

Synergism between cysteinyl leukotrienes and thromboxane A₂ to induce allergic late phase nasal blockage in guinea pigs

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Abstract

We examined whether cysteinyl leukotrienes (CysLTs) and thromboxane (TX) A₂ are synergistically involved in a cedar pollen-induced allergic late phase nasal blockage in guinea pigs. Sensitized animals were repeatedly challenged by pollen inhalation once every week. Combined treatment with pranlukast (a CysLT antagonist) and seratrodist (a TXA₂ antagonist) inhibited late phase nasal blockage, but the magnitude of inhibition (approximately 50%) was equal to those of the respective single treatments, suggesting that CysLTs produced late after challenge induces TXA₂ production in the nasal tissue, as in the case of the lung of this species. However, pranlukast did not affect TXB₂ increase in the nasal tissue. In contrast, combined intranasal instillation of LTD₄ and U-46619 (a TXA₂ mimetic) produced much greater nasal blockage than single administration of each agonist in sensitized animals. Therefore, allergic late phase nasal blockage should be induced by synergistic activity of CysLTs and TXA₂ at the effector organ.

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1. Introduction

Nasal blockage is considered the most serious problem for patients suffering from allergic rhinitis. When a specific allergen is applied to the nasal cavities of patients, over 90% show an immediate response of sneezing, rhinorrhea and nasal blockage. In addition, approximately 50% develop a late phase reaction, with the predominant symptom being nasal blockage [1]. The details of the mechanisms of induction of nasal blockage are unclear. 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. Plasma leakage in the capillary vessels, at the nasal mucosa, is also involved in the induction of nasal blockage via the formation of mucosal edema that accompanies increase in blood flow.

Several chemical mediators that participate in induction of allergic nasal blockage have been described. Studies indicate that cysteinyl leukotrienes (CysLTs) and thromboxane (TX) A₂, which are arachidonic acid metabolites, are important mediators in induction of nasal blockage [2–8]. CysLT₁ receptor antagonists and TP receptor antagonists have been used clinically as therapeutic drugs for allergic rhinitis [9–11]. In previous experimental animal studies, we found that a specific antagonist against CysLT₁ and TP receptors, pranlukast [12,13] and seratrodast [14,15], respectively, effectively suppressed late phase nasal blockage in cedar pollen-induced allergic rhinitis of guinea pigs [16,17]. We also observed a late phase peak in generation of CysLTs and TXA₂ in nasal tissue, during induction of late phase nasal blockage [17], indicating that CysLTs and TXA₂ contribute to the induction of late response. More interestingly, the sum of inhibitory rates produced by respective single treatments with pranlukast and seratrodast has been calculated to be greater than 100% [17], implying interaction between CysLTs and TXA₂ in inducing late phase nasal blockage. However, there have been no reported studies of the mechanisms of interaction between CysLTs and TXA₂ in induction of nasal blockage.

In the present study, we first evaluated the effect of combined treatment with the antagonist pranlukast and seratrodast on allergen-induced nasal blockage in an experimental model. This combined treatment produced no additive inhibition of late phase nasal blockage. Based on this finding, we hypothesized two mechanisms of interaction between CysLTs and TXA₂ in induction of nasal blockage: (1) CysLT-induced nasal blockage at the late phase is totally dependent on TXA₂ generation because it has been well known that LTD₄ can induce TXA₂ production in the lung of guinea pigs [18,19]; and (2) CysLTs and TXA₂ synergistically act on different effector sites in the nasal tissue to induce nasal blockage. To test the first hypothesis, we examined the effects of pranlukast on TXA₂ production in nasal tissue during late phase. To test the second hypothesis, we examined whether intranasal instillation of LTD₄ and U-46619 (a TXA₂ mimetic) synergistically induces nasal blockage.

