Suplatast tosilate ameliorates airway hyperreactivity and inflammation through inhibition of the GATA-3/IL-5 signaling pathway in asthmatic rats

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Abstract. Airway hyperreactivity and inflammation are important factors in the aggravation of lung function. Suplatast tosilate (IPD) is a novel and unique anti-asthma clinical compound. However, the mechanisms of IPD action in the inhibition of asthma remain to be elucidated. The present study aimed to investigate the role of the GATA binding protein 3 (GATA-3)/interleukin (IL)-5 signaling pathway in IPD-induced inhibition of asthma. Sprague-Dawley rats were sensitized by intraperitoneal injection with ovalbumin (OVA) to establish an animal model of asthma. IPD was administered continuously (C-IPD) or at a later stage (L-IPD). Budesonide (BUD) was used as a positive control. Airway resistance and the expression of genes at the mRNA and protein levels were measured. Morphological changes in lung tissue and the percentage of eosinophils (EOS) in peripheral blood were observed and correlation analysis was performed. The results revealed that sensitization by OVA significantly increased airway resistance and the percentage of EOS in peripheral blood and induced significant inflammatory changes in lung tissue, as demonstrated by thick epithelium, goblet cell hyperplasia and submucosal cell infiltration. In addition, sensitization by OVA was found to markedly upregulate IL-5 mRNA and protein expression. Airway resistance was found to positively correlate with the expression of IL-5 in the rat lung tissues. Sensitization by OVA was also observed to markedly enhance GATA-3 protein expression and GATA-3 levels were found to positively correlate with airway resistance and IL-5 levels. Similar to the effect of BUD, treatment with C-IPD or L-IPD was found to significantly attenuate OVA-induced increases in airway resistance and the percentage of EOS in peripheral blood. Notably, treatment with C-IPD or L-IPD markedly reduced the OVA-induced expression of IL-5 and GATA-3. In the present study, IPD intervention was demonstrated to ameliorate airway hyperreactivity and inflammation and the mechanisms may involve inhibition of the GATA-3/IL-5 signaling pathway.

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Introduction

Asthma is a major cause of chronic morbidity and mortality worldwide. The prevalence of asthma has increased considerably over the past two decades, particularly in children (1). Chronic inflammatory disorders and consequent airway hyperreactivity are critical processes in asthma. The inflammatory response and mucus plugs in the airway cause airflow obstruction, and consequently induce the onset of asthma (2). The development of asthma is associated with the production of immunoglobulin E (IgE) and Th2 cytokines, including interleukin (IL)-4, -5 and -13 (3). In a previous study, IL-5 deficiency was found to eliminate airway hyperreactivity in mice with asthma (4). The disequilibrium between Thl and Th2, usually demonstrated by Th2 cell hyperfunction, is considered to be one of the most important immunological pathogeneses of bronchial asthma. Th2 cell-derived cytokines and chemokines, including IL-4, -5, -6, -9 and -13 and granulocyte-macrophage colony-stimulating factor, promote the recruitment and activation of eosinophils (EOS), the production of IgE, and thus cause chronic inflammation and higher reactivity of airways (3). IL-5 functions as an important cytokine in the effector phase of asthma, and inhibition of the expression or function of IL-5 has been hypothesized to represent a promising mechanism for asthma treatment (5,6). GATA binding protein 3 (GATA-3) is a zinc finger transcription factor, which enhances IL-5 expression by binding to a specific motif of the IL-5 promoter in Th2 cells (7-9). Changes in GATA-3 expression and function are commonly accompanied by altered levels of IL-5, indicating that GATA-3 may represent an important agent for the regulation of IL-5 expression (10).

