Ozone-induced nasal hyperresponsiveness to tachykinins in guinea pigs

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ABSTRACT

Objective: To assess role of hydroxyl radicals in the ozone-induced upper airway hyper-responsiveness to tachykinins.

Methods: A prospective, controlled, animal model ($n = 96$) was performed. Half of them exposed to air (A-group, placebo) and the other half exposed to 3 ppm ozone (O-group) for 2 h. Two hours post air/ozone exposure, animals were anesthetized and equally randomized to be pretreated with one of the three treatments, including saline vehicle, dimethylthiourea (DMTU; 500 mg/kg *m*, a hydroxyl radical scavenger), or phosphoramidon (Phos; 2 mg/kg, an inhibitor for neutral endopeptidase). Ten minutes after pretreatment, half of the animals in each group were *i.v.* injected with capsaicin (2 μ g/kg), and the other half were *i.v.* injected with substance P (10 μ g/kg) to produce Evans blue dye extravasation.

Results: Nasal exudative response to capsaicin or substance P in O-group was found to be significantly greater than that in A-group. This ozone-induced nasal airway hyperresponsiveness was largely prevented by DMTU. Phosphoramidon produced a similar nasal airway hyperresponsiveness in the A-group, but failed to alter ozone-induced nasal airway hyperresponsiveness in O-group. In sharp contrast, only substance P, but not capsaicin, produced a laryngeal exudative response in the A-group, which was similar to that in the O-group. The laryngeal exudative response to substance P was not significantly affected by DMTU or Phos.

Conclusion: In the guinea-pig model, hydroxyl radicals play a vital role in the development of ozone-induced nasal airway hyperresponsiveness to tachykinins. It is possibly mediated through the suppressive action of ozone on the tachykinin degradation.

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Key words: Neutral endopeptidase, oxygen radicals, ozone, phosphoramidon, substance P, tachykinins, upper airway hyper-reactivity

Ozone is a major pollutant in urban areas and its ambient concentration is associated with the occurrence of asthma.^{1,2,3,4} Consequently, many animal studies have been conducted to investigate the pathophysiological mechanisms underlying ozone-induced lower airway hyperreactivity.^{5,6} However, ~40% of inhaled ozone is taken up in the nasal passages in healthy human subjects.⁷ The upper airway includes the nose and larynx. They have a close anatomic relationship and yet possess quite distinct physiologic regulations.^{8,9,10,11} Upper airway hyperreactivity may lead to sneezing, rhinorrhea, nasal obstruction, cough, and laryngeal spasm resulting in an increase in airway resistance to respiratory flow. Can ozone induce upper airway hyperreactivity? If it does, is it clinically significant?

Airway C-fiber afferents are chemosensitive sensory nerve endings that play an important role in the reflex regulation of airway functions.^{12,13} Their nerve endings contain tachykinins, a family of proinflammatory neuropeptides. The release of tachykinins may cause various pathophysiological consequences including vasodilation, plasma exudation, and bronchoconstriction.^{14,15,16,17,18}

Released tachykinins are mainly degraded by neutral endopeptidase. Ozone exposure has been shown to enhance the excitabilities of airway C-fiber nerve endings¹⁹ and inhibit airway neutral endopeptidase.^{20,21} Both actions may cause excess tachykinins in airway tissues. Thus, dysfunction of airway C-fiber afferents and inactivation of neutral endopeptidase have been largely implicated in the ozone-induced lower airway hyperreactivity.^{19,22,23,24} Furthermore, because ozone is an oxidant pollutant, its ability to induce lower airway hyperreactivity is linked to the effects of oxygen radicals.²⁵ Although airway C-fiber afferents are known to innervate the upper airway,²⁶ the possible involvement of these pathophysiological mechanisms in the ozone-induced upper airway hyperreactivity remains to be investigated.

