



Inhibition of phosphodiesterase activity, airway inflammation and hyperresponsiveness by PDE4 inhibitor and glucocorticoid in a murine model of allergic asthma

Jian-gang Sun, Yang-mei Deng, Ximei Wu, Hui-fang Tang, Jun-fang Deng, Ji-qiang Chen, Shui-you Yang, Qiang-min Xie *

Zhejiang Respiratory Drugs Research Laboratory of State Food and Drug Administration, Medical Science College of Zhejiang University, Hangzhou 310031, PR China

Received 10 September 2005; accepted 3 July 2006

Abstract

Phosphodiesterase 4 (PDE4) isozyme plays important roles in inflammatory and immunomodulatory cells. In this study, piclamilast, a selective PDE4 inhibitor, was used to investigate the role of PDE4 in respiratory function and inflammation in a murine asthma model. Sensitized mice were challenged with aerosolized ovalbumin for 7 days, piclamilast (1, 3 and 10 mg/kg) and dexamethasone (2 mg/kg) were orally administered once daily during the period of challenge. Twenty-four hours after the last challenge, airway hyperresponsiveness to methacholine was determined by whole-body plethysmography, airway inflammation and mucus secretion by histomorphometry, pulmonary cAMP-PDE activity by HPLC, cytokine levels in bronchoalveolar lavage fluid and their mRNA expression in lung by ELISA and RT-PCR, respectively. In control mice, significant induction of cAMP-PDE activity was parallel to the increases of hyperresponsiveness, inflammatory cells, cytokine levels, mRNA expression as well as goblet cell hyperplasia. However, piclamilast dose-dependently and significantly improved airway resistance and dynamic compliance, and the maximal effect was similar to that of dexamethasone. Piclamilast treatment dose-dependently and significantly prevented the increase in inflammatory cell number and goblet cell hyperplasia, as well as production of cytokines, including eotaxin, TNF α and IL-4. Piclamilast exerted a weaker inhibitory effect than dexamethasone on eosinophils and neutrophils, had no effect on lymphocyte accumulation. Moreover, piclamilast inhibited up-regulation of cAMP-PDE activity and cytokine mRNA expression; the maximal inhibition of cAMP-PDE was greater than that exerted by dexamethasone, and was similar to dexamethasone on cytokine mRNA expression. This study suggests that inhibition of PDE4 by piclamilast robustly improves the pulmonary function, airway inflammation and goblet cell hyperplasia in murine allergenic asthma. © 2006 Elsevier Inc. All rights reserved.

Keywords: Phosphodiesterase 4; Piclamilast; Asthma; Airway hyperresponsiveness; Airway inflammation

Introduction

Bronchial asthma is a chronic airway inflammatory disease characterized by airway hyperresponsiveness (AHR) to non-specific stimuli such as air pollutants, cold air, exercise, aller-

gens, and infections. Each of these stimuli evokes airway narrowing indirectly through the release of mediators from a variety of effector cells, including inflammatory cells, epithelial cells, and neurons. Among these mediators, proinflammatory mediators released from mast cells and eosinophils orchestrated by T cells have been identified as being particularly relevant to the development of asthma (Holgate et al., 1999). The potent efficacy of corticosteroids in asthma has been suggested to result from suppression of the production of Th2 cytokines, which underpins mast cell, eosinophil and basophil responses (Georas, 2004). In the past decade, inhibitors of cyclic

* Corresponding author. Zhejiang Respiratory Drugs Research Laboratory of State Food and Drug Administration, Medical Science College, Zhejiang University, 353 Yanan Rd. Hangzhou City, Zhejiang Province 310031, P.R. China. Tel./fax: +86 571 8721 7380.

E-mail address: xieqm@zju.edu.cn (Q. Xie).

nucleotide phosphodiesterase 4 (PDE4) isoenzymes have been investigated in several preclinical airway disease models of inflammation and in patients with asthma and chronic obstructive pulmonary disease (COPD); because of their potent anti-inflammatory effects, selective PDE4 inhibitors were expected to be developed as an alternative to glucocorticoids in the treatment of asthma (Houslay and Adams, 2003; Spina, 2003.)

PDEs hydrolyze cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) to their inactive monophosphates, and inhibition of PDEs allows cAMP/cGMP to be elevated within cells. Among the PDEs, PDE4, PDE7 and PDE8 are specific for cAMP. It was established that PDE4 isoenzymes play a particularly important role in inflammatory and immunomodulatory cells and are the predominant PDE in inflammatory cells, including mast cells, neutrophils, basophils, eosinophils, T lymphocytes, macrophages, and structural cells such as sensory nerves and epithelial cells (Torphy, 1998). PDE4 inhibitors block the hydrolysis of cAMP, leading to elevation of intracellular cAMP levels and subsequent suppression of inflammatory activity and decrease of mediator release from these inflammatory cells. Although PDE4 inhibitors are under development as potential therapeutic agents for patients with asthma or COPD, and two orally active PDE4 inhibitors (cilomilast and roflumilast) are in the late phase III of clinical development (Lipworth, 2005), the precise roles of PDE4 and the mechanism action of PDE4 inhibitors for treatment of asthma remain obscure.

Piclamilast (RP73401) is a PDE4 inhibitor with equal affinity for the high-affinity rolipram binding site (HARBS) and low-affinity rolipram binding site (LARBS) (Zhao et al., 2003). In selective inhibition of human neutrophil function in vitro, piclamilast was found to be roughly equipotent to roflumilast, but it showed potency more than 100 times greater than cilomilast or rolipram (Hatzelmann and Schudt, 2001). The rank order of potency for PDE4 inhibitors was similar in human eosinophils among roflumilast, cilomilast, rolipram and piclamilast (Hatzelmann and Schudt, 2001). The in vivo potency of piclamilast has also been investigated in classic animal models: for inhibition of the allergen-induced early airway reaction in guinea pigs, piclamilast was approximately 8-fold less potent than roflumilast, 4- and 6.5-fold greater than rolipram and cilomilast, respectively (Bundschuh et al., 2001). Moreover, in antigen-induced late inflammatory response in sensitized brown Norway rats, orally administered piclamilast was substantially less potent than roflumilast for inhibiting infiltration of eosinophils, neutrophils, and lymphocytes, protein accumulation, and release of tumor necrosis factor α (TNF α). Orally administered piclamilast inhibited lipopolysaccharide-induced release of TNF α : it was 8-fold less potent than roflumilast, 3.1-fold more potent than rolipram and 38.5-fold more potent than cilomilast (Wollin et al., in press). Although much is known about the pharmacological effects of piclamilast on asthma, its development for the treatment of inflammatory diseases has been discontinued due to its important side effects. Nonetheless, piclamilast is still a useful tool for studying the roles of PDE4 in various diseases. To extend our understanding of PDE4 in asthma, in the present study, we used piclamilast to

specifically inhibit PDE4 activity, and to evaluate the roles of PDE4 in cAMP-PDE enzyme activity in lungs, secretion of airway mucus, production of proinflammatory mediators and airway responsiveness in a murine asthma model.

