The role of PDE4 in pulmonary inflammation and goblet cell hyperplasia in allergic rats

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Abstract

Phosphodiesterase 4 (PDE4) has been suggested to a critical factor in the pathogenesis of inflammation by metabolizing cAMP in human leukocytes, endothelium and epithelium. The present study aimed at evaluating the PDE4 activity and expression, the relationship between the inflammation and cAMP-activity in the lungs, and potential interventions of PDE inhibitors and antiinflammatory drugs in the reduction of lung inflammation and goblet cell hyperplasia in allergic rats. The total leukocyte number and eosinophil number in bronchoalveolar lavegar fluid and PDE4 activity and expression in lungs significantly increased in OVA-sensitized and challenged allergic rat. Lung histology showed an increased infiltration of inflammatory cells in the perivascular and peribronchial spaces, structure changes and goblet cell hyperplasia in the OVA-sensitized and -challenged allergic rats. A significant correlation was observed between the increases in cAMP-PDE activity and inflammation in the lung. Those OVA-induced changes were prevented by pretreatment with PDE inhibitor in a dose-related patterns and with glucocorticosteroid. We found an increase in the proportion of PDE4 and PDE4 gene expression, while a decrease in the proportion of PDE3 in the lung of allergic rats. Incubation with different PDE inhibitors down-regulated OVA-induced cAMP hydrolysis. Our data suggest that PDE4C may play an important role in the airway inflammation, remodeling and goblet cell hyperplasia after repeated challenge of sensitized rats.

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1. Introduction

Cyclic nucleotide phosphodiesterases (PDEs) represent critical pathways for the degradation of the ubiquitous intracellular second messenger 3′, 5′-cyclic adenosine monophosphate (cAMP) and/or 3′, 5′-cyclic guanosine monophosphate (cGMP), involved in regulation of cellular functions [1]. PDEs comprise eleven biochemically and pharmacologically distinct enzyme families (PDEs 1–11), of which PDE4 is cAMP-specific and encoded by four genes (A, B, C, D) [2]. Various isoforms of PDE4 have been shown to interact with other proteins and lipids, allowing specific isoforms to be targeted to distinct intracellular sites and signaling complexes within cells [3]. Experimental and clinical studies demonstrated that inhibition of PDE4 can attenuate cell inflammatory response and bronchial hyperresponsiveness [4–8]. It has been suggested that PDE4 inhibitors may be a potential therapy for chronic airway diseases such as asthma and chronic obstructive lung disease, since treatment with PDE4 inhibitors showed better effects of antiinflammation and immunomodulation [2,9].

Although PDE4 activity was noted to be increased in circulating leukocytes in asthmatic or allergic patients [10–15], the role of PDE4 activity in the pathogenesis of asthma and/or allergy remains unclear. While others observed no significant change of PDE4 activity in such patients [16–18]. Recently, Singh SP et al. reported that prenatal exposure to
cigarette smoke affected the airway reactivity by modulating levels of cAMP in the lung through changes in PDE-4D activity [19], indicating the importance of altered PDE4 activity. However, there is still a need for understanding the role of lung PDE4 activity in the development of allergic asthma. Our previous study demonstrated that cAMP-PDE activity was elevated in lung tissue harvested from rats sensitized and challenged with ovalbumin (OVA) [20]. In order to understand the involvement of PDE4 in antigen-induced airway inflammation and remodeling, the mRNA expression of PDE4 subtypes and PDE4 activity in the lung were investigated in a rat model of asthma after intratracheal exposure of OVA. We also evaluate the potential effects of cAMP-PDE or cyclooxygenase activity on the development of antigen-induced airway inflammation, goblet cell hyperplasia, and lung damage, by using a selective PDE4 inhibitor piclamilast (RP73401, N-(3,5-dichloropyrid-4-yl)-3-cyclopentyl-oxy-4-methoxy benzamide) and a non-selective cyclooxygenase inhibitor indomethacin, to compare with those of antiinflammatory glucocorticosteroid dexamethasone.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats, weighing 150–170 g (Shanghai Laboratory Animal Center, Chinese Academy Sciences, Grade II, certificate No., 22-9601018), were maintained on diets free of OVA. All experimental animals used in this study were under a protocol approved by the Zhejiang Medical Laboratorial Animal Administration Committee of China.

