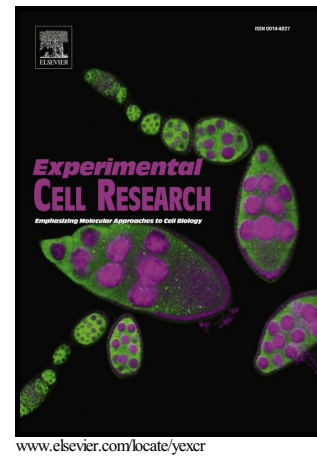


Vasoactive intestinal peptide inhibits airway smooth muscle cell proliferation in a mouse model of asthma via the ERK1/2 signaling pathway

Juan Wang, Yun-Xiao Shang, Xu-Xu Cai, Li-Yun Liu



PII: S0014-4827(18)30064-8
DOI: <https://doi.org/10.1016/j.yexcr.2018.01.042>
Reference: YEXCR10913

To appear in: *Experimental Cell Research*

Received date: 14 October 2017
Revised date: 20 January 2018
Accepted date: 31 January 2018

Cite this article as: Juan Wang, Yun-Xiao Shang, Xu-Xu Cai and Li-Yun Liu, Vasoactive intestinal peptide inhibits airway smooth muscle cell proliferation in a mouse model of asthma via the ERK1/2 signaling pathway, *Experimental Cell Research*, <https://doi.org/10.1016/j.yexcr.2018.01.042>

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting galley proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Vasoactive intestinal peptide inhibits airway smooth muscle cell proliferation in a mouse model of asthma via the ERK1/2 signaling pathway

Juan Wang, Yun-Xiao Shang, Xu-Xu Cai, Li-Yun Liu

Department of Pediatrics, Shengjing Hospital of China Medical University, Shenyang, China.

Abstract

Asthma is a heterogeneous clinical syndrome characterized by airway inflammation, hyper-responsiveness and remodeling. Airway remodeling is irreversible by current antiasthmatic drugs, and it is the main cause of severe asthma. Airway smooth muscle cells (ASMCs) act as the main effector cells for airway remodeling; the proliferation and hypertrophy of which are involved in airway remodeling. Caveolin (Cav)-1 is present on the surface of ASMCs, which is involved in cell cycle and signal transduction regulation, allowing ASMCs to change from proliferation to apoptosis. The extracellular signal-regulated kinase (ERK)1/2 signaling pathway is a common pathway regulated by various proliferative factors, which demonstrates a regulatory role in airway remodeling of asthma. There have been many studies on the correlation between vasoactive intestinal peptide (VIP) and airway reactivity and inflammation in asthma, but the functions and related mechanisms of ASMCs remain unclear. In this study, we established an airway remodeling model in asthmatic mice, and concluded that VIP inhibits airway remodeling *in vivo*. The *in vitro* effect of VIP on interleukin-13-induced proliferation of ASMCs was studied by examining the effects of VIP on expression of ERK1/2, phospho-ERK1/2 and Cav-1 in ASMCs, as well as changes in cell cycle distribution. VIP inhibited phosphorylation of the ERK1/2 signaling pathway and expression of Cav-1 on ASMCs and decreased the proportion of S phase cells in the cell cycle, thus inhibiting the proliferation of ASMCs. This study provides a novel therapeutic mechanism for the treatment of asthma.

Key words:

Vasoactive intestinal peptide; airway remodeling; ERK1/2 signaling pathway; caveolin-1; cell cycle

1. Introduction

Asthma is a heterogeneous clinical syndrome characterized by airway inflammation, hyper-responsiveness and remodeling, the symptoms of which include paroxysmal or persistent wheezing, dyspnea and coughing. Airway smooth muscle cells (ASMCs) act as the main effector cells of airway remodeling, and their proliferation and hypertrophy lead to irreversible pathological changes in the airway. Cultured ASMCs from asthma patients show significant proliferation [1]. Therefore, how to inhibit the proliferation of ASMCs and the occurrence of airway remodeling have become hot topics in asthma research.

As an inhibitory non-adrenergic non-cholinergic neuropeptide, vasoactive intestinal peptide (VIP) was initially considered to be a gastrointestinal hormone, regulating physiological activity of the digestive system. With more in-depth research, VIP has been recognized to play a more important role in the neuroendocrine-immune response and participate in a variety of biological effects such as metabolic processes, cell differentiation, smooth muscle relaxation and regulation of immune responses [2], as well as the maintenance of immune homeostasis, which is closely related to diseases such as asthma, chronic obstructive pulmonary disease, pulmonary hypertension, septic shock, diabetes, rheumatism and multiple sclerosis. It is suggested that VIP inhibits neutrophil chemotaxis [3] and promotes T helper (Th)1 to Th2 transformation [4]. Moreover, VIP has a strong effect on bronchial relaxation [5] and alleviating airway inflammation [6], showing its potential in asthma treatment. VIP plays a role in inhibiting vascular remodeling in patients with pulmonary hypertension [7], but it is not clear if it can inhibit airway remodeling and its mechanism.