Materials and methods

Reagents

Piclamilast and dexamethasone were kindly provided by Beijing Joynn Drug Research Center (Beijing, China) and Henrui Pharmaceutical Co. Ltd. (Jiangsu, China), respectively. Ovalbumin

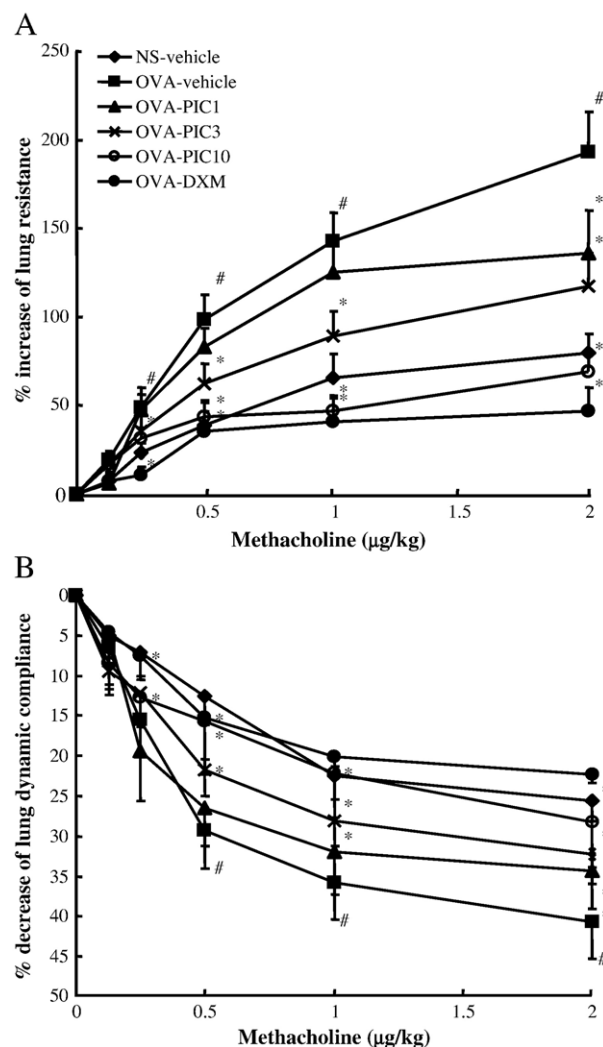


Fig. 1. Dose-dependent response of R_L and C_{dyn} to methacholine. OVA-sensitized mice were orally administered with piclamilast at the doses of 1 (PIC1), 3 (PIC3) and 10 mg/kg (PIC10), dexamethasone at the dose of 2 mg/kg (DEX) or vehicle once a day for 7 days. One hour after administration of piclamilast, dexamethasone or vehicle, mice were challenged with aerosolized antigen or normal saline (NS) for 30 min daily. Twenty-four hours after the last OVA challenge, R_L and C_{dyn} against intravenous methacholine was determined as described in Materials and methods. Data are expressed as mean $\pm$ SEM. #: $p < 0.05$, versus NS-challenged and vehicle-treated mice ($n = 8$, NS-vehicle group); *: $P < 0.05$, OVA-challenged and PIC1—($n = 9$, OVA-PIC1 group), PIC3—($n = 8$, OVA-PIC3 group), PIC10—($n = 9$, OVA-PIC10 group), DEX—($n = 8$, OVA-DEX group) treated mice versus OVA-challenged and vehicle-treated mice ($n = 12$, OVA-vehicle group).

(OVA), cyclic adenosine monophosphate (cAMP), β -mercaptoethanol, pepstatin A, periodic acid-Schiff stain kit and methacholine (MCh) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). TRIzol and Taq DNA polymerase were from GIBCO (Carlsbad, CA, USA). Reverse Transcription System kit was provided by MBI Fermentas (Vilnius, Lithuania). Oligonucleotide primers for eotaxin, interleukin 4 (IL-4), TNF α , interferon γ (INF- γ) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized by Shanghai Sangon Biological Engineering Technology and Services Co. Ltd. (Shanghai, China). TNF α , INF- γ and IL-4 ELISA kits were from eBioscience Co. (San Diego, CA, USA), and eotaxin ELISA kit was from R and D Systems, Inc. (Minneapolis, MN, USA). All other chemicals were of reagent grade.

Animals

Ten-week-old female BALB/c mice, purchased from Shanghai SLAC Laboratory Animal CO. Ltd. (Shanghai, China), were maintained in the animal facility under standard indoor conditions with a 12-h light–dark cycle (lights on from 8:00 am to 8:00 pm) and were given free access to water and rodent chow. In all experiments, all animal care and handling procedures were approved by the Institutional Animal Care and Use Committee of Zhejiang University.

Sensitization and treatment

On days 0 and 10, mice were systemically sensitized with a subcutaneous injection of 20 μ g/mouse OVA emulsified in 2 mg aluminum hydroxide adjuvant at footpad, neck, back, and groin. From days 22 to 28, before antigen challenge, OVA-sensitized mice were orally administered with piclamilast or dexamethasone once a day, at the doses of 1, 3 and 10 mg/kg/day for

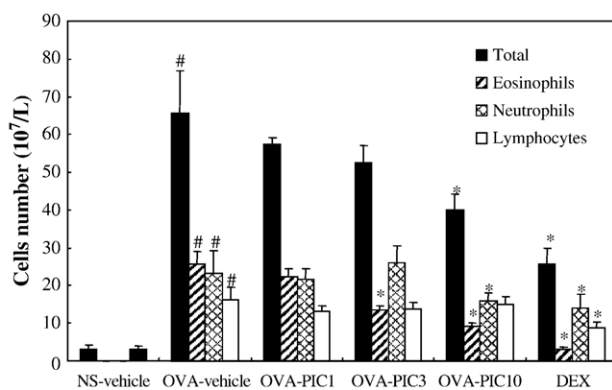


Fig. 2. Effects of piclamilast and dexamethasone on inflammatory cell levels in BALF. Mice were processed as described in Fig. 1, and BALF was harvested 24 h after the last OVA challenge. Total inflammatory cell numbers in BALF were counted, and cell classification was performed on a minimum of 200 cells to classify eosinophils, neutrophils, and lymphocytes. Data are expressed as mean $\pm$ SEM. #: $p < 0.05$, versus NS-challenged and vehicle-treated mice ($n = 8$, NS-vehicle group); *: $p < 0.05$, OVA-challenged and PIC1—($n = 11$, OVA-PIC1 group), PIC3—($n = 11$, OVA-PIC3 group), PIC10—($n = 11$, OVA-PIC10 group), DEX—($n = 11$, OVA-DEX group) treated mice versus OVA-challenged and vehicle-treated mice ($n = 14$, OVA-vehicle group).

