Markedly Increased Nasal Blockage by Intranasal Leukotriene D₄ in an Experimental Allergic Rhinitis Model: Contribution of Dilated Mucosal Blood Vessels

Nobuaki Mizutani¹, Takeshi Nabe¹, Aki Imai¹, Hiromu Sakurai², Hiroshi Takenaka³ and Shigekatsu Kohno¹,*

¹Department of Pharmacology and ²Analytical and Bioinorganic Chemistry, Kyoto Pharmaceutical University, 5 Nakauchi, Misasagi, Yamashina, Kyoto 607-8414, Japan
³Department of Otorhinolaryngology, Osaka Medical College, Takatsuki, Osaka 569-8686, Japan

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ABSTRACT—We examined whether nasal hyperresponsiveness to leukotriene (LT) D₄ is seen in our allergic rhinitis model, which showed sneezing and biphasic nasal blockage by repeated antigen inhalation challenge, and whether a dilatation of mucosal blood vessels contributes to this hyperresponsiveness. Nasal blockage [increase of specific airway resistance (sRaw)] was indexed as nasal (hyper)responsiveness. The sensitized – challenged guinea pig showed a remarkable dose-dependent increase in sRaw by intranasal instillation of LTD₄ (10⁻⁶–10⁻⁸ M) at 10 h and 2 days but not 7 days after the challenge. The increase in sRaw induced by LTD₄ was largely blocked by pranlukast or naphazoline, and this was dose-dependently suppressed by Nω-nitro-L-arginine methyl ester. Sodium nitroprusside induced an elevation of sRaw in the sensitized – challenged animal in the hyperresponsiveness state, but the degree did not differ from that in the non-sensitized – non-challenged group. The amount of NO₂⁻ and NO₃⁻ in nasal cavity lavage fluid after LTD₄ instillation in the sensitized – challenged animal in the hyperresponsiveness state was significantly greater than that before the instillation. These results demonstrate that the hyperresponsiveness to LTD₄ acquired by repeated antigen challenge is mainly due to dilatation of nasal blood vessels, which can be related to hyperproduction of nitric oxide through cysteinyL LT₁-receptor activation.

Keywords: Hyperresponsiveness, Allergic rhinitis, Leukotriene D₄, Nasal blockage, Nitric oxide

Cysteinyl leukotrienes (CysLTs: LTC₄, LTD₄ and LTE₄) are a family of potent inflammatory mediators that appear to contribute to the pathophysiologic features of allergic rhinitis. It has been reported that CysLTs were detected in the nasal washings following antigen challenge of subjects with allergic rhinitis (1, 2) and that treatment with a 5-lipoxygenase inhibitor and a CysLT₁-receptor antagonist modified allergen-induced nasal mucosa swelling via dilatation of nasal capacitance blood vessel and increases in nasal capillary permeability (3 – 5), suggesting that CysLTs act as significant inflammatory mediators in allergic rhinitis and that allergen-induced vascular changes are predominantly induced by CysLTs.

To date, clinical examinations revealed that allergic rhinitis patients show obviously increased nasal blockage in response to topical LTD₄, a phenomenon known as nasal hyperresponsiveness (6 – 8). On the other hand, although very high doses of topical LTD₄ have been reported to increase either nasal blockage or nasal vascular permeability in non-sensitized animals (9, 10), it has not been determined whether a higher response to the agonist occurs in the allergic rhinitis model. Due to the limitation of clinical research and insufficient information obtained from normal animals, the mechanism of nasal hyperresponsiveness to LTD₄ in allergic rhinitis patients remains unclear. The use of an experimental allergic rhinitis model that closely resembles the human case indispensably resolves such drawbacks in the analysis of the nasal hyperresponsiveness to LTD₄.

Recently, we reported an allergic rhinitis model that showed anaphylactic biphasic elevation of specific airway resistance (sRaw) (11) and considerable nasal hyperresponsiveness to histamine following repetitive quantitative inhalation challenge using Japanese cedar pollen in the guinea pig (12). Furthermore, these symptoms in this model are similar to those observed in clinical situations, indicating that our model is extremely effective in determining whether...
Nasal Hyperresponsiveness to LTD\textsubscript{4} is also observed.

In the present study, we first evaluated the effect of pranlukast, a CysLT\textsubscript{1}-receptor antagonist, on biphasic elevation of sRaw induced by the antigen challenge in the sensitized – challenged guinea pig. Then, we examined whether repetitive inhalations of antigen in the sensitized – challenged guinea pig lead to nasal hyperresponsiveness to LTD\textsubscript{4} and whether dilatation of the mucosal blood vessel contributes to the nasal hyperresponsiveness.

MATERIALS AND METHODS

Animals

Male, 3-week-old, Hartley guinea pigs weighing 250 – 300 g were purchased from Japan SLC, Hamamatsu. The animals were housed in a temperature-controlled room at 23 ± 1°C and 60 ± 10% humidity, illuminated from 08:00 – 20:00 h. They were fed a standard laboratory diet and given water ad libitum. The first sensitization was started after 2 weeks.

This animal study was approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University.

