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Tachykinins *via* Tachykinin NK_2 receptor activation mediate ozone-induced increase in the permeability of the tracheal mucosa in guinea-pigs

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1 Acute exposure to ozone is known to cause airway hyperresponsiveness, which, at least in part, seems to result from an increase in the permeability of the airway mucosa. Recently, we demonstrated that depletion of sensory neuropeptides inhibits the ozone-induced increase in the permeability of the tracheal mucosa in guinea-pigs. The aim of this study was to determine whether tachykinins mediate ozone-induced increase in the permeability of the tracheal mucosa in guinea-pigs.

2 Anaesthetized guinea-pigs were exposed to either 3 p.p.m. ozone or filtered air for 30 min. Immediately after exposure, a tracheal segment was isolated *in vivo* and administered with horseradish peroxidase (HRP). The permeability was assessed by monitoring the appearance of HRP in the blood.

3 A low dose of NKA increased the permeability of the tracheal mucosa, whereas a low dose of SP was without effect. Low and high doses of the selective NK_3 receptor agonist, senktide, were also without effect. The effect of a low dose of NKA was abolished by the NK_2 receptor antagonist, SR-48,968. A high dose of SP increased the permeability in a manner reversible by the NK_1 receptor antagonist, CP-96,345.

4 Pretreatment with SR-48,968 completely inhibited the ozone-induced increase in the permeability, whereas CP-96,345 had no effect.

5 It is thus concluded that endogenous tachykinins mediate the ozone-induced increase in the permeability of the tracheal mucosa in guinea-pigs mainly *via* NK_2 receptor activation.
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Abbreviations: CGRP, calcitonin gene-related peptide; HRP, horseradish peroxidase; NKA, neurokinin A; NKB, neurokinin B; SP, substance P

Introduction

Acute exposure to ozone, a principal component of photochemical smog is known to cause airway inflammation and hyperresponsiveness (Holtzman *et al*., 1983; Kaneko *et al*., 1994; Seltzer *et al*., 1986). This ozone-induced airway hyperresponsiveness may result, at least in part, by an increase in the permeability of the airway mucosa, which has also been shown to occur after ozone exposure (Kehrl *et al*., 1987; Miller *et al*., 1986; Nishiyama *et al*., 1998). However, the mediators of the ozone-induced increase in the permeability of the airway mucosa remains unknown.

Tachykinins, a group of neuropeptides including substance P (SP), neurokinin A (NKA), and neurokinin B (NKB) are known to be present in sensory nerves in the airways of various mammalian species, including man (Lundberg *et al*., 1984a; Martling, 1987; Regoli *et al*., 1994). SP, NKA, and NKB preferentially interact with the tachykinin NK_1, NK_2, and NK_3 receptors, respectively. These tachykinins are released by a range of chemical or physical stimuli which have pathophysiological importance, such as toluene diisocyanate (Thompson *et al*., 1987), histamine (Martins *et al*., 1991), cigarette smoke (Lundberg *et al*., 1984b), viral infection (Jacoby *et al*., 1988), and allergen (Bertrand *et al*., 1993). Recently, we demonstrated that capsaicin desensitization inhibits the ozone-induced increase in the permeability of the tracheal mucosa in guinea-pigs (Nishiyama *et al*., 1998). Capsaicin has been shown to selectively excite or (at higher doses) desensitize a specific subset of primary sensory nerves. Tachykinins *via* activation of these tachykinin receptors, cause a series of inflammatory responses collectively referred to as neurogenic inflammation. However, the other sensory neuropeptide, calcitonin gene-related peptide (CGRP) does not produce relevant proinflammatory effects in the airways. Thus, it is possible that endogenous tachykinins mediate the ozone-induced increase in the permeability of airway mucosa *via* activation of tachykinin receptors.

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The aim of this study was to determine whether tachykinins mediate the ozone-induced increase in the permeability of the tracheal mucosa in guinea-pigs. For this purpose we examined the effect of exogenous administration of the NK_1, NK_2, and NK_3, receptor agonists on the permeability and then we examined the effect of selective tachykinin receptor antagonists on the ozone-induced increase in the permeability of the tracheal mucosa in guinea-pigs.

