

The Effect of Leukotriene D₄ Inhalation on the Antigen-Induced Airway Hyperresponsiveness and Inflammation in 5-Lipoxygenase Gene-Deficient Mice

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Key Words

Leukotriene D₄ · Airway hyperresponsiveness ·
5-Lipoxygenase · Eosinophil · IgE

Abstract

Background: The role of 5-lipoxygenase (5-LO) products in the asthmatic bronchoconstriction is evident. However, the role of 5-LO products in airway hyperresponsiveness (AHR) and airway inflammation is still under discussion. The aim of the present study is to investigate the role of leukotriene D₄ (LTD₄) in AHR and allergic airway eosinophilia in mice. **Methods:** The effect of LTD₄ inhalation on antigen-induced AHR and airway eosinophilia was investigated in 5-LO gene-deficient mice. **Results:** After three inhalations of LTD₄, airway responsiveness to acetylcholine was not altered in normal or allergic wild-type and 5-LO knockout (KO) mice. In contrast, the number of eosinophils in 5-LO KO allergic mice increased to the level of wild-type allergic mice after the inhalation of LTD₄. These observations were confirmed by a histopathological study of the lungs. No change in the cytokine levels in bronchoalveolar lavage fluid and serum immunoglobulin levels was shown after LTD₄

inhalation. **Conclusion:** These findings suggest that LTD₄ plays a role in eosinophilic airway inflammation but not in AHR in mice.

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Introduction

5-Lipoxygenase (5-LO) is an important enzyme which converts arachidonic acid to leukotriene B₄ (LTB₄), LTC₄, LTD₄ and LTE₄ [1–4]. LTC₄ and its metabolic products, LTD₄ and LTE₄, have the ability to mediate airway narrowing, mucus secretion and vascular leakage via specific receptors. Thus, leukotrienes (LTs) are potent bioactive lipids that have been conventionally viewed as paracrine mediators in inflammatory disease processes such as bronchial asthma [5, 6].

The existence of 5-LO was indicated in certain inflammatory cells, including polymorphonuclear leukocytes, eosinophils, mast cells and macrophages [1, 7]. Antigen challenge causes an immediate asthmatic response through the cross-linking of IgE molecules on the surface of mast cells and the subsequent release of histamine, prostaglandins and 5-LO products (LTs) [8, 9]. After an

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1018–2438/01/1264–0309\$17.50/0

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acute bronchospasm (immediate asthmatic response), there often occurs a more severe airway obstruction, or late asthmatic response, which is characterized by increased mucus production and persistent chest tightness. Repeated airway obstruction due to airway inflammation leads to airway hyperresponsiveness (AHR) [10].

There have been many investigations about the role of 5-LO products in the development of bronchial asthma [2–4]. The pathophysiological role of 5-LO products was mainly investigated by employing pharmacological agents [11–15]. Currently, selective antagonists against 5-LO products are available, but the analysis of the pathological role of a specific substance in a particular disease has limitations when using a pharmacological agent. We have already indicated the efficacy of some LT antagonists on late asthmatic response (LPR) in guinea pigs [11, 12]; however, the effect of LT antagonists on AHR and eosinophilia is inconsistent. Many investigators have reported conflicting results [13–15]. Despite extensive investigations, the precise role of 5-LO products in the onset of AHR and airway inflammation is still obscure.

Recently, significant advances were obtained in the area of *in vivo* manipulation of the mammalian genome through gene targeting. This kind of approach has been extensively carried out to investigate the role of target molecules in specific diseases. In 1997, Irvin et al. [16] reported the lack of an allergic airway inflammation and AHR in 5-LO gene-deficient mice. However, there is no information about the dominant molecule of 5-LO products. The present study was, therefore, conducted to investigate the effect of LTD₄ on the antigen-induced AHR and airway inflammation in 5-LO knockout (KO) mice by inhalation of vaporized LTD₄.

Material and Methods

Animals

Seven-week-old male 5-LO KO mice (129 Sv background) weighing 25–30 g were obtained from Jackson Laboratory. Age-matched male wild-type animals (129 Sv background) were used as control. The animals were housed in plastic cages in an air-conditioned room at 22 ± 1 °C with a relative humidity of 60 ± 1%, fed a standard laboratory diet and given water *ad libitum*. Experiments were performed following the Guidelines for the Care and Use of Experimental Animals of the Japanese Association for Laboratory Animals Science established in 1987.