Reagents

Reagents and their sources were as follows: LTD\textsubscript{4} and pranlukast hemihydrate (Ono Pharm., Co., Ltd., Osaka); AA-861 (donated from the laboratory of Takeda Chem. Ind., Osaka); indomethacin, mepyramine, naphazoline hydrochloride, N\textsuperscript{ω}-nitro-L-arginine methyl ester (L-NAME) hydrochloride, N\textsuperscript{ω}-nitro-D-arginine methyl ester (D-NAME) hydrochloride, L-arginine hydrochloride and sodium nitroprusside (SNP) dihydrate (Sigma Chem., St. Louis, MO, USA); Evans blue (Merck, Darmstadt, Germany); sodium pentobarbital (Abbott Lab, North Chicago, IL, USA); methylcellulose and polyoxyethylene [20] sorbitan monolaurate (Wako Pure Chem., Osaka); and lidocaine hydrochloride (Fujisawa Pharm. Co. Ltd., Osaka). All other reagents used were the highest grade of commercial products available.

LTD\textsubscript{4} in physiological saline containing 0.5% ethanol and SNP in physiological saline were prepared. Pranlukast, mepyramine and indomethacin were suspended in 0.5% methylcellulose, AA-861 was suspended in physiologic saline containing 1% polyoxyethylene [20] sorbitan monolaurate and all other drugs were dissolved in physiological saline.

We harvested the Japanese cedar (Cryptomeria japonica) pollens. Al(OH)\textsubscript{3} gels were prepared from 0.5 M NaOH and 1/12 M Al\textsubscript{2}(SO\textsubscript{4})\textsubscript{3} as previously described (13).

Preparation of Japanese cedar pollen extracts

Cedar pollen extracts used for sensitization were prepared as previously described (11). In brief, pollens were suspended in physiologic saline at 100 mg/ml and allowed to stand at 4°C for 18 h under mild stirring. The suspension was then centrifuged (1,700 × g, 15 min), and the resultant supernatant was used as the sensitization antigen; this was stored at −80°C until use. The protein concentration in the solution was estimated to be 500 μg protein/ml according to the method of Bensadoun and Weinstein (14).

Sensitization and challenge

As previously described (11), bilateral intranasal sensitization of guinea pigs was induced by the instillation of cedar pollen extracts adsorbed on Al(OH)\textsubscript{3} gel of 0.3 μg protein · 0.3 mg Al(OH)\textsubscript{3} · 0.3 mg Al(OH)\textsubscript{3} per each nostril 2 times a day for 7 days. Prior to each sensitization, the upper airway mucosal surface was topically anesthetized by subjecting the animal to a 5-min inhalation of a 4% lidocaine hydrochloride mist, which was generated with an ultrasonic nebulizer (NE-U12; Omron, Osaka). This procedure, providing effective sensitization by prolonged retention of the antigen Al(OH)\textsubscript{3} in the nasal cavity, was employed based on the research findings that lidocaine reduces the ciliary beat frequency of the guinea pig airway in vitro (15) and that topical anesthetic drugs do not decrease mucosal absorbency (16). Then, the sensitized animal was bilaterally intranasally challenged once every week by quantitative inhalation of the cedar pollen at a dose of 1.8 mg/each nostril using a hand-made inhalation apparatus (Fig. 1). Upon application of the pollen to both nostrils of the spontaneously breathing guinea pig, almost all inhaled pollens were trapped in the upper airways (17).

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**Fig. 1.** Schedule for sensitization with the cedar pollen extracts and subsequent nasal challenge by inhalation of the cedar pollens in the guinea pig.
Four groups of guinea pigs were employed for the experiments: non-sensitized – non-challenged; sensitized – challenged; sensitized – non-challenged; non-sensitized – repeatedly challenged (2 days after the 7th antigen inhalation challenge without sensitization).

**Effects of drugs on the biphasic increased sRaw induced by pollen inhalation challenge**

Pranlukast (30 mg/kg, p.o.), a CysLT₁-receptor antagonist, and mepyramine (10 mg/kg, p.o.), an H₁-receptor antagonist, were administered 1 h before the 4th pollen inhalation challenge. sRaw was measured before and 10 min, 1, 2, 3, 4, 6, 8 and 10 h after the pollen challenge. The respective magnitudes of the early and late responses were calculated as the area under the response curve (AUC) from 0 (before) to 3 h and 3 to 10 h after antigen challenge of vehicle- or drug-treated animals.

