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Neutrophil elastase inhibitor, ONO-5046 suppresses ozone-induced airway mucus hypersecretion in guinea pigs

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Abstract

To investigate the role of neutrophil elastase in ozone-induced airway hypersecretion, we measured goblet cell secretion by using a semiquantitative morphometric technique in guinea pigs. The magnitude of mucus discharge was estimated from the mucus score, which is inversely related to the degree of mucus discharge in histological sections of trachea stained for mucus glycoprotein with periodic acid Schiff/Alcian blue. Mucus hypersecretion of goblet cells was induced by ozone exposure and persisted for up to 5 h after exposure. Pretreatment with *N*-[2-{4-(2,2-dimetyl-propionyloxy) phenyl-sulfonylamino} benzoyl] aminoacetic acid (ONO-5046), a specific neutrophil elastase inhibitor (200 mg/kg, intraperitoneally), significantly inhibited goblet cell hypersecretion both just after and 5 h after ozone-exposure, but the latter inhibition was not complete. In bronchoalveolar lavage fluid, ozone exposure significantly increased the number of neutrophils just after and 5 h after exposure, while ONO-5046 significantly inhibited the increase in neutrophils only 5 h after ozone-exposure. These results indicate that neutrophil elastase may play an important role in the ozone-induced tracheal goblet cell hypersecretion and influx of neutrophils. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Neutrophil elastase inhibitor; Ozone-exposure; Mucus score; Goblet cell hypersecretion

1. Introduction

Increased secretion of mucus is an important feature commonly seen in many inflammatory airway diseases such as bronchial asthma, chronic bronchitis, bronchiectasis and cystic fibrosis. Mucus hypersecretion may contribute to airway obstruction and increase the risk of bacterial infection of the airway (Lundgren, 1992), which therefore exacerbates the underlying diseases. While it may be essential to control mucus hypersecretion for the treatment of these diseases, the mechanisms of hypersecretion have yet to be fully clarified.

Previous studies have shown that airway hypersecretion is induced by acute exposure to ozone in animal models (Trewer et al., 1959; Last et al., 1977). In dogs and guinea pigs, ozone-induced hypersecretion is associated with an increase in the number of neutrophils observed both in biopsies of the airway epithelium and in bronchoalveolar lavage (Trewer et al., 1959; Last et al., 1977). Although there is an abundance of data showing that neutrophils increase mucus secretion, the precise mechanism of such secretion has not yet been elucidated.

Elastase, that causes airway inflammation (Janoff, 1985), is a protein contained in the azurophilic granules of neutrophils, along with several other enzymes. The elastase activity in sputum collected from patients with secretagenous diseases has also been reported to increase (Stockley et al., 1984; Buttle et al., 1990). Furthermore, a previous study showed that both concentration and activity of neutrophil elastase significantly increases in the

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bronchoalveolar lavage fluid of ozone-exposed normal subjects (Koren et al., 1989). We therefore undertook the present study to determine whether or not neutrophil elastase plays an important role in the goblet cell hypersecretion induced by ozone-exposure in guinea pigs, using a semiquantitative morphometric technique (mucus score).

2. Materials and methods

2.1. Study design

Twenty-five Hartley-strain guinea pigs (400–600 g) were used for the assessment of goblet cell secretion. Five of these animals were treated with vehicle (saline) and measured without ozone exposure. Ten animals were exposed to ozone with vehicle, and five were measured just after ozone exposure. The remainder were measured 5 h after ozone exposure. The other 10 animals were treated with ONO-5046 (200 mg/kg), a specific neutrophil elastase inhibitor (Kawabata et al., 1991), intraperitoneally 30 min before ozone exposure. ONO-5046 has been reported to inhibit guinea pig neutrophil elastase activity in a concentration-dependent manner (IC_{50} value of 23.2 ± 1.2 nM) and the reported inhibition was competitive, with a K_i value of 7.65 ± 0.62 nM by Dixon analysis (Sakamaki et al., 1996). Five of these animals were measured just after ozone exposure and the remainder were measured 5 h after ozone exposure.