The present study was conducted in guinea pigs to investigate whether ozone exposure may augment nasal and laryngeal exudative and edematous responses to endogenous and exogenous tachykinins, and if so, to assess the role of hydroxyl radicals and neutral endopeptidase in this ozone-induced upper airway hyperreactivity. To accomplish these objectives, capsaicin and substance P were employed to induce nasal and laryngeal exudative responses. The former drug stimulates C-fiber afferents to cause the release of endogenous tachykinins, whereas the latter is an exogenous tachykinin.

MATERIALS AND METHODS

Generation of Ozone and Ozone Exposure

Ozone was generated using ultraviolet lamp (model 03V1-0, Orec, Akron, OH). The main airflow was then turbu-

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lently mixed with a small flow and routed to a 24-L Plexiglas exposure chamber. The air/ozone flow was 2 L/min in the exposure box. The ozone concentration inside the chamber was monitored by an ultraviolet monitor (model 1003-AH, Dasibi, Glendale, CA) and its accuracy was calibrated by the potassium iodide method. Male Hartley guinea pigs (body weight, 367 ± 12 g; $n = 96$) were exposed to either clean air or 3.0 ppm ozone for a 2-hour period, according to our previous work¹⁹ After exposure, animals were removed from the exposure chamber and held in an animal cage for one hour for the subsequent experiments.

Animal Preparation

Animals were anesthetized with an i.p. injection of α -chloralose (100 mg/kg; Sigma, St. Louis, MO) and urethane (500 mg/kg; Sigma). The carotid artery and jugular vein were cannulated for recording arterial blood pressure and for i.v. administration of pharmacological agents, respectively. During the course of the experiments, supplemental doses of α -chloralose (20 mg/kg/h) and urethane (100 mg/kg/h) were administered to maintain the abolition of pain reflexes induced by pinching the animal's hindpaw. The animal was fixed in a supine position and the trachea was cannulated below the larynx with a short tracheal cannula *via* a tracheotomy, through which the animal breathed spontaneously. Animal preparation took ~ 1 hr. Throughout the experiment, the body temperature of the animal was maintained at $\sim 36^\circ\text{C}$ by means of a servo heating blanket. All protocols were in accordance with the guidelines for the care and use of laboratory animals published by the National Institutes of Health, Bethesda, MD and were approved by the Committee of the National Science Council, Taipei, Taiwan.

Measurements of Plasma Exudation and Tissue Edema

The magnitude of plasma exudation was quantified by measuring the extravasation of Evans blue dye, using the aforementioned method.²⁷ In brief, Evans blue dye (25 mg/kg) was injected i.v. 5 minutes before injecting capsaicin or substance P. Five minutes later, the thorax was quickly opened. The left ventricle was opened and the systemic circulation was perfused with 200 mL of saline at a pressure of 120 mmHg to remove the intravascular dye.

After vascular perfusion, the tissues of the nasal cavity, paranasal sinus, and whole larynx were excised and each was then separated into two parts. Tissue Evans blue dye was extracted by incubation in 1.5 mL of formamide (Sigma) at 40°C for 24 hours and its concentration was measured by light absorbance (Multiscan spectrophotometer, U-100. Hitachi, Tokyo, Japan) at 620 nm. The tissue content of Evans blue dye was calculated by interpolation on a standard curve of dye concentrations in the range of $0.05 \mu\text{g/mL}$ and was expressed as ng of dye/mg of tissue.

Pharmacological Agents

A stock solution of capsaicin ($500 \mu\text{g/mL}$; Sigma) was prepared in a vehicle of 10% Tween 80, 10% ethanol, and 80% saline. The injected capsaicin solution ($500 \mu\text{g/mL}$) was prepared daily by diluting the stock solution with isotonic saline. Dimethylthiourea (500 mg/mL; Sigma), phosphoramidon (2

mg/mL; Sigma) and substance P ($10 \mu\text{g/mL}$; Sigma) were prepared using isotonic saline.

