Original Article
Correlation of c-fos protein expression with neuropeptide content in the lung of bronchial asthmatic rat

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Abstract: Objective: To investigate and analyze the correlation between the c-fos protein expression and neuropeptide content in the lung of bronchial asthmatic rats. Methods: Thirty-two (32) SD rats were randomly allocated into 4 groups of the normal control, the non-acute asthma, the acute asthma and the dexamethasone intervention. Immunohistochemistry was performed for histological observation, and substance P (SP) and vasoactive intestinal peptide (VIP) concentrations in the bronchoalveolar lavage fluid were measured by enzyme-linked immunosorbent assay (ELISA). Results: SP concentration in the alveolar lavage of asthmatic rat was significantly higher than that in the normal control group ($P < 0.0001$), whereas VIP concentration was significantly lower ($P < 0.0001$). The optical density of c-fos protein in the lung tissues of groups of the non-acute asthma, the acute asthma and the dexamethasone intervention was positively correlated with SP concentration in the bronchoalveolar lavage fluid (r = 0.908, r = 0.967, r = 0.865), and negatively correlated with the VIP concentration in the alveolar lavage (r = -0.974, r = -0.949, r = -0.962). Conclusion: The c-fos protein expression and neuropeptide content in the lungs of asthmatic rats are related with asthma attacks.

Keywords: Bronchial asthma, c-fos protein, neuropeptides

Introduction

Bronchial asthma is a common chronic allergic airway inflammation whose pathogenesis is complex, involving a variety of inflammatory cells and inflammatory mediators. It has been demonstrated that c-fos is involved in the airway inflammation [4]. The c-fos is an immediate early gene, which is expressed at low levels in the physiological state. It regulates the growth and differentiation of many cell types, and is involved in the signal transduction and regulation process of important brain functions. Thus, c-fos has been commonly recognized as one of the positioning markers of the functional activity of brain morphology [26, 32]. Moreover, in recent years, many studies have confirmed that the substance of neural peptides which is widely distributed in the lung tissues plays a role as neurotransmitter or neuromodulator, thereby affecting the functions of the airways and pulmonary vessels. Li & Xu have reported that "many inflammatory and functional effects of neuropeptide are correlated with asthma". This study investigated the relationship between the c-fos protein expression and the neuropeptide substance P (SP) and vasoactive intestinal peptide (VIP) in mouse with bronchial asthma in exacerbation, in an aim to provide references for further investigations of the pathogenesis of bronchial asthma.

The neuropeptide S (NPS)-NPS receptor 1 (NPSR1) pathway has recently been implicated in the pathogenesis of asthma [33]. A TMM microarray database comparison suggested a common co-regulated pathway, which includes JUN/FOS oncogene homologs, early growth response genes, nuclear receptor subfamily 4 members and dual specificity phosphatases. The proteins encoded by FOS family genes contain a leucine zipper domain and they can

dimerize with proteins of the JUN family forming the transcription factor complex AP-1 [1, 29, 41]. The AP-1 components, FOS and FOSL1 (= FRA), are also shown to play a role in the activation of IL8 [12]. Clinically, asthma and chronic obstructive pulmonary diseases represent chronic inflammation, which is regulated by both the immune and neuroendocrine systems. The central nervous system (CNS) signals the immune system through the autonomic nerves and the hypothalamic-pituitary-adrenal (HPA) axis. HPA activation into the lateral ventricle induces Fos expression in neurons that produce corticotropin-releasing hormone (CRH) in the hypothalamic paraventricular nucleus (PVN) [38]. Neuropeptide exerts potent actions within the PVN including the regulation of feeding, influence of cardiovascular and GI functioning as well as a myriad of neuroendocrine actions [40]. Previous research has suggested that *c-fos* is critically involved in neuronal excitability and survival [42]. The immediate early gene (IEG) c-fos has been used as a marker of neuronal activity, and correlates with an increase in electrical and metabolic activity in brain cells by pathological situations, also involved in phenomena of neuronal plasticity, amongst others. C-fos is expressed in response to a wide range of stimuli and is implicated in processes such as transcription of genes, apoptosis or proliferation [25, 27]. *c-fos* is critical in the up-regulation of neuropeptide expression in the granule cell layer of dentate gyrus in response to kainic acid administration. As neuropeptide is an important endogenous anti-epileptic agent, previous research has raised the hypothesis that the neuroprotective function of *c-fos* is mediated in part by the regulation of neuropeptide expression [36].