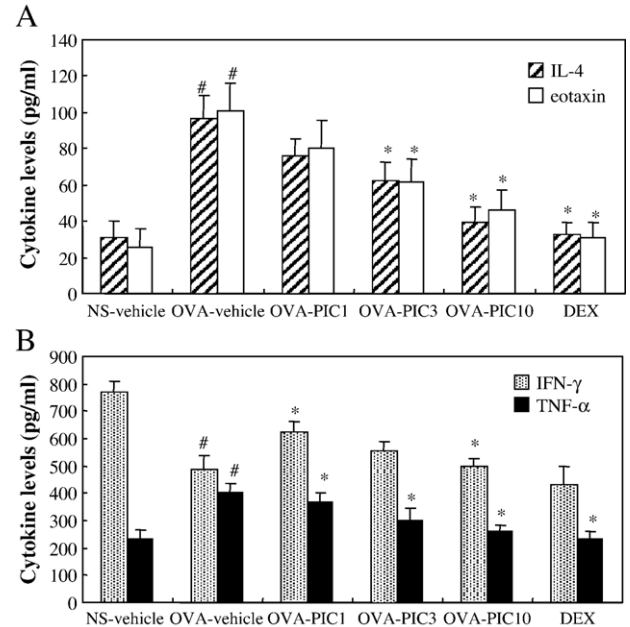


Fig. 3. Effects of piclamilast and dexamethasone on cytokine and chemokine levels in BALF. Mice were processed and treated as described in Fig. 1, and BALF was harvested 24 h after the last OVA challenge. Levels of IL-4, eotaxin, TNF α and IFN- γ in BALF were analyzed by ELISA. $n = 10$ (each group). Results are shown as mean $\pm$ SEM. #: $p < 0.05$, versus NS-vehicle group; *: $p < 0.05$, versus OVA-vehicle group.

piclamilast and 2 mg/kg/day for dexamethasone. Control mice were orally administered with the same volume of 2% hydroxymethylcellulose vehicle. One hour after administration of piclamilast, dexamethasone or vehicle, mice were challenged with antigen as described previously (Xie et al., 2003). Briefly, all mice in a plastic box were challenged with aerosolized 1% OVA or an equal volume of normal saline (NS) by a jet nebulizer (BARI Co. Ltd., Germany) for 30 min. The doses of piclamilast were based on its anti-inflammatory activity in a model of antigen-induced airway inflammatory in rats and on pharmacokinetic results showing that administration of 5 mg/kg/day for 7 days gave therapeutically relevant blood levels in rats and dogs, and that 1 mg/kg/day was the lowest dose for inhibition of inflammatory response (Beijing Joinn Drug Research Center, unpublished data). The dose of dexamethasone used in the present study is similar to those used in other investigations based on its anti-inflammatory effects (Trifilieff et al., 2000).

Measurement of respiratory function

Airway resistance (R_L) and dynamic compliance (C_{dyn}) were determined as changes of airway function after intravenous MCh challenge. The lung function of anesthetized and tracheostomized mice was assessed as described previously (Xie et al., 2002). Briefly, each anesthetized mouse was placed supine inside a plexiglas whole-body plethysmograph. The flow rate was monitored with a Fisher tube connected to the airways in a pressure transducer, and the changes in lung volume were measured by detecting pressure changes in the plethysmographic chamber through a port in the connecting tube with a pressure

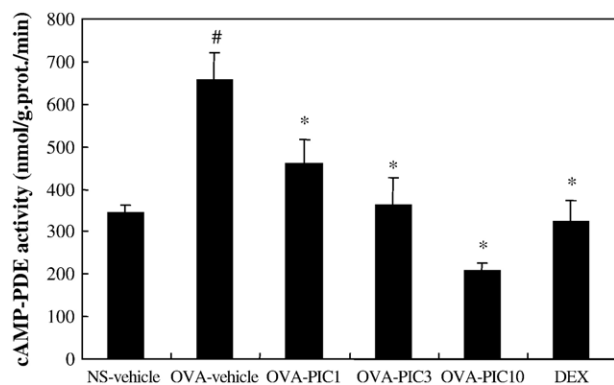


Fig. 4. cAMP-phosphodiesterase activity in the lung tissues of asthmatic mice. Mice were processed and treated as described in Fig. 1; lung tissues were harvested 24 h after the last OVA challenge and used for determination of cAMP-PDE activity as described in Materials and methods. Data are expressed as mean ± SEM. #: $p < 0.05$, versus NS-challenged and vehicle-treated mice ($n = 6$, NS-vehicle group); *: $p < 0.05$, OVA-challenged and PIC1—($n = 10$, OVA-PIC1 group), PIC3—($n = 8$, OVA-PIC3 group), PIC10—($n = 9$, OVA-PIC10 group), DEX—($n = 10$, OVA-DEX group) treated mice versus OVA-challenged and vehicle-treated mice ($n = 10$, OVA-vehicle group).

transducer. To measure pleural pressure, a needle (No. 12) with a multiholed tip was directly inserted into pleural cavity 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 signals from all pressure transducers were continuously processed (MedLab, Nanjing Biotech Instruments, China) by fitting flow, volume, and pressure to an equation of motion. Changes in R_L and C_{dyn} from MCh provocation were analyzed by using a dose–response curve to calculate the PD_{50} and PD_{20} values of MCh (the provocative doses of MCh required to increase R_L by 50% and decrease C_{dyn} by 20%, as compared with the baseline). MCh dosages (0.125 mg/kg to 2 mg/kg, i.v.) were described previously (Henry et al., 2002). The effects of piclamilast and dexamethasone were determined by comparing the changes in R_L and C_{dyn} after drug treatment with the mean of MCh response alone in the same mouse on previous and successive control periods.

Preparation of bronchoalveolar lavage fluids

After the last OVA challenge, mice were anesthetized with urethane (2 g/kg, i.p.), and bronchoalveolar lavage fluids (BALF) were obtained via tracheal tube and washing the lung with 0.5 ml of sterilized normal saline containing 1% bovine serum albumin (BSA) and 5000 IU/l heparin three times. BALF was diluted with 1.5 ml of Hank's balanced salt solution (HBSS) containing 2% fetal calf serum, and was centrifuged at $500 \times g$ at 4 °C for 10 min. The supernatants were harvested and stored at –80 °C until measurement of cytokines and chemokines, the pellets were resuspended with HBSS for cell count and classification. Two hundred cells from the cell suspension were stained by Wright–Giemsa and classified under light microscope. The results are expressed as the numbers of each type of cell population in one liter of BALF.

Cytokine and chemokine assays

The concentrations of eotaxin, $TNF\alpha$, $IFN-\gamma$ and IL-4 in BALF were measured by sandwich ELISA using paired matched antibodies according to the manufacturer's instructions. The limits of detection were 4 pg/ml for IL-4, 15.6 pg/ml for $IFN-\gamma$, 10 pg/ml for $TNF\alpha$ and 2 pg/ml for eotaxin.