2.2. Drugs

Piclamilast, Siguazodan (SK and F 94836) and theophylline were kindly provided by Beijing Joinn Drug Research Center, Professor Han-Liang Zhou (Medical School of Zhejiang University), and MiniSheng Pharmaceutical Co Ltd. (HangZhou, China). Rolipram, indomethacin, ovalbumin (Grade V), cAMP, cGMP, phenyl methyl sulfonyl fluoride (PMSF), pepstatin A were purchased from Sigma (Shanghai, China). Dexamethasone sodium phosphate (Dex) was purchased from Zhejiang Xuanju Pharmaceutical Co Ltd., Xuanju, China, methylhydroxypropyl cellulose (MC) from Zhongmei Huadong Pharmaceutical Co Ltd. (HangZhou, China), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) from Sangon Biological Engineering Technology and Service Co Ltd. (Shanghai, China), Coomassie brilliant blue protein reagents from Jiancheng Bio-Tek Corporation (Nanjing, China), and methanol from Siyou Pharmaceutical Co Ltd. (Tianjing, China).

2.3. OVA sensitization and challenge

The procedure of animal sensitization and challenge was modified on the basis of the previous study [21] and the experimental protocol is shown in Fig. 1. In short, 0.5 mL of an allergen solution containing 0.2% OVA mixed with 10% aluminium hydroxide gel was injected intraperitoneally, while another 0.5 mL was divided nine intracutaneous injection sites in the proximity of lymph nodes in the paws, lumbar regions and the neck on day 0. For the preparation of the drugs suspensions, the solution of 1% MC was used. The solution of piclamilast (0.1, 1, 3 or 10 mg/kg, respectively), dexamethasone (0.3 mg/kg), indomethacin (3 mg/kg), or 1% MC for same volume was intratracheally administered at the volume of 2 mL/100 g (body weight) for 7 days once a day from 14 days and on after the sensitization of OVA. Thirty minutes after each administration of pretreatments, rats were placed in an arosoil chamber and arosolly exposed to either saline (NS) or 1% OVA, generated in a jet nebulizer (particle size 1–5 μm; PARI MASTER, Starnberg, Germany) for 20 min.

2.4. Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed by gently instilling D-Hank's solution into the lung via a tracheal catheter followed by withdrawal. Such process was repeated three times with 5 mL D-Hank's as total volume. Total BAL cells counts were determined with a hemacytometer. Differential cells were counted after slides were prepared by centrifuged at 1500 rpm for 10 min and staining with Wright stain. The number of total cells for each sample was then determined according to the volume of BAL recovered.

2.5. Histology

The lungs were harvested and fixed in the buffered formalin (10%, pH 7.0) for 48 h. The samples were embedded in paraffin and dehydrated in 70 to 100% ethanol/xylene. Sections were cut at 2–4 μm thickness, deparaffinized, and stained with hematoxylin and eosin. Pathological changes in the lungs were assessed, including the number of eosinophil influx, tissue edema, and epithelial lesion. Inflammatory changes were graded as normal (scale 0), rare (scale 1), mild (scale 2), moderate (scale 3), severe (scale 4), as described previously [21]. To analyze airway remodeling, the thickness of the bronchiolar wall was measured by a pathologic diagram-writing analyzing system (HPIAS-1000, Wuhan Champion Image Technology Corporation Limited, Wuhan, China). Goblet cells containing the mucous were identified by staining with Alcian Blue-periodic acid Schiff (AB/PAS). Positive cells with purple/magenta color were counted by using Olympus CH-20 photomicroscope at ×400 and expressed as the number of goblet cells per mm of epithelial layer. Histopathological assessment was performed blind on randomized sections.