Another 25 guinea pigs (400–600 g) were also used for measurement of bronchoalveolar lavage fluid. These animals were divided into the same groups as those described above.

These procedures were in accordance with the Helsinki convention for the use and care of animals and were approved by the ethical committee of Kyushu University for animal experiments.

2.2. Assessment of goblet cell secretion

2.2.1. Tissue preparation

Goblet cell secretion was assessed as described previously (Tokuyama et al., 1990). Briefly, the guinea pigs were killed by exanguination and the chest was opened. The lung was inflated until the pleural margins were sharply defined by injecting 10% formaline in saline through a tracheostomy. After inflation, the upper trachea was ligated to prevent any outflow of fixative. To increase the efficacy of fixation, the systemic circulation was perfused with fixative as follows. The left ventricle was incised, a blunt-ended needle was inserted into the aorta, and the ventricles were cross-clamped. After the blood was expelled from the incised right atrium with heparinized saline at 100 mm Hg pressure until the perfusate was clear, systemic fixation was performed with 10% formaline in saline (approximately 150 ml infused). The trachea and main bronchi were removed and attached with thread to a card to preserve their shape and relative orientations, and were fixed for at least 24 h in 10% formaline in saline. Three-micrometer-thick sections of the trachea and main bronchi were cut longitudinally in the coronal plane, and stained with Alcian blue and periodic acid Schiff (Alcian blue, pH 2.5, periodic acid Schiff) in sequence to demonstrate the acidic and neutral intracellular glycoprotein of the secretory cells. The slides were then observed at a magnification of 400 × using a microscope.

2.2.2. The mucus score

The glycoprotein-containing cells in the epithelium were graded according to the amount of intracellular mucus as follows: grade 1: the vertical distance of the stained area was within one-third of the epithelial layer, measured from the basement membrane to the cell apex; grade 2: the vertical distance of the stained area exceeded one-third of the epithelial layer (Fig. 1a,b).

Grade 1, therefore, represented goblet cells that had discharged their mucus, whereas grade 2 represented cells that had not discharged any mucus. The stained areas were graded in 20 consecutive high-power fields along the two ''walls'' of the lower trachea (as seen in a longitudinal

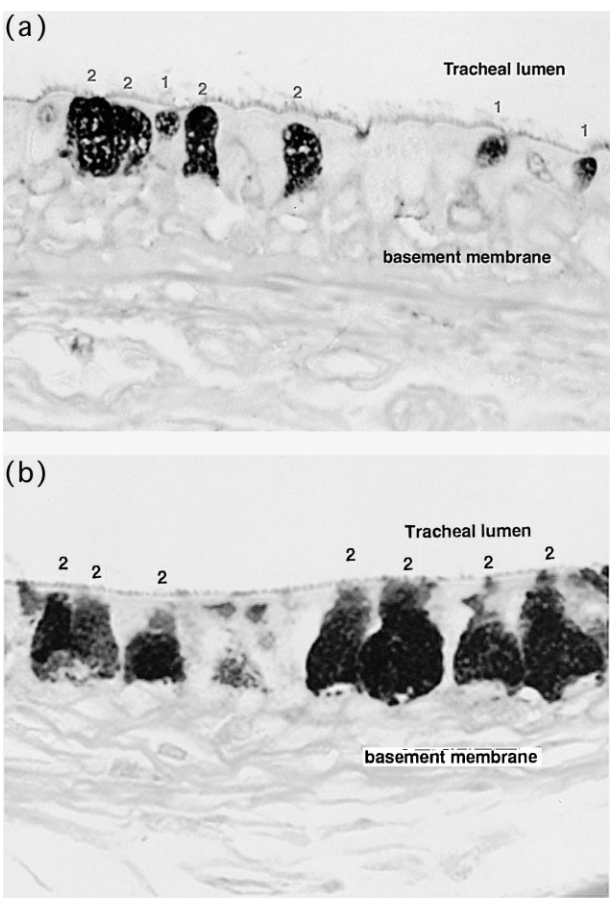

Fig. 1. An example of the epithelium from the lower trachea in a guinea pig ((a) was from an ozone-challenged animal, (b) was from a control animal.). The tissue specimen was stained with Alcian blue pH 2.5 and periodic acid Schiff. The numbers indicate the mucus score grading. Number 1 represents goblet cells that had discharged their mucin while number 2 represents cells that have not yet discharged their mucin.

