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Magnetic Resonance Imaging of Protease-Mediated Lung Tissue Inflammation and Injury

Angélique Rivot, Natacha Jugniot, Samuel Jacoutot, Nicolas Vanthuyne, Philippe Massot, Philippe Mellet,* Sylvain R.A. Marque, Gérard Audran, Pierre Voisin, Marie Delles, Gilles Devouassoux, Eric Thiaudiere, Abderrazzak Bentaher, and Elodie Parzy



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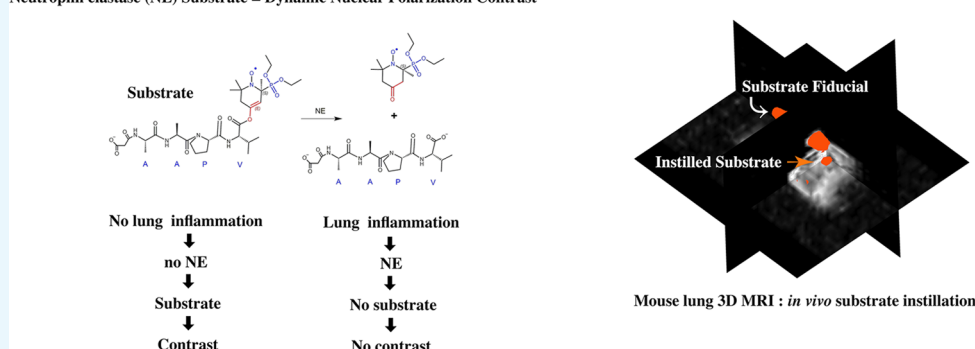


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Neutrophil elastase (NE) Substrate = Dynamic Nuclear Polarization Contrast



ABSTRACT: Pulmonary inflammation usually involves strong neutrophil recruitment with a marked release of proteases such as neutrophil elastase (NE). Noninvasive *in vivo* assessment of unregulated elastase activity in the lungs would provide a valuable diagnostic tool. Here, it is proposed to use Overhauser-enhanced magnetic resonance imaging (OMRI) in mice where inflammation was induced by the instillation of lipopolysaccharide (LPS). OMRI contrast in the lungs was generated by a dedicated NE free radical substrate. The free radical decayed more rapidly in LPS-treated mouse lungs than in control mice, indicating the occurrence of increased proteolysis under inflammation. Preclinical detection of abnormal proteolysis opens the way for new diagnosis modality and antiprotease testing *in vivo*.

INTRODUCTION

Pulmonary inflammatory diseases such as chronic obstructive pulmonary diseases (COPD) with or without exacerbations and cystic fibrosis (CF) are a major concern worldwide. A common hallmark of these pathologies is the massive influx of polymorphonuclear neutrophils (PMN) and the local extracellular discharge of proteases, particularly the readily active neutrophil elastase (NE). Patients deficient in alpha-1 antitrypsin, the main physiological inhibitor of NE, are at high risk of developing emphysema, a main component of COPD.¹ Also, abnormal concentrations of active NE are detected in CF patient lungs.² These clinical observations along with experimental animal studies strongly suggest that an imbalance of proteases and their physiological inhibitors contributes to tissue degradation and loss of lung architecture. Therapeutically, early diagnosis of overwhelming proteolysis and the use of specific inhibitors could therefore block protease-mediated disease progression. A judicious noninvasive imaging approach where diseased lungs could be specifically monitored by proteolysis imaging is sought after.

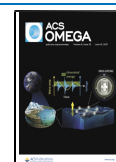
To this end, we have previously reported an original approach, namely, Overhauser-enhanced magnetic resonance imaging (OMRI)³ that could be used to assess digestive proteolysis in living mice.⁴ Next, we developed novel, stable, and nontoxic nitroxide probes whose OMRI visibility was made highly specific to NE-mediated hydrolysis.^{5,6}

The goal of this study is to assess the ability of OMRI for the molecular imaging of NE extracellular proteolytic activity in neutrophil-rich inflamed lungs of living mice. Mice were intranasally (*i.n.*) administered with endotoxin lipopolysaccharide (LPS), and their lungs were investigated/diagnosed by OMRI at various time points, following nitroxide instillation.