2.6. Tissue homogenates

The lung right lobes were removed and homogenated (DY89-I Homogenater, Xinzhi SCI TECH research institute, Linbo, China) in ice-cold hypotonic homogenization buffer and centrifuged (Eppendorf Centrifuge, Xinzhi SCITECH research institute, Linbo, China) in ice-cold hypotonic homogenization buffer and centrifuged (Eppendorf Centrifuge, 5804R, Germany) at 12,850×g for 30 min at 4 °C. The buffer solution contained 30 mmol/L HEPES, pH 7.4, 1 mmol/L Ethylenediaminetetraacetic Acid (EDTA), 1 mmol/L β-mercaptoethanol, 2 mmol/L PMSF and 10 g/L pepstatin A (0.1% Triton X-100). The supernatants were collected and stored at −80 °C until the further assay PDE activity.

2.7. Assay of PDE activity

The PDE activity was analyzed by a modified method of the previous study [20] at RP-HPLC (HP1100, agilent, USA). A 10-μL sample was added to the Eppendorf tubes. Each sample has an inactive copy for control. After addition of PBS (mmol/L−1: NaCl 137, KCl 2.7, Na2HPO4 8.8, KH2PO4 1.5, CaCl2 1.0, and MgCl2 1.0; pH=7.4), and 10 mmol/L cAMP and CCM 20 μL, respectively, the reaction was allowed to proceed under the incubation at 37 °C for 10 min and stopped in boiling water for 2 min. The supernatants were collected, and centrifuged at 12,850×g for 30 min at 4 °C. The amount of cAMP present in 50 μL supernatant was determined by RP-HPLC analysis (Hypersil ODS 4.0×250
mm, Hewlett-Packard, Palo Alto, CA) using a standard curve of cAMP. The PDE inhibitors, theophylline (non-selective), rolipram (PDE4-selective) and siguazodan (PDE3-selective) were used to inhibit the cAMP-PDE activity to analyze the component of cAMP-PDE activity in the lung. Protein was determined by the Bradford method with bovine serum albumin as a standard [22]. The results were expressed as nanomolar of PDE activity per milligram of protein.

2.8. Analysis of PDE4 subtype mRNAs

Total RNA was isolated from the tissue using TRizol Reagent (Invitrogen, Carlsbad, CA). Preparation of first-strand cDNA from rat was performed using First-strand cDNA Synthesis kit (Shanghai Sangon Biological Engineering Technology and Service, Shanghai, China). Sequences of PCR primer sets used for PDE4 were used as reported previously, each of the PCR primer sets was able to detect all known variants derived from the appropriate PDE4 gene [23,24]. PCR amplification was performed in a PCR buffer [10 mM Tris–HCl, pH 9.0, 100 mM KCl, 80 mM (NH4)2SO4 and 0.1% NP-40] containing each dNTP at 0.2 mM, 1.5 mM MgCl2, each primer at 500 nM, and 1 U of Taq DNA polymerase (Sangon) in a total reaction volume of 25 μL for 30 cycles, with the following cycle parameters: denaturing 94 °C for 45 s, annealing 56 °C for 70 s and extension 72 °C for 2 min. After PCR amplification, 8 μL of each reaction mixture was resolved by electrophoresis on a 1.5% agarose gel containing ethidium bromide, and the PCR product bands were quantified by using UVP Gel Documentation system (UVP, Upland, CA). The levels of PDE4 mRNAs were calculated relative to β-actin. Under the condition, the PCR product accumulation did not reach plateau levels (data not shown).

2.9. Statistics

All data were presented as Mean±S.E.M. One-way ANOVA analysis was used to evaluate the levels of cAMP-PDE activities, bronchial wall thickness and goblet cell hyperplasia. Statistical Package for the Social Sciences (SPSS) 11.0 was used to evaluate the histological score. A probability of P<0.05 was considered significant.

