

Effect and Mechanism of Ligustrazine on Th1/Th2 Cytokines in a Rat Asthma Model

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Abstract: Ligustrazine is an alkaloid isolated from the rhizome of Chuanxiong (*Ligusticum chuanxiong Hort*), which is known to possess antioxidant, anti-inflammatory, anti-fibrosis and immunomodulative effects. It is used clinically to treat asthma as an assistant therapy of glucocorticoid. The purpose of this study was to explore the effects of intraperitoneal ligustrazine on Th1/Th2 cytokines in a rat asthma model and the underlying mechanism. SD rats were sensitized and challenged with ovalbumin (OVA) to establish an asthmatic model. Within 24 hours after the last ovalbumin challenge, changes in airway histology were observed. The concentrations of IL-4 and IFN- γ in bronchoalveolar lavage fluid (BALF) were measured by enzyme linked immunosorbent assay (ELISA). The protein expressions of GATA-3 and T-bet in lung were measured by Western blot. The results showed that an increase of Th2 cytokine and an inhibition of Th1 cytokine were accompanied by an increased expression of GATA-3 protein and a decreased expression of T-bet protein in rat asthmatic airways compared to those in normal control group. Intraperitoneal ligustrazine administration could significantly lower the level of IL-4 in BALF and the expression of GATA-3 protein in lung and also increase the level of IFN- γ and T-bet in asthmatic rats, resulting in a decreased percentage of eosinophils (EOS) in BALF and ameliorated airway inflammatory cell infiltration. In conclusion, ligustrazine inhibits OVA induced airway inflammation by modulating key master switches GATA-3 and T-bet that result in reversing the Th2 cytokine patterns in asthma.

Keywords: Ligustrazine; Asthma; GATA-3; T-bet.

Introduction

Allergic asthma is a complex disorder characterized by reversible and variable degrees of airway bronchoconstriction, hyper-responsiveness, and chronic inflammation and remodeling of the airways (Holgate, 1999; Drazen, 2003). In particular, it has been shown, in patients with asthma and in animal models, that allergic airway inflammation is associated with increased T helper (Th) 2 cytokine production and decreased Th1 cytokine production (Holgate, 1999). Allergic asthma is therefore considered a Th2-driven disease in which the presence of Th2 cells correlates with IgE production, mast cell hyperplasia, and eosinophil infiltration, determining the severity of the disease (Savelkoul, 2000).

The differentiation of naive CD4⁺ T cells into Th1 or Th2 effector cells is a critical process during immune responses. The existence of two different types of CD4⁺ T cells was described based on the different cytokine profiles produced (Abbas *et al.*, 1996). Th1 cells produce interferon (IFN)- γ , promoting cell-mediated immunity and control of intracellular pathogens. In contrast, Th2 cells produce interleukin (IL)-4, IL-5 and IL-13, which promote humoral immunity, allergic reactions to environmental antigens, and resistance to helminthic infections. Several studies have shown that T-bet and GATA-3 are the key transcription factors for differentiation toward Th1 and Th2 cells (Zheng and Flavell, 1997; Szabo, *et al.*, 2000; Ho and Glimcher, 2002). Previous studies in transgenic mice have revealed that expression of a dominant negative form of GATA-3 can prevent allergic airway inflammation and Finotto *et al.* suggest that T-bet might protect from asthma (Zhang *et al.*, 1999; Finotto *et al.*, 2002).

Ligustrazine is an alkaloid isolated from the rhizome of *Ligusticum chuanxiong* Hort, which is known as Chuanxiong in Chinese. The alkaloid ligustrazine has the chemical name 2,3,5,6-tetramethylpyrazine (because it is a pyrazine ring with four symmetrically placed methyl groups) (Fig. 1). Isolated alkaloids from Chuanxiong, and purified synthetic ligustrazine, have been used in China as medicinal agents for 30 years. Ligustrazine was reported to possess a very broad spectrum of pharmacological actions, such as antioxidant, anti-inflammatory (Zheng *et al.*, 2006), anti-fibrosis (Yan *et al.*, 1996), antitumor and immunomodulative effects (Wei *et al.*, 2004). A recent study indicated that ligustrazine could suppress airway inflammation in asthmatic rats (Chen *et al.*, 2005). However, the mechanism remains unclear. In this study, we used a murine model of asthma in which rats were initially sensitized with ovalbumin (OVA) and subsequently stimulated by inhalation of aerosolized OVA. The therapy consisted of 2 weeks of intraperitoneal ligustrazine hydrochloride injection in order to investigate the effects of ligustrazine on Th1/Th2 cytokines in a rat asthma model and explore the underlying mechanisms.