Agents

Ovalbumin (OA, Seikagaku Kogyo, Tokyo, Japan), acetylcholine chloride (Ach, Nacalai Tesque, Kyoto, Japan), bovine serum albumin (BSA; Seikagaku Kogyo), Türk's solution (Wako Pure Chemical Industries, Osaka, Japan), pancuronium bromide (Sigma, St. Louis,

Mo., USA), pentobarbital sodium (Abbott Lab., Chicago, Ill., USA), disodium ethylenediaminetetraacetic acid (EDTA-2Na, Nacalai Tesque), Diff-Quick solution (International Reagent, Kobe, Japan), monoclonal anti-mouse IgE antibody (LO-ME-3, Serotek, Oxford, UK) and peroxidase-conjugated streptavidin (Dakopatts, Glostrup, Denmark) were purchased commercially.

Protocol for Antigen Exposure

Mice were actively sensitized by intraperitoneal injections of 50 µg OA with 1 mg alum on days 0 and 12. Starting on day 22, they were exposed to aerosolized OA (1% w/v diluted in sterile saline) for 30 min, 3 times every 4th day according to a previously reported method [17]. Control animals were exposed to saline in a similar manner. In naive control animals, saline was first injected, and then they were exposed to vaporized saline in a similar manner. The aerosol (particle size: 2.0–6.0 µm) was generated by a nebulizer (Ultrasonic nebulizer TUR-3200, Nihon Koden, Tokyo, Japan) by filling a perspex cylinder chamber (diameter 5.5 cm, height 12 cm) with a vaporized solution. To examine the effect of LTD₄, animals were exposed to vaporized LTD₄ solution. From the several preliminary experiments, mice were exposed to 10 ng/ml LTD₄ saline solution for 30 min after each exposure of antigen. When sensitized mice were challenged with aerosolized antigen, 60–100 pg/ml LTD₄ was detected in bronchoalveolar lavage fluid (BALF). Similar amounts of LTD₄ were detected in the BALF after the inhalation of 10 ng/ml LTD₄ for 30 min. This was the reason for determining the concentration of LTD₄ in the present experiments.

Measurement of Airway Response to Ach

Measurement of bronchial responsiveness to Ach was carried out according to previously described modified methods [17]. Briefly, to measure bronchial responsiveness to Ach, mice were anesthetized with sodium pentobarbitone (80 mg/kg *i.p.*) and the jugular vein was cannulated for intravenous injection of Ach. Mice were injected with pancuronium bromide (0.1 mg/kg, *i.v.*) to suppress spontaneous respiration, and ventilated with a rodent ventilator (New England Medical Instruments, Medway, Mass., USA) with oxygen-supplemented air at 60 strokes/min at a stroke volume of 0.6 ml/animal. Bronchoconstriction was measured according to the overflow method, using a bronchospasm transducer (Ugo Basil 7020, Milano, Italy) connected to the tracheal cannula. To measure bronchial responsiveness to Ach, changes in respiratory overflow volume were measured using increasing doses of Ach. The increase in the respiratory overflow volume induced by Ach was represented as a percentage of the maximal overflow volume (100%) obtained by clamping the tracheal cannula. The area under the curve (AUC) was calculated from the dose-response curve data for Ach (range: 31.25–2,000 µg/kg) [18].

BAL Study

To measure airway inflammation, we studied the accumulation of inflammatory cells in BALF. Experiments were carried out according to previously described methods [19]. Briefly, 24 h after the last inhalation of antigen (30 days after the first immunization), animals were sacrificed with an intraperitoneal injection of sodium pentobarbitone (100 mg/kg). The trachea was cannulated and the air lumen was washed 4 times with 1 ml of calcium- and magnesium-free phosphate-buffered saline containing 0.1% BSA and 0.05 mM EDTA-2Na, and the procedure was repeated 3 times (total volumes: 3 ml, recovery >85%). BALF from each animal was collected in a plastic tube, cooled on ice, and centrifuged (150 g) at 4 °C for 10 min. Cell

pellets were resuspended in the same buffer (1 ml). BALF was stained with Türk's solution and the number of nucleated cells was counted in a Bürker chamber. A differential count was made on a smear prepared with a cytocentrifuge (Cytospine II, Shandon, UK), and stained with Diff-Quik solution (based on standard morphological criteria) of at least 300 cells (magnification $\times 500$). The amount of IL-5 in BALF was measured using enzyme-linked immunosorbent assay (ELISA; Endogen, Cambridge, Mass., USA). In our preliminary experiments studying the correlation between the number of BALF cells and lung cells, a good correlation between the number of cells at 24 h after the antigen challenge was indicated. Therefore, we counted the number of cells in BALF 24 h after the antigen provocation.

