Cysteinyl Leukotrienes Induce Nasal Symptoms of Allergic Rhinitis via a Receptor-Mediated Mechanism in Guinea Pigs

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ABSTRACT—To examine whether cysteinyl leukotrienes (cysLTs: LTC₄, LTD₄ and LTE₄) induce symptoms of allergic rhinitis via their receptors, we studied the following: i) the specific binding of radiolabeled cysLTs to guinea pig nasal mucosa membrane and ii) effects of nasal LTD₄ challenge in normal guinea pigs. The binding study indicated that there was a single population of binding sites for LTC₄, LTD₄ and LTE₄ with Kᵦ and Bₘₐₓ values of 34.9±2.0, 0.252±0.015 and 0.589±0.039 nM and 10, 140±490, 122±11 and 306±23 fmol/mg protein, respectively. The in vivo study showed that topical nasal challenge of LTD₄ (0.1–30 µg/nose) increased nasal secretion, nasal airway resistance and nasal eosinophil infiltration without inducing sneezing. While the increases in nasal secretion and nasal airway resistance were transient, peaking 10 to 20 min after LTD₄ challenge, nasal eosinophil infiltration persisted at least until 24 hr post-challenge. These nasal symptoms were dose-dependently suppressed by oral administrations of pranlukast (0.3–3 mg/kg). The results suggest that cysLTs cause not only early-phase symptoms but also nasal eosinophil migration, a characteristic associated with the late-phase symptom of allergic rhinitis, via a receptor-mediated mechanism. Cysteinyl leukotrienes, thus, may be important mediators in allergic rhinitis.

Keywords: Cysteinyl leukotriene, Rhinitis, Eosinophil, Receptor, Pranlukast

Cysteinyl leukotrienes (cysLTs: LTC₄, LTD₄ and LTE₄) are potent allergic mediators released from various inflammatory cells such as mast cells and eosinophils (1). The action of cysLTs is mediated by their specific receptors that trigger activation/recruitment of inflammatory cells, enhancement of mucous secretion, vascular permeability and smooth-muscle tone (reviewed in ref. 2). Because of these activities, cysLTs have been implicated in allergic rhinitis (3). Recent human studies have shown that nasal provocation of LTD₄ induce rhinorrhea and nasal congestion, as indicated by increases in nasal secretion and nasal airway resistance (NAR). The threshold concentration of LTD₄ required to induce these symptoms is approximately 5000-fold lower than that of histamine, a known mediator of allergic rhinitis (4). Increased cysLTs have also been demonstrated in nasal lavage fluid in patients with allergic rhinitis after antigen provocation (5). These studies further support the involvement of cysLTs in allergic rhinitis.

Although it is hypothesized that cysLTs participate in allergic rhinitis via a receptor-mediated mechanism, convincing evidence has yet to be established. Firstly, existence of cysLTs-receptors has been demonstrated in nasal mucosa of neither humans nor animals. Secondly, the prevention of cysLTs-induced symptoms of rhinitis by specific cysLT receptor antagonists has not been reported yet. This study was designed to examine if nasal mucosae had specific cysLT-receptors and whether pranlukast, a cysLT antagonist, abolished LTD₄-induced rhinitis symptoms in guinea pigs. Symptoms such as nasal secretion, increase in NAR and nasal eosinophil infiltration were evaluated. Pranlukast has previously been demonstrated as a potent in vitro (6) and in vivo (7) antagonist against LTC₄, LTD₄ and LTE₄.

MATERIALS AND METHODS

Animals
Male Hartley guinea pigs (Nihon Rabbit Co., Osaka) weighing 300 to 600 g and 500 to 900 g were used for animal and radioligand binding studies, respectively. The animals were housed in an air-conditioned room at 23±2°C, 55±5% humidity with alternating 12-hr light/dark cycles. Animals were given food and water ad libi-
were determined by the method of Lowry.

and stored at 80°C until use. Protein concentrations (pH 7.4) with a final protein concentration of 10 mg/ml

pellet was resuspended in 10 mM HEPES-NaOH buffer

higher centrifugation (40,000 X g for 30 min at 4°C). The

After centrifuging the homogenate (1,500 x g for 15 min

Physcotron (Niti-on, Chiba) at 21,000 rpm for 20 sec.

