Role of neutrophil elastase in ozone-induced airway responses in guinea-pigs

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ABSTRACT: Ozone-induced airway hyperresponsiveness occurs concurrently with neutrophilic inflammation and epithelial injury in various species including humans. The mechanism of neutrophil-induced airway hyperresponsiveness, however, has not yet been fully clarified. Neutrophil elastase (NE) is a multipotent protease released from activated neutrophils, which may play a role in ozone-induced airway hyperresponsiveness. In order to address this issue, the effects of ONO-5046, a specific NE inhibitor, were investigated in ozone-exposed guinea-pigs.

Awake animals were exposed to ozone at 3 parts per million for 2 h, airway responsiveness to acetylcholine (ACh) measured and examination of bronchoalveolar lavage fluid (BALF) performed.

Ozone exposure increased airway responsiveness to both inhaled and intravenous ACh, the concentration of NE in BALF and the number of neutrophils and airway epithelial cells in BALF. Although pretreatment with ONO-5046 (200 mg·kg⁻¹, *i.p.*) had no effect on these changes immediately after the exposure, it significantly inhibited airway hyperresponsiveness to inhaled ACh, whilst decreasing the number of neutrophils and epithelial cells in BALF 3–5 h after the exposure. In contrast, ONO-5046 showed no significant effect on airway hyperresponsiveness to intravenous ACh at any time.

These results suggest that neutrophil elastase contributes to ozone-induced airway hyperresponsiveness developing during the hours after exposure, presumably by means of inducing epithelial injury.

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Airway hyperresponsiveness and inflammation are characteristic features of asthma. Although eosinophilic airway inflammation is generally observed in patients with asthma [1], infiltration of neutrophils is also detected in the airways of patients with acute severe asthma [2, 3]. However, the pathophysiological role of neutrophils in airway hyperresponsiveness has not yet been fully clarified [4]. Ozone exposure causes airway hyperresponsiveness associated with neutrophilic airway inflammation in various species including humans [5–12]. Such evidence strongly raises the possibility that neutrophilic inflammation may contribute to the development of ozone-induced airway hyperresponsiveness, although the precise mechanism of this is still unknown. This possibility remains controversial since several studies have reported the lack of evidence for a contribution of neutrophils to ozone-induced airway hyperresponsiveness [13, 14].

A previous study reported that both the concentration and activity of neutrophil elastase (NE) significantly increased in the bronchoalveolar lavage fluid (BALF) of ozone-exposed normal subjects [10]. Recent studies have shown NE concentration to increase in the sputum and bronchial lavage fluid of asthmatics, in particular, in patients with noninfectious status asthmaticus [3, 15]. In addition, aerosolized human NE has been reported to induce airway hyperresponsiveness in guinea-pigs, and

histological examination of intrapulmonary bronchi revealed marked epithelial injury [16]. These studies suggest that NE may play a pivotal role in airway hyperresponsiveness in both ozone-exposed subjects and asthmatics.

The integrity of the airway epithelial layer may be a key determinant of airway responsiveness. The loss of epithelial barrier function induces airway hyperresponsiveness by facilitating the diffusion of inhaled bronchoconstrictors to the airway smooth muscle [17]. Furthermore, the epithelium has been known to release inhibitory modulators of airway responsiveness, such as prostaglandin E₂ (PGE₂), nitric oxide, and epithelium-derived relaxing factors (EpDRFs) [18–22]. Owing to the fact that general desquamation of epithelium is another feature of ozone-exposed airways, it is conceivable that NE may contribute to the development of ozone-induced airway hyperresponsiveness, possibly by inducing epithelial injury [23, 24].

In the present study, the effects of ONO-5046, a currently available inhibitor of human NE, on the airway inflammation and hyperresponsiveness induced by ozone exposure in guinea-pigs were examined [25]. In order to determine whether NE is responsible for the epithelial injury, the effects of ONO-5046 on airway hyperresponsiveness between animals administered with inhaled and

intravenous-acetylcholine (ACh) were compared. This approach was based on previous work showing that the disturbance of epithelial integrity is reflected in the difference in airway responsiveness to inhaled and to intravenous bronchoconstrictors [26, 27].