The extracellular signal-regulated kinase (ERK)1/2 signaling pathway is a member of the mitogen-activated protein kinase (MAPK) family, the activation of which transduces signals from the cell membrane surface receptor to the nucleus, and it is a common pathway regulated by various proliferative factors. ERK1/2 are widely distributed in ASMCs. Phospho (p)-ERK1/2 are involved in the proliferation of ASMCs and have a regulatory role in airway remodeling of asthma [8].

As non-coated cell capsules/vesicles, caveolae often form clusters on the cell surface or as monomers in cell lacunae and cell membrane occlusion. Caveolin (Cav)-1, as the main component of the caveolae, exists on the surface of ASMCs and

it is involved in regulation of the cell cycle and signal transduction, with anti-angiogenic functions and negative regulatory activity on growth factors [9]. Negative regulation of platelet-derived growth factor (PDGF) can lead to transformation from proliferation to apoptosis in SMCs [10].

Interleukin (IL)-13, as a Th2 type cytokine, plays an important role in the pathogenesis of asthma and it is involved in the regulation of airway inflammation, airway hyper-responsiveness and bronchial contraction [11]. IL-13 is also involved in the phosphorylation of various signaling pathways, including ERK, in cultured ASMCs [12].

The current study aimed to establish an airway remodeling model in asthmatic mice, allowing study of the effect of VIP on airway remodeling *in vivo*, and the *in vitro* effects and possible mechanisms of VIP on IL-13-induced proliferation in ASMCs extracted from asthmatic mice, providing a new perspective for the clinical application of VIP in asthma treatment.

2. Materials and Methods

2.1 Experimental animals

Thirty SPF female Balb/c mice (18–22 g) aged 6–8 weeks were provided by the Experimental Animal Center of Shengjing Hospital, China Medical University. All animal experiments were conducted in accordance with the Guidelines for Animal Care and Use (NIH publication, 8th Edition, 2011) and were approved by the Animal Care and Use Committee of China Medical University.

2.2 Reagents

Ovalbumin VIP, Cav-1 antagonist β -cyclodextrin(β -CD), and ERK1/2 inhibitor PD-98059 were purchased from Sigma (St. Louis, MO, USA). Aluminum hydroxide powder was purchased from Shanghai Meixing Chemical Co. Ltd. Antibodies to ERK, p-ERK, β -actin and α -smooth muscle actin (SMA) were purchased from Beijing Bioss. IL-13 was purchased from PeProTech (Rocky Hill, NJ, USA).

2.3 Model of airway remodeling in asthmatic mice

The airway remodeling model was established as previously described [13-15]. After 1-week adaptation, intraperitoneal (i.p.) injection of 0.2 ml sensitizer [100 μ g

ovalbumin (OVA) + 1 mg aluminum hydroxide powder] was administered on days 1, 7 and 14. On day 21, asthma was induced by atomization excitation: mice were kept in a $20 \times 20 \times 20$ cm homemade atomization container and subjected to atomized 2% OVA solution for 30 min every other day, for 8 consecutive weeks. Mice were randomly divided into 3 groups: control, asthma and VIP treatment groups, with 10 in each group. Mice in the control group received i.p. injection of phosphate-buffered saline (PBS) and were subjected to atomized PBS. For VIP treatment, mice received i.p. injection of 150 $\mu\text{g/kg}$ VIP 30 min before atomization excitation.

2.4 Histological examination

The mice were killed 24 h after the last excitation. The middle right lobe of the lung was excised and fixed with 4% paraformaldehyde, then stained with hematoxylin and eosin (HE). Identification of airway remodeling by pathological image analysis was performed as previously described[15]. The thickness of the bronchial wall (WAt) and smooth muscle thickness (WAs) was measured under a light microscope (200 $\times$). Three small and medium bronchial tubules with complete cross-sections were selected for each slice. An image acquisition system was used to obtain images, and the area of the basement membrane (Pbm), total area of the bronchus (WAt₁), lumen area (WAt₂), smooth muscle margin area (WAs₁) and smooth muscle inner area (WAs₂) were measured by image analysis software (NIS-Elements Br3.0), and normalized by Pbm, that is, using (WAt₁ – WAt₂)/Pbm and (WAs₁ – WAs₂)/Pbm to represent WAt ($\mu\text{m}^2/\mu\text{m}$) and Was ($\mu\text{m}^2/\mu\text{m}$).

2.5 Cell culture

The mice were killed at 24 h after the last excitation. As previously described [16], under sterile conditions, the bronchial tissue was separated and mashed using ophthalmic scissors. D-Hanks solution was used to clean the tissue, and the small pieces of bronchial tissue were transferred into type I collagenase, trypsin and papain solution, and incubated in a 37°C water bath for 40 min with shaking. The digested cell suspension was then added to an equal volume of complete medium to terminate the reaction, and the undigested tissue mass was removed. The cells were then seeded onto 12-well plates at 5×10^5 cells/well, and passaged when they reached 90% confluence. The ASMCs were purified by differential incubation time for adherence,

and experiments were conducted using cells with 4–6 passages. Immunocytochemical staining was used to identify ASMCs.