During lung inflammation, a variety of neural, epithelial, endothelial, and phagocytic cells produce inflammatory mediators that activate airway nocisensors. These include: IL-1beta, TNF-alpha, tachykinins, such as neuropeptide substance P (SP) [38]. Their effects involve airway smooth muscle contraction, mucus secretion, increased vascular permeability, and inflammatory cell activation. During airway mucosal inflammation, tachykinin content in nociceptive afferents increases [9]. SP causes airway vasodilatation and protein extravasation into the mucosa by increasing vascular permeability. Electrical stimulation of vagal afferents produc-

es neurogenic inflammation by releasing neuropeptides from nociceptive endings [20]. Neurogenic inflammation is a non-specific mechanism, which may serve as an amplifier for inflammatory processes. During pathogen invasion, allergic reaction, or traumatic injury, the nocisensor is activated and the inflammatory process is enhanced. Sensory activation releases neuropeptides like SP that cause airway epithelial cells, mast cells, and macrophages to release inflammatory mediators including TNF [30]. Activation of neurokinin receptors up regulates inflammatory cytokine expression [7]. Inflammatory cytokines such as IL-1beta may induce airway hyper-responsiveness by enhancing SP expression in airway nerves [35]; nocisensors and resident cells that release mediators and chemokines are further stimulated. Chemokines attract phagocytes, such as macrophages and neutrophils, to engulf pathogens and destroy the invading organisms. Thus, sensory activation forms a positive feedback loop to amplify the initial stimulatory effects and promote innate host defense. Furthermore, SP stimulates T cells to produce interferon-gamma [2] to exert cellular immunity and also act as chemoattractant for dendritic cells and recruit them into inflammatory sites [16]. Dendritic cells are professional antigen presenting cells that stimulate naïve T cell proliferation. Thus, by releasing neuropeptides, airway sensors may also facilitate adaptive immune response. Indeed, morphological studies show close contact between airway sensory nerves and dendritic cells [34].

The development of intrapulmonary immune complexes results in inflammation, characterized by microvascular permeability and polymorphonuclear neutrophil influx with resultant localized edema [3]. Neurogenic inflammation also involves the release of substance P from sensory nerve endings in response to pain or infection. When the irritant capsaicin was applied to skin, edema occurred as a result of sensory neuropeptide release, including substance P [13]. The effect of substance P in edema formation could be blocked by substance P antagonists that previously were characterized as blockers of neurogenic inflammation [11]. Neurogenic inflammation was also examined in mice with immune complex-mediated acute lung injury and associated microvascular permeability. Vascular permeability is

strongly mediated by substance P, and substance P-containing C-fibers are part of the mucosal epithelium lining of the lungs [3]. When mice deficient in the receptors for both substance P and complement factor C5a (intrinsic to the inflammatory response) were studied for protection of the lung from immune-complex injury, either deficiency alone provided protection [3]. This suggested that both substance P and C5a bound to their G protein-coupled receptors were essential for immune-complex inflammatory lung injury. Substance P is critical to the inflammatory cascade in amplifying immune-complex injury to the lung by acting at the microvasculature of the airway mucosa. The results of these studies suggest that neuropeptide regulation of neurohumoral responses is at least partially mediated by substance P [6].

c-fos, a proto-oncogene regulating the transcription of many genes, plays a critical role in the cell cycle and differentiation and may be involved in the regulation of inflammation in asthma. Very low levels of c-fos are detectable in most human cells, and its expression is rapidly and transiently increased by multiple factors, some of which are involved in the airways inflammation of asthma (histamine, eicosanoids, and cytokines) [24]. Several studies have raised the idea that c-fos may also have tumor-suppressor activity, that it might be able to promote as well as suppress tumorigenesis. c-fos induces a loss of cell polarity and epithelial-mesenchymal transition, leading to invasive and metastatic growth in mammary epithelial cells [8]. [17] have demonstrated that Corticosteroid-resistant bronchial asthma is associated with increased c-fos expression in monocytes and T lymphocytes.

