



Involvement of peroxynitrite in pollen-induced nasal blockage in guinea pigs

Nobuaki Mizutani ^{a,b}, Takeshi Nabe ^{a,*}, Masanori Fujii ^a, Shin Yoshino ^b, Shigekatsu Kohno ^a

^a Department of Pharmacology, Kyoto Pharmaceutical University, 5 Nakauchi, Misasagi, Yamashina, Kyoto 607-8414, Japan

^b Department of Pharmacology, Kobe Pharmaceutical University, 4-19-1 Motoyamakita, Higashinada, Kobe 658-8558, Japan

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Abstract

Nitric oxide (NO) has been implicated in early and late phase nasal blockage in a Japanese cedar pollen-induced experimental allergic rhinitis guinea pig model. In this study, we investigated the role of peroxynitrite, which is formed by a rapid reaction of NO with superoxide anion, in the antigen-induced biphasic nasal blockage. Sensitized guinea pigs were repeatedly challenged by pollen inhalation once every week. The peroxynitrite scavenger, ebselen (30 mg/kg), or the xanthine oxidase inhibitor, allopurinol (50 mg/kg), was intraperitoneally administered 30 min before the antigen challenge. The late phase nasal blockage induced 4 h after the challenge was largely suppressed by ebselen (57% inhibition; $P < 0.05$) and allopurinol (47% inhibition; $P < 0.05$), but neither ebselen nor allopurinol influenced the early phase response. On the other hand, the intranasal instillation of peroxynitrite (10^{-3} and 10^{-2} M, 10 μ l/nostril) caused a remarkable dose-dependent nasal blockage in the sensitized guinea pig. These results suggest that peroxynitrite plays a major role in the late phase nasal blockage induced by the antigen challenge in sensitized guinea pigs.

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1. Introduction

Nasal blockage is considered to be the most serious problem for patients suffering from allergic rhinitis. When a specific allergen is applied to the nasal cavities of allergic rhinitis patients, over 90% show an immediate sneezing, rhinorrhea and nasal blockage response. In addition, approximately 50% develop a late phase reaction, with the predominant symptom being nasal blockage (Ilipoulos et al., 1990). However, detailed mechanisms underlying nasal blockage are unclear. The nasal mucosa contains venous sinusoids, and an increase in blood flow into the sinusoids produces a rapid reduction in the volume of the nasal airway cavity. Thus, it is strongly suggested that nasal blockage is mainly induced by dilation of the blood vessels in the sinusoids (Eccles, 1995).

Nitric oxide (NO) is a powerful vasodilator that modulates systemic vascular tone (Rees et al., 1989). One study has shown in healthy subjects that the large amount of NO that originated

from the paranasal sinuses was continuously produced in the nasal cavities (Lundberg et al., 1995). Furthermore, Amal et al. (1997) and Kharitonov et al. (1997) reported that the NO concentration in exhaled air was elevated in patients with allergic rhinitis compared to that in normal subjects. On the other hand, NO rapidly reacts with superoxide anions, which are released from inflammatory cells such as neutrophils (Salman-Tabcheh et al., 1995; McCall et al., 1989; Carreras et al., 1994; Sutherland et al., 1993; Weiss, 1989), macrophages (Avron and Gallily, 1995; Rodenas et al., 1995), eosinophils (Pincus et al., 1982; Nabe et al., 1998a; Hashimoto et al., 2003), and endothelial cells (Szabo et al., 1995; Kooy Poyall, 1994), and results in the formation of a highly proinflammatory molecule, peroxynitrite, suggesting that peroxynitrite may be involved in the nasal blockage of allergic rhinitis. However, involvement of peroxynitrite in allergic rhinitis has not been demonstrated to date.

We developed a Japanese cedar pollen-induced allergic rhinitis model in sensitized guinea pigs, in which marked biphasic nasal blockage was induced following repeated pollen challenge (Nabe et al., 1998b). We have indicated that NO

* Corresponding author. Tel.: +81 75 595 4668; fax: +81 75 595 4764.

