Inhibition of Human Neutrophil Elastase-Induced Acute Lung Injury in Hamsters by Recombinant Human Pre-elafin (Trappin-2)*

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Study objectives: Pre-elafin, also known as trappin-2, is an elastase-specific inhibitor that could be an ideal candidate for the treatment of neutrophil elastase-driven lung diseases. The inhibitory activity of pre-elafin resides in the COOH-terminal region that can be released as mature elafin. The NH2-terminal moiety of pre-elafin is characterized by the presence of a specific repeating sequence, termed cementoin, believed to immobilize the inhibitor to lung protein components and restrict its diffusion from the desired sites of action. This property should confer an advantage to pre-elafin compared to elafin in the treatment of neutrophil elastase-driven lung diseases.

Measurements: The inhibitory effect of recombinant human pre-elafin was assessed in a human neutrophil elastase-induced acute lung injury model in Golden Syrian hamsters. BAL fluid hemoglobin content was used as a marker of lung injury.

Results: Recombinant human pre-elafin administered intratraehally 1 h prior to neutrophil elastase dose-dependently inhibited the lung hemorrhage with a calculated half-effective dose of 8.1 μg/kg (0.7 nmol/kg). Pre-elafin was equally efficient when administered 3 h before neutrophil elastase. In contrast to pre-elafin, commercial synthetic elafin was ineffective in inhibiting neutrophil elastase-induced lung hemorrhage even at a dose of 4.45 nmol/kg.

Conclusions: Our results suggest that pre-elafin may be eventually used in the treatment of neutrophil elastase-driven lung diseases.

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Key words: BAL fluid; hamsters; leukocyte elastase; lung; pulmonary emphysema; recombinant fusion proteins; serine proteinase inhibitors

Abbreviations: ED50 = half-effective dose; mOD = milli-optical density; SLPI = secretory leukocyte protease inhibitor

Neutrophil elastase (EC 3.4.21.37) is a potent serine protease involved in host defense. However, neutrophil elastase is a double-edged sword that, if uncontrolled, can degrade numerous host extracellular matrix components, especially elastin, and jeopardize the lung fragile architecture.1 α1-Antitrypsin, secretory leukocyte protease inhibitor (SLPI), and elafin are endogenous elastase inhibitors present in the peripheral lung that normally protect tissue from such unwanted elastolysis.2,3 A tight balance between elastase and its inhibitors is the cornerstone of the elastase-antielastase hypothesis.4,5 According to this hypothesis, an inefficient antineutrophil elastase protection contributes to the pathogenesis of numerous lung diseases, including emphysema, chronic bronchitis, and cystic fibrosis.1,6–8

A logical strategy to boost the overwhelmed natural antielastase system is augmentation therapy by administering a neutrophil elastase inhibitor. This is done in premature emphysema resulting from α1-antitrypsin deficiency, a genetic disorder affecting 1 in 1,600 to 1,800 live births in most Northern European and North American populations.9 De-
spite its clinical success,\textsuperscript{10} the administration of purified human $\alpha_1$-antitrypsin (Prolastin; Bayer Corporation; West Haven, CT) is problematic because the supply cannot currently meet patient demand.\textsuperscript{11} As an alternative to $\alpha_1$-antitrypsin, other neutrophil elastase inhibitors are being designed and evaluated.\textsuperscript{12} The rationale for targeting neutrophil elastase with such inhibitors in the treatment of different lung diseases has been reviewed extensively.\textsuperscript{12,13}