To summarize the above, both the neuropeptide substance P and the c-fos gene are significantly implicated in asthma and other pulmonary inflammatory processes, yet the correlation between the neuropeptide substance P and the c-fos gene in the pathogenesis of asthma has yet to be thoroughly investigated. Neuropeptide SP augments the secretion of pro-inflammatory cytokines to mediate vascular permeability and airway edema formation. c-fos gene, as a member of the proto-oncogene FOS family, regulates cell cycle progression and

apoptosis in a variety of pathological conditions including lung cancer. c-fos is a component of the transcription factor AP-1, which mediates cell proliferation in response to external growth signals in the form of peptide growth factors. Does c-fos up-regulate the expression of neuropeptide SP in the inflammatory process of an asthmatic rat model? Our exploratory study aims to elucidate this connection to provide intellectual inspirations for future research on this topic.

Materials and methods

Animals and their grouping

Thirty-two (32) SD rats of 6 to 8 weeks old were selected, in which there were 16 males and 16 females, weighing 33 g each. They were purchased from the Experimental Animal Center of the Fourth Military Medical University. These rats were randomly allocated into four groups, i.e. the normal control group, the non-acute asthma group, the asthma of acute-onset group and the dexamethasone intervention group, with 8 rats in each group. This study has been approved by the institutional ethics committee regarding the proper use and handling of animals.

### Establishment of the asthma model [19, 28, 37]

OVA was purchased from Sigma (USA), and rabbit anti-rat c-fos monoclonal antibody from Xi'an Zhongshan Biotechnology Ltd. After 2 weeks adaptive feeding, rats in the non-acute asthma group, the acute asthma group and the dexamethasone group were intraperitoneally injected with 1 mL of ovalbumin suspension for immunization (1.0 mg OVA + 50.0 mg Alum dissolved in PBS) on the 1st day and the 8th day, and rats in the control group were intraperitoneally injected with 1 mL of PBS. After 14 days, rats in the non-acute asthma group, the acute asthma group and the dexamethasone intervention group were nasally dripped with 50 μL of 1.0 mg/mL OVA-PBS suspension, once a day for seven days. The acute asthma group was again induced for another seven days, once every other day. Rats in the control group were administered with PBS solution, with the same procedures as above. The dexamethasone
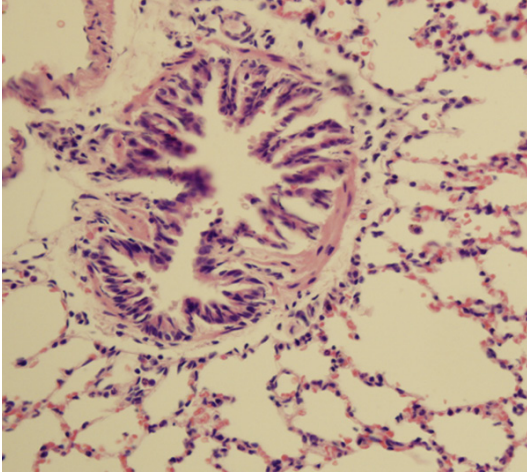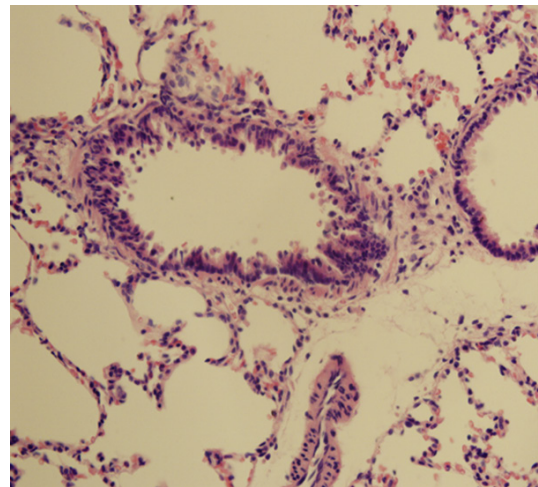
Figure 1. Lung tissue of the normal control group.
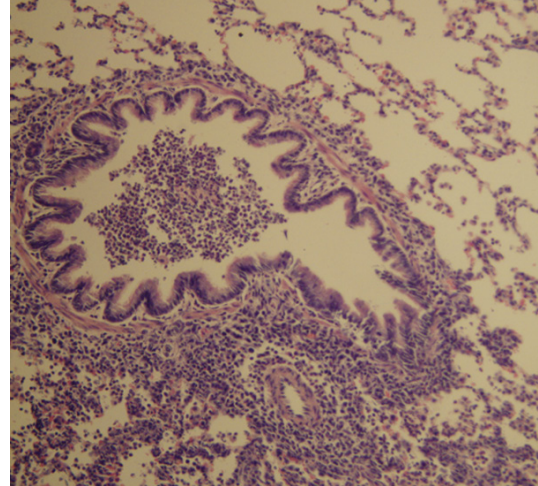
Figure 3. Lung tissue of the acute asthma group.
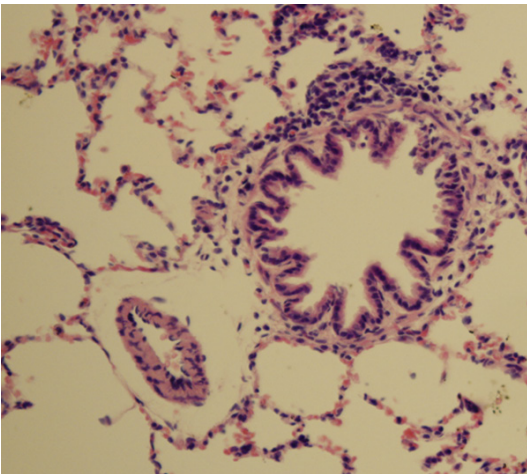
Figure 2. Lung tissue of the non-acute asthma group.