2.6 Western blotting

Total protein was extracted and quantified. Protein was separated by 10% SDS-PAGE and transferred to nitrocellulose (polyvinylidene difluoride; PVDF) membranes. Primary antibodies anti-ERK1/2 (1: 500), anti-p-ERK1 / 2 (1: 200), and β -actin (1: 5000) were diluted with 5% (m/v) skimmed milk and loaded onto the PVDF membrane for incubation at 4°C overnight. The nitrocellulose membrane was removed from the primary antibody and placed in goat anti-rabbit IgG-horseradish peroxidase (1: 5000) at 37°C for a further 2 h, followed by electrochemiluminescence. A gel image analysis system(WD-9413B,Liuyi,Beijing,China) was used to scan and analyze results. The protein content was analyzed by density, and the relative gray value was used to represent the protein expression. Relative gray value was calculated as the gray value of the protein of interest divided by the gray value of β -actin in the same group.

2.7 Cell proliferation

Proliferation of ASMCs was detected by MTT assay. The ASMCs of the 4th passage from the asthma group were cultured with serum-free Dulbecco's modified Eagle's medium (DMEM) for 24 h to synchronize the cells. After adding the intervention factor for 48 h, MTT was added at a concentration of 0.2 mg ml, followed by 4 h incubation at 37°C. Dimethylsulfoxide (200 μ l) was then added to dissolve the crystals. The OD value was measured at 490 nm using a microplate reader(ELX-800,BIOTEK,USA).

2.8 Cell cycle analysis

The ASMCs of the 4th passage from the asthma model group were cultured with serum-free DMEM for 24 h to synchronize the cells at G0 phase. Cells were collected after 48 h of intervention. Pre-cooled 70% ethanol was used to fix the cells at 4°C for 2 h. Staining buffer (500 μ l) was added slowly and cells were fully resuspended. After addition of 25 μ l propidium iodide staining solution and 10 μ l RNase A, cells were kept at 4°C for 30 min. Flow cytometry analysis was performed after incubation, and

S phase ratio was determined.

2.9 Statistical analysis

All data was presented as mean $\pm$ standard deviation, and statistical analysis was performed using SPSS version 23.0. Analysis of variance was used to compare the homogeneity of variance in each group. Further comparison within each group was conducted using a least significant difference *t* test. Differences with $p < 0.05$ were considered statistically significant.

3. Results

3.1 Effects of VIP on airway remodeling

By measurement of the thickness of the bronchial wall and smooth muscle layer using lung tissue HE staining and pathological imaging analysis (Figure 1d), we found that, in the control group, the bronchial morphology was regular, and the airway epithelium was normal, without loss of cilia. Alveolar wall was intact, and the alveolae and bronchi demonstrated no inflammatory exudate or inflammatory cell infiltration around the bronchioles and vascular walls (Figure 1a). In contrast, in the asthma group, inflammatory cell infiltration was seen around the alveolar cavity, bronchioles and vascular wall, with cilia and epithelial cell shedding, thickening of ASM and vascular smooth muscle (VSM), and even damage of some of the alveolar structure (Figure 1b). In the VIP treatment group, inflammatory cell infiltration and thickening of smooth muscle were relieved compared to the asthma group (Figure 1c).

3.2 Identification of ASMCs

Cultured ASMCs were identified by immunocytochemistry. Under the inverted microscope, ASMCs were spindle-shaped, growing in parallel, and arranged in bundles, showing a "peak/valley" shape at the border of dense and sparse growth (Figure 2a). Immunocytochemistry indicated that the brown positive signal of α -SMA was filamentous (Figure 2b), which indicated successful culture of ASMCs.

3.3 Cell proliferation

3.3.1 Optimal concentration and time of VIP treatment of ASMCs

To determine the optimal concentration and time of VIP treatment on ASMCs,

cells were treated with 10^{-5} , 10^{-6} and 10^{-7} mM VIP for 12, 24, 48 and 72 h. Cell proliferation was determined by MTT assay. Cell viability was significantly lower in the 10^{-6} and 10^{-5} mM VIP groups than in the untreated group, but there was no significant difference between cell viability in the 10^{-7} mM VIP group and the untreated group ($p>0.05$). There was no significant difference between the 10^{-6} and 10^{-5} mM VIP groups ($p>0.05$) (Figure 3a). Different concentrations of VIP all showed the highest inhibition rate at 48 h post-treatment (Figure 3b). Therefore, for the follow-up experiments, we used 10^{-6} mM VIP for 48 h.