E-mail address: nabe@mb.kyoto-phu.ac.jp (T. Nabe).

produced by activation of constitutive NO synthase (cNOS), rather than of inducible NOS, is involved in the pollen-induced early and late phase nasal blockage (Imai et al., 2001), and that the late phase nasal blockage can be related to the production of NO through cysteinyl leukotriene (CysLT) receptor activation (Mizutani et al., 2001).

The aim of this study was to examine the role of peroxynitrite in nasal blockage in this experimental allergic rhinitis model. We pharmacologically assessed involvement of peroxynitrite and superoxide anion on the allergic biphasic nasal blockage and the LTD₄-induced nasal blockage using each of the synthase inhibitors or scavengers. We also evaluated whether the instillation of peroxynitrite induces nasal blockage.

2. Materials and methods

2.1. Animals

Male 3-week-old Hartley guinea pigs (weight, 200–250 g) were purchased from Japan SLC (Hamamatsu, Japan). Animals were housed in an air-conditioned room at 23 ± 1 °C and 60 ± 10% humidity, with lights on from 08:00 to 20:00. Access to standard laboratory diet (Labo G standard, Norsan, Yokohama, Japan) and water was provided *ad libitum*. All study protocols were approved by the Experimental Animal Research Committee at Kyoto Pharmaceutical University.

2.2. Reagents

Japanese cedar (*Cryptomeria japonica*) pollen was harvested in Gifu and Shiga prefectures in 1998. LTD₄ was obtained from Cayman Chemical (Ann Arbor, MI, USA). *N*^ω-Nitro-L-arginine methyl ester (L-NAME) was purchased from Wako Pure Chemical (Osaka, Japan). Ebselen and allopurinol were purchased from Sigma Aldrich Chemical (St. Louis, MO, USA). Peroxynitrite was obtained from Dojindo Laboratory (Kumamoto, Japan).

Al(OH)₃ gel was prepared using 0.25 N NaOH and 0.25 N Al₂(SO₄)₃, as described elsewhere (Nabe et al., 1997a).

The cedar pollen extract used for sensitization was prepared as described elsewhere (Nabe et al., 1998b). Briefly, pollen was suspended in phosphate-buffered saline at 100 mg/ml and kept at 4 °C for 18 h with mild stirring. The suspension was then centrifuged (1700 × g, 15 min), and the supernatant was stored at –80 °C until used as the sensitization antigen.

L-NAME was dissolved in saline. Ebselen and allopurinol were dissolved in dimethyl sulfoxide.

Peroxynitrite (10^{–3} and 10^{–2} M) was diluted in phosphate-buffered saline 1 min before the intranasal instillation. For the negative control, decomposed peroxynitrite was prepared by leaving the solution at room temperature for more than 1 month.

2.3. Sensitization and challenge with cedar pollen

As previously described (Nabe et al., 1998b), guinea pigs were sensitized by bilateral intranasal instillation of cedar pollen extracts adsorbed onto Al(OH)₃ gel (0.3 μg protein/0.3 mg

Al(OH)₃) at a volume of 3 μl/each nostril twice a day for 7 days. To prevent the rapid elimination of the antigen by ciliary movement, prior to each sensitization, the upper airway mucosal surface was anesthetized by using a 5-min inhalation of a mist of 4% lidocaine hydrochloride solution that was generated by an ultrasonic nebulizer (NE-U12; Omron, Osaka, Japan). The sensitized animals were then intranasally challenged once every week by inhalation of 1.8 mg of cedar pollen (3.6 mg/animal) using a handmade inhalation apparatus (Nabe et al., 1997b).

2.4. Measurement of nasal blockage

To evaluate the degree of nasal blockage, specific airway resistance (sRaw) was measured by a two chambered, double-flow plethysmograph system (Pulmos-I; MIPS, Osaka, Japan) according to the method of Pennock et al. (1979). Briefly, each animal was placed with their neck extended through the partition of a 2-chambered box. Sensors in the front and rear chambers detected airflow, and sRaw was measured using a Pulmos-I analyzer. The change in sRaw was expressed as the value obtained minus the pre-challenge baseline value in sRaw (before).