Pre-elafin, also known as trappin-2, is a 117-amino acid (including a 22-amino acid signal peptide) elastase-specific inhibitor. Pre-elafin is composed of two structurally distinct COOH-terminal and NH$_2$-terminal regions, each responsible for a specific function.\textsuperscript{14} The antielastase activity of pre-elafin resides in a four-disulfide bridge core in the COOH-terminal region that can be proteolytically released as mature elafin. The NH$_2$-terminal moiety of pre-elafin is characterized by the presence of a specific repeating sequence rich in glutamine and lysine residues termed cementoin.\textsuperscript{15} In addition to neutrophil elastase, pre-elafin and elafin inhibit pancreatic elastase, proteinase 3, and endogenous vascular elastase, but not other serine proteases such as trypsin, chymotrypsin, cathepsin G, plasmin, and granzymes.\textsuperscript{16–22} Theoretically, the properties of pre-elafin make it an ideal candidate for the treatment of neutrophil elastase-driven lung diseases.\textsuperscript{14,23,24} Of special interest is the presence of the transglutaminase NH$_2$-terminal domain (cementoin) that could act as molecular glue by immobilizing the inhibitor to lung protein components and restricting its diffusion from the desired sites of action.\textsuperscript{15} This property should confer an advantage to pre-elafin compared to elafin in the treatment of neutrophil elastase-driven lung diseases. We have recently produced fully active full-length human pre-elafin, and showed that it compares favorably to elafin in inhibiting human neutrophil elastase in vitro.\textsuperscript{25} We hypothesized that recombinant human pre-elafin might show a therapeutic potential in the treatment of neutrophil elastase-driven lung diseases. Moreover, we postulated that pre-elafin would be more potent than elafin in this regard. In the present study, we have evaluated the effect of recombinant human pre-elafin on human neutrophil elastase-induced acute lung injury in hamsters and compared this to the effect of elafin.

Materials and Methods

Materials

Recombinant human pre-elafin was produced in a yapsin 1-deficient strain of yeast as we described previously.\textsuperscript{25} Synthetic human elafin and human neutrophil elastase were purchased from Peptide Institute (Osaka, Japan) and Elastin Products Company (Owensville, MO), respectively. The chromogenic substrate N-methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide was obtained from Sigma Chemical Company (St. Louis, MO).

Human Neutrophil Elastase-Induced Acute Lung Injury

The experimental model used in the present study has been described extensively.\textsuperscript{26–28} Briefly, male Syrian Golden hamsters (mean ± SEM weight, 110.2 ± 1.0 g), obtained from Charles River Canada (Saint-Constant, QC, Canada), were anesthetized with ketamine, 87 mg/kg, and xylazine, 13 mg/kg, intraperitoneally. Animals were endotracheally intubated by direct laryngoscopy with a 1.3 × 48-mm IV catheter (Inysye; Becton Dickinson; Sandy, UT), as previously described.\textsuperscript{29} The hamsters were instilled with the indicated doses of inhibitors, dissolved in sterile 0.9% NaCl in a final volume of 100 μL, 1 to 12 h before receiving 100 μL of human neutrophil elastase (500 μg/mL in sterile 0.9% NaCl) via the same route. Fewer animals were used for the highest doses of inhibitors because of the quantity of material requested. Control animals received twice the vehicle only, while one group received saline solution and then neutrophil elastase. No animals died from the interventions. All of the animals were killed with pentobarbital, 70 mg/kg, intraperitoneally 3 h after the last instillation, and BAL was performed with 2.5 mL of saline solution repeatedly instilled three times.\textsuperscript{26} The hemoglobin content of BAL fluid was quantified as a marker of acute lung injury.\textsuperscript{26} All animal experimentation was approved by the Comité de Protection des Animaux de l’Université Laval.

Elafin and Pre-elafin Inhibitory Assays

The functional inhibitory activity of pre-elafin and elafin was assessed by measuring the residual hydrolytic activity of human neutrophil elastase after incubation with the inhibitors, as previously described.\textsuperscript{25} Briefly, 20 pmol of neutrophil elastase and 20 pmol of pre-elafin or elafin were incubated in a final volume of 20 μL of Tris-NaCl buffer (0.1 mol/L Tris, 0.5 mol/L NaCl, pH 7.5) at 37°C for 15 min. Then, 18 μL of this mixture was added to 100 μL of 0.2 mM N-methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide in Tris-NaCl buffer in 96-well ELISA plate. Kinetic changes in absorbance were measured at 405 nm every minute for 10 min with a microplate reader (THERMOMax; Molecular Devices; Sunnyvale, CA).

Statistical Analysis

All results are expressed as mean ± SEM. Comparisons were performed using analysis of variance followed by a Student-Newman-Keuls test. In some cases, when SDs were proportional to the means, a logarithmic transformation of the raw data was done prior to the analysis of variance to equalize the variances.\textsuperscript{20} In all cases, $p < 0.05$ was considered significant.