3.3.2 Effects of VIP treatment on proliferation of ASMCs

MTT assay showed that IL-13 significantly induced the proliferation of ASMCs *in vitro* after 48 h treatment ($p<0.05$), while the proliferation of ASMCs was significantly decreased after VIP treatment ($p<0.05$). After treatment with PD98059, cell proliferation was significantly lower than in the VIP group ($p<0.05$). After treatment with β -CD, cell proliferation was significantly lower than in the IL-13 treatment group, but higher than in the VIP group (Figure 3c).

3.4 Effect of VIP on Cav-1 expression in ASMCs

Expression of Cav-1 in ASMCs was detected by western blotting after each treatment. Expression of Cav-1 was significantly decreased after IL-13 treatment ($p<0.05$), and expression of Cav-1 was significantly higher after treatment with VIP and PD98059 than that in the IL-13-treated group ($p<0.05$) (Figure 4).

3.5 Effect of VIP on ERK1/2 and p-ERK1/2 expression in ASMCs

After 48h of IL-13 treatment, expression of ERK1/2 and p-ERK1/2 was significantly increased as detected by western blotting ($p<0.05$). However, after treatment with VIP or PD98059, the expression level of p-ERK1/2 was significantly lower than in the IL-13 treatment group ($p<0.05$) (Figure 5).

3.6 Cell cycle analysis

The cell cycle distribution of each group of cells after 48 h treatment showed that in IL-13-treated ASMCs, the proportion of cells in S phase was significantly higher than in the control group ($p<0.05$). For the VIP and PD98059 treatment groups, the

proportion of cells in S phase was significantly lower than in the IL-13-treated group ($p < 0.05$). This suggests that VIP reduced the proportion of ASMCs in S phase, thereby reducing intracellular DNA synthesis and inhibiting cell proliferation (Figure 6).

4. Discussion

Asthma is a chronic airway disease characterized by airway inflammation, hyper-responsiveness and remodeling. The airway remodeling includes structural changes, such as subepithelial fibrosis, proliferation of fibroblasts and myoblasts, and increased smooth muscle fibers. These changes lead to irreversible airway obstruction. The traditional treatment for asthma includes glucocorticoids and β -agonists, which can reduce airway inflammation, diastolic bronchial, and relieve symptoms of asthma. However, they cannot prevent or treat airway remodeling caused by persistent inflammation [17]. Even inhalation of high doses of glucocorticoids or systemic glucocorticoids do not alleviate asthma symptoms in about 5–10% of patients with severe or refractory asthma [18], which is closely associated with airway remodeling. Therefore, how to control the process of airway remodeling effectively has become a difficult topic for asthma research [19, 20].

Many animal and clinical studies have confirmed that, as a non-adrenergic non-cholinergic neurotransmitter, VIP can affect various aspects of asthma, such as reducing airway inflammation and hyper-reactivity, and inhibition of airway mucus secretion. ASMCs, as the main effector cells of asthma, mediate airway hyper-responsiveness, airway remodeling and fibrosis by inducing airway contraction and production of extracellular matrix and pro-inflammatory and anti-inflammatory factors [21]. St Hilaire et al. have confirmed that VIP can inhibit VSMC proliferation [22]. However, it remains elusive whether VIP inhibits the proliferation of ASMCs. In this study, we confirmed the inhibitory effect of VIP on the proliferation of ASMCs induced by IL-13 *in vitro*.

IL-13 is a key cytokine in the Th2 response and it is involved in IgE synthesis, eosinophil activation and mucus secretion, induction of airway hyper-responsiveness, production of vascular endothelial growth factor by structural cells, and participation in airway inflammation and remodeling [23-25]. Thus, in this study, we examined the effect of VIP on IL-13-induced proliferation of ASMCs and the associated signaling

pathways.

ERK1/2 is an important intracellular signaling molecule that transcribes extracellular signals into the nucleus and regulates gene expression, which is a common pathway regulated by different growth factors [26]. ERK1/2 regulates ASMC secretion [27,28], proliferation [29], and hyper-responsiveness [30]. As the main signaling pathway regulating the proliferation of ASMCs [24,31,32], ERK1/2 regulate cell growth by promoting cell cycle transitions as cells exit quiescence and progress through G1 into S phase[33],and is involved in the pathogenesis of asthma, chronic airway inflammation, airway hyper-responsiveness [34, 35], and ASMC proliferation in chronic airway remodeling. In a rat model of chronic asthma, ERK1/2 is highly activated. Therefore, inhibition of the ERK1/2 signaling pathway may be effective in the treatment of airway remodeling in asthma [15]. The results of the present study were consistent with this hypothesis, which confirmed that ERK1/2 is involved in signal transduction of VIP to inhibit proliferation of ASMCs.