2.5. Effects of L-NAME, ebselen and allopurinol on biphasic nasal blockage induced by pollen inhalation challenge

A NOS inhibitor, L-NAME (30 mg/kg) was intravenously administered 15 min before the 15th challenge. A peroxynitrite scavenger, ebselen (30 mg/kg) or a xanthine oxidase (XO) inhibitor, allopurinol (50 mg/kg) was intraperitoneally administered 30 min before the 15th antigen challenge (Fig. 1).

The dose of each inhibitor or scavenger has been reported to cause an almost maximal inhibition (Imai et al., 2001; Miyamoto et al., 1996; Takasago et al., 1997).

2.6. Effects of ebselen and allopurinol on nasal blockage induced by LTD₄ instillation

Nasal responsiveness tests described previously (Mizutani et al., 2001) were performed according to the following procedures: doses of LTD₄ (10^{–8} and 10^{–6} M, 10 μl/nostril) were consecutively instilled at 20 min intervals to the sensitized guinea pig 2 days after the 20th antigen challenge (Fig. 1). sRaw

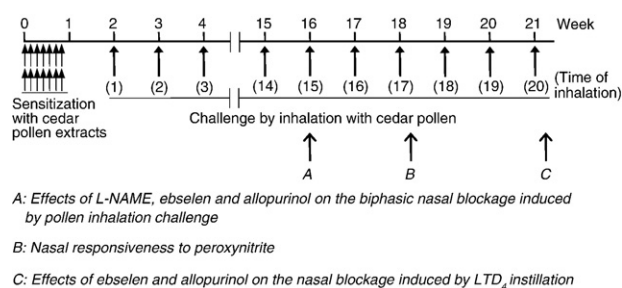


Fig. 1. Experimental study protocol. L-NAME, *N*^ω-Nitro-L-arginine methyl ester.

was measured 10 min after the respective instillation. Ebselen (30 mg/kg) and allopurinol (50 mg/kg) were administered intraperitoneally 30 min before LTD₄ (10^{-8} M) instillation.

2.7. Nasal responsiveness to peroxynitrite

To estimate the nasal responsiveness to peroxynitrite, sRaw was measured before and after an intranasal instillation of a peroxynitrite solution (10 μ l/nostril) (Fig. 1).

To measure the time-course changes in sRaw elevation by peroxynitrite, a fixed concentration of peroxynitrite (10^{-3} M) was applied to sensitized-challenged guinea pigs 2 days after the 17th pollen inhalation challenge, and sRaw was monitored for 20 min.

To induce sRaw elevation by 2 doses of peroxynitrite, peroxynitrite (10^{-3} and 10^{-2} M, 10 μ l/nostril) was intranasally instilled at a 20-min interval to the sensitized-challenged guinea pigs 2 days after the 17th antigen challenge. sRaw was measured 10 min after the respective instillation.

2.8. Statistical analyses

Data are represented as means $\pm$ S.E.M. Main statistical analyses were performed with a Student's *t*-test when two groups were compared. Comparison of the effects of ebselen and allopurinol on the nasal blockage induced by LTD₄ (Fig. 3) was performed by one-way analysis of variance (ANOVA). When a significant difference was detected, individual group differences were determined by Bonferroni's multiple test. A probability value of $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Effects of L-NAME, ebselen and allopurinol on the biphasic nasal blockage

Consistent with our previous finding (Nabe et al., 1998b), in sensitized-challenged guinea pigs, there were two peaks (1 and 4 h after the antigen challenge) during the time-course changes

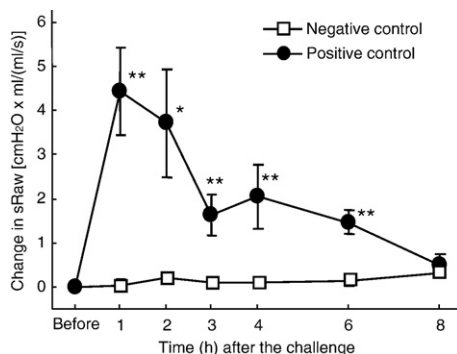


Fig. 2. Time-course changes in the biphasic nasal blockage induced by the 15th antigen challenge in the sensitized guinea pig. Each point represents the mean $\pm$ S.E.M. of 6 animals. * $P < 0.05$ and ** $P < 0.01$ compared with normal. Negative control, nonsensitized-challenged; positive control, sensitized-challenged.