2. Materials and methods

2.1. Animals

Male, 3-week-old Hartley guinea pigs weighing 200–250 g were purchased from Japan SLC (Hamamatsu, Japan). They were housed in an air-conditioned room at $23 \pm 1^\circ\text{C}$ and

60 ± 10% humidity, with lights on from 08:00 to 20:00 h. The animals were fed a standard laboratory diet and given water ad libitum. The study was 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. Lidocaine hydrochloride was obtained from Fujisawa Pharmaceutical Co., Osaka, Japan. Leukotriene (LT) D₄ and U-46619 were obtained from Cayman Chem., Ann Arbor, MI, USA. Pranlukast hemihydrate was supplied by Dr. H. Ohno, Ono Pharm. Com., Osaka, Japan. Seratrodast was supplied by Dr. Y. Ashida, Takeda Pharm. Ind., Osaka, Japan. LTD₄ and U-46619 were diluted with physiological saline containing 0.5% ethanol. Pranlukast and seratrodast were suspended in 0.5% methylcellulose.

Aluminum hydroxide [Al(OH)₃] gel was prepared with 0.25N NaOH and 0.25N Al₂(SO₄)₃, as described elsewhere [20].

The cedar pollen extract used for sensitization was prepared as described elsewhere [21]. Briefly, the 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.

2.3. Sensitization and challenge with cedar pollen

Guinea pigs were sensitized by intranasal instillation of cedar pollen extract adsorbed on Al(OH)₃ gel into the bilateral cavities at a dose of 0.3 µg protein per 0.3 mg Al(OH)₃ per 3 µl per nostril twice each day for 7 days [22]. Prior to each sensitization, the upper airway mucosal surface was topically anesthetized by a 5-min inhalation of 4% lidocaine mist, generated using an ultrasonic nebulizer (NE-U12, Omron, Osaka, Japan), to prevent rapid elimination of antigen by ciliary movement. The sensitized animal was intranasally challenged once every week by inhalation of 1.8 mg of cedar pollen per nostril (3.6 mg/animal) using a hand-made inhalation apparatus [21] until the 13th challenge. We previously reported that such cedar pollen challenge induced almost reproducible biphasic nasal blockage from the 4th to 10th challenges [22].

Upon application to both nostrils of a spontaneously breathing guinea pig, almost all inhaled pollen was trapped in the upper airways [21].

2.4. Measurement of specific airway resistance (sRaw)

As an indicator of respiratory function (nasal blockage), sRaw was measured in conscious guinea pigs using a two-chambered, double-flow plethysmograph system (Pulmos-I-II-III, M.I.P.S., Osaka, Japan) as described by Pennock et al. [23]. Briefly, an animal was placed with its neck extending through the partition of a two-chambered box. Sensors in the front and rear chambers detected airflow, and sRaw was measured using a Pulmos-I-II-III data analyzer.

Because guinea pigs functionally respire through the nose but not through the mouth, sRaw can be considered the total resistance of the upper and lower airways. However, in a

previous study, when spontaneously breathing conscious guinea pigs were forced to inhale pollen as described above, almost all of the inhaled pollen was trapped in the upper airways, with less than 0.001% reaching to the lower airways [21]. Furthermore, although antigen inhalation-induced, early bronchoconstrictor response is characterized by rapid and shallow breathing in another guinea pig model of asthma [24], previous findings show that in the present model, the pollen inhalation challenge-induced elevation of sRaw correlates well with the decrease in respiratory frequency [22]. In addition, eosinophil accumulation in the lung, which is a characteristic feature of asthmatic response, was not observed at 5 h after the pollen inhalation challenge in the present model [17], although eosinophils were markedly increased in the nasal cavity lavage fluid (NCLF) at 5 h after the challenge [25]. These findings indicate that the change in sRaw induced by pollen challenge in the present model almost exclusively reflects upper airway obstruction.