Histological Study

The mice were sacrificed 24 h after the third antigen inhalation, and whole lungs were distended by injection of 10% buffered formalin via the trachea, removed, and immersed in the same fixative with the trachea clamped for 24 h. The tissue was sliced and embedded in paraffin, and 6- μ m sections were stained with hematoxylin and eosin for light microscopy examination.

Measurement of Immunoglobulins

Immediately before the last antigen challenge, blood was collected, and each serum was obtained by centrifugation and stored at -80°C . Antigen-specific IgE in mouse serum was measured using the ELISA by the method previously described [18]. Briefly, serum OA-specific IgE was measured by coating the plate with monoclonal rat anti-mouse IgE antibody (LO-ME-3) at a concentration of 5 $\mu\text{g}/\text{ml}$. After blocking with 1% BSA, serum dilutions were incubated for 1 h followed by biotinylated OA and peroxidase-conjugated streptavidin. Serially diluted monoclonal anti-OA IgE was used as a standard. Optical densities of the enzymatic reactions were read using an automatic ELISA plate reader (Titertek Multiscan MCC/340, Lab Systems Oy, Helsinki, Finland) at 450 nm (reference 690 nm). The detection limit was 10 ng/ml.

Statistical Analysis

Values are presented as the mean $\pm$ SEM. The significance between saline-inhaled and OA-inhaled animals was estimated using Student's *t* test or the Aspin-Welch test. To define statistically significant differences among the groups, data were subjected to one-way analysis of variance (ANOVA) followed by Dunnett's multiple range test. *p* values less than 0.05 were considered significant.

Results

Airway-Responsiveness to Ach after Repeated Antigen Challenge and the Effect of LTD₄ Inhalation

The effect of 5-LO gene deficiency on antigen-induced AHR is indicated in figure 1A. Inhaling antigen 3 times caused a significant increase in AUC in wild-type mice but not in 5-LO KO mice. Table 1 indicates the concentrations of Ach that caused 40% narrowing of airways (PC40) in each group. A marked decrease of PC40 was observed in wild-type mice but not in 5-LO KO mice by

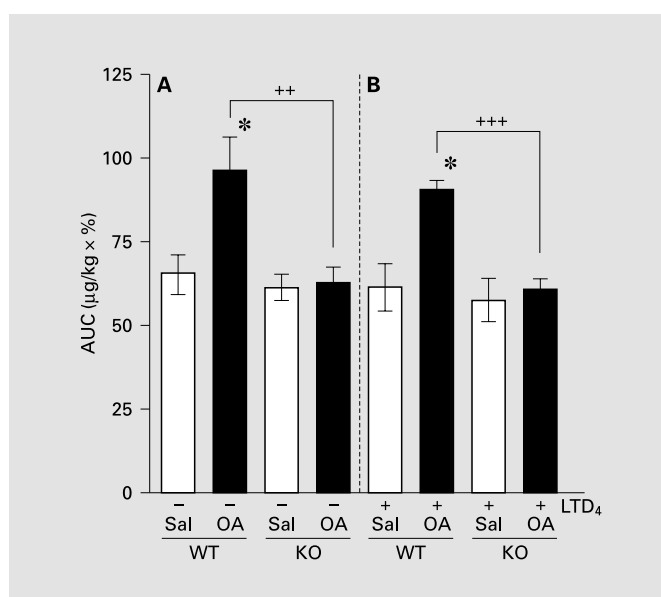


Fig. 1. Airway responsiveness to Ach in wild-type (WT; 129 Sv) and 5-LO KO mice. Twenty-four hours after the final antigen challenge, bronchial responsiveness to Ach was assessed. LTD₄ was inhaled 3 times during each antigen challenge. Each column represents the mean $\pm$ SEM of 6–9 animals. **A** Saline inhalation. **B** LTD₄ inhalation. * *p* < 0.05 from saline-treated animals; ++ *p* < 0.01, +++ *p* < 0.005 from wild-type animals. Sal = Saline.

Table 1. Average concentration of Ach for causing 40% contraction of the maximum contraction (PC40; $\mu\text{g}/\text{ml}$)

	Inhalation	
	saline	LTD ₄
Wild-type		
Saline	225	226
OA	90	95
5-LO KO		
Saline	228	228
OA	230	231

the provocation with antigen. The effect of LTD₄ inhalation is shown in figure 1B and table 1. The inhalation of LTD₄ did not affect the AUC of wild-type and 5-LO KO mice which were treated with saline or inhaled antigen. In addition, as indicated in table 1, the PC40 of each group was not altered by the inhalation of LTD₄. These findings indicate that the 5-LO gene deficiency caused the loss of antigen-induced AHR in mice, but AHR was not affected by the inhalation of LTD₄.