Suplatast tosilate (IPD), (2-(4-(3-ethoxy-2-hydroxypropoxy)phenylcarbamoyl)ethyl)dimethylsulfonium p-toluenesulfonate, is a novel and unique compound

that suppresses cytokine production from Th2 cells (11). The compound has been reported to inhibit IL-5 and IL-4 synthesis and EOS inflammation, reduce IgE antibody titer, attenuate labrocyte degranulation and therefore improve the inflammatory response and hyperreactivity of airways (11-13). IPD has also been found to induce a marked effect through its metabolic product, 4-(3-ethoxy-2-hydroxypropoxy) acrylanilide (M-1) (14,15). In addition, IPD has been revealed to intercept histoleucocyte to dendritic cell differentiation, and thus also dendritic cell maturity and function, and induces Th1 reaction reinforcement in patients with asthma (16). Its effective target may be associated with a chloride channel located on the blood EOS surface (17). Clinical analysis has confirmed that IPD reduces asthma (including hormone-dependent and cough variant asthma) symptom score, improves lung function and upregulates β2 receptor levels resulting in a smaller dose of β2-agonist being applied, which was named 'saving hormone' (18).

To date, the specific molecular mechanisms of IPD action in asthma treatment remain unclear. The current study aimed to observe the effect of IPD on asthma and determine the mechanisms associated with the GATA-3/IL-5 signaling pathway in rat models by intraperitoneal injection with ovalbumin (OVA). Airway resistance, the percentage of EOS in peripheral blood and expression of GATA-3 and IL-5 were observed. To ensure the results of this study were accurate, the classical anti-asthma glucocorticoid drug, budesonide (BUD), was used as a comparison.

Materials and methods

Animals and drugs. Four-week-old healthy male Sprague-Dawley rats of a specific pathogen-free grade, weighing 200±20 g, were purchased from the Animal Department of Hunan Agricultural University (Changsha, China). IPD was synthesized and supplied by Beijing Sinocro PharmaScience Co., Ltd. (Beijing, China). BUN was supplied by AstraZeneca (London, UK). IL-5 and GATA-3 antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). OVA was provided by Sigma-Aldrich (St. Louis, MO, USA). An enzyme-linked immunosorbent assay (ELISA) kit was obtained from Wuhan Boster Biological Technology, Ltd. (Wuhan, China). The study was approved by the ethics committee of Hunan Provincial People's Hospital, The First Affiliated Hospital of Hunan Normal University (Changsha, China).

Experimental group and protocol. Rats were divided randomly into 5 groups: control, OVA, BUD, continuous intervention with IPD (C-IPD) and late intervention with IPD (L-IPD).

Sensitization was performed as described previously, with certain modifications (19). All the groups, with the exception of control group rats, were sensitized by intraperitoneal injection with 100 mg OVA, 100 mg aluminum hydroxide and the $5x10^9$ inactive *pertussis bacillus*, on days 1 and 8. Control rats were administered physiological normal saline (NS) instead of OVA. In the C-IPD group, the rats were intragastrically administered IPD (50 mg/kg/day; dissolved in NS) from day 1, while rats in the remaining groups were treated with the same doses of NS, once per day for 2 weeks.

Following 2 weeks, sensitized rats were treated as follows: control rats were administered 2 ml NS by pump atomization for 10 min; OVA rats received 2 ml 2% OVA by pump atomization for 10 min to maintain asthma; BUD rats were administered 0.64 ml/kg BUD solution, followed by 2 ml 2% OVA by pump atomization 1 h later; C-IPD and L-IPD rats received 50 mg/kg IPD intragastrically, followed by 2 ml 2% OVA by pump atomization 1 h later. All the treatments were performed once per day for 7 days.

Measurement of airway resistance. Following the indicated treatments, airway resistance was measured by recording respiratory pressure curves using whole body plethysmography (Buxco Research Systems, Wilmington, NC, USA), as described previously (20). Rats were anesthetized by intraperitoneal injection of 10% chloral hydrate solution (4 ml/kg body weight). The anesthetized rats were fixed in a supine position on a wooden fixation plate. The trachea was exposed by centrally cutting the skin at the neck and endotracheal intubation was performed. The rats were put into a sealed container to monitor the airway pressure and baseline resistance was recorded for 5 min. After an inhalation aerosol delivery system was embedded, rats were exposed to NS or various concentrations of histamine dihydrochloride (0.32-2.56 mg/ml) for 20 sec and airway resistance values were recorded. Prior to recording the next pressure curve, resistance values were allowed to return The data displayed in whole body plethysmography were collected and transformed into airway resistance values.

