

Rho-kinase inhibitor fasudil reduces allergic airway inflammation and mucus hypersecretion by regulating STAT6 and NFκB

T. Xie^{1,*}, GY. Luo^{2,*}, Y. Zhang¹, X. Wang¹, XY. Wang¹, M. Wu³ and GP. Li^{1,4}

¹Inflammations & Allergic Diseases Research Unit, First Affiliated Hospital of Sichuan Medical University, ²Staff Health Clinic, Sichuan Medical University, Luzhou, Sichuan, China, ³Department of Basic Biomedical Sciences, School of Medicine and Health Sciences, University of North Dakota, Grand Forks, ND, USA, and ⁴State Key Laboratory of Respiratory Disease for Allergy, School of Medicine, Shenzhen University, Shenzhen, Guangdong, China

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Summary

Background Airway mucus hypersecretion is a key pathophysiological feature in asthma. Fasudil, a selective Rho-A/Rho kinase inhibitor, has been used in clinical trials to treat pulmonary hypertension. However, its function in modulating airway mucus hypersecretion in asthma remains undefined.

Objective We examined whether fasudil, a selective Rho-A/Rho kinase inhibitor, affects the mucus hypersecretion by suppressing MUC5AC via signal transducer and activator of transcription factor 6 (STAT6) and nuclear factor-kappa B (NFκB) in mice and cells.

Methods We measured mucus secretion and the expression of Rho-kinase in the airway tissue of patients with asthma. BALB/c mice were sensitized and challenged with ovalbumin (OVA) followed with fasudil treatment. The lung tissues were assessed for airway inflammation and mucus secretion. Cytokine levels and airway responsiveness were determined. STAT6 and NFκB were quantified by Western blot. 16HBE cells were stimulated with house dust mite (HDM) extracts. MUC5AC and *muc5ac* promoter activities were measured. Using siRNA to knockdown STAT6 in epithelial cells, we determined the impact of STAT6 on *muc5ac* promoter activity. NFκB nuclear translocation was observed with immunostaining.

Results Fasudil administration significantly decreased the number of inflammatory cells, inflammation index in the lung and airway responsiveness. Fasudil also reduced mucous secretion and MUC5AC expression in OVA-challenged mice. Fasudil down-regulated the levels of IL-17, IL-4 and IL-13 in the lung tissue of OVA-challenged mice. Fasudil also decreased the expression and phosphorylation of NFκB and STAT6 as well as the nuclear translocation of NFκB. In addition, human airway epithelial cells (16HBE) were challenged with HDM extracts and then treated with fasudil. Fasudil inhibited HDM extract-induced MUC5AC expression, which is associated with a reduction in STAT6 and NFκB in epithelial cells.

Conclusions and Clinical Relevance These findings indicate that the Rho-A/Rho kinase inhibitor, fasudil, plays a negative regulatory role in allergen-induced mucus secretion and MUC5AC expression by regulating STAT6 and NFκB.

Keywords fasudil, MUC5AC, mucus hypersecretion, NFκB, Rho-kinase inhibitor, STAT6

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Correspondence:

Guoping Li, Inflammations & Allergic Diseases Research Unit, First Affiliated Hospital of Sichuan Medical University, Luzhou 646000, Sichuan, China.

E-mail: lzlzp@163.com and Min Wu, Department of Basic Biomedical Sciences, School of Medicine and Health Sciences, University of North Dakota, 501 N Columbia Rd, Grand Forks, ND 58203-9037, USA.

E-mail: min.wu@med.und.edu

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Introduction

Airway mucus hypersecretion is now recognised as a key pathophysiological feature in asthma [1]. Mucus secretion is required for airway defence, but mucus

hypersecretion is a principal cause of severe asthma and, ultimately, mortality [2, 3]. Mucous metaplasia in asthma increases the susceptibility of the airway epithelium to rhinovirus infection [4]. Inhaled corticosteroids are the evidence-based, guideline-recommended, first-line treatment for controlling persistent or chronic asthma [5]. However, conventional therapies are

*These authors contributed equally to this work.

ineffective for severe asthma. Mucus hypersecretion is difficult to treat and is dependent upon secretion clearance from the airway [6]. Consequently, it is important to develop novel drugs that can effectively inhibit mucus hypersecretion in severe asthma.

The mucociliary system consists of mucus-secreting goblet cells and ciliated cells and generates a continuously overturning layer of protective mucus that lines the airway epithelium. There are currently no effective treatments for excessive mucus production. Goblet cell differentiation and mucus production are subject to extensive control [7]. Mucus hypersecretion can be a critical contributor to asthma exacerbation and can be initiated by multiple intracellular signalling pathways [8, 9]. MUC5AC and MUC5B are evolutionarily conserved genes that encode structurally related mucin glycoproteins, the principal macromolecules in airway mucus [3]. Overproduction of MUC5AC is also an additional disease feature of asthma. MUC5AC is a central effector of allergic inflammation that is required for airway hyper-reactivity (AHR). Inhibiting MUC5AC may be an effective way of treating asthma [10]. It has been reported that induction of MUC5AC is dependent upon two important signalling pathways, the IL-13/IL-4 receptor- α complex and the epidermal growth factor receptor (EGFR). Signal transducer and activator of transcription 6 (STAT6) is necessary and sufficient for inducing *muc5ac* gene [2, 11, 12]. STAT family proteins are transcription factors, which play critical roles in cell growth and survival. They are activated by phosphorylation as a response to extracellular stimuli or by intracellular non-receptor tyrosine kinases [13]. We found that decreased STAT6 and NF κ B expression is related to reduced mucous secretion in Der p 2 DNA vaccine-immunized mice [14, 15].

