

Phosphatidylinositol 3-kinase inhibitor suppresses inducible nitric oxide synthase expression in bronchiole epithelial cells in asthmatic rats

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Abstract Inducible nitric oxide synthase (iNOS) is known to produce nitric oxide (NO), which is a main contributor to asthmatic airway inflammation. Recent studies have shown that phosphatidylinositol 3-kinase (PI3K) is ubiquitously expressed in airway epithelial cells and its inhibition could relieve airway inflammation and hyperresponsiveness. This study aimed to explore the interaction of PI3K and NO signaling in allergic asthma. We investigated the effects of PI3K inhibitor wortmannin on iNOS expression in bronchiole epithelial cells and NO, IL-4 and IFN- γ levels in lung tissues of asthmatic rat model, which was prepared by 10% OVA solution sensitization and 1% OVA aerosol challenge. Our results showed that the ratio of eosinophils to total cells in BALF, PI3K activity, NO and IL-4 levels in lung tissues was increased after OVA sensitization and challenge, but then was attenuated by the administration of wortmannin. In contrast, IFN- γ level in lung tissues was decreased after OVA sensitization and challenge and increased after the administration of wortmannin. The expression of iNOS protein in bronchiole epithelial cells, iNOS mRNA level and iNOS activity in lung tissues was markedly upregulated after OVA sensitization and challenge, but the

upregulation was significantly antagonized by wortmannin. Taken together, these data provide evidence that PI3K functions upstream to modulate iNOS/NO signaling, which then promotes the development of airway inflammation in asthmatic animal model. PI3K inhibitor wortmannin could lead to reduced iNOS expression and NO production, therefore inhibiting airway inflammatory responses.

Keywords Phosphatidylinositol 3-kinase · Asthma · Nitric oxide · Inducible nitric oxide synthase · Airway inflammation

Introduction

Nitric oxide (NO) is an important signal molecule generated from L-arginine by a family of nitric oxide synthases (NOSs), which consist of three independent enzymes: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). iNOS can be stimulated by proinflammatory cytokines such as IL-4 and IFN- γ [1]. Airway inflammation in asthma involves many inflammatory cells, structural cells and some cellular components. Under the inflammatory conditions, the inflammatory cells are induced to express iNOS and release NO [2]. Excessive release of fractional exhaled nitric oxide (FeNO) is detected in asthma patients and experimental asthma models and has been confirmed to be correlated with the severity of airway inflammation [3–5].

Phosphatidylinositol 3-kinase (PI3K) is a key enzyme, which phosphorylates phosphatidylinositol at the D-3 hydroxyl of the inositol ring, forming the phosphatidylinositides PI 3-phosphate (PIP), PI 3,4-diphosphate (PIP₂) and PI 3,4,5-triphosphate (PIP₃) [6]. Based on their structure and substrate specificity, PI3K isoforms are classified

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into three groups, namely class IA and IB, class II and class III [7]. PI3K IA is ubiquitously expressed in airway smooth cells, epithelial cells, vascular smooth cells and inflammatory cells [8]. Recent studies suggested that PI3K plays an essential role in airway inflammatory response [9–11]. PI3K activity increases significantly in a murine model of allergic asthma challenged with aerosolized ovalbumin (OVA), and administration with wortmannin or LY294002, two inhibitors of PI3K, may decrease airway inflammation and hyperresponsiveness in the same model [12, 13].

In this study, we investigated the expression of iNOS at protein and mRNA levels and NO production in an allergic asthmatic rat model. We further examined whether wortmannin, a PI3K inhibitor, could affect the expression of iNOS, as well as the production of NO, IL4 and IFN- γ . Our results demonstrated that PI3K inhibitor could affect iNOS expression and NO production and inhibit airway inflammatory response in airway of asthmatic rats.

Materials and methods

Materials

The rats were obtained from animal laboratory center, Wenzhou Medical College (Wenzhou, China). Ovalbumin was purchased from Sigma (USA). Wortmannin was from Biomol (USA), and rabbit anti-rat iNOS polyclone antibody was from Wuhan Boster (China). NO and iNOS assay kits were purchased from Nanjin Jiancheng Bioengineering Institute (China). PIP₃ ELISA kit was purchased from Usenlife Science & Technology Company (USA). ELISA test kits for IL-4 and IFN- γ were purchased from Sigma (USA).

