



Effects of ciclamilast, a new PDE 4 PDE4 inhibitor, on airway hyperresponsiveness, PDE4D expression and airway inflammation in a murine model of asthma

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Received 12 December 2005; received in revised form 28 April 2006; accepted 4 July 2006

Abstract

PDE4 (phosphodiesterase-4) plays a critical role in pathogenesis of allergic asthma and chronic obstructive pulmonary disease (COPD). PDE4 inhibitors are presently under clinical development for the treatment of asthma and/or COPD. Ciclamilast, a new PDE4 inhibitor, is a piclamilast (RP 73401) structural analogue, but has a more potent inhibitory effect on PDE4 and inflammation in the airway tissues and less side effects than that of piclamilast. In this study, we elucidate primarily on the roles of compound on PDE4 enzyme in physiological and pathological processes in a mouse model of asthma. The sensitized/challenged mice were reexposed to ovalbumin and airway response to inhaled methacholine was monitored. Orally administration of ciclamilast, in a dose-dependent manner, significantly inhibited changes in lung resistance and lung dynamic compliance, as well as upregulation of cAMP-PDE activity, increase of PDE4D mRNA expression, but not PDE4B from lung tissue in the murine model. In addition, the compound dose-dependently reduced mRNA expression of eotaxin, tumor necrosis factor (TNF)- α and interleukin (IL)-4, but slightly increased mRNA expression of interferon (IFN)- γ from lung tissue. Further, levels of eotaxin, TNF- α and IL-4, and eosinophil and neutrophil accumulation in bronchoalveolar lavage fluid were also significantly reduced. Pathological examination, goblet cell hyperplasia and inflammatory cells infiltration in lung tissue were suppressed by treatment with ciclamilast. A significant correlation was observed between the increases in PDE4D mRNA expression and airway hyperresponsiveness. These studies confirm that inhibitory effect of ciclamilast on airway hyperresponsiveness includes its inhibiting PDE4D mRNA expression, down-modulating PDE4 activity, anti-inflammation and anti-mucus hypersecretion, and ciclamilast may have therapeutic potential for the treatment of asthma.

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Keywords: Phosphodiesterase-4; PDE4D; Ciclamilast; Dexamethasone; Inflammation; Cytokines; Goblet cell; Airways hyperresponsiveness; Asthma

1. Introduction

Asthma is a chronic inflammatory disease of the respiratory tract that is tightly associated with airway hyperresponsiveness, increased mucus production, and an infiltration of the bronchial mucosa with inflammatory cells such as mast cells, eosinophils, neutrophils and lymphocytes (CD4⁺ T cells) (National Institutes of Health, 2002). There is an evidence of an altered local T cell

response in favour of T helper (Th)2 cytokine of release interleukin (IL)-4, IL-5 and IL-13 resulting in B cell isotype switching to IgE, mast cell, eosinophil and neutrophil recruitment and production of a wide range of inflammatory mediators (Kay, 2001). The resulting pulmonary inflammation leads to bronchoconstriction and ultimately to airway remodeling (Holtgate et al., 2000).

Phosphodiesterase (PDE) isoenzymes control the degradation of cyclic AMP and thus play key roles in regulating compartmentalized signaling responses to intracellular gradients of cyclic AMP (Houslay and Adams, 2003). The PDE4 family of enzymes, encoded by four PDE4 genes, which generate at least 16 different isoforms, exclusively hydrolyzes cyclic AMP and accounts for most of this activity within cells (Houslay and

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Adams, 2003). Selective inhibitors of PDE4, which are directly against the catalytically active site, inhibit the function of a variety of inflammatory and immunomodulatory cells. PDE4 inhibitors may also be useful as alternatives or adjuncts to glucocorticoids in the treatment of asthma because they exhibit significant anti-inflammatory activity in a variety of in vivo models of allergic pulmonary inflammation (Spina, 2003). The potential value of selective PDE4 inhibitors in the treatment of inflammatory disease of the airways has been demonstrated in clinical studies of their use in asthma and chronic obstructive pulmonary disease (COPD) (Lipworth, 2005). However, whether or not PDE4 is upregulated in blood cells and tissues in asthma and/or allergy remain controversial. Many early clinical investigations reported a significant decrease in cAMP or an impaired beta-adrenergic function and decreased receptor numbers in patients with asthma or allergy as compared with healthy individuals (Koeter et al., 1982; Sato et al., 1990; Polson et al., 1988; Weth et al., 1985). Although some investigations reported a significant increase in PDE4 activity of T lymphocytes and mononuclear leucocytes in patients with asthma or allergy as compared with healthy individuals (Crocker et al., 2000; Hanifin and Chan, 1995; Mue et al., 1976; Sawai et al., 1998), others observed no significant change in PDE4 activity in such patients (Coulson et al., 1989; Gantner et al., 1997; Landells et al., 2000, 2001). There has been no reported study on PDE4 regulation in the lung in any animal model of asthma or in patients with asthma except for a recently current reported rat model (Tang et al., 2005).

Our recent studies have demonstrated that ciclamilast, piclamilast, and rolipram could inhibit the purified human PDE4A activity expressed in yeast cell GL62 in a concentration-dependent manner. Ciclamilast showed higher potency against PDE4A than rolipram and piclamilast, with an IC_{50} value (95% confidence limits) of 1.27 (0.84–1.91) $\mu\text{mol/L}$ compared to 66.4 (33.3–132.2) and 3.73 (2.51–5.53) $\mu\text{mol/L}$ for piclamilast and rolipram, respectively. This result suggested ciclamilast might be a selective PDE4 inhibitor, similar to piclamilast and rolipram (Chen et al., 2004). In the comparison of piclamilast with ciclamilast in bronchodilating and antiallergic effects, ciclamilast showed more potent bronchorelaxant effects in guinea pig airway smooth muscle on resting tension, a synergetic effect on the bronchodilating effect of isoproterenol in vitro, and more potent inhibition of slow reaction substance of anaphylaxis (SRS-A) released from lung tissues of the sensitized guinea pigs and ovalbumin-induced bronchoconstriction (Schultz–Dale reaction) (Ji et al., 2003). To investigate whether or not the cAMP-PDE activity elevation was caused by enhanced PDE4 gene expression, we examined lung mRNA levels of various PDE4 subtypes in normal and allergic rats. The results showed that ovalbumin sensitization and challenge significantly increased the levels of PDE4B, PDE4C, and PDE4D mRNAs, as compared with normal rats. Moreover, these changes were suppressed by ciclamilast, piclamilast, rolipram, and dexamethasone (unpublished data), but ciclamilast had a more potent inhibitory effects than piclamilast. These results strongly suggest that the increased cAMP-PDE activity might be, at least in part, caused by enhanced PDE4 gene expression by antigen sensitization and challenge.

In the side effects, oral administration of ciclamilast in beagle dogs was well tolerated up to 10 mg/kg, but at 12 mg/kg i.g. in one of four dogs a single vomit was observed 3 and 4 h after drug administration, respectively. In the other dogs, slight salivation and chewing were observed. The minimal emetic dose was set at 10 mg/kg i.g. However, oral administration of piclamilast in beagle dogs was not tolerated up to 10 mg/kg, in three of four dogs a single vomit was observed 1 and 2 h after drug administration, respectively. In all dogs, severity salivation and chewing were observed. Above data was kindly provided by Prof. Cong-lin Zuo from Beijing Joinn Drug Research Center.