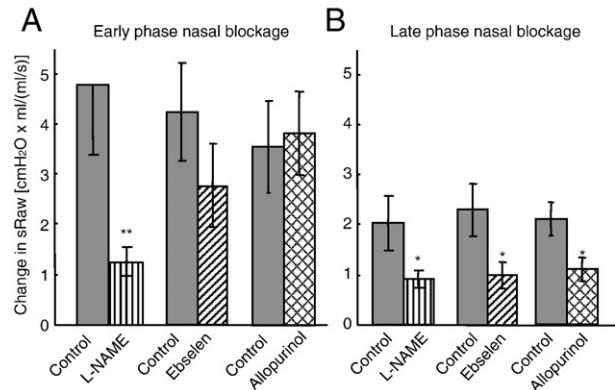


Fig. 3. Effects of L-NAME, ebselen and allopurinol on the early (A) and late phase (B) nasal blockage induced by the 15th antigen challenge in the sensitized guinea pig. Each column represents the mean $\pm$ S.E.M. of 6 animals. * $P < 0.05$ and ** $P < 0.01$ compared with control. Control, sensitized-challenged.

for sRaw after the challenge. This indicates that the antigen-induced increase in sRaw consisted of both an early and a late phase nasal blockage response (Fig. 2). In contrast, sRaw did not change after pollen inhalation in nonsensitized guinea pigs (Fig. 2).

After an antigen challenge, we investigated the effects of L-NAME, ebselen and allopurinol on the early and late phase nasal blockages at 1 and 4 h, respectively. As previously reported (Imai et al., 2001), L-NAME strongly and significantly inhibited both early and late phase nasal blockages (Fig. 3A and B). On the other hand, ebselen and allopurinol significantly suppressed the late phase nasal blockage at 4 h after the antigen challenge but not the early phase response (Fig. 3A and B).

3.2. Effects of ebselen and allopurinol on LTD₄-induced nasal blockage

Fig. 4 shows the effects of ebselen and allopurinol on the nasal blockage induced by LTD₄ instillation. In the sensitized

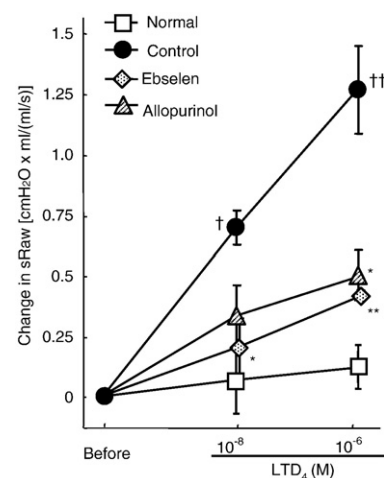


Fig. 4. Effects of ebselen and allopurinol on the nasal blockage induced by the instillation of LTD₄ 2 days after the 20th antigen challenge in the sensitized guinea pigs. Each point represents the mean $\pm$ S.E.M. of 6 animals. † $P < 0.05$ compared with normal. * $P < 0.05$ and ** $P < 0.01$ compared with control. Normal, nonsensitized-challenged; control, sensitized-challenged.