Materials and Methods

Animals

Specific pathogen-free (SPF) six-week old male SD rats (200 $\pm$ 20 g) were purchased from the Experimental Animal Center of Tongji Medical College, Huazhong University

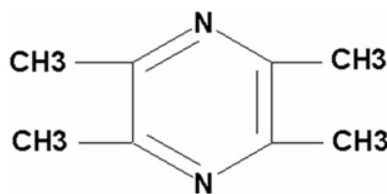


Figure 1. The structure of 2,3,5,6-tetramethylpyrazine.

of Science and Technology (Wuhan, China). The rats were kept in standard rat cages and allowed to feed and drink freely. Constant temperature and humidity were maintained with a fixed 12-hour light-dark cycle. All animal protocols were approved by the Animal Care Committee of Tongji Medical College, Huazhong University of Science and Technology.

Reagents

Ligustrazine hydrochloride injection (20 mg/ml) was purchased from Zhengzhou Zhuofeng pharmaceutical factory (Zhengzhou, China). Dexamethasone sodium phosphate injection (5 mg/ml) was purchased from Wuhan Double-Crane Pharmaceutical Co. Ltd. (Wuhan, China). Chicken egg ovalbumin (OVA) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Heat-killed *Bordetella pertussis* bacilli were kindly donated by the manufacturers, Wuhan Institute of Biological Products. Enzyme linked immunosorbent assay (ELISA) kits for rat recombinant IL-4 and IFN- γ were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Mouse monoclonal antibody anti-GATA-3, anti-T-bet and anti- β -actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Horseradish peroxidase conjugated goat antimouse IgG were purchased from Pierce Chemical Co. (Rockford, IL, USA). BCA protein assay kit was purchased from Beijing BioTeke Biotechnologies, Inc. (Beijing, China).

Establishment of Animal Models

SD rats were sensitized and challenged with OVA by the methods of Liao *et al.* (2006) to establish an asthmatic model. Forty rats were randomly divided equally into a normal control group (N group, 8 rats), an asthmatic group (A group, 8 rats), an asthmatic control group (AC group, 8 rats), a ligustrazine group (L group, 8 rats) and a dexamethasone (Dex) group (D group, 8 rats).

Thirty-two rats received an intraperitoneal injection of 1 ml 10% OVA (100 mg OVA in 1 ml normal saline) mixed with 100 mg aluminum oxyhydrogen and 5×10^9 heat-killed *Bordetella pertussis* bacilli on day 1. Two weeks after the sensitization, the rats inhaled 1% OVA for 30 min in an exposure chamber (50 cm $\times$ 30 cm $\times$ 25 cm) for 14 successive days. For inhalation challenge, OVA was dissolved in normal saline, nebulized, and delivered at 1 ml/min. The 8 sensitized rats of the asthma control group

received a 0.8 ml intraperitoneal injection of normal saline 30 min before OVA challenge. The ligustrazine group and dexamethasone group received an intraperitoneal injection of ligustrazine hydrochloride injection (80 mg/kg) and dexamethasone sodium phosphate injection (0.5 mg/kg), respectively, 30 min before OVA challenge. The 8 rats of the normal control group received an intraperitoneal injection of 1 ml normal saline on day 1 and inhaled saline aerosol.