Ciclamilast [*N*-(3,5-dichloropyrid-4-yl)-3-cyclopentyl-oxy-4-methylbenzamid] is a piclamilast (RP 73401) structurally analogous compound. In the present study, we investigated direct inhibitory effects of ciclamilast on lung function, cAMP-PDE activity and PDE4 subtypes expression of lung tissues, cytokines, inflammation and airway mucus secretion in an asthmatic mouse model.

2. Materials and methods

2.1. Sensitization and treatment

All animal treatments were strictly in accordance with the European Community guidelines for the use of experimental animals and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). Female BALB/c mice (20 ± 2 g, purchased from Shanghai Slac Laboratory Animal Co. Ltd Certificate No. SCXK 2003-0003) were studied in all experiments. All animals were housed in Plexiglas cages and kept on a 12/12 h light–dark cycle in temperature and humidity controlled rooms. Food was withheld 8 h before the experiments, with free access to water. Unless otherwise indicated in the text, standard laboratory food and water were provided ad libitum.

To sensitize the mice, 20 μg ovalbumin (Grade V, Sigma Chemical Co., St. Louis, MO) adsorbed in 2 mg alum aluminium hydroxide adjuvant was subcutaneously injected at footpad, neck, back, and groin on days 0 and 10. Negative (Sham) control mice were injected with saline following the same protocol. To test the treatment effects of ciclamilast and piclamilast (Beijing Joinn Drug Research Center, China) and dexamethasone (Jiangsu Henrui Pharmaceutical Co. Ltd, China), mice (treatment group) were administered with ciclamilast 1, 3 and 10 mg/kg/day or piclamilast 1, 3 and 10 mg/kg/day or dexamethasone 2 mg/kg/day (suspended in 2% hydroxymethylcellulose solution) by gavage 1 h before the antigen challenge daily for 7 consecutive days. Negative (Sham) control mice were treated by gavage with only the vehicle (2% hydroxymethylcellulose solution). After the treatment, all animals were placed in a plastic box and challenged with an antigen via the airways with aerosolized ovalbumin (1% in saline) or control (equal volume saline) in a jet nebulizer (BARI Co. Ltd, Germany) for 30 min, on days 22–28, as previously described (Xie et al., 2003). The doses of ciclamilast were determined by studies of its anti-inflammatory activity in an

antigen-induced airway inflammatory model in rats and by its kinetic studies that showed 5 mg/kg/day ciclamilast produces therapeutically relevant blood levels in rats and dogs (Beijing Joynn Drug Research Center, China, unpublished data). The dose of dexamethasone in this investigation is similar to other investigations of its anti-inflammatory effects (Trifileff et al., 2000). For ciclamilast a dose of 1 mg/kg, was the lowest dose that was effective in inhibiting an inflammatory response.

2.2. Evaluation of lung resistance and dynamic compliance

Lung resistance and lung dynamic compliance were used to evaluate the changes in airway function after intravenously methacholine (Sigma, St. Louis, USA) for provocation. The lung function of anesthetized and tracheostomized mice was assessed as described (Xie et al., 2002). Briefly, each anaesthetized animal was placed supine inside a Plexiglas whole-body plethysmograph. The flow rate was monitored with a Fleisch tube connected to the airway in a pressure transducer. Changes in lung volume were measured by detecting pressure changes in the plethysmographic chamber through a port in the connecting tube with a pressure transducer. To measure pleural pressure, a needle (No. 12) with a multiholed tip was directly inserted into the pleural cavity (between fourth and fifth rib at left thorax) through a port in the connecting tube with a differential pressure transducer. Transpulmonary pressure was calculated as the difference between mouth and pleural pressure. The signal of all pressure transducers were continuously computed (MedLab, Nanjing Biotech Instruments, China) by fitting flow, volume, and pressure to an equation of motion. Cumulative doses-effect curves were thus established and the provocation dose (PD) of methacholine which cumulatively produced a 50% increase in lung resistance (PD₅₀) or a 20% fall in lung dynamic compliance (PD₂₀) was determined for each animal. These values were used as indices of airway responsiveness. Cumulative doses of 0.125–2 mg/kg (i.v. via a cannula is placed in jugular vein) were administered for a period of 2–5 min per dose with lung function measurements taken between each dose (Henry et al., 2002).

2.3. Assay for cAMP-PDE Activity

cAMP-PDE activity was assessed as described in details in our previous study (Tang et al., 2005). Briefly, frozen lungs were thawed and cut into small cubes. Twenty-five milligrams lung tissues were homogenized in ice-cold hypotonic homogenization buffer (30 mmol/L HEPES, 1 mmol/L EDTA, 1 mmol/L β -mercaptoethanol, 2 mmol/L phenylmethyl sulfonyl fluoride and 10 g/l pepstatin A, 0.1% Trion X-100, pH 7.4). The cAMP-PDE assay mixture (200 μ l) in phosphate-buffered saline (PBS, pH 7.4) contained 137 mM NaCl, 2.7 mM KCl, 8.8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 1 mM CaCl₂, 1 mM MgCl₂, 1 μ M cAMP (Sigma, St. Louis, USA) and lung homogenate. The PDE reaction was started by the addition of 10 μ l lung homogenate and was performed at 37 °C for 10 min. The reaction was stopped by boiling the mixture for 3 min. The assay mixture was cooled on ice, followed by centrifugation at 14,650 $\times$ g for 30 min at 4 °C. The amount of cAMP present in the supernatant

was determined by high performance liquid chromatography (HPLC, Hewlett-Packard, Palo Alto, CA) using a standard curve of cAMP. The mobile phase used in this separation consisted of 60% water, 10% methanol and 30% PBS (pH=6.6). The active and inactive forms of each sample (50 μ l) were injected into the HPLC system, respectively. The remnant cAMP was analyzed by a Hypersil ODS 4.0 $\times$ 250 column equilibrated with mobile phase, and separated by isocratic elution for 10 min followed by mobile phases at 0.8 ml/min. The chromatographic profile of each sample was compared with that obtained for standard samples containing cAMP (retention time=7.8 min) at an absorbance of 254 nm, AUF=0.02, by comparing peak areas with a standard curve and used to estimate PDE activity. Protein was determined by Bradford method with bovine serum albumin as the standard. The results were expressed as nmol of PDE activity per milligram of protein.

2.4. Analysis of PDE4B and PDE4D-specific mRNAs

PDE4B and PDE4D-specific mRNA was determined by reverse transcriptase–polymerase chain reaction (RT–PCR) (Jin and Conti, 2002; Singh et al., 2003). Briefly, total RNA was isolated from each tissue using TRIzol Reagent (Invitrogen, Carlsbad, CA). RNA purification and RT–PCR were performed using commercial reagents. First-strand cDNA was generated from 5 μ g of total RNA using oligo-dT to prime the reverse transcription according to the supplied protocol (Invitrogen). The completed first-strand cDNA reaction product (2 μ l) was directly amplified by PCR after the addition of 200 ng of specific primers and 1 U of Taq DNA polymerase (Shanghai Sangon Biological Engineering Technology and Service, China). PDE4B and PDE4D-specific primers for the PCR were as follows: PDE4B forward, 5'-TGGAAATCCTGGCTGCCAT-3' and reverse, 5'-TCCACAGAAGCTGTGTGCT-3'; PDE4D forward, 5'-CGGAAGTGCCTTGATGT ACAAC-3', and reverse, 5'-AGAGGCGTTGTGCTTGTCACAC-3'. Commercially available primers were used to amplify glyceraldehyde phosphate dehydrogenase to serve as control. After the hot start at 94 °C for 10 min, the PCR was run for 35 cycles (94 °C, 30 s; 60 °C, 30 s; and 72 °C, 30 s) and a final extension at 72 °C for 5 min in a gradient thermal cycler PCR machine (Eppendorf, Germany). Aliquots of polymerase chain reaction products were separated by electrophoresis on 1.5% agarose gels and visualized with ethidium bromide, and the PCR product bands were quantified by using UVP Gel Documentation system (UVP, Upland, CA).