Animals and experimental protocol

Twenty-four pathogen-free male rats weighting about 200–250 g were randomly divided into three groups, namely, control group, asthmatic group and PI3K inhibitor wortmannin group. The rats were sensitized on days 1 and 8 by intraperitoneal injection of 10% (wt/vol) OVA mixed with 10% alumin hydroxide solution in a total volume of 1 mL. From day 15 after the initial sensitization, the animals were challenged for 30 min with an aerosol of 1% OVA in normal saline using an ultrasonic nebulizer, twice per week for 8 weeks. As a control, OVA was replaced by saline during sensitization and challenge. In the PI3K inhibitor treatment group, rats were injected with wortmannin (15 μ g/kg/day, from day 15 to 56) intraperitoneally 30 min before the challenge. All animals were killed by exsanguination after the last challenge.

Cell counts in bronchoalveolar lavage fluid

BALF was obtained from the right lung of the animals before they were killed. After being centrifuged at 1,200g for 10 min, BALF was prepared for counting total cells in 2% ice acetic acid solution and differential cells (eosinophil, neutrophil, lymphocyte and macrophage) in Rye's solution. The ratios of differential cells to total cells were calculated, respectively.

Immunohistochemistry

Left lung tissue was cut as cross-sections approximately 3 mm $\times$ 3 mm $\times$ 3 mm in size, then fixed in 4% paraformaldehyde and embedded in paraffin. The sections (5 μ m thick) were loaded onto poly-L-lysine-coated glass slide, dewaxed and treated with 3% H₂O₂-methanol. After blocking with 10% normal goat serum for 15 min at 37°C, the sections were incubated with 1:200 rabbit anti-rat iNOS poly-clone primary antibody for 1 h at 37°C. For negative control, normal rabbit serum was used instead of primary antibody. After washing in 0.01 mM PBS for 10 min, the sections were incubated with horseradish peroxidase-conjugated secondary antibody (dilution 1:100) for 10 min at 37°C and peroxidase activity was stained with diaminobenzidine. Finally, the sections were counterstained with hematoxylin and mounted with neutral balsam and coverslips. Brown staining revealed iNOS protein expression. Five to ten bronchioles per slide were chosen and analyzed to determine the mean densitometric value.

RT-PCR analysis

Total RNA was isolated from about 100 mg lung tissue using Trizol (Invitrogen). cDNA was synthesized from total RNA by reverse transcription with oligo-primers. The cDNA was amplified by PCR with iNOS and GAPDH primers synthesized by Shanghai Sangon (China). The sequences of the primers were as follows: iNOS 5'-TG GGACCCGCACCACTAC-3' and 5'-GGCATTCGCGAC AAA-3'; GAPDH 5'-GACAAGATGGTAAGGTCCG-3' and 5'-CACGGAAGGCCATGCCAGTGC-3. PCR parameters were as follows: predenaturing at 94°C for 5 min, then 30 cycles of denaturing at 94°C for 0.5 min, annealing at 58°C for 1 min and extension at 72°C for 1 min. The amplified products were analyzed by electrophoresis in 1.5% agarose gels and visualized by ethidium bromide. The gels were autoradiographed by MUVB-20 image analysis system, and then, the absorbance was analyzed.

PI3K activity assay

About 100 mg lung tissue was homogenized in 1 mL solution containing 50 mmol/L Tris pH 7.5, 40 mmol/L β -glycerophosphate, 100 mmol/L NaCl, 2 mmol/L EDTA, 50 mmol/L NaF, 200 μ mol/L Na_5VO_4 , 20 μ mol/L PMSF and 1% Triton X-100. After ultrasonication in ice bath solution, the homogenate was centrifuged at 14,000g for 20 min. The amount of PIP_3 produced was quantified by PIP_3 competition enzyme immunoassays according to manufacturer's instructions. The protein amount was determined by Bradford reagents. PI3K activity was expressed as pmol PIP_3 produced by 1-mg lung tissue extract containing equal amounts of total proteins [14].