Methods

Experimental protocol

Male Hartley guinea-pigs (500–550 g) were used in this study. Airway responsiveness to inhaled and to intravenous ACh was measured, and examinations of BALF performed, in separate animals in order to eliminate the possible interference between the procedures. Each measurement was performed using five animals. The measurements were made before, immediately after, and 3 and 5 h after ozone exposure. ONO-5046 was used as an NE inhibitor, because it has been reported to inhibit guinea-pig NE activity in a concentration-dependent manner (inhibitory concentration of 50% IC₅₀) 23.2±1.2 nM), and the reported inhibition was competitive, with an inhibitory constant (*K_i*) of 7.65±0.62 nM by means of Dixon analysis [28]. Vehicle (0.9% saline) or ONO-5046 (200 mg·kg⁻¹) was intraperitoneally administered to the animals 30 min prior to ozone exposure.

Ozone exposure was performed as previously described [12]. The animals inhaled dry air or 3.0±0.1 parts per million (ppm) (mean±SD) ozone for 2 h while awake and breathing spontaneously in a 24 L exposure chamber. Ozone was generated by passing 100% oxygen through an ozonator (Model 0-1-2; Nihon Ozone, Tokyo, Japan), which was regulated by means of a variable voltage supply. The concentration of ozone in the chamber was continuously monitored using an ultraviolet analyser (Model 1500; Dasibi, Glendale, CA, USA).

Measurement of airway responsiveness

The guinea-pigs were anaesthetized with 50 mg·kg⁻¹ pentobarbital sodium administered intraperitoneally. The animals were intubated *via* a tracheotomy and were then mechanically ventilated using a respirator (Model No. 680; Harvard Apparatus; South Natick, MA, USA) at a constant tidal volume of 7 mL·kg⁻¹ and a frequency of 60 breaths·min⁻¹. A catheter was introduced into a jugular vein to administer the drugs. Another catheter was inserted into a carotid artery, through which the blood pressure was measured *via* an electric manometer (LPU-0.1; Nihon Kohden, Tokyo, Japan). In order to estimate pleural pressure, a fluid-filled catheter was introduced into the oesophagus at a point at which the maximal amplitude of pressure was obtained. The animals were placed supine in a flow-type body plethysmograph made of plexiglas and with 2.8 L of dead space (customized; Chest Medical, Tokyo, Japan). The plethysmograph airflow was measured using a Fleisch pneumotachograph (TV-132T; Nihon Kohden) and a differential pressure transducer (TP-602T; Nihon Kohden). The transpulmonary pressure was estimated from the difference between the oesophageal and airway opening pressure, measured by means of a differential pressure transducer (TP-603T; Nihon Kohden). The total pulmonary resistance (*RL*) was calculated from transpulmonary pressure and plethysmograph airflow.

Airway responsiveness to inhaled ACh was determined by inhalation of an increasing concentration of ACh administered *via* the endotracheal tube. The ACh aerosol (output 1.5 mL·min⁻¹) was generated by means of an ultrasonic nebulizer, (TUR-3200; Nihon Kohden) placed in line with the ventilator. The dose/response curves were constructed as follows: saline aerosol was given for 15 breaths, and the subsequent *RL* value was used as baseline. The ACh aerosol was administered for 15 breaths, separated by 5-min intervals. The concentration of ACh was increased for each series of 15 breaths. *RL* was monitored for 5 min after each nebulization, and the maximum value was plotted against the ACh concentration. To achieve a constant-volume history, hyperinflation (three tidal volumes) was induced between each ACh challenge. The challenge was stopped when *RL* exceeded 200% of baseline. The provocative concentration of ACh required to produce a 200% increase in *RL* (PC₂₀₀) was calculated by log-linear interpolation in individual animals.

Airway responsiveness to intravenous ACh was measured using a protocol similar to that used for inhaled ACh. Briefly, the dose/response curves were constructed as follows: 1 mL·kg⁻¹ saline was given for 5 s, and the subsequent *RL* value used as baseline. The ACh solution, 1 mL·kg⁻¹, was administered for 5 s, separated by 5-min intervals. The concentration of ACh was increased for each series of administration. *RL* was monitored for 5 min after each administration, and the maximum value was plotted against the ACh concentration. The PC₂₀₀ was calculated by log-linear interpolation in individual animals.