2.5. Nasal cavity lavage (NCL)

The nasal cavity of the sensitized animal was lavaged with physiologic saline before, and 5 h after the 8th antigen challenge. NCL was performed once for each guinea pig, as previously described [21,25]. Briefly, each guinea pig was anesthetized i.p. with sodium pentobarbital (30 mg/kg) at the predetermined time point: before, or 5 h after the antigen challenge. Silicon tubing connected to an air pump was positioned in the left nostril, which was then kept under slightly reduced pressure. Washing with 1 ml of physiologic saline prewarmed to 37 °C was performed from the right nostril to the left nostril. The NCLF recovered from the left nostril was centrifuged at $120 \times g$ for 5 min at 4 °C. The resultant supernatant was stored at –80 °C until assayed for concentration of CysLTs and TXB₂.

2.6. Measurement of CysLTs and TXB₂

CysLTs and TXB₂ in NCLF were measured by enzyme immunoassay after extraction of the sample as previously reported [17]. A 1.6 ml volume of ice-chilled ethanol was added to 0.4 ml of sample, and the mixture was allowed to stand at 0 °C for 30 min. After centrifugation at $15,000 \times g$ for 10 min at 4 °C, 40 µl of 0.2% gelatin solution was added to 1.8 ml of the supernatant. After standing at –20 °C for 1 h, the solution was evaporated to dryness under reduced pressure. The residue was then dissolved in 0.36 ml of 0.1 M phosphate buffer (pH 7.5) containing 0.1% bovine serum albumin. Amounts of CysLTs and TXB₂ in the solution were measured using a leukotriene C₄/D₄/E₄ enzyme-immunoassay system (Amersham International plc, Buckinghamshire, UK) and a TXB₂ enzyme immunoassay kit (Cayman Chemical Co., Ann Arbor, MI, USA), respectively.

2.7. Intranasal instillation of LTD₄ and U-46619-induced nasal blockage

LTD₄ (10^{-12} and 10^{-10} M) and U-46619 (10^{-9} and 10^{-7} M) were intranasally instilled singly or in combination in sensitized challenged guinea pigs on day two after the 10th pollen challenge, as previously described [17,26–30]. Magnitude of nasal blockage after instillation of LTD₄ and U-46619 in sensitized challenged guinea pigs was compared with

blockage in non-sensitized non-challenged animals. Previously, we found no differences in response to intranasal instillation of LTD₄ (10^{-8} and 10^{-6} M) among non-sensitized challenged, sensitized non-challenged and non-sensitized non-challenged guinea pigs [26].

When single treatment was performed, 10 μ l/nostril of 10^{-12} and 10^{-10} M LTD₄ or 10^{-9} and 10^{-7} M U-46619 was consecutively instilled into the bilateral nasal cavities at an interval of 20 min. The sRaw value was measured before and 10 min after each set of two LTD₄ or U-46619 instillations. In combined treatment with LTD₄ and U-46619, both 10^{-12} M LTD₄ and 10^{-9} M U-46619 were intranasally instilled, followed by instillation of 10^{-10} M LTD₄ and 10^{-7} M U-46619.

In previous studies [26,30], we found that administration of LTD₄, a potent bronchoconstrictor, into the nasal cavities of non-sensitized guinea pigs produced no elevation of sRaw, even at 10^{-6} M. Narita et al. [4] reported that most Evans blue dye instilled intranasally was found within the nasal cavity. These indicate that the changes in sRaw induced by instillation of LTD₄ and U-46619 in the present method exclusively reflect the nasal response but not the lower airway response.

2.8. Drug administration

The effect of combined treatment with pranlukast and seratrodist on allergen-induced nasal blockage was evaluated and compared with effects of respective single treatments. In one experiment, pranlukast (30 mg/kg) and/or seratrodist (20 mg/kg) were p.o. administered 1 h before the 6th antigen challenge. In another experiment, pranlukast or seratrodist was singly p.o. administered 2 h after the challenge.

In order to evaluate effects of pranlukast and seratrodist on production of CysLTs and TXB₂ in NCLF, each drug was p.o. administered 1 h before the 10th challenge.

2.9. Statistical analyses

Data were statistically evaluated by one-way analysis of variance. If difference appeared significant, the individual group difference was determined using Bonferroni's multiple tests. A *P*-value of <0.05 was considered to indicate statistical significance.