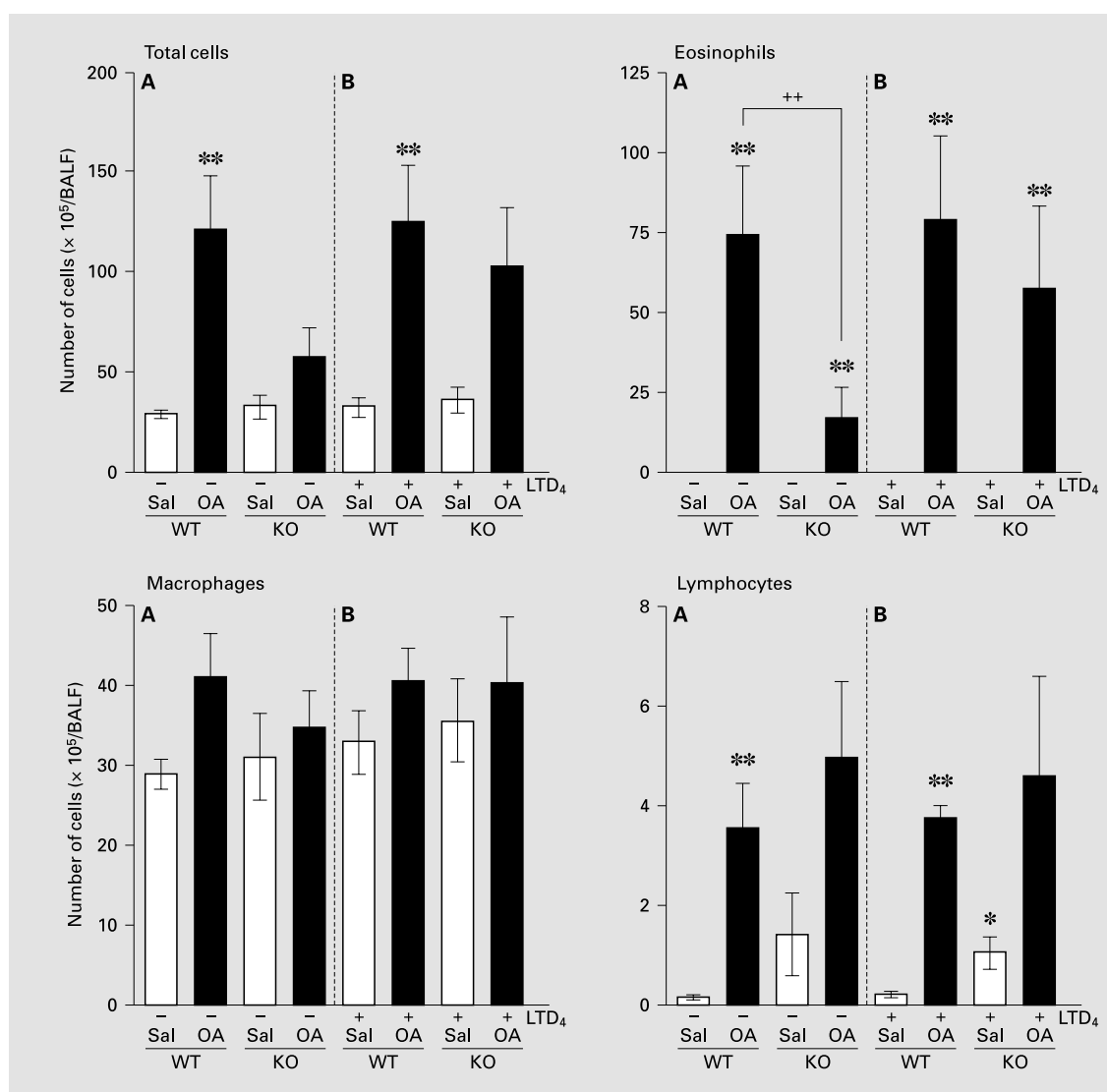


Fig. 2. Antigen-induced increases in the number of inflammatory cells in BALF from wild-type (WT) and 5-LO KO mice. Mice were immunized by injection with OA and vaporized antigen was inhaled 3 times. LTD₄ was inhaled 3 times during each antigen challenge. BALF was obtained 24 h after the final antigen challenge. Each column represents the mean $\pm$ SEM of 6–9 animals. **A** Saline inhalation. **B** LTD₄ inhalation. * $p < 0.05$; ** $p < 0.01$ from saline-treated animals; ++ $p < 0.01$ from wild-type animals. Sal = Saline.