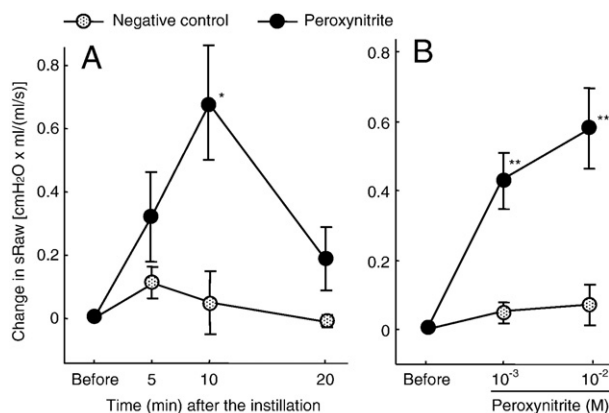


Fig. 5. Time-course changes in nasal blockage induced by the instillation of peroxynitrite (10^{-3} M) (A) and nasal blockage response to two doses of peroxynitrite (10^{-3} and 10^{-2} M) (B) 2 days after the 17th antigen challenge in the sensitized guinea pigs. Each point represents the mean $\pm$ S.E.M. of 6 animals. * $P < 0.05$ and ** $P < 0.01$ compared with negative control. Negative control, sensitized-challenged-peroxynitrite decomposition.

guinea pig, a dose-dependent nasal blockage was observed at 10^{-8} and 10^{-6} M LTD₄ 2 days after the antigen challenge, whereas the nonsensitized guinea pig did not show any nasal blockage response to LTD₄ even at 10^{-6} M. The nasal blockage induced by LTD₄ in the sensitized animal was significantly inhibited by ebselen and allopurinol.

3.3. Change in nasal airway after intranasal peroxynitrite application

Fig. 5A shows the time-course change in sRaw after instillation of peroxynitrite into both nasal cavities of the sensitized guinea pigs 2 days after the pollen inhalation challenge. Instillation of peroxynitrite caused a swift elevation of sRaw that peaked at 10 min followed by a diminution at 20 min. Thus, sRaw was measured 10 min after the peroxynitrite instillation in the experiments that followed.

Nasal responsiveness to peroxynitrite was evaluated 2 days after the antigen challenge. As a result, a dose-dependent nasal blockage was recognized at 10^{-3} and 10^{-2} M peroxynitrite (Fig. 5B). In contrast, no changes were noted for sRaw after the instillation of a solution containing decomposed peroxynitrite in a sensitized-challenged animal.

4. Discussion

Previous studies indicated that NO is largely involved in the pathogenesis of the pollen-induced biphasic nasal blockage in the present experimental allergic rhinitis model (Imai et al., 2001). On the other hand, the most harmful effects caused by the high levels of NO originate from the reaction of NO with superoxide anions that subsequently forms peroxynitrite. Thus, it will be quite interesting to discover whether peroxynitrite is actually involved in the pathogenesis of nasal blockage in the sensitized guinea pig.

We examined whether a peroxynitrite scavenger, ebselen, inhibits the early and late phase nasal blockages after the pollen

inhalation challenge in the allergic rhinitis model. Ebselen significantly inhibited the late phase nasal blockage, but the early phase nasal blockage was not significantly affected by it. Since ebselen efficiently scavenges peroxynitrite (Haenen et al., 1997), the reduction of peroxynitrite should account for the effectiveness of ebselen during late phase nasal blockage. On the other hand, it has been reported that NO rapidly reacts to superoxide anions, resulting in the production of peroxynitrite (Huie and Padmaja, 1993). Therefore, it is of interest to examine the role of superoxide anions in biphasic nasal blockage induced by the antigen challenge. We found that the xanthine oxidase inhibitor, allopurinol, significantly reduced the antigen-induced late phase nasal blockage but not the early phase response. This indicates that the inhibitory effect of allopurinol on the late phase nasal blockage might be related to the decrease in the production of peroxynitrite that is accompanied by the reduction of superoxide anion. Overall, these results suggest that both peroxynitrite and superoxide anions as well as NO are the key molecules that lead to the late phase nasal blockage.