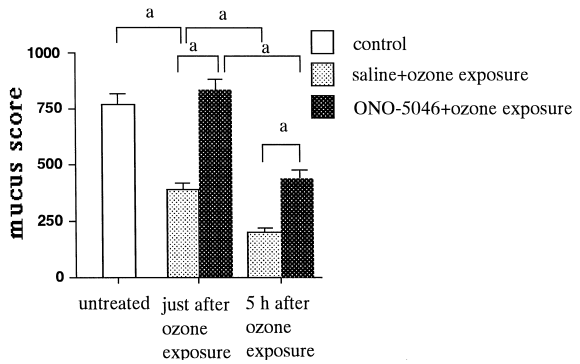

Fig. 2. Goblet cell secretion indicated by the mucus score. The values represent the means ± S.E. The mucus score decreased significantly after ozone exposure. Pretreatment with neutrophil elastase inhibitor significantly inhibited these decreases just after and 5 h after ozone exposure. $a = P < 0.05$.

section), starting at the carina and then moving cranially (a total of 40 high-power fields). The diameter of one of the high-power fields was 0.57 mm (measured using a calibrated eyepiece graticule).

Consequently, a distance of 20 high-power fields represented an 11.4 mm length of epithelium. The length of the trachea in guinea pigs in the weight range used in the present study was ~2.5 cm. As a result, the discharge of the goblet cells was assessed in the lower half of the trachea. Mucous score was calculated as

$$(1 \times n_1) + (2 \times n_2)$$

where 1 and 2 were the gradings, and n_1 and n_2 the total number of cells in each grade, respectively. Any stained areas that were agranular or had ill-defined boundaries were not included in the counts.

2.3. Bronchoalveolar lavage

Bronchoalveolar lavage was performed as previously mentioned (Koto et al., 1995). Briefly, after ozone exposure these animals were given pentobarbital (50 mg/kg intraperitoneally). The lung was lavaged three times in situ with isotonic saline at a constant hydrostatic pressure (25 cm H_2O) to avoid barotrauma. Approximately 10 ml/kg of saline was introduced at each lavage. Following an assessment of the total cell count, a total of 10^5 cells was suspended in 1 ml of normal saline and centrifuged onto glass slides using a centrifugal cell collector ($700 \times g$ for 5 min). After air-drying, the slides were fixed in methanol and then stained with May-Giemsa. A 500-cell differential count was then performed. The results are expressed as cell counts per milliliter of recovered bronchoalveolar lavage fluid.

2.4. Ozone exposure

Ozone exposure was performed as previously described (Koto et al., 1995). The animals inhaled 3.0 ± 0.1 ppm (mean ± S.D.) of ozone for 2 h while they were awake and breathing spontaneously in a 2.4-l exposure chamber. The ozone was generated by passing 100% O_2 through an ozonator (Model 0-1-2; Nihon Ozone, Tokyo, Japan) regulated by a variable-voltage supply. The concentration of ozone was monitored continuously with an ultraviolet (UV) analyzer (Model 1500; Dasibi, Glendale, CA).

2.5. Reagents

The drugs used in this study were ONO-5046 (Ono, Tokyo, Japan), and pentobarbital sodium (Abbot Laboratories, North Chicago, IL).

2.6. Statistics

The Mann–Whitney *U*-test was used to compare the mucus score and cell counts obtained by bronchoalveolar lavage. All data were reported as the means ± S.E.M. A *P*-value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Assessment of the goblet cell secretion

The results of goblet cell secretion as assessed from the mucus score are shown in Fig. 2. The mucus score de-

Table 1
Bronchoalveolar lavage profile
Definition of abbreviations: ONO-5046 = neutrophil elastase inhibitor Mϕs = alveolar macrophages; Ns = neutrophils; Epith = airway epithelial cells. Values represent means ± S.E.