Methods

The experimental protocol followed the 'Guiding Principles in the Care and Use of Animals' published by the Council of the American Physiological Society and the 'Guide for the Care and Use of Laboratory Animals' published by NIH. The protocol was approved by the Committee on Animal Research of the Yokohama City University School of Medicine.

Animals and animal preparation

Male Hartley strain guinea-pigs (Japan SLC, Shizuoka, Japan) weighing 450~550 g were used. Guinea-pigs were anaesthetized with an initial dose of 40 mg kg^{-1} sodium pentobarbital intraperitoneally and laid supine. Additional sodium pentobarbital was given as required to maintain anaesthesia. A catheter (0.51-mm ID × 0.94 mm-OD; Dow Corning, Midland, MI, U.S.A.) filled with heparinized saline was inserted into the femoral vein of each guinea-pig for administration of drugs and for drawing blood. As soon as the catheter had been inserted, anaesthetized guinea-pigs were exposed for 30 min to 3 p.p.m. ozone or filtered air in a 23.5-liter acrylic chamber.

Ozone exposure

Ozone was produced by an ozone generator (MOT-001A type; Nippon Ozone, Tokyo, Japan). Ozone concentration in the chamber was monitored continuously by use of an ultraviolet ozone analyzer (Model DY-1500; Nippon Ozone) and kept at 3 p.p.m. This dose of ozone was chosen because it has been shown to significantly increase the permeability of the tracheal mucosa in guinea-pigs (Nishiyama *et al*., 1998).

Measurement of the permeability of the tracheal mucosa

The permeability of the tracheal mucosa was measured by monitoring the appearance in the blood of horseradish peroxidase (HRP) that had been instilled into the isolated tracheal segment as previously reported (Nishiyama *et al*., 1998). Immediately after exposure to ozone or filtered air, a tracheal segment was isolated *in vivo* between two polyethylene cannulae (Disposable Multi-purpose Tube; ATOM Co., Tokyo, Japan) that were inserted by making tracheostomies. Fifteen minutes after the 30-min exposure to ozone or filtered air, HRP solution (50 mg ml^{-1}) was instilled very slowly to fill the lumen of the isolated tracheal segment. Blood samples were drawn before and 10, 20, 30 and 40 min after the instillation of HRP *via* the catheter, previously inserted into the femoral vein. The withdrawn blood was replaced with an equal volume of heparinized saline. Blood samples were immediately centrifuged, and the plasma was stored frozen at −20°C until assayed. Plasma HRP levels were measured by an ELISA as previously described (Nishiyama *et al*., 1998).

Effect of exogenous NK_1, NK_2, and NK_3 receptor agonists on the permeability of the tracheal mucosa

The effect of exogenously administered SP, NKA, and a selective NK_3 receptor agonist, senktide, on the permeability of the tracheal mucosa was examined using seven groups of five guinea-pigs each. After isolation of the tracheal segment, six groups were pretreated with phosphoramidon (1 mg kg^{-1} i.v.), and 5 min later, were administered with SP (1 or 10 nmol kg^{-1} i.v.), NKA (0.1 or 1 nmol kg^{-1} i.v.), or senktide (1 or 10 nmol kg^{-1} i.v.). The remaining group (control) was pretreated with phosphoramidon and then administered with the vehicle (normal saline). Plasma HRP was measured before and 10, 20, 30, and 40 min after tachykinin administration. The effect of the antagonists on SP- or NKA-induced increase in the permeability was investigated by pretreating two groups of guinea-pigs with either CP-96,345 (3 mg kg^{-1} i.v.) or SR-48,968 (300 μg kg^{-1} i. v.), 20 min before tachykinin injection.