Collection and Analysis of Bronchoalveolar Lavage Fluid (BALF)

Within 24 hours after the last aerosolization with either OVA or saline at day 28, BALF of the left lung was performed after tying off the right lung at the mainstem bronchus. The rats were anesthetized by intraperitoneal injection of 2% sodium pentobarbital (40 mg/kg) and then the left lungs were lavaged three times with 4 ml normal saline at room temperature, using polyethylene tubing inserting into the left mainstem bronchus. Sixty to eighty percent of the 4 ml input volume was retrieved. Total leukocytes were determined by hemacytometer counting using crystal violet stain. The lavage fluid collected was centrifuged at 3,000 r/min for 10 min at 4°C, and the supernatant was stored at -80°C until it was evaluated. The cell precipitation was cytospun on a slide and stained with Wright-Giemsa stain. A total of at least 200 cells/slide were counted. The eosinophils (EOS), macrophages, lymphocytes, neutrophils were counted separately to determine the percent of each cell type. The levels of IL-4 and IFN- γ in BALF were detected by ELISA kits following the manufacturer's instructions.

Measurement of GATA-3 and T-bet Protein by Western Blot

After left lung being lavaged, the rats' right lung tissue samples were immediately frozen in liquid nitrogen except the middle lobe. Frozen lung tissue was homogenized in lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM DTT, 0.1% SDS, 1 x protease inhibitor cocktail), and protein concentration was determined using the BCA protein assay kit. 50 μ g protein was solubilized in SDS sample buffer (50 mM Tris-HCl, pH 6.8, containing 5% 2-mercaptoethanol, 2% SDS, 10% glycerol, and 0.01% bromophenol blue), separated on 7.5% SDS-PAGE, and transferred onto a nitrocellulose membrane at 100 V for 2 hours. The membrane was then blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 hour at room temperature and subsequently exposed to 1 μ g/ml of monoclonal antibodies anti-GATA-3, anti-T-bet or anti- β -actin overnight at 4°C. The next day, the membrane was incubated with horseradish peroxidase conjugated goat antimouse IgG (1:2,000) for 1 hour at 37°C. Tagged proteins were detected using enhanced chemiluminescence (ECL) assay and recorded on X-ray films. The optical density (OD) value of the protein bands on the X-ray films were quantitated using Kodak Digital Science 1D Image Analysis Software. The relative OD value of GATA-3 protein and T-bet protein were defined as the ratio of OD_{GATA-3}/OD _{β -actin}, OD_{T-bet}/OD _{β -actin}, respectively.

Histopathological Examination

After left lung being lavaged, the middle lobe of the right lung tissues excised from each rat were fixed in 10% formalin and embedded in paraffin. Sections 3–4 μm thin from blocks were stained with hematoxylin-eosin (H&E).

Statistical Analysis

All data were expressed as means $\pm$ standard deviation. Statistical analyses were performed using one-way ANOVA with SPSS12.0 software. The significance of the difference between the groups was assessed by student's two-tailed t-test. $p < 0.05$ was considered significant.

Results

Symptoms

There were no asthmatic symptoms in N group. But A group and AC group showed typical symptoms of asthma such as dysphoria, tachypnea, wheezing, muscles of abdomen ticking, incontinence, etc. However, L group and D group did not show such asthmatic symptoms.

Ligustrazine Decreased the Number of Total Cells, EOS Percentage and Neutrophil Percentage in BALF of OVA Sensitized Rats

The number of total cells, percentage of EOS and percentage of neutrophil in the BALF of A group and AC group were both significantly increased compared to N group. However, they were significantly reduced after administration of ligustrazine compared with the asthmatic rats, but were significantly increased compared to the normal control rats ($p < 0.01$, Table 1). The number of total cells, percentages of EOS, neutrophil and lymphocyte were all decreased in D group. There was no significant difference between A and AC group ($p > 0.05$, Table 1).

Table 1. Cellular Composition in the BALF of the Five Groups

Groups	N	Total Cells ($\times 10^4/\text{ml}$)	Eosinophils (%)	Macrophages (%)	Lymphocytes (%)	Neutrophils (%)
N	8	7.81 ± 1.27	0	89.49 ± 0.70	6.30 ± 0.43	4.21 ± 0.37
A	8	$67.16 \pm 10.64^*$	$7.17 \pm 0.22^*$	$56.46 \pm 1.99^*$	$15.14 \pm 1.01^*$	$21.23 \pm 2.01^*$
AC	8	$69.81 \pm 10.04^*$	$7.06 \pm 0.19^*$	$57.00 \pm 1.96^*$	$14.77 \pm 1.09^*$	$21.18 \pm 2.02^*$
D	8	$34.06 \pm 5.33^{*\Delta}$	$3.25 \pm 0.08^{*\Delta}$	$90.37 \pm 0.66^\Delta$	$1.02 \pm 0.17^\Delta$	$5.36 \pm 0.55^{*\Delta}$
L	8	$44.94 \pm 5.86^{*\Delta}$	$3.32 \pm 0.05^{*\Delta}$	$66.85 \pm 1.78^\Delta$	15.61 ± 1.03	$14.23 \pm 1.02^{*\Delta}$