2.5. mRNA expression of eotaxin, TNF- α , IL-4 and interferon (IFN)- γ in lung tissue

A coupled RT–PCR was used to semi-quantify the RNA levels in lung tissue (Wong et al., 1994). The 100 mg lung tissues were homogenized and RNA was extracted in 1 ml TRIzol (Gibco, USA) according to the manufacturer's instructions. 1 μ g aliquots of the total RNA sample were reverse transcribed in 25 μ l final volume with Avian Myeloblastosis Virus (AMV) reverse transcriptase first-strand cDNA synthesis kit (Gibco,

USA) according to the manufacturer's instructions. The PCR amplification was performed in the total volume of 25 μ l (1.25 U of LA Taq DNA polymerase; Gibco, USA), 0.2 μ M sense and antisense primers, and 200 μ M dATP, dTTP, dGTP and dCTP. The following oligonucleotide primers (the number in parentheses refers to the nucleotide region of the sequence) were used: specific forward primer for eotaxin, 5'-AGA GGC TGA GAT CCA AGC AG-3' (sense) and specific reverse primer for eotaxin, 5'-CAG ATC TTC TTG CCC AAC CT-3' (antisense), calculated length of amplicon 263 bp; specific forward primer for tumor necrosis factor(TNF)- α , 5'-AAC TAG TGG TGC CAG CCG AT-3' (sense) and specific reverse primer for TNF- α , 5'-CTT CAC AGA GCA ATG ACT CC-3' (antisense), calculated length of amplicon 325 bp; specific forward primer for IL-4, 5'-GCA TGT ACC AGC AAC TTC GT-3' (sense) and specific reverse primer for IL-4, 5'-TTG GCT TCA TTC ACA GAA CAG-3' (antisense), calculated length of amplicon 287 bp; specific forward primer for IFN- γ , 5'-CCT CAG ACT CTT TGA AGT CT-3' (sense) and specific reverse primer for IFN- γ , 5'-CAG CGA CTC CTT TTC CTC TT-3' (antisense), and calculated length of amplicon 225 bp; specific forward primer for the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-CTGGTGCTGAGTATGTCGTG-3' (sense) and specific reverse primer for GAPDH, 5'-CAGTCTTCTGAGTGGCAGTG-3' (antisense), and calculated length of amplicon 296 bp. Amplification was then performed in a gradient thermal cycler PCR machine (Eppendorf, Germany) as follows: 3 min at 95 °C, followed by up to 35 cycles of 30 s each at 94 °C, 30 s at 55 °C, 45 s at 72 °C, and a final elongation at 72 °C for 10 min. The amplified DNA was analyzed by electrophoresis on 1.5% agarose gel containing 0.5 μ g/ml ethidium bromide. Photographs of UV-transilluminated ethidium bromide-stained agarose gels were digitized using an imaging system (UVP company limited, England). Results were expressed as a ratio of OD signal for eotaxin and cytokines to that for GAPDH.

2.6. Preparation of bronchoalveolar lavage fluids

Seven days after the challenge, mice were anaesthetized with urethane (2 g/kg, i.p.). Bronchoalveolar lavage fluids were obtained by cannulating the trachea through a small incision with 1.5 ml of 0.9% sterile saline containing 1% bovine serum albumin and 5000 IU/l heparin. bronchoalveolar lavage fluid cells were centrifuged once with Hank's balanced salt solution (HBSS) containing 2% fetal calf serum at 500 g for 10 min at 4 °C. The supernatant was stored at -80 °C until measurement of cytokines and chemokine. The pelleted bronchoalveolar lavage fluid cells were resuspended in HBSS and the total number of leucocytes were counted by a Neubauer chamber. Two hundred cells in cytocentrifuged preparation of bronchoalveolar lavage fluids stained with Wright-Giemsa were differentiated under a light microscope by classical cell morphology. The total number of each cell type was determined by multiplying the percentage by the total number of cells. The results were expressed as the number of each cell population in 1 l of bronchoalveolar lavage fluids.

2.7. Cytokine and chemokine assays

The bronchoalveolar lavage fluids were centrifuged at 2000 g for 60 min at 4 °C. The concentrations of eotaxin, TNF- α , IFN- γ and IL-4 in bronchoalveolar lavage fluids supernatants were measured by sandwich enzyme linked immunosorbent assay (ELISA) using paired matched Abs according to the manufacturer's instructions. For TNF- α , IFN- γ and IL-4 detection, the ELISA kits were obtained from BD, eBioscience. Eotaxin ELISA kit was purchased from R&D Systems (Minneapolis, MN). The limits of detection were as follows: IL-4, 4 pg/ml; IFN- γ , 15.6 pg/ml and TNF- α , 10 pg/ml; and eotaxin, 2 pg/ml.

2.8. Histologic examination

The lungs were infused via trachea with 1 ml of 10% neutral formalin and immersed in the same fixative for seven days. Tissues were paraffinized, and 5 μ m sections were cut and stained with Alcian blue-periodic acid-Schiff stain (Sigma, St. Louis, USA) for measuring mucus production and H&E for examining cell infiltration under a light microscope. To determine the extent of mucus production, goblet cell hyperplasia in the airway epithelium was semi-quantified blind using a 5 point grading system described by Kuperman et al. (2002). Briefly, the adopted grading system was: 0, no goblet cells; 1, <25%; 2, 25–50%; 3, 50–75%; 4, >75%. To determine the severity of inflammatory cell infiltration, peribronchial cell counts were performed blind based on a 5 point scoring system described by Duan et al. (2004). Briefly, the scoring system was: 0, no cells; 1, a few cells; 2, a ring of cells 1 cell layer deep; 3, a ring of cells 2–4 cells deep; 4, a ring of cells >4 cells deep. Scoring of goblet cells and inflammatory cells was performed in at least three different fields for each lung section. Mean scores were obtained from six animals.