Results

Potency of Recombinant Human Pre-elafin To Inhibit Neutrophil Elastase-Induced Acute Lung Injury

The potency of recombinant human pre-elafin to inhibit neutrophil elastase-induced acute lung injury was first evaluated by administrating different doses of the inhibitor. The instillation of neutrophil elastase induced a massive lung hemorrhage resulting in

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a BAL hemoglobin content of 294.5 ± 74.4 milli-optical density (mOD). In contrast, sham-exposed animals only showed minimal damages (12.8 ± 1.8 mOD). Pre-elafin, administered intratracheally 1 h before the instillation of neutrophil elastase, dose-dependently inhibited the acute lung injury as assessed by measuring the BAL content of hemoglobin 3 h after neutrophil elastase (Fig 1). Doses of pre-elafin of 0.1, 1, 10, 25, and 250 µg/kg inhibited the lung hemorrhage by 18.5%, 27.3%, 59.4%, 59.9%, and 73.2%, respectively. From data shown in Figure 1, we calculated a half-effective dose (ED50) of pre-elafin of 8.1 µg/kg (0.7 nmol/kg). For comparison, the ED50 of pre-elafin is shown in Table 1, together with published ED50 of other neutrophil elastase inhibitors also assessed in human neutrophil elastase-induced lung hemorrhage in Golden Syrian hamsters. Since the dose of 10 µg/kg was as effective as higher doses in the dose-response study (Fig 1), it was therefore chosen for the following studies.

We next addressed the question of whether the treatment was effective when administered at longer intervals before neutrophil elastase instillation. To do so, hamsters were instilled with pre-elafin, 10 µg/kg, at 12, 6, 3, and 1 h before neutrophil elastase instillation. As shown in Figure 2, pre-elafin instilled 3 h before neutrophil elastase was as effective as when instilled 1 h before resulting in BAL hemoglobin contents of 128.2 ± 41.8 mOD and 107.0 ± 64.6 mOD, respectively. On the contrary, animals receiving pre-elafin 6 h before neutrophil elastase showed a significantly more pronounced hemorrhage (227.8 ± 70.4 mOD), while those instilled with pre-elafin 24 h before neutrophil elastase had a BAL hemoglobin content of 315.8 ± 27.2 mOD, similar to those receiving neutrophil elastase alone (Fig 1).

**Comparison of the Potency of Pre-elafin and Elafin to Inhibit Neutrophil Elastase-Induced Acute Lung Injury**

We showed that a dose of 10 µg/kg of recombinant human pre-elafin was effective in inhibiting neutrophil elastase-induced lung hemorrhage when administered 1 h prior to the instillation of the enzyme. This dose represents an instillation of 0.59 nmol/kg of the recombinant inhibitor based on a molecular weight of 11.273 kd. We were then interested to test whether elafin was also effective using the same protocol. Hamsters were instilled with recombinant human pre-elafin, 10 µg/kg; an equimolar dose of commercial synthetic elafin, 5.3 µg/kg (0.89 nmol/kg); or a fivefold higher dose of commercial synthetic elafin, 26.5 µg/kg (4.45 nmol/kg). Only the treatment with recombinant human pre-elafin resulted in a significant decrease of the BAL hemoglobin content compared to neutrophil elastase alone (Fig 3). We had previously shown that recombinant human pre-elafin compared favorably to commercial synthetic elafin to inhibit neutrophil elastase in vitro.\(^{25}\) To eliminate the possibility that the synthetic elafin preparation used for the animal study was not active, we compared several frozen aliquots of the same batch of recombinant human pre-elafin and commercial synthetic elafin used in the animal study. We consistently showed that commercial synthetic elafin was as effective as recombinant human pre-elafin in inhibiting neutrophil elastase in vitro (Fig 4).

**DISCUSSION**

As one of the most abundant and destructive enzymes in the human body, neutrophil elastase is believed to play an important role in numerous lung diseases. Not surprisingly, the search for potent neutrophil elastase inhibitors, including native inhibitors or their recombinant counterparts, is a very active field.\(^{12,24,31}\) For instance, purified human α1-antitrypsin is used in the treatment of α1-antitrypsin deficiency.\(^{10}\) Recombinant SLPI aerosolized to normal volunteers increases the antineutrophil elastase activity in BAL fluid and suppresses the neutrophil elastase burden in individuals with cystic fibrosis.\(^{32}\)

![Figure 1](http://journal.publications.chestnet.org/pdfaccess.ashx?url=/data/journals/chest/21973/ on 06/21/2017)
Currently, SLPI is in phase II clinical trials for cystic fibrosis. However, the potency of pre-elafin to treat lung diseases involving neutrophil elastase has never been evaluated despite specific properties making it a very attractive candidate for such treatment.