0.01 mM leupeptin hemisulfate salt and 0.01 mM pep

containing protease inhibitors (2 mM EDTA 4Na, 100

PBS (pH 7.4). The tissues were then suspended in 5 vol.

mucosae were removed. The tissues were dissected on ice,

Preparation of nasal mucosa membranes in guinea pigs

Membrane fractions of guinea pig nasal mucosa were

preparated by a previously described method (6) with slight

modification. Briefly, animals were decapitated and nasal

mucosae were removed. The tissues were dissected on ice, weighed and washed twice with 25 ml of ice-cold 1/15 M PBS (pH 7.4). The tissues were then suspended in 5 vol.

of 10 mM HEPES-NaOH/0.25 M sucrose buffer (pH 7.4) containing protease inhibitors (2 mM EDTA 4Na, 100 

pg/ml soybean trypsin inhibitor, 100 pg/ml bacitracin, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 0.01 mM leupeptin hemisulfate salt and 0.01 mM pepstatin A). Tissues were homogenized thrice on ice by a

Physcotron (Niti-on, Chiba) at 21,000 rpm for 20 sec. After centrifuging the homogenate (1,500 × g for 15 min at 4°C), the supernatant was collected and subjected to higher centrifugation (40,000 × g for 30 min at 4°C). The pellet was resuspended in 10 mM HEPES-NaOH buffer (pH 7.4) with a final protein concentration of 10 mg/ml and stored at −80°C until use. Protein concentrations were determined by the method of Lowry.

Radioligand binding study

The binding study was carried out in 10 mM HEPES-NaOH buffer (pH 7.4) containing 10 mM CaCl₂ and 10 mM MgCl₂. For the [³H]LTC₄ and [³H]LTD₄ binding study, 20 mM L-penicillamine/50 mM L-serine borate and 20 mM L-penicillamine were respectively added to the buffer to prevent the conversion of LTC₄ to LTD₄ and LTE₄. In the saturation study, various concentrations of [³H]cysLTs were incubated with aliquots of nasal membranes (50, 100 and 100 µg protein/ml, respectively, for [³H]LTC₄, [³H]LTD₄ and [³H]LTE₄) at 25°C for 30 min. In the competition study, [³H]cysLTs ([³H]LTC₄, 0.5 nM; [³H]LTD₄, 1 nM; [³H]LTE₄, 1 nM) were incubated with aliquots of nasal membranes (50, 100 and 100 µg protein/ml, respectively for [³H]LTC₄, [³H]LTD₄ and [³H]LTE₄) at 25°C for 30 min in the presence of various concentrations of competing drugs. Binding reactions were terminated by rapid filtration of the sample through Whatman GF/C glass microfiber filter strips with a Brandel Cell Harvester followed by 4 repetitions of rapid washing with 2.5-ml aliquots of ice-cold 10 mM HEPES-NaOH/100 mM NaCl buffer (pH 7.4). Residual [³H]-LTC₄, [³H]LTD₄ and [³H]LTE₄ counts bound to the filters were determined by a liquid scintillation counter (LSC-5100; Aloka, Tokyo) after the addition of 8 ml ACS II (Amersham, Buckinghamshire, England). The specific binding was defined as the difference between total binding and nonspecific binding determined in the presence of 5 µM of LTC₄, 1 µM of LTD₄ and 1 µM of LTE₄, respectively.

Nasal symptoms and nasal eosinophil infiltration

Various concentrations of 20 µl LTD₄ or histamine solution were dripped into each nostril of either conscious or anesthetized normal guinea pigs and changes in nasal symptoms (NAR, nasal secretion and sneezing) and nasal eosinophil infiltration were observed as described below. All experiments were performed in an air-conditioned room.

NAR: Conscious animals were placed in a transparent body chamber and challenged with LTD₄ as described above. Using non-invasive respiratory measurements (Buxco Electronics, Inc., Sharon, CT, USA), the total airway resistance (TAR) measured by the double flow-plethysmograph technique (8) was considered as the NAR (9). The change was measured at 0, 10, 20 and 30 min post-challenge.