**Nasal sRaw responsiveness to LTD₄ and SNP**

The following five examinations were carried out to evaluate nasal airway responsiveness to LTD₄ and SNP in the conscious guinea pig. Either 10 μl/nostril of LTD₄ or SNP was instilled, and sRaw was indexed as nasal airway responsiveness. Non-sensitized – non-challenged guinea pigs acted as controls in some experiments. 1) Time-course changes in sRaw elevation by LTD₄ and SNP: a fixed concentration of LTD₄ (10⁻⁴ M) or SNP (10⁻³ M) was applied to the sensitized – challenged animal 2 days after the 20th or 30th pollen inhalation challenge, and sRaw was monitored for the subsequent 120 min or 10 min. 2) Occurrence and disappearance of sRaw elevation by LTD₄ after an antigen inhalation challenge: various doses of LTD₄ (10⁻¹² – 10⁻⁴ M) were instilled at 20-min intervals to the sensitized – challenged animal 10 h, 2 days or 7 days after the 20th pollen inhalation challenge. sRaw was measured 10 min after the respective instillations. 3) Dose-sRaw responsiveness to SNP: various doses of SNP (10⁻¹⁰ – 10⁻² M) were instilled at 10-min intervals to the sensitized – challenged animal 2 days after the 30th pollen inhalation challenge. sRaw was measured 2 min after the respective instillations. 4) Change in sRaw elevation by LTD₄ in response to increased number of pollen inhalation challenges: a fixed dose of LTD₄ (10⁻⁸ M) was instilled to the sensitized – challenged animal 2 days after the respective 1st to 30th pollen inhalation challenges. sRaw was measured 10 min after instillation. 5) Influence of sensitization without challenge or non-sensitization – pollen inhalation challenge on nasal responsiveness to LTD₄; two doses of LTD₄ (10⁻⁴ and 10⁻⁶ M) were instilled at 20-min intervals to the sensitized – non-challenged or to the non-sensitized – repeatedly challenged animal.

In the text, “hyperresponsiveness” indicates the state that the sensitized – challenged animal showed significantly elevated sRaw (nasal blockage) induced by intranasal LTD₄ or agonist instillation.

**Measurement of sRaw**

sRaw was measured by a two-chambered, double-flow plethysmograph system according to the method of Pennock et al. (18). In brief, the animal was placed with its neck extended through the partition of a two-chambered box, and sRaw was measured using the Data analyzer Pulmos-I (M.I.P.S., Osaka) and a PC 9801 FA computer (NEC, Tokyo) after detection of the airflow by sensors attached to both the front and rear chambers.

**Measurement of the change in diameter of the nasal blood vessel**

Time-course change in the diameter of nasal blood vessels following application of LTD₄ solution onto the nasal mucosa was evaluated 2 days after the 30th pollen inhalation challenge in the sensitized – challenged guinea pig.

Non-sensitized – non-challenged and sensitized – challenged guinea pigs were anesthetized by sodium pentobarbital (40 mg/kg, i.p.) and set on a heating pad to avoid any decrease in body temperature. Nasal mucosa was exposed surgically and observed under a stereoscopic microscope (SZX12; Olympus Optical Co., Ltd., Tokyo) at a magnification of 50. Ten microliters of LTD₄ (10⁻⁴ M) was dropped onto the exposed mucosal surface followed by observation for 40 min. Nasal mucosa was photographed by a camera (C-35AD-4, Olympus Optical Co., Ltd.) equipped with the microscope, before and 2, 5, 10, 20 and 40 min after LTD₄ application. The main blood vessels in the mucosa on the photographs were veins, which were 50 – 80 μm in diameter before the LTD₄ application and formed irregular networks. The diameters of the randomly selected veins were measured before and after the application.

**Measurement of the change of vascular permeability**

Plasma extravasation into the nasal tissue as a result of LTD₄ instillation was evaluated. Evans blue (10 mg/ml, kg⁻¹, i.v.) was administered to the sensitized – challenged guinea pig 2 days after the 30th pollen inhalation challenge.

One hour later, 10⁻³ M LTD₄ was instilled into both nasal cavities in the same manner as described above. Ten or forty minutes after the instillation, the guinea pig was sacrificed by exsanguination under pentobarbital anesthesia (40 mg/kg, i.v.). To quantify the dye in the nasal tissue, nasal mucosal tissue was removed after perfusion of the head using 40 ml/animal of saline through both the right and left carotid arteries. The tissue was treated with an alkaline solution (1.2 M KOH, 37°C for 18 h, 2 ml/animal), the suspension was centrifuged at 1,700 × g for 10 min, and the dye in the supernatant was calorimetrically measured at 620 nm, according to the method of Katayama.
et al. (19). Non-sensitized – non-challenged – saline-instilled and non-sensitized – non-challenged – LTD$_4$-instilled groups acted as controls.

**Effects of drugs on the increased sRaw induced by LTD$_4$**

Pranlukast (30 mg/kg, p.o.), a CysLT$_1$-receptor antagonist, was administered 1 h before the application of LTD$_4$ (10$^{-4}$ M) on the 2nd day after the 20th antigen challenge. Measurement of NO$_2$ synthase inhibitor, or its inactive enantiomer AA-861 (100 mg/kg, i.p.), a 5-lipoxygenase (5-LO) inhibitor, was administered 1 h and 30 min, respectively, before LTD$_4$ instillation.

On the 2nd day after the 20th antigen challenge, naphazoline (0.1 mg/kg, i.v.), an $\alpha$-stimulant, was administered 8 or 35 min after the application of LTD$_4$ (10$^{-4}$ M). sRaw was measured immediately before and 2 or 5 min after $\alpha$-stimulant treatment.

L-NAME (3, 10 and 30 mg/kg, i.v.), a nitric oxide (NO) synthase inhibitor, or its inactive enantiomer D-NAME (10 mg/kg, i.v.) was administered 15 min before LTD$_4$ instillation on the 2nd day after the 20th challenge. Furthermore, in order to investigate the reversal effect of L-arginine on L-NAME-induced suppression of LTD$_4$-induced sRaw elevation, L-arginine (600 mg/kg, i.v.) was co-administered with L-NAME 15 min before LTD$_4$ instillation. sRaw was measured 10 min after LTD$_4$ instillation.