Figure 4. Lung tissue of the dexamethasone group.

group was intraperitoneally injected with 0.2 mg/kg dexamethasone 1 hour before the atomization attack from the 21st day for consecutive 7 days.

Specimen collection and preparation

Twenty-four (24) hours after the last attack, rats in each group were ether-anesthetized and weighed. The right main bronchus was occluded by line tying treatment after DEPC. 3 ml of saline was injected from the trachea into the left lung for lavage, and the lavage fluid was recycled for three times (with recycling rate of 87% to 97%). Thorax was cut from the thyroid cartilage under a sterile state, where the trachea was sheared, and lung tissues isolated and weighed. The lung tissues of the right lower lobe were taken out, and fixed by 10% formalin for 24 h. A suction needle is used to extract gas from the lung tissues, which were then dehydrated by alcohol, and paraffin-embedded to make 5 μm thick sections. Sections were then applied with HE staining and immunohistochemical staining (ABC staining).

Immunohistochemical staining and image analysis

Infiltration of airway inflammation, mucus plug blocked airway stenosis and shedding of airway epithelial cells in each group were observed under the microscope. Mean optical density value was determined by computer image analysis system after the immunohistochemical staining. The absorbance value of the positive

Table 1. Between-groups comparison of the optical density of c-fos protein in the lung tissue of the rats and the SP and VIP concentrations in the bronchoalveolar lavage fluid ($\bar{x} \pm S$)

Group	c-fos	SP (pg/ml)	VIP (pg/ml)
Normal control	6.298 ± 0.631	65.316 ± 3.546	32.390 ± 2.632
Non-acute asthma	20.377 ± 1.500	224.212 ± 5.480	19.648 ± 3.025
Acute asthma	29.727 ± 1.193	326.634 ± 6.070	8.225 ± 1.286
Dexamethasone	12.694 ± 1.900	146.841 ± 8.836	27.008 ± 2.327
F	819.79	4561.59	426.70
P	< 0.0001	< 0.0001	< 0.0001

parts was achieved by subtracting the optical density value of the positive parts from the optical density value of the background. Five small airways were sampled from each animal, and the optical density value of a particular specimen was the average value of these five small airways.

Measurement SP and VIP contents in the alveolar lavage fluid

Alveolar lavage fluid was centrifuged (3000 r/min, 4°C, 10 min), and its supernatant was measured using radioimmunoassay, in strict accordance with the requirements of the time box.

Statistical analysis

Excel database was established and statistical analysis was performed with SPSS19.0 statistical package. The statistical methods of this study include single factor analysis of variance and correlation analysis.

Results

Observation of the general conditions

The asthma group, after being excited, appeared shortness of breath, cyanosis of lips, abdominal incitement, irritability, and restlessness.

Pathological observation

The normal control group showed bronchopulmonary structural integrity and epithelial integrity and smoothness, with no obvious wrinkles and loss; occasional goblet cells between the ciliated columnar epithelium; no significant infiltrations of macrophages and addicted acidic granulocytes in the bronchial mucosa and its peripherals and the perivascular areas; no

hyperplasia in the airway and vascular smooth muscle; normal alveolar septa, without inflammatory cell infiltration. Bronchial wall, alveoli, pulmonary artery, and pulmonary vein were all in a normal state (**Figure 1**).