$\text{NO}_2^-/\text{NO}_3^-$ content and iNOS activity assay

About 100 mg lung tissue was homogenized in 1 mL solution containing 50 mmol/L Tris pH 7.6 and 1 mmol/L EDTA, then centrifuged at 3000 rpm for 10 min. The concentration of nitrite (NO_2^-)/nitrate (NO_3^-) in the supernatants was determined using a $\text{NO}_2^-/\text{NO}_3^-$ assay kit according to manufacturer's instructions.

To examine the effect of the calcium level on NOS activity, CaCl_2 or calmodulin antagonist trifluoperazine was added to the homogenates, respectively. The concentration of iNOS was spectrophotometrically analyzed using an iNOS commercial kit according to the manufacturer's instructions.

ELISA assay for cytokines

For cytokine determinations, the homogenate of lung tissue was harvested. IL-4 and IFN- γ in lung homogenate were measured using commercially available ELISA kits according to manufacturer's instructions.

Statistical analysis

Data were presented as means $\pm$ standard error of the mean (SEM). Statistical comparisons of means were made with analysis of variance (ANOVA) followed by the Student–Newman–Keuls post hoc test when ANOVA indicated a significant difference. A value of $P < 0.05$ was accepted as statistically significant.

Results

Ratios of differential cells to total cells in BALF

In BALF from OVA-sensitized/challenged asthmatic rat, the ratio of eosinophils to total cells was significantly

elevated compared to the control. In contrast, the ratio of eosinophils to total cells was decreased after the administration of PI3K inhibitor wortmannin (Fig. 1).

iNOS expression in bronchial epithelial cells

Immunohistochemistry analysis revealed that iNOS protein was stained weakly or negatively in bronchial epithelial cells of control rat (Fig. 2a). In contrast, iNOS immunostaining was obviously strong in OVA-sensitized/challenged rat (Fig. 2b). However, after the administration of wortmannin, the strong iNOS protein immunostaining in OVA-sensitized/challenged rat was significantly decreased (Fig. 2c).

To confirm that iNOS expression is modulated by PI3K inhibitor, we performed RT-PCR analysis to examine iNOS expression at mRNA level. The results showed that iNOS mRNA level was weak in the lung tissues of control rat. In contrast, iNOS mRNA level was strongly upregulated in lung tissue of OVA-sensitized/challenged rat. However, wortmannin could inhibit the upregulation of iNOS mRNA expression in OVA-sensitized/challenged rat (Fig. 3).

$\text{NO}_2^-/\text{NO}_3^-$ content and iNOS activity in lung tissue

Next, we examined NO production in lung tissue homogenate and found that $\text{NO}_2^-/\text{NO}_3^-$ content in OVA-sensitized/challenged rat was elevated compared to the control. Upon the administration of wortmannin, $\text{NO}_2^-/\text{NO}_3^-$ content in OVA-sensitized/challenged rat was decreased

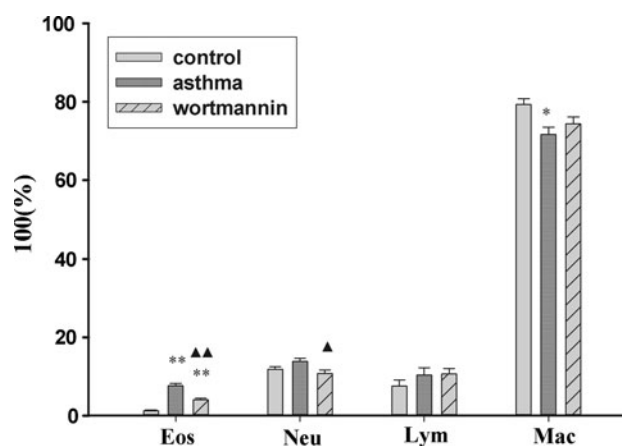


Fig. 1 Effect of wortmannin on the counts of total cells and the ratios of differential cells to total cells in rat BALF. The ratios of eosinophils, neutrophils, lymphocytes and macrophages to total cells in BALF were shown for three groups: saline-control, OVA-sensitized/challenged asthmatic and administration of wortmannin (15 μ g/kg/day, from day 15 to 56) rats. Values shown were means $\pm$ SEM from eight independent experiments. * $P < 0.05$, ** $P < 0.01$ versus control group; ▲ $P < 0.05$, ▲▲ $P < 0.01$ versus asthmatic group