2.9. Statistical analysis

Data are presented as means $\pm$ S.E.M. SPSS version 12 (SPSS Inc, Chicago) was used for statistical analysis. To avoid inflation of the type I error because of multiple comparison in

Table 1
Effects of ciclamilast and dexamethasone on methacholine PD₅₀ (lung resistance, R_L) and PD₂₀ (lung dynamic compliance, C_{dyn}) in model of asthmatic mice

Groups	<i>n</i>	R_L PC ₅₀ (95% CI) μ g/kg, i.v.	C_{dyn} PC ₂₀ (95% CI) μ g/kg, i.v.
Control	8	1.37 (1.12–1.69)	0.89 (0.74–1.08)
Model	14	0.22 (0.20–0.24)	0.27 (0.23–0.31)
Ciclamilast	9	0.30 (0.27–0.34)	0.37 (0.30–0.46)
1 mg/g			
Ciclamilast	10	0.35 (0.30–0.41)	0.57 (0.46–0.71)
3 mg/kg			
Ciclamilast	10	0.59 (0.51–0.69)	1.24 (0.88–1.76)
10 mg/kg			
Dexamethasone	11	1.42 (1.04–1.94)	1.36 (0.90–2.06)
2 mg/kg			

CI: confidence interval.

figure one, differences in dose-response curves to methacholine stimulation were assessed with variance analysis of repeated-measures method (with Greenhouse–Geisser epsilon correction and Tukey test), and PD_{50} and PD_{20} calculated by weighted probit analysis of Bliss method. Other figures, one-way analysis of variance followed by Dunnett test was used to determine significant differences between treatment groups. The critical level for significance was set at $P < 0.05$.

3. Results

3.1. Ciclamilast and dexamethasone inhibit allergen-induced airway responsiveness to methacholine

The ovalbumin-sensitized mice were challenged with aerosolized ovalbumin repeatedly for 7 consecutive days and lung

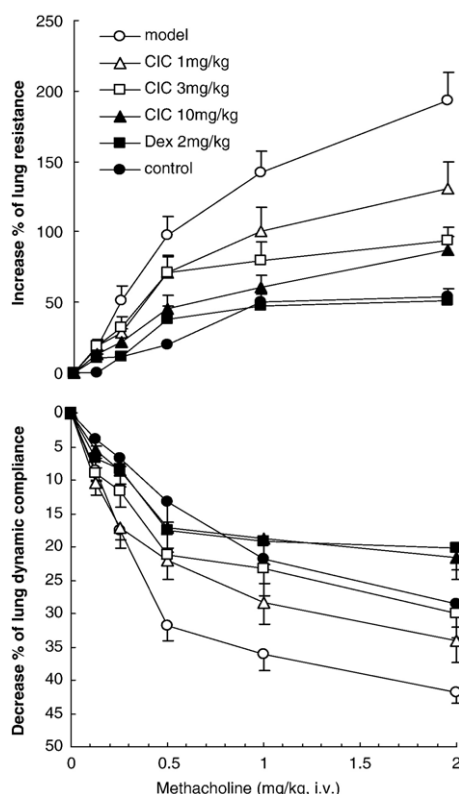


Fig. 1. Effects of ciclamilast or dexamethasone on methacholine induced increase in lung resistance and decrease in lung dynamic compliance. Relative changes in percentage of baseline control before methacholine provocation are given in the legend. Intravenous administration of bolus doses of methacholine induced airway hyperresponsiveness 24 h after the last ovalbumin challenge. Control group ($n=8$), model group ($n=14$), ciclamilast (CIC) 1 mg/kg ($n=9$), 3 mg/kg ($n=10$) and 10 mg/kg ($n=9$), or dexamethasone (Dex) 2 mg/kg ($n=11$). Data shown as mean $\pm$ S.E.M. The curve changes of lung resistance with Greenhouse–Geisser correction, between-subjects effects ($F=9.28$, $P<0.001$); within-subjects effects ($F=96.16$, $P<0.001$); subjects-by-doses interaction ($F=5.39$, $P<0.001$). The curve changes of lung dynamic compliance, between-subjects effects ($F=4.46$, $P<0.05$); within-subjects effects ($F=119.22$, $P<0.001$); subjects-by-doses interaction ($F=2.36$, $P<0.05$). With Tukey HSD test, significant differences of ciclamilast 3 mg/kg and 10 mg/kg, dexamethasone 2 mg/kg and control group compared with model group, $P<0.05$, $P<0.001$, $P<0.001$ and $P<0.001$, respectively.

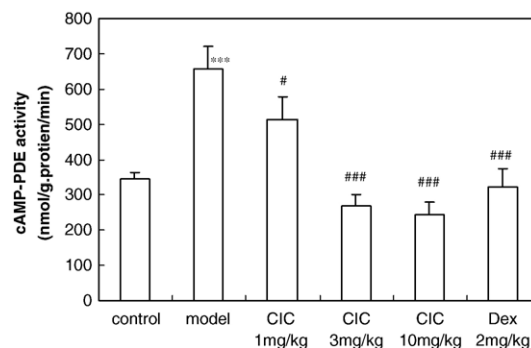


Fig. 2. cAMP-phosphodiesterase (PDE) activity in lung tissue. Ovalbumin ($n=10$) induced a significant increase in cAMP-PDE activity in the sensitized mice in comparison with control mice ($n=6$). Ciclamilast (CIC) 1 mg/kg ($n=10$), 3 mg/kg ($n=10$), 10 mg/kg ($n=8$) and dexamethasone (Dex) 2 mg/kg ($n=10$) partially or completely reverse the ovalbumin-induced increase in cAMP-PDE activity when they were applied 1 h before allergen challenge, once a day for 7 consecutive days. Data are shown as mean $\pm$ S.E.M. Statistically significant differences at *** $P<0.001$, as compared with control. Statistically significant differences at # $P<0.05$ and ### $P<0.001$, as compared with model.

resistance and lung dynamic compliance were measured before and after each dose of intravenous methacholine. In animals of the model group, airway hyperresponsiveness was demonstrated by a significant decrease in lung resistance PD_{50} value in addition to a significant increase in maximum lung resistance. Similarly, the cumulative dose-effect curves for methacholine had a significantly lower lung dynamic compliance PD_{20} value with a decreased minimum dynamic compliance (Table 1 and Fig. 1). These changes in airway responsiveness to methacholine were quantitatively observed as a 6.2 fold decrease in lung resistance PD_{50} and a 3.3 fold decrease in lung dynamic compliance PD_{20} (Table 1). Ciclamilast dose-dependently suppressed airway hyperresponsiveness to methacholine as compared to the model group. The provocative doses of methacholine of ciclamilast 10 mg/kg group were elevated by approximately three-fold than that observed in model group. Dexamethasone 2 mg/kg developed a more potent suppressant effect than that of ciclamilast. Results of the statistical significance tests within the repeated measurement with variance analysis of Greenhouse–Geisser correction model for the relative changes of the dose-response curves compared with baseline data are given in Fig. 1 for lung resistance and lung dynamic compliance parameters to methacholine stimulation. The curve changes of lung resistance with Greenhouse–Geisser correction, between-subjects effects ($F=9.28$, $P<0.001$); within-subjects effects ($F=96.16$, $P<0.001$); subjects-by-doses interaction ($F=5.39$, $P<0.001$). The curve changes of lung dynamic compliance with Greenhouse–Geisser correction, between-subjects effects ($F=4.46$, $P<0.05$); within-subjects effects ($F=119.22$, $P<0.001$); subjects-by-doses interaction ($F=2.36$, $P<0.05$).