On the 2nd day after the 20th challenge, indomethacin (10 mg/kg, p.o.), a cyclooxygenase (COX) inhibitor, and AA-861 (100 mg/kg, i.p.), a 5-lipoxygenase (5-LO) inhibitor, were administered 1 h and 30 min, respectively, before LTD$_4$ instillation.

**Measurement of NO$_2$ and NO$_3$ in nasal cavity lavage fluid (NCLF)**

The NCL was conducted according to the previously described method (17) in sensitized–challenged and non-sensitized–non-challenged guinea pigs. Briefly, after anesthetization with sodium pentobarbital (40 mg/kg, i.p.), one end of the silicone tubing, the other end of which was connected to an air pump, was properly positioned in the right nostril under reduced pressure by the air pump; then 1 ml saline prewarmed at 37°C was aspirated from the left nostril via a tube.

Three initial NCLs were performed to remove pre-existing NO$_2$ and NO$_3$ at 20-min intervals to non-sensitized–non-challenged or sensitized–challenged guinea pigs, and the 3rd NCLF served as a baseline. LTD$_4$ (10$^{-4}$ M) was intranasally instilled 10 min after the 3rd NCL and then NCL was performed 10 min after the LTD$_4$ instillation.

The NCLF recovered was centrifuged at 10,000 r.p.m. for 40 min at 4°C. The resultant supernatant was used for the NO$_2$ and NO$_3$ assay. The amounts of NO$_2$ and NO$_3$ were measured by NO$_2$ and NO$_3$ assay kit-F (fluorometric) (Dojindo Lab. Co., Ltd., Kumamoto).

**Statistical analyses**

Statistical analyses were performed by one-way analysis of variance (ANOVA). Individual group differences were determined by Bonferroni’s multiple test when a significant difference was detected. Comparison of the amounts of NO$_2$ and NO$_3$ before and after LTD$_4$ instillation were performed by the paired t-test. Statistical significance was established at the P<0.05 level.

**RESULTS**

**Effects of pranlukast and mepyramine on the biphasic increased sRaw induced by the pollen inhalation challenge**

When the effects of pranlukast and mepyramine on the increase in sRaw at the early and late phases induced by the 4th challenge were compared by AUCs, pranlukast effectively suppressed the late sRaw elevation, but did not influence the early response. On the other hand, mepyramine inhibited neither the early nor the late sRaw elevation (Fig. 2). Similar results were also observed at the 7th, 10th

![Fig. 2. Effect of pranlukast and mepyramine on the area under the response curve (AUC) for the increase in specific airway resistance (sRaw) at the early (0–3 h) (a) and late (3–10 h) (b) phase after inhalation challenge using cedar pollen in the sensitized–challenged guinea pig. The experiment was performed at the 4th cedar pollen inhalation challenge. Pranlukast (30 mg/kg) and mepyramine (10 mg/kg) were administered orally 1 h before the inhalation challenge. Data represent the mean±S.E.M. of 12 animals. Open column: non-sensitized–non-challenged, closed column: sensitized–challenged, striped column: sensitized–challenged–pranlukast-treated, shaded column: sensitized–challenged–mepyramine-treated. **P<0.01 compared to the sensitized–challenged group. ††P<0.01, compared to the non-sensitized–non-challenged group.](image-url)
and 13th challenges (data not shown).

Time-course changes in sRaw after LTD₄ instillation and nasal responsiveness to LTD₄ after the pollen inhalation challenge

Figure 3A shows the time-course change in sRaw after instillation of 10⁻⁸ M LTD₄ into both nasal cavities of the sensitized–challenged guinea pig 2 days after the 20th pollen inhalation challenge. Instillation of LTD₄ caused a swift elevation of sRaw that peaked at 10 min followed by a diminution at 20 min. However, a moderate increase in sRaw was still recognized at 20 to 120 min. sRaw was consequently measured 10 min after LTD₄ instillation in the following experiments.

Nasal responsiveness of the sensitized–challenged guinea pig to LTD₄ was evaluated 10 h, 2 days and 7 days after the 20th antigen challenge and compared to that of the non-sensitized–non-challenged group. In the sensitized–challenged animal, 10⁻¹² M LTD₄ tended to elevate sRaw, and dose-dependent increases were recognized at 10⁻¹⁰ to 10⁻⁸ M, with the increases being significant at 10 h and 2 days after the antigen challenge, whereas the non-sensitized–non-challenged animal showed no sRaw increase by the agonist at 10⁻⁶ M. However, this outstanding hyperresponsiveness almost disappeared on the 7th day (Fig. 3B).