Mean $\pm$ SD, * $p < 0.01$ versus the N group; $^\Delta p < 0.01$ versus A and AC group.

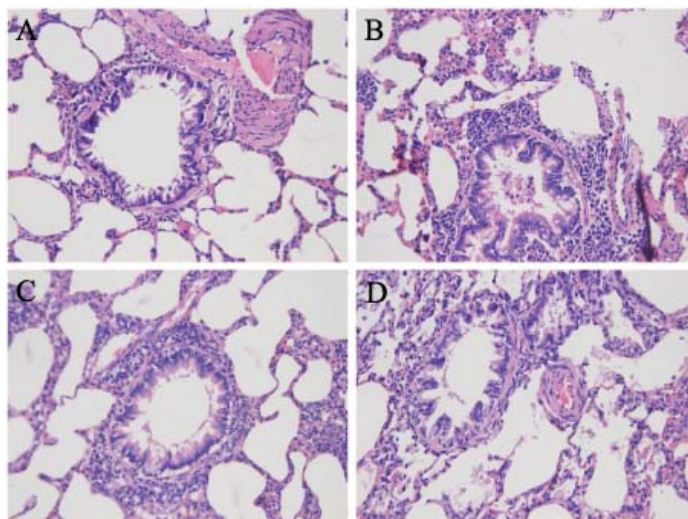


Figure 2. The H&E staining of lung tissues of (A) the N group (200 $\times$), (B) the A group (200 $\times$), (C) the L group (200 $\times$), and (D) the D group (200 $\times$).

Ligustrazine Induced Morphological Changes in the Lung Tissue of OVA Sensitized Rats

Figure 2 indicates that in the asthmatic group, a massive peribronchial infiltration of inflammatory cells, thickening of bronchial wall and area of smooth muscle, and occlusion of the bronchial lumen by mucus and inflammatory cells. However, ligustrazine treatment resulted in an intact bronchial epithelial layer and few inflammatory cells infiltration, the same features shown in the dexamethasone group.

Ligustrazine Inhibited Th2 Cytokine and Increased Th1 Cytokine Level in BALF of OVA Sensitized Rats

Compared with N group, the concentration of IL-4 in the BALF of A group and AC group was significantly increased ($p < 0.01$, Table 2), while the concentration of IFN- γ was decreased ($p < 0.01$, Table 2), and thus the IL-4/IFN- γ ratio was increased ($p < 0.01$, Table 2). The concentration of IL-4 in the BALF was significantly suppressed by ligustrazine administration ($p < 0.01$, Table 2), and the concentration of IFN- γ was increased ($p < 0.01$, Table 2) after administration of ligustrazine. However, after dexamethasone administration, the concentration of IL-4 and IFN- γ in the BALF was both decreased ($p < 0.01$, Table 2). The IL-4/IFN- γ ratio in L and D group were both lower than those in A group and AC group ($p < 0.01$, Table 2). There was no significant difference between A group and AC group in the levels of IL-4, IFN- γ and IL-4/IFN- γ ratio ($p > 0.05$, Table 2).