Rho-A/Rho-kinase signalling plays important roles in diverse cell functions, such as cell migration and proliferation, smooth muscle cell contraction and gene expression [16]. Rho-kinases (ROCK-I or ROCK- β and ROCK-II or ROCK- α) are effector molecules of Rho-A and are involved in STAT transcriptional activity. Rho family GTPases are required for the activation of Jak/STAT signalling by G protein-coupled receptors [17, 18]. Recent studies demonstrated that the Rho-A signalling cascade leads to STAT3 activation induced by cytotoxic necrotising factors (CNFs) and CNF3 intoxication [13]. Reports also demonstrated that Rho-kinase inhibition attenuates airway responsiveness, inflammation, matrix remodelling and oxidative stress activation induced by chronic inflammation [19]. Rho-A/Rho kinase inhibitors regulate smooth muscle relaxation via the inhibition of myosin light chain phosphorylation and actin depolymerisation [20]. Bronchial smooth muscle Rho-A expression may be related to IL-13-JAK1-STAT6 signalling [21]. However, it remains

unknown whether the Rho-A/Rho-kinase pathway modulates mucus secretion via STAT6.

Fasudil, a selective Rho-A/Rho kinase (ROCK) inhibitor, has been used in clinical trials for several cardiovascular diseases and pulmonary hypertension. Oral administration of fasudil inhibits eosinophil recruitment, goblet cell hyperplasia and airway hyper-reactivity by suppressing IL-5 and IL-13 [22]. However, it remains unknown whether Rho-A/Rho kinase modulates mucin *muc5ac* in airway allergic inflammatory diseases and cultured human epithelial cells. Therefore, we examined the effect of fasudil on mucus secretion and MUC5AC in allergen-challenged mice and cells.

Methods

Reagents and animals

The antibodies NF κ B (Clone #: F-6, Cat #: sc-8008), STAT6 (Clone #: S-20, Cat #: sc-621), β -actin (Clone #: N-21, Cat #: sc-130656), phospho-NF κ B (Clone #: Ser337, Cat #: sc-330220-R), phospho-STAT6 (Clone #: Tyr641, Cat #: sc-11762), MUC5AC (mucin 5AC, Clone #: K-20, Cat #: sc-16903) and Rho-A (Cat #: sc-179) (Santa Cruz Biotechnology, CA, USA). STAT6 siRNA (Cat #: sc-29497) were purchased from Santa Cruz Biotechnology (Delaware, CA). The enzyme-linked immunosorbent assay (ELISA) kits for IL-17, IL-4 and IL-13 were purchased from R&D Systems (Minneapolis, MD, USA). The MUC5AC-pGL3 luciferase vector was constructed by our laboratory based on the pGL3 luciferase reporter vector (Promega, Madison, WI, USA) following the manufacturer's instructions. BALB/c mice were maintained under specific pathogen-free conditions and were 6–8 weeks of age. The mice were housed in the Animal Experimental Center of Sichuan Medical University under pathogen-free conditions. All animal experiments were performed in accordance with the guidelines of the Animal Experiments Center of Luzhou Medical College.

Immunisation and challenge protocol

BALB/c mice were randomly grouped and sensitized by intraperitoneal (i.p.) injection with 20 μ g ovalbumin (OVA) with 1 mg aluminium hydroxide on days 0 and 7. The mice were challenged with intranasal (i.n.) instillation of 10 μ g OVA in 100 μ L phosphate-buffered saline (PBS) from days 14 to 21. The mice treated with fasudil were intraperitoneally administered 10 mg/kg fasudil hydrochloride (Tianjin Chase Sun Pharmaceutical Co, Ltd, Tianjin, China) from days 14 to 21. PBS control mice were treated with intranasal instillation of 100 μ L PBS from days 14 to 21. At 24 h after intranasal challenge, airway responsiveness was measured with a Buxco whole-body plethysmography (WBP) system

(Buxco Research Company, St. Paul, USA) in response to inhaled methacholine (acetyl- β -methyl-choline chloride; Sigma, Saint Louis, USA). Airway responsiveness was expressed as the enhanced pause (Penh), described in detail previously [23]. The mice were sacrificed with an overdose of ketamine. Lung tissues were stored in liquid nitrogen or fixed in 10% neutral-buffered formalin and embedded in paraffin.

Histological evaluation

The mouse trachea was cannulated and lavaged twice with 1.0 mL PBS. Bronchoalveolar lavage fluid (BALF) was centrifuged (500 g for 5 min at 4°C) and resuspended. The cells were used for cytopins onto slides. The cells in the BAL fluid were fixed and stained as described previously [14]. Differential cell counts were performed in duplicate on coded slides for 200 cells from each sample. The lung tissues were fixed in 10% neutral-buffered formalin and embedded in paraffin. Lung sections were stained with standard haematoxylin–eosin (H&E) staining methods to evaluate the tissue inflammation and were also stained with periodic acid–Schiff (PAS) reagent to detect airway mucus production. The degree of cellular infiltration was scored using previously described methods [24]. A score ranging from 0 to 3 was applied to each observed bronchus, with approximately 10 areas total scored.

Human lung tissue

The study included patients with asthma and control subjects who were recruited in an ongoing study at the Affiliated Hospital of Sichuan Medical University (Sichuan, China) between January 2012 and October 2014. The diagnosis of asthma was based on our previous reports [24]. Control subjects with left pulmonary shadow (but no asthma symptoms) were examined by bronchoscopy, and subjects with asthma or other chronic airway diseases were excluded from control group. Bronchoscopies were performed according to standard procedures. Biopsy specimens were collected from the right middle lobar bronchi, fixed in 4% paraformaldehyde and embedded in paraffin. The Sichuan Medical University Ethics Committee approved this human study.

Cell culture and luciferase assay

Human airway epithelial cells (16HBE) were cultured in DMEM/F12 culture medium with 10% of foetal calf serum (FCS) at 37°C in 5% CO₂. After the 16HBE cells reached 85% confluence in 6-well plastic plates, the medium was replaced with serum-free DMEM/F12 culture medium. The cells were then treated with house

dust mite (HDM) extract or fasudil in a serum-free culture medium.

The 16HBE cells were transfected with STAT6 siRNA and LipofectAmine 2000 according to the manufacturer's instruction. For the luciferase assay, a 3.7-kb segment of the 5' flanking region of the human MUC5AC gene (nucleotides from 23752/+7) was cloned into the pGL3-Basic Luciferase Vector (Promega). A PLR-TK vector was used as a control plasmid to measure transfection efficiency. Luciferase activity was measured according to our previous report [24].