The caveolar structure is present on the cell surface, and mostly on SMCs, fibroblasts, adipocytes, alveolar cells, epidermal cells and skeletal muscle cells. As the main component of the caveolae, Cav-1 is involved in regulating the cell cycle and signal transduction, which can initiate apoptosis [36]and negatively affect cell growth through the interactions with ERK1/2 signaling pathway[37]. Previous studies have shown that Cav-1 knockout mice have significant airway hyper-responsiveness [38]and exhibit increase of 25–30% in the S phase population compared to the wild-type cells[39]. Cav-1 mRNA expression in the lungs of OVA-sensitized mice was reduced [40]. Inhibition of the down-regulation of Cav-1 expression can inhibit allergen-induced airway remodeling [41]. Clinical studies have also shown decreased airway expression of Cav-1 in patients with asthma [42]. These studies have shown that the expression of Cav-1 negatively regulates asthma, but the mechanism of its effect on the proliferation of ASMCs is not clear. Our study is believed to be the first to demonstrate that VIP inhibits the down-regulation of Cav-1 expression, and therefore IL-13-induced proliferation of ASMCs in asthma.

The cell cycle is mainly divided into 4 phases: the early stage of DNA synthesis (G1 phase), DNA synthesis phase (S phase), late stage of DNA synthesis (G2 phase), and cell division stage (M phase). Cell cycle distribution is also an important indicator of cell proliferation [43]. In patients with asthma as well as in asthma animal models,

the proportion of ASMCs in S and G2/M phases is significantly increased, and the proportion in G0/G1 phase is decreased, suggesting that ASMCs are over-proliferating during asthma[44,45]. In the present study, the percentage of ASMCs in S phase after IL-13 stimulation was significantly increased compared to the control group, which was consistent with previous reports. After VIP treatment, the proportion of cells in S phase decreased significantly. After treatment with ERK1/2 pathway inhibitor PD98059, the proportion of cells in S phase decreased more significantly (Figure 6). These results suggest that VIP inhibits ASMC proliferation by reducing the proportion of cells in S phase; that is, reducing the proportion of cells in the proliferative phase, and this inhibition is associated with the ERK1/2 signaling pathway. During asthma, VIP immune response fibers are reduced in the airways, and serum VIP level is decreased. The anti-proliferative effect of VIP on ASMCs is weakened, which may exacerbate airway remodeling.

Our study demonstrated that VIP inhibits the proliferation of ASMCs by inhibiting the down-regulation of Cav-1, inhibiting phosphorylation of the ERK signaling pathway, and decreasing the proportion of cells in S phase, therefore inhibiting the occurrence of OVA-induced airway remodeling in mice.

In summary, VIP has strong effects in bronchial relaxation, inhibiting airway inflammation and ASMC proliferation, and thus inhibiting airway remodeling. Our study provides a theoretical basis for novel drug development for asthma.

Conflicts of interest

The authors declare that they have no competing interests.

References

- [1] Johnson PR, Roth M, Tamm M, Hughes M, Ge Q, King G, Burgess JK, Black JL, Airway smooth muscle cell proliferation is increased in asthma, *Am J Respir Crit Care Med.* 164(2001)474–477.
- [2] Gonzalez-Rey E, Delgado M, Vasoactive intestinal peptide and regulatory T-cell induction: a new mechanism and therapeutic potential for immune homeostasis, *Trends Mol Med.* 13(2007) 241-251.
- [3] Sergejeva S, Hoshino H, Yoshihara S, Kashimoto K, Lötvall J, Lindén A, A synthetic VIP peptide analogue inhibits neutrophil recruitment in rat airways in vivo, *Regul Pept.* 117(2004)

149-154.

- [4] Voice JK, Grinninger C, Kong Y, Bangale Y, Paul S, Goetzl EJ, Roles of vasoactive intestinal peptide (VIP) in the expression of different immune phenotypes by wild-type mice and T celltargeted type II VIP receptor transgenic mice, *J Immunol.* 170(2003)308-314.
- [5] Hasaneen NA, Foda HD, Said SI, Nitric oxide and vasoactive intestinal peptide as co-transmitters of airway smooth-muscle relaxation: analysis in neuronal nitric oxide synthase knockout mice, *Chest.* 124(2003)1067-1072.
- [6] Anderson P, Gonzalez-Rey E, Vasoactive intestinal peptide induces cell cycle arrest and regulatory functions in human T cells at multiple levels, *Mol Cell Biol.* 30(2010)2537-2551.
- [7] Said SI, The vasoactive intestinal peptide gene is a key modulator of pulmonary vascular remodeling and inflammation, *Ann NY Acad Sci.* 1144(2008)148-153.
- [8] Dai Y, Li F, Wu L, Wang R, Li P, Yan S, Xu H, Xia M, Bai C, Roxithromycin treatment inhibits TGF- β 1-induced activation of ERK and AKT and down-regulation of Caveolin-1 in rat airway smooth muscle cells, *Respir Res.* 15(2014)96.
- [9] Kasper M , Barth K, Bleomycin and its role in inducing apoptosis and senescence in lung cells- modulating effects of caveolin -1, *Curr Cancer Drug Targets.* 9(2009)341-353 .
- [10] Halayko AJ , Tran T , Gosens R, Phenotype and functional plasticity of airway smooth muscle: Role of caveolae and caveolins, *Proc Am Thorac Soc.* 5(2008)80-88 .
- [11] Goto K, Chiba Y, Misawa M. IL-13 induces translocation of NFkappaB in cultured human bronchial smooth muscle cells, *Cytokine.*2009;46:96-9.
- [12] Moynihan B, Tolloczko B, Michoud MC, Tamaoka M, Ferraro P, Martin JG. MAP kinases mediate interleukin-13 effects on calcium signaling in human airway smooth muscle cells, *Am J Physiol Lung Cell Mol Physiol.* 2008;295:L171-7.
- [13] Miller M, Cho JY, McElwain K, McElwain S, Shim JY, Manni M, Baek JS, Broide DH, Corticosteroids prevent myofibroblast accumulation and airway remodeling in mice, *Am J Physiol Lung Cell Mol Physiol .* 290(2006)L162–L169.
- [14] Wei B, Liu Y, Yue X, Li Y, Shang Y, Biological characteristics of tracheal smooth muscle cells regulated by NK-1R in asthmatic rat with airway remodeling, *Int J Clin Exp Med.* 8(2015);14718-14726.
- [15] Xie M, Liu XS, Xu YJ, Zhang ZX, Bai J, Ni W, Chen SX, ERK1/2 Signaling Pathway Modulates the Airway Smooth Muscle Cell Phenotype in the Rat Model of Chronic Asthma, *Respiration.*74(2007)680–690.
- [16] Li M, Shang Y, Wei B, Yang Y, The effect of substance P on asthmatic rat airway smooth