Table 2. IL-4 and IFN- γ Concentrations in the BALF and IL-4/IFN- γ Ratio of the Five Groups

Groups	N	IL-4 (pg/ml)	IFN- γ (pg/ml)	IL-4/IFN- γ
N	8	9.88 $\pm$ 1.31	69.36 $\pm$ 3.37	0.14 $\pm$ 0.02
A	8	24.88 $\pm$ 3.92*	23.41 $\pm$ 2.75*	1.07 $\pm$ 0.18*
AC	8	23.68 $\pm$ 3.56*	22.73 $\pm$ 2.34*	1.05 $\pm$ 0.19*
D	8	12.97 $\pm$ 1.73 $^{\Delta}$	17.68 $\pm$ 1.86 $^{\Delta}$	0.74 $\pm$ 0.18 $^{\Delta}$
L	8	13.30 $\pm$ 1.75 $^{\Delta}$	45.57 $\pm$ 3.42 $^{\Delta}$	0.29 $\pm$ 0.04 $^{\Delta}$

Mean $\pm$ SD, *p < 0.01 versus the N group; $^{\Delta}$ p < 0.01 versus A and AC group.

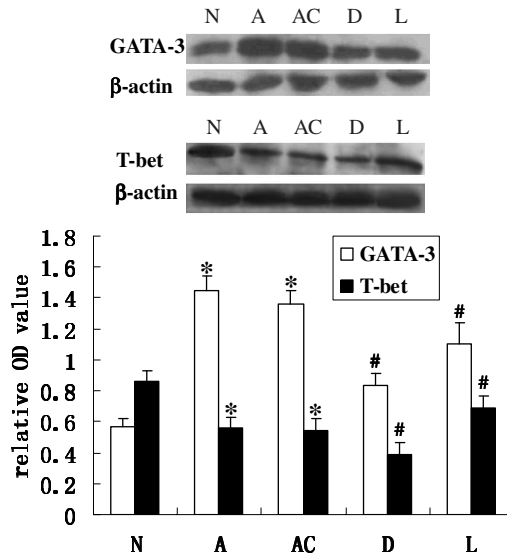


Figure 3. Relative optical density (OD) value of GATA-3 and T-bet Western blot in each group. *p < 0.01 versus the N group; #p < 0.01 versus A and AC group.

Ligustrazine Decreased GATA-3 and Increased T-bet Protein Expression in the Lung Tissue of OVA Sensitized Rats

The rats in A and AC group expressed higher GATA-3 and lower T-bet protein than the normal control rats (p < 0.01, Fig. 3). Ligustrazine significantly suppressed GATA-3 protein and increased T-bet protein expression (p < 0.01, Fig. 3). While dexamethasone suppressed both GATA-3 and T-bet protein expressions (p < 0.01, Fig. 3). There was no significant difference between A and AC group (p > 0.05, Fig. 3).

Discussion

In the past two decades, a dramatic increase in the prevalence of asthma has been witnessed worldwide. Asthma is a chronic disease characterized by reversible airway

obstruction, airway hyper-responsiveness (AHR) and airway inflammation and remodeling. In most cases, the asthmatic inflammatory process results from inappropriate immune responses to common environmental antigens in a genetically susceptible individual (Wills-Karp, 1999). These inappropriate immune responses are orchestrated by a subset of CD4⁺ T helper cells termed as T helper 2 (Th2) cells. CD4⁺ T cells can be divided into two subsets, Th1 cells and Th2 cells, according to their characteristic cytokine production profiles. Th1 cells predominantly promote cell-mediated immune response, whereas Th2 cells suppress the Th1-inflammatory response (Romagnani, 1997). The functional differences between Th subsets are explained primarily through the activities of the cytokines they secrete. IFN- γ is the characteristic cytokine of Th1 cells, which also produce IL-2 and TNF- β . IL-4 is the corresponding cytokine of Th2 cells, which also secrete IL-5, IL-6, IL-9, IL-13 and IL-10. IL-4 is a key cytokine in the development of allergic inflammation. It promotes eosinophil survival and proliferation. It is now widely accepted that T cells, particularly Th2 cells, play a critical role in the pathogenesis of asthma (Herrick and Bottomly, 2003).