3.2. Allergen-induced increase of cAMP-PDE activity in the lung of allergic mice and effects of ciclamilast and dexamethasone

The lung cAMP-PDE activity in model mice was significantly elevated, resulting in an almost two-fold higher cAMP-

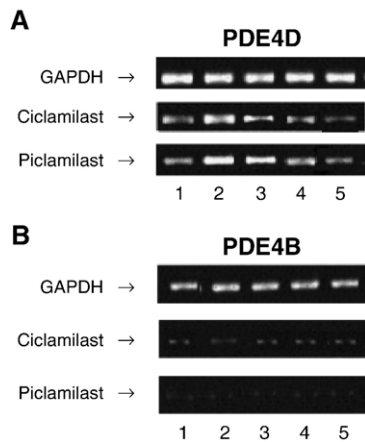


Fig. 3. PDE4B or PDE4D mRNA expression in lungs (representative data from six mice per group tested for PDE4B or PDE4D mRNA expression). GAPDH = glyceraldehyde phosphate dehydrogenase, a housekeeping gene. (A) Model mice had significantly higher expression levels of PDE4D mRNA (approximately twofold) than control mice. Treatment with ciclamilast or piclamilast dose-dependently inhibited PDE4D-specific mRNA expression in comparison with model mice. (B) Model mice had a weak expression levels of PDE4B mRNA, and no significantly higher expression levels of PDE4B mRNA than control mice. Line 1: control mice, Line 2: model mice, Line 3: ciclamilast or piclamilast 1 mg/kg, Line 4: ciclamilast or piclamilast 3 mg/kg, Line 5: ciclamilast or piclamilast 10 mg/kg.

PDE activity than that of normal animals (Fig. 2). Rolipram-sensitive (100 μ M) cAMP-PDE activity (i.e., PDE4 activity) was approximately 1.8 fold higher in model mice than in control mice. PDE3 is another cAMP-PDE known to play a role in the regulation of airway contraction (Torphy, 1998). However, relative PDE3 activity (determined as the activity sensitive to the PDE3-selective inhibitor siguazodan) in total cAMP-PDE activity was conversely lower in model mice than in control mice (data not shown). PDE5, a cGMP-specific PDE, is also

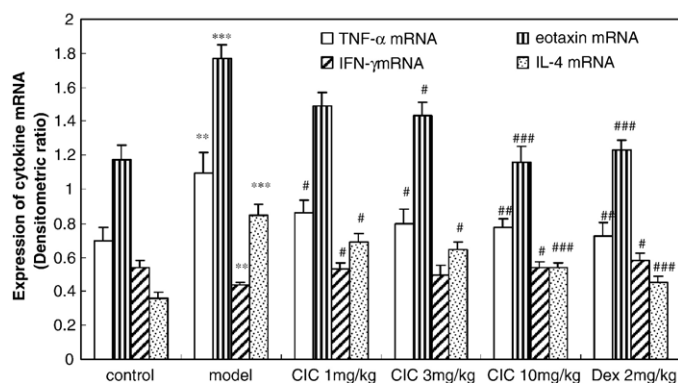


Fig. 4. Expression of IL-4, eotaxin, IFN- γ and TNF- α mRNA in lung tissue and effects of ciclamilast and dexamethasone. Mice were sensitized by ovalbumin and ciclamilast (CIC) or dexamethasone (Dex) treatment was applied once a day for 7 days ($n=6$). PCR products of TNF- α , eotaxin, IFN- γ and IL-4 were visualized by electrophoresis in a 1.5% agarose gel containing 0.5 μ g/ml ethidium bromide. Densitometric analyses of the gel were made and results were expressed as a ratio of quantified IL-4, eotaxin, IFN- γ and TNF- α product over GAPDH product. Data are shown as the mean $\pm$ S.E.M. Statistically significant differences at $**P<0.01$ and $***P<0.001$, as compared with control group. Statistically significant differences at $^{\#}P<0.05$; $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$, as compared with model group.

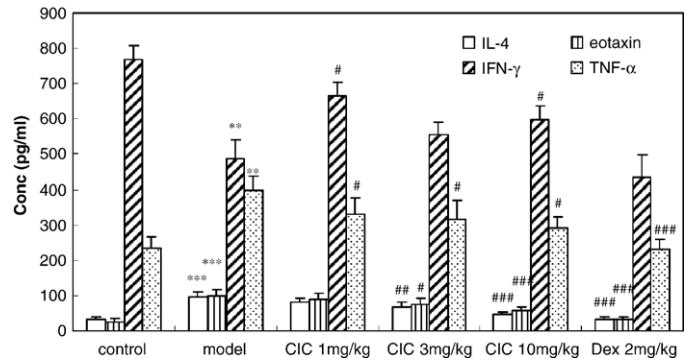


Fig. 5. Effects of ciclamilast and dexamethasone on cytokine and chemokine levels in bronchoalveolar lavage fluids. Bronchoalveolar lavage fluids were collected 24 h after the last ovalbumin challenge. Levels of IL-4, eotaxin, TNF- α and IFN- γ were analyzed using ELISA ($n=10$). Ciclamilast (CIC) and dexamethasone (Dex) were applied once a day for 7 days during the challenge. Data were shown as mean $\pm$ S.E.M. Statistically significant differences at $**P<0.01$ and $***P<0.001$, as compared with control group. Statistically significant differences at $^{\#}P<0.05$; $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$, as compared with model group.

abundantly present in lung (Tang et al., 2005; Francis et al., 2001). However, the PDE5-selective inhibitor zaprinast did not significantly affect the cAMP-PDE activity in either control mice or model mice (data not shown). These results suggest that the increase in total cAMP-PDE activity by ovalbumin sensitization and challenge might be primarily caused by upregulation of PDE4. We then tested the effect of the selective PDE4 inhibitor ciclamilast on the allergen-induced increase in cAMP-PDE activity and meanwhile, we also tested the effect of dexamethasone, a glucocorticoid for comparison. Treatment with 1, 3, 10 mg/kg ciclamilast significantly reduced the cAMP-PDE activity in a dose-dependent manner (ID_{50} of 3.52 mg/kg), (Fig. 1). Ciclamilast 10 mg/kg and dexamethasone 2 mg/kg fully inhibited the increase of cAMP-PDE activity induced by

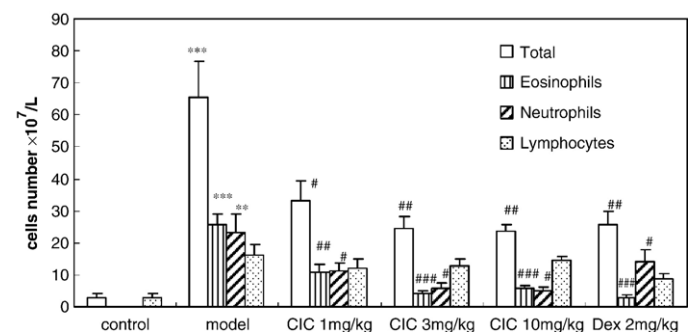


Fig. 6. Effects of ciclamilast and dexamethasone on bronchoalveolar lavage fluid cell infiltration. Bronchoalveolar lavage fluids were collected 24 h after the last ovalbumin challenge. Inflammatory cell counts in bronchoalveolar lavage fluid obtained from sensitized mice 24 h after the last saline aerosol or 1% ovalbumin aerosol challenge (control group, $n=8$; model group, $n=12$; CIC 1 mg/kg, $n=9$; CIC 3 mg/kg, $n=12$; CIC 10 mg/kg, $n=11$; Dex 2 mg/kg, $n=8$). Differential cell count was performed on a minimum of 200 cells to identify eosinophils, neutrophils, and lymphocytes. Data were shown as mean $\pm$ S.E.M. Statistically significant differences at $**P<0.01$ and $***P<0.001$, as compared with control group. Statistically significant differences at $^{\#}P<0.05$; $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$, as compared with model group.