3. Results

3.1. Effect of combined treatment with pranlukast and seratrodist on early and late phase nasal blockage

In sensitized, vehicle-treated, challenged guinea pigs (control), there were two peaks (1 and 4 h after the antigen challenge) in the time-course of the changes in sRaw at the 8th challenge, indicating that the antigen-induced increase in sRaw consisted of early and late phase nasal blockage responses (Fig. 1). In contrast, as previously reported [17,22], sRaw did not change after pollen inhalation in non-sensitized guinea pigs.

First, we evaluated effects of combined pre-treatment with pranlukast and seratrodist on the 6th challenge-induced early and late phase nasal blockage, and compared the mag-

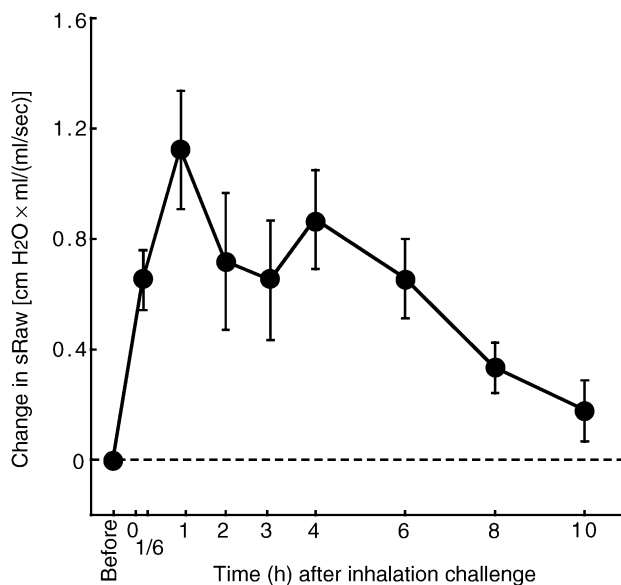


Fig. 1. Time-course changes in specific airway resistance (sRaw) after the 8th inhalation challenge with cedar pollen in sensitized guinea pigs. Each point represents the mean $\pm$ S.E. of 11 or 12 animals.

nitude of the inhibition with that obtained by respective single treatment with pranlukast or seratrodist (Fig. 2). Similar to our previous findings [17], single administration of pranlukast or seratrodist 1 h before the allergen challenge significantly suppressed the late phase nasal blockage (4 and 6 h after the challenge), although they had no significant effect on the early phase response (1 h after the challenge). The combined pre-treatment with pranlukast and seratrodist also effectively suppressed the late phase increase in sRaw at 4 and 6 h after the challenge. The degree of inhibition was almost the same as that produced by respective single pranlukast or seratrodist treatment. However, the combined treatment did not significantly affect the early phase nasal blockage at 1 h after the challenge (Fig. 2).

3.2. Effect of post-treatment with pranlukast and seratrodist on biphasic nasal blockage

Fig. 3 shows the effect of pranlukast and seratrodist administered 2 h after (post-treatment) the 8th antigen challenge on the early (1 h) and late (4 and 6 h) phase nasal blockage. Also, the degree of inhibition produced by post-treatment was compared with single pre-treatment with pranlukast or seratrodist. Post-treatment with pranlukast inhibited the late phase nasal blockage, and the magnitude of inhibition tended to be slightly smaller than that produced by pre-treatment with pranlukast. Post-treatment with seratrodist also attenuated the late phase, and the degree of inhibition was almost equal to that of pre-treatment with seratrodist (Fig. 3).