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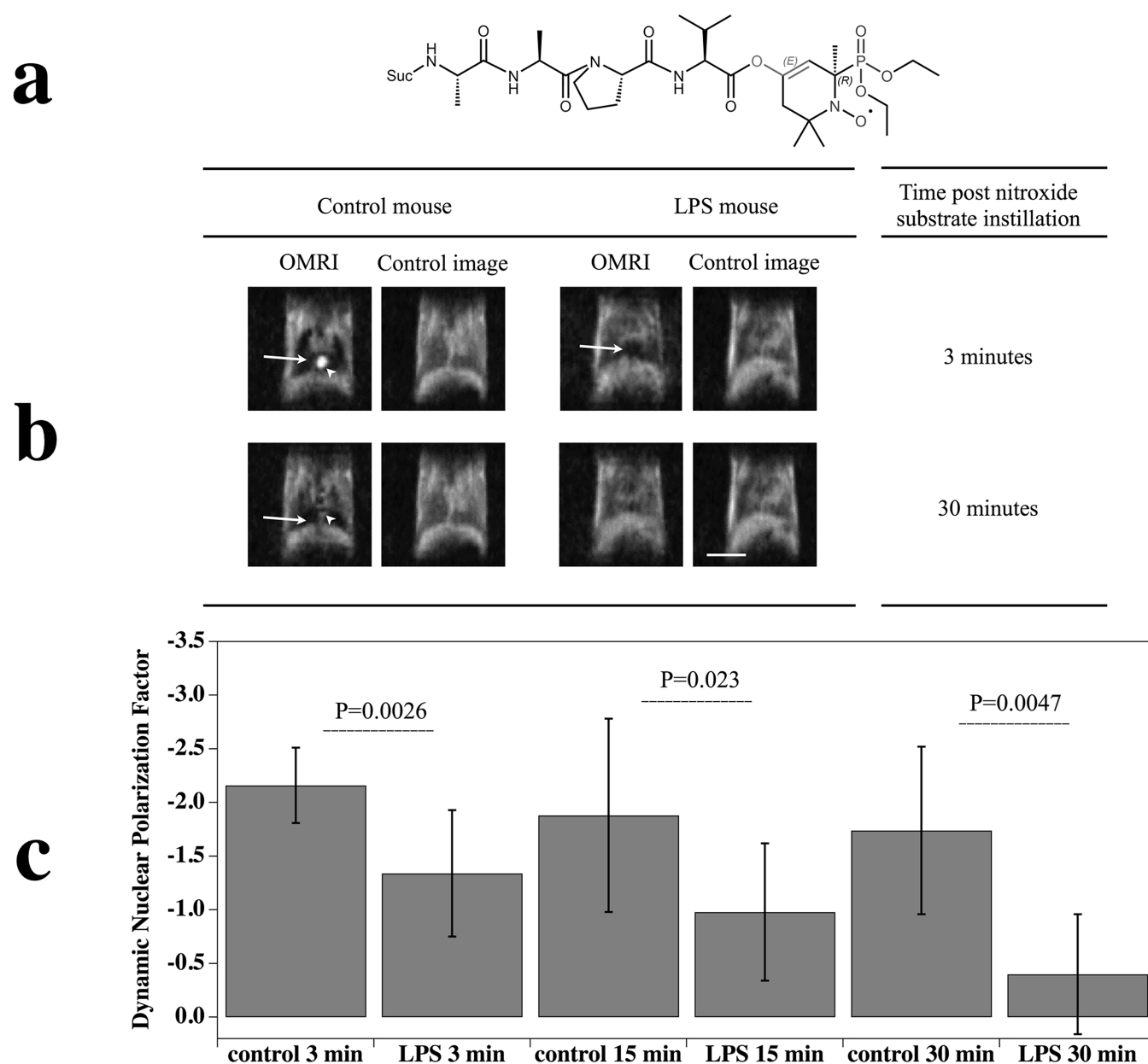


Figure 1. (a) Chemical structure of the nitroxide substrate. (b) Overhauser-enhanced chest MRI of NE substrate nitroxide in representative untreated and LPS-treated mice. Coronal images were taken 3 min and 30 min postnitroxide substrate instillation. Lung area in control images appears as a hyposignal, and the liver appears brighter in the bottom part. Arrows and arrowhead indicate the areas of effect in the presence of EPR. No significant Overhauser effect was observed in the lung of the LPS-treated mouse 30 min postinstillation. Scale bar is 1 cm. The displayed slices are extracted from 3D matrices in all cases (matrix size: $64 \times 32 \times 32$; field of view: $64 \text{ mm} \times 32 \text{ mm} \times 32 \text{ mm}$. Acquisition parameters : repetition time (TR) : 600 ms, inter echo time : 9 ms ; and number of echoes per TR : 8. (c) Maximal magnitude of the DNPF measured in mouse lungs in control and LPS-treated mice at 3, 15, and 30 min postnitroxide substrate instillation. Error bars represent standard deviation.

The kinetics of substrate decay were monitored and compared to those of unchallenged mice.