Assay for cAMP-PDE activity

Cyclic AMP-PDE Activity was assessed as described previously (Tang et al., 2005). Briefly, frozen lungs were thawed and cut into small cubes, and 25 mg (wet weight) of lung tissue was homogenized in ice-cold hypotonic buffer (30 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM β -mercaptoethanol, 2 mM PMSF, 10 g/L pepstatin A, and 0.1% Triton X-100). The cAMP-PDE assay mixture (200 μ l) contains 0.1 M phosphate-buffered saline (pH 7.4, PBS), 1 μ M cAMP, and lung homogenate. The reaction was initiated by addition of 10 μ l lung homogenate and incubation at 37 °C; 10 min later, the reaction was stopped by boiling the mixture for 3 min. Then, 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 HPLC (Hewlett–Packard, Palo Alto, CA, USA). The mobile phase used in this separation consisted of 60% water,

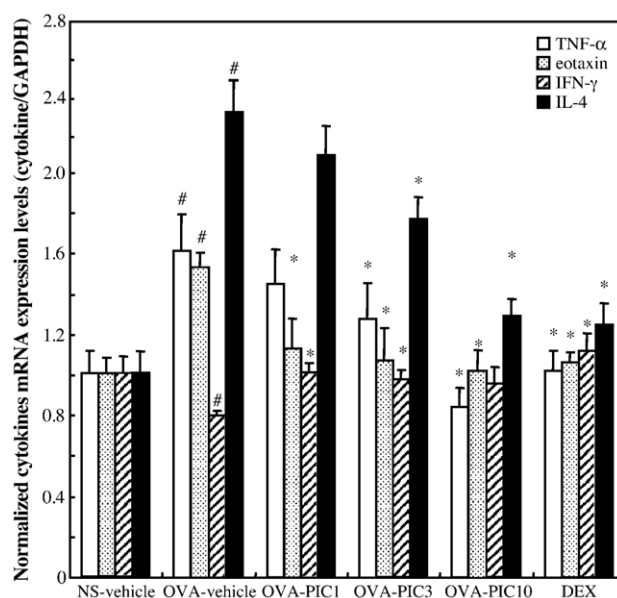


Fig. 5. Effects of piclamilast and dexamethasone on mRNA expression of IL-4, eotaxin, $IFN-\gamma$ and $TNF\alpha$ in the lungs of asthmatic mice. Mice were processed and treated as described in Fig. 1. Twenty-four hours after the last OVA challenge, 100 mg of lung tissues from each mouse were harvested for isolation of total RNA, and 1 μ g aliquots of the total RNA sample was used for reverse transcriptional synthesis of first-strand that was used as template of PCR amplification. The PCR product was analyzed by electrophoresis on 1.5% agarose gels and specific bands were digitized using imaging system. PCR reactions were done in triplicates, and the relative expression of mRNAs from independent triplicate experiments was normalized by dividing the eotaxin and cytokine values by the respective GAPDH values, and the mean levels of mRNA expression from NS-vehicle mice were defined as 1. Results are shown as mean ± SEM. #: $p < 0.05$, versus NS-vehicle group; *: $p < 0.05$, versus OVA-vehicle group.

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 which was 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 standard samples

containing cAMP (retention time, 7.8 min) at 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 the Bradford method using BSA as a standard. The results are expressed as nmol of PDE activity per milligram of protein.

Messenger RNA expression of eotaxin, TNF α , IL-4 and IFN- γ in lungs

A coupled reverse transcription polymerase-chain reaction (RT-PCR) was used to semi-quantify the RNA levels (Fulton et al., 2000) in lung tissues ($n=6$ in each group). The 100 mg lung tissues were homogenized and the total RNA was isolated in 1 ml TRIzol (GIBCO) according to the manufacturer's instructions. For synthesis of first-strand, 1 μ g aliquots of the total RNA sample was used for reverse transcription in 25 μ l final volume with Avian Myeloblastosis Virus (AMV) reverse transcriptase first-strand cDNA synthesis kit (GIBCO) 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) containing 0.2 μ m sense and antisense primers and 200 μ m dNTP mixture, respectively. The following oligonucleotide primers were used: forward primer for eotaxin, 5'-AGAGGCTGAGATCCAAGCAG-3' and reverse primer for eotaxin, 5'-CAGATCTTCTTGCCCAACCT-3'; forward primer for TNF α , 5'-AACTAGTGGTGCCAGCCGAT-3' and reverse primer for TNF α , 5'-CTTCACAGAGCAATGACTCC-3'; forward primer for IL-4, 5'-GCATGTACCAGCAACTTCGT-3' and reverse primer for IL-4, 5'-TTGGCTTCATTACAGAA CAG-3'; forward primer for IFN- γ , 5'-CCTCAGACTCTTT GAAGTCT-3' and reverse primer for IFN- γ , 5'-CAGCGACT CCTTTTCCTCTT-3'; forward primer for housekeeping gene, GAPDH, 5'-CTGGTGCTGAGTATGTCGTG-3' and reverse primer for GAPDH, 5'-CAGTCTTCTGAGTGGCAGTG-3'. Amplification was performed in a thermocycler (Eppendorf, Germany) as follows: 3 min at 95 $^{\circ}$ C, followed by up to 35 cycles of 30 s each at 94 $^{\circ}$ C, 30 s at 55 $^{\circ}$ C, 45 s at 72 $^{\circ}$ C, and a final elongation at 72 $^{\circ}$ C for 10 min. The PCR product was analyzed by electrophoresis on 1.5% agarose gel containing 0.5 μ g/ml of ethidium bromide. Specific bands from UV-transilluminated ethidium bromide-stained agarose gels were digitized by imaging system (UVP Co., England). PCR reactions were done in triplicates, and the relative expression of mRNAs from independent triplicate experiments was normalized by dividing the eotaxin and cytokines values by the respective GAPDH values, and the mean levels of mRNA expression from NS-vehicle mice were defined as 1.

Histological examination

The lungs were infused via trachea with 1 ml of 10% neutral formalin and immersed in the same fixative for 7 days. After tissues were paraffinized, 5 μ m sections were prepared and stained with alician blue-periodic acid-Schiff for identification of mucus containing cells, and with hematoxylin-eosin for observation of cell infiltration under light microscope. To determine the extent of mucus production, goblet cell hyperplasia