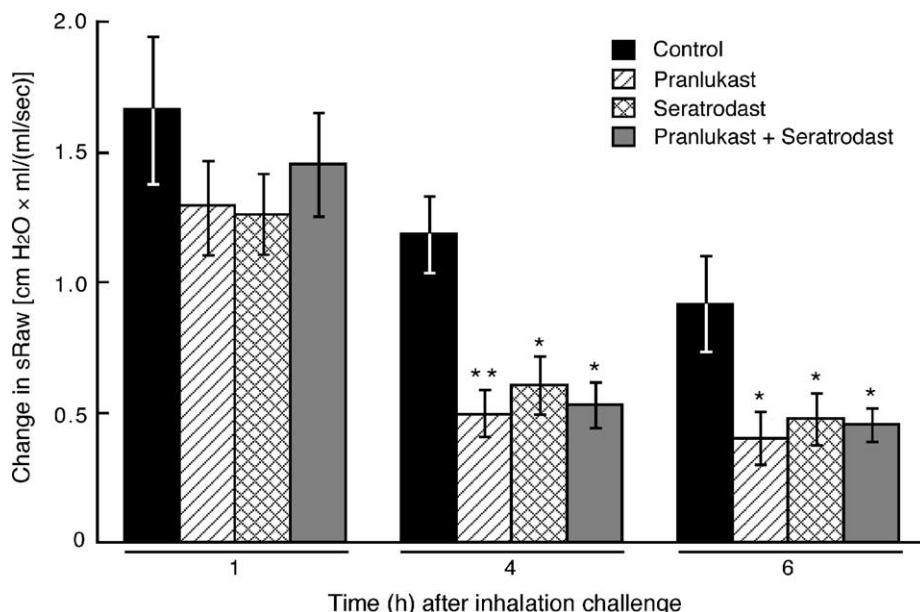


Fig. 2. Effects of pranlukast, seratrodest and combined treatment with pranlukast and seratrodest on changes in specific airway resistance (sRaw) after the 6th inhalation challenge with cedar pollen in sensitized guinea pigs. Pranlukast (30 mg/kg), seratrodest (20 mg/kg) and pranlukast (30 mg/kg) + seratrodest (20 mg/kg) were administered 1 h before the antigen challenge. Each column represents the mean $\pm$ S.E. of 20–26 animals. (*) $P < 0.05$ and (**) $P < 0.01$, compared to control.

3.3. Effect of pranlukast and seratrodest on the increase in CysLTs and TXB₂ in NCLF induced by antigen challenge

We previously reported that amounts of both CysLTs and TXB₂ in NCLF increased in a biphasic fashion after allergen challenge (two peaks, at 20 min and 5 h after challenge) [17].

The increases in CysLTs and TXB₂ in NCLF at 5 h after the 10th challenge, when the late phase nasal blockage response peaked, were not affected by administration of pranlukast or seratrodest (Fig. 4).

3.4. Nasal blockage induced by intranasal instillation of LTD₄ and/or U-46619

Changes in sRaw induced by single or combined treatment with LTD₄ (10^{-12} and 10^{-10} M) and U-46619 (10^{-9} and 10^{-7} M) in sensitised challenged guinea pigs were evaluated two days after the 12th antigen challenge, and compared with those in non-sensitized non-challenged animals (Fig. 5). In non-sensitized non-challenged animals, there was no increase in sRaw with single or combined treatment with LTD₄ and U-46619. In sensitized challenged animals, single treatment with 10^{-12} M LTD₄ or 10^{-9} M U-46619 induced no change in sRaw, but sRaw was significantly increased by treatment with 10^{-10} M LTD₄