Nasal secretion: The surgically exposed trachea of animals, anesthetized with sodium nembutal (30 mg/kg, i.p.), were incisioned and a cannula was inserted in the lung-side section to maintain spontaneous respiration. Another cannula was inserted in the nasal-side section. At 10, 20 and 30 min post-challenge, cotton threads (weigh-
ing approximately 10 mg, 3-cm-long and 1-mm-diameter) were placed in both nostrils and nasal secretion was flushed out with compressed air-flow from the trachea to nostrils for 1 min. After flushing, weight increases of the threads due to nasal secretion were quantitatively evaluated as nasal secretion.

**Sneezing:** Conscious animals were challenged with LTD₄ as described above. The frequency of sneezing was counted at 10-min intervals for 30 min after the challenge of mediators.

**Eosinophil infiltration:** Conscious animals were challenged with LTD₄ or histamine as described above. Animals were sacrificed by transection of the abdominal aorta under urethane anesthesia (50 mg/kg, i.p.) at various times after post-challenge, and a cannula was inserted in the nasal side-section for nasal lavage. Nasal cavities were washed five times with 5 ml of saline containing 0.1% bovine serum albumin and the nasal lavage fluid (NLF) was collected. The NLF was centrifuged at 100 x g for 10 min at 4°C. Centrifuged, cell-pellets were suspended in 0.5 ml saline containing 0.1% bovine serum albumin. Cells were then smeared on glass slides from aliquots of cell suspension with cytocentrifuge (50 x g, 5 min, room temperature) before staining with May-Grundwald-Giemsa solution for eosinophil count under light microscopy.

**Drug administration**

Pranlukast and epinastine were orally administered 1 hr before LTD₄ challenge in animals fasted overnight.

**Statistical analyses**

Data from the radioligand binding assay were subjected to computer analysis using programs of Equilibrium Binding Data Analysis (EBDA by McPherson, Elsevier-BIOSOFT, 1983). The equilibrium dissociation constant (Kₐ) and the maximum number of binding sites (Bₐₘₐₓ) were determined by Scatchard plot analysis of the saturation binding data. The competitive activities of the analogues were expressed as the inhibition constant (Kᵢ), defined as 

\[ Kᵢ = IC₅₀ / (1 + [3H]cysLTs / Kₐ) \]

where [3H]cysLTs was the concentration of radioligand used. All data were expressed as the mean ± S.E.M. Statistical significance was determined either by Student's t-test or one-way analysis of variance followed by Dunnett's t-test. P values of less than 0.05 were considered to be significantly different.

# RESULTS

**Characteristics of [3H]cysLTs binding to nasal mucosa membranes**

The specific binding of [3H]LTC₄, [3H]LTD₄ and [3H]LTE₄ to fractions of nasal mucosa membrane was saturatable. Scatchard plot analyses showed the presence of a homogeneous population of binding sites with the Kₐ and Bₐₘₐₓ values shown in Table 1. There were no significant differences among the Hill coefficients (0.944 ± 0.018, 0.943 ± 0.027 and 0.945 ± 0.019, for [3H]-LTC₄, [3H]-LTD₄ and [3H]-LTE₄, respectively; n = 5), indicating no mutual interaction among the cysLTs.

**Table 1.** Kₐ and Bₐₘₐₓ value for [3H]LTC₄, [3H]LTD₄ and [3H]-LTE₄ specific binding to a fraction of guinea pig nasal mucosa membranes

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Kₐ (nM)</th>
<th>Bₐₘₐₓ (fmol/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTC₄</td>
<td>34.9±2.0</td>
<td>10,140±490</td>
</tr>
<tr>
<td>LTD₄</td>
<td>0.252±0.015</td>
<td>122±11</td>
</tr>
<tr>
<td>LTE₄</td>
<td>0.589±0.039</td>
<td>306±23</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M. of five separate experiments.

