Research Article
Ligustrazine Inhibits Lung Phosphodiesterase Activity in a Rat Model of Allergic Asthma

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Objectives. This study sought to examine whether ligustrazine was capable of inhibiting phosphodiesterase (PDE) activity and improving lung function in a rat model of asthma.

Methods. Rats were initially sensitized using ovalbumin (OVA) and then were challenged daily with aerosolized OVA beginning 14 days later (30 min/day) to generate a rat model of asthma. Changes in airway function following methacholine (MCh) injection were evaluated by monitoring lung resistance ($R_L$) and dynamic lung compliance ($C_{dyn}$) values using an AniRes2005 analytic system. In addition, serum IgE was measured via ELISA, while PDE expression was evaluated via qPCR and western blotting.

Key Findings. Ligustrazine significantly impaired allergen-induced lung hyperresponsivity and inflammation in this asthma model system. Ligustrazine treatment was also associated with reduced expression of PDEs including PDE4 in the lungs of these rats.

Conclusions. Ligustrazine suppresses airway inflammation and bronchial hyperresponsivity in this rat model system, and these changes are associated with decreased PDE expression at the protein and mRNA levels.

1. Introduction

Asthma is a very common form of respiratory disease wherein immunological dysfunction can lead to chronic airway inflammation, leading to aberrant airway constriction, mucus production, and breathing difficulties in affected individuals. There are over 300 million individuals worldwide affected by asthma, and data from the Global Initiative for Asthma (GINA) suggest that there will be as many as 400 million cases by the year 2025. Individuals affected by asthma can, in some cases, suffer from severe airway constriction that can be life threatening [1, 2]. Asthma and associated inflammation are closely associated with the infiltration of inflammatory cells including neutrophils and eosinophils into the airways. This inflammation coincides with inflammatory activity linked to cAMP degradation [3] and the production of characteristic cytokines produced by type 2 helper T cells (Th2 cells) [4]. Asthma symptoms can generally be managed effectively using combinations of corticosteroids and $\beta_2$ agonists, but no cures for this condition are available at present. This has led many researchers to explore the potential value of traditional Chinese medicine (TCM) to treat asthma. Indeed, TCM has been increasingly incorporated into established treatment regimens as a complementary and alternative medicine (CAM) approach. However, this approach has led to a relatively limited scientific basis for the medicinal use of many TCM formulations. As such, there has been increasing interest in the pursuit of more evidence-based studies of TCM in an effort to narrow the information gap pertaining to these treatments, thereby offering patients potential treatments that may yield better therapeutic outcomes [5–7].

Phosphodiesterases (PDEs) are key enzyme which hydrolyzes cAMP and cGMP, resulting in nucleotide inactivation [8]. When PDEs are inhibited, levels of cAMP and cGMP rise significantly, resulting in several phenotypic
changes including a reduction in immune-mediated inflammation and the relaxation of airway smooth muscle. In contrast, lowered levels of cAMP are thus often studied in the context of asthma treatment, including production of cAMP are thus often studied in the context of asthma pathogenesis [9–11]. Drugs capable of inducing the production of cAMP are thus often studied in the context of asthma treatment, including $\beta_{2}$-adrenoceptor agonists and theophylline [12, 13]. Many different extracellular stimuli are capable of promoting cAMP production within cells in response to either PDE inhibition or adenylyl cyclase (AC) activation. Compounds capable of targeting these enzymes may thus have therapeutic value for the treatment of asthma. Consistent with such a mode, PDE4 inhibitors have been shown to decrease airway inflammation and increase cAMP levels, with this mechanism being linked to an inhibition of Th2 cellular function and associated inflammatory cytokine secretion [14–18].

Ligustrazine is an alkaloid derivative of the Chuanxiong rhizome (Ligusticum Chuanxiong Hort). It has been shown to possess an array of anti-inflammatory, antiinflammatory, antibacterial, and immunomodulatory activities. Notably, recent work suggests that ligustrazine can induce cAMP production and modulate IL-4 secretion in a PAMP-dependent manner [19]. We, therefore, hypothesized that ligustrazine would be able to protect against allergen-induced asthma in rats via modulating PDE activity and thereby altering cAMP-related signaling. To that end, we sensitized Sprague-Dawley (SD) rats using ovalbumin (OVA) before challenging them with aerosolized OVA to establish an animal model of asthma. These rats were then injected with ligustrazine, and airway inflammation and reactivity were analyzed. In addition, we evaluated the impact of ligustrazine treatment on lung Th1/Th2 cytokine levels and PDE expression in this asthma model system.