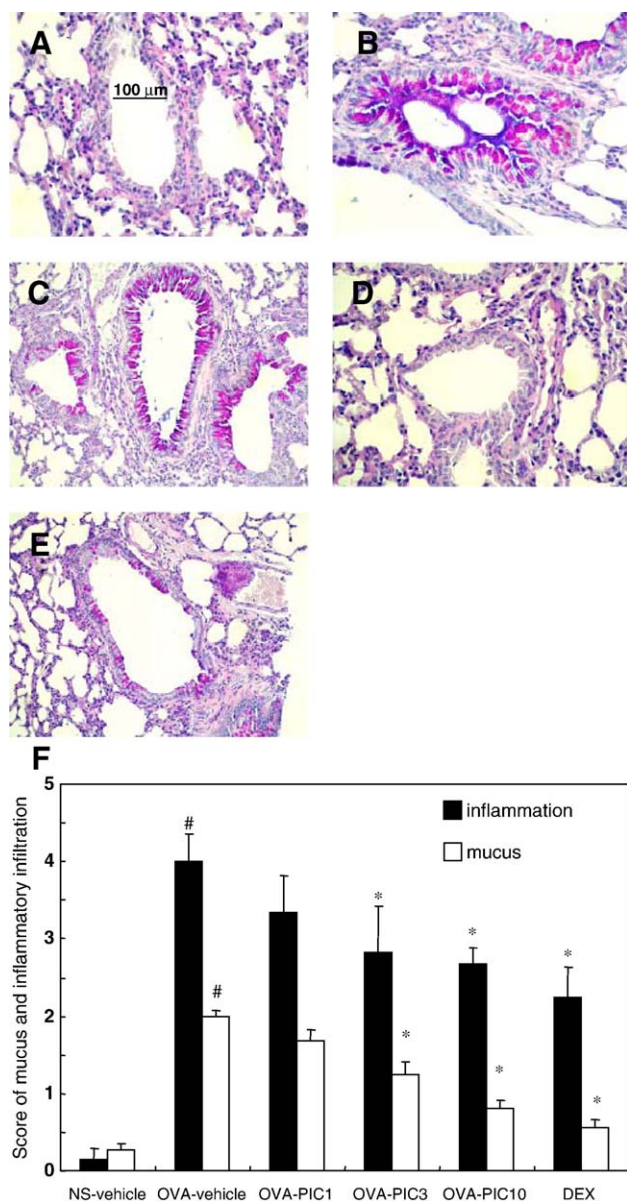


Fig. 6. Effects of piclamilast and dexamethasone on mucus production and eosinophil infiltration in lungs. Histological examination of lung tissue mucus secretion (A–E) and eosinophil infiltration were performed 24 h after the last allergen challenge. A: NS-vehicle group; B: OVA-vehicle group; C: OVA-PIC1 group; D: OVA-PIC10 group; E: OVA-DEX group. Semi-quantitative analysis of mucus production and inflammatory cell infiltration (F) in lung sections were performed as described in Materials and methods. Lung tissues were fixed, sectioned at 5 μ m thickness, and stained with periodic acid-Schiff for mucus production (red are positive for mucus) or H and E for tissue eosinophils infiltration. Results are shown as mean $\pm$ SEM, $n=6$ in each group #: $p<0.05$, versus NS-vehicle group; *: $p<0.05$, versus OVA-vehicle group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in the airway epithelium was blindly semi-quantified using a 5 point grading system described previously (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 inflammatory cell number was blindly counted and the severity was evaluated by a 5 point scoring system described previously (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 derived from six animals.

Statistical analysis

Numerical data are presented as means $\pm$ SEM from six mice. Statistical calculations were performed using SigmaStat software (SigmaStat 2.0, SPSS Inc., Chicago, IL, USA), ANOVA and Student–Newman–Keuls multiple comparisons test were used for calculate the difference of respiratory function (Fig. 1), inflammatory cells (Fig. 2) and cytokine levels (Fig. 3) in BALF, cAMP-PDE activity (Fig. 4), mRNA expression (Fig. 5) in lungs between each group. A non-parametric test, Mann–Whitney *U*-test was used to compare the difference of goblet cell hyperplasia and infiltration of inflammatory cells in airway (Fig. 6F). Significance was assessed at the $p < 0.05$ level. Experiments were performed independently at least twice; results were qualitatively identical, and representative results are shown.

Results

Piclamilast inhibited allergen-induced airway hyperresponsiveness to methacholine

The effect of piclamilast on allergen-induced airway hyperresponsiveness to intravenous MCh was investigated in actively sensitized mice. OVA-sensitized mice were challenged with an aerosol of normal saline (NS) or OVA on seven consecutive days; the airway responsiveness to intravenous MCh at the doses of 0, 0.5, 1.0, 1.5 and 2.0 $\mu\text{g/kg}$ was assessed 24 h after the last challenge. The responsiveness to MCh in NS-challenged mice (NS-vehicle group) showed little change in airway resistance (R_L) and lung dynamic compliance (C_{dyn}); however, OVA-challenged mice (OVA-vehicle group) exhibited an obvious AHR with a significant and dose-dependent increase in R_L and decrease in C_{dyn} as compared with NS-challenged mice (Fig. 1A, B and Table 1). Oral pretreatment with piclamilast at the doses of 1 (OVA-PIC1 group), 3 (OVA-PIC3 group) and 10 mg/kg (OVA-PIC10 group) significantly reduced AHR provoked by MCh in a dose-dependent manner. As a positive control, dexamethasone at the dose of 2 mg/kg (OVA-DEX group) also had a robust effect on reduction of AHR (Fig. 1 and Table 1). The potency of piclamilast at the dose of 10 mg/kg in inhibition of AHR was similar to that of dexamethasone at the dose of 2 mg/kg.

Table 1

Inhibitory effects of piclamilast and dexamethasone on AHR in OVA-sensitized mice

Groups	Dose (mg/kg)	<i>n</i>	R_L PD ₅₀ (95% CL) MCh ($\mu\text{g/kg}$)	C_{dyn} PD ₂₀ (95% CL) MCh ($\mu\text{g/kg}$)
NS-vehicle		8	0.67 (0.57–0.78)	0.99 (0.78–1.24)
OVA-vehicle		14	0.22 (0.20–0.24)	0.30 (0.27–0.35)
OVA-PIC1	1	11	0.28 (0.25–0.30)	0.38 (0.32–0.45)
OVA-PIC3	3	10	0.34 (0.30–0.39)	0.46 (0.38–0.55)
OVA-PIC10	10	11	0.82 (0.63–1.07)	0.73 (0.57–0.94)
Dexamethasone	2	11	1.78 (1.24–2.56)	1.22 (0.93–1.60)

Effects of oral administration of piclamilast and dexamethasone on methacholine (MCh)-induced increase of airway resistance (PD₅₀) and reduction of lung dynamic compliance (PD₂₀) in actively sensitized mice. Sensitized mice react at smaller doses of intravenous methacholine, indicating airway hyperresponsiveness. Data are given as mean (95% confidence limits).

Piclamilast inhibited airway eosinophilia and lung inflammation

To observe the effect of piclamilast on airway inflammatory cell infiltration in antigen-induced and OVA-sensitized mice, 24 h after the last OVA challenge, numbers of inflammatory cells including eosinophils, neutrophils and lymphocytes in the BALF were counted. The total number of inflammatory cells in BALF of OVA-vehicle mice was 22-fold greater than that in NS-vehicle mice. Piclamilast treatment at the doses of 1 and 3 mg/kg did not significantly inhibit the infiltration of inflammatory cells in airways, whereas at the dose of 10 mg/kg, it significantly decreased the total inflammatory cell numbers in BALF, this effect being slightly smaller than seen in DEX-treated mice. Classification of these inflammatory cells indicated that in OVA-vehicle mice, numbers of eosinophils, neutrophils and lymphocytes in the BALF increased 462-, 512- and 6-fold, respectively, as compared with those in NS-vehicle mice (Fig. 2). Pretreatment of OVA-sensitized and -challenged mice with varying doses of piclamilast for 7 days significantly decreased eosinophil and neutrophil numbers in dose-dependent manners. The inhibitory effect of piclamilast at 10 mg/kg on eosinophils was smaller than that of DEX; however, piclamilast at 10 mg/kg had an inhibitory effect on neutrophil numbers similar to DEX at 2 mg/kg. On lymphocytes, piclamilast did not show any obvious inhibitory effect, whereas DEX did significantly decrease lymphocytes in BALF (Fig. 2).