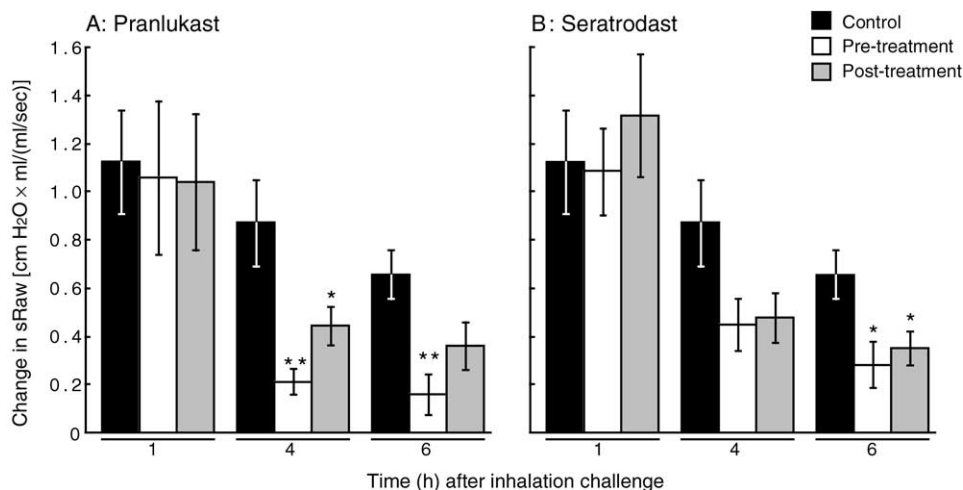


Fig. 3. Effects of pre- and post-treatment with (A) pranlukast and (B) seratrodoast on changes in specific airway resistance (sRaw) after the 8th inhalation challenge with cedar pollen in sensitized guinea pigs. Pranlukast (30 mg/kg) and seratrodoast (20 mg/kg) were administered 1 h before (pre-treatment) or 2 h after (post-treatment) the antigen challenge. Each column represents the mean $\pm$ S.E. of 11 or 12 animals. (*) $P < 0.05$ and (**) $P < 0.01$, compared to control.

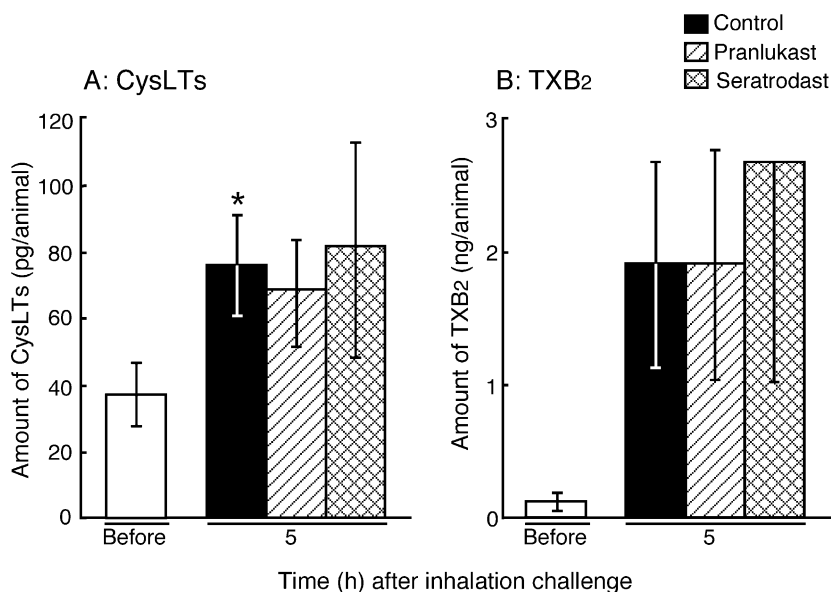


Fig. 4. Effects of pranlukast and seratrodoast on late-phase release of cysteinyl leukotrienes (CysLTs) (A) and thromboxane (TX) B₂ (B) after inhalation challenge with cedar pollen in sensitized guinea pigs. Pranlukast (30 mg/kg) or seratrodoast (20 mg/kg) was administered 1 h before the 10th antigen challenge, and nasal cavity lavage fluid (NCLF) was collected 5 h after the challenge. Each column represents the mean $\pm$ S.E. of 5–8 animals. (*) $P < 0.05$, compared to before challenge.