Bronchoalveolar lavage

Guinea-pigs were sacrificed and bronchoalveolar lavage performed as previously described [12]. Both lungs were gently lavaged three times with 10 mL 0.9% saline *via* the tracheal cannula at a pressure of 25 cmH₂O. The total cell counts including desquamated epithelial cells were determined using light microscopy and a standard haemocytometer. The lavage fluid was immediately centrifuged for 10 min at 200 × *g* at 4°C. The cell pellet was resuspended in saline, and Cytospin preparations (Cytospin 3; Shandon, Pittsburgh, PA, USA) were carried out. Differential counts on 500 cells were performed using light microscopy and a single-blind method following a modified Wright-Giemsa stain (Diff-Quick; Baxter, McGaw Park, IL, USA). The viability of the recovered cells was evaluated using the trypan blue exclusion test. In the supernatant of the BALF from the animals treated with vehicle, the concentration of NE- α_1 -protease inhibitor (α_1 -PI) complex (NE-PI) was measured by means of an enzyme immunoassay as described below.

Measurement of neutrophil elastase in bronchoalveolar lavage fluid

NE was measured by means of an enzyme immunoassay as a complex with α_1 -PI (Merck, Darmstadt, Germany). The samples were added to wells coated with sheep anti-NE elastase immunoglobulin G (IgG). This antibody does not cross-react with cathepsin G or other neutrophil proteinases. After incubation and washing, the solid phase-bound NE-PI was further incubated with alkaline phosphatase-labelled rabbit, anti-NE-PI IgG. After further

washing, *p*-nitrophenylphosphate was added to measure the amount of solid phase-bound NE-PI. This assay was calibrated using a standard solution of known NE-PI concentration. The lower detection limit of this assay was $25 \mu\text{g}\cdot\text{L}^{-1}$. All data were expressed as the mean values of duplicate determinations.

Measurement of ONO-5046 concentration in plasma

Venous blood (1 mL) was collected, using a heparinized syringe, from animals pretreated with ONO-5046. The blood samples were centrifuged for 30 min at $1500 \times g$ at 4°C , and then their supernatants collected as plasma. The plasma concentration of ONO-5046 was measured by means of high-performance liquid chromatography (HPLC). Briefly, the sample (0.5 mL) was diluted with distilled water and 1 N HCl, and then applied to a Sep-Pak C_{18} short column (Waters, Milford, MA, USA). The sample and standard solutions of ONO-5046 were eluted with methanol from the short column, and the methanol solution was applied to a Bond Elut SAX short column (Varian, Horbor City, CA). The sample and standard solutions from the short column and the compounds in this solution were extracted using ethylacetate. The organic phase was evaporated to dryness and the residue was dissolved in 150 μL of mobile phase and subjected to HPLC (LC-module-1; Waters, Milford, MA, USA). An aliquot (50 μL) was injected through an autosampler on to an analytical column and the mobile phase composition changed from 0.02 M KH_2PO_4 (pH 3.8)/ CH_3CN (4:1, volume (vol)/vol) to 0.02 M KH_2PO_4 (pH 3.8)/ CH_3CN (5:4, vol/vol) in the linear gradient mode over 40 min. The separation was completed within 55 min for each sample, and quantification of ONO-5046 in each sample were carried out on the basis of the calibration plots obtained from the peak area ratio for each standard solution. The lower detection limit of this assay was $0.15 \mu\text{g}\cdot\text{mL}^{-1}$.

Drugs

ONO-5046 was a gift from Ono Pharmaceutical (Osaka, Japan) and was suspended in 0.9% saline at a concentration of $100 \text{ mg}\cdot\text{mL}^{-1}$. Pentobarbital sodium was obtained from Abbott (North Chicago, IL, USA). ACh chloride was obtained from Sigma Chemical (St Louis, MO, USA), and was dissolved in 0.9% saline.

