Effect of Human Neutrophil Elastase on Tracheal Mucociliary Transport in Anesthetized Quails

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Received June 30, 1997 Accepted October 2, 1997

ABSTRACT—We investigated the effect of human neutrophil elastase (HNE) on tracheal mucociliary transport in anesthetized quails. Topical application of HNE (30–300 μg/kg) to tracheal mucosa dose-dependently decreased mucociliary transport velocity (MCTV). The HNE (300 μg/kg)-induced decrease in MCTV was blocked by ONO-5046 ¥Na (sodium N-[2-[4-(2,2-dimethylpropionyloxy)phenyl-sulfonylamino]-benzoyl]aminoacetate tetrahydrate) (3–30 mg/kg, i.m.), a specific neutrophil elastase inhibitor. Furthermore, we found that HNE increased DNA, fucose and protein contents of tracheal lavages, and the increases were also reverted by ONO-5046 ¥Na. These results indicated that HNE decreased tracheal mucociliary transport, and the decrease may be, at least in part, ascribed to the deterioration of tracheal secretions.

Keywords: Tracheal mucociliary transport, Tracheal secretion, Human neutrophil elastase

Airway inflammation and obstruction are prominent features in chronic obstructive pulmonary diseases (COPD). In the patients with COPD, both the increase in mucus cells and decrease in ciliated cells, together with the associated increase in viscous secretions, result in a marked impaired mucociliary transport responsible for airway obstruction (1). A large number of neutrophils and high concentrations of active proteases are known to exist in the sputa of patients with COPD (2). Many of the pathological changes present in human COPD can be reproduced in animals by intratracheal administration of lysosomal enzymes (3) or by neutrophil recruitment into the tracheobronchial tree (4). Recent studies stressed the potential importance of neutrophil and mast cell proteases in stimulating secretion from airway gland serous cells (5). These studies indicate that neutrophil elastase, cathepsin G and chymase are the most potent secretagogues known. So far, there are several in vitro reports that porcine pancreatic elastase impaired mucociliary transport (6), but little evidence has been presented regarding the in vivo effect of human neutrophil elastase (HNE) on mucociliary transport. Accordingly, in the present study, we examined in vivo whether HNE affected tracheal mucociliary transport velocity (MCTV).

Male quails, weighing 100–120 g, obtained from Kyudo Co., Ltd. (Fukuoka), were anesthetized with 20% urethane given intraperitoneally at a dose of 1 g/kg body weight (7). The anesthetized quail was fixed on its back and then the feathers on the larynx were cut off. The neck skin was incised along the frontal median line, and the traumatic margins were clipped with two celpins to keep the incision slightly open. The local blood vessels and connective tissues were carefully separated from the trachea to expose the trachea. A thread was laid under the exposed trachea so that the trachea could be taken up to make the following operation easy. An approximately 4 x 3 mm² of aperture was made in the trachea with a burned surgical blade. As soon as the operation was over, the quail was inserted into an observation box. In the observation box, the quail trachea was kept under about 38°C and approximately 100% relative humidity by use of a constant humidity generator (AHC-1; ACE Scientific Laboratory, Fukuoka). Ash powders made from paper ash were placed on the caudal side of the tracheal mucosa to select the site where the powders were carried at the fastest speed, and MCTV was measured at the same site throughout the experiment. When a steady MCTV was achieved, the time taken for the powder to move 2 mm was measured.

HNE (elastase, from human sputum, 875 units/mg
protein) was obtained from Elastin Products Co., Inc. (Owensville, MO, USA). The HNE stock was dissolved in phosphate-buffered saline without Ca$^{2+}$ and Mg$^{2+}$ (PBS(--), PH 7.4) and used to prepare solutions of the appropriate concentrations. In the observation box, a 2-$\mu$l aliquot of HNE solution was splashed directly to the tracheal mucosa through a 21-gauge needle with a plastic sprayer on its tip, by applying 0.25 kg/cm$^2$ of air pressure for 30 msec. The application of air pressure was switched on by an electro-magnetic valve controlled by an electronic stimulator (SEN-3201; Nihon Kohden, Co., Ltd., Tokyo). In sham-treated animals, PBS(--) solution was applied. HNE or PBS(--) solution was applied 15 min after the MCTV measurement started. ONO-5046 • Na (sodium N-[2-[4-(2,2-dimethylpropionyloxy)phenyl-sulfonylamino]benzoyl]aminooacetate tetrahydrate; Ono Pharmaceutical Co., Ltd., Osaka), a specific neutrophil elastase inhibitor (8), was dissolved freshly in 0.12% Na$_2$CO$_3$ solution (PH 7.8) and administered into the femoral muscle 15 min before HNE or PBS(--) solution was applied. MCTV was measured for 75 min through a microscope in each experiment. The results were calculated by the following equation: \(\frac{V_t}{V_o} \times 100\%\) (\(V_t\) = the average velocity in every 5 min and \(V_o\) = the average velocity in the last 5 min before application).