Determination of the percentage of EOS in peripheral blood. Following the indicated treatments, rats of all groups were stimulated with 2.56 mg/ml histamine dihydrochloride. Whole peripheral blood was collected via the rat vena caudalis and EOS percentage in the blood was investigated at day 1 (pre-experiment) and day 21 (post-experiment), respectively. Subsequently, 100 μl whole peripheral blood was dropped on a slide and dispersed uniformly. Giemsa staining solution (50 μl) was added to the slide and the same dose of balanced solution was added. After the blood on the slide was dry, 100 leucocytes were observed under an optical microscope and the number of EOS was recorded.

Hematoxylin and eosin (H&E) staining. Lung tissue was removed and fixed with 4% paraformaldehyde. Resin-embedded specimens were cut into 2-μm slices, mounted onto slides and stained with H&E, as described previously (21). The stained slices were examined under a light microscope (BX-41; Olympus, Tokyo, Japan). Images of the intrapulmonary airway epithelium (large airway) were recorded from 5 consecutive high-power fields.

Western blot analysis for IL-5 and GATA-3 expression. Lung tissue homogenates were dissociated in 1 ml RIPA buffer for 30 min and then centrifuged at 12,000 x g for 15 min at 4˚C. Following quantification with a BCA protein assay kit (KangChen Bio-tech Inc., Shanghai, China), 10 μg total protein from each sample was mixed with loading buffer. Samples were separated by 10% SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked with 5% fat-free milk in TBS-T for 1 h at room temperature, followed by incubation with primary antibodies against IL-5 or GATA-3 for

Table I. Effect of various treatments on airway resistance in histamine dihydrochloride stimulated-rats

			Histamine dihydrochloride (mg/ml)			
Group	n	Saline	0.32	0.64	1.28	2.56
Control	10	1	1.09±0.03	1.35±0.21	1.90±0.10	2.07±0.12
OVA	10	1	1.62±0.17[a]	2.24±0.24[a]	3.04±0.23[a]	4.27±0.42[a]
BUD	10	1	1.23±0.12[c]	1.71±0.30[c]	2.10±0.16[c]	2.93±0.38[c]
C-IPD	9	1	1.26±0.10[c]	1.85±0.31[c]	2.16±0.18[c]	2.97±0.39[c]
L-IPD	10	1	1.34±0.17[b]	1.97±0.22[b]	2.27±0.16[c]	3.20±0.30[c]

Data are presented as the mean ± SD. [a]P<0.01 vs. control; [b]P<0.05 and [c]P<0.01 vs. OVA. OVA, ovalbumin; BUD, budesonide; IPD, suplatast tosilate; C, continuous; L, late-stage.

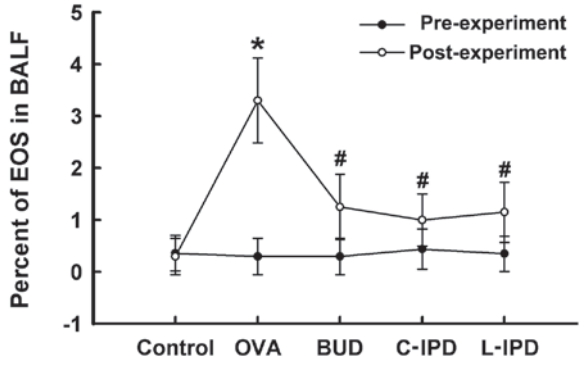

Figure 1. Percentage of EOS in peripheral blood. Rats were administered the indicated treatments. Prior to and following the experiment, the rats were stimulated by 1.28 mg/ml histamine dihydrochloride to determine the percentage of EOS in peripheral blood. Data are presented as the mean ± SD, n=10. *P<0.01 vs. control; #P<0.05 vs. OVA. EOS, eosinophils; IPD, suplatast tosilate; OVA, ovalbumin; BUD, budesonide; C, continuous; L, late stage; BALF, bronchoalveolar lavage fluid.