Statistical analysis

All data are expressed as mean $\pm$ SEM. PC200 was calculated by log-linear interpolation in individual animals, based on a previous study [24]. The effects of ONO-5046 on PC200 and on cell counts in BALF were compared by analysis of variance (ANOVA), and the significance of differences between the values was assessed using the Bonferroni correction for multiple comparisons. The time-dependent effects of ozone exposure on NE-PI concentration in BALF were assessed by multiple linear regression. The time course of the plasma concentration of ONO-5046 was assessed by ANOVA. A *p*-value <0.05 was considered to indicate statistical significance.

Results

Effects of ozone exposure on neutrophil elastase- α_1 protease inhibitor concentration in bronchoalveolar lavage fluid

Figure 1 shows the time-dependent effects of ozone exposure on NE-PI concentration in BALF. In sham-exposed animals, the concentration of NE-PI in BALF was below the detection limit. Immediately after ozone exposure, the NE-PI concentration increased to $124\pm10 \mu\text{g}\cdot\text{L}^{-1}$, and further increased to $186\pm17 \mu\text{g}\cdot\text{L}^{-1}$ 3 h after the exposure ($p<0.05$, immediately after *versus* 3 h after exposure). The NE-PI concentration was $160\pm18 \mu\text{g}\cdot\text{L}^{-1}$ 5 h after the exposure, which was still higher than that of the sham-exposed control.

Effects of ONO-5046 on cell counts in bronchoalveolar lavage fluid

Figure 2a shows the effects of ONO-5046 on neutrophil counts in BALF after ozone exposure. The number of neutrophils in BALF increased time-dependently after ozone exposure. Pretreatment with ONO-5046 showed no effect on neutrophil number either immediately or 3 h after ozone exposure, but significantly reduced it 5 h after ozone exposure ($p<0.05$, ONO-5046-treated *versus* vehicle-treated animals).

Figure 2b shows the effects of ONO-5046 on epithelial cell counts in BALF. The number of epithelial cells also increased time-dependently after ozone exposure; thus, suggesting the development of epithelial desquamation. Although pretreatment with ONO-5046 did not affect the increase in epithelial cell number immediately after ozone exposure, it significantly suppressed the further progression of epithelial desquamation 3 and 5 h after ozone

Fig. 1. – Effects of ozone exposure on neutrophil elastase- α_1 -protease inhibitor complex (NE-PI) concentration in bronchoalveolar lavage fluid. In sham-exposed animals, the concentration of Ne-PI in BALF was below the detection limit (– – –; $<25 \mu\text{g}\cdot\text{L}^{-1}$). The NE-PI concentration in ozone-exposed animals was measured immediately and 3 and 5 h after exposure. The values represent mean $\pm$ SEM ($n=5$). *: $p<0.05$.

Fig. 2. – Effects of ONO-5046 on: a) neutrophil; and b) epithelial cell counts in BALF after ozone exposure. □ : vehicle pretreatment; ▨: ONO-5046 pretreatment (200 mg·kg⁻¹). The values represent mean±SEM (n=5). Ozone exposure resulted in significantly increased cell number at all time points (p<0.05 compared to sham-exposed animals); *: p<0.05 compared to vehicle-treated ozone-exposed animals.

exposure (both p<0.05, ONO-5046-treated *versus* vehicle-treated animals).

The number of macrophages, but not of lymphocytes nor eosinophils, was significantly suppressed immediately and 3 h after ozone exposure (both p<0.05, ozone *versus* sham-exposed animals) (table 1). Pretreatment with ONO-5046 showed no significant effect on the number of macrophages, lymphocytes and eosinophils. Pretreatment with ONO-5046 in air exposed animals had no effect on differential cell counts at any time points (data not shown).