- muscle cell proliferation, migration, and cytoplasmic calcium concentration in vitro, *Journal of Inflammation*. 8(2011)18.
- [17] Elias JA, Lee CG, Zheng T, Ma B, Homer RJ, Zhu Z, New insights into the pathogenesis of asthma, *J Clin Invest*. 111(2003)291-297.
- [18] Chung KF, Wenzel SE, Brozek JL, Bush A, Castro M, Sterk PJ, Adcock IM, Bateman ED, Bel EH, Bleecker ER, Boulet LP, Brightling C, Chanez P, Dahlen SE, Djukanovic R, Frey U, Gaga M, Gibson P, Hamid Q, Jajour NN, Mauad T, Sorkness RL, Teague WG, International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma, *Eur Respirat J*. 43(2014)343–373.
- [19] Lee HY, Hur J, Kim IK, Kang JY, Yoon HK, Lee SY, Kwon SS, Kim YK, Rhee CK, Effect of nintedanib on airway inflammation and remodeling in a murine chronic asthma model, *Exp Lung Res*. 11(2017)1-10. doi: 10.1080/01902148.2017.1339141.
- [20] Lindén A, Hansson L, Andersson A, Palmqvist M, Arvidsson P, Löfdahl CG, Larsson P, Lötvall J, Bronchodilation by an inhaled VPAC(2) receptor agonist in patients with stable asthma, *Thorax*. 58(2003) 217-221.
- [21] Prakash YS, Airway smooth muscle in airway reactivity and remodeling: what have we learned? *Am J Physiol Lung Cell Mol Physiol*. 305(2013)L912–L933.
- [22] St Hilaire RC, Murthy SN, Kadowitz PJ, Jeter JR Jr, Role of VPAC1 and VPAC2 in VIP mediated inhibition of rat pulmonary artery and aortic smooth muscle cell proliferation, *Peptides*. 31(2010)1517-22.
- [23] Ribatti D, Puxeddu I, Crivellato E, Nico B, Vacca A, Levi-Schaffer F, Angiogenesis in asthma, *Clin Exp Allergy*. 39(2009)1815-1821.
- [24] Pei QM, Jiang P, Yang M, Qian XJ, Liu JB, Kim SH, Roxithromycin inhibits VEGF-induced human airway smooth muscle cell proliferation: Opportunities for the treatment of asthma, *Exp Cell Res*. 347(2016)378–384.
- [25] Bagnasco D, Ferrando M, Varricchi G, Passalacqua G, Canonica GW, A critical evaluation of anti-IL-13 and anti-IL-4 strategies in severe asthma, *Int Arch Allergy Immunol*. 170(2016)122–131.
- [26] Grassian AR, Metallo CM, Colloff JL, Stephanopoulos G, Brugge JS, Erk regulation of pyruvate dehydrogenase flux through PDK4 modulates cell proliferation, *Genes Dev*. 25(2011) 1716-1733.
- [27] Wuyts WA, Vanaudenaerde BM, Dupont LJ, Demedts MG, Verleden GM, Involvement of p38 MAPK, JNK, p42/ p44 ERK and NF- κ B in IL-1 induced chemokine release in human