Immunostaining

Frozen lung tissues fixed with OCT were sectioned, and immunohistochemical staining was performed on glass slides using standard histological methods. The 16HBE cells or sections were fixed in acetone and blocked at room temperature. The cells or tissue sections were incubated with either NF κ B or MUC5AC antibodies (Santa Cruz Biotechnology). A mouse isotype serum instead of the primary Abs was used as a negative control. FITC-conjugated goat anti-rabbit Abs or FITC-conjugated anti-mouse secondary Abs was used to probe the primary Abs. The nucleus was stained with DAPI reagent. Tissue sections were viewed with a Leica TCS SP5 confocal microscope (Leica, Mannheim, Germany). The fluorescence intensity was calculated in 10 randomly selected airway cells.

Cytokine assays and Western blot

Lung tissues were homogenised in RIPA lysis buffer for cytokine assays and Western blot analysis. Cytokine levels were determined in triplicate lung lysates using ELISA kits for IL-13, IL-4 and IL-17. Lysates (20 μ g) were electrophoresed on 10% SDS polyacrylamide gels at 100 V for 2 h and transferred to a microporous polyvinylidene difluoride (PVDF) membrane at 100 mA for 2 h. The membranes were blotted with NF κ B antibodies (1 : 1000), and phospho-NF κ B rabbit polyclonal antibodies (Ser311 of p65, 1 : 1000), STAT6 polyclonal antibodies, phospho-STAT6 polyclonal antibodies and Rho-A polyclonal antibodies and processed using enhanced chemiluminescence (Pierce, Rockford, U.S.A.).

Statistical analysis

The data are expressed as the mean $\pm$ standard error. The statistical analysis was performed using ANOVA (Tukey's *post hoc*) or Student's *t*-test. All statistical analyses were performed using the SPSS 13.0 software (SPSS, Chicago, IL, USA). The level of significance was defined as $P < 0.05$ between any two groups.

Results

Patients with asthma exhibit increased Rho-kinase expression in airway tissue

To evaluate the clinical relevance of Rho-kinase in airway mucus secretion, we measured mucus secretion and the expression of Rho-kinase in the airway tissue of patients with asthma and normal individuals collected by bronchoscopy. The number of airway goblet cells in patients with asthma increased compared to normal control individuals (Figs 1a and c; $n = 4$). Patients with asthma exhibited increased goblet cell metaplasia as determined by PAS staining. In addition, the expression of Rho-kinase increased ~ 3.0 -fold in the airway of patients with asthma compared to control individuals (Figs 1b and d; $n = 4$).

Rho/Rho-kinase inhibitor decreases airway inflammation in ovalbumin-challenged mice

To investigate the effect of the Rho-kinase inhibitor, fasudil, in airway inflammation and airway hyperresponsiveness, we performed intraperitoneal injection of fasudil followed by intranasal instillation to induce airway inflammation (Fig. 2a). These fasudil treatments resulted in a significant decrease in total inflammatory cells and eosinophils in the BAL fluid compared to

OVA-challenged mice ($P = 0.03$ and $P = 0.001$) (Fig. 2b) and a decreased inflammation index in the lung (Fig. 2c, $P = 0.002$). Histological inflammatory responses were also observed in the lungs and airways (Fig. 2d). There was reduced inflammatory cell infiltration in the airway, around blood vessels and in the alveoli of fasudil-treated mice. Penh, a marker of airway responsiveness, was increased in OVA-challenged mice. However, Penh levels were decreased by fasudil in mice that received a methacholine dose of 12.5, 25 and 50 mg/mL compared to OVA-challenged mice ($P = 0.001$, $P = 0.048$, $P = 0.001$, respectively) (Fig. 2e).

Rho/Rho-kinase inhibitor decreases mucus secretion in ovalbumin-challenged mice

Airway mucus hypersecretion contributes to morbidity and mortality in many patients with asthma [9]. However, the effect of fasudil on mucus secretion remains unclear. We observed that OVA exposure caused mucous secretion in OVA-challenged mice. PAS staining demonstrated a 0.6-fold decrease in mucous secretion in the airway of fasudil-treated mice compared to OVA-challenged mice (Fig. 3a, $P = 0.001$). The number of MUC5AC-positive epithelial cells also decreased by approximately 0.55-fold in fasudil-treated mice compared to OVA-challenged mice (Fig. 3b, $P = 0.043$).

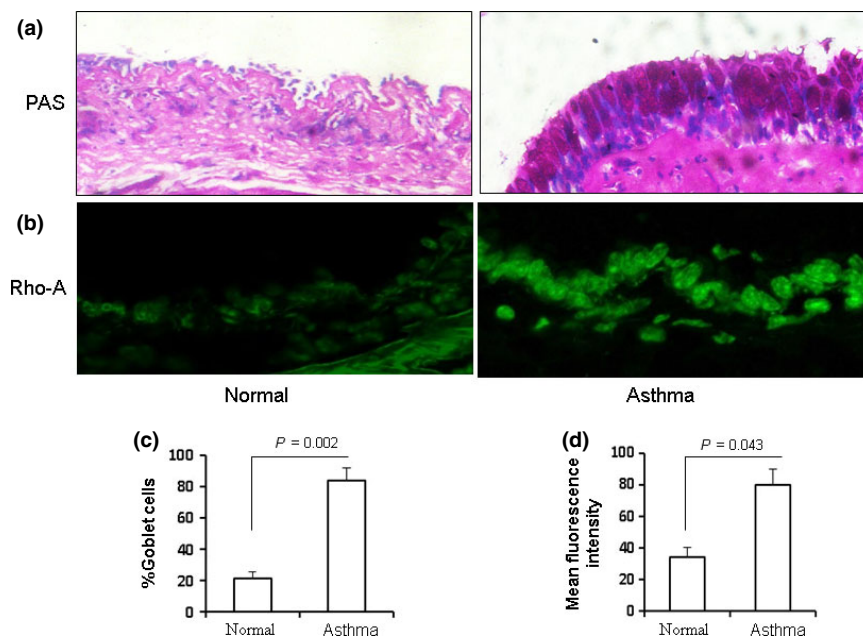


Fig. 1. Asthma exhibits mucus hypersecretion and increased Rho-A expression in airway tissue. (a) PAS staining of normal airway tissue and airway tissue from patients with asthma (original magnification, $\times 200$). (b) Immunostaining for the expression of Rho-A ($\times 400$). (c) The PAS-positive cell percentage was quantified in 10 random fields (original magnification, $\times 200$). (d) The fluorescence intensity was quantified per micrometre of Rho-A in 10 random fields (original magnification, $\times 400$). Data are the mean $\pm$ SEM of four patients (three males and one female) and four control subjects (two males and two females). Ages of patients: years, mean $\pm$ SEM, 42.12 ± 9.14 ; normal subjects, 38.12 ± 11.2 .

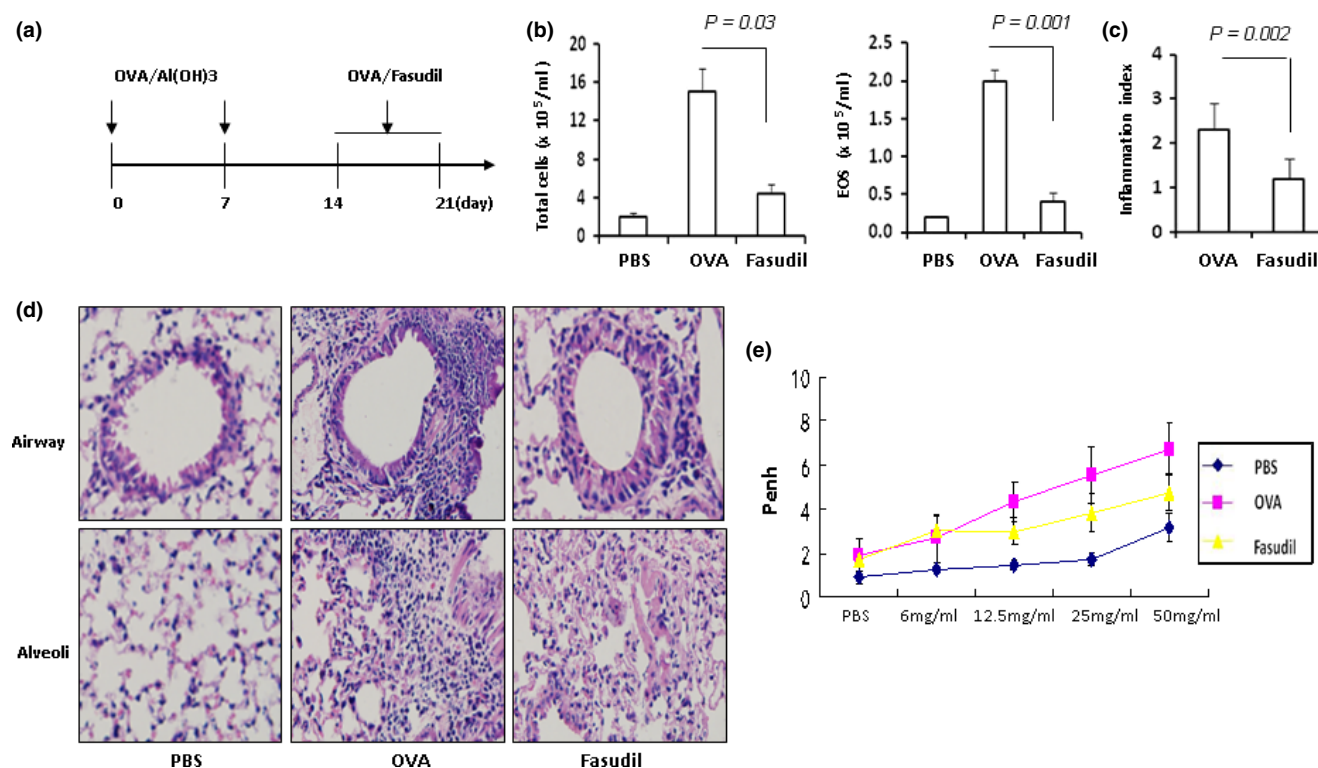


Fig. 2. Fasudil decreases airway inflammation and airway responsiveness in OVA-challenged mice. (a) Sensitization and challenge protocol for OVA-inducing asthmatic mice ($n = 8$ mice for each group). i.p. for intraperitoneal; i.n. for intranasal. (b) The number of total cells and eosinophils (Eos) in the BAL of mice was determined by differential cell analysis. (c) The inflammatory cell infiltration index was determined in the lungs. (d) Lung tissues were stained using H&E (original magnification, $\times 200$). (e) Airway hyperresponsiveness was determined by measuring the enhanced pause (Penh) ($n = 8$ each group).

Rho/Rho-kinase inhibitor down-regulates NF κ B, STAT6 and IL-4/IL-13 in ovalbumin-challenged mice

To determine whether Rho/Rho-kinase plays a role in regulating the expression and activity of NF κ B and STAT6 in allergic inflammation, Western blot analysis and immunostaining were performed using the lung tissue of OVA mice. As shown in Fig. 4a, OVA challenge resulted in increased expression and phosphorylation of NF κ B and STAT6 in OVA-challenged mice, which was inhibited by fasudil. We then used immunohistochemistry to detect the expression and localisation of NF κ B in the airway space. In OVA-challenged mice, there was significantly increased expression of NF κ B in the airway. OVA challenge also increased the nuclear translocation of NF κ B in OVA-challenged mice compared to control mice. However, fasudil application in mice inhibited the expression of NF κ B in the airway epithelium compared to control mice. The nuclear translocation of NF κ B in fasudil-treated mice significantly decreased compared to control mice (Fig. 4b). OVA-challenged mice showed increased levels of IL-17, IL-4 and IL-13 compared to PBS control mice as determined by ELISA. Fasudil treatment decreased the levels of

IL-17, IL-4 and IL-13 compared to control mice (Fig. 4c). Collectively, these data suggest a role for fasudil in the negative regulation of NF κ B, STAT6, IL-17, IL-4 and IL-13 in OVA-challenged mice.

Rho/Rho-kinase inhibitor down-regulates MUC5AC in house dust mite-induced cells

House dust mite significantly induces MUC5AC gene expression [24]. To further elucidate the effect of the Rho/Rho-kinase inhibitor on MUC5AC in airway epithelial cells, MUC5AC expression was assessed in dust mite induced-16HBE cells using immunostaining and a MUC5AC PGL3 luciferase reporter vector *in vitro*. We treated 16HBE cells with 50 μ g HDM and found that the levels of MUC5AC were increased compared to the PBS control. After 45 μ mol/L fasudil pretreatment for 2 h, the expression of MUC5AC in 16HBE cells exposed to HDM significantly decreased compared to control cells (Figs 5a and b, $P = 0.006$). Using luciferase activity analysis, we found that HDM significantly induced MUC5AC gene expression in 16HBE cells compared to PBS controls ($P = 0.003$), whereas fasudil pretreatment significantly decreased

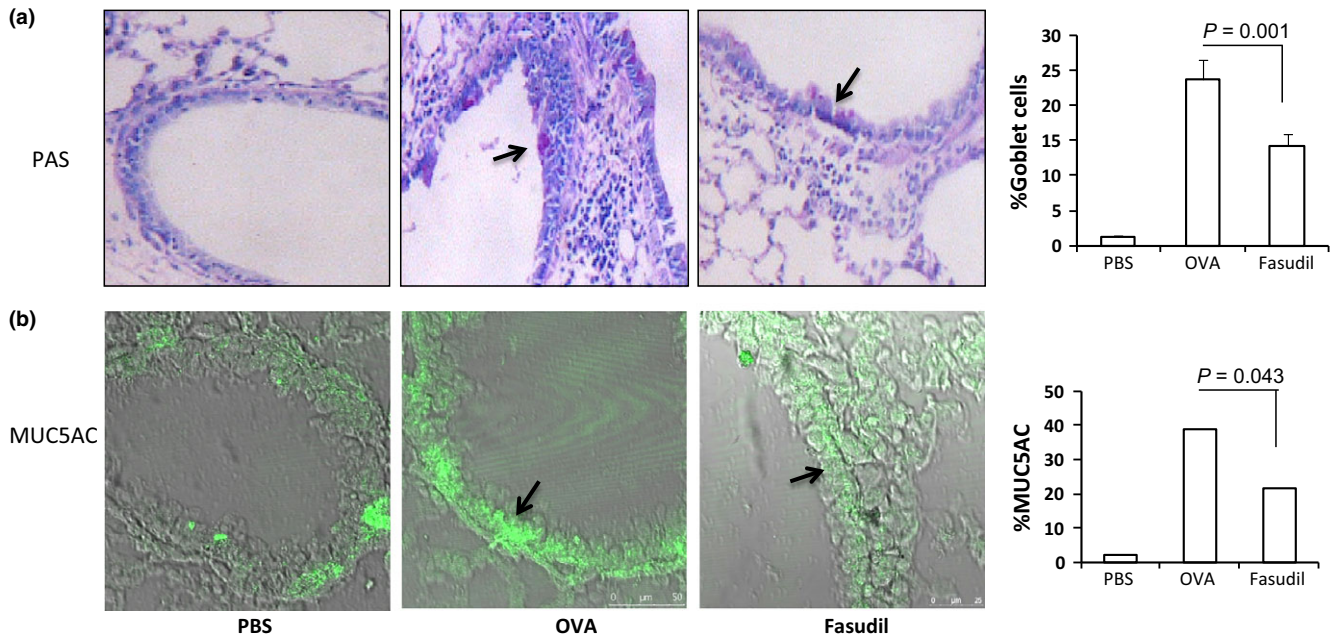


Fig. 3. Fasudil decreases mucus hypersecretion in OVA-challenged mice. (a) PAS staining of epithelial goblet cells in the mouse lungs. The PAS-positive cell percentage was quantified in 10 random airway epithelial cells fields (original magnification, $\times 200$). (b) MUC5AC immunostaining in the mouse lungs (merged images). The percentages of MUC5AC staining positive cells were counted in 10 random airway epithelial cells fields and presented as the mean $\pm$ SEM (Original magnification, $\times 200$).

HDM-induced MUC5AC gene expression (Fig. 5c, $P = 0.0001$).

Rho/Rho-kinase inhibitor down-regulated NF κ B and STAT6 in house dust mite-induced cells

Ovalbumin-induced Rho-associated coiled-coil-forming kinases (ROCKs) activation occurs in OVA-induced allergic airway inflammation mouse models [25]. However, the role of Rho-kinase in mucous secretion in asthma remains unclear. To further confirm the role of Rho-kinase and the related pathway, we treated 16HBE cells with HDM and showed an increase in Rho-A expression in HDM-induced 16HBE cells. HDM treatment also increased the expression and phosphorylation of STAT6 and NF κ B in 16HBE cells compared to PBS-treated cells. After fasudil treatment, the expression of Rho-A, STAT6 and NF κ B, as well as phosphorylation of STAT6 and NF κ B significantly decreased compared to HDM-induced control 16HBE cells (Fig. 6a). HDM treatment induced significant NF κ B nuclear translocation, which was abolished by fasudil (Fig. 6b). These results suggest that Rho-kinase regulates STAT6 and NF κ B activation. Finally, based on the luciferase activity of HDM-treated 16HBE cells pre-transfected with STAT6 siRNA, MUC5AC gene expression was decreased compared to sham control-transfected cells (Fig. 6c). Taken together, our studies demonstrate that Rho-kinase may

critically regulate airway inflammation by the key cytokines, IL-4 and IL-13, and MUC5AC secretion via STAT6/NF κ B (Fig. 6e).

Discussion

The major features of allergic asthma include airway inflammation, airway hyperresponsiveness (AHR) and goblet cell metaplasia. The aberrant secretion and accumulation of mucus in the airway lumen are clinical hallmarks associated with various lung diseases. Mucus hypersecretion is also a pathophysiological feature of asthma, contributing to morbidity and mortality. In our present studies, patients with asthma exhibited increased goblet cell metaplasia. In addition, the expression of Rho-kinase increased in the airway of patients with asthma. These results indicate that increased Rho-kinase may be associated with goblet cell metaplasia.

Rho kinase is a serine/threonine protein kinase. Increased Rho-A/ROCK activities have been shown in allergic models of asthma [26]. The Rho-A/ROCK signalling pathway is important for the increased Ca^{2+} -sensitivity of smooth muscle. ROCK inhibitors are effective as bronchodilators for treating asthma [27]. Recent studies demonstrated that ROCK2 and ROCK1 regulate AHR and that ROCK2, but not ROCK1, plays a role in allergen-induced goblet cell hyperplasia [28]. ROCK1

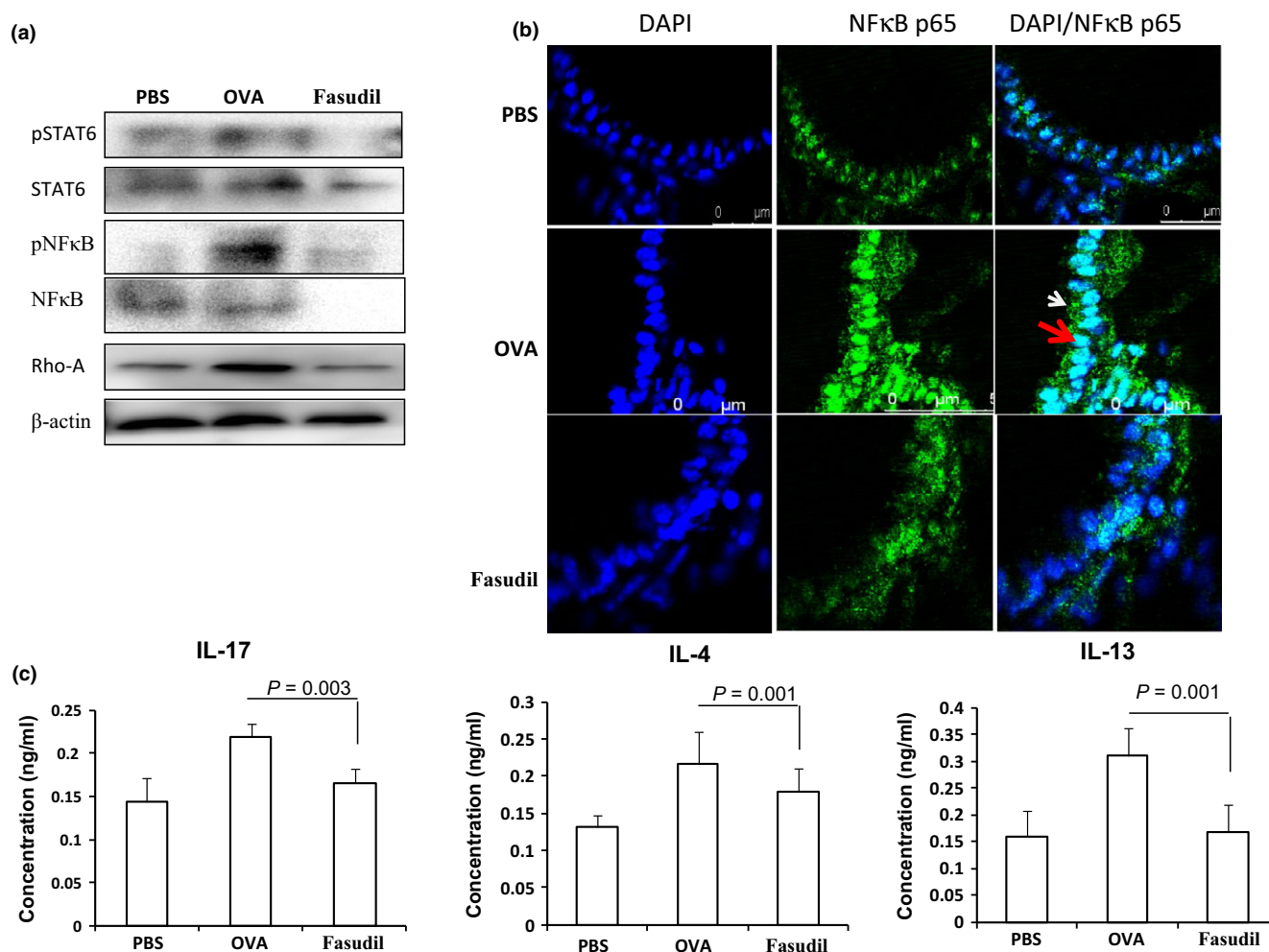


Fig. 4. Fasudil modulates NFκB, STAT6 and cytokines in OVA-challenged mice. (a) The expression and phosphorylation of STAT6 and NFκB in lung homogenates from mice were determined by Western blotting. (b) NFκB nuclear translocation was assessed by immunofluorescence staining with the NFκB p65 antibody. Arrows indicate relevant markers in positively stained cells. FITC-conjugated secondary Abs ($\times 200$). (c) Standard ELISA was performed to determine the levels of IL-4, IL-13 and IL-17 in the lung tissue.

and ROCK2 are important for regulating eosinophil inflammation and goblet cell hyperplasia [25]. Rho-A signalling through the platelet P2Y₁ receptor controls leucocyte recruitment in allergic mice [29]. Fasudil, a selective Rho-A/Rho kinase inhibitor, decreased the level of ROCK 1, ROCK 2 and Rho-A in the cardiac tissue of fasudil-treated rats [30]. Fasudil attenuates the elevated ROCK activity in transgenic mice overexpressing the enzyme, copper-zinc superoxide dismutase 1 [31]. We found that fasudil treatment significantly decreased inflammatory cells and the inflammation index in lung. Our observations are consistent with a recent report on a modulatory role for Rho kinase in airway inflammation [32].

The airway response to inflammation or bronchoprovocation is associated with increased mucus secretion. Mucus hypersecretion may be related to

activation of the extracellular regulated kinase 1/2, signalling through the epidermal growth factor receptor (EGF-R) or secretory phospholipases A2 [6]. EGF-R and the Th2 cytokine pathways were identified as possible targets for the pharmacotherapy of airway mucus hypersecretion in asthma [8, 9]. Inhibition of the STAT6, STAT3 and EGFR signalling pathways significantly attenuated the expression of the airway mucins, MUC5AC and MUC5B [33]. In our study, we also confirmed that OVA exposure caused mucous secretion and MUC5AC expression. Fasudil decreased mucous secretion and MUC5AC expression. This result indicates that Rho-A/Rho kinase plays a critical role in regulating allergen-induced mucus hypersecretion.

Th2 cytokine signalling, such as IL-13 and IL-4, plays a critical role in goblet cell metaplasia and

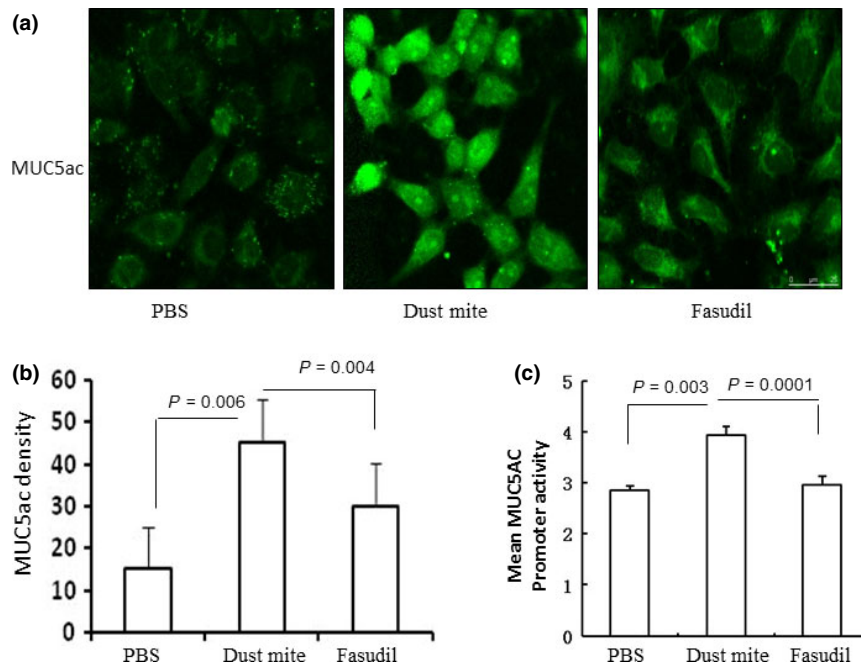


Fig. 5. Fasudil regulates MUC5AC in HDM-induced cells. (a) Immunofluorescence staining with the MUC5AC antibody. FITC-conjugated secondary Abs ($\times 400$). (b) The MUC5AC level is represented by the fluorescence intensity. (c) MUC5AC activation was determined by luciferase promoter assay. The 16HBE cells were transfected with the MUC5AC promoter luciferase plasmid. The cells were then stimulated with HDM, and the luciferase activity was measured. Data are representative of three experiments performed in triplicate and are expressed as the mean $\pm$ SEM.

mucus production. Inhibition of Th2 cytokines may represent a protective strategy to prevent mucus congestion of distal airways [21, 34]. However, corticosteroids (dexamethasone) did not inhibit the effects of IL-13 on goblet cell differentiation in severe asthma [35]. IL-17 is a potent stimulator of *muc5ac* gene expression in well-differentiated human bronchial epithelial cells. NF κ B is involved in cytokine-induced MUC5AC expression. IL-17 promoted NF κ B translocation and binding to the NF κ B-binding sequences of the *muc5ac* promoter [36–38]. In this study, OVA-challenged mice also showed increased levels of IL-17, IL-4 and IL-13. The Rho-A/Rho kinase inhibitor, fasudil, decreased the levels of IL-17, IL-4 and IL-13 in the lung tissue. These results indicate that the Rho-A/Rho kinase is associated with MUC5AC expression. IL-13/STAT6 signalling is critical for the development of antigen-induced bronchial smooth muscle hyperresponsiveness together with the up-regulation of the Rho-A protein [39]. In our study, OVA-challenged mice exhibited increased expression of the Rho-A protein, as well as phosphorylation of NF κ B and STAT6. The nuclear translocation of NF κ B in the airway increased in OVA-challenged mice. Moreover, not only did the Rho-A/Rho kinase inhibitor fasudil inhibit the expression and phosphorylation of NF κ B and STAT6, fasudil also decreased the

nuclear translocation of NF κ B. These data suggest a role for fasudil in the down-regulation of NF κ B and STAT6 in OVA-challenged mice.

House dust mite is a common allergen of allergic asthma. HDM extracts contain many proteins of known and unknown characteristics. HDM has cysteine protease activity that may cause the release of cytokines. HDM-induced interleukin-13 expression is dependent on the activation of NF κ B [40, 41]. HDM also induces granulocyte-macrophage colony-stimulating factor, IL-6, IL-8, monocyte chemotactic protein-1 and macrophage inflammatory protein-3 α . NF κ B regulates the release of cytokines and chemokines [42]. In previous studies, we observed a similar cytokine profile induced by HDM. HDM extracts induced IL-2, IL-4 and IL-17 in cultured lung epithelial cells. We also showed that NF κ B was phosphorylated and that HDM extracts induced STAT6 expression in HDM extract-treated lung epithelial cells [43]. In the present studies, we confirmed that HDM increased *muc5ac* gene and MUC5AC expression in cultured human epithelial cells. MUC5AC expression is related to NF κ B, STAT6 and histone deacetylase 2 (HDAC2) [12]. The NF κ B-binding site is at -3594/-3582 in the *muc5ac* promoter. NF κ B directly regulates cytokine-enhanced MUC5AC expression [36]. In cultured cells, we also found that fasudil decreased *muc5ac* gene and MUC5AC expression

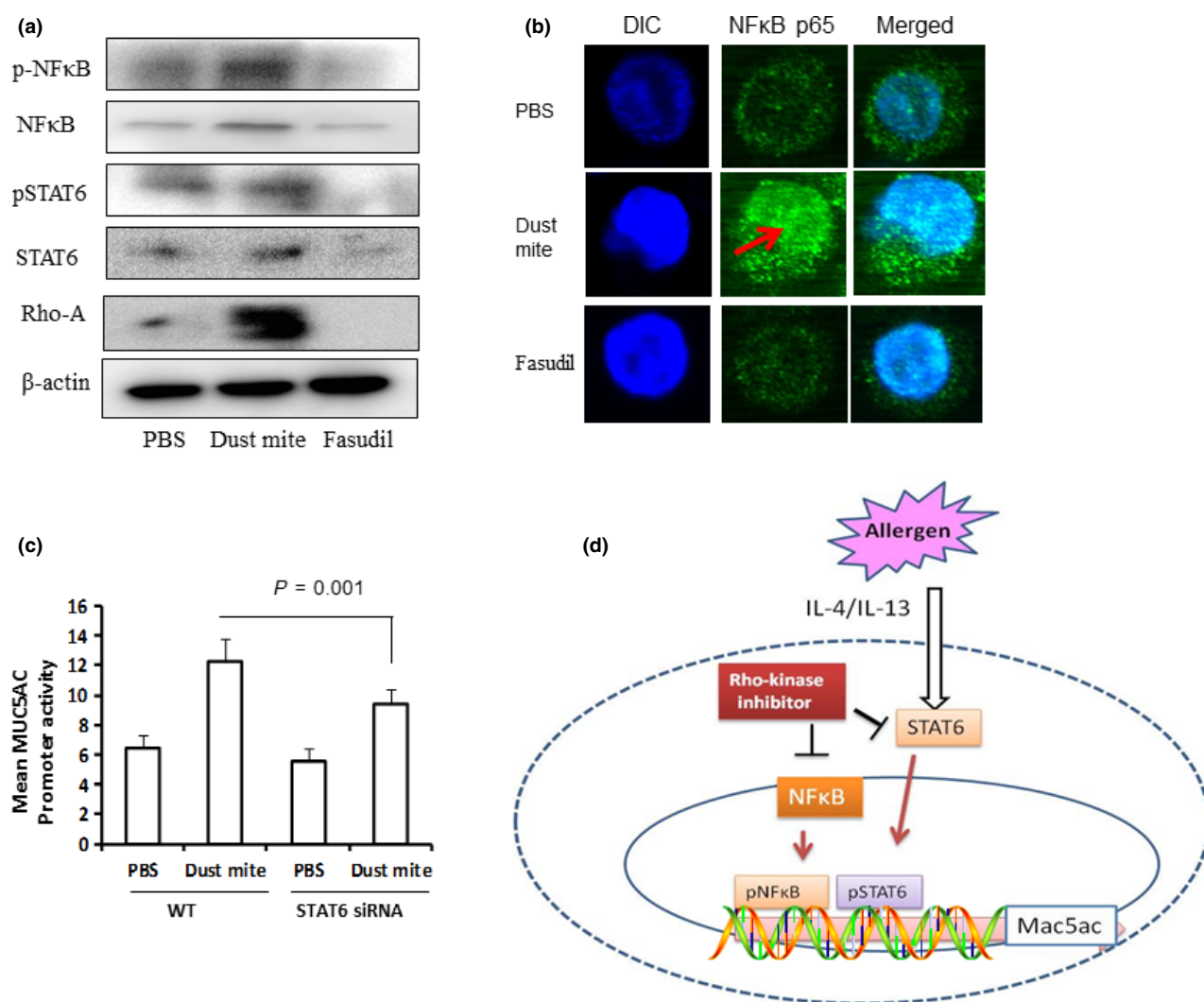


Fig. 6. Fasudil down-regulates NFκB and STAT6 in HDM-induced cells. (a) The expression and phosphorylation of STAT6 and NFκB in 16HBE cells were determined by Western blotting. (b) NFκB nuclear translocation was assessed by immunofluorescence staining with the NFκB p65 antibody. FITC-conjugated secondary Abs ($\times 400$). (c) MUC5AC activation was determined by luciferase promoter assay. The 16HBE cells were cotransfected with the MUC5AC promoter luciferase plasmid and STAT6 siRNA vs. the control vector and scrambled siRNA. (d) Diagram showing the cell signalling pathway with the Rho/Rho-kinase inhibitor.

associated with the expression and phosphorylation of NFκB and STAT6. Fasudil decreased the nuclear translocation of NFκB. Furthermore, in cultured human epithelial cells, knockdown of STAT6 resulted in a decrease in MUC5AC. We also found that HDM extracts enhanced the binding of STAT6 to the flanking region around the distal STAT6-binding site of the *MUC5AC* promoter for IL-4/IL-13-induced *MUC5AC* expression (data not shown).

In summary, we demonstrate that Rho-kinase is associated with airway mucus secretion in patients with asthma. We further show that the Rho-A/Rho kinase inhibitor, fasudil, is involved in the regulation of allergic inflammation, airway hyperresponsiveness

and mucus secretion in a mouse model of allergic asthma. Fasudil treatment decreased mucus secretion and *muc5ac* expression. We showed that the activation of NFκB and STAT6 is an essential mechanism in both the OVA-induced mouse model of allergic asthma and HDM-induced MUC5AC expression in human airway epithelial cells, and we identified a regulatory effect of fasudil on the activation of NFκB and STAT6 that is essential for mucus hypersecretion. This study highlights an important negative regulatory role in allergen-induced mucus secretion and MUC5AC expression via the regulation of STAT6 and NFκB and may provide new strategies for controlling mucus over production in asthma.

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Author contributions

Guoping Li and Nanshan Zhong designed the study. Tao Xie, Ghuangyan Luo, Yun Zhang, Xing Wang and

Xiaoyun Wang performed the experiments. Ghuangyan Luo, Min Wu and Guoping Li analysed the data and wrote the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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