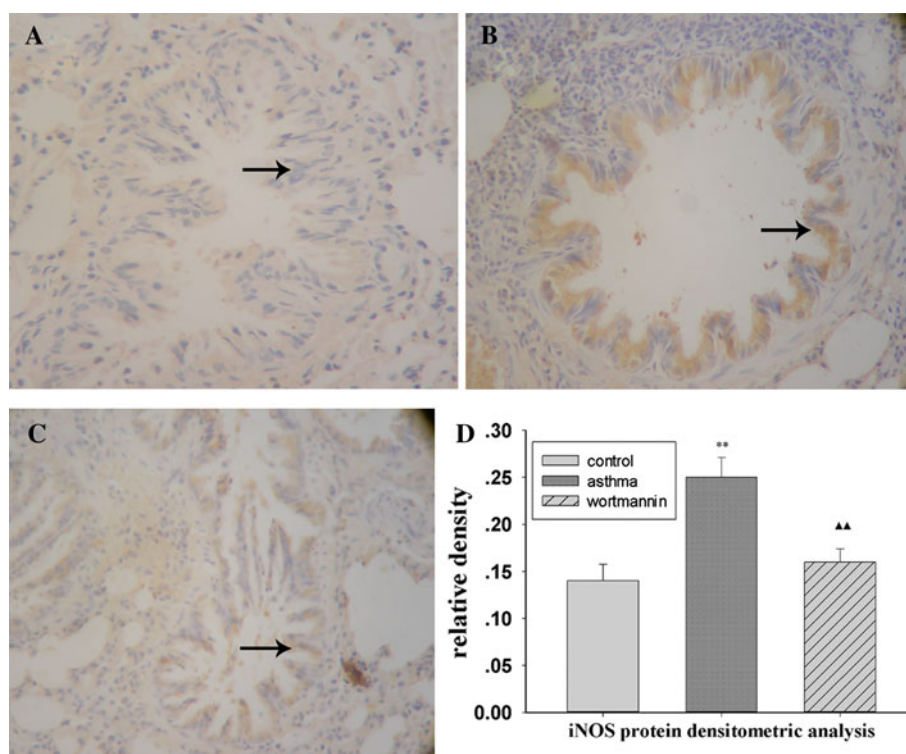


Fig. 2 Effect of wortmannin on iNOS protein expression in rat lung. iNOS immunostaining of 5- μ m sections of paraffin-embedded bronchiole epithelial cells was shown for three groups: saline-control, OVA-sensitized/challenged asthmatic and administration of wortmannin (15 μ g/kg/day, from day 15 to 56) rats. *Brown* indicated positive staining of iNOS protein. Original magnifications, $\times 200$. **a** Nearly negative expression of iNOS in bronchiole epithelial cells of control rats. **b** Strongly positive expression of iNOS in bronchiole epithelial

cells of OVA-sensitized/challenged asthmatic rats. **c** Weakly positive expression of iNOS in bronchiole epithelial cells of wortmannin-administrated rats. **d** Data were represented as histograms by densitometric analysis of iNOS immunoactive staining. Values shown were means $\pm$ SEM from 5 to 10 bronchioles in every eight independent experiments. ** $P < 0.01$ versus control group; ▲▲ $P < 0.01$ versus asthmatic group. (Color figure online)

