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Original Article

Late airway obstruction and neutrophil infiltration in sensitized mice after antigen provocation were suppressed by selective and non-selective phosphodiesterase inhibitors

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Abstract

Suppression of antigen-induced late airway obstruction associated with neutrophilic inflammation by selective and non-selective phosphodiesterase (PDE) inhibitors was investigated in mice. Respiratory resistance (Rrs) increased in sensitized BDF1 mice 4–6 h after antigen provocation, whereas no obvious immediate reaction was observed. This reaction was associated with marked airway neutrophilia without significant infiltration of eosinophils. A selective PDE IV inhibitor, T-440 (10–30 mg/kg), and a non-selective PDE inhibitor, theophylline (10 mg/kg), significantly inhibited airway obstruction and neutrophilia when administered orally. An anti-allergic drug, ketotifen (1 mg/kg), caused slight inhibition of airway obstruction, whereas it did not affect airway neutrophilia. These results suggest that neutrophilic inflammation plays a role in the airway obstructive reaction and that PDE has a regulatory role in obstructive airway disease associated with airway inflammation.

Key words: airway inflammation, airway obstruction, mouse, neutrophil, phosphodiesterase inhibitor

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Introduction

Accumulation of neutrophils in the tissue is a characteristic feature of inflammatory disease. In some obstructive airway diseases associated with airway inflammation, such as asthma and chronic obstructive pulmonary disease (COPD), large numbers of neutrophils can be detected in the bronchial mucosa or washings.[1–4] Therefore, a possible regulatory role of neutrophilic inflammation in obstructive airway disease is suggested.

Nevertheless, especially in asthma, locally accumulated and activated eosinophils play a central role in late-phase airway obstruction. Antigen provocation in asthmatic patients increases the number of eosinophils in bronchoalveolar lavage fluid (BALF),[5,6] sputum[7] and peripheral blood.[8] The concentration of eosinophil granule protein in the sputum of asthmatic patients was correlated with the degree of airway obstruction.[9] Eosinophils produce eicosanoids derived from the 5- and 15-lipoxygenase pathways, especially leukotriene (LT) C_4/LTD_4, which shows potent bronchoconstrictor activity.[10,11]

Many animal models have been developed to confirm the role of eosinophils in producing antigen-induced late-phase airway obstruction. In sheep and rabbits, antigen challenge caused early and late bronchoconstriction, the latter event being associated with the influx of eosinophils into the bronchial lumen.[12,13] In guinea pigs, antigen challenge produced a late

bronchoconstrictor response with prominent infiltration of eosinophils into the airways.[14,15] However, results from most current models are not sufficient to confirm whether late airway obstruction is mediated by eosinophils alone, because other inflammatory cells, especially neutrophils, are also observed in the airway lumen at the time of late airway obstruction. As eosinophils undoubtedly play a central role in inducing late airway obstruction, the question remains as to whether neutrophil infiltration contributes to obstruction of the airway or not.

The animal model we developed and used in the present study may answer this question. Antigen provocation in sensitized mice produced delayed airway obstruction associated with marked infiltration of neutrophils, but not eosinophils, into the airway.

It has been reported that neutrophil functions are modulated by the intracellular cyclic nucleotide level. An increase in intracellular cAMP levels in neutrophils is associated with a decrease in several neutrophil functions, including chemotaxis, respiratory burst and lysosomal enzyme release.[16–18] Intracellular cAMP levels of neutrophils are regulated by enzyme phosphodiesterases (PDE). Currently, at least seven different PDE isozyme gene families are recognized in many types of cells.[19–21] In particular, the PDE isozyme responsible for hydrolyzing cAMP in neutrophils has been reported to be predominantly of the cAMP-specific type (PDE IV) and the neutrophil respiratory burst was inhibited by the PDE IV inhibitors rolipram and Ro 20–1724.[22]

Therefore, the present study was performed to define the role of neutrophils in the development of airway obstruction by means of pharmacological modulation using a selective PDE IV inhibitor (T-440) and a non-selective PDE inhibitor (theophylline). The effect of the anti-allergic drug ketotifen, which has been reported to prevent late airway obstruction in guinea pigs[23] and rats,[24] was also investigated.