2. Materials and Methods

2.1. Allergen Model. We obtained male SD rats (220–240 g) from Shanghai BK Laboratory Animal Limited Company (Shanghai, China). Rats were housed in a standard climate-controlled animal facility (23 ± 2°C, 50% ± 10% humidity, 12 h light/dark cycle) with free food and water access. Food was withheld from animals for 8 h before experiment initiation. The Institutional Animal Care of the Anhui University of Chinese Medicine approved all animal studies. Rat sensitization was achieved by 1 mL of aluminum hydroxide (10%) containing 0.2% OVA (Sigma, MO, USA): subcutaneously injecting rats with 0.5 mL in ten points (0.05 mL in each point) as neck (one point), footpads (four points), back (left, center, and right, three points), and bilateral groin (two points) and intraperitoneal injecting rats with remaining 0.5 mL. The sensitization was performed on day 0, as in prior studies [20, 21]. Beginning on day 14 postsensitization, animals were exposed once per day for 7 days to aerosolized OVA (1% in saline) for 30 minutes using a jet nebulizer (Master; Pari GmbH, Starnberg, Germany; droplet diameter: 1-5 μm) (Figure 1).

2.2. Animal Treatments. Rats were separated into 6 treatment groups ($n = 12$/group). Saline was used for both the sensitization and challenge of control animals, whereas all other model animals were treated with OVA. Rats in the ligustrazine groups were sensitized and challenged using OVA, after which they were intraperitoneally injected once per day with 40 or 80 mg/kg ligustrazine (Harbin Sanlian Pharmaceutical Limited Company, Harbin, China, 79H1505) from day 14 to 21, as in prior studies [22]. Theophylline (40 mg/kg, Shanghai Xinyijinzhuzhu Pharmaceuticals Co., LTD., Shanghai, China) or dexamethasone (1 mg/kg, Zhejiang Xianju Pharmaceuticals Co., LTD., Hangzhou, China) was intraperitoneally injected into positive control group animals using the same dosing schedule as for animals treated with ligustrazine. All of these compound doses were selected in light of our preliminary data and prior studies [20–22]. Theophylline was selected as a nonselective PDE inhibitor [23], while dexamethasone was used because it can serve as a prophylactic asthma treatment [24]. On day 21 postsensitization, we euthanized all animals, collected their lung tissues, snap-froze them, and stored them at -80°C.

2.3. Airway Hyperresponsiveness Analyses. Lung resistance ($R_l$) and dynamic lung compliance ($C_{dyn}$) were assessed based on airway functionality changes following intravenous methacholine (MCh) injection [25]. Rats were first anesthetized; after which they were positioned within a plethysmograph whole-body plethysmograph in a supine position. An airway-connected fisher tube in a pressure transducer was used for monitoring expiratory flow rates, while lung volume changes were also assessed based upon pressure changes within the plethysmographic chamber. Pleural pressure was evaluated by inserting a needle with multiple holes in its tip into the pleural cavity via a port connecting it to a differential pressure transducer. Differences between pleural and mouth pressure were then used to determine transpulmonary pressure, with all pressure transducer signals being continuously processed (AniRes2005 animal lung function analytic system, Beijing, China) based upon the fitting of pressure, flow, and volume data to a motion equation. MCh-associated changes in $R_l$ and $C_{dyn}$ were assessed using a dose-response curve in response to MCh doses of 0.5-8 g/L (inhaled), as these doses were found to provoke airway hyperreactivity but not death in preliminary experiments. How ligustrazine, theophylline, and dexamethasone affected these airway responses was determined based on a comparison of $R_l$ and $C_{dyn}$ changes following drug treatment to mean MCh responses in the same rat during prior and successive control periods.

2.4. Serum IgE Measurement. Serum separator tubes were used to collect blood samples from individual rats. After allowing 30 minutes for clotting to occur, tubes were spun at 3000 × g for 10 minutes. Serum was then collected and maintained at -80°C. For analysis of serum IgE levels, serum samples were first spun for 30 minutes at 12000 × g at 4°C, after which supernatants were analyzed with a rat IgE-specific ELISA kit (13015R, Yuanye Biotech Co. Ltd.).