Antigen-Induced Airway Inflammation

To measure the magnitude of the airway inflammation, the changes in the number of inflammatory cells and cytokine (IL-4 and IL-5) levels in BALF and the histopathological changes in the lungs were examined. The effect of 5-LO gene deficiency is shown in figure 2A. The total number of leukocytes, eosinophils and lymphocytes in BALF increased significantly after three antigen inhalations in wild-type mice. The magnitude of the increase in

eosinophilia in 5-LO KO mice was, however, significantly lower than that in wild-type mice. The antigen-treated wild-type and 5-LO KO mice showed a tendency to have an increased number of macrophages. The effect of LTD₄ inhalation on the changing of inflammatory cells in BALF is shown in figure 2B. LTD₄ did not cause any airway eosinophilia in saline-treated wild-type and 5-LO KO mice. After the inhalation of LTD₄, the attenuated eosinophilia in antigen-treated 5-LO KO mice was recovered.

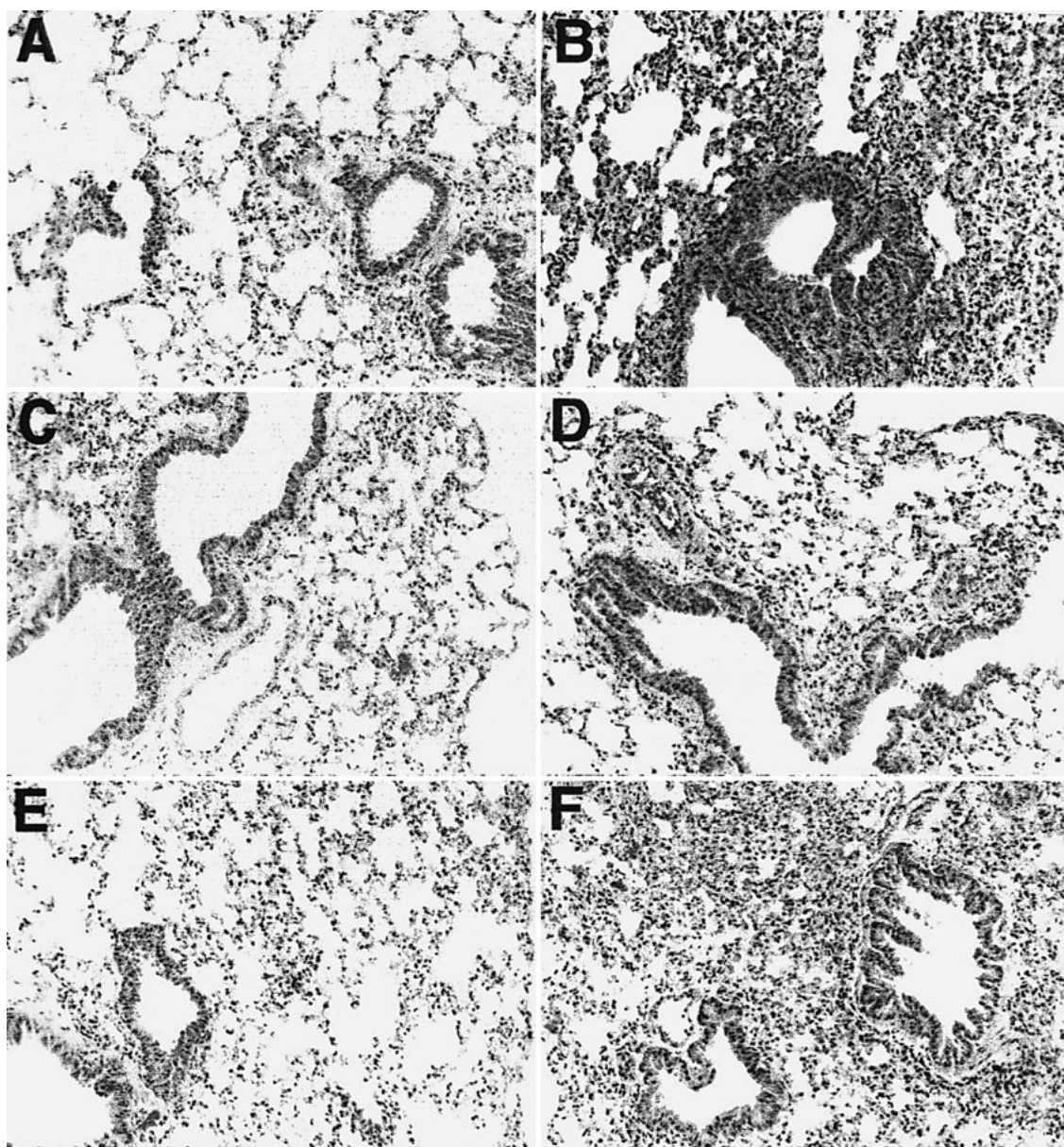


Fig. 3. Histological analysis of lung sections from wild-type mice (**A, B**) and 5-LO KO mice (**C–F**) 24 h after antigen challenge. Twenty-four hours after the final antigen challenge, the whole lungs were