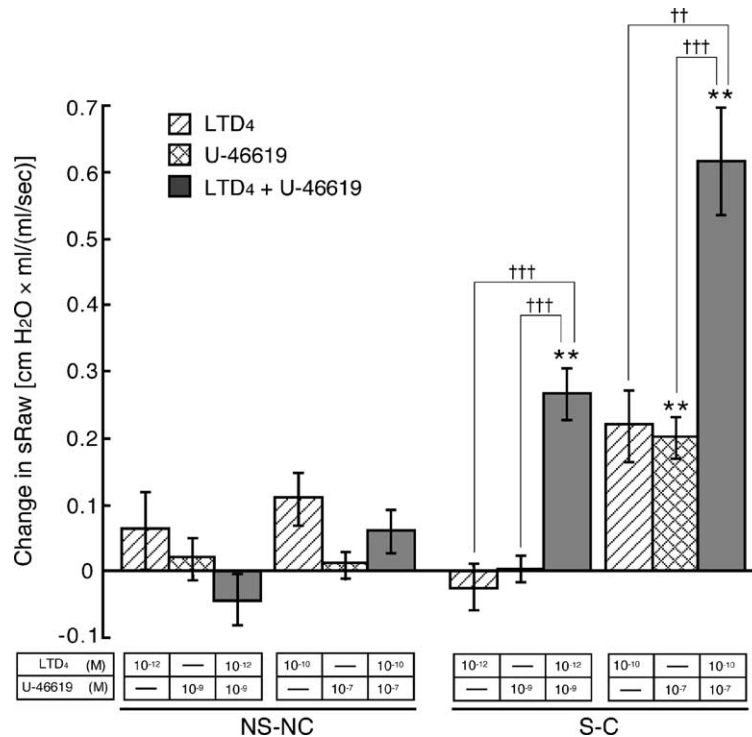


Fig. 5. Increase in specific airway resistance (sRaw) induced by intranasal application of leukotriene (LT) D₄, U-46619 and a combination of LTD₄ and U-46619 in non-sensitized and sensitized guinea pigs. The experiment was performed two days after the 12th inhalation challenge with cedar pollen. Each point represents the mean $\pm$ S.E. of 7 or 8 animals. (**) $P < 0.01$, compared to non-sensitized non-challenged (NS-NC). S-C: sensitized challenged. (†) $P < 0.01$ and (††) $P < 0.001$.

or 10⁻⁷ M U-46619. Interestingly, combined treatment with 10⁻¹² M LTD₄ and 10⁻⁹ M U-46619 clearly increased sRaw in sensitised challenged animals, and a greater increase in sRaw was induced by combined application of 10⁻¹⁰ M LTD₄ and 10⁻⁷ M U-46619. Responses produced by combined treatment with LTD₄ and U-46619 were larger than the sum of those induced by respective single administrations of LTD₄ or U-46619 (Fig. 5).

4. Discussion

Consistent with our previous finding [17], when specific antagonist of CysLTs or TXA₂, pranlukast [12,13] or seratrodist [14,15], respectively, was singly administered 1 h before the pollen challenge, it suppressed induction of late phase nasal blockage. Interestingly, however, the combined treatment with two antagonists produced no additive inhibition of late phase nasal blockage. This suggests two possible interactions between CysLTs and TXA₂: (1) CysLT-induced nasal blockage at the late phase is totally dependent on TXA₂

generation because it has been well known that LTD₄ can induce TXA₂ production in the lung of guinea pigs [18,19]; and (2) CysLTs and TXA₂ synergistically act on different effectors sites in the nasal tissue to induce nasal blockage.

We previously reported that in sensitized challenged guinea pigs, levels of CysLTs and TXB₂ increase in NCLF in a biphasic fashion, with peaks at 20 min and 5 h after challenge [17]. Thus, before evaluating whether CysLTs produce TXA₂ to induce late phase nasal blockage, we examined whether increases of CysLTs and TXA₂ during the late phase depend on their productions at the early phase. Single post-treatment with pranlukast or seratrodist clearly suppressed the late phase nasal blockage, indicating that late generation of CysLTs and TXA₂ is independent of their respective productions at the early phase, and implying that cellular sources of CysLTs and TXA₂ during the late phase are not identical to those at the early phase.