Throughout the above experiments, no significant differences were found between the sRaw of the two groups before LTD₄ instillation: mean ± S.E.M. of sRaw values 10 h, 2 days and 7 days after the 20th antigen challenge in the

Fig. 3. Time-course of the increase in specific airway resistance (sRaw) induced by intranasal instillation of leukotriene (LT) D₄ (10⁻⁸ M) (A), and nasal responsiveness to LTD₄ 10 h (a), 2 days (b) and 7 days (c) after the 20th cedar pollen inhalation challenge (B) in the sensitized–challenged guinea pig. The experiment was performed 2 days after the 20th cedar pollen inhalation challenge. Data represent the mean ± S.E.M. of 8 to 10 animals. Open circle: non-sensitized–non-challenged, closed square: sensitized–challenged. **P<0.01, compared to the non-sensitized–non-challenged group.
sensitized–challenged and non-sensitized – non-challenged groups were 1.72 ± 0.22 and 1.58 ± 0.15, 1.51 ± 0.13 and 1.58 ± 0.15, and 1.59 ± 0.13 and 1.50 ± 0.13 (n = 12) cmH2O × ml/(ml/s), respectively.

Nasal responsiveness to LTD₄ during the course of repetitive challenges

Figure 4 shows the results of nasal responsiveness to 10⁻⁸ M LTD₄ two days after the respective 1st–30th pollen challenges in the sensitized–challenged guinea pig. Although no increase in sRaw was observed in the sensitized–challenged group at the 1st pollen inhalation challenge, significant hyperresponsiveness was obtained at the 4th pollen challenge. The degree of hyperresponsiveness was elevated at an antigen-challenging time-dependent fashion until the 30th antigen challenge.

Influence of sensitization–pollen inhalation challenge and non-sensitization–pollen inhalation challenge on nasal responsiveness to LTD₄

In sensitized–challenged animals, a dose-dependent increase was recognized at 10⁻⁸ and 10⁻⁶ M LTD₄ 2 days after the 7th antigen challenge, which was significant in comparison with the non-sensitized–non-challenged group. The non-sensitized–repeatedly challenged and sensitized–non-challenged animals also showed dose-dependent increases in sRaw in response to LTD₄, but their magnitudes were marginal and not significantly different from the non-sensitized–non-challenged group (Fig. 5).

Effect of pranlukast on the increased sRaw induced by LTD₄

Figure 6A shows the effect of pranlukast on the increased sRaw induced by LTD₄ instillation. When pranlukast (30 mg/kg, p.o.) was administered 1 h before LTD₄, sRaw elevation was potently suppressed to a level similar to that of the non-sensitized–non-challenged group.

Effect of naphazoline on the increased sRaw induced by LTD₄

Intravenous naphazoline dramatically lowered spontaneous sRaw in both the non-sensitized–non-challenged and sensitized–challenged groups. Meanwhile, the increased sRaw induced by LTD₄ (10⁻⁶ M) appeared to be completely blocked and was further lowered to below normal levels at 10 and 40 min after LTD₄ instillation by the drug. However, the reduced levels of sRaws in response to naphazoline at 10 and 40 min after LTD₄ application did not reach the level of sRaw after naphazoline in the non-sensitized–non-challenged and/or sensitized–challenged group without

**Fig. 4.** Changes in nasal responsiveness to leukotriene (LT) D₄ (10⁻⁸ M) during the course of repetitive challenges in guinea pigs 2 days after respective pollen inhalation challenges. sRaw was measured 10 min after LTD₄ instillation. Data represent the mean ± S.E.M. of 10 animals. Open circle: non-sensitized–non-challenged, closed square: sensitized–challenged. *P<0.05, **P<0.01, compared to the non-sensitized–non-challenged group.

**Fig. 5.** Nasal responsiveness to leukotriene (LT) D₄ in the non-sensitized–repeatedly challenged and the sensitized–non-challenged guinea pigs. Two doses of LTD₄ (10⁻⁸ and 10⁻⁶ M) were instilled at 20-min intervals into the non-sensitized–repeatedly challenged animals 2 days after the 7th pollen inhalation challenge and the sensitized–non-challenged animal. sRaw was measured 10 min after LTD₄ instillation. Data represent the mean ± S.E.M. of 7 or 8 animals. Open circle: non-sensitized–non-challenged, closed circle: non-sensitized–challenged, open square: sensitized–non-challenged, closed square: sensitized–challenged. **P<0.01, compared to the non-sensitized–non-challenged group.
LTD₄: the reduced sRaw in response to naphazoline at 10 min after the LTD₄ nasal drop was not significant, whereas that at 40 min was significantly weaker than the level in the non-sensitized – non-challenged group (Fig. 6B).

Intranasal administration (10 μl/nostril, 10⁻⁴ M) of naphazoline induced a dramatic lowering of spontaneous sRaw and was comparable to that by intravenous administration (0.1 mg/kg) in the non-sensitized – non-challenged animals:

changes in sRaw 5 min after the intranasal and intravenous treatment were −0.54 ± 0.13 (n = 5) and −0.52 ± 0.11 (n = 5) cmH₂O × ml/(ml/s), respectively. The effect of intranasal naphazoline on the sRaw elevation induced by LTD₄ was unreliable because of the elimination of naphazoline from the nasal cavity by the increased secretion of the nasal discharge by LTD₄.