Early phase nasal blockage induced by the antigen challenge was inhibited by L-NAME but not ebselen, indicating that NO but not peroxynitrite was relevant to the induction of the early phase response. The difference in the effects on the early phase nasal blockage between L-NAME and ebselen may be related to the time of the production of the superoxide anion. It has been reported that superoxide anion is produced by inflammatory cells such as neutrophils (Salman-Tabcheh et al., 1995; McCall et al., 1989; Carreras et al., 1994; Sutherland et al., 1993; Weiss, 1989), macrophages (Avron and Gallily, 1995; Rodenas et al., 1995), eosinophils (Pincus et al., 1982; Nabe et al., 1998a; Hashimoto et al., 2003), and endothelial cells (Szabo et al., 1995; Kooy and Poyall, 1994). We previously reported that there was infiltration of inflammatory cells including mononuclear cells, neutrophils and eosinophils into the nasal tissue during the late phase nasal blockage in our experimental model (Yamasaki et al., 2001). This suggested that the inflammatory cell infiltrate produced superoxide anion, with a subsequent production of peroxynitrite by the reaction of NO with the superoxide anion that developed during the late phase nasal blockage in the sensitized guinea pig. On the other hand, we have demonstrated that with an almost complete reduction of eosinophils by an anti-IL-5 antibody treatment, there was no effect on the late phase nasal blockage (Yamasaki et al., 2002). This indicates that eosinophils can be excluded as a candidate for a cellular source of the superoxide anion that is closely associated with the induction of the late phase nasal blockage.

On the other hand, late phase, but not early phase, nasal blockage was significantly attenuated by pranlukast, a CysLT₁ receptor antagonist (Mizutani et al., 2001). Furthermore, we found that marked nasal hyperresponsiveness (nasal blockage) to intranasal instillation of LTD₄ was observed 2 days after the antigen challenge in the sensitized guinea pig, and that the LTD₄-induced nasal blockage was strongly inhibited by L-NAME (Mizutani et al., 2001), indicating that the late phase nasal blockage is at least in part due to vasodilatation, and most likely due to NO production by cNOS via the CysLT₁ receptor activation in the endothelial cells of the nasal mucosa vessel.

Therefore, in a subsequent experiment, we examined the role of peroxynitrite on the nasal blockage induced by LTD₄ instillation. Consistent with the inhibitory effects seen during the late phase nasal blockage that was induced by the antigen challenge, LTD₄-induced nasal blockage was also inhibited by ebselen and allopurinol. These results suggest that NO produced by LTD₄ instillation may react with superoxide anion, which is produced by the LTD₄ stimulation or progressively produced after the antigen challenge in the nasal mucosa of the sensitized guinea pigs, leading to the formation of peroxynitrite. In addition, the formation of peroxynitrite should be related to the development of LTD₄-induced nasal blockage. When taken together, development of the late phase nasal blockage induced by the antigen challenge may be related to the peroxynitrite that is produced by the activation of CysLT₁ receptors in the sensitized guinea pigs.

It has been reported that levels of nitrotyrosine, which are formed after peroxynitrite attacks tyrosine residues, are markedly elevated in the nasal mucosa of patients with perennial allergic rhinitis, but absent in nonallergic patients (Sato et al., 1998). However, at the present, there are no studies that support the increased nitrotyrosine that we observed in this experimental allergic rhinitis model. Thus, in the future, whether there are increased levels of nitrotyrosine in the nasal mucosa in this model needs to be investigated.

On the other hand, when a sensitized guinea pig was treated with the instillation of peroxynitrite, nasal blockage was clearly induced. In our preliminary study, we examined the in vitro response of nasal mucosa to peroxynitrite in guinea pigs. The nasal mucosal preparation was moderately pre-contracted by a treatment with norepinephrine. The addition of peroxynitrite (10^{-7} – 10^{-5} M) concentration-dependently relaxed the pre-contracted mucosal tension (Mizutani et al., unpublished data). As no muscle tissue is present other than the vascular smooth muscle in the nasal mucosa, the contraction and relaxation of the nasal mucosa should be almost completely reflected by the movement of the vascular smooth muscle cells. Based on these observations, nasal blockage induced by the instillation of peroxynitrite may be the reason for its vasodilative effect.

In conclusion, peroxynitrite produced by reaction of NO with superoxide anion is an important molecule in the late phase nasal blockage in the sensitized guinea pigs.

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