3. Results

There was no significant difference in histological measurements between animals sensitized and challenged with saline and challenged with OVA or sensitized with OVA and challenged with saline (data not shown). Data from those animals were pooled as controls. The number of total cells (Fig. 2A) and eosinophils (Fig. 2B) in BALF significantly increased in OVA-sensitized and challenged rats pretreated with MC, as compared to controls (P<0.01, respectively). Pretreatment with piclamilast and dexamethasone significantly prevented from OVA-induced increased number of both total leukocytes and eosinophils in BAL fluid (P<0.05 or less), as compared to OVA-sensitized and challenged animals pretreated with MC, while indomethacin showed inhibitory effects only on number of leukocytes (Fig. 2A). Number of total leukocytes and eosinophils in BAL fluid of animals pretreated with piclamilast at the concentrations of 0.3 mg/kg and indomethacin was significantly higher than that in controls. Number of infiltrated eosinophils (Fig. 3A), scores of tissue edema (Fig. 3B) and epithelial damage (Fig. 3C) in the lung tissue significantly increased in OVA-sensitized and challenged animals pretreated with MC, as compared to controls (P<0.05, respectively). Pretreatment with piclamilast showed significant inhibitory effects on OVA induced these changes at the concentrations of 1 and 3 mg/kg with a dose-related pattern. OVA-animals pretreated with dexamethasone had similar levels to controls, significantly different from OVA-sensitized and -challenged animals pretreated with MC (P<0.01, Fig. 3A, B and C). Animals with indomethacin had significantly lower number of infiltrated eosinophils than OVA-animals with MC (Fig. 3A), but still significantly higher than controls (P<0.05, respectively). Pretreatment with indomethacin had no effects on OVA-induced lung tissue edema (Fig. 3B) and epithelial damage (Fig. 3C).

Levels of bronchial wall thickness and the number of goblet cells were significantly higher in OVA-challenged animals pretreated with MC, as compared to controls (P<0.05, Table 1), while pretreatment with dexamethasone significantly prevented from OVA-induced bronchial thickness (P<0.05, vs. OVA-challenged rats pretreated with MC), rather than by piclamilast at the low concentrations and indomethacin. Pretreatment with either piclamilast or dexamethasone significantly prevented OVA-induced increased number of goblet cells, as compared to OVA-challenged animals pretreated with MC (P<0.05 or 0.01, respectively, Table 1).

In the lung histology, increased number of infiltrated eosinophils was observed in peri-bronchial (Fig. 4B) and perivascular (Fig. 4F) tissues of OVA-challenged animals pretreated with MC. In OVA-animals with MC, monolayer of epithelial cells on the small airway became multiple and thicker (Fig. 5B) and goblet cell hyperplasia contained both acid (purple color) and neutral (magenta) mucins (Fig. 5F). These OVA-induced histological alterations were obviously prevented by pretreatment with dexamethasone (Figs. 4D and H, 5D and H). Piclamist at higher doses (3 mg/kg) inhibited the influx of inflammatory cells in the lung tissue (Fig. 4C and G) and thickening of the bronchiolar wall in small bronchial (Fig. 5C). Pretreatment with piclamilast at all doses had partially inhibitory effects on goblet cell hyperplasia (Fig. 5G), while indomethacin did not have inhibitory effects on all changes (Figs. 4E and I, 5E and I).

The cAMP-PDE activity significantly increased in the lung tissue of OVA-sensitized and challenged rats pretreated with MC, compared with controls (P<0.01, Fig. 6). Pretreatment with piclamilast showed an inhibitory effects on lung cAMP-PDE with a dose-dependent pattern. The similar effects of dexamethasone (0.3 mg/kg) to piclamilast at the dose of 3 mg/kg on the inhibition of lung cAMP-PDE activities were noted with a significant different from OVA rats with MC (P<0.05, respectively, Fig. 6), but not indomethacin. In order to investigate the potential effects of PDE inhibitors on OVA-induced cAMP-PDE activities, theophylline as a non-selective PDE inhibitor, rolipram as a PDE4-selective inhibitor and siguazodan as a PDE3-selective inhibitor were incubated with the lung homogenates from controls or OVA-sensitized and challenged rats pretreated with MC. Fig. 7 shows that these compounds significantly inhibited cAMP-PDE activity at a concentration-related pattern. Theophylline inhibited the hydrolysis of homogenates in the lung from controls or OVA-challenged rats with a parallel potency. The inhibited hydrolysis of cAMP was 69% and 84% by theophylline at the
concentration of $10^{-3}$ M (Fig. 7A) in controls and OVA-challenged rats, 40% and 70% by rolipram at $10^{-4}$ M (Fig. 7B), and 47% and 20% by siguazodan at $10^{-4}$ M (Fig. 7C), respectively.