**Fig. 6.** Effect of pranlukast (A) and naphazoline (B) on leukotriene (LT) D₄-induced increase of specific airway resistance (sRaw) in the sensitized – challenged guinea pig. The experiment was performed 2 days after the 20th cedar pollen inhalation challenge. A: Pranlukast (30 mg/kg) was administered orally 1 h prior to LTD₄ instillation. sRaw was measured 10 min after LTD₄ instillation. Data represent the mean ± S.E.M. of 8 animals. Open circle: non-sensitized – non-challenged, closed square: sensitized – challenged, striped square: sensitized – challenged – pranlukast-treated. **P < 0.01, compared to the sensitized – challenged group. ††P < 0.01, compared to the non-sensitized – non-challenged group.**

B: Naphazoline (0.1 mg/kg) was administered intravenously 8 or 35 min after intranasal instillation of LTD₄. Changes in sRaw were measured 10 (a) or 40 (b) min after LTD₄ treatment. Data represent the mean ± S.E.M. of 8 animals. †P < 0.05, ††P < 0.01, compared to the naphazoline-treated or non-treated in the sensitized – challenged group or to the non-sensitized – non-challenged group.
Time-course changes in the diameter of nasal blood vessels due to LTD₄ application

Figure 7A shows the time-course changes in the diameter of blood vessels due to exposure to 10⁻⁸ M LTD₄. LTD₄ application caused a swift dilatation of the vessels, which peaked at 10 min, followed by recovery at 40 min to the same level as that before the drop in LTD₄. In the non-sensitized – non-challenged-LTD₄-instilled group, no changes were observed at any time. Figure 7B represents the stereoscopic microscope pictures of nasal blood vessels in the sensitized – challenged animal: before (a) and 10 min after LTD₄ instillation (b).

Nasal vascular permeability response to LTD₄

Table 1 shows the nasal vascular permeability induced by 10⁻⁸ M LTD₄. When LTD₄ was instilled into the nasal cavities of the non-sensitized – non-challenged guinea pigs, no increase in the amount of dye in the nasal tissue was observed. However, LTD₄ instilled in the sensitized – challenged group induced a significant increase in vascular permeability, which returned to the normal (non-sensitized – non-challenged-saline-instilled group) level 40 min after the LTD₄ instillation.

Effect of L-NAME on the increased sRaw induced by LTD₄

Effect of L-NAME on the increased sRaw induced by LTD₄ instillation is shown in Fig. 8a. Increased sRaw due to LTD₄ instillation in the sensitized – challenged group was dose-dependently inhibited by pretreatment with L-NAME (3 – 30 mg/kg, i.v.), but not with D-NAME (10 mg/kg, i.v.). However, as shown in Table 2, neither L-NAME nor L-arginine affected baseline sRaw.

Figure 8b shows the reversal effect of L-arginine (600 mg/kg, i.v.) on L-NAME (10 mg/kg, i.v.)-induced

Fig. 7. Time-course of changes in the diameter of nasal blood vessels induced by dropping of leukotriene (LT) D₄ (10⁻⁸ M) (A) and microscopic photographs of nasal blood vessels before (a) and 10 min after (b) dropping of LTD₄ (B) in the sensitized – challenged guinea pig. The experiment was performed 2 days after 30th pollen inhalation challenge. Data represent the mean ± S.E.M. of 3 animals. Open circle: non-sensitized – non-challenged, closed square: sensitized – challenged. *P<0.05, compared to the non-sensitized – non-challenged group. Bar scales in B (a and b) indicate 100 μm.
Table 1. Amount of Evans blue in nasal tissue following intranasal instillation of leukotriene (LT) D₄ in the guinea pig

<table>
<thead>
<tr>
<th>Time (min) after saline or LTD₄ instillation</th>
<th>Amount of Evans blue (μg/animal)</th>
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<tbody>
<tr>
<td>10</td>
<td>21.2 ± 1.1</td>
</tr>
<tr>
<td>40</td>
<td>21.7 ± 2.4</td>
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Ten microliters/nostril of saline or 10⁻⁸ M LTD₄ was instilled into both nasal cavities of the guinea pig 1 h after Evans blue (10 mg·ml⁻¹·kg⁻¹, i.v.) administration on the 2nd day after the 30th pollen inhalation. Data are the mean ± S.E.M. from 10 animals. *P<0.05, compared to the non-sensitized – non-challenged – saline-instilled group. †P<0.05, compared to the non-sensitized – non-challenged – LTD₄-instilled group. N.D.: not determined.

Table 2. Influence of N⁶-nitro-l-arginine methyl ester (L-NAME) and l-arginine on baseline specific airway resistance (sRaw) of the sensitized – challenged guinea pig

<table>
<thead>
<tr>
<th>Time (min) after drug administration</th>
<th>sRaw [cmH₂O × ml/(ml/s)]</th>
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<tbody>
<tr>
<td>0</td>
<td>1.49 ± 0.05</td>
</tr>
<tr>
<td>15</td>
<td>1.58 ± 0.05</td>
</tr>
</tbody>
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L-NAME (10 mg/kg, i.v.) or l-arginine (600 mg/kg, i.v.) was administered to the sensitized – challenged guinea pig. Data are the mean ± S.E.M. of 8 animals.
suppression of the sRaw elevation by LTD₄ in the sensitized–challenged guinea pig. The inhibitory effect of L-NAME was significantly reversed by co-administration of L-arginine, although L-arginine alone showed no effect on the increase of sRaw induced by LTD₄.