Induction of the transcription factors GATA-3 (GATA-binding protein-3) and T-bet (T-box expressed in T cells) are critical for differentiation of naive CD4⁺ T cells into Th2 and Th1 cells, respectively (Ho and Glimcher, 2002). GATA-3, a member of the GATA family of zinc finger transcription factors, which bind to the WGATAR (W = A/T; R = A/G) DNA sequence through a highly conserved C4 zinc-finger domain, has been identified as a key regulator of Th2 development (Zheng and Flavell, 1997). Naive CD4⁺ T cells express low levels of GATA-3 mRNA. The expression of GATA-3 is, however, markedly upregulated in cells differentiating along the Th2 lineage, and is down-regulated in cells differentiating along the Th1 pathway. Blocking of GATA-3 with a dominant-negative construct or antisense DNA prevents Th2 cytokine activation and eosinophilia in allergen-challenge mouse models (Zhang *et al.*, 1999; Finotto *et al.*, 2001). GATA-3 is the only known Th2 transcription factor with autoactivating properties, and it also has the capacity to inhibit IFN- γ expression, resulting in repression of Th1 and development of a Th2 phenotype (Ferber *et al.*, 1999). On the other hand, T-bet, a member of the T-box family of transcription factors, is a key controller of Th1 differentiation (Afkarian *et al.*, 2002). T-bet is induced by STAT-1 mediated signals, which strongly promote IFN- γ and IL-12R β 2 expression for differentiation into Th1 cells (Mullen *et al.*, 2001). T-bet expression is strongly correlated with IFN- γ expression and is specifically up-regulated in primary Th cells that differentiate along the Th1 pathway (Szabo *et al.*, 2000). T-bet-deficient mice develop spontaneous airway changes consistent with major signs of human asthma (Finotto *et al.*, 2002), and an increased number of GATA-3-expressing cells, and fewer cells positive for T-bet have been observed in bronchial biopsy specimens from patients with asthma (Finotto *et al.*, 2002; Nakamura *et al.*, 1999). These findings suggested that T-bet might protect from asthma by predisposing the cells to differentiate to Th1 (rather than Th2) and further establishing and maintaining the Th1 phenotype.

Ligustrazine is an alkaloid isolated from the rhizome of *Ligusticum chuanxiong* Hort. It has been shown to be an immune response modifier in both animal models and human subjects, as well as an effective anti-fibrosis and antitumor agent (Wei *et al.*, 2004).

Previous studies showed that ligustrazine could enhance IFN- γ production by Th1 cells, while inhibiting Th2 cytokine production (Wei *et al.*, 2002). However, it remains unclear whether ligustrazine plays a role in the effector phase of allergic airway inflammation and whether it inhibits airway inflammation by modulating the expression of GATA-3 and T-bet. In our experiments, we observed an increase of Th2 cytokine and an inhibition of Th1 cytokine were accompanied with an increased expression of GATA-3 protein and a decreased expression of T-bet protein in rat asthmatic airways compared to those in the normal control group. Ligustrazine markedly lowered the level of IL-4 in BALF, while increased the level of IFN- γ in asthmatic rats. Likewise, in asthmatic rats, the expressions of GATA-3 and T-bet protein in lung tissue were lowered and increased, respectively, which revealed that modulating the expressions of GATA-3 and T-bet might be the mechanism of ligustrazine on Th1/Th2 cytokines. Meanwhile, after ligustrazine administration, the percentage of EOS in BALF was significantly decreased, as well as the thickness of the bronchial wall and the accumulation of inflammatory cells. These data demonstrated that ligustrazine significantly reduced airway inflammation in a rat model of allergic asthma. These effects were comparable to dexamethasone but were not accompanied by the suppression of Th1 responses seen with dexamethasone. Glucocorticoid is the most effective anti-inflammatory agent for asthma despite their potentially serious adverse effects. In our study, dexamethasone reversed the ratio of IL-4 and IFN- γ by inhibiting both GATA-3 and T-bet. The decreasing degree of GATA-3 was more than that of T-bet, resulting in a reversing status of Th2 polarization.

In conclusion, ligustrazine, in contrast to the overall suppression of GATA-3 and T-bet by dexamethasone, exhibits immunomodulatory actions on Th1/Th2 cytokines caused, at least partially, by down-regulation of GATA-3 and up-regulation of T-bet. GATA-3 and T-bet might be prospective targets for ligustrazine in the treatment of asthma. Using ligustrazine as an assistant therapy of glucocorticoid to treat asthma might decrease the dosage of glucocorticoid and ameliorate the adverse effects of glucocorticoid therapy.

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