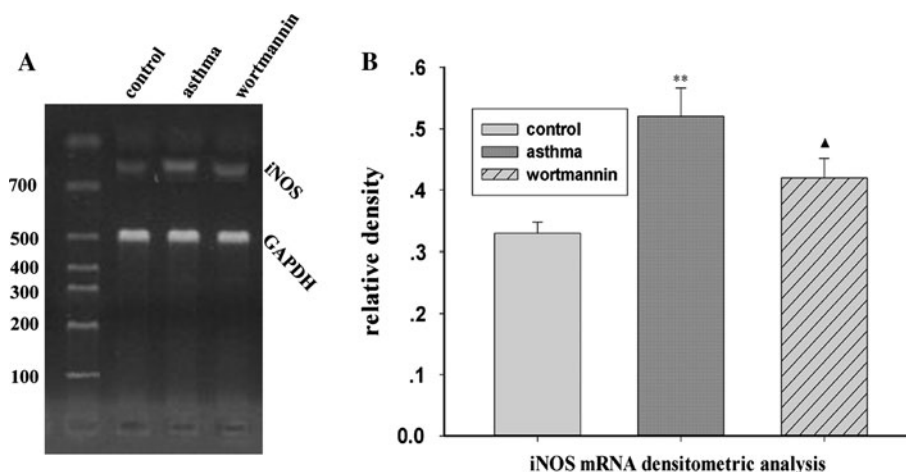


Fig. 3 Effect of wortmannin on iNOS mRNA expression in rat lung. Expression of iNOS mRNA in lung tissues was shown for three groups: saline-control, OVA-sensitized/challenged and administration of wortmannin (15 μ g/kg/day, from day 15 to 56) rats. **a** Representative RT/PCR results analyzed by electrophoresis gels. GAPDH

served as an internal control. **b** Data were represented as histograms by densitometric analysis of iNOS/GAPDH mRNA in lung tissues. Values shown were means $\pm$ SEM from eight independent experiments. ** $P < 0.01$ versus control group; ▲ $P < 0.05$ versus asthmatic group

(Fig. 4a). Since it is known that excessive NO is derived by iNOS under inflammatory condition, we further examined iNOS activity. The results showed that iNOS activity in

lung tissue of OVA-sensitized/challenged rat was elevated compared to the control, but was inhibited by the administration of wortmannin (Fig. 4b).

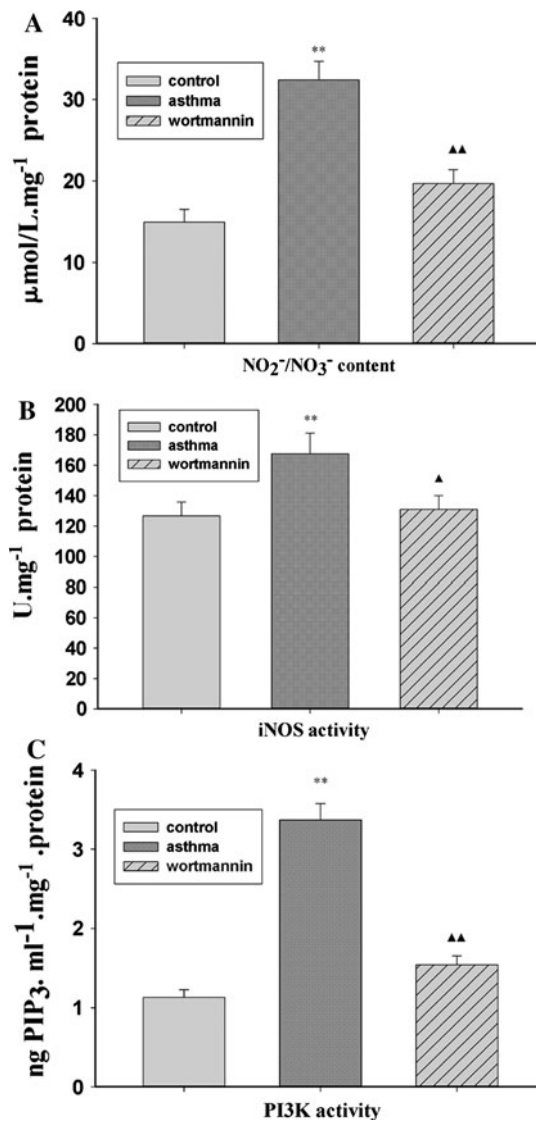


Fig. 4 Effect of wortmannin on NO₂⁻/NO₃⁻, iNOS and PI3K activities in rat lung. NO₂⁻/NO₃⁻ content, iNOS activity and PI3K activity in lung tissues were shown for three groups: saline-control, OVA-sensitized/challenged and administration of wortmannin (15 μg/kg/day, from day 15 to 56) rats. **a** NO₂⁻/NO₃⁻ content in lung tissues. **b** iNOS activity assay in lung tissues. **c** PI3K activity in lung tissues. Values shown were means ± SEM from eight independent experiments. ***P* < 0.01 versus control group; ▲*P* < 0.05, ▲▲*P* < 0.01 versus asthmatic group