The non-acute asthma group showed mild thickening of bronchial epithelium and wall, and mild epithelial hyperplasia with a few lesions. Goblet cell hyperplasia was relatively moderate compared with the acute asthma group. Large amounts of lymphocyte infiltration were seen around the airway, but the inflammation was significantly reduced compared with the acute asthma group. And mild hyperplasia was shown in the bronchial smooth muscle (**Figure 2**).

The acute asthma group showed thickening of the bronchial epithelium and wall, and obvious epithelial damage and shedding. Mucus plug obstruction was seen in the lumen. Large amounts of eosinophil and lymphocyte infiltration could be seen around the medium and large airways and blood vessels. Mild hyperplasia was shown in the bronchial smooth muscle, and alveolar septa were significantly widened (**Figure 3**).

The dexamethasone intervention group showed normal bronchial epithelium and wall, no damage or loss of epithelium, and normal lumen, with occasional goblet cells. No hyperplasia was seen in the bronchial and vascular smooth muscle. Lymphocytic infiltration was seen around the medium and large airways and perivascular areas. There was no thickening of the bronchial epithelium, and almost no inflammation shown on the wall. The lumen was basically normal, with increased interstitial pulmonary inflammatory cells (**Figure 4**).

Optical density of c-fos protein and the concentration of SP and VIP in the bronchoalveolar lavage fluid

Between groups comparison shows that differences in the optical density values of c-fos protein in the lung tissues of the rats and the SP and VIP concentrations in the bronchoalveolar lavage fluid were statistically significant (P <

Table 2. Correlation of the optical density of the c-fos protein in the lung tissues of the rats and the concentrations of SP and VIP in the bronchoalveolar lavage fluid in each group

Group	c-fos and SP		c-fos and VIP	
	r	P	r	P
Non-acute asthma	0.908	0.002	-0.974	< 0.001
Acute asthma	0.967	0.006	-0.949	0.001
Dexamethasone	0.865	< 0.001	-0.962	0.001

0.0001). The optical density value of c-fos protein and the SP concentration in the bronchoalveolar lavage fluid in the acute asthma group were significantly higher than those in the normal control group, the dexamethasone group and the non-acute asthma group, whereas the VIP concentration in the bronchoalveolar lavage fluid of the acute asthma group were lower than that of the other groups (**Table 1**).

Correlation of the optical density of the c-fos protein in the lung tissues of the rats and the concentrations of SP and VIP in the bronchoalveolar lavage fluid in the non-acute asthma group, the acute asthma group and dexamethasone group

For the non-acute asthma group, the acute asthma group and the dexamethasone group, the optical density of the c-fos protein in the lung tissues of the rats and the concentration of SP in the bronchoalveolar lavage fluid were positively correlated, with correlation coefficients higher than 0.850, and the differences were statistically significant ($P < 0.05$); whereas the optical densities of the c-fos protein in the above three groups was negatively correlated with the VIP concentration in the bronchoalveolar lavage fluid, with correlation coefficients higher than 0.900, and the differences were statistically significant ($P < 0.05$), as shown in **Table 2**.

Discussion

Bronchial asthma is a chronic disorder of airway inflammation and airway hyperresponsiveness. In addition to eosinophils, mast cells, macrophages and other inflammatory cell infiltration, its main pathological changes also include shedding of airway mucosal epithelial cells, and hypertrophy of airway smooth muscle. The c-fos is an immediate oncogene, locat-