Fig. 8 demonstrates lung mRNA levels of PDE4 subtypes measured by semi-quantitative PCR in controls and OVA-sensitized and challenged rats. A moderate elevation of PDE4A and 4B mRNA expression in the lung harvested from OVA-sensitized and challenged rats, as shown in Fig. 8A. Expression of PDE4C mRNA significantly increased after OVA sensitization and challenge, as compared with controls ($P < 0.01$, Fig. 8B). There was a significant correlation between lung cAMP-PDE activity and the number of leukocytes and eosinophils in BAL fluid (Spearman’s Correlation Coefficient=0.836, $P < 0.01$) and in the lung tissue ($P < 0.05$).

4. Discussion

Airway inflammation is a critical feature of asthma, involving mediator release from mast cells and eosinophils and orchestrating by T cells. Eosinophil infiltration is a prominent feature in the pathophysiology of asthma. In addition, asthma is also characterized by pronounced structural changes of the bronchial wall associated with inflammatory process and termed as airway remodeling, such as damage and shedding of the airway surface epithelium, thickness of the epithelial reticular basement membrane [25,26]. While excess mucus secretion not only obstructs airways but also contributes to airway hyperresponsiveness [27,38]. Potential role of PDE4 in the development of allergic diseases has been investigated only in human blood leukocytes. Our previous study demonstrated that cAMP-PDE activity was significant increased in lung homogenate harvested from antigen-sensitized and challenged rats [20]. The results from the present study furthermore suggested that the increase of cAMP-PDE activity in the lung of OVA-sensitized and -challenged rat may result from the increased activity of PDE4, since the significant increase in PDE4 mRNA expression was noted after antigen challenges.

Little has been known about the mechanism by which PDE4 gene expression was up-regulated and about the expression of various PDE4 subtype genes in the lungs after antigen challenges, although it was shown that different PDE4 subtype genes can undergo distinct regulation [28–30]. cAMP has been found to induce the expression of various PDE4 subtype genes including 4D5 [28]. In transiently transfected

Table 1

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose</th>
<th>n</th>
<th>Bronchial wall thickness (μm)</th>
<th>Number of Goblet cell/mm epithelium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>Saline 4 mL/kg</td>
<td>7</td>
<td>0.4±0.0</td>
<td>4.9±1.7</td>
</tr>
<tr>
<td>MC-treated OVA rats</td>
<td>Saline 4 mL/kg</td>
<td>7</td>
<td>0.6±0.0</td>
<td>38.8±7.1**</td>
</tr>
<tr>
<td>Pilocamist-treated OVA rats</td>
<td>0.3 mg/kg</td>
<td>6</td>
<td>0.6±0.1</td>
<td>22.9±2.8*</td>
</tr>
<tr>
<td></td>
<td>1 mg/kg</td>
<td>6</td>
<td>0.5±0.0</td>
<td>18.1±5.3*</td>
</tr>
<tr>
<td></td>
<td>3 mg/kg</td>
<td>6</td>
<td>0.4±0.0</td>
<td>23.2±3.8*</td>
</tr>
<tr>
<td>Dexamethasone-treated OVA rats</td>
<td>0.3 mg/kg</td>
<td>6</td>
<td>0.3±0.0*</td>
<td>12.2±1.8**</td>
</tr>
<tr>
<td>Indomethacin-treated OVA rats</td>
<td>3 mg/kg</td>
<td>7</td>
<td>0.7±0.1</td>
<td>46.1±4.2</td>
</tr>
</tbody>
</table>