The present study demonstrates that recombinant human pre-elafin exerts a significant protective effect against neutrophil elastase-induced acute lung injury in hamsters. This effect was characterized by a dose-dependent decrease in the BAL fluid hemoglobin content of treated animals, most likely due to the inactivation of instilled elastase. In contrast to pre-elafin, elafin did not show such a protective effect.

We calculated an ED50 of 8.1 μg/kg (0.7 nmol/kg) for recombinant human pre-elafin. Beside elafin, we did not directly compare pre-elafin to other neutrophil elastase proteinaceous inhibitors. Using exactly the same model, others have determined an ED50 of 35 μg/kg (6.3 nmol/kg) for truncated (58Arg-107Ala) SLPI, which represents a ninefold higher dose than pre-elafin on a molar basis. In a similar model, in which α1-antitrypsin was administered via the same route but 5 min prior to neutrophil elastase, others consistently showed an ED50 of approximately 2.85 mg/kg (54.8 nmol/kg). This represents a

![Figure 2](http://journal.publications.chestnet.org/pdfaccess.ashx?url=/data/journals/chest/21973/)  
**Figure 2.** Time-dependent effect of recombinant human pre-elafin on human neutrophil elastase-induced acute lung injury in hamsters. Animals received an intratracheal instillation of recombinant human pre-elafin, 10 μg/kg, at the indicated times before the instillation of neutrophil elastase, 50 μg. Each value is the mean ± SEM of the BAL hemoglobin content. *Significantly different from 12 h; **Significantly different from 12 h and 6 h (Student-Newman-Keuls test, p < 0.05). See Figure 1 legend for expansion of abbreviations.

![Figure 3](http://journal.publications.chestnet.org/pdfaccess.ashx?url=/data/journals/chest/21973/)  
**Figure 3.** Comparative effect of recombinant human pre-elafin and commercial synthetic elafin on human neutrophil elastase-induced acute lung injury in hamsters. Animals received an intratracheal instillation of recombinant human pre-elafin, 10 μg/kg (0.89 nmol/kg); elafin, 5.3 μg/kg (0.89 nmol/kg); or fivefold higher dose (5X) of elafin, 26.5 μg/kg (4.45 nmol/kg) 1 h before the instillation of neutrophil elastase, 50 μg. Each value is the mean ± SEM of the BAL hemoglobin content. *Significantly different from human neutrophil elastase only (Student-Newman-Keuls test, p < 0.05). See Figure 1 legend for expansion of abbreviations.

### Table 1—Published ED50 of Different Neutrophil Elastase Inhibitors in Human Neutrophil Elastase-Induced Lung Hemorrhage in Golden Syrian Hamsters

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Molecular Weight, daltons</th>
<th>ED50, μg/kg</th>
<th>ED50, nmol/kg</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEI-8362†</td>
<td>541</td>
<td>45</td>
<td>83.3</td>
<td>Mitsuhashi et al 34</td>
</tr>
<tr>
<td>α1-Antitrypsin†</td>
<td>52,000</td>
<td>2850</td>
<td>54.8</td>
<td>Shinguh et al 26; Fujie et al 28; Shinguh et al 33</td>
</tr>
<tr>
<td>FK706†</td>
<td>593</td>
<td>30</td>
<td>50.6</td>
<td>Shinguh et al 26</td>
</tr>
<tr>
<td>FR1340433†</td>
<td>1,166</td>
<td>39</td>
<td>33.8</td>
<td>Shinguh et al 33</td>
</tr>
<tr>
<td>FR901277†</td>
<td>962</td>
<td>19</td>
<td>19.7</td>
<td>Fujie et al 28</td>
</tr>
<tr>
<td>Half SLPI†</td>
<td>5,514</td>
<td>35</td>
<td>6.3</td>
<td>Mitsuhashi et al 27</td>
</tr>
<tr>
<td>Pre-elafin†</td>
<td>11,273</td>
<td>8</td>
<td>0.7</td>
<td>This study</td>
</tr>
</tbody>
</table>

