the cells, they

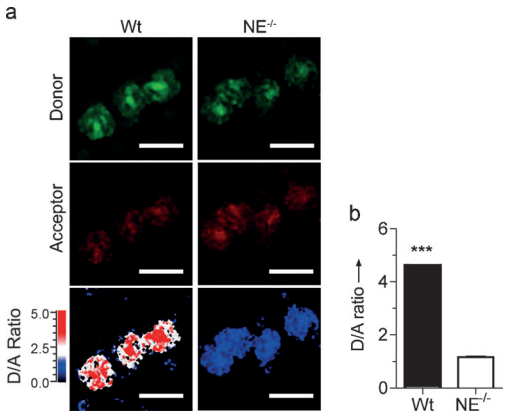


Figure 3. Measurement of NE activity on primary mouse airway neutrophils from LPS-treated mice. Airway neutrophils from wildtype (Wt) or NE-deficient ($NE^{-/-}$) mice were incubated with NEmo-2 and spread on glass slides. a) Donor fluorescence and sensitized acceptor emission was imaged and D/A ratio images were calculated. NEmo-2 was rapidly cleaved on Wt neutrophils but no cleavage was found on control $NE^{-/-}$ neutrophils. Blue = low D/A ratio, intact NEmo-2; red = high D/A ratio, indicative of cleaved NEmo-2. b) D/A ratios showed a fourfold increase for Wt over $NE^{-/-}$ neutrophils. Data is shown as mean ± SEM; n = six mice per group. Scale bar: 20 μm.

were allowed to settle in cell culture dishes, incubated with NEmo-2, and imaged directly. We observed defined plasma membrane staining comparable to that of cultured RAW macrophages. NE activity was confined to the membrane of Wt neutrophils, but not found on $NE^{-/-}$ neutrophils (Figure S6). On cytospin $NE^{-/-}$ neutrophils but not on Wt neutrophils, direct acceptor excitation showed a full signal, indicating that NEmo-2 resided exclusively on the outer plasma membrane allowing the TAMRA-containing part to diffuse away after cleavage. In addition, incubation of Wt neutrophils with A1AT blocked cleavage of the reporter, further supporting the exclusive measurement of membrane-associated NE activity (Figure S7). This result demonstrates that the NE that retains activity on the cell surface of neutrophils likely reflects a combination of membrane-bound and freshly secreted NE located in the vicinity of the plasma membrane.

Taken together, our results suggest that in lung inflammation, NE is predominantly active on the surface of the infiltrating neutrophil but not in extracellular fluids lining the airway surfaces. This indicates that proteolytic degradation is restricted to areas with direct physical contact to neutrophils. A pool of membrane-associated NE activity might allow neutrophils to interact with target tissue and preserve NE activity despite high concentrations of endogenous inhibitors

by creating a microenvironment shielded from inhibitor access. Therefore, future treatment strategies for chronic neutrophilic inflammatory diseases such as COPD and CF, for which NE has become a marker in clinical studies,[4,9,24] may benefit from taking into account the local activity distribution of the enzyme. Ideally, drug candidates should target neutrophils directly and insert themselves into the outer leaflet of the plasma membrane to inhibit NE on the cell surface. Alternatively, compounds that restrict the attachment of neutrophils to the extracellular matrix might reduce proteolysis of matrix proteins.

With the novel ratiometric fluorescent NE reporters, we now have the tools to analyze two major proteases implicated in chronic inflammation and proteolytic destruction of the lung, MMP12 and NE. Surprisingly, both seem to be predominantly active on the surface of the respective enzyme-secreting cell. This spatial restriction might be a prerequisite to protect the lung and potentially other organs from excessive proteolysis and destruction of the extracellular matrix.