distended by injection of 10% buffered formalin via the trachea, removed and immersed for 24 h. Tissue was sliced and embedded in paraffin, and 6- μ m sections were stained with hematoxylin and eosin ($\times 50$). **A, C, E** Group injected with saline and exposed to saline. **B, D, F** Group injected with OA and having inhaled OA. **E, F** LTD₄ inhalation group. **A, C, E** Normal features. **B, D, F** After inhalation of OA, inflammatory infiltrates, especially eosinophils and mononuclear cells, are present around the peribronchial and perivascular region in wild-type and 5-LO KO mice; the magnitude of eosinophil infiltration in **F** is greater than in **D**.

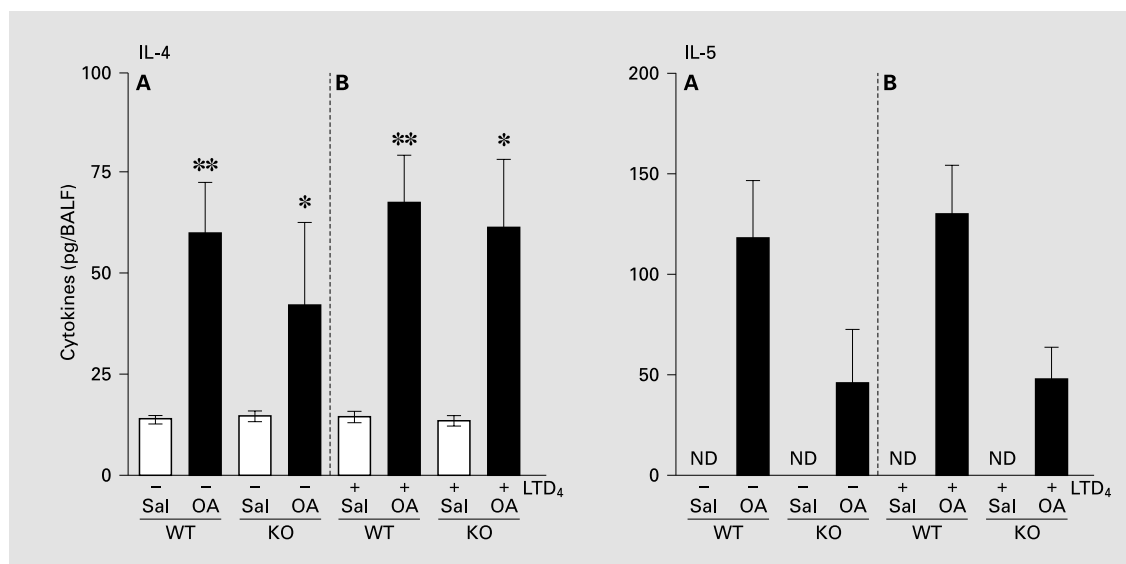


Fig. 4. Antigen-induced increases in the level of cytokines in BALF from wild-type (WT) and 5-LO KO mice. Mice were immunized by injection with OA and vaporized antigen was inhaled 3 times. LTD₄ was inhaled 3 times during each antigen challenge. BALF was obtained 24 h after the final antigen challenge. Each cytokine was measured by an ELISA. Values are means $\pm$ SEM of 6–7 animals. **A** Saline inhalation. **B** LTD₄ inhalation. * $p < 0.05$; ** $p < 0.01$ from saline-treated animals. Sal = Saline.