*In all studies, human neutrophil elastase (50 μg) was administered intratracheally to Syrian Golden hamsters, and the animals were killed 3 h later. Hemoglobin content was then determined in BAL fluid as described in the “Materials and Methods” section. Neutrophil elastase inhibitors were either instilled via the intratracheal route 1 h(†) or 5 min(‡) prior to neutrophil elastase.
78-fold higher dose than pre-elafin on a molar basis. It has been suggested\textsuperscript{26} that low-molecular-weight synthetic inhibitors would have an advantage over proteinaceous inhibitors. However, recombinant human pre-elafin appears to be 30-fold to 120-fold more potent on a molar basis (two to sixfold more potent on a weight basis) than small synthetic inhibitors (molecular weight, 541 to 1,166 d) such as FK706, FR134043, FR901277, and TEI-8362, based on published studies\textsuperscript{26,28,33,34} using a similar experimental model. Therefore, the potency of recombinant human pre-elafin to inhibit neutrophil elastase-induced lung hemorrhage compares more than favorably to the potency of other neutrophil elastase inhibitors either proteinaceous or synthetic.

The experimental model of acute lung injury used in the present study has been well described.\textsuperscript{26–28} The main advantage of this commonly used model is that it allows the rapid evaluation of the capacity of any given elastase inhibitor to efficiently inhibit neutrophil elastase in the complex setting of the lung environment. We are well aware of the limitations of this model. For instance, the quantification of other parameters in BAL fluid is meaningless considering the massive contamination by peripheral blood cells and proteins. Also, it is believed that the assessment of the ability of an elastase inhibitor to suppress neutrophil elastase-induced pulmonary hemorrhage is not predictive of its ability to prevent emphysema.\textsuperscript{35} Finally, a single instillation of human neutrophil elastase only elicits slight changes in pulmonary function,\textsuperscript{28} precluding any determination of this important physiologic readout in the evaluation of the tested inhibitor potency. Despite the above-mentioned limitations, we firmly believe that the neutrophil elastase-induced acute lung injury model gives valuable information and serves as a stepping stone to further investigations on the therapeutic potential of pre-elafin.

It has been suggested that evaluating the potency of a neutrophil elastase inhibitor to inhibit elastase-induced lung damage is inappropriate since such an inhibitor will obviously inhibit the enzyme.\textsuperscript{27} However, our results clearly showed that the evaluation of the inhibitory potential of an inhibitor \textit{in vitro} does not predict the outcome in the \textit{in vivo} situation. Indeed, while pre-elafin and elafin are comparable in their ability to inhibit neutrophil elastase \textit{in vitro} (this article and Bourbonnais and colleagues\textsuperscript{25}), pre-elafin clearly is more potent than elafin once instilled in the lung. At the moment, we can only speculate on the mechanisms explaining the observed difference in the respective \textit{in vivo} potency of pre-elafin and elafin. This could be due to a rapid inactivation of free mature elafin. In support of this concept is the observation that no inhibitory activity attributable to elafin was detected in human BAL fluid,\textsuperscript{36} despite the obvious presence of elafin in this biological fluid.\textsuperscript{2} In contrast, pre-elafin, being attached to extracellular matrix components via its cementoin domain,\textsuperscript{15} may avoid such inactivation process and remain active. Alternatively, elafin may bind to cells. Indeed, Efremov and colleagues\textsuperscript{37} have shown that the 15-amino acid N-terminal extremity of elafin, which has no defined spatial structure in crystal nor in solution models, forms a short α-helix region once in a membrane-like environment and might act as a cell membrane anchor. Such binding could be part of a clearance process. We are currently directing our efforts to elucidate these important questions.

In the present study, we did not compare the effectiveness of intratracheal instillation of recombinant human pre-elafin to its systemic (IV) administration. As others, we believe that the most interesting approach to deliver a neutrophil elastase inhibitor in human is aerosolization. This requires considerably less inhibitor than systemic administration, allows a more efficient delivery of active inhibitor, and is easier to administer to patients.\textsuperscript{38,39} In order to mimic an eventual administration of pre-elafin to the lung in humans, intratracheal administration was used in the present study to assess the therapeutic potential of pre-elafin to protect from pulmonary hemorrhage.
neutrophil elastase-induced acute lung injury. Considering the difference in bioavailability and deposition between intratracheal instillation and aerosolization, which has been shown for many therapeutic agents, the effect of aerosolized pre-elafin will also have to be evaluated in animals.

In summary, our results are the first to show a protective effect of pre-elafin on neutrophil elastase-induced acute lung injury in hamsters. This suggests that pre-elafin may be eventually used in the treatment of neutrophil elastase-driven lung diseases. In addition, pre-elafin could be used as a tool for studying the role of neutrophil elastase in other diseases of the lung or other organs.

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