+ and ++ stand for the $P$ values less than 0.05 and 0.01, respectively, as compared to the control group. * and ** stand for the $P$ values less than 0.05 and 0.01, respectively, as compared to OVA-challenged animals treated with vehicle.
into β2 adrenoreceptor-expressing CHO-K1 cell line, Houslay et al. demonstrated that the PDE4D5 promoter up-regulated reporter gene expression in response to increased cell cAMP [3]. Increased cAMP-dependent induction of PDE4D5 transcript and activity in primary cultured human airway smooth muscle cells (hASMs) led to an up-regulation of functional activity [28]. The inflammatory mediators, lipopolysaccharide, specifically activates PDE4B gene and PDE4 activity in peripheral monocytes and neutrophils harvested from mice deficient in PDE4 (type 4 cAMP-specific PDE)-B and PDE4D [29,30]. Such response was completely absent in mice deficient in PDE4B but not PDE4D, indicating that PDE4B gene activation by LPS may constitute a feedback regulation essential for an efficient immune response. Furthermore, experimental studies also demonstrated that cigarette smoking induced chronic pulmonary pathophysiological features in mice, accompanied by an increased PDE4D gene expression in the lung [31]. It is possible that the expression and activation of PDE4 subtypes are highly associated with the pathological situations. Our results showed that challenge with antigen could obviously up-regulate expression of the PDE 4C gene, rather than 4A, 4B and 4D genes. It indicates that the mechanism by which antigen stimulates these gene expressions via cAMP signaling might be distinct from other
challenges. The present study furthermore confirmed the up-regulation of gene expression and overactivation of PDE4 in the lung tissues harvested from antigen challenged rats, obviously correlated with the development of antigen-induced lung inflammation. Of its subtypes, PDE4C maybe play an important role in PDE4-involved inflammation.

Piclamilast as a selective PDE 4 inhibitor has been shown to inhibit eosinophil adhesion and migration in the in vitro system with a dose-dependent pattern [32], have potent and long-acting effects on relaxation of human bronchial muscle [33], and modulate tissue remodeling associated with lung inflammatory processes including asthma [34]. The results from the present study demonstrated that pretreatment with piclamilast significantly attenuated eosinophilic infiltration and sequential goblet cell hyperplasia induced by OVA in a dose-related manner. The inhibitory effect of piclamilast at the high concentration was equivalent to that of dexamethasone on OVA-induced eosinophil infiltration, while more potent than dexamethasone on cAMP-PDE activity. It suggests that selective PDE4 inhibitor can be competitive to bind the active site of PDE4 and inhibit the activity, but dexamethasone potently suppressed the PDE activity by other mechanisms, e.g., inhibition of protein synthesis [35]. Our results also indicate that cAMP-PDE activity plays, at least partially, an important role in the development of antigen-induced goblet cell hyperplasia. Although there is evidence that COX-2 may be a key element in the pathophysiological process of asthma [36,37], the present study failed to show preventive effects of indomethacin on PDE activity and goblet cell hyperplasia.

Our results demonstrate that goblet cell hyperplasia is associated with the infiltration and activation of eosinophils, similar to the previous findings [20]. Furthermore, cAMP-PDE activity is critical in the development of lung inflammation, injury and remodeling, since PDE inhibition prevented from eosinophil infiltration from the circulation to the lung tissue and alveolar space, lung edema, and increased airway wall thickness.

![Graph](image-url)
and number of goblet cells. Of PDEs, PDE4 may play a key role in antigen-induced alterations, evidenced by that PDE4 comprises a higher proportion of the cAMP hydrolyzing activity in homogenates from OVA-sensitized and challenged rats. The increased cAMP-PDE activity might be, at least in part, due to enhanced PDE4 gene expression by antigen sensitization and challenge.

In conclusion, our results demonstrate that the inhibition of cAMP-PDE activity prevented from antigen-induced eosinophil infiltration, lung tissue injury, increased airway wall thickness, and goblet cell hyperplasia. Pretreatment with piclamilast could inhibit OVA-induced cAMP-PDE activity in the lung with a dose-related pattern. This study suggests that PDE4 may be an important therapeutic target for antigen-associated lung inflammation and tissue remodeling.

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References