- airway smooth muscle cells, *Respir Med* .97(2003)811–817.
- [28] Peng Q, Matsuda T, Hirst SJ, Signaling pathways regulating interleukin-13-stimulated chemokine release from airway smooth muscle, *Am J Respir Crit Care Med*.169(2004) 596–603.
- [29] Zhai W, Eynott PR, Oltmanns U, Oltmanns, Leung SY, Chung KE, Mitogen-activated protein kinase signalling pathways in IL-1 dependent rat airway smooth muscle proliferation, *Br J Pharmacol* .143(2004)1042–1049.
- [30] Inoue H, Kato R, Fukuyama S, Nonami A, Taniguchi K, Matsumoto K, Nakano T, Tsuda M, Matsumura M, Kubo M, Ishikawa F, Moon BG, Takatsu K, Nakanishi Y, Yoshimura , A.Spred-1 negatively regulates allergen-induced airway eosinophilia and hyperresponsiveness, *J Exp Med*.201(2005)73–82.
- [31] Johnson GL, Lapadat R, Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases, *Science*.298(2002)1911-1912.
- [32] Stewart AG, Airway wall remodelling and hyperresponsiveness: modelling remodelling in vitro and in vivo, *Pulm Pharmacol Ther*. 14(2001)255-265.
- [33] Cha H, Shapiro P, Tyrosine-phosphorylated extracellular signal regulated kinase associates with the Golgi complex during G2/M phase of the cell cycle: evidence for regulation of Golgi structure, *J Cell Biol*. 153(2001)1355-1367.
- [34] Duan W , Chan JH , Wong CH , Leung BP, Wong WS, Anti-inflammatory effects of mitogen-activated protein kinase kinase inhibitor U0126 in an asthma mouse model, *J Immunol*.172(2004)7053-7059 .
- [35] Zhang Y , Adner M , Cardell L, Interleukin-1 beta attenuates endothelin B receptor-mediated airway contractions in a murine in vitro model of asthma: roles of endothelin converting enzyme and mitogen-activated protein kinase pathways, *Clin Exp Allergy*. 34(2004) 1480-1487 .
- [36] Jablonski EM , Hughes FM Jr, The potential role of caveolin-1 in inhibition of aquaporins during the AVD, *Biol Cell*. 98(2006)33-42 .
- [37] Sun MZ, Guan Z, Liu S, Zhou X, Wang N, Shao S, Lin D, Caveolin-1 interferes cell growth of lung cancer NCI-H446 cell through the interactions with phospho-ERK1/2, estrogen receptor and progesterin receptor, *Biomed Pharmacother*.66 (2012)242-248.
- [38] Aravamudan B, Vanoosten SK, Meuchel LW, Vohra P, Thompson M, Sieck GC, Prakash YS, Pabelick CM, Caveolin-1 knockout mice exhibit airway hyperreactivity, *Am J Physiol Lung Cell Mol Physiol*. 303(2012)L669–681.

- [39] Razani B, Engelman JA, Wang XB, Schubert W, Zhang XL, Marks CB, Macaluso F, Russell RG, Li M, Pestell RG, Di Vizio D, Hou H Jr, Kneitz B, Lagaud G, Christ GJ, Edelmann W, Lisanti MP, Caveolin-1 null mice are viable but show evidence of hyper proliferative and vascular abnormalities, *J Biol Chem.* 276(2001)38121–38138.
- [40] Chen CM, Wu MY, Chou HC, Lang YD, Wang LF, Downregulation of caveolin-1 in a murine model of acute allergic airway disease, *Pediatr Neonatol.* 52(2011)5–10.
- [41] Le Saux CJ, Teeters K, Miyasato SK, Hoffmann PR, Bollt O, Douet V, Shohet RV, Broide DH, Tam EK, Down-regulation of Caveolin-1, an Inhibitor of Transforming Growth Factor- β Signaling, in Acute Allergen-induced Airway Remodeling, *J Biol Chem.* 283(2008)5760-5768.
- [42] Bains SN, Tourkina E, Atkinson C, Joseph K, Tholanikunnel B, Chu HW, Riemer EC, Martin R, Hoffman S, Loss of caveolin-1 from bronchial epithelial cells and monocytes in human subjects with asthma, *Allergy.* 67(2012)1601–1604.
- [43] Ding J, He G, Gong W, Wen W, Sun W, Ning B, Huang S, Wu K, Huang C, Wu M, Xie W, Wang H, Effects of nickel on cyclin expression, cell cycle progression and cell proliferation in human pulmonary cells, *Cancer Epidemiol Biomarkers Prev.* 18(2009)1720-1729.
- [44] Hassan M, Jo T, Risse PA, Tolloczko B, Lemi re C, Olivenstein R, Hamid Q, Martin JG, Airway smooth muscle remodeling is a dynamic process in severe long-standing asthma, *J Allergy Clin Immunol.* 125(2010)1037-1045.
- [45] Du CL, Xu YJ, Liu XS, Xie JG, Xie M, Zhang ZX, Zhang J, Qiao LF, Up-regulation of cyclin D1 expression in asthma serum-sensitized human airway smooth muscle promotes proliferation via protein kinase C α , *Exp Lung Res.* 36(2010) 201-210.

Highlights

- VIP inhibited the occurrence of OVA-induced airway remodeling in mice.
- VIP inhibited the proliferation of ASMCs by inhibiting the down-regulation of Cav-1.
- VIP inhibited the proliferation of ASMCs via the ERK1/2 signaling pathway and decreased the proportion of cells in S phase.