Effects of ONO-5046 on airway responsiveness to acetylcholine

Figure 3a shows the effects of ONO-5046 on airway responsiveness to inhaled ACh after ozone exposure. Immediately after exposure, the log PC₂₀₀ in ozone-exposed guinea pigs (1.37±1.09 µg·mL⁻¹) was significantly lower than that in sham-exposed animals (3.21±1.08 µg·mL⁻¹) (p<0.01), suggesting that ozone exposure caused airway hyperresponsiveness. Subsequently, 3 and 5 h after exposure (3 h: 1.89±1.06 µg·mL⁻¹; 5 h: 1.73±1.14 µg·mL⁻¹), this airway hyperresponsiveness to ACh returned to near baseline values, but remained elevated (both p<0.05). Pretreatment with ONO-5046 had no effect on log PC₂₀₀ immediately after ozone exposure (0.37±0.09 (vehicle-treated) *versus* 0.47±0.10 µg·mL⁻¹ (ONO-5046-treated); NS). However, 3 and 5 h after exposure, the log PC₂₀₀ in animals pretreated with ONO-5046 (3 h: 2.37±1.16 µg·mL⁻¹, 5 h: 2.23±1.11 µg·mL⁻¹) were significantly higher than those in animals pretreated with vehicle at each time point (3 h: 1.89±1.06 µg·mL⁻¹; 5 h: 1.73±1.14 µg·mL⁻¹) (both p<0.05).

Figure 3b shows the effects of ONO-5046 on airway responsiveness to intravenous ACh after ozone exposure. The log PC₂₀₀ in ozone-exposed animals were significantly lower at all time points than those in sham-exposed animals (0 h: 0.93±0.07, 3 h: 1.20±0.09, 5 h: 1.13±0.05 *versus* sham-exposed: 1.58±0.08 µg·mL⁻¹) (all p<0.05). In contrast to the data regarding inhaled ACh, ONO-5046 had no significant effect on log PC₂₀₀ in ozone-exposed animals at any time point [0 h: 1.11±0.06 (ONO-5046) *versus* 0.93±0.07 µg·mL⁻¹ (vehicle 3 h: 1.24±0.10 (ONO-5046) *versus* 1.20±0.09 µg·mL⁻¹ (vehicle 5 h: 1.07±0.03 (ONO-5046)) *versus* 1.13±0.05 (vehicle all ns). Pretreatment with ONO-5046 in air-exposed animals had no effect on airway responsiveness to inhaled nor intravenous ACh at any time points (data not shown).

Plasma concentration of ONO-5046

The plasma concentrations of ONO-5046 were 3.89±1.84 µg·mL⁻¹ immediately after ozone exposure, 0.75±0.35 µg·mL⁻¹ 3 h after exposure and below the detection limit (0.15 µg·mL⁻¹) 5 h after exposure.

Discussion

The present study demonstrated that ozone exposure induced neutrophilic inflammation and epithelial injury in guinea-pig airways. In addition, ozone exposure increased

Table 1. – Effects of ONO-5046 pretreatment on cell counts in bronchoalveolar lavage fluid after ozone exposure

	Cell number 10 ⁵ cells·mL ⁻¹						
	Sham	Ozone-exposed					
		0 h		3 h		5 h	
		Vehicle	ONO-5046	Vehicle	ONO-5046	Vehicle	ONO-5046
Macrophages	3.32±0.43	1.62±0.51*	2.20±0.62*	2.16±0.20*	1.94±0.58*	2.30±0.45	2.68±0.14
Lymphocytes	0.38±0.07	0.16±0.04	0.16±0.05	0.17±0.03	0.26±0.07	0.37±0.07	0.39±0.12
Eosinophils	0.22±0.10	0.26±0.13	0.10±0.04	0.36±0.15	0.21±0.06	0.46±0.23	0.37±0.11

*: p<0.05 compared to sham-exposed control.

Fig. 3. – Effects of ONO-5046 on airway responsiveness after ozone exposure to acetylcholine: a) inhaled; and b) intravenous. □ : vehicle pretreatment; ▨ : ONO-5046 pretreatment (200 mg·kg⁻¹). The values represent mean±SEM (n=5). Ozone exposure resulted in significantly decreased airway responsiveness at all time points. p<0.05 compared to sham-exposed animals; *: p<0.05 compared to vehicle-treated ozone-exposed animals.

the concentration of NE in BALF and airway responsiveness to both inhaled and intravenous ACh. Although an NE inhibitor, ONO-5046, had no effect on these changes immediately after the exposure, it significantly inhibited the degree of epithelial injury and airway hyperresponsiveness to inhaled ACh 3–5 h after ozone exposure. In contrast, ONO-5046 had no effect on airway hyperresponsiveness to intravenous ACh at any time.