Effect of NK_1 and NK_2 receptor antagonists on the ozone-induced increase in the permeability of the tracheal mucosa

The effect of a selective NK_1 receptor antagonist CP-96,345 and a selective NK_2 receptor antagonist SR-48,968 on the ozone-induced increase in the permeability of the tracheal mucosa was examined. Guinea-pigs were divided into six groups of five guinea-pigs each based on the drugs administered and exposure to filtered air or ozone: Groups 1 and 2 were pretreated with CP-96,345 (3 mg kg^{-1} i.v.) or its vehicle, respectively, and exposed to ozone; Group 3 was pretreated with the vehicle of CP-96345 and exposed to filtered air; Groups 4 and 5 were pretreated with SR-48,968 (300 μg kg^{-1} i.v.) or its vehicle, respectively, and exposed to ozone; and Group 6 was pretreated with the vehicle of SR-48,968 and exposed to filtered air. The doses of CP-96,345 and SR-48,968 were chosen because the doses of these drugs showed selective blockade of NK_1 or NK_2 receptors, respectively, in the previous studies (Emonds-Alt *et al*., 1992; Solway *et al*., 1993). Both drugs were administrated intravenously *via* the femoral catheter 15 min before exposure to either ozone or filtered air.

To examine whether tachykinin antagonists affect the permeability of the tracheal mucosa in the absence of ozone exposure, two additional groups of five guinea-pigs each were pretreated with either CP-96,345 (3 mg kg^{-1} i.v.) or SR-48,968 (300 μg kg^{-1} i.v.) and exposed to filtered air.

Statistical analysis

The mean values of HRP concentration in the plasma, normalized by the tracheal surface area (ng ml^{-1} cm^{-2}), were expressed as the arithmetic mean ± s.e.mean. Between-group differences in HRP concentration were assessed using two-way repeated-measure analysis of variance followed by the contrast method (means/regression coefficient comparisons). Probability values of $P<0.05$ were considered significant.

Drugs

The drugs used in this study were sodium pentobarbital (Abbott Laboratories, North Chicago, IL, U.S.A.), HRP (Zymed Laboratories, San Francisco, CA, U.S.A.), Rabbit anti-HRP polyclonal antibody (EY Laboratories, San Mateo, CA, U.S.A.), NKA, and senktide (Sigma, St. Louis, MO, U.S.A.). CP-96,345 and SR-48,968 were kindly provided by the Pfizer Central Research Division (Groton, CT, U.S.A.) and Sanofi Recherche (Montpellier Cedex, France), respectively.

Results

Effect of exogenous NK$_1$, NK$_2$, and NK$_3$ receptor agonists on the permeability of the tracheal mucosa

Administration of SP (1 nmol kg^{-1} i.v.) did not cause any change in the permeability of the tracheal mucosa (Figure 1). However, a higher dose of SP (10 nmol kg^{-1}) significantly increased the permeability of the tracheal mucosa. This increase was completely inhibited by pretreatment with the NK$_1$ receptor antagonist CP-96,345.

A dose as low as 0.1 nmol kg^{-1} i.v. of NKA significantly increased the permeability of the tracheal mucosa (Figure 2). Whereas a lower dose of NKA (0.01 nmol kg^{-1} i.v.) did not show any effect (data not shown). The increase in the permeability induced by NKA (1 nmol kg^{-1} i.v.) was completely inhibited by pretreatment with the NK$_2$ receptor antagonist SR-48,968.

On the other hand, administration of senktide did not cause any change in the permeability of the tracheal mucosa even in a higher dose (10 nmol kg^{-1}) (Figure 3).

Effect of NK$_1$ and NK$_2$ receptor antagonists on the ozone-induced increase in the permeability of the tracheal mucosa

Acute exposure for 30 min to 3 p.p.m. ozone increased significantly the permeability of the tracheal mucosa to HRP as compared to exposure to filtered air as indicated by the measurement of plasma HRP levels (Figure 4).

Pretreatment with the NK$_1$ receptor antagonist CP-96,345 (3 mg kg^{-1} i.v.) did not affect the ozone-induced increase in the permeability of the tracheal mucosa (Figure 4). In contrast, pretreatment with the NK$_2$ receptor antagonist SR-48,968 (300 μg kg^{-1} i.v.) completely inhibited this increase (Figure 5). CP-96,345 (3 mg kg^{-1}) or SR-48,968 (300 μg kg^{-1}) did not affect plasma HRP levels in guinea-pigs exposed to filtered air (data not shown).