**Fig. 1.** Effects of LTD₄ on changes in NAR (A) and nasal secretion (B). Animals were nasally challenged with various concentrations of LTD₄. Changes in NAR are expressed as area under the curve (AUC) from 0 to 30 min post-challenge. Nasal secretion was measured 10 min after challenge. Each point represents the mean ± S.E.M. of 6 to 10 animals. *P < 0.05 and **P < 0.01 vs vehicle (Dunnett’s t-test).
Inhibition of $[^{3}H]$cysLTs binding to nasal mucosa membranes by pranlukast

Unlabeled ligands (LTC$_4$, LTD$_4$, and LTE$_4$) concentration-dependently inhibited $[^{3}H]$LTC$_4$, $[^{3}H]$LTD$_4$, and $[^{3}H]$LTE$_4$ bindings to nasal membrane fractions of guinea pigs with $K_i$ values of 45.2±1.7, 0.59±0.09 and 1.44±0.16 nM, respectively (n=5). These bindings were also concentration-dependently inhibited by a cysLT antagonist, pranlukast, with $K_i$ values of 7,700±570, 0.98±0.16 and 1.70±0.22 nM, respectively (n=5). The inhibitions of pranlukast on $[^{3}H]$LTD$_4$ and $[^{3}H]$LTE$_4$ bindings were approximately 7900-fold and 4500-fold more potent than that on $[^{3}H]$LTC$_4$. An anti-histamine, epinastine, had no effects on $[^{3}H]$cysLT binding even at 100 μM.

Effects of LTD$_4$ on nasal symptoms

A preliminary time-course study showed that LTD$_4$ challenge (3 pg/site) transiently increased both NAR and nasal secretion, with these levels returning to the basal level within the subsequent 20 min and no changes were observed at least until 8 hr after the challenge. However, whereas nasal secretion peaked at 10 min post-challenge, NAR peaked 10 to 20 min post-challenge. Therefore we compared the area under the curve (AUC) of NAR from 0 to 30 min post-challenge and amount of nasal secretion at 10 min post challenge in the subsequent experiments. As shown in Fig. 1, nasal LTD$_4$ challenge concentration-dependently increased both NAR and nasal secretion significantly at concentrations higher than 10 pg/site and 1 pg/site, respectively, in normal guinea pigs. The respective increases in NAR and nasal secretion were approximately 3- and 2-fold at the highest concentration when compared with those of animals treated with vehicle. The challenge, however, did not induce sneezing even at 30 μg/site (5.3±1.0 counts vs 8.5±1.4 counts in vehicle and LTD$_4$, respectively, during the first 10 min after the challenge).

Effects of LTD$_4$ and histamine on nasal inflammatory cell infiltration

Nasal LTD$_4$ (30 μg/site) challenge time-dependently increased eosinophil counts in NLF. The infiltration peaked at 8 hr and persisted for not less than 24 hr after the challenge. Histamine, in contrast, did not have such an effect even at 3 mg/site within the experimental period (Fig. 2). A concentration-response study showed that the response of LTD$_4$ (1–30 μg/site) at 8 hr after post-challenge was concentration-dependent, with significant responses at concentrations higher than 3 μg/site (Fig. 3). Although lymphocyte counts were also significantly increased at the highest concentration of LTD$_4$ (1.75±0.33×10$^4$ and 5.44±1.30×10$^4$ cell/NLF in vehicle and LTD$_4$ challenge, respectively; P<0.01 with Dunnett's t-test), no concentration-responses were observed in the total cells, neutrophils or macrophages.

Effects of pranlukast on LTD$_4$-induced nasal symptoms

Nasal LTD$_4$ (10 μg and 1 μg/site for NAR and nasal secretion, respectively) challenge significantly increased AUC of NAR from 0 to 30 min post-challenge and nasal secretion at 10 min post-challenge in normal guinea pigs. These changes were reduced by oral administrations of pranlukast (0.3–3 mg/kg) in a dose-dependent fashion with significant inhibitions at doses higher than 1 mg/kg in NAR and at 3 mg/kg in nasal secretion. However, these symptoms were not affected by an anti-histaminergic, epinastine, even at 30 mg/kg (Fig. 4), a dose that was sufficient to totally suppress histamine-induced responses (data not shown).
Fig. 3. Concentration-response of LTD₄ on nasal eosinophil infiltration. Guinea pigs were nasally challenged with various concentrations of LTD₄ and eosinophils in NLF at 8 hr post-challenge were counted as described in Fig. 2. Each point represents the mean ± S.E.M. of 9 to 10 animals. *P < 0.05 and **P < 0.01 vs vehicle (Dunnett's t-test).