12 h at 4˚C. Next, the membrane was incubated for 1 h at room temperature with specific horseradish peroxidase-conjugated secondary antibodies. Signals were detected using an enhanced chemiluminescence system according to the manufacturer's instructions (Applygen Technologies, Peking, China). Protein expression was quantified by scanning the X-ray films and analyzing with ImageJ 1.47d software (22).

Reverse transcription polymerase chain reaction (RT-PCR). Following the indicated treatments, lung tissue was collected from the rats. Total RNA was extracted with TRIzol reagent and then reverse-transcribed into cDNA. IL-5 mRNA splicing was indirectly assessed by PCR. Primers spanning the splice site were designed as follows: forward, TGCTTCTGTGCT TGAACGTTCTAAC and reverse, TTCTCTTTTTGTCCGT CAATGTATTTC. The primers for GAPDH were designed as follows: forward, CAAGGTCCATGACAACTTTG and reverse, GTCCACCCTGTTGCTGTAG. PCR products were electrophoretically resolved on 2% agarose gel and observed under UV illumination.

Detection of IL-5 in bronchoalveolar lavage fluid (BALF) with ELISA. Phosphate-buffered saline (0.5 ml) was injected into the trachea via a cannula and this procedure was repeated 3 times. BALF was collected and stored at -80˚C until use. The concentration of IL-5 in BALF and specific concentration of standard IL-5 provided by the kit was determined by ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). The optical density was determined using a microplate reader (EL340; BioTek Instruments, Inc., Winooski, VT, USA) at 450 nm.

Statistical analysis. All data are presented as the mean ± SD. Statistically significant differences between groups were analyzed by one-way analysis of variance with SPSS 16.0 (SPSS Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

IPD inhibits airway hyperreactivity in asthmatic rats. As demonstrated in Table I, following the indicated treatments, rats were exposed to histamine dihydrochloride at increasing concentrations via an embedded inhalation aerosol delivery system. Airway resistance of the OVA-injected rats was found to be significantly augmented compared with the control rats. Under the same challenge concentrations, airway resistance levels were markedly decreased by the administration of BUD, a widely used anti-asthmatic agent. Notably, treatment with C-IPD or L-IPD markedly alleviated OVA-induced airway resistance elevation. No statistical significance was observed between airway resistance levels in C-IPD and L-IPD groups (P>0.05).

Rats were administered with the indicated treatments and stimulated by various concentrations of histamine dihydrochloride prior to recording airway resistance levels.

IPD decreases the percentage of EOS in peripheral blood. Prior to sensitization by OVA, the percentage of EOS in peripheral blood among the groups was not found to be statistically significant (P>0.05). However, following sensitization, the percentage of EOS in peripheral blood was markedly increased compared with that in the control group (P<0.01). C-IPD and L-IPD were found to markedly reduce the percentage of EOS caused by OVA exposure, which was similar to the effect of BUD (Fig. 1).