In this study, ozone exposure markedly increased the concentration of NE-PI in BALF from guinea-pigs, and this finding was consistent with a previous report in which the concentration of NE increased in BALF from normal subjects exposed to 0.4 ppm of ozone for 2 h [10]. As a causal mechanism of the effect of NE on airway hyperresponsiveness, this study focused on the concurrent epithelial injury which is generally observed in ozone-exposed animals. A disorder of epithelial function induces airway hyperresponsiveness by multiple mechanisms such as facilitating the diffusion of stimuli to the airway tissue [17], and decreases in the concentrations of various epithelial-derived modulators [18–22]. NE is a multipotent serine protease capable of degrading not only elastin but also type IV collagen, fibronectin, laminin and proteoglycans, all of which are components of the basement membrane and of epithelial integrity [29]. In the phy-

siological state, NE activity is neutralized by various antiproteases, such as α_1 -proteinase inhibitor, α_2 -macroglobulin and secretory leukoprotease inhibitor [29], but these antiproteases are easily inactivated by oxidative stress. Indeed, ozone *per se* acts as a strong radical and contributes to the secondary formation of other oxygen radicals [30–32]. The protection by ONO-5046 against an increase in epithelial desquamation and airway hyperresponsiveness to inhaled ACh suggests that intrinsic NE contributes to ozone-induced disorders at least 3 h after the exposure.

The inhibitory effects of ONO-5046 on ozone-induced epithelial injury and airway hyperresponsiveness to inhaled ACh do not provide any direct evidence for the contribution of NE-induced epithelial injury to airway hyperresponsiveness. The possibility remains that these events might be independent from each other. In this regard, a previous study demonstrated the degree of airway hyperresponsiveness to be higher in ozone-exposed guinea-pigs to which ACh was administered by inhalation rather than intravenously [26]. This difference between the administration routes indicates epithelial factors to be involved in ozone-induced airway hyperresponsiveness [23, 24]. If epithelial injury plays an important role in airway hyperresponsiveness and the effect of NE is localized on the epithelium, the effect of ONO-5046 on airway responsiveness should differ in animals receiving ACh administered *via* either the inhaled or the intravenous route. Therefore, the effect of ONO-5046 on ozone-induced airway hyperresponsiveness to intravenous ACh was examined. The lack of any inhibitory effect of ONO-5046 on airway hyperresponsiveness to intravenous ACh strongly suggests NE-mediated epithelial injury to play a pivotal role in airway hyperresponsiveness in this experimental model.

Although ONO-5046 significantly inhibited ozone-induced airway hyperresponsiveness to inhaled ACh, it did not completely inhibit it. This compound seems to account for one-third of the effect observed at 3 and 5 h after ozone exposure. One possibility is that the dose of ONO-5046 administered was not sufficient to abolish NE activity. However, this possibility is unlikely for the following reason. According to the manufacturer's written specification, ONO-5046 shows excellent distribution from plasma into lung tissue (personal communication). In addition, ONO-5046 inhibits guinea-pig NE activity in a concentration-dependent manner (IC₅₀ 23.2±1.2 nM) [28]. An IC₅₀ of 23.2 nM is equivalent to 1.23 × 10⁻⁴ µg·mL⁻¹, which is far lower than the plasma concentrations of ONO-5046 measured in animals immediately and 3 h after ozone exposure (3.89±1.84 and 0.75±0.35 µg·mL⁻¹, respectively). Although the concentration 5 h after exposure was below the detection limit (<0.15 µg·mL⁻¹), it remains possible that the concentration was still sufficient to block NE activity. Therefore, it is strongly suggested that intrinsic NE activity was effectively inhibited by ONO-5046 in this study at all time points. Another explanation for the incomplete inhibition of airway hyperresponsiveness by ONO-5046 is that there may be other mechanisms accounting for the hyperresponsiveness to inhaled ACh. The observation that airway hyperresponsiveness to intravenous ACh was not affected by ONO-5046 supports, at least in part, this latter explanation. It may suggest the

existence of a common pathway in ozone-induced airway hyperresponsiveness to inhaled and intravenous ACh.