Experimental Procedures

A total of 96 male guinea pigs were evenly divided into 6 groups. Animals were first exposed to air ($n = 48$) or ozone ($n = 48$) for a 2-hour period. Two hours later, animals were anesthetized and pretreated with saline vehicle, dimethylthiourea (DMTU; 500 mg/kg; a hydroxyl radical scavenger), or phosphoramidon (Phos; 2 mg/kg; an inhibitor for neutral endopeptidase) to study the possible role of hydroxyl radical and endopeptidase in the ozone-induced airway hyperreactivity. Thus, the animals were divided into 6 treatment groups, Air+Saline, Ozone+Saline, Air+DMTU, Ozone+DMTU, Air+Phos, and Ozone+Phos groups. Ten minutes after pretreatment, half of the animals in each group were i.v. injected with capsaicin (CAP; $2 \mu\text{g/kg}$, a stimulator to increase endogenous tachykinins), and the other half were i.v. injected with substance P (SP; $10 \mu\text{g/kg}$, an exogenous tachykinin) to produce Evans blue dye extravasation.

Statistical Analysis

Results of exudative responses of nasal and laryngeal tissues were analyzed by one-way analysis of variance adjusted for multiple comparisons by Duncan's method when appropriate. A value of $p < 0.05$ was considered significant. All data were presented as means $\pm$ SE.

RESULTS

In the Air+Saline group, challenge of either capsaicin (Fig. 1) or substance P (Fig. 2) produced plasma exudation in nasal tissues as evidenced by a significant increase in Evans blue concentration. In the Ozone+Saline group, challenge of the same dose of either capsaicin (Fig. 1) or substance P (Fig. 2) produced an augmented nasal exudative response that was significantly greater than the response from the Air+Saline group. The Ozone+DMTU group had significantly greater nasal exudative response to challenge of capsaicin (Fig. 1) and substance P (Fig. 2) compared to the Air+Saline group, but was similar compared to the Ozone+Saline group. The nasal exudative responses to challenge of either capsaicin (Fig. 1) or substance P (Fig. 2) in both the Air+Phos and Ozone+Phos groups were significantly greater than the Air+Saline and the Ozone+DMTU group, but they were not significantly different from the Ozone+Saline group.

In contrast to the results obtained from nasal tissues, challenge of capsaicin failed to increase Evans blue concentration of laryngeal tissues in all study groups (Fig. 3). Challenge of substance P produced a significant increase in Evans blue concentration of laryngeal tissues in the Air+Saline group (Fig. 4). However, the laryngeal exudative response to substance P did not vary among the Air+Saline, Ozone+Saline, Ozone+DMTU, Air+Phos, and Ozone+Phos groups (Fig. 4).

DISCUSSION

Using a guinea-pig model, we demonstrated that both capsaicin- and substance P-induced nasal exudative responses, whereas only substance P caused laryngeal exudative responses. Capsaicin is known to stimulate C-fiber afferents to cause the release of endogenous tachykinins. Substance P, in contrast, is an

Figure 1. Evans blue dye contents of nasal tissue excised from animals receiving i.v. injection of capsaicin (Cap) after air or ozone exposure. Animals received pretreatment with saline vehicle, dimethylthiourea (DMTU), or phosphoramidan (Phos). a: Significantly different from the Air+Saline group; b: Significantly different from the Air+Saline+Cap group; c: Significantly different from the Ozone+Saline+Cap group; d: Significantly different from the Ozone+DMTU+Cap group. Data in each group are means $\pm$ SE for 8 animals.

Figure 2. Evans blue dye contents of nasal tissue excised from animals receiving i.v. injection of substance P (SP) after air or ozone exposure. Data in each group are means $\pm$ SE for 8 animals. See legend of Fig. 1 for detail descriptions of animal groups and statistical comparisons.

exogenous tachykinin. Ozone exposure significantly augmented nasal exudative responses to both challenges. However, it does not have significant effect on the laryngeal exudative responses to substance P. These results suggest that ozone exposure selec-

Figure 3. Evans blue dye contents of laryngeal tissue excised from animals receiving i.v. injection of capsaicin (Cap) after air or ozone exposure. See legend of Fig. 1 for detail descriptions of animal groups. No significance could be detected between any two groups. Data in each group are means $\pm$ SE for 8 animals.