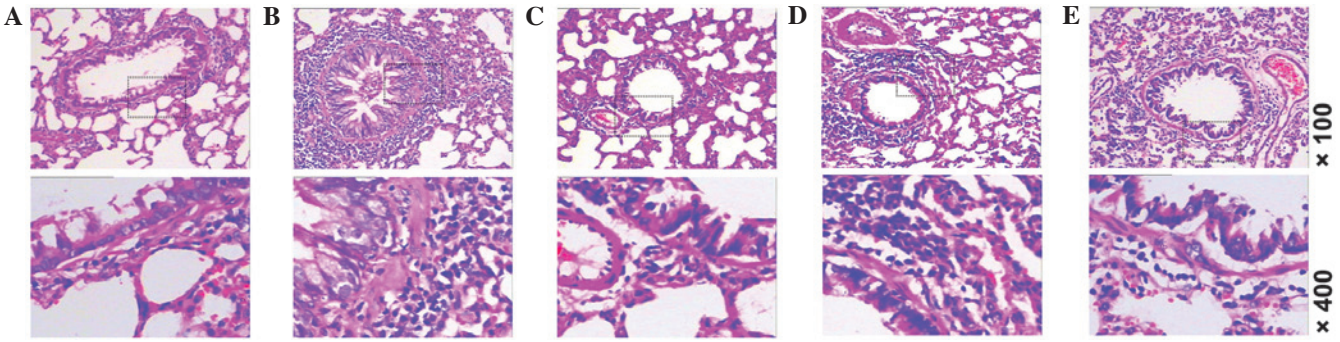

Figure 2. Morphological changes in lung tissue, as detected by H&E staining. (A) Control, (B) OVA, (C) BUD, (D) C-IPD and (E) L-IPD (top row magnification, x100; bottom row, x400). H&E, hematoxylin and eosin; IPD, suplatast tosilate; OVA, ovalbumin; BUD, budesonide; C, continuous; L, late-stage.