To know whether HNE affected the components of tracheal secretions, the tracheal secretions were analyzed by the following method: In the observation box, HNE (300 $\mu$g/kg) or PBS(--) solution was applied directly to the tracheal mucosa 30 min after the trachea was exposed as described above. ONO-5046•Na solution was administered into the femoral muscle 15 min before HNE or PBS(--) solution was applied. The quail was taken out from the observation box 25 min after the topical application. The trachea was knotted tightly with the thread 10 mm below the aperture. A tracheal segment from the thread knot to the aperture was disconnected and removed from the quail. The tracheal secretion in the segment was collected by tracheal lavage with 200 $\mu$l of PBS(--) solution. Insoluble materials in the tracheal lavage sample were precipitated by centrifugation. A 100-$\mu$l aliquot of the supernatant was mixed immediately with 100 $\mu$l of phenol solution to avert further degradation of the DNA, and 100 $\mu$l of the supernatant was stored at -30°C for fucose and protein analyses. DNA was isolated from each lavage sample by two series of phenol extraction, precipitated with ethanol and then redissolved in 70 $\mu$l of distilled water. The DNA content was determined at 260 nm by spectrophotometry (Gene Quant; Amersham Pharmacia Biotech, Uppsala, Sweden).

The fucose content was quantitated by the Gibbons method (9), and the protein was assayed by the Bradford method (10).

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**Fig. 1.** Effect of topically applied HNE on mucociliary transport velocity (MCTV) in anesthetized quails. Values are expressed as percentages of the respective preapplication values. Each point and vertical bar represent a mean value and S.E. of six animals. ○: Control, △: Vehicle (PBS(--)), ●: 30 $\mu$g/kg of HNE, ▲: 100 $\mu$g/kg of HNE, ■: 300 $\mu$g/kg of HNE. *P<0.05, **P<0.01, statistically significant difference from the vehicle value (ANOVA).

All the values were expressed as means±S.E. Statistical analyses were performed by Fisher PLSD (ANOVA), and a P value <0.05 was considered significant.

The application of 30 to 300 $\mu$g/kg of HNE to the tracheal mucosa significantly decreased MCTV in a dose-dependent manner, whereas PBS(--) had little effect on MCTV (Fig. 1). The HNE-induced decrease reached its maximum 25 min after application and gradually reverted. The decrease induced by 300 $\mu$g/kg of HNE was dose-dependently inhibited by pretreatment with 3–30 mg/kg of ONO-5046•Na, while ONO-5046•Na alone showed no effect on MCTV (Fig. 2).

The DNA, fucose and protein contents in the tracheal lavages from the HNE (300 $\mu$g/kg)-treated group were significantly higher than those from the PBS(--) control group (Table 1). Moreover, the HNE-induced increases in DNA, fucose and protein contents were significantly reverted by ONO-5046•Na, whereas ONO-5046•Na itself had little effect on the contents.

Mucociliary clearance mainly depends on the frequency and coordination of ciliary beat and the components in airway secretions (e.g., DNA, mucins, etc.). Impaired mucociliary clearance induced by hypersecretion, which may be caused by secretagogues, is a typical characteristic of COPD (1, 11). The secretagogues are mainly the pro-
teases released from the mast cells and neutrophils. It has been demonstrated in both animals and humans that in vitro proteases (e.g., HNE, cathepsin G, etc.) can inhibit ciliary beat frequency and stimulate airway mucin secretion (5, 12). In the present study, we observed that 300 pg/kg of HNE significantly increased DNA, fucose and protein contents in the tracheal secretions. In the same model, 30–300 μg/kg of HNE significantly decreased MCTV.