Discussion

Recently, we demonstrated that neuropeptide depletion by capsaicin pretreatment inhibits the ozone-induced increase in the permeability of the tracheal mucosa in guinea-pigs (Nishiyama *et al*., 1998). Because CGRP does not produce relevant proinflammatory responses in rodent airways, the present observation supported the hypothesis that ozone exposure releases tachykinins from the sensory nerve endings, and thereby increases the permeability of the tracheal mucosa. The most important finding of the present study is that a selective NK$_2$ receptor antagonist inhibited completely the increase in the permeability, induced by ozone in the tracheal mucosa of guinea-pigs, thus indicating that tachykinins mediate this response.

SP and NKA are co-released from sensory nerve endings in similar amounts. Thus, the ability of tachykinins to produce a given response is dictated by the receptor type expressed on the target cell and by the relative affinity of the endogenous agonist for that receptor. In this study, the dose of SP required to increase the permeability of the tracheal mucosa in guinea-pigs was two log higher than that of NKA. This observation further suggests that the receptor mainly involved in the tachykinin-induced increase in the perme-

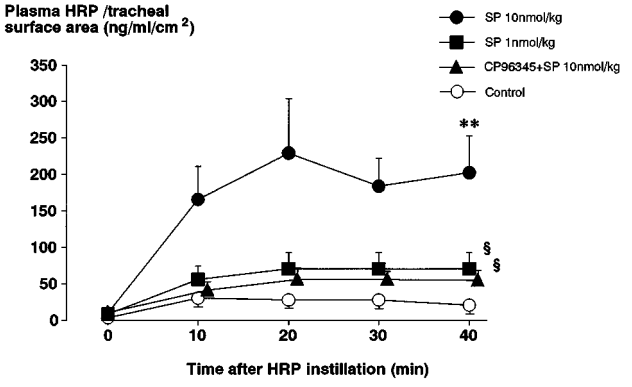

Figure 1 Effects of substance P (SP) on the permeability of the tracheal mucosa. Shown are plasma levels of horseradish peroxidase (HRP) in guinea-pigs administered 10 nmol kg^{-1} i.v. of SP ($n=5$, closed circles) and 1 nmol kg^{-1} i.v. of SP ($n=5$, closed squares), in guinea-pigs pretreated with CP-96,345 and then administered 10 nmol kg^{-1} i.v. of SP ($n=5$, closed triangles), and in controls ($n=5$, open circles). The time point 0 indicates the point of instillation of the HRP solution into the tracheal segment. $^{**}P<0.01$, as compared with the control group. $^{\S}P<0.01$, as compared with the group administered 10 nmol kg^{-1} i.v. of SP.

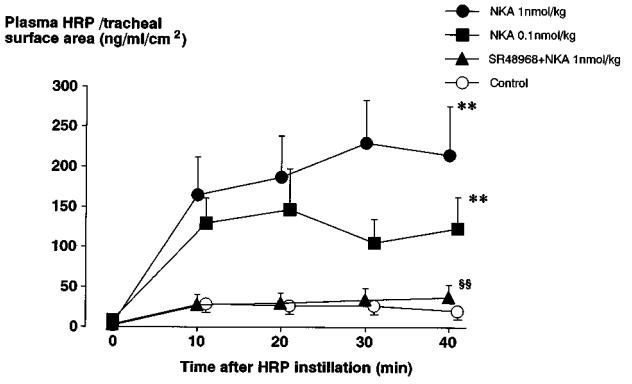

Figure 2 Effect of neurokinin A (NKA) on the permeability of the tracheal mucosa. Shown are plasma levels of horseradish peroxidase (HRP) in guinea-pigs administered 1 nmol kg^{-1} i.v. of NKA ($n=5$, closed circles) and 0.1 nmol kg^{-1} i.v. of NKA ($n=5$, closed squares), in guinea-pigs pretreated with SR-48,968 and then administered 1 nmol kg^{-1} i.v. of NKA ($n=5$, closed triangles), and in controls ($n=5$, open circles). The time point 0 indicates the point of instillation of the HRP solution into the tracheal segment. $^{**}P<0.01$, as compared with the control group. $^{\S}P<0.01$, as compared with the group administered 1 nmol kg^{-1} i.v. of NKA.

ability is the NK_2 receptor because NKA shows a better affinity for this receptor subtype (Maggi & Schwartz, 1997). This conclusion is in line with the observation that a selective NK_1 antagonist did not inhibit the ozone-induced increase in the permeability of the tracheal mucosa.