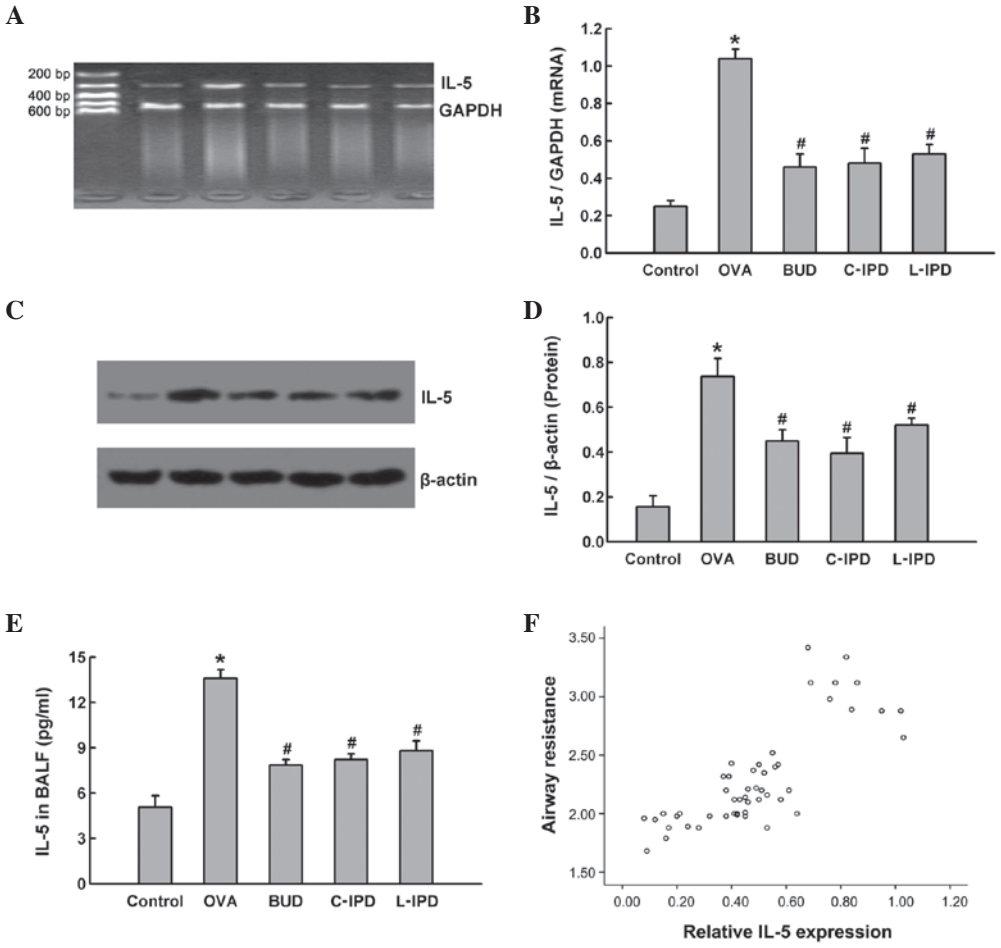

Figure 3. Role of IL-5 in the anti-asthma effects of IPD. Rats were divided into 5 groups: control, OVA, BUD, C-IPD and L-IPD. Following treatment, lung tissue was removed to extract RNA and protein. IL-5 mRNA levels were detected by (A) RT-PCR and (B) quantified. IL-5 protein levels were measured by (C) western blot analysis and (D) analyzed by densitometry. (E) IL-5 levels were detected in BALF by ELISA. (F) Correlation analysis between the expression of IL-5 in lung tissue and airway resistance was performed. Data are presented as the mean ± SD, n=10. *P<0.01 vs. control; #P<0.05 vs. OVA. IPD, suplatast tosilate; OVA, ovalbumin; BUD, budesonide; C, continuous; L, late-stage; IL, interleukin; BALF, bronchoalveolar lavage fluid.

IPD improves morphological changes of lung tissue in asthmatic rats. As sensitization by OVA markedly enhanced levels of airway resistance, we determined whether airway hyperreactivity is due to changes in the structure of lung tissue using H&E staining. As demonstrated in Fig. 2, lung tissue samples obtained from rats intraperitoneally injected with OVA demonstrated marked thickening of the epithelium, goblet cell hyperplasia and submucosal cell infiltration (Fig. 2B). Similar to the effect of intervention with BUD on the injured lung tissue (Fig. 2C), intervention with C-IPD (Fig. 2D) or L-IPD (Fig. 2E) markedly reduced these structural changes.

IL-5 is involved in the action of IPD in ameliorating OVA-induced airway hyperreactivity. IL-5 expression in lung