The combination treatment with indomethacin (10 mg/kg, p.o.) and AA-861 (100 mg/kg, i.p.) barely influenced the increased sRaw induced by LTD₄ instillation in the sensitized–challenged guinea pig (data not shown).

Nasal responsiveness to SNP

Figure 9a shows the time-course change in sRaw after instillation of 10⁻⁶ M SNP. SNP caused a prompt elevation of sRaw that peaked at 2 min, followed by diminution until 10 min after instillation. According to the results obtained, sRaw was measured 2 min after SNP instillation in the following experiments.

Nasal responsiveness to SNP of sensitized–challenged guinea pigs was evaluated 2 days after the 30th pollen challenge and compared to that of non-sensitized–non-challenged animals. No apparent differences in the concentration-related changes in sRaw were observed between the two groups (Fig. 9b).

Amount of NO₂⁻ and NO₃⁻ in NCLF induced by LTD₄

In the sensitized–challenged group, LTD₄ application caused a significant increase in NO₂⁻ and NO₃⁻ in NCLF compared with that before treatment. On the other hand, these levels in the non-sensitized–non-challenged group showed only a slight tendency to increase in response to...
LTD₄. The increased production of NOₓ and NO₃ by LTD₄ in the sensitized – challenged group was significantly larger than that in the non-sensitized – non-challenged group (Fig. 10).

DISCUSSION

In the present experiments, sRaw was selected as the index of nasal obstructive response to intranasal instillation of LTD₄. We consider that sRaw reflects total (upper and lower airway) airway resistance, because the guinea pig functionally respirates through the nose but not through the mouth. The early bronchoconstrictor response is reportedly characterized by rapid and shallow breathing in a guinea pig asthma model (20). We previously reported that the pollen inhalation challenge-induced elevation of sRaw correlated well with a decrease in respiratory frequency in our experimental allergic rhinitis model (11). These reports indicate that allergic responses of the upper and lower airways are characteristically different in conscious guinea pigs. In our allergic rhinitis model, intranasal instillation of histamine and LTD₄ decrease respiratory frequency, whereas forced inhalation of a fine mist of histamine, approx. 80% of which was trapped in and acted on the bronchi, induces rapid and shallow breathing (S. Kohno et al., unpublished data). In addition, administration of LTD₄, a well-known potent bronchoconstrictor, into the nasal cavities of the non-sensitized guinea pig produced no elevation of sRaw even at 10⁻⁴ M. Furthermore, Narita et al. (21) reported that most Evans blue dye instilled intranasally was found within the nasal cavity. Thus, it can be concluded that the changes in sRaw induced by intranasal instillation of agonists in the present manner entirely reflect the nasal response (nasal airway patency) and not the lower airway response.

We previously reported that our allergic rhinitis model come to show nasal hyperresponsiveness to histamine (12) with the elevation of serum IgG₁ and IgE levels by repetitive antigen inhalation challenges (11). In the present study, we demonstrated that remarkable hyperresponsiveness to LTD₄, which was much stronger than that to histamine, was observed using this model. In agreement with the features of the hyperresponsiveness to histamine (12), the intensity of the nasal hyperresponsiveness to LTD₄ increased with the number of antigen provocations in sensitized – challenged animals, and the hyperresponsiveness was evident at 10 h and on the 2nd day, but not on the 7th day after the pollen challenge. Thus, the hyperresponsiveness to these agonists might be acquired at least partly through the same mechanisms. Recent experiments revealed that the nasal hyperresponsiveness to histamine had occurred already 4 h after antigen inhalation challenge and that the amount of CysLTs in nasal cavity lavage fluid after antigen inhalation challenge showed a biphasic increase, the time of which corresponded to that of the biphasic sRaw elevation (M. Yamasaki et al., submitted manuscript). It appears that the former result is applicable in the case of the hyperresponsiveness to LTD₄. Thus, the late sRaw elevation can be induced by the late production of CysLTs combined with the hyperresponsiveness. However, CysLTs hardly contributes to the early sRaw elevation, because the CysLT₁-receptor antagonist did not influence it (Fig. 2). On the other hand, Horworth and Holgate (22) reported that an H₁-receptor antagonist is effective for sneezing, itching and nasal secretion, but not for nasal blockage in allergic rhinitis patients. In accordance with the clinical results, mepyramine did not inhibit both the early and the late responses (Fig. 2), but sneeze was significantly suppressed by the drug (data not shown) in our model, strongly suggesting that the model is potentially useful for research on allergic rhinitis. Since the early sRaw elevation was inhibited by neither the H₁- nor the CysLT₁-receptor antagonist as described above, other mediator(s) than histamine and CysLTs must take part in the elevation.

The exposure frequency-dependent increase and restoration pattern of nasal hyperresponsiveness closely resemble clinical allergic rhinitis. It is accepted that nasal hyperresponsiveness in patients diagnosed with perennial allergic rhinitis is more evident than that in individuals with seasonal rhinitis (23). Furthermore, seasonal allergic rhinitis patients exhibit more marked nasal symptoms in the pollen season than in the off-season (24). Consequently, we believe that our allergic rhinitis model of guinea pigs is effective in the research of detailed mechanisms of the induction of nasal hyperresponsiveness.