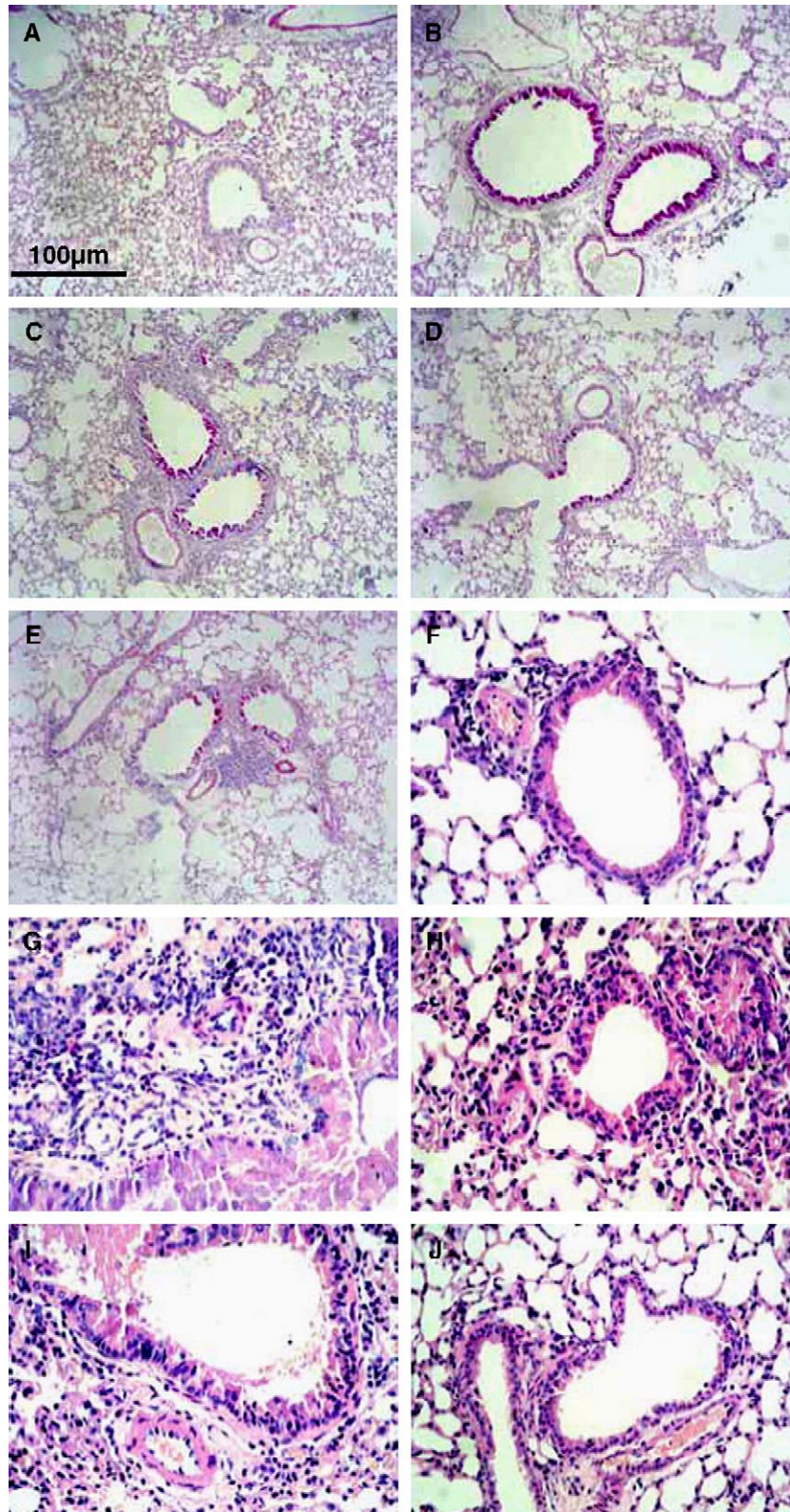


Fig. 7. Effects of ciclamilast and dexamethasone on lung tissue eosinophilia and mucus production. Representative histological image of lung tissue mucus secretion (A–E) and eosinophilia (F–J) 24 h after the last challenge of ovalbumin. (A) Mucus secretion in control. (B) Mucus secretion in model. (C–E) Mucus secretion in ovalbumin-sensitized mice treated with CIC 3 mg/kg (C), CIC 10 mg/kg (D) or Dex 2 mg/kg (E). (F) Eosinophilia in sham-vehicle. (G) Eosinophilia in OVA-OVA. (H–J) Eosinophilia in ovalbumin-sensitized mice treated with ciclamilast (CIC) 3 mg/kg (H), CIC 10 mg/kg (I) or dexamethasone (Dex) 2 mg/kg (J). (K) Using semi-quantitative analyses, a summarized data show the ovalbumin-induced changes of mucus production and eosinophilia and effects of ciclamilast or dexamethasone on them ($n=6$ in each group). Lung tissues were fixed, sectioned at 5 μ m thickness, and stained with periodic acid-Schiff for mucus production (magenta-staining epithelial cells are positive for mucus) or H and E for tissue eosinophilia. Data were shown as mean $\pm$ S.E.M. Statistically significant differences at *** $P<0.001$, as compared with control. Statistically significant differences at # $P<0.05$ and ## $P<0.01$, as compared with model.

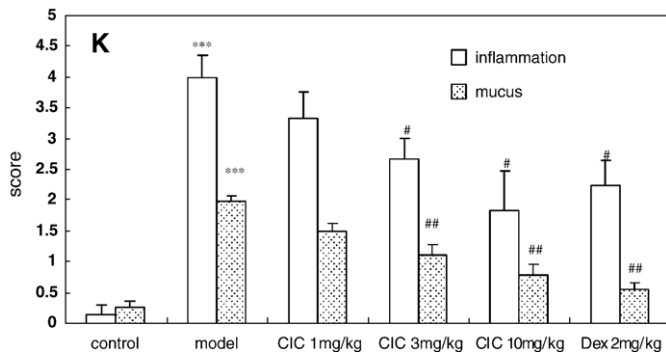


Fig. 7 (continued).

ovalbumin-sensitized and ovalbumin challenged ($P < 0.001$), (Fig. 1).

3.3. Effects of ciclamilast and piclamilast on allergen-induced PDE4B and PDE4D mRNA expression from lung tissues

To examine whether the higher lung PDE4 activity in model mice reflected increased transcription of the PDE4B or PDE4D gene, we isolated RNA from the lungs of model mice and control mice and analyzed the RNA for PDE4B and PDE4D-specific mRNA expression by RT-PCR. Model mice had significantly higher levels of PDE4D mRNA (approximately twofold) than control mice, but not PDE4B mRNA (Fig. 3). Treatment with ciclamilast 1, 3, 10 mg/kg respectively inhibited PDE4D-specific mRNA expression by $61.5 \pm 15.2\%$, $69.8 \pm 13.5\%$, $81.6 \pm 19.5\%$ ($P < 0.01$), as compared with model group. However, piclamilast 1, 3, 10 mg/kg respectively inhibited 1 mg/kg inhibited PDE4D-specific mRNA expression only by $45 \pm 12.9\%$, $55.8 \pm 21.5\%$, $62.8 \pm 19.5\%$ ($P < 0.05$ – $P < 0.01$) in comparison with model group. There was a significant correlation between lung PDE4D mRNA expression and the lung resistance increase (Correlation Coefficient $R = 0.929$, $P < 0.05$) and dynamic lung compliance decrease inhibited by ciclamilast (Correlation Coefficient $R = 0.908$, $P < 0.05$).