2.5. Bronchoalveolar Lavage Fluid (BALF) Analysis. At 24 h following the final OVA challenge, urethane (1 g/kg, i.p.)
was utilized to anesthetize rats. The lungs of these animals were then lavaged thrice using a 5 mL volume of normal saline containing 1% BSA and 5000 IU heparin. These lavage samples were then pooled and spun for 10 minutes at 500 \( \times \) g at 4°C, and pelleted cells were resuspended in HBSS for counting and analysis. Briefly, 200 cells per sample were stained using Wright-Giemsa, after which cells within these samples were evaluated via light microscopy. Data were expressed as cells per liter of BALF.

2.6. Histological Examination. Following collection, the lungs were fixed for 7 days using 10% neutral-buffered formalin. Tissues were then paraffinized and cut into 5 \( \mu \)m sections, which were stained using hematoxylin and eosin (H&E) prior to the light microscopy-mediated assessment of inflammatory cell infiltration.

2.7. Quantitative PCR (qPCR). Trizol (Sigma) was used to extract total tissue RNA based on provided directions, after which a cDNA synthesis kit (Shanghai Sangon Biological Engineering Technology and Service Co. Ltd., Shanghai, China) was used for cDNA preparation. Next, qPCR was conducted in 20 \( \mu \)L reaction volumes that contained 2x Goldstar TaqMan mixture (10 mL), primers (F+R; each at 10 mM in 0.4 mL), probes (10 mM, 0.4 mL), cDNA (2 mL), and nuclease-free dH\(_2\)O (6.8 \( \mu \)L). Thermocycler settings were as follows: 10 min at 95°C, 40 cycles of 15 s at 95°C, 1 min at 60°C. Primer and probe sequences are shown in Table 1.

2.8. Protein Quantification. A coomassie blue staining protein assay kit (Nanjing Jianshen Bioengineering Institute, Nanjing, China) was used to evaluate protein levels via the Bradford approach, with BSA serving as a reference standard.

2.9. Western Blotting. A total of 40 \( \mu \)g of protein extracted from lung samples was electrophoretically separated (10% SDS-PAGE) and transferred onto a nitrocellulose membrane. This blot was then blocked using 5% nonfat milk in TBST for 1 h prior to overnight incubation with primary anti-PDE (1:500; Santa Cruz), anti-PDE4 (1:500; Santa Cruz), and anti-\( \beta \)-actin (1:1000; Sigma). Blots were then probed with secondary antibodies, and protein bands were detected via chemiluminescent exposure prior to densitometric analysis. \( \beta \)-Actin was used to normalize protein expression (arbitrary units), with all values in individual experiments being normalized against control group samples.

2.10. Statistical Analysis. Data are means \( \pm \) SD and were compared using nonparametric tests and two-tailed \( t \)-tests, as appropriate. Relative mRNA expression was assessed using the 2\(^{-\Delta\Delta Ct}\) approach. \( P < 0.05 \) was the significance threshold.
threshold, and all data are representative of two or more independent experiments.

3. Results

3.1. Ligustrazine Suppresses Airway Hyperreactivity in a Rat Asthma Model. In the present study, we utilized a rat model of allergic asthma wherein sensitization was associated with significant reductions in $C_{\text{dyn}}$ and significant increases in $R_L$ relative to control animals ($P < 0.05$). When these animals were treated using ligustrazine, they exhibited reductions in airway hyperreactivity in response to sensitization and MCh concerning $C_{\text{dyn}}$ and $R_L$ values, although these differences were not significant ($P > 0.05$) (Figure 2(a)). In contrast, treatment with theophylline and dexamethasone significantly decreased airway hyperreactivity in these OVA-sensitized rats ($P < 0.05$) (Figure 2(b)). This may suggest that ligustrazine can reduce airway hyperresponsiveness in an asthma model system via suppressing the inflammation associated with OVA sensitization and subsequent aerosolized OVA challenge.

3.2. Ligustrazine Decreases Serum IgE Levels in Asthmatic Rats. IgE is a key driver of bronchial hyperreactivity in models of asthma, and as such, the measurement of total serum IgE can offer insight into asthma disease status. As such, we next measured total serum IgE in our different rat treatment groups (Figure 3). We found that OVA sensitization and challenge were associated with elevated serum IgE levels in these rats, whereas ligustrazine (40 mg/kg) and theophylline treatment was associated with significant reductions in these serum IgE levels.

3.3. Ligustrazine Suppresses Allergen-Induced Inflammatory Cell Infiltration in the Lungs. Using this same model of allergen-induced asthma, we next collected BALF samples from rats at