In terms of the pathophysiological action of LTD₄ on nasal mucosa, LTD₄ induces dilatation of nasal capacitance blood vessels and increases nasal capillary permeability. We evaluated the contribution of nasal blood vessel dilatation to the LTD₄-induced elevation of sRaw in the sensitized – challenged animal using naphazoline, a potent vasoconstrictive α-agonist. The result showed that the drug appeared to completely suppress the increase in sRaw by LTD₄ and lowered sRaw to below spontaneous levels. Therefore, it was strongly suggested that the blood vessel dilatation was directly involved in the sRaw elevation induced by LTD₄, which prompted us to examine LTD₄-induced changes in nasal mucosal blood vessels using a stereoscopic microscope. Microscopy demonstrated that superficial veins of 50 to 80 μm in diameter, which are occasionally located in the upper stream of capacitance blood vessels, dilated immediately in response to LTD₄. This dilatation of the upper stream vein strongly suggests progressive distension of the venous sinusoid forming the capacitance vessels, which are reported to mainly reflect nasal patency (25). In the present study, maximum dilata-
Nasal Hyperresponsiveness to LTD₄

...ation occurred at 10 min after LTD₄ application, which was identical to that recorded for maximum sRaw induced by LTD₄. From these results, it can be concluded that nasal blood vessel dilatation largely contributes to the sRaw elevation by instilled LTD₄, and the results explain why naphazoline potently lowered the elevated sRaw by LTD₄.

Next, we examined whether the elevation of sRaw induced by intranasal instillation of LTD₄ is induced via CysLT₁-receptor stimulation. Pranlukast (30 mg/kg, p.o.), a CysLT₁-receptor antagonist, was administered at a dose that specifically inhibits bronchoconstrictive responses induced by CysLTS, but not those by other agonists in guinea pigs in vivo (26), and was found to strongly inhibit the response. Consequently, the dilatation of nasal blood vessels induced by LTD₄ is solely mediated by CysLT₁-receptor activation.

LTD₄-induced dilatation of nasal blood vessels by CysLT₁-receptor activation is potentially induced by two distinct endogenous substances, which are the products of the L-arginine enzymatic pathway and the metabolites of arachidonic acid via the COX enzymatic pathway. The increase in sRaw by LTD₄ was potently blocked by an NO synthase (NOS) inhibitor, L-NAME, and the inhibitory effect of L-NAME was completely reversed by the co-administration of the substrate for NOS, L-arginine. On the other hand, the combination treatment with COX inhibitor, indomethacin, and 5-LO inhibitor, AA-861, had no inhibitory effect on the response. These results suggest that intranasally instilled LTD₄ mainly induces the increase in sRaw by NO, and not metabolites of arachidonic acid, through the activation of CysLT₁-receptors on the surface of the endothelial cells of nasal blood vessels. LTD₄ instillation caused a significant increase in NO⁻ and NO₃⁻ in NCLF compared with that before LTD₄ instillation in the sensitized–challenged guinea pig, but not in the non-sensitized–non-challenged animal; and the dose-sRaw elevation curve of nasal SNP, an NO donor, in the sensitized–challenged animal was almost equivalent to that in the non-sensitized–non-challenged group. Therefore, it is conceivable that nasal vessel dilatation by LTD₄ results from the acquired hyperproduction of NO from the vascular endothelial cells and not from the direct action of LTD₄ on the vascular smooth muscle cells themselves. As is well known, NO produced by constitutive NOS induces relaxation of the smooth muscle cells through an activation of cytosolic guanylate cyclase and an increase in the intracellular guanosine 3',5'-cyclic monophosphate level. Although NO is known to be a powerful vasodilator and to control the filling of nasal capacitance vessels (27), baseline sRaw was not affected by L-NAME, which is in contrast to the effect of naphazoline. Based upon the present study’s result and a report that L-NAME did not change nasal airway resistance from baseline in healthy subjects (28), NO may not substantially act as a vasodilator in nasal airways under unstimulated conditions.

On the other hand, the extent of the naphazoline-induced decrease in sRaw in the sensitized–challenged–LTD₄-treated animal, particularly at 40 min after LTD₄ instillation, was smaller than that of the non-sensitized–non-challenged–sRaw elevation (10 min) induced by LTD₄, but nasal capillary permeability is not involved at a relatively late period (40 min or later). LTD₄ instillation into the nasal cavities of allergic rhinitis patients has been reported to cause an increase in nasal secretion (7). During the present experiments, we found that viscous secreted material had accumulated intranasally in the sensitized–challenged–LTD₄-treated guinea pig but not in the non-sensitized–challenged–LTD₄-treated animals, although at present we cannot quantitatively measure this secretion. Thus, LTD₄ may contribute to a persistent increase in sRaw on the basis of the enhanced secretion.

In conclusion, we demonstrated that repetitive exposure of sensitized–challenged guinea pigs to cedar pollen results in remarkable but reversible hyperresponsiveness to LTD₄. Hyperresponsiveness in sensitized–challenged animals is mainly due to an increased dilatative response of the nasal blood vessels to the agonist, which can be related to hyperproduction of NO.

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REFERENCES