NKA has been shown to produce a diversity of physiological functions in the airways, including bronchoconstriction (Martling, 1987) and acceleration of the mucociliary movement (Kondo *et al*., 1990). However, the effect of NKA on the permeability of the airway mucosa has not been described before. Recently, it was reported that NKA increases the permeability of the duodenal mucosa in rats (Hällgren *et al*., 1997), an effect that was measured as the blood-to-lumen movement of EDTA. The direction of this movement was thus the opposite of that investigated here. To our knowledge, the present study is the first to show the involvement of NKA and NK_2 receptors in the enhancement of the permeability of the mucosa as measured by the lumen-to-blood movement of macromolecules.

In our study, we used a tracheal pouch system to assess the mucosal permeability (Nishiyama *et al*., 1998). The advantage of this method is that all tracers are equally distributed to the entire tracheal lumen of the pouch. Thus, the permeability is normalized for the tracheal surface area, which was estimated from calculations based on the volume and length of the tracheal pouch of each animal. However, mucosal permeability assessed as the lumen-to-blood transfer of a macromolecule *in vivo*, can be affected by additional factors, such as blood flow in the lumen, mucous secretion, and endothelial permeability of the vessels.

We observed that SP, a non-selective ligand for the three tachykinin receptors, produced an increase in the permeability of the tracheal mucosa that was abolished by a tachykinin NK_1 receptor antagonist. This finding suggests that NK_1 receptors may increase the permeability of the tracheal mucosa if appropriate doses of the agonist are given. However, these pharmacological doses probably exceed the physiological range and the present observation unmasks the presence and the function of a receptor, that does not exhibit any physiological or physiopathological role, regarding the ozone-induced increase in the permeability of tracheal mucosa. NK_1 receptors mediate airway vascular permeability in different tissues including the airways (Rogers *et al*., 1988). However, the permeability of the airway mucosa is a quite different process from vascular permeability. The permeability of the tracheal mucosa measures the diffusion of macromolecules from the tracheal lumen to the blood stream, whereas vascular permeability assesses the leakage of plasma proteins into the interstitial space. Thus, it is possible that different receptors are involved in completely diverse processes.

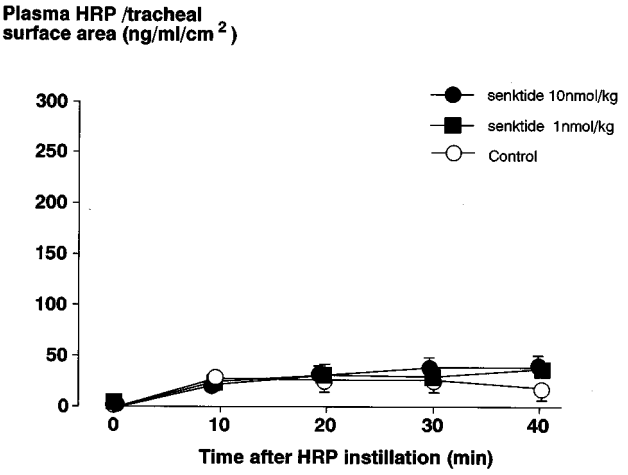

Figure 3 Effects of senktide on the permeability of the tracheal mucosa. Shown are plasma levels of horseradish peroxidase (HRP) in guinea-pigs administered 10 nmol kg^{-1} i.v. of senktide ($n=5$, closed circles) and 1 nmol kg^{-1} i.v. of senktide ($n=5$, closed squares), and in controls ($n=5$, open circles). The time point 0 indicates the point of instillation of the HRP solution into the tracheal segment.