3.4. Effect of ciclamilast and dexamethasone on cytokine and chemokine expression in the lung tissue of asthmatic mice

Since cytokines and chemokines plays a critical role in the pathogenesis of allergic airway diseases, the mRNA expression of IL-4, eotaxin, TNF- α and IFN- γ in the lungs of asthmatic mice were measured. The lung tissue samples were collected 24 h after the last ovalbumin challenge. IL-4, eotaxin, TNF- α , and IFN- γ expression were measured using RT-PCR. TNF- α , eotaxin, and IL-4 expression were upregulated and IFN- γ expression was downregulated after ovalbumin sensitization. Both ciclamilast and dexamethasone significantly inhibit the upregulation of IL-4, eotaxin, and TNF- α and downregulation of IFN- γ induced by ovalbumin. Though ciclamilast reduced IL-4, eotaxin, and TNF- α expression in a dose-dependent manner, it had mild effects on IFN- γ expression in lung tissue as compared to IL-4, eotaxin, and TNF- α . (Fig. 4).

3.5. Effect of ciclamilast and dexamethasone on cytokine and chemokine levels in bronchoalveolar lavage fluids

To determine the levels of cytokines and chemokine in vivo, we also directly measured the levels of IL-4, eotaxin, TNF- α and IFN- γ from bronchoalveolar lavage fluids using ELISA. Bronchoalveolar lavage fluids samples were collected 24 h after the last ovalbumin challenge. As shown in Fig. 5, ovalbumin sensitization significantly increased the levels of IL-4, eotaxin, and TNF- α in bronchoalveolar lavage fluids in model mice as compared with control mice ($P < 0.01$ or 0.001). The IFN- γ levels decreased in model mice compared to control mice ($P < 0.01$). Treatment with ciclamilast (1 mg/kg, 3 mg/kg or 10 mg/kg) or dexamethasone (2 mg/kg) decreased the IL-4, eotaxin, and TNF- α levels and increased the IFN- γ level increased in bronchoalveolar lavage fluids as compared to model mice. Though ciclamilast reduced IL-4, eotaxin, and TNF- α levels in a dose-dependent manner, it had mild effects on IFN- γ levels in bronchoalveolar lavage fluids as compared to IL-4, eotaxin, and TNF- α levels.

3.6. Ciclamilast and dexamethasone inhibit airway eosinophilia and lung inflammation

One day after the last airway ovalbumin challenge, the number of total cells, eosinophils, neutrophils and lymphocytes in the bronchoalveolar lavage fluids increased 22-, 462-, 512- and 6-fold, respectively, compared to those of control (Fig. 6). Daily administration of difference doses of ciclamilast from day 22 (the first day of challenge with aerosol ovalbumin) to day 28 (the last day of challenge) reduced significantly airway infiltration of cells in a dose-dependent manner, which was indicated by the significant reduction in the number of total cells, eosinophils, and neutrophils. Dexamethasone at the dose of 2 mg/kg also inhibited the increase of total cells and eosinophils, and had a similar potency to ciclamilast 10 mg/kg (Fig. 6). However, ciclamilast 3 mg/kg and 10 mg/kg were more potent in inhibiting the increase of neutrophils compared to dexamethasone 2 mg/kg after ovalbumin challenge ($P < 0.05$).

3.7. Effects of ciclamilast and dexamethasone on antigen-induced mucus production and eosinophil infiltration

Lung tissue was collected 24 h after the last ovalbumin challenge. Model mice (Fig. 7B) developed a marked goblet cell hyperplasia and mucus hypersecretion within the bronchi in the lung as compared with control (Fig. 7A). Ciclamilast 3 mg/kg (Fig. 7C), 10 mg/kg (Fig. 7D) and dexamethasone 2 mg/kg (Fig. 7E) markedly attenuated ovalbumin-induced mucus secretion as compared with model (Fig. 7B). Similarly, ovalbumin aerosol challenge (Fig. 7F) induced marked infiltration of inflammatory cells into the peribronchiolar and perivascular connective tissues as compared with control (Fig. 7G). Ciclamilast 3 mg/kg (Fig. 7H), 10 mg/kg (Fig. 7I) and dexamethasone 2 mg/kg (Fig. 7J) markedly inhibited ovalbumin-induced the eosinophil-rich leukocyte infiltration as compared with model (Fig. 7G). Semi-quantitative analyses showed that

both ciclamilast and dexamethasone significantly abated the ovalbumin-induced mucus secretion and the eosinophil-rich leukocyte infiltration (Fig. 7K).

4. Discussion

In the present study, we investigated the effects of ciclamilast and dexamethasone on changes of airway hyperresponsiveness, lung PDE4 activity, PDE4 subtypes mRNA expression, inflammation, and mucus secretion in a model of mouse asthma. In this mouse model of allergic asthma, antigen sensitization and repeated challenge induced airway hyperresponsiveness, airway inflammation, airway goblet cell hyperplasia, and an increase in cAMP-PDE enzyme activity and mRNA expression of PDE4D, eotaxin, TNF- α , and IL-4, and a decrease in mRNA expression for IFN- γ . A parallel change in the levels of eotaxin, TNF- α , IL-4, and IFN- γ in the bronchoalveolar lavage fluids was also shown. These changes were suppressed by ciclamilast or dexamethasone. Ciclamilast showed higher potent against PDE4D mRNA expression than that of piclamilast.

Airway hyperresponsiveness is one of the hallmark features of asthma resulting in episodic bronchoconstriction and perception of symptoms. It is defined by episodes of excessive airway narrowing in response to a provoking stimulus. The mechanism of airway hyperresponsiveness in asthma remains unclear, but it is clear that excess mucus, edema airway narrowing and increases in the airway wall thickness not only obstructs airways but also contributes to airway hyperresponsiveness (Spina, 2003; Grönke et al., 2002; Pare, 2003). Recently, two studies show that mice lacking PDE4D are poorly responsive to cholinergic stimulation (Hansen et al., 2000), and in isolated murine tracheal rings from PDE4D-deficient mice, responsiveness to carbachol was markedly attenuated, but was restored by pre-treating tissues with indomethacin or the protein kinase A inhibitor RpcAMP (Mehats et al., 2003). These studies identify PDE4D as an important regulatory molecule capable of modulating agonist-induced airway smooth muscle responsiveness, raising the possibility that airway hyperresponsiveness to cholinergic stimulation may also be modulated by activation and/or expression of PDE4D in airway smooth muscle. In the present study, we found a significant parallel change between airway hyperresponsiveness with upregulation of lung PDE4 activity and expression increase in PDE4D, cytokines, airway mucus hypersecretion, and inflammatory cells infiltration in a mouse model. Ciclamilast and dexamethasone significantly attenuated airway hyperresponsiveness in a dose-dependent manner. These results suggest the inhibitory effect of ciclamilast on airway hyperresponsiveness includes modulating PDE4 activity, anti-inflammation, and anti-mucus hypersecretion.