Figure 4. Evans blue dye contents of laryngeal tissue excised from animals receiving i.v. injection of substance P (SP) after air or ozone exposure. See legend of Fig. 1 for detail descriptions of animal groups and statistical comparisons. Data in each group are means $\pm$ SE for 8 animals.

tively induces nasal airway hyperresponsiveness to endogenous and exogenous tachykinins.

The exact mechanisms by which ozone could augment nasal exudative responses to capsaicin or substance P are unclear. Ozone is shown to increase the excitabilities of airway C-fiber nerve endings.¹⁹ It also caused airway epithelial injury,^{28,29,30} and the consequent epithelial desquamation may make sensory nerve endings more accessible to exogenous stimuli. Thus, the same dose of capsaicin could cause a larger release of endogenous tachykinins from the sensitized C-fiber

nerve endings and a greater exudative response in the Ozone+Saline group compared to the Air+Saline group. In addition, ozone is shown to inhibit neutral endopeptidase,^{20,21} which may result in excess tachykinin in nasal tissues and augmented exudative responses to either capsaicin or substance P. In this study, pretreatment with phosphoramidon, an inhibitor for neutral endopeptidase, produced augmented nasal exudative responses in the Air+Saline group that mimicked the effect of ozone on nasal exudative responses in the Ozone+Saline group. It did not further increase nasal exudative responses in the Ozone+Saline group. These observations provide evidence of the involvement of nasal neutral endopeptidase inhibition in the development of ozone-induced nasal airway hyperresponsiveness to tachykinins. It is found that the ozone-induced augmentation of nasal exudative responses could be totally prevented by pretreatment with dimethylthiourea, a hydroxyl radical scavenger. This finding suggests that the hydroxyl radical plays a vital role in the pathogenesis of this nasal airway hyperreactivity. This is not surprising because the hydroxyl radical has been shown to inhibit airway neutral endopeptidase.^{31,32,33,34} Thus, pretreatment with dimethylthiourea may lower the oxidative stress of nasal tissues after ozone exposure and prevent the inhibition of nasal neutral endopeptidase by ozone.

In this study, challenge of substance P produced a significant increase in Evans blue concentration of laryngeal tissues, but challenge of capsaicin failed to increase Evans blue concentration of laryngeal tissues in the Air+Saline group. This observation shows that substance P activated tachykinin receptors in the laryngeal tissue, but this concentration of capsaicin could not activate laryngeal C-fibers. Furthermore, there was a different response to ozone exposure in the nose and larynx. The capsaicin- or substance P-induced nasal exudative response could be enhanced by ozone exposure. In contrast, substance P-induced laryngeal exudative response dose not significantly increase with ozone exposure. The apneic response induced by nasal chemical stimulus could be enhanced by ozone exposure, but the apneic response evoked by laryngeal chemical stimulus was inhibited by ozone exposure (data not shown). The upper airway includes the nose and larynx. Although they have a very close anatomic relationship, their physiologic regulations are quite different. Previous studies showed that laryngeal exposure to wood smoke triggers an apneic response that does not depend on laryngeal C-fiber afferents.^{35,36} In contrast, nasal exposure to wood smoke can evoke apneic or sniff-like responses and C-fiber afferents play an important role in this smoke-induced nasal response.³⁷ Different pathophysiologic responses between nose and larynx is demonstrated.^{36,37}

In summery, in the guinea-pig model, hydroxyl radicals play a vital role in the development of ozone-induced nasal airway hyperresponsiveness to tachykinins, which is possibly mediated through the suppressive action of ozone on the tachykinin degradation. Further research is needed to gain knowledge on the underline mechanisms of ozone-induced nasal hyperresponsiveness. Knowing this underlying mechanism might clarify the reasons for increasing prevalence of allergic rhinitis and the search for new therapeutic agents for allergic rhinitis.

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