	Cell counts × 10^5/ml of recovered bronchoalveolar lavage fluid			
	Total cells	Mϕs	Ns	Epith
Control	4.46 ± 0.44	3.32 ± 0.43	0.12 ± 0.03	0.42 ± 0.09
Just after ozone	3.67 ± 0.84	1.62 ± 0.51	0.43 ± 0.09[a]	1.19 ± 0.18[a]
5 h after ozone	7.77 ± 1.22[a,b]	2.30 ± 0.45	1.68 ± 0.20[a,b]	2.96 ± 0.48[a,b]
ONO-5046 + just after ozone	4.06 ± 0.85	2.20 ± 0.62	0.43 ± 0.17[a]	1.17 ± 0.24[a]
ONO-5046 + 5 h after ozone	5.90 ± 0.26	2.68 ± 0.14	0.74 ± 0.18[a,c]	1.72 ± 0.17[a,c]

[a] $P < 0.05$ compared with control.
[b] $P < 0.05$ compared with just after ozone.
[c] $P < 0.05$ compared with 5 h after ozone.

creased significantly after ozone exposure, indicating that ozone exposure increased the mucus secretion of the goblet cell. ONO-5046 completely abolished the decrease in the mucus score induced by ozone just after exposure. In addition, 5 h after ozone-exposure, ONO-5046 significantly inhibited hypersecretion, but did not completely abolish it.

3.2. Bronchoalveolar lavage

Table 1 shows the changes in the cell counts in bronchoalveolar lavage fluid before and after ozone exposure. The total cell counts increased significantly 5 h after ozone-exposure ($P < 0.05$), and this increase was inhibited by pretreatment with ONO-5046.

Significant increases in neutrophils were noted both just after and 5 h after ozone exposure in the animals that received saline pretreatment ($P < 0.05$, $P < 0.05$, respectively). ONO-5046 pretreatment significantly inhibited the neutrophilia 5 h after ozone exposure ($P < 0.05$), but not just after. The airway epithelial cells in bronchoalveolar lavage fluid increased both just after and 5 h after ozone-exposure in comparison to the controls($P < 0.05$, $P < 0.05$, respectively), and the latter increase was inhibited by pretreatment with ONO-5046.

No other cell counts differed significantly among either the control, ozone-exposed with saline pretreatment, and ozone-exposed with ONO-5046 pretreatment groups.

4. Discussion

We now demonstrated that pretreatment with neutrophil elastase inhibitor (ONO-5046) prevented the development of mucus hypersecretion from goblet cells induced by ozone exposure in guinea pigs. At 5 h after ozone-exposure either the neutrophil influx to the airway or epithelial cell desquamation was also inhibited by pretreatment with ONO-5046. These results suggest that both the recruited neutrophils and neutrophil elastase might play an important role in the ozone-induced mucus hypersecretion from goblet cells.

Airway mucus is derived from two principal sources (Basbaum and Finkbeiner, 1994); goblet cells in the surface of epithelium and sero-mucus glands in the subepithelium. Due to the greater mass of the gland cells (Nadel et al., 1985), the mechanism of gland secretion has been investigated in many studies (Ueki et al., 1980; Nadel et al., 1984). In contrast, the secretion from goblet cells is difficult to distinguish from that from other sources. In the present study, we employed a semiquantitative morphometric measurement system to assess the degree of goblet cell discharge based on the mucus score (Tokuyama et al., 1990). Goblet cell granules occupy the apical third of the cell just before discharge (grade 2), and are retained at the apex after discharge (grade 1). As a result, in this method the decrease in the mucus score indicates an increase in goblet cell discharge. The mucus score is reported to identify the goblet cell discharge relatively quickly and easily compared to the other methods used previously (Florey et al., 1932; Lamb., 1975; McDonald., 1988). This method also proved to be highly reproducible and agreed closely with the quantitative measurement of goblet cell discharge with a digitizing tablet as used in a previous study (Tokuyama et al., 1990).