Although upregulation of lung PDE4 has been demonstrated in a rat allergic model (Tang et al., 2005), lung PDE4 upregulation in a mouse model of asthma has not been reported to our knowledge. Furthermore, increased PDE4D gene expression in the lung was observed in a cigarette smoking-induced mouse model of chronic obstructive pulmonary disease (Singh et al., 2003). However, our results also demonstrated lung PDE4D gene expression is increased in the mouse model. As far as why PDE4 enzyme

activity was upregulated during asthma, except for PDE4 gene expression, studies of signal pathways are need. Some cytokines, such as IL-1 β might up-regulate PDE 4B2 expression in human myometrial cells through a prostaglandin E2- and cyclic adenosine 3',5'-monophosphate-dependent pathway (Oger et al., 2002). IL-3 and IL-4 activate PDE3 and PDE4 by different mechanisms in FDCP2 myeloid cells. The mitogen-activated protein kinase kinase (MEK)-1 inhibitor PD98059 inhibited IL-4-, IL-3-, and PMA-induced activation of MEK and PDE4, but not IL-4-induced activation of PDE3 (Ahmad et al., 1999). Corticosteroid (Almawi et al., 1996) and PDE4 inhibitors (Spina, 2003) might down-regulate PDE4 activity by inhibiting production of the various cytokines. A recent report suggested dexamethasone also might directly reduce PDE4 gene expression (Ahlstrom et al., 2005). In the present study, inhibitory effects of ciclamilast and dexamethasone on mRNA expression for eotaxin, TNF- α , and IL-4 in the lung, and the levels of eotaxin, TNF- α and IL-4 in the bronchoalveolar lavage fluids suggests that both drugs down-regulate PDE4 activity by inhibiting production of the various cytokines and cannot be completely ruled out. Regarding PDE4 inhibitors reducing production of the various cytokines, there were numerous studies during the last decade (Spina, 2003; Ouagued et al., 2005; Eskandari et al., 2004).

Most of these T cell functions derive from the production of cytokines, chemokines, and other mediators. Asthma is often associated with a Th2-mediated inflammatory reaction in the airway. The major Th2 cytokines measured in bronchoalveolar lavage fluids of patients with asthma are IL-4, IL-5, IL-9, and IL-13, although some Th1 cytokines such as IFN- γ can also be found in bronchoalveolar lavage fluids (Prieto et al., 2001). Our data are in similar to several reports demonstrating that PDE4 inhibitors can attenuate production of IL-4 and IL-5 production in various stimulation paradigms. SB 207499 has been reported to inhibit oxazolone-induced IL-4 production in a mouse model of ear inflammation (Griswold et al., 1998). Roflumilast inhibits IL-4 and IL-5 production in CD3/CD28 costimulated human T cells (Hatzelmann and Schudt, 2001). Rolipram has also been shown to inhibit IL-5 formation in ovalbumin challenged mouse splenic cells in vitro and IL-4 and IL-5 from bronchoalveolar lavage fluids in ovalbumin challenged mice (Foissier et al., 1996). However, L-826,141 transcriptionally down-regulates production of TNF- α in peripheral blood mononuclear cell and whole blood assays. Profiling the effect of this compound on various cytokines in the signaling cascade attenuated by cAMP elevation demonstrates that L-826,141 is also a potent inhibitor of IL-12, granulocyte macrophage-colony stimulating factor (GM-CSF), and IFN- γ as well as TNF- α formation. The data demonstrate that specific inhibition of L-826,141 preferentially blocks the production of Th1 versus Th2 effector cytokines in vitro (Claveau et al., 2004). In contrast, our data demonstrate that specific inhibition of ciclamilast preferentially blocks the production of Th2 versus Th1 effector cytokines in a mouse model of asthma.

Eotaxin is an eosinophil-specific chemokine associated with the recruitment of eosinophils to the site of allergic inflammation. In addition to its chemoattractant effect, eotaxin can also stimulate the rapid release of eosinophils and their progenitors

from the bone marrow and induce degranulation of eosinophil via high affinity binding to CCR3 (Minshall et al., 1997; Gorski et al., 2002; Rankin et al., 2000). Because of its action on eosinophils, eotaxin and its receptor might be attractive therapeutic targets for the treatment of allergic diseases (Erin et al., 2002). Therefore, we examined the expression of eotaxin mRNA in tissues. Our results demonstrated that eotaxin mRNA expression was markedly enhanced in lung tissues of the model group. In addition, the eotaxin mRNA expression in lung tissues was significantly suppressed after ciclamilast treatment. These results suggest that ciclamilast is beneficial to allergic inflammation via inhibiting the expression of eotaxin mRNA. Further study, such as whether ciclamilast can affect the level of CCR3, will be investigated.

Current guidelines on asthma management highlight mucus plugging alongside bronchoconstriction and inflammation as a cause of airway obstruction and airflow limitation (National Institutes of Health, 2002; Sidebotham and Roche, 2003; Rogers, 2002). Consequently, it is important to understand the pathophysiology of mucus hypersecretion in asthma. This should allow identification of therapeutic targets and subsequent rational development of pharmacotherapeutic drugs. Airway mucus hypersecretion in asthma has characteristic pathophysiological features. Many of these features, such as sputum production and goblet cell hyperplasia, are common to other hypersecretory respiratory diseases; for example, COPD and cystic fibrosis. Pharmacotherapy of airway mucus hypersecretion in asthma has been discussed in detail recently (Rogers, 2004). Pharmacotherapy can be divided into two sections: first, anti-inflammatory treatment of airway inflammation, probably the most beneficial therapy overall; and second, therapies directed specifically at different levels of the pathophysiological ‘cascade’, from secretagogues to clinical symptoms. However, with the exception of glucocorticosteroids, it is unlikely that any single class of compound will provide comprehensive treatment of mucus hypersecretion in asthma. Though it is not clear exactly whether PDE4 inhibitors attenuated mucus hypersecretion or sputum volume in patients with asthma or COPD (Lipworth, 2005), our results, and recently other studies, suggest PDE4 inhibitors and glucocorticoid attenuate airway mucus secretion in a model of allergen challenge (Kanehiro et al., 2001; Kasper et al., 2002; McMillan et al., 2005), and decreased MUC5AC expression induced by epidermal growth factor in human airway epithelial cells (Mata et al., 2005).

In conclusion, it was found that inhibitory effect of ciclamilast on airway hyperresponsiveness includes its inhibiting PDE4 mRNA expression, down-modulating PDE4 activity, anti-inflammation and anti-mucus hypersecretion. Ciclamilast may have therapeutic potential for the treatment of allergic asthma.

Acknowledgements

We thank Prof. Cong-lin Zuo and Beijing Joinn Drug Research Center for kindly supplying ciclamilast and piclamilast. We also thank Dr. Shelli L. Kirstein from the Departments of Pharmacology and Medicine, University of California for the manuscript

revision. This work was supported by the National Natural Science Foundation of China, No. 30371314 and the Science and Technology Department of Zhejiang Province (No. 2006C23009, No. 2005C21072).

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