ed on the 14q24.3~q31 of human autosomal genes [14]. The protein product of c-fos is combined with the c-Jun protein to form a stable heterodimer AP-1 (activator protein-1), which could bind with the AP-1 binding site, a specific sequence of the DNA molecules, and is a transcription factor. Therefore c-fos gene is closely related to the growth, proliferation and activation of cells [23]. Oncogene c-fos is expressed in low levels in most normal cells. It can be affected by a variety of inflammatory mediators, inflammatory cells and cytokines. When activated, the oncogene c-fos can activate a variety of mediators and cytokines [22], and thus plays an important role in asthma. Studies have reported that in airway inflammatory diseases, histamine stimulation can enhance the expression of c-fos [22]. Demoly is the first to report that the airway epithelial cells in the bronchial biopsy have a rather high expression of c-fos protein [4], and later finds out that the degree of positive reaction of c-fos in the epithelial cells is positively correlated with the extent of epithelial cell shedding. Our study confirms that positive c-fos protein was mainly distributed in the cytoplasm of airway epithelial cells through immunohistochemical methods. The asthma symptoms were significant in the acute asthma group and the non-acute asthma group, evidenced by the optical microscopic examination of damages in the bronchial epithelium of rats, and the epithelial cell loss and necrosis. By contrast, epithelial cells in the dexamethasone group and the control group remained intact. The c-fos protein expression in the airway epithelium of the bronchioles of rats with acute asthma was significantly higher than that of the dexamethasone group and the control group, indicating that the onset of asthma is related with the involvement of c-fos. The c-fos takes the airway epithelial cells as target cells, thereby resulting in the activation and loss of epithelial cells. This is consistent with the reports of [5] and [17] that the enhancement in the expression of c-fos gene might not only be associated with cell growth and proliferation, but also affect the activity of epithelial cells and cause the damage of epithelial cells. The epithelial cells, after being activated and damaged, could release a variety of inflammatory mediators and cytokines, leading to the aggravation of inflammation. And the shedding of airway epithelial cells would result in the exposure of a large number of nerve fibers,

leading to the hyperresponsiveness of airway, in the meanwhile the repair process of the airway epithelial damage can also allow fibroblast proliferation of epithelial cells, airway wall thickening, and airway remodeling, resulting in further narrowing of the airway, which induce and aggravate asthma. [31] have demonstrated that lung lavage levels of inflammatory cytokines such as TNF-alpha, IL-1beta, IL-6, IL-10, MIP-2, and IFN-gamma are correlated with c-fos m-RNA expression in an isolated rat lung model. Our results also show that the c-fos protein expression in the airway epithelial cells in the dexamethasone group was significantly lower than that in the acute asthma group, and this is consistent with the report of Lane SJ that the c-fos protein expression might be down-regulated by adrenal corticosteroids in asthma to achieve the anti-inflammatory effect.

SP is an important member of the tachykinin family, and is one of the major excitatory neurotransmitters of the non-adrenergic non-cholinergic (NANC) nervous system within the lung. SP-containing nerve fibers are widely distributed in all levels of the bronchial branches of the lung, and are occasionally stretched into the alveolar walls, and located in the airway epithelium, the basal underside of the epithelium, the airway smooth muscle, and around the submucosal glands and blood vessels. SP plays a role in regulating the contraction functions of the small airway walls [10, 15, 21], and the small airways are the major places of lesions of asthma and chronic obstructive pulmonary diseases. The release of SP enables the contraction of airway smooth muscle, the dilation of blood vessels and the increased permeability of the vascular walls, and activates the inflammatory cells. These coincide with the clinical and pathological features of asthma. In this study, we have observed that the number of SP-positive fibers in the small airways of the lungs of asthmatic rats was significantly higher than that of the control group, and a large number of SP-positive fibers can be seen around the alveolar, suggesting that the increase in SP-positive fibers may be closely associated with the onset of asthma. The increase in SP-positive fibers may also be a direct cause of the increase in SP content in the patients' blood, bronchoalveolar lavage fluid, and even sputum. VIP is a major neurotransmitter of intrapulmonary I-NANC nerves, and is the strongest endogenous bronchodilator currently available. VIP, after being inhaled or injected to humans, has a protective role to the bronchoconstriction induced by such neuropeptides as histamine, 5-serotonin, or leukotriene D4. VIP can also inhibit the secretion of macromolecules by bronchial glands, and inhibit of the release of the mediators of mast cells induced by antigen-antibodies, thus reducing the airway hypersensitivity [39]. The results of this study show that the VIP contents in the bronchoalveolar lavage fluid of the acute asthma group and the non-acute asthma group were significantly lower than that of the control group. This might be explained that the reduced VIP content in the lung tissues allows it to play a lesser or weakening role in airway dilation and anti-inflammation. Thus its "brake" mechanism to the cholinergic nerve is also restricted, leading to enhanced airway constriction, and finally the onset or aggravation of asthma. Our study also shows that the optical density of c-fos protein was positively correlated with the concentration of SP in the bronchoalveolar lavage fluid, and negatively correlated with the VIP concentrations, which is presumably due to the fact that enhanced expression of c-fos gene is one of the reasons of the increase in SP concentration and decrease in VIP concentration in the bronchoalveolar lavage fluid.

Disclosure of conflict of interest

None.

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