Fig. 4. Effects of pranlukast and epinastine on LTD₄-induced increases in NAR (A) and nasal secretion (B). Animals were nasally challenged with either 10 μg or 1 μg/site of LTD₄ before NAR or nasal secretion were measured as described in Fig. 1. Drugs were orally administered 1 hr before LTD₄ challenge. #P < 0.05 and ##P < 0.01 vs vehicle (Student's t-test) and *P < 0.05 and **P < 0.01 vs control (Dunnett’s t-test).
Effects of pranlukast on LTD₄-induced nasal eosinophil infiltration

Nasal LTD₄ (3 μg/site) challenges significantly increased eosinophil counts in NLF 8 hr after the challenge. This effect was inhibited by oral administrations of pranlukast (0.3–3 mg/kg) in a dose-dependent fashion. The suppression was significant at doses higher than 1 mg/kg. Epinastine (30 mg/kg, p.o.) did not affect the LTD₄-induced change (Fig. 5).

Fig. 5. Effects of pranlukast and epinastine on LTD₄-induced nasal eosinophil infiltration. Animals were nasally challenged with LTD₄ (3 μg/site), and eosinophils in NLF were counted at 8 hr post-challenge as described in Fig. 3. Drugs were orally administered 1 hr before LTD₄ challenge. **P<0.01 vs vehicle (Student’s t-test) and *P<0.05 vs control (Dunnett’s t-test).

DISCUSSION

This study shows that the membrane fraction of guinea pig nasal mucosae had specific binding sites for [³H]cys-LTs, and rhinitis symptoms induced by nasal LTD₄ challenge were abolished by a cysLT receptor antagonist, pranlukast. These results support the current hypothesis that cysLTs are involved in allergic rhinitis via a receptor mediated mechanism.

The radioligand binding assay indicated the existence of a single class of high affinity and saturable binding sites for cysLTs in membrane fractions of the guinea pig nasal mucosa. These binding sites are cysLT receptors because pranlukast inhibited the binding of [³H]cysLTs to their specific binding sites in the nasal mucosa membrane. This is the first demonstration of the existence of cysLTs receptor in the nasal mucosa. It should be noted that Kᵅ and Bₘ₉₉ values for [³H]LTC₄, [³H]LTD₄ and [³H]LTE₄ in this study are similar to those previously reported with membrane fractions of guinea pig lung (6). Therefore, it appears that the affinity and distribution of receptors for cys-LTs is similar between upper and lower airways.

Analyses of Kᵅ and Bₘ₉₉ values in our study indicate that LTD₄ and LTE₄ receptors had similar affinity and distribution, while LTC₄ receptors displayed an affinity 60- to 140-fold lower and a density 30- to 80-fold higher than those of LTD₄ and LTE₄ in the guinea pig nasal mucosa, respectively. These findings are consistent with other biochemical (10) and pharmacological (11) studies and support the recent hypothesis that LTC₄ receptor is biochemically different from LTD₄ and LTE₄ receptors (10). The similarity in affinity and distribution between LTD₄ and LTE₄ receptors may be explained by the fact that LTE₄ binds to a subset population of high-affinity LTD₄ receptors (12). Thus, LTC₄ receptors may be different from those of LTD₄ and LTE₄, although there is a possibility that LTD₄ and LTE₄ share the same binding site in guinea pig nasal mucosa.

In this study, we used LTD₄ as a representative of cys-LTs. Since LTC₄ is rapidly converted to LTD₄ and LTE₄ by γ-glutamyl transpeptidase (13), it is very difficult to study the specific action of LTC₄, LTD₄ and LTE₄, respectively, in vivo. Therefore, many investigators have used LTD₄ in investigating actions of cys-LTs (3). Although our study does not exclude a possible contribution of LTE₄, it may be useful in understanding the actions of cys-LTs.