We next examined whether CysLTs and TXA₂ influence each other's production when the late phase nasal blockage is induced. Resultantly, production of CysLTs or TXB₂ was not affected by seratrodist or pranlukast, respectively, suggesting that these metabolites are independently produced during the late phase, and that there is no interaction between production of CysLTs and TXA₂ at the late phase. LTD₄ can induce TXA₂ production in lungs of guinea pigs [18,19], indicating that allergen-induced TXA₂ production is mediated by CysLT production in the lower airway of this species. In agreement with these reports [18,19], it has also been reported that TP receptor antagonists including seratrodist inhibit LTD₄-induced airflow obstruction and plasma leakage in the lower airway of guinea pigs [14,31,32] although pranlukast shows no inhibitory effect on U-46619-induced bronchoconstriction in guinea pigs [12]. In contrast to these reports indicating CysLT-induced TXA₂ generation [12,14,18,19,31,32], the late phase production of TXA₂ in the nasal tissue was independent of CysLTs produced in this allergic rhinitis model. Therefore, the absence of additive inhibition by pranlukast and seratrodist on the late phase response should be due to other mechanisms than interaction between production of CysLTs and TXA₂.

In the next experiment, we examined whether CysLTs and TXA₂ interact with each other at the effector organ cell to induce nasal blockage. We previously found that although non-sensitized guinea pigs exhibit little or no increase of sRaw in response to intranasal application of histamine and LTD₄, sensitized repeatedly challenged guinea pigs show marked nasal blockage response to histamine and LTD₄ intranasally instilled [16,26]. In those studies, the nasal hyper-responsiveness to histamine and LTD₄ was exhibited 4 h to 2 days after an allergen challenge, but was repressed by day 7 [16,26]. Details of the mechanisms underlying development of nasal hyper-responsiveness are unknown, but nasal blockage responses to mediators in sensitized challenged guinea pigs can be used to analyze whether CysLTs and TXA₂ interact with each other at the effector organ.

Similar to previous findings of hyper-responsiveness to histamine and LTD₄ [16,26], intranasal instillation of U-46619 (a TXA₂ mimetic) also induced more marked nasal blockage in sensitized challenged guinea pigs than in non-sensitized animals. More interestingly, combined application of LTD₄ and U-46619 produced synergistic action in induction of nasal blockage. This result strongly suggests that LTD₄ and U-46619 synergistically cooperate in nasal tissue to induce nasal blockage during the late phase reaction in the allergic rhinitis model.

Mechanisms of the cooperation between CysLTs and TXA₂, to induce the late phase nasal blockage, are unclear at present. We previously found evidence suggesting that LTD₄-induced nasal blockage, in sensitized guinea pigs, is mainly mediated by dilatation of nasal blood vessels via activation of CysLT₁ receptors, and that this dilatation is induced by nitric oxide (NO) derived from constitutive-type endothelial NO synthase [26,33]. On the other hand, details of the mechanism of TXA₂-induced nasal blockage are unknown, despite the effectiveness of TP receptor antagonists in clearing allergic nasal blockage. However, it has been reported that U-46619 can increase vascular permeability at the conjunctiva [34] and lung [35,36] of guinea pigs. Similar to effects on these tissues, intranasal application of U-46619 clearly produces plasma leakage in nasal tissue of guinea pigs [6,8]. In addition, the finding that TXA₂ is a potent vasoconstrictor [37,38] suggests that it induces mucosal edema via ischemia reperfusion injury. The available evidences suggest that LTD₄ and TXA₂ synergistically cooperate in nasal blood vessels to cause nasal blockage, probably acting at different sites, with LTD₄ inducing vasodilatation at the capacitance vessels via NO production following CysLT₁ receptor activation, and TXA₂ increasing vascular permeability at capillary vessels or induce vascular injury via TP receptor activation.

In conclusion, it was strongly suggested that CysLTs and TXA₂, which are generated in the late phase of responses to allergens, synergistically cooperate in nasal blood vessels to cause the late phase nasal blockage in the present experimental model of allergic rhinitis.

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