METHODS

Materials

Ovalbumin (Sigma Chemical Co., St Louis, MO, USA), sodium pentobarbital (Dainabot, North Chicago, IL, USA), complete Freund's adjuvant (CFA; Difco, Detroit, MI, USA), Tween 80 (Nacalai Tesque, Kyoto, Japan), theophylline (Sigma Chemical Co.), ketotifen (Sandoz Pharmaceutical Co., Tokyo, Japan), Diffu-gen™ RID plate (Tago, Burlingame, CA, USA) and a mouse IgE enzyme immunoassay (EIA) kit (Yamasa, Tokyo, Japan) were purchased. T-440 was synthesized by the Lead Optimization Research Laboratory, Tanabe Seiyaku Co. (Osaka, Japan).

Sensitization

BDF1 mice (25–35 g, Japan KBL) were immunized by injecting 10 μg ovalbumin emulsified with 200 μL CFA (50%) four times every other week. The first injection was given into both sides of the foot pad and the other injections were given intraperitoneally. Ten days after the last immunization, total IgE, IgG_1, IgG_{2a} and IgG_{2b} levels in the serum were measured using the mouse IgE EIA kit and the Diffu-gen™ RID plates according to the manufacturers' directions. In a separate experiment, these animals were challenged with inhaled antigen.

Drug administration and antigen challenge

All test compounds were dissolved or suspended in distilled water with 1% Tween 80. Vehicle and these drugs were orally administered twice, at 30 min before and at 2 h after the antigen challenge. Saline or ovalbumin solution (10%) were aerosolized with a pressure nebulizer (Pulmo-Aide 5650D; Devilbiss, PA, USA) which generates an aerosol with a median diameter of 5 μm. The output of the nebulizer was 16 L/min. Aerosol from the nebulizer was directed into an animal chamber (30 × 30 × 30 cm). Animals were challenged by exposure to the aerosol for 20 min.

Measurement of respiratory function

To analyze the pulmonary mechanics, Rrs was measured by a forced oscillation technique according to the method described by Iijima *et al.*[25] and Arima *et al.*[26] In brief, the mouse was placed inside a body box and a 30 Hz sine wave oscillation (peak to peak, 2 cmH_2O) was applied to its body surface. Oscillating pressure was obtained with a 10 cm loudspeaker driven by a sine wave generator and a power amplifier. Body box pressure was measured by a flow-resistant tube (TV-241T; Nihon Koden, Tokyo, Japan) and a differential pressure transducer (TP-602T; Nihon Koden). A plastic mask connected to the flow-resistant tube was snugly applied to the face. The respiratory volume of each animal was monitored with the same transducer. The Rrs was calculated as the ratio of body box pressure to respiratory volume and was expressed as the mean of four

continuous respirations. Measurements of Rrs were made before and after administration of drugs and 5 min and 2, 4, 5, 6 and 24 h after antigen challenge. The peak late increase in Rrs was defined as the maximum percentage increase in Rrs between 4 and 6 h after challenge for each animal.

Bronchoalveolar lavage and histologic examination

Saline- or ovalbumin-challenged mice were killed by intraperitoneal administration of an excess dose of sodium pentobarbital 6 h after challenge. The trachea was cannulated with a polyethylene tube through which the lungs were lavaged with 0.5 mL Hank's balanced salt solution (HBSS) four times (2 mL total). Bronchoalveolar lavage fluid was centrifuged at 500 g for 5 min. The pellet obtained was immediately suspended in 250 μL HBSS and the total cell number in BALF was counted by an automatic cell counter (Celltac MEK-5158; Nihon Koden). Differentiation of the cells was conducted by microscopy using centrifuged preparations stained with May-Giemsa, counting 200 cells in each animal. For histopathologic examination, the lungs of other mice were fixed by intratracheal instillation of 10% neutral-buffered formalin at a distending pressure of 15 cmH_2O followed by external fixation in 10% neutral-buffered formalin for 1 week. After that, tissues were embedded in paraffin, sectioned at 4–5 μm and stained with hematoxylin–eosin (H&E).