RESULTS

The NE nitroxide substrate used here was characterized in a previous study.⁶ It could be observed through OMRI in vitro with electron paramagnetic resonance (EPR) irradiation at 5426 MHz. The observed dynamic nuclear polarization factor (DNPF) was in the range of -10 at around 1 mM nitroxide, which made it attractive for further detection of NE in the context of lung inflammation. Figure 1b displays typical lung images extracted from three-dimensional (3D) matrices measured at 3 and 30 min postnitroxide administration in

unchallenged or LPS-treated mice. In the absence of EPR irradiation, the signal-to-noise ratio in the lung region was around 4, which made it possible to evaluate the Overhauser effect brought by the presence of the nitroxide substrate. Thanks to animal contention, no significant respiration artifact was observed in the field of view. When EPR was applied, either the signal was significantly increased (positive contrast, $\epsilon < -1$, e.g., at 3 min postinstillation in control mice) or decreased, (negative contrast, $-1 < \epsilon < 1$, e.g., in LPS-treated mice at 3 min postinstillation), depending on the substrate concentration. Clearly, the Overhauser effect measured in the mouse lungs did not appear homogeneous in space. This feature could be attributed to the protocol of instillation which

used a catheter under atmospheric pressure, thus preventing the production of spray. Therefore, it could be anticipated that solution droplets could not be homogeneously spread over the whole lung volume. This assumption could be somehow confirmed by the fact that positive Overhauser effects were prominently observed in the accessory lobe, as shown in Figure 1b. Importantly, positive contrasts were mainly observed in control mice at 3 min postinstillation, whereas negative contrasts or the absence of the Overhauser effect were mostly observed in LPS-treated mice, indicating faster consumption of the nitroxide substrate in the inflammatory state. Notably, a significant Overhauser effect was measured in control mice 30 min postinstillation, showing a remarkable lifetime before the clearance of the nitroxide from the lungs.

More quantitatively, the maximal magnitude of the DNPF, derived from the MRI signal and phase with and without EPR, was calculated in all mice over time. Results are shown in Figure 1c.

The decay of the magnitude of the DNPF was significantly faster in LPS-treated mice, highlighting the hydrolysis of the NE substrate in the setting of inflammation.

In a parallel experiment, PMN cell counts in bronchoalveolar lavages of control ($n = 10$) and LPS-treated ($n = 10$) mice revealed a striking neutrophil influx only for LPS-challenged mice. Thus, also in line with our previous *in vitro* studies,⁶ faster nitroxide substrate decay would be mainly attributed to the presence of PMN-derived active NE, and to a lesser extent, proteinase 3 (data not shown). Of relevance, *in vitro* EPR experiments showed that neither hydrogen peroxide nor hypochloric acid (in the millimolar range) altered succinyl-(ala)₂-pro-val-nitroxide, ruling out the *in vivo* impact of the oxidative system on the substrate (data not shown).

DISCUSSION

Despite the use of the low-field MRI system together with low gradient strength, the image quality was sufficient to permit the observation of the Overhauser effect in the mouse lung and also to calculate the DNPF in the 3D images at a millimetric resolution. A limitation of the present study was the low signal-to-noise ratio in the magnitude images, which could prevent the accurate calculation of DNPF, as seen from data spread. Fortunately, phase measurements were of critical help to unambiguously identify regions where the nitroxide was actually present. As such, data reported in Figure 1c at 15 or 30 min postinstillation in LPS-treated mice could simply be interpreted as the absence of nitroxide (no phase shift, no signal change upon EPR), which was obviously not the case in control mice. The specificity of the substrate toward NE has been previously studied.⁶ For instance, bacterial proteases such as *Pseudomonas aeruginosa* metallo-elastase that is found in CF cannot cleave the nitroxylated substrate.⁶ Clearly, the performed OMRI on inflamed mouse lungs postnitroxide substrate instillation unveiled that substrate consumption was due to active NE. It must be emphasized that while the present study focused on the OMRI detection of substrate consumption, ongoing studies aim to design a substrate that allow for the detection of this latter product as well. Nevertheless, the present results pave the way to a number of potential applications. Work is in progress to assess the sensitivity of the method on other inflammatory models, for example, a cigarette smoke exposure model. The MRI of protease-mediated pulmonary inflammatory diseases could be invaluable for testing inhibitor-based therapeutic strategies in

preclinical studies. The applicability of such molecular imaging modality to examine the protease/inhibitor balance in other inflamed organs or to target other suspected proteases with highly specific nitroxide-peptide molecules could be envisioned. Finally, translation to clinical practice awaits the development of MRI at lower fields.