In a preliminary experiment, to determine the HNE concentration in the tracheal secretions after administration, we collected the secretions from 30 mm of trachea because about 30 mm of trachea might be involved in administration of HNE. The secretions weighed about 42.0 mg. So, the HNE concentration in the tracheal secretions of the quails administered with 100 μg/kg was about 23.8 μg/100 μl, which is similar to the HNE concentration (25.4 μg/100 μl) in the sputa of the COPD patients (2). Therefore, as in humans with COPD, the delayed mucociliary transport in this experiment may be partly ascribed to the hypersecretion.

We also found that 30 mg/kg of ONO-5046·Na significantly abolished the HNE-induced decrease in MCTV and that ONO-5046·Na decreased HNE-induced increases in DNA, fucose and protein contents. In addition, our previous results in a primary culture of hamster tracheal epithelial cells (13) showed that HNE increased mucus secretion, and the increase was significantly attenuated by ONO-5046·Na. Taken together, it is probable that ONO-5046·Na may block the HNE-induced decrease in MCTV partly through its inhibitory effect on the HNE-induced increase in macromolecule secretion.

Our previous results showed that DNA at concentrations of 1, 3 and 10 μg/ml remarkably decreased MCTV and that a close correlation between DNA concentration and MCTV was demonstrated (data not shown). Based on the present results that HNE increased DNA content accompanied with a decrease in MCTV, it seems to us that DNA may play an important role in mucociliary transport among airway secretion components.

### Table 1. Effect of ONO-5046·Na on HNE-induced increases of DNA, fucose and protein contents in tracheal secretions of anesthetized quails

<table>
<thead>
<tr>
<th>Dose</th>
<th>Contents (μg/T.S.)</th>
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<tbody>
<tr>
<td></td>
<td>DNA</td>
</tr>
<tr>
<td>PBS (−)</td>
<td>0.97±0.10</td>
</tr>
<tr>
<td>PBS (−) ONO-5046·Na 30 mg/kg</td>
<td>0.99±0.04</td>
</tr>
<tr>
<td>HNE 300 μg/kg</td>
<td>2.31±0.13**</td>
</tr>
<tr>
<td>HNE ONO-5046·Na 300 μg/kg</td>
<td>1.13±0.02††</td>
</tr>
</tbody>
</table>

The tracheal lavage was collected 25 min after the HNE treatment and 40 min after ONO-5046·Na pretreatment, respectively. The DNA, fucose and protein contents are expressed as μg/10 mm of tracheal segment (T.S.). Each value represents the mean±S.E. of 5 animals. **P<0.01 vs PBS(−) group. †P<0.05, ‡P<0.01 vs HNE group.
DNA in airway secretions may originate from inflammatory cells, other exfoliated cells and infecting organisms. Since neutrophil elastase has been reported to cause cytotoxic changes and even cause cell death (12), the high DNA content in tracheal secretions may be mainly attributed to a massive release of DNA from the HNE caused disintegrated cells and a large scale accumulation of DNA in the deteriorated tracheal secretions. DNA persistence in the viscous tracheal secretions resulted from the difficulty of eradicating its complexes, which were associated with other molecules such as phospholipids, may also contribute to the high DNA content in the secretions. In the scanning electronic microscopic analysis, however, we did not find the exfoliated epithelial cells in the HNE (300 μg/kg)-treated group, although we found the damaged ciliary cells in the HNE (1 mg/kg)-treated group (data not shown). Therefore, at present, it remains unclear what is the origin of DNA in the HNE (300 μg/kg)-treated group. Since some investigators have found in vitro that HNE stimulates mucin secretion by goblet cells and submucosal gland cells in both animals and humans (1, 14), the high contents of fucose and protein in the tracheal secretions may be explained by the hypersecretion of mucin.

In conclusion, we demonstrated that HNE decreased the mucociliary transport function, which may be caused by excessive release or secretion, accumulation and persistence of macromolecules, especially DNA. Other investigators have found that neutrophil elastase is present in noninfected human sputum but is enzymatically inactive, and that during infection, neutrophil elastase concentration is significantly increased with its enzymatic activity only partly inactivated (2, 15). Therefore, if HNE can partly contribute to the impaired mucociliary transport function, neutrophil elastase inhibitors such as ONO-5046 Na may possess some preventive role in the impaired function. With respect to human COPD, however, the significance of the present results in quails remains to be tested by experiments involving patients and normal subjects.

REFERENCES