Statistics

All data are presented as the mean±SEM. Statistical analysis was performed by the Student's *t*-test for comparison between two groups and by one-way analysis of variance and Bonferroni's method for three groups or more. Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

Time course of airway obstruction after antigen challenge

In the sensitization group, all the serum levels of IgE, IgG_1, IgG_{2a} and IgG_{2b} were significantly elevated (Table 1). There was no significant difference in baseline Rrs between the groups challenged with saline (210±14 cmH_2O/L per s; $n = 8$) and ovalbumin (230±17 cmH_2O/L per s; $n = 14$). The percentage changes in Rrs following challenge with saline or ovalbumin are shown in Fig. 1. Although Rrs did not change at all 5 min after challenge, an obvious increase in Rrs was observed at 2 h. This reaction reached a maximum and was statistically significant at 4–6 h. When Rrs increased, mice exhibited apparent signs of dyspnea, such as labored respiration, panting and a decrease in body temperature. The Rrs returned to baseline levels by 24 h after challenge (Fig. 1).

Histologic examination

A representative photomicrograph of the lung from a control mouse is shown in Fig. 2a. The bronchial mucosal surface remained smooth and neither smooth

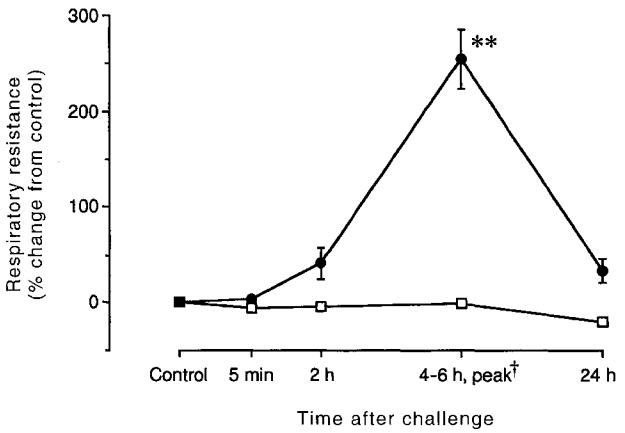

Fig. 1 Antigen-induced late airway obstruction in sensitized mice. Per cent changes in respiratory resistance following challenge with saline (□; $n=8$) or ovalbumin (●; $n=14$) are shown. $^{**}P<0.01$ compared with saline-challenged mice (Student's *t*-test). †Peak value between 4 and 6 h after challenge.

Table 1. Serum levels of IgE, IgG_1, IgG_{2a} and IgG_{2b} in ovalbumin-sensitized mice

	Immunoglobulin concentration	
	Non-sensitized	Sensitized
IgE (μg/mL)	0.10±0.03	5.60±2.20**
IgG_1 (mg/mL)	0.37±0.06	11.00±0.68**
IgG_{2a} (mg/mL)	0.22±0.01	3.20±0.34**
IgG_{2b} (mg/mL)	0.07±0.01	0.56±0.04**

BDF1 mice were sensitized by injecting 10 μg/mL ovalbumin emulsified with 200 μL complete Freund's adjuvant (50%) four times every other week. Ten days after sensitization, serum levels of total IgE, IgG_1, IgG_{2a} and IgG_{2b} in mice were measured ($n=5$). $^{**}P<0.01$ compared with non-sensitized mice.