PI3K activity in lung tissue

To confirm the involvement of PI3K in airway inflammatory response, we detected PI3K activity. As shown in Fig. 4c, PI3K activity in lung tissue of OVA-sensitized/challenged asthmatic rat was significantly elevated compared to the control. But upon the administration of wortmannin, PI3K activity in lung tissue of OVA-sensitized/challenged rat was decreased.

IL-4 and IFN-γ contents in lung tissue

IL-4 and IFN-γ represent a pair of well-known cytokines involved in the modulation of inflammatory responses. Therefore, we further determined whether the production of these two cytokines is modulated by PI3K signaling. Compared to the control, IL-4 level was elevated and IFN-γ level was decreased in lung tissue of OVA-sensitized/challenged rat. Notably, with the administration of wortmannin, IL-4 level was decreased and IFN-γ level was increased in lung tissue of OVA-sensitized/challenged rat (Fig. 5).

Discussion

It remains controversial whether NO plays an important role in human asthma and in animal models of allergic airway inflammation. In addition, the relative contribution of NOS isoforms to NO production in these conditions is elusive. For example, Xiong et al. [15] provided evidence that supported the role of iNOS in inflammatory process in asthma. In contrast, De Sanctis et al. [16] reported that iNOS was not important in the development of airway inflammation. iNOS, unlike eNOS or nNOS, is regulated at the transcriptional level in response to endotoxins or certain proinflammatory cytokines. It has been proposed that iNOS induced in airway epithelial cells by inflammatory cytokines acts as the major source of increased NO in asthmatic subjects [17]. Recently, NO release has also been reported in human small airway epithelial cells [18].

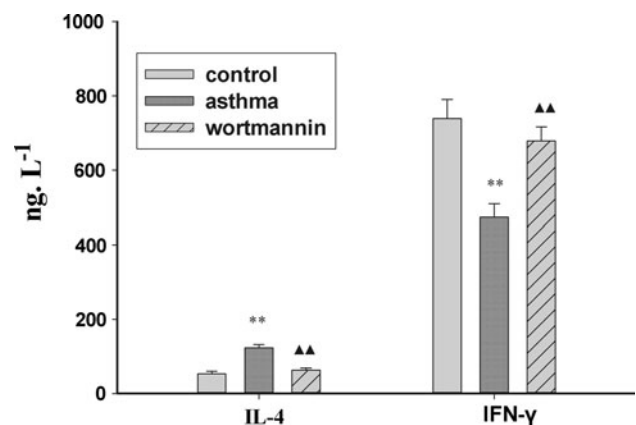


Fig. 5 Effect of wortmannin on IL-4 and IFN-γ levels in rat lung. IL-4 and IFN-γ contents detected by ELISA assay in lung tissues were shown for three groups: saline-control, OVA-sensitized/challenged asthmatic and administration of wortmannin (15 μg/kg/day, from day 15 to 56) rats. Values shown were means ± SEM from eight independent experiments. ***P* < 0.01 versus control group; ▲▲*P* < 0.01 versus asthmatic group

In this study, we found that iNOS expression at both protein and mRNA levels in airway epithelial cells was upregulated, and iNOS activity and NO production in lung tissue were correspondingly increased in OVA-sensitized/challenged allergic rat. These results were consistent with that reported by Takemoto et al. [19] who observed upregulation of iNOS mRNA and protein expression in NC/Nga mice asthmatic model.