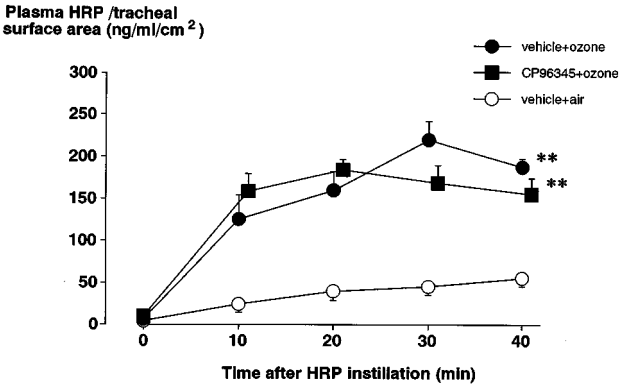

Figure 4 Effect of the NK_1 receptor antagonist, CP-96,345, on the ozone-induced increase in the permeability of the tracheal mucosa. Plasma levels of horseradish peroxidase (HRP) during a 40-min sampling period in guinea-pigs pretreated with CP-96,345 and then exposed for 30 min to 3 p.p.m. of ozone ($n=5$, closed squares), and in those pretreated with the vehicle and then exposed to either ozone ($n=5$, closed circles) or filtered air ($n=5$, open circles), are shown. The time point 0 indicates the point of instillation of the HRP solution into the tracheal segment. $^{**}P<0.01$, as compared with the vehicle-treated, air-exposed group.

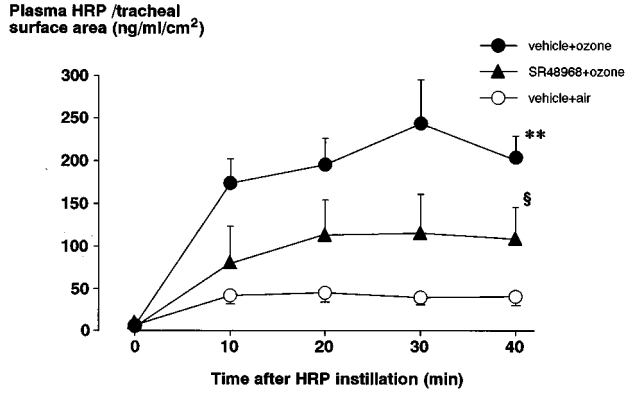

Figure 5 Effect of the NK_2 receptor antagonist, SR-48,968, on the ozone-induced increase in the permeability of the tracheal mucosa. Plasma levels of horseradish peroxidase (HRP) during a 40-min sampling period in guinea-pigs pretreated with SR-48,968 and then exposed for 30 min to 3 p.p.m. of ozone ($n=5$, closed triangles), and in those pretreated with the vehicle and then exposed to either ozone ($n=5$, closed circles) or filtered air ($n=5$, open circles), are shown. The time point 0 indicates the point of instillation of the HRP solution into the tracheal segment. $^{**}P<0.01$, as compared with the vehicle-treated, air-exposed group. $^{\S}P<0.01$, as compared with the vehicle-treated, ozone-exposed group.

Plasma levels of HRP in guinea-pigs pretreated with the NK_2 receptor antagonist and exposed to ozone showed a tendency to exceed those found in guinea-pigs pretreated with its vehicle and exposed to filtered air. However, the difference was not statistically significant. It is possible that additional mechanisms, other than tachykinins/NK_2 receptors, are involved in the ozone-induced permeability. Inflammatory mediators such as platelet-activating factor and arachidonic acid products, which could be released from airway epithelium during ozone exposure, might also be important (Kaneko *et al*., 1995; Samet *et al*., 1992).

In contrast to our *in vivo* study, a previous study suggested that NK_1 receptor activation reduces the permeability caused by ozone in cultured airway epithelial cells (Yu *et al*., 1996). If this mechanism occurs *in vivo*, the NK_1 receptor antagonist should potentiate the ozone-induced increase in the permeability. However, results of our present study showed that NK_1 receptor antagonist had no effect. Their results involving the NK_1 receptors were obtained only after the use of very high doses of SP. In addition, their study assessed the permeability by measuring the tracer flux in the submucosal to luminal direction, which is opposite to ours. These differences may account for its different results. It is suggested that *in vitro* models of airway mucosal permeability may not be good predictors of the same event when it occurs *in vivo*.

In conclusion, the present study in guinea-pigs demonstrated that activation of NK_2 receptors by endogenous tachykinins mediate the ozone-induced increase in the permeability of the tracheal mucosa.

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