Piclamilast decreased cytokines and chemokine levels in BALF

To determine the levels of cytokines in BALF, BALF was harvested 24 h after the last OVA challenge. IL-4, eotaxin, TNF α , and IFN- γ levels were measured by ELISA. As shown in Fig. 3, in OVA-vehicle mice, IL-4, eotaxin and TNF α levels in BALF increased to 3.1-, 4.2- and 1.8-fold compared to those in NS-vehicle mice. However, IFN- γ level in BALF decreased to 0.6-fold in NS-vehicle mice. Treatment of OVA-sensitized and -challenged mice with either piclamilast or DEX significantly and dose-dependently reduced the IL-4, eotaxin and TNF α levels, and increased IFN- γ level. Inhibition of IL-4 and TNF α levels by piclamilast at 10 mg/kg was similar to that afforded by DEX at 2 mg/kg.

Piclamilast decreased allergen-induced elevation of cAMP-PDE activity in lungs

OVA challenge in actively OVA-sensitized mice leads to an approximately 2-fold increase of cAMP-PDE activity in lungs, as compared with NS-challenged and vehicle-treated mice. Piclamilast inhibited allergen-induced cAMP-PDE activity in a dose-dependent manner, and piclamilast at the dose of 3 mg/kg almost completely abolished the allergen-induced elevation of cAMP-PDE activity, which is similar to the effect of DEX at the dose of 2 mg/kg. The inhibitory effect of piclamilast at 10 mg/kg was greater than that of DEX at 2 mg/kg, and the ID₅₀ of piclamilast for inhibition of cAMP-PDE activity was approximately 3.52 mg/kg (Fig. 4).

Piclamilast inhibited cytokine and chemokine mRNA expression in the lungs

To measure cytokine and chemokine mRNA expression in vivo, lung tissues were harvested 24 h after the last OVA challenge. IL-4, eotaxin, TNF α and IFN- γ mRNA expression were measured by RT-PCR. As shown in Fig. 5, both piclamilast and dexamethasone significantly inhibited the up-regulation of IL-4, eotaxin and TNF α mRNA expression, and the down-regulation of IFN- γ mRNA expression as compared with vehicle-treated mice. The inhibitory effect of piclamilast at 10 mg/kg on IL-4 and eotaxin mRNA expression was similar to that of DEX at 2 mg/kg; inhibition of TNF α mRNA expression was greater than DEX, and up-regulation of IFN- γ mRNA expression was less than DEX at 2 mg/kg.

Piclamilast inhibited OVA-induced mucus production and eosinophil infiltration

Lung tissue was harvested 24 h after the last OVA challenge. OVA-vehicle mice (Fig. 6B) exhibited an obvious goblet cell hyperplasia and mucus hypersecretion within the bronchi in the lung as compared with NS-vehicle mice (Fig. 6A). Piclamilast at the dose of 10 mg/kg (Fig. 6D) and dexamethasone at the dose of 2 mg/kg (Fig. 6E) markedly attenuated OVA-induced mucus secretion as compared with that in vehicle-treated mice (Fig. 6B). Piclamilast at the dose of 1 mg/kg did not show any obvious effect on mucus production (Fig. 6C). Similarly, OVA challenge increased infiltration of inflammatory cells into the peribronchiolar and perivascular connective tissues as compared with in NS-vehicle mice. Piclamilast at the doses of 1 and 10 mg/kg, and dexamethasone at 2 mg/kg markedly inhibited the OVA-induced eosinophil-rich leukocyte infiltration as compared with control mice (Fig. 6A–E). All results indicated that the OVA-induced mucus secretion and the eosinophil-rich inflammatory cell infiltration were significantly reduced by piclamilast at 10 mg/kg and dexamethasone at 2 mg/kg (Fig. 6F).

Discussion

Cyclic AMP plays an important regulatory role in all cell types, and particularly is involved in the pathogenesis of asthma,

as cAMP broadly suppresses the activity of immune and inflammatory cells (Chung, 2006). Intracellular cAMP levels reflect the balance between the generation of cAMP from ATP by adenylyl cyclase and degradation by PDE, and PDE4 has been identified as a predominant PDE in many inflammatory cells, including eosinophils, T cells, and neutrophils. Consequently, PDE4 inhibitors may have potency as a therapeutic intervention in allergic disorders such as asthma (Kanehiro et al., 2001). In the present study, we used a murine model of allergen-induced AHR in previously sensitized mice which closely reflects several important features of human asthma (Drazen et al., 1999; Vargaftig, 1999; Epstein, 2004) to assess the roles of PDE4 in cAMP-PDE enzyme activity, secretion of airway mucus, production of proinflammatory mediators and airway responsiveness of asthma by using a selective PDE4 inhibitor, piclamilast.

The effect of piclamilast on airway function was monitored by changes in airway responsiveness to intravenous MCh, and the results were compared with the effects of administration of DEX. Airway challenge of sensitized mice triggered significant increases in R_L and reductions in C_{dyn} to MCh in a dose-dependent manner. Under this condition, oral pretreatment with piclamilast significantly and dose-dependently prevented increases in R_L and decreases in C_{dyn} , and the effect of piclamilast at the dose of 10 mg/kg was similar to that of DEX at the dose of 2 mg/kg. Our results were consistent with previous study, indicating that piclamilast attenuated allergen-induced bronchoconstriction in guinea pigs in vivo (Bundschuh et al., 2001), and synergized with isoproterenol to attenuate the OVA-induced bronchoconstriction of sensitized guinea pigs, in vitro (Ji et al., 2003).

The precise mechanism whereby PDE4 inhibitors attenuate allergen-induced AHR and inflammation is still not clear. Based on our results, we suggest that the bronchodilatory effect of piclamilast might not result from direct decrease of smooth muscle tension, though a previous study indicated that piclamilast decreased resting tension of bronchial smooth muscle in guinea pigs, in vitro (Ji et al., 2003). However, another previous study showed that piclamilast did not prevent serotonin-induced direct bronchoconstriction in vivo (Wollin et al., in press). We assumed that the bronchodilatory effect of piclamilast resulted from a decreased infiltration of inflammatory cells, and from a consequent decrease of proinflammatory mediator release from inflammatory cells, as some proinflammatory mediators are potent bronchoconstrictors, such as histamine, leukotrienes, ILs and TNF α . This assumption was strongly supported by our observation that in the BALF of allergen-challenged mice, the total number of inflammatory cells, eosinophils, neutrophils and lymphocytes, was markedly increased as compared with that in control mice, and pretreatment with piclamilast significantly and dose-dependently reduced the number of inflammatory cells in BALF.

The inhibitory effect on eosinophils was much greater than that on neutrophils and lymphocytes. Inhibition of eosinophils was further evidenced by histological examination, indicating that piclamilast dose-dependently and significantly decreased infiltration of inflammatory cells into the peribronchiolar and

perivascular connective tissues. The potency of piclamilast at 10 mg/kg on neutrophils is similar to that of DEX at 2 mg/kg; however, piclamilast had no obvious effect on lymphocytes. These results correspond to the concept that asthma is an inflammatory disorder of the airways involving mediator release from mast cells and eosinophils and orchestrated by T cells (Mehats et al., 2003).