MATERIALS AND METHODS

Chemicals. Succinyl-(ala)₂-pro-val-nitroxide (Figure 1a) was synthesized and purified, as already described,⁶ and dissolved in saline buffer. This nitroxide is a substrate of NE. LPS was purchased from Sigma (Saint-Quentin-Fallavier, France) and dissolved in saline buffer at a final concentration of 0.2 mg/mL.

Animal Preparation. C57BL/6 mice (female, $n = 8$ /group, 20 ± 2 g) (Janvier Labs, Le Genest Saint Isle, France) were *i.n.*-instilled with 50 μ L of saline buffer alone or 50 μ L of LPS at 0.2 mg/mL (animal study committee authorization # 2018112816448743–V12). Previous studies showed that the *i.n.* instillation of LPS at 10 μ g per mouse induced massive inflammation over the whole lung, as seen from a huge recruitment of PMN.^{7–9}

After 24 h postchallenge, mice were anesthetized with isoflurane (Virbac, France), instilled intratracheally with 70 μ L of succinyl-(ala)₂-pro-val-nitroxide, a NE substrate that is also a contrast agent for OMRI,^{5,6} at 25 mM and introduced in the MRI system at 0.2 T.³ Animal contention was operated with a dedicated bed constrained in the lung region, allowing abdominal breathing. An air-filled balloon was used to monitor respiration throughout the experiment. Animal temperature was also controlled and was maintained at 38 °C constantly.

OMRI System and Acquisition. MRI was performed with a MRI-Tech open-magnet system operating at 0.194 T and capable of 20 mT/m gradient strength (MRI-Tech, Canada Inc. Edmonton, Alberta). The system was further equipped with a tunable microwave cavity operating at ca. 5.4 GHz where living mice could be positioned.⁵ The microwave frequency used in this study, namely, 5426 MHz corresponded to the fourth (out of 6) EPR line of the nitroxide substrate observed with a classical X-band EPR spectrometer. Upon catalyzed hydrolysis, the peptide enol ester-nitroxide substrate is converted into a ketone nitroxide, the EPR lines of which are shifted.⁵ The EPR cavity was also equipped with a saddle-shaped NMR coil (28 mm in diameter) operating at 8.25 MHz. Fast spin echo 3D MRI without (control images) and with the OMRI EPR saturation of the nitroxide substrate line at 5426 MHz was acquired within 38 s at different times postnitroxide instillation: 3, 15, and 30 min. Acquisition parameters: repetition time (TR): 600 ms, inter echo time: 9 ms; number of echoes per TR: 8; matrix size: $64 \times 32 \times 32$; and field of view: $64 \times 32 \times 32$ mm. In order to avoid unwanted heating, EPR saturation was applied for 19 s, corresponding to the central lines of the k -space.

Data Processing. Magnitude and phase images were used to calculate the DNPF defined as follows:

$$\text{DNPF} = \varepsilon - 1 \text{ with } \varepsilon = \frac{\langle I_z \rangle}{I_0}$$

where $\langle I_z \rangle$ and I_0 are the proton polarizations with and without EPR saturation, respectively. In liquids, where the electron-proton coupling factor is usually positive and lower or equal to 0.5, ε could theoretically range from 1 (no Overhauser

effect) to -55 , depending on nitroxide concentration and taking into account the number of EPR lines. The negative values of ε were assessed by observing a phase shift of π in the phase image.

For each time postinstillation, the ratio of the image signal was calculated to derive the value of ε in every pixel, taking into account the phase shift. Prior to this calculation, a mask was generated by thresholding the signal at four times the standard deviation of the noise measured in air.

Statistical Analysis. For each postinstillation time, the DNPF was compared between the control and LPS-treated mice using a nonparametric paired Wilcoxon rank test. Significant difference was considered for $P < 0.05$.

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Author Contributions

A.R. performed animal preparation, MRI developments, and MRI data collection and analyses. N.J. performed EPR data collection and analyses. S.J. performed chemical synthesis. N.V. performed enantiomer purification. P.M. was responsible for the MRI setup. P.M. helped design the study and edited the manuscript. S.R.A.M. and G.A. designed the chemical probes.

P.V. helped design the study. M.D. was in charge of the bronchoalveolar lavages. G.D. helped design the study and edited the manuscript. E.T. helped design the study, performed MRI analysis, drafted, and edited the manuscript. A.B. provided the animal model and edited the manuscript. E.P. helped design the study and performed MRI data collection. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

CF: cystic fibrosis
COPD: chronic obstructive pulmonary disease
DNPF: dynamic nuclear polarization factor
LPS: lipopolysaccharide
NE: neutrophil elastase
OMRI: Overhauser magnetic resonance imaging
ROS: reactive oxygen species

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