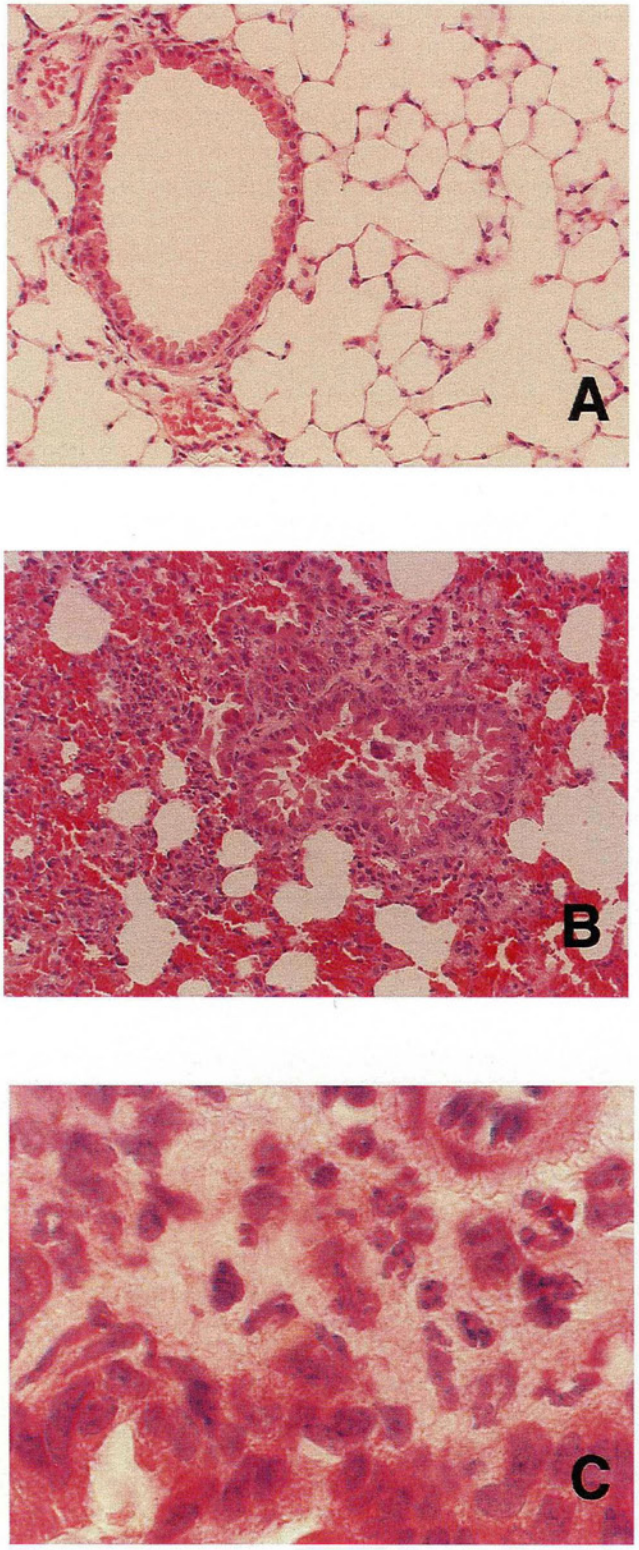

Fig. 2 Antigen-induced airway neutrophilia in sensitized mice. Representative photomicrographs of the lung from mice 6 h after challenge with (a) saline or (b,c) ovalbumin are shown. Original magnification ×200 (a,b), ×1000 (c); H&E stain.

muscle contraction nor inflammatory cell infiltration were observed. In contrast, submucosal and peribronchial edema and marked hemorrhage were noted 6 h after challenge (Fig. 2b,c). There was no obvious bronchial smooth muscle contraction, while the bronchial lumen was plugged with exudate and red blood cells. A large number of neutrophils infiltrated the bronchial wall and peribronchial tissue, whereas eosinophils were not observed in any of the tissues (Fig. 2b,c). These histopathologic findings coincided with the findings of BAL examination (Table 1).

Effects of test compounds

The effects of T-440, theophylline and ketotifen on antigen-induced airway obstruction and infiltration of inflammatory cells in sensitized mice were examined. Administration of these compounds did not affect baseline Rrs (data not shown). A marked increase in Rrs was observed in control mice 4–6 h after challenge as described above (Table 1). T-440 (10–30 mg/kg) and theophylline (10 mg/kg) significantly inhibited late airway obstruction. Slight but not significant inhibition of this reaction was obtained by the administration of ketotifen at 1 mg/kg (Table 2).

As shown in Table 2, the number of total cells and neutrophils in BALF significantly increased 6 h after challenge. The neutrophil accumulation was a specific reaction as no obvious infiltration of eosinophils was observed and the number of mononuclear cells significantly decreased at that time. This reaction was accompanied by a significant increase in BALF red blood cells ($1.51 \pm 0.22 \times 10^8$ *vs* $0.28 \pm 0.05 \times 10^8$ /BALF in saline-challenged control; $P < 0.01$). Oral administration of T-440 dose-dependently (10–30 mg/kg) inhibited the increase in total cells and neutrophils in BALF. Theophylline also suppressed the number of total cells and neutrophils at 10 mg/kg. Ketotifen did not show any inhibition at 1 mg/kg. None of the test compounds affected the eosinophil and mononuclear cell number (Table 2).