Furthermore, we suggested that infiltration of a large amount of inflammatory cells into the airways resulted in excessive production of proinflammatory mediators including cytokines, chemokines and leukotrienes (Grönke et al., 2002). Our data showed that in BALF of allergen-challenged mice, levels of IL-4 and TNF α were strongly increased, and the IFN- γ level was significantly decreased as compared with those in control mice. It is well known that IL-4 plays a crucial role as an inflammatory mediator in allergic asthma through induction of Th2 inflammation and IgE synthesis (Eskandari et al., 2004). TNF α is an important pathogenic mediator of asthma; TNF α regulates the transcription of a host of proinflammatory cytokines, chemokines and adhesion molecules in various leukocytes through nuclear transcription factor nuclear factor- κ B, and further leads to inflammatory cell chemotaxis and increased proinflammatory cell accumulation in the airways, as well as activation of proinflammatory cells and release of cytokines (Thomas, 2001). In contrast to TNF α and IL-4, IFN- γ has been shown in vitro to relax rather than to constrict airway smooth muscle. Eotaxin, one of the chemokines, is a potent eosinophil chemoattractant and is strongly implicated in the pathogenesis of asthma.

Our RT-PCR results indicated that both piclamilast and DEX dose-dependently and significantly down-regulated TNF α , IL-4 and eotaxin mRNA expression, and up-regulated IFN- γ mRNA expression in lungs. Therefore, we suggest that suppression of TNF α , IL-4, eotaxin mRNA expression and induction of IFN- γ mRNA expression resulted in the decrease of IL-4, TNF α and eotaxin protein levels, and increase of IFN- γ protein levels in BALF. However, the precise mechanism of action of piclamilast on these mRNA expressions remains obscure.

We supposed that inhibition of PDE4 activity by piclamilast in inflammatory and immune cells led to elevation of cAMP contents in these cells, which subsequently influenced the activity of inflammatory and immune cells and mRNA expression of inflammatory mediators. Although up-regulation of lung PDE4 has been demonstrated in allergic rats (Tang et al., 2005), lung PDE up-regulation in the mouse model of asthma has not been reported to our knowledge. Our cAMP-PDE measurement in lung suggested that in allergen-challenged mice, cAMP-PDE activity was significantly increased, and pretreatment with piclamilast dose-dependently and significantly decreased cAMP-PDE activity. Although the decrease of cAMP levels in allergen-challenged mice may result from the up-regulation of PDE activities in general, pretreatment with the selective PDE4 inhibitor piclamilast at 10 mg/kg completely abolished allergen-induced PDE activity, suggesting that in allergen-challenged mice, PDE4 played a predominant role in inflammatory reaction rather than other PDE subtypes.

Nothing is known about the role of cAMP-PDE enzyme activity in lung in asthmatic progression; however, our result in the mouse and rat model (Tang et al., 2005), demonstrated that PDE4 activity was up-regulated in the allergic asthmatic lung, evidenced by using a selective PDE4 inhibitor, piclamilast, suggesting that PDE4 played key roles in regulating compartmentalized signaling responses to intracellular gradients of cAMP (Houslay and Adams, 2003). In the rat model, the up-regulation of PDE4 mRNAs suggested that the increased lung cAMP-PDE activity was, at least in part, caused by increased PDE4 gene expression (Tang et al., 2005). In the mouse model, though it is not exactly known how piclamilast or dexamethasone attenuated the PDE enzyme activity, one possibility for both drugs was that they might reduce PDE gene expression and/or activation through their extensive anti-inflammatory activities. Further studies should be designed to elucidate this hypothesis.

In allergen-challenged mice, obvious increases of IL-4 levels in BALF and IL-4 mRNA expression in lungs were observed. It has been reported that IL-4 is essential for mucus production in sensitized and challenged mice (Tomkinson et al., 1999), and that induction of goblet cell hyperplasia might be an important factor in the development of altered dynamic compliance (Miotla et al., 1998; Blyth et al., 1998). Our histological results suggested that piclamilast significantly inhibited the mucus production in lungs, which corresponded previous reports that PDE4 inhibitor and glucocorticoid attenuated 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). Though it is not clear whether PDE4 inhibitors attenuated mucus hypersecretion or sputum volume in patients with asthma or COPD (Lipworth, 2005), we suggest that the suppressive effect of piclamilast on IL-4 production in the airways is likely to modulate the changes in the epithelium of peripheral airways and goblet cell hyperplasia, which resulted in the attenuation of hypersecretion in asthmatic airways.

Conclusion

In the present study, we investigated the roles of PDE4 in AHR, inflammation and goblet cell hyperplasia by using piclamilast, a selective PDE4 inhibitor, in an antigen-challenged and previously sensitized murine model of asthma. In this asthma model, significant increase of cAMP-PDE activity in lung was parallel to the production of proinflammatory mediators, to the increase of AHR, airway goblet cell hyperplasia and inflammatory cell infiltration. Inhibition of PDE4 activity by piclamilast exhibited novel effects in terms of improvement of respiratory function, anti-inflammation and decrease of goblet cell hyperplasia. This study suggested that PDE4 plays a key role in the pathogenesis of allergen-induced murine asthma, and provides a rationale for the treatment of asthma by using selective PDE4 inhibitors.

Acknowledgements

This work was supported by the National Natural Science Foundation of China, No.30371314. We are grateful for the piclamilast from Prof. Cong-lin Zuo and Beijing Joinn Drug Research Center. We also thank Dr. M. J. Hilton for manuscript revision.