ONO-5046 had no effect on airway hyperresponsiveness or epithelial injury immediately after ozone exposure. Based on previous findings, it is speculated that ozone-induced airway hyperresponsiveness involves multiple mechanisms, including the release of eicosanoids and airway neural activation in addition to epithelial injury [9, 12, 24]. Mechanisms other than epithelial injury by NE may also play a role immediately after ozone exposure. For example, it was previously shown that both the ozone-induced airway hyperresponsiveness and neutrophilia in BALF immediately after ozone exposure are inhibited *via* a depletion of tachykinins by systemic capsaicin pretreatment [12]. In the early phase of ozone exposure, tachykinins, but not NE, may play a crucial role in airway hyperresponsiveness and neutrophilic inflammation. As to epithelial damage in the early phase, ozone and related free radicals may directly injure the epithelial layer. This speculation is supported by a previous report showing that oxidants directly reduce epithelial barrier functions by damaging the tight junctions of cultured human tracheal epithelial cells [33]. The following release of NE from recruited neutrophils may exacerbate the epithelial injury, which was observed in the late phase of the present study. The knowledge that the enzymatic activity of NE is potentiated in the presence of oxygen radicals further supports this possibility [34].

The contribution of neutrophils to ozone-induced airway hyperresponsiveness in guinea-pigs has been questioned by some investigators [13, 14]. A previous study showed ozone exposure to cause airway hyperresponsiveness even in animals depleted of neutrophils by cyclophosphamide [14]. In that study, airway hyperresponsiveness was evaluated by means of intravenous ACh administration. In the present study, ozone-induced airway hyperresponsiveness to intravenous ACh was not affected by ONO-5046 pretreatment, but airway hyperresponsiveness to inhaled ACh was significantly suppressed by ONO-5046. These results may, thus, not conflict with the findings of previous reports, if the neutrophils and NE contribute only to airway hyperresponsiveness to inhaled bronchoconstrictors.

The finding that ONO-5046 inhibited the recruitment of neutrophils in BALF 5 h after ozone exposure may provide *in vivo* evidence for the significance of intrinsic NE as a mediator which causes a subsequent accumulation of neutrophils. Aerosolized NE has been reported to induce airway hyperresponsiveness and the recruitment of polymorphonuclear cells to the airway epithelium in intrapulmonary bronchi [16]. A previous *in vitro* study reported that treatment with NE, using concentrations at which there was no fatal toxic effect on the cells, increased production of interleukin-8 (IL-8), a potent chemokine for neutrophils, in cultured airway epithelial cells [35]. The interactions between activated neutrophils and epithelial cells may constitute the NE- and IL-8-mediated vicious cycle, thus contributing to the persistence of neutrophilic airway inflammation.

Recently, HILTMANN *et al.* [36] reported the effect of recombinant antileukoprotease (rALP) on ozone-induced airway hyperresponsiveness in patients with asthma. They observed that airway responsiveness to inhaled methacholine was increased 16 h after ozone exposure, and

was accompanied by neutrophilic airway inflammation. However, rALP treatment did not protect against ozone-induced airway hyperresponsiveness. The role of neutrophil-derived proteases in asthmatics remains uncertain; thus, further studies are needed.

In summary, ozone exposure induced neutrophilic inflammation and epithelial injury in guinea-pig airways. Ozone exposure increased the concentration of neutrophil elastase in bronchoalveolar lavage fluid and airway responsiveness to both inhaled and intravenous acetylcholine. Although the neutrophil elastase inhibitor, ONO-5046, significantly inhibited epithelial injury and airway hyperresponsiveness to inhaled acetylcholine, it had no effect on airway hyperresponsiveness to intravenous acetylcholine.

These results, therefore, suggest that neutrophil elastase contributes to ozone-induced airway hyperresponsiveness, presumably by inducing epithelial injury.

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