Received: December 29, 2011
Revised: February 24, 2012
Published online: May 3, 2012

Keywords: fluorescent probes · FRET · lung emphysema · medicinal chemistry · proteases

[1] B. Korkmaz, M. S. Horwitz, D. E. Jenne, F. Gauthier, *Pharmacol. Rev.* **2010**, *62*, 726 – 759.
[2] A. Belaaouaj, R. McCarthy, M. Baumann, Z. Gao, T. J. Ley, S. N. Abraham, S. D. Shapiro, *Nat. Med.* **1998**, *4*, 615 – 618.
[3] S. D. Shapiro, N. M. Goldstein, A. M. Houghton, D. K. Kobayashi, D. Kelley, A. Belaaouaj, *Am. J. Pathol.* **2003**, *163*, 2329 – 2335.
[4] P. D. Sly, S. Brennan, C. Gangell, N. de Klerk, C. Murray, L. Mott, S. M. Stick, P. J. Robinson, C. F. Robertson, S. C. Ranganathan, *Am. J. Respir. Crit. Care Med.* **2009**, *180*, 146 – 152.
[5] P. Birrer, N. G. McElvaney, A. Rudeberg, C. W. Sommer, S. Liechti-Gallati, R. Kraemer, R. Hubbard, R. G. Crystal, *Am. J. Respir. Crit. Care Med.* **1994**, *150*, 207 – 213.
[6] C. A. Owen, *Int. J. Chronic Obstruct. Pulm. Dis.* **2008**, 3, 253 – 268.
[7] U. Demkow, F. J. van Overveld, *Eur. J. Med. Res.* **2010**, *15*, 27 – 35.
[8] P. O. Ganrot, C. B. Laurell, S. Eriksson, *Scand. J. Clin. Lab. Invest.* **1967**, *19*, 205 – 208.
[9] T. Z. Khan, J. S. Wagener, T. Bost, J. Martinez, F. J. Accurso, D. W. Riches, *Am. J. Respir. Crit. Care Med.* **1995**, *151*, 1075 – 1082.
[10] M. J. Castillo, K. Nakajima, M. Zimmerman, J. C. Powers, *Anal. Biochem.* **1979**, *99*, 53 – 64.
[11] K. Nakajima, J. C. Powers, B. M. Ashe, M. Zimmerman, *J. Biol. Chem.* **1979**, *254*, 4027 – 4032.
[12] O. Wiesner, R. D. Litwiller, A. M. Hummel, M. A. Viss, C. J. McDonald, D. E. Jenne, D. N. Fass, U. Specks, *FEBS Lett.* **2005**, *579*, 5305 – 5312.
[13] C. A. Owen, M. A. Campbell, P. L. Sannes, S. S. Boukedes, E. J. Campbell, *J. Cell. Biol.* **1995**, *131*, 775 – 789.
[14] B. Korkmaz, S. Attucci, M. A. Juliano, T. Kalupov, M. L. Jourdan, L. Juliano, F. Gauthier, *Nat. Protoc.* **2008**, *3*, 991 – 1000.
[15] E. J. Campbell, C. Owen, *J. Biol. Chem.* **2007**, *282*, 14645 – 14654.
[16] T. Kalupov, M. Brillard-Bourdet, S. Dade, H. Serrano, J. Wartelle, N. Guyot, L. Juliano, T. Moreau, A. Belaaouaj, F. Gauthier, *J. Biol. Chem.* **2009**, *284*, 34084 – 34091.
[17] A. Cobos-Correa, J. B. Trojanek, S. Diemer, M. A. Mall, C. Schultz, *Nat. Chem. Biol.* **2009**, *5*, 628 – 630.
[18] Nomenclature from: I. Schechter, A. Berger, *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157 – 162.
[19] G. Matute-Bello, C. W. Frevert, T. R. Martin, *Am. J. Physiol. Lung Cell. Mol. Physiol.* **2008**, *295*, L379 – L399.
[20] B. Starcher, I. Williams, *Lab. Anim.* **1989**, *23*, 234 – 240.
[21] E. Remold-O'Donnell, *J. Exp. Med.* **1985**, *162*, 2142 – 2155.
[22] T. Sugimori, J. Cooley, J. R. Hoidal, E. Remold-O'Donnell, *Am. J. Respir. Cell Mol. Biol.* **1995**, *13*, 314 – 322.
[23] B. Faller, M. Cadene, J. G. Bieth, *Biochemistry* **1993**, *32*, 9230 – 9235.
[24] M. L. Bartoli, A. Di Franco, B. Vagaggini, E. Bacci, S. Cianchetti, F. L. Dente, M. Tonelli, P. L. Paggiaro, *Respiration* **2009**, *77*, 265 – 272.