References

- Blyth, D.I., Pedrick, M.S., Savage, T.J., Bright, H., Beesley, J.E., Sanjar, S., 1998. Induction, duration, and resolution of airway goblet cell hyperplasia in a murine model of atopic asthma: effect of concurrent infection with respiratory syncytial virus and response to dexamethasone. *American Journal of Respiratory Cell and Molecular Biology* 19 (1), 38–54.
- Bundschuh, D.S., Eltze, M., Barsig, J., Wollin, L., Hatzelmann, A., Beume, R., 2001. In vivo efficacy in airway disease models of roflumilast, a novel orally active PDE4 inhibitor. *Journal of Pharmacology and Experimental Therapeutics* 297 (1), 280–290.
- Chung, K.F., 2006. Phosphodiesterase inhibitors in airway diseases. *European Journal of Pharmacology* 533 (1), 110–117.
- Drazen, J.M., Takebayashi, T.N., Long, C., De Sanctis, G.T., Shore, S.A., 1999. Animal models of asthma and chronic bronchitis. *Clinical and Experimental Allergy* 29 (Suppl 2), 37–47.
- Duan, W., Chan, J.H., Wong, C.H., Leung, B.P., Wong, W.S., 2004. Anti-inflammatory effects of mitogen-activated protein kinase inhibitor U0126 in an asthma mouse model. *The Journal of Immunology* 172 (11), 7053–7059.
- Epstein, M.M., 2004. Do mouse models of allergic asthma mimic clinical disease? *International Archives of Allergy and Applied Immunology* 133 (1), 84–100.
- Eskandari, N., Wickramasinghe, T., Peachell, P.T., 2004. Effects of phosphodiesterase inhibitors on interleukin-4 and interleukin-13 generation from human basophils. *British Journal of Pharmacology* 142 (8), 1265–1272.
- Fulton, S.A., Martin, T.D., Redline, R.W., Henry Boom, W., 2000. Pulmonary immune responses during primary mycobacterium bovis–Calmette–Guerin bacillus infection in C57Bl/6 mice. *American Journal of Respiratory Cell and Molecular Biology* 22 (3), 333–343.
- Georas, S.N., 2004. Inhaled glucocorticoids, lymphocytes, and dendritic cells in asthma and obstructive lung diseases. *Proceedings of American Thoracic Society* 1 (3), 215–221.
- Grönke, L., Kanniss, F., Holz, O.R., Jörres, A., Magnussen, H., 2002. The relationship between airway hyper-responsiveness, markers of inflammation and lung function depends on the duration of the asthmatic disease. *Clinical and Experimental Allergy* 32 (1), 57–63.
- Hatzelmann, A., Schudt, C., 2001. Anti-inflammatory and immunomodulatory potential of the novel PDE4 inhibitor roflumilast in vitro. *Journal of Pharmacology and Experimental Therapeutics* 297 (1), 267–279.
- Henry, P.J., Mann, T.S., D'Aprile, A.C., Self, G.J., Goldie, R.G., 2002. An endothelin receptor antagonist, SB-217242, inhibits airway hyperresponsiveness in allergic mice. *American Journal of Physiology. Lung Cellular and Molecular Physiology* 283 (5), L1072–L1078.
- Holgate, S.T., Lackie, P.M., Davies, D.E., Roche, W.R., Walls, A.F., 1999. The bronchial epithelium as a key regulator of airway inflammation and remodeling in asthma. *Clinical and Experimental Allergy* 29 (S2), 90–95.
- Houslay, M.D., Adams, R.D., 2003. PDE4 cAMP phosphodiesterases, modular enzymes that orchestrate signalling cross-talk, desensitization and compartmentalization. *The Biochemical Journal* 370 (Pt 1), 1–18.
- Ji, H., Xie, Q.M., Chen, J.Q., 2003. Comparison of piclamilast with ciclamilast in bronchodilating and antiallergic effects. *Zhejiang Da Xue Xue Bao Yi Xue Ban* 32 (4), 274–278.
- Kanehiro, A., Ikemura, T., Makela, M.J., Lahn, M., Joetham, A., Dakhama, A., Gelfand, E.W., 2001. Inhibition of phosphodiesterase 4 attenuates airway hyperresponsiveness and airway inflammation in a model of secondary allergen challenge. *American Journal of Respiratory and Critical Care Medicine* 163 (1), 173–184.
- Kasper, M., Sims, G., Koslowski, R., 2002. Increased surfactant protein D in rat airway goblet and Clara cells during ovalbumin-induced allergic airway inflammation. *Clinical & Experimental Allergy* 32 (8), 1251–1258.
- Kuperman, D.A., Huang, X.L., Koth, L., Chang, G.H., Dolganov, G.H., Zhu, Z., Elias, J.A., Sheppard, D., Erle, D.J., 2002. Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. *Nature Medicine* 8 (8), 885–889.
- Lipworth, B.J., 2005. Phosphodiesterase-4 inhibitors for asthma and chronic obstructive pulmonary disease. *Lancet* 365 (9454), 167–175.
- Mata, M., Sarria, B., Buenestado, A., Cortijo, J., Cerda, M., Morcillo, E.J., 2005. Phosphodiesterase 4 inhibition decreases MUC5AC expression induced by epidermal growth factor in human airway epithelial cells. *Thorax* 60 (2), 144–152.
- McMillan, S.J., Xanthou, G., Lloyd, C.M., 2005. Therapeutic administration of Budesonide ameliorates allergen-induced airway remodelling. *Clinical & Experimental Allergy* 35 (3), 388–396.
- Mehats, C., Jin, S.L., Wahlstrom, J., Law, E.D., Umetsu, T., Conti, M., 2003. PDE4D plays a critical role in the control of airway smooth muscle contraction. *The FASEB Journal* 17 (13), 1831–1841.
- Miotla, J.M., Teixeira, M.M., Hellewell, P.G., 1998. Suppression of acute lung injury in mice by an inhibitor of phosphodiesterase type 4. *American Journal of Respiratory Cell and Molecular Biology* 18 (3), 411–420.
- Spina, D., 2003. Phosphodiesterase-4 inhibitors in the treatment of inflammatory lung disease. *Drugs* 63 (23), 2575–2594.
- Tang, H.F., Song, Y.H., Chen, J.C., Chen, J.Q., Wang, P., 2005. Upregulation of phosphodiesterase-4 in the lung of allergic rats. *American Journal of Respiratory and Critical Care Medicine* 171 (8), 823–828.
- Thomas, P.S., 2001. Tumor necrosis factor- α : the role of this multifunctional cytokine in asthma. *Immunology and Cell Biology* 79 (2), 132–140.
- Tomkinson, A., Kanehiro, A., Rabinovitch, N., Joetham, A., Cieslewica, G., Gelfand, E.W., 1999. The failure of STAT6 deficient mice to develop airway eosinophilia and airway hyperresponsiveness is overcome by IL-5. *American Journal of Respiratory and Critical Care Medicine* 160 (4), 1283–1291.
- Torphy, T.J., 1998. Phosphodiesterase isozymes: molecular targets for novel antiasthma agents. *American Journal of Respiratory and Critical Care Medicine* 157 (2), 351–370.
- Trifilieff, A., El-Hashim, A., Bertrand, C., 2000. Time course of inflammatory and remodeling events in a murine model of asthma, effect of steroid treatment. *American Journal of Physiology. Lung Cellular and Molecular Physiology* 279 (6), L1120–L1128.
- Vargaftig, B.B., 1999. What can we learn from murine models of asthma? *Clinical and Experimental Allergy* 29 (Suppl 1), 9–13.
- Wollin, N., Bundschuh, D.S., Wohlsen, A., Marx, D., Beume, R., in press. Inhibition of airway hyperresponsiveness and pulmonary inflammation by roflumilast and other PDE4 inhibitors. *Pulmonary Pharmacology and Therapeutics* (Electronic publication ahead of print, 27 Oct., 2005).
- Xie, Q.M., Chen, J.Q., Shen, W.H., Yang, Q.H., Bian, R.L., 2002. Effects of cyclosporin A by aerosol on airway hyperresponsiveness and inflammation in guinea pigs. *Acta Pharmacologica Sinica* 23 (3), 243–247.
- Xie, Q.M., Chen, J.Q., Shen, W.H., Yang, Q.H., Bian, R.L., 2003. Comparison of bronchodilating and antiinflammatory activities of oral formoterol and its (R,R)-enantiomers. *Acta Pharmacologica Sinica* 24 (3), 277–282.
- Zhao, Y., Zhang, H.T., O'Donnell, J.M., 2003. Inhibitor binding to type 4 phosphodiesterase (PDE4) assessed using [^{3} H]piclamilast and [^{3} H]rolipram. *Journal of Pharmacology and Experimental Therapeutics* 305 (2), 565–572.