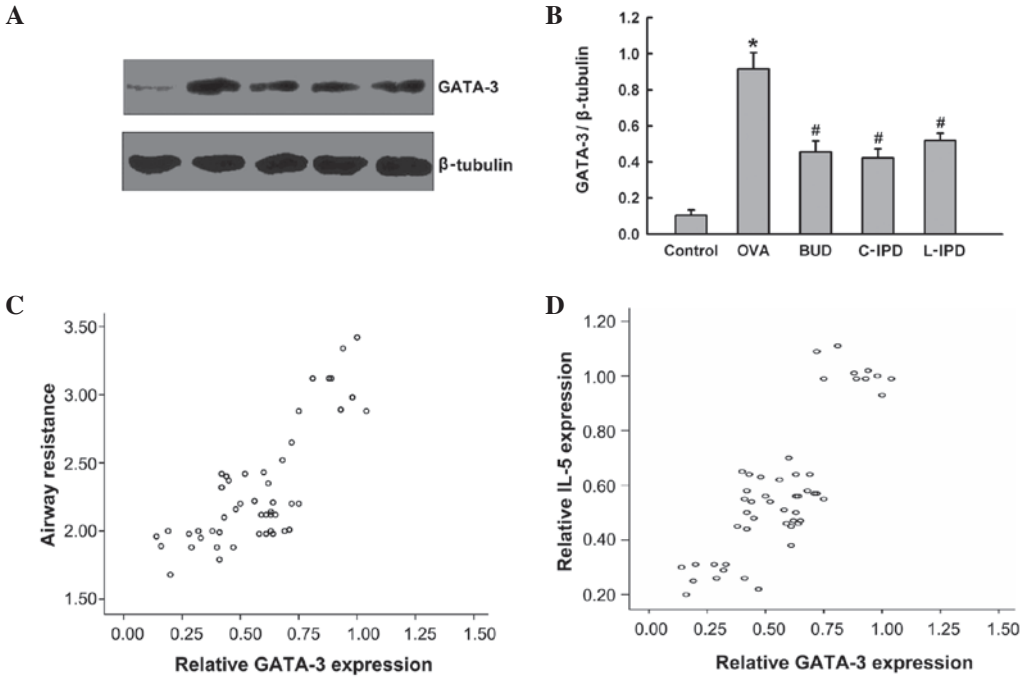

Figure 4. Role of GATA-3 in the anti-asthma effects of IPD. Rats were divided into 5 groups: control, OVA, BUD, C-IPD and L-IPD. Following treatment, nuclear proteins from lung tissues were extracted and (A) GATA-3 expression was determined by western blot analysis and (B) analyzed by densitometry. The correlation between GATA-3 expression and (C) airway resistance or (D) IL-5 expression was determined. Data are presented as the mean ± SD, n=10. *P<0.01 vs. control; #P<0.05 vs. OVA. GATA-3, GATA binding protein 3; IPD, suplatast tosilate; OVA, ovalbumin; BUD, budesonide; C, continuous; L, late-stage; IL, interleukin.

tissue at the transcription and translation level was detected and results indicated that, following sensitization by OVA, IL-5 mRNA (Fig. 3A and B) and protein (Fig. 3C and D) levels were markedly enhanced in the lung tissue of OVA group rats. Increased expression of mRNA and protein was significantly attenuated by the administration of C-IPD or L-IPD, as well as BUD.

Next, BALF samples were analyzed for IL-5 levels in BALF using ELISA. Following sensitization, IL-5 levels in the BALF of OVA group rats were markedly increased and this elevation was found to be partially blocked by C-IPD, L-IPD and BUD (Fig. 3E). These results indicated that OVA not only induced IL-5 expression, but also enhanced its secretion, and treatment with IPD suppressed IL-5 expression and secretion.

In addition, the association between increased levels of IL-5 and airway hyperreactivity was investigated by correlation analysis between IL-5 protein expression in lung tissue and airway resistance. The correlation coefficient was 0.909 (Fig. 3F), indicating that airway hyperreactivity may be associated with increased IL-5 levels, and the inhibition of IL-5 by IPD ameliorates the OVA-induced airway hyperreactivity.

GATA-3 protein regulates IL-5 expression in the OVA-induced asthma model. To determine which transcription factors regulate IL-5 expression, GATA-3 expression was analyzed at the protein level. Following sensitization by OVA, GATA-3 expression was markedly increased, and this was found to be reduced by intervention with C-IPD, L-IPD or BUD (Fig. 4A and B). The correlation between GATA-3 expression in the lungs and airway hyperreactivity or IL-5 expression was determined and the correlation coefficients were 0.775 (Fig. 4C) and 0.828 (Fig. 4D), respectively. These results indicated that increased airway resistance and IL-5 expression may be mediated by increased GATA-3 expression, and inhibition of GATA-3 expression by C-IPD, L-IPD or BUD ameliorates airway hyperreactivity and IL-5 expression.

Discussion

Bronchial asthma is one of the most common allergic disorders in humans and in recent years, disease incidence and mortality rates have increased rapidly. Therefore, understanding the pathogenesis of bronchial asthma is extremely important for the development of methods to prevent and treat this disease. Studies in animals are useful for the identification of etiological factors, exploration of disease mechanisms and evaluation of therapeutic effects (23,24). In the current study, Sprague-Dawley rats were treated according to a previous study by Matsumoto *et al* (19) to establish a model of asthma. OVA was injected intraperitoneally as an anaphylactogen, combined with aluminum hydroxide and inactive *Bacillus pertussis* (an immune adjuvant) at days 1 and 8 to sensitize the rats (25). Following sensitization, severe symptoms of asthma were observed in rats of the OVA group only. In addition, airway resistance was markedly increased compared with levels prior to sensitization, indicating hyperreactivity in the airways. The percentage of EOS in peripheral blood was also increased. Pathological analysis of lung tissue was performed, revealing increased levels of IL-5 and GATA-3, indicative of a successful model of asthma.