In the present study, after ozone-exposure, the number of discharged cells increased, and the mucus score decreased. Since these effects were inhibited by the pretreatment with ONO-5046, neutrophil elastase may play a role in the hypersecretion of goblet cells in guinea pigs. Neutrophil elastase is one of the proteolytic enzymes in the azure granules in neutrophils. Fahy et al. (1992) reported that sputum from patients with bronchiectasis contained a high concentration of neutrophil elastase, which induced a very large secretory response in bovine submucosal gland cells in vitro. This secretory response was inhibited by the pretreatment with neutrophil elastase inhibitor (Fahy et al., 1992). It has also been reported that human neutrophil elastase has a secretagenous effect associated with degranulation of the airway submucosal glands of ferrets, dogs, and humans, and that these responses are inhibited by a selective human neutrophil elastase inhibitor, 4-(4-bromophenylsulfonylcarbamoyl) benzoyl-L-varyl-L-proline 1 (RS)-(1-trifluroacetyl-2-methyl-propyl) amide (ICI 200,355) (Bassett et al., 1989; Schuster et al., 1992). The secretory response to neutrophil elastase was not due to any cell destruction caused by elastase, but was due to its specific effect (Schuster et al., 1992). A morphometric analysis of canine tracheal submucosal gland cells showed a 40% decrease in the cytoplasm occupied by secretory granules after stimulation with neutrophil elastase (Schuster et al., 1992). Goblet cell secretion may thus have the same effect as submucosal gland secretion. However, the exact mechanism by which neutrophil elastase stimulates goblet cells is still unknown.

Acute exposure to high concentrations of ozone causes an increase in the activity of goblet cells (Trewer et al., 1959; Last et al., 1977). Ozone accelerated the rate of goblet cell secretion in rat tracheal cells in vitro (Last et al., 1977), without any morphological changes. In the present study, the secretion of goblet cells increased further 5 h after ozone exposure, and closely paralleled the increase in neutrophils. ONO-5046 inhibited these changes 5 h after ozone exposure. These results suggest that neutrophil elastase may play an important role in goblet cell secretion, and that neutrophil elastase also simultaneously stimulated neutrophil influx to the airway.

Neutrophil elastase was reported to degrade the capillary basement membrane collagen (Mainardi et al., 1980) and solubilize alveolar extracellular matrix (Palmgren et

al., 1992) so that the neutrophils migrate. The neutrophils infiltrated into the tissue may release elastase, which thus further facilitates neutrophil migration from capillaries through the interstitium into the intra-alveolar spaces. Another possible explanation is that neutrophil elastase may stimulate the activity of such chemotactic factors as interleukin-8 (IL-8) (McElvaney and Nakamura, 1992; Nakamura et al., 1992), tumor necrosis factor-α (TNF-α) (Schuderi, 1989) and cytokine-induced neutrophil chemoattractant (Yamaguchi et al., 1997). Moreover, neutrophil elastase has been reported to promote the rate of leukocyte adherence and extravasation induced by reperfusion of the ischemic bowel (Zimmerman and Granger, 1990).

In the present study, we did not measure the concentration of neutrophil elastase in bronchoalveolar lavage fluid. We previously showed that ozone exposure significantly increased the concentration of neutrophil elastase. In that study we measured the concentration of neutrophil elastase using an enzyme immunoassay as a complex with α-1 protease inhibitor (Matsumoto et al., 1999). We could not use this method to assess the effect of ONO-5046 on neutrophil elastase in bronchoalveolar lavage. However, ONO-5046 has been reported to inhibit guinea pig neutrophil elastase activity in a concentration-dependent manner (Sakamaki et al., 1996) as mentioned before. It is likely that ONO-5046 may suppress airway secretion through the inhibition of neutrophil elastase.

In summary, ONO-5046, a novel elastase inhibitor specific for neutrophil elastase was found to reduce significantly the ozone-induced hypersecretion of goblet cells in guinea pigs. It also inhibited neutrophil accumulation, and epithelial cell desquamation. The evidence presented herein thus supports the possibility that both recruited neutrophils and neutrophil elastase play an important role in goblet cell hypersecretion in the airway with inflammation.

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