It has been well defined that iNOS expression is induced by IL-4 and IFN- γ via regulatory factor (IRF)-1 and signal transducers and activators of transcription (STAT)-6 [20, 21]. The elevated IL-4 and decreased IFN- γ levels shown in our study could provide the explanation for the epithelial iNOS overexpression in OVA-sensitized/challenged allergic rat. Furthermore, our study showed that inflammatory cells such as eosinophils were recruited in BALF from OVA-sensitized/challenged allergic rat, which suggested the development of airway inflammation. Several studies based on iNOS-deficient mice model confirmed that iNOS deficiency could result in the suppression of OVA allergic airway inflammation [15, 22]. On the contrary, Feder et al. [23] demonstrated that a selective iNOS inhibitor had no effect on the influx of eosinophils into the lung of allergen-challenged mice. Moreover, it was reported that airway responsiveness was not significantly different between iNOS-deficient and wild-type mice [16].

Although the role of NO in airway homeostasis and the pathogenesis of asthma are still controversial, it is clear that NO is a marker of airway inflammation or hyperresponsiveness. Studies with NOS inhibitors, NO donors and NOS knockout mice have helped define the role of NO in airway inflammation [15, 16]. High level of NO produced by iNOS appeared to mediate the pathologic development of airway inflammation in mice [24]. Similarly, decreased NO level was found to be associated with the reversal of airway inflammation in asthma [25].

PI3K signaling is actively involved in the modulation of cell survival, proliferation, differentiation, apoptosis, chemotaxis and production of inflammatory mediators [9]. Recent studies have shown that PI3K was associated with the pathogenesis of asthma. PI3K- γ and PI3K- δ are especially involved in allergic responses, and they were expected to be new therapeutic targets for asthma [10]. In this study, we demonstrated that upregulation of PI3K enzyme activity occurred in lung tissue of OVA-sensitized/challenged allergic rat. Kwak et al. [26] also reported that PI3K activity in lung tissue increased significantly after OVA challenge in a murine model of asthma. PI3K not only participates in the degranulation of bronchial eosinophils and allergen-induced airway hyperresponsiveness, but also regulates smooth cell growth [27]. Additionally, administration of a biologically inactive protein (dominant-negative PI3K p85 protein) or a pharmacological inhibitor

wormannin could reduce airway eosinophilic inflammation and hyperresponsiveness [27]. While some studies revealed that wortmannin prevented the degranulation of eosinophils rather than their accumulation in allergen-sensitized/challenged rat lung [13, 28], other study reported that wortmannin almost completely abolished Sephadex-induced BALF eosinophils accumulation [13]. The discrepancies may be due to significant differences in the sensitization and challenge protocols.

Although the previous reports have described the effects of PI3K inhibitors on NO synthesis, those conclusions are not completely consistent. We therefore investigated the effects of PI3K inhibitor wortmannin on iNOS expression and NO production in lung tissue. Wortmannin is a selective inhibitor of PI3K, and its anti-inflammatory effect has been demonstrated in human eosinophils and in allergic animal models [29]. With the administration of wortmannin in OVA-sensitized/challenged rat, we found that iNOS protein expression in bronchial epithelial cells, iNOS mRNA expression and NO release in lung tissue were significantly attenuated. In addition, the ratio of eosinophils to total cells in BALF was decreased. It has been confirmed that PI3K/Akt could induce iNOS expression and NO release in vascular smooth muscle cells and macrophages [30, 31]. Recent study has shown that the activation of PI3K/Akt pathway through tyrosine kinase, which in turn induces p38-MAPK activation and initiates NF- κ B, seemed to be crucial to the upregulation of iNOS expression [32]. Additionally, PI3K could induce the dimerization of iNOS and promoted enzyme activity of iNOS [33]. In this study, we further observed that the elevated IL-4 and decreased IFN- γ levels in OVA-sensitized/challenged allergic rat were reversed by the inhibition of PI3K, which could impair iNOS expression [34, 35]. Taken together, these data indicate that decreased PI3K activity is responsible for the suppression of iNOS/NO pathway in allergic rat after administration of wortmannin.

In summary, the present study provides evidence that PI3K-iNOS/NO signaling may play an important role in the development of airway inflammation in OVA-sensitized/challenged asthmatic animal model. PI3K inhibitor wortmannin could lead to reduced iNOS expression and NO production, therefore inhibiting airway inflammatory responses.

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