Nasal LTD₄ challenges significantly increased nasal secretion and NAR without inducing sneezing in normal guinea pigs. This observation is similar to that in an
earlier human study (4). On the other hand, while the threshold concentration in inducing these changes were different in this study, that in the human study is the same. This discrepancy may be attributed in large part to the condition of the recipients: we used anesthetized guinea pigs for nasal secretion and conscious guinea pigs for NAR while the investigators used conscious patients in the earlier study. The precise mechanism by which LTD₄ induces these changes is unclear. However, it appears to be mediated by cysLTs receptor on nasal mucosa, because these changes were abolished only by pranlukast but not by epinastine, which can antagonize the action of histamine, a known mediator to increase both nasal secretion and NAR (4).

While the increase of NAR caused by LTD₄ in this study was transient, peaking 10 to 20 min after the challenge, that in humans persisted for 8 hr after the challenge (4). The discrepancy between these two results is unclear. This may be attributed to species differences, method for LTD₄ challenge and/or subject condition (i.e., allergic or non allergic). In this study, LTD₄ was nasally instilled into normal guinea pigs, whereas in the human study, LTD₄-soaked paper discs were placed in the noses of patients with allergic rhinitis. Therefore, the longer contact period of LTD₄ to the nasal mucosa and use of allergic recipient could have changed the NAR response of recipients to LTD₄. In fact, it has been demonstrated that allergic persons have an enhanced sensitivity to LTD₄ (14).

In addition to rhinitis symptoms, nasal eosinophil infiltration is one of the characteristics associated with allergic rhinitis. We therefore studied if LTD₄ caused nasal eosinophil infiltration. We found that nasal LTD₄, but not histamine, challenge induced significant eosinophil infiltration into guinea pigs that persisted until 24 hr after LTD₄ challenge. It has been reported that LTD₄ (30 μg/ml) inhalation caused prolonged eosinophil infiltration into guinea pig lungs (15, 16). These results collectively suggest that LTD₄ potently induces eosinophil infiltration. Since the infiltration was totally inhibited by pranlukast, the action is probably mediated by cysLTs receptor as well. However, in contrast to another report (17) that LTD₄ as low as 10⁻¹⁰ M displays chemotactic activity against human eosinophils, we found that LTD₄ up to 10 μM had no apparent chemotactic activity against guinea pig eosinophils in vitro (data not shown). Therefore, induction of eosinophil infiltration is probably not a direct effect of LTD₄ on guinea pig eosinophils. It is possible, however, that cells other than eosinophils and/or organs may produce eosinophil chemoattractant in response to LTD₄ via a receptor-mediated mechanism. One of the candidate mediators would be thromboxane A₂ (TXA₂), because LTD₄ induces TXA₂ synthesis (18), and TXA₂ has been suggested to be associated with nasal eosinophil migration in a model of allergic rhinitis in guinea pigs (19). Further studies are warranted to elucidate the mechanisms by which LTD₄ induces eosinophil infiltration in vivo.

The symptoms of allergic rhinitis often display a biphasic pattern characterized by early-phase symptoms (sneezing, rhinorrhea and nasal obstruction) and late-phase symptom (nasal congestion) accompanied by eosinophil infiltration into nasal mucosae. Although the role of eosinophils in allergic rhinitis is not clear, it has been suggested that eosinophils are an important cellular source of chemical mediators, including cysLTs in the late-phase (20). Our results suggest that cysLTs induce not only early-phase symptoms but also nasal eosinophil infiltration, a characteristic associated with the late-phase, via cysLT receptor-mediated mechanisms. Since eosinophils are a cellular source of cysLTs (21), it may be rational to speculate that cysLTs production in the late-phase is enhanced by eosinophil recruitment of cysLTs. This concept is supported by the fact that cysLTs are detectable in not only the early- but the late-phase (5) as well. Therefore, in addition to induction of the early-phase symptoms in allergic rhinitis, cysLTs may modulate events in the late-phase response.

In conclusion, as cysLTs induced symptoms typical of allergic rhinitis via a receptor-mediated mechanism, they may thus have an important role in allergic rhinitis.

REFERENCES