Discussion

The present study clearly demonstrates that late airway obstruction accompanied by marked infiltration of neutrophils is induced by antigen provocation in sensitized mice. It is surprising that eosinophils, being scarcely observed in the airway when Rrs increased, did

Table 2. Effects of T-440, theophylline and ketotifen on antigen-induced inflammatory cell infiltration in bronchoalveolar lavage fluid and late airway obstruction in sensitized mice

Challenge	Drug*	Dose (mg/kg)	Rrs (%)†	n	Cell number (×10⁵ /BALF)‡ Total cells	Neutrophils	Eosinophils	Mononuclear cells	n
Saline	–	–	−1.8±4.2§§	6	1.64±0.13§§	0.02±0.00§§	0.00±0.00	1.61±0.11§§	14
Ovalbumin	–		250±31	14	4.83±0.55	4.25±0.47	0.06±0.02	0.38±0.02	14
	T-440	10	130±20§§	7	3.31±0.32§	2.89±0.28§	0.02±0.01	0.36±0.03	14
		30	82±22§§	7	3.09±0.36§§	2.59±0.27§§	0.02±0.00	0.36±0.02	14
	Theophylline	10	87±20§§	7	3.64±0.42	3.10±0.31§	0.06±0.01	0.31±0.03	14
	Ketotifen	1	180±54	4	5.30±0.75	4.55±0.64	0.10±0.03	0.47±0.04	14

*Each test compound was administered orally 30 min before and 2 h after antigen challenge.

†Maximum per cent increase in respiratory resistance (Rrs) betweem 4 and 6 h after the antigen challenge are shown.

‡Numbers of total cells, neutrophils, eosinophils and mononuclear cells in bronchoalveolar lavage fluid (BALF) 6 h after antigen challenge are shown.

§$P<0.05$, §§$P<0.01$ compared with ovalbumin-challenged control (Bonferroni's method).

not seem to have much effect on airway obstruction. Therefore, our model seems to be very convenient for analyzing the role of neutrophils in airway obstruction associated with inflammation.

In the present study we used CFA as an adjuvant for sensitization. It has been reported that the predominant immunoglobulin synthesized in such animals is IgG rather than IgE.[27] However, the serum IgE level was clearly elevated along with obvious increases in IgG_1, IgG_{2a} and IgG_{2b} in our model. Repeated long-term sensitization may induce IgE synthesis. Kurup *et al.* have reported that the serum level of IgE, as well as IgG_1, was significantly elevated when mice were sensitized with alum.[28] These facts suggest that the humoral reactions that occurred in mice after antigen provocation were essentially the same in both models. However, potent eosinophilic inflammation was observed in the lung after antigen provocation in alum-sensitized mice.[28,29] The reason for the discrepancy is not yet clear, but some possibilities are as follows:

(1) It was reported that depletion of $CD4^+$ T cells completely abrogated eosinophilic inflammation,[30,31] indicating that eosinophil inflammation was essentially dependent on $CD4^+$ T cells. Complete Freund's adjuvant has been reported to direct the Th1 type reaction, whereas alum potentiated the Th2 reaction.[32] Th2-type cytokines, such as interleukin (IL)-4 and IL-5, were key factors in the development of eosinophilic inflammation.[31,33,34] Thus, the lack of eosinophil accumulation in our model may be due to the absence of a Th2 response.

(2) Kennedy *et al.* have reported that eosinophil infiltration in the airway was a reaction with a slow onset, being detected from 24 h after antigen challenge and peaking at 72 h, whereas the peak of neutrophils was at 6–24 h.[29] Therefore, even in our model, eosinophil recruitment may be detected at 24 h or later. Taken together, the eosinophils, which did not exist in the airway when Rrs increased, do not seem to have much effect on airway obstruction in this model.

Lung neutrophilia was accompanied by submucosal and peribronchial edema and marked hemorrhage, suggesting the occurrence of inflammation and tissue damage. Neutrophils are associated with tissue injury in many inflammatory conditions.[35] Some inflammatory diseases related to immune complexes showed antigen-specific chemotaxis and activation of neutrophils.[36] Irvin *et al.* have observed complement-dependent airway hyperreactivity and marked neutrophilia in rabbits.[37] In addition, contributions of neutrophil-derived oxygen metabolites, proteases and cationic materials to tissue injury were also suggested.[35] Therefore, these mechanisms may play a role in antigen-induced airway neutrophilia and injury. Further investigation will be required, for example to determine the effects of anti-inflammatory drugs on the antigen-induced increase in BALF red blood cells.