Histological changes in the lungs are shown in figure 3. In histological sections of lung tissue from wild-type mice which inhaled OA, severe pulmonary eosinophilic inflammation was observed (fig. 3B). The severity of eosinophilia in 5-LO KO mice was clearly lower when compared with that in wild-type mice (fig. 3D). Infiltrating eosinophils and mononuclear cells were mainly seen in the peribronchial and perivascular regions of the lungs. When LTD₄ was inhaled, the total number of leukocytes, eosinophils, macrophages and lymphocytes was not altered in the saline-treated group (fig. 3E). In 5-LO KO mice which inhaled antigen, the number of eosinophils increased after LTD₄ treatment (fig. 3F).

The levels of IL-4 and IL-5 in BALF are shown in figure 4. The effect of 5-LO gene deficiency is shown in figure 4A and the effect of LTD₄ is indicated in figure 4B. Both IL-4 and IL-5 increased significantly in wild-type and 5-LO KO mice after the inhalation of the antigen. The level of IL-5 showed a tendency to decrease by the depletion of the 5-LO gene.

Serum Immunoglobulin Levels

To measure the serum immunoglobulin level, serum was obtained from mice on day 30, immediately before the final antigen challenge. Significant increases in anti-

gen-specific IgE were observed in both wild-type and 5-LO KO mice (fig. 5). The elevation in antigen-specific IgE was slightly lower in the 5-LO KO mice. As for total IgG and total IgE serum levels, a significant elevation in each immunoglobulin was observed in wild-type mice after antigen provocation (data not shown). The 5-LO gene deficiency and LTD₄ inhalation had no effect on the increase of each serum immunoglobulin.

Discussion

In the present study, we demonstrated an important role of 5-LO during the onset of antigen-induced airway eosinophilic inflammation and AHR. The 5-LO gene deficiency clearly decreased the antigen-induced AHR and airway eosinophilia in mice. However, LTD₄ inhalation did not affect the airway responsiveness to Ach and the number of inflammatory cells in BALF in saline-treated wild-type mice. In contrast, LTD₄ inhalation accelerated the accumulation of eosinophils in the BALF after the antigen inhalation in 5-LO KO mice but not in wild-type mice. These findings suggest that the 5-LO and LTD₄ have an important role for the onset of allergic airway inflammation. Since inhaled LTD₄ has a small effect

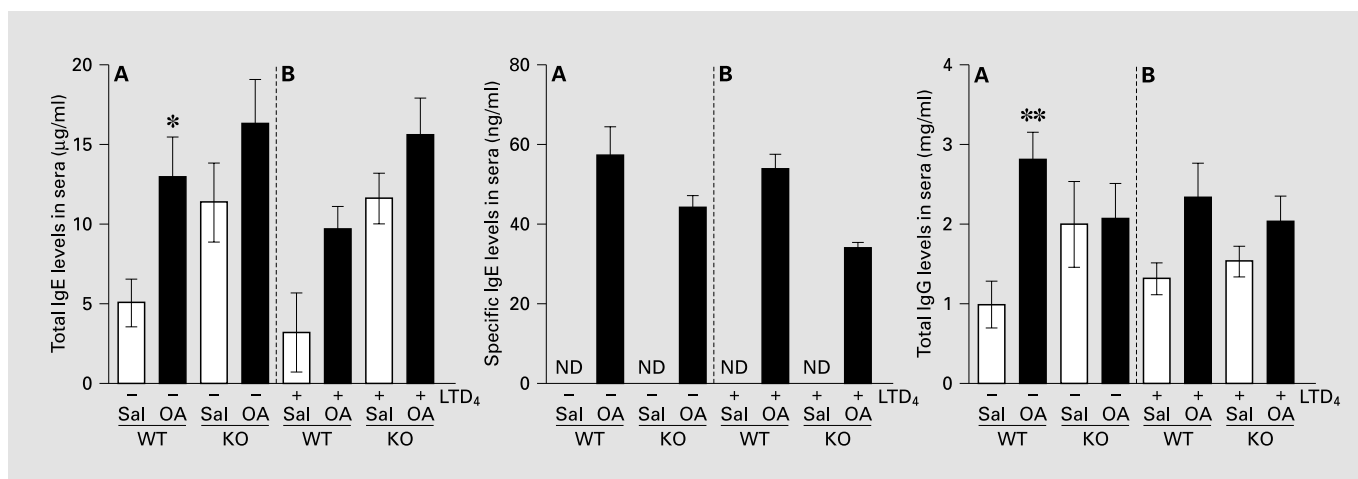


Fig. 5. Changes in serum IgE and IgG levels in wild-type (WT) and 5-LO KO mice. Immediately before the final antigen challenge, serum was obtained. Each immunoglobulin level was measured by an ELISA. Values are the means $\pm$ SEM of 6–7 animals. **A** Saline inhalation. **B** LTD₄ inhalation. * $p < 0.05$; ** $p < 0.01$ from saline-treated animals. Sal = Saline.

in the allergic AHR in mice, the role of intrinsic LTD₄ has to be investigated. In our previous studies employing this system, three (ONO-1078, BAY-7195 and ICI-204,219) of five LT antagonists showed a clear inhibition of AHR. But two of them are ineffective. More extensive experiments will be necessary to confirm the role of LTs in AHR.

Recently, Irvin et al. [16] reported that genetic deficiency of 5-LO was effective in suppressing antigen-induced AHR and airway eosinophilia. The present study confirms their findings. In addition, many studies have reported the efficacy of some 5-LO inhibitors on allergic AHR and eosinophilia in humans and mice [20–23]. These results indicate the importance of 5-LO products for the onset of AHR and airway eosinophilia. However, despite extensive studies, what kind of 5-LO product is important for the onset of allergic inflammation and AHR remains obscure.

Regarding the role of 5-LO products in the airway eosinophilic inflammation, there are many studies reporting the participation of LTs [4, 11–13]. Three different kinds of experiments could support the above findings. The first involves examining the effect of 5-LO gene depletion, the second involves testing the effect of inhalation of aerosolized LTs, and the third involves investigating the effect of 5-LO inhibitors or LT antagonists on allergic airway inflammation.

In experiments employing 5-LO gene-deficient mice, we confirmed Irvin's previous findings [16] indicating the lack of allergic airway eosinophilia in 5-LO KO mice. The present experiments using vaporized LTD₄ also suggested the participation of LTD₄ in the onset of allergic eosinophilia. Underwood et al. [24] demonstrated that aerosolized LTD₄ induced airway eosinophilia in guinea pigs but not in rats. Mulder et al. [25] also reported no significant effect of inhaled LTD₄ on airway eosinophilia in mild asthmatic patients. These findings indicate species differences for the inhaled LTD₄ effect. The present findings indicate no role of inhaled LTD₄ in causing eosinophilia in wild-type saline-treated mice but a marked effect in allergic 5-LO KO mice. These findings suggest a participation of LTD₄ and other allergic mediators in allergic airway eosinophilia in mice. Although the participation is partial, the role of LTs in the airway eosinophilia is supported by the pharmacological efficacy of anti-LT agents on allergic airway eosinophilia [26–30]. In these pharmacological studies, the efficacy of both LTD₄ and LTB₄ antagonists was reported. The role of LTB₄ in addition to LTD₄ has to be examined in future studies.

With respect to the role of the 5-LO during the onset of AHR, the present findings indicate the importance of 5-LO. As shown in the results, allergic AHR was diminished by 5-LO gene depletion, but LTD₄ inhalation did not affect the airway response in any groups of mice examined. Irvin et al. [16] also reported the ablation of

allergic AHR in 5-LO gene-deficient mice. There are some findings that indicate the suppression of the onset of an allergic bronchial response by 5-LO inhibitors in humans and experimental animals [4, 20–23]. However, the effect of LT receptor antagonists on the allergic AHR is inconsistent. Some studies reported the efficacy of LTD₄ antagonists on AHR [11, 23, 28], whereas another one did not [15]. Similar results were obtained in our laboratory. These contradictions may be due to the different experimental conditions and different drug potencies. In addition, there has been no opportunity to clarify the potency of LTD₄ inhalation during the onset of AHR in mice. However, inhaled LTD₄ did not cause AHR in humans [25]. These previous findings and present observations suggest a small role for extrinsic LTD₄ during the onset of AHR. At present, there is some evidence to suggest the effectiveness of LTD₄ antagonists on nonallergic AHR

including tobacco, aspirin or bacterial lipopolysaccharide-induced AHR [31–35]. Therefore, LTD₄ may play an important role in the onset of nonallergic AHR. Further experiments will be necessary to confirm the role of LTs in the onset of AHR.

In addition, the present results suggest the low possibility of the participation of 5-LO products in the changes of immunological molecules including T helper 2 cytokines and immunoglobulins. An antigen-induced increase of IL-4 and IL-5 in BALF and serum immunoglobulins was not affected by 5-LO gene depletion and LTD₄ inhalation. These findings indicate the low participation of 5-LO products in immunological phenomena.

In conclusion, the present findings indicate that 5-LO plays an important role in the development of allergic AHR and airway eosinophilia in mice. In addition, LTD₄ plays an important role in airway eosinophilia.

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