Previous studies have demonstrated that the blockade of Th2 cytokines may represent a major therapeutic target in asthma. Inhibition of Th2 cytokines using monoclonal antibodies against IL-5 and IL-3 and an IL-4 receptor antagonist was demonstrated to improve asthma symptoms (26,27). In addition, IPD has been reported to suppress Th2 cytokines, including IL-5 and IL-4, and inhibit EOS inflammation, resulting in a decrease in IgE antibody titer (20). Results of the current study revealed that C-IPD and L-IPD treatment attenuated airway resistance, similar to the classical anti-asthma drug, BUD, indicating that hyperreactivity of the airway was ameliorated. In addition, the systemic inflammatory response was analyzed by calculating the percentage of EOS in the peripheral blood, and C-IPD and L-IPD treatments were found to reduce the percentage of EOS. H&E staining also revealed that IPD inhibited labrocyte degranulation and mediators of inflammation secretion. Previously, Sano *et al* (12) reported that IPD represses Th2 cytokine-induced allergic inflammation. Results of the present study were consistent with previous studies (13-15). In addition, IPD has been administered in clinical studies and was found to improve the clinical symptoms and lung function of patients. Early IPD treatment was also revealed to prevent food allergies or sensitization dermatitis (28). Consistent with these observations, results of the current study indicated that the late administration of IPD has the same effect on asthma as continuous intervention with IPD.

Since IPD was found to suppress the airway inflammatory response and hyperreactivity associated with Th2 cytokines, and as IL-5 is one of the most important Th2 cytokines, the effect of IL-5 inhibition on IPD-induced decreases in airway resistance and the percentage of EOS in peripheral blood was investigated by analysis of IL-5 mRNA and protein expression in the lung tissue. The results demonstrated that, following sensitization with OVA, IL-5 mRNA and protein levels were markedly elevated. In addition, IL-5 levels in BALF were analyzed by ELISA, and IL-5 release levels were also observed to be enhanced. Notably, treatment with continuous or late-stage IPD blocked IL-5 expression and its release induced by OVA. To confirm that IPD-induced IL-5 downregulation is associated with ameliorated OVA-induced hyperreactivity of the airway, a correlation analysis was performed between IL-5 expression in the lung tissue and airway resistance. The results revealed that airway resistance positively correlated with IL-5 expression, indicating that IPD-induced attenuation of airway hyperreactivity was due to inhibition of IL-5 expression. In a previous study, IL-5 expression was markedly enhanced during inflammation in atopic asthma (29). Till *et al* (30) also found that IL-5 and EOS levels were increased in the peripheral blood and BALF of asthma patients and there was a positive correlation between the two parameters. In addition, Shi *et al* (31) reported that, following the inhalation of IL-5, the percentage of EOS and airway hyperreactivity were significantly increased in asthma patients. These observations and the present study indicate that increased IL-5 levels mediate the inflammatory response in asthma patients and the inhibition of IL-5 biosynthesis and release represents an important mechanism by which IPD treats asthma. To ensure the results of this study were accurate, rats were exposed to BUD by pump atomization and BUD was also found to reduce IL-5 expression and release, similar to treatment with IPD.

GATA-3 is an important transcription factor that regulates the expression of a number of genes at the transcription level in Th2 cells (32-34). A GATA-3 binding site was previously identified in the promotor region of the IL-5 gene (35) and therefore, we hypothesized that OVA-induced asthma and the upregulation of IL-5 in rats is associated with GATA-3. To examine this hypothesis, the expression of GATA-3 was analyzed in the lung tissue and treatment with OVA was found to markedly enhance GATA-3 protein expression in rats. Notably, increased GATA-3 levels were found to positively correlate with airway resistance and IL-5 expression. The results indicated that the upregulation of IL-5 and hyperreactivity may be triggered by GATA-3. However, following continuous or late-stage treatment with IPD, GATA-3 protein expression in the lung tissue was observed to be markedly reduced. Therefore, the inhibition of GATA-3 expression appears to function as an upstream mechanism by which IPD targets asthma and represses IL-5 expression.

In conclusion, the present study demonstrated that IPD treatment alleviated OVA-induced asthma in rats by inhibition of the GATA-3/IL-5 signaling pathway. These results provide novel insights into the mechanisms underlying the anti-asthmatic action of IPD and represent preliminary evidence for the clinical application of IPD.

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