Immediately after challenge, no significant change in Rrs was observed. It was reported that the airway smooth muscle layer of the mouse is thinner and less sensitive to many types of spasmogen than that of the rat, hamster, guinea pig and rabbit.[38] Additionally, mice do not have respiratory bronchioles, which play an important role in airway obstruction.[39] Therefore, mast cell-derived mediators such as histamine, prostaglandins and leukotrienes do not seem to produce any potent airway smooth muscle contraction in sensitized mice, although mast cells in the bronchial mucosa may be degranulated by antigen challenge through the IgE signaling pathway.

A significant increase in Rrs occurred at 4–6 h after challenge. In chronic airway inflammation, mucosal and submucosal edema and mucus hypersecretion as well as airway smooth muscle contraction can contribute to airway obstruction.[40] In the present study, submucosal edema and occlusion of the bronchial lumen with exudate but no obvious airway smooth muscle contraction were recognized in the lung in accordance with airway obstruction. Therefore, this edematous and exudative change seems to be related to airway obstruction. Examination of the effect of a typical drug causing relaxation of bronchial smooth muscle, such as a β_2-adrenoceptor agonist, may be an effective method for further delineation and this investigation is currently underway.

We have previously reported that T-440 inhibited PDE IV purified from guinea pig lung with an IC_{50} of 0.057 μmol/L, but it did not inhibit PDE I, II, III and V, even at 10 μmol/L.[41] The effects of T-440 and its structurally related compounds on PDE IV activity correlated closely with the inhibition of antigen- and chemical mediator-induced bronchoconstriction *in vivo*.[42] The bioavailability of T-440 in mice is unknown. However, in the present study this drug inhibited airway obstruction and neutrophil infiltration, suggesting that T-440 exerts inhibitory activity on PDE IV at the time when both reactions occurred. A non-selective PDE inhibitor, theophylline, also inhibited airway obstruction and neutrophil infiltration. Phosphodiesterase IV is responsible for hydrolyzing cAMP in neutrophils.[22] In fact, a PDE IV inhibitor[22] and theophylline[43] have been reported to inhibit the activation of neutrophils. Therefore, inhibition of PDE IV activity by T-440 and theophylline may be involved in the suppression of airway neutrophilia. The effects of both drugs on airway obstruction were essentially the same as those on airway neutrophilia, suggesting a possible relationship between airway obstruction and neutrophilic inflammation. As mononuclear cells decreased after antigen challenge, this change was not affected by treatment with any drug. Therefore, the direct role of mononuclear cells in the development of airway obstruction appears to be negligible.

Submucosal edema may contribute to this airway obstruction, as described earlier. Vascular permeability is regulated by cAMP[44] and selective and non-selective PDE inhibitors are reported to inhibit airway microvascular leakage.[45] These facts suggest that the inhibition of airway obstruction by T-440 and theophylline is mediated by additive effects on neutrophil infiltration and submucosal edema. For further analysis of the relationship between neutrophilic inflammation and airway obstruction, additional investigations will be needed, for example to determine the time course of airway edematous change by morphometric analysis.

The suppressive effects of ketotifen on late airway obstruction using guinea pigs[23] and rats[24] have been reported. Our present findings, that ketotifen slightly inhibited late airway obstruction, are consistent with previous reports. In addition, this drug has a potent antagonistic action against histamine H_1-receptors.[46] Histamine is reported to modulate airway vascular permeability,[47] suggesting the possible contribution of histamine to airway obstruction. Ketotifen may attenuate airway obstruction via suppression of histamine-mediated airway submucosal edema. The lack of effect of ketotifen on airway neutrophilia suggests that histamine is not the main mediator of this reaction.

In conclusion, we have developed a unique animal model of late airway obstruction associated with neutrophilic inflammation in mice. T-440 and theophylline inhibited airway obstruction, as well as neutrophil infiltration, suggesting a possible relationship between neutrophilic inflammation and airway obstruction. These results also implicate the possible regulatory role of PDE in obstructive airway disease associated with airway inflammation.

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