- VIP inhibited airway remodeling and therefore provided a new approach for the treatment of asthma.

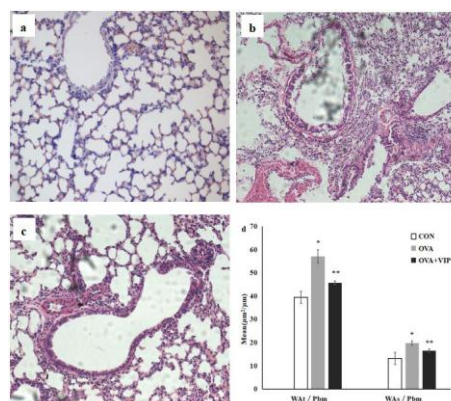


Figure 1. HE staining of bronchial and lung tissue in mice. 40 $\times$. Pictures represent bronchial and lung tissue in control group (a), asthma group (b) and VIP treatment group (c). The airway wall and SMC of the asthma group were significantly thicker than those of the control group ($p<0.05$). The pathological changes in the VIP treatment group were milder than in the asthma group. (d) Relative area of airway base circumference (Pbm), airway wall (WAt) and airway smooth muscle (WAs). * $p<0.05$, compared with control group; ** $p<0.05$ compared with asthma group.

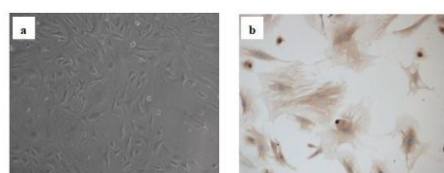


Figure 2. Immunocytochemistry was used to identify cultured ASM cells. Under the inverted microscope (a, 100 $\times$), ASM cells were spindle-shaped, growing in parallel and arranged in bundles, showing peak/valley shape at the border of dense growth and sparse growth. ASMC immunocytochemical staining (b, 200 $\times$) showed α -SMA as brown filaments.

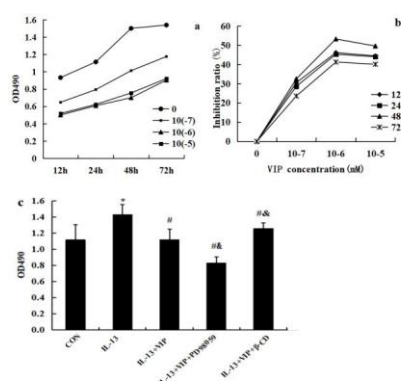


Figure 3. Effect of VIP on ASM proliferation. The effect of VIP on proliferation of ASM cells was concentration and time dependent. Cell viability was significantly lower in the 10⁻⁶ and 10⁻⁵ mM VIP groups than in the untreated group ($p<0.05$) (a). The peak inhibition treatment time duration was 48 h (b). MTT assay was used to detect the inhibitory effect of VIP on IL-13-induced ASM proliferation (c). * $p<0.05$, compared with the control group; # $p<0.05$, compared with IL-13 group; & $p<0.05$, compared with IL-13 + VIP group.

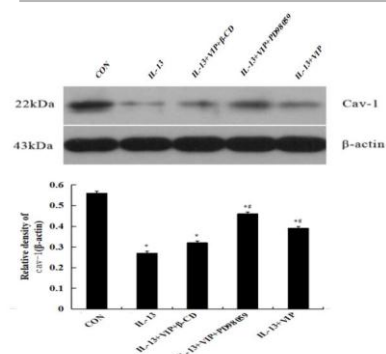


Figure 4. Western blotting was used to detect the effect of VIP on expression of Cav-1 in ASMCs. n = 3. *p<0.05, compared with control group; #p<0.05, compared with IL-13 group.

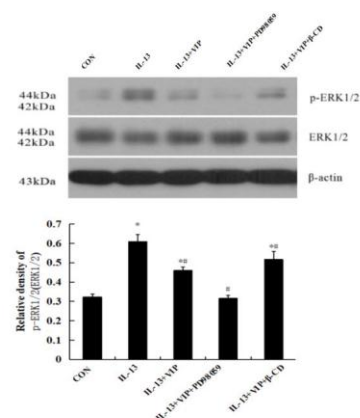


Figure 5. Western blotting was used to detect expression of p-ERK1/2 and ERK1/2 protein. n = 3. *p<0.05, compared with control group; #p<0.05, compared with IL-13 group.

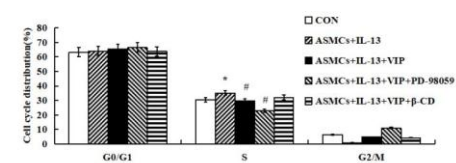


Figure 6. Effect of VIP on cell cycle distribution of ASMCs was detected by flow cytometry. *p<0.05, compared with control group at the same stage; #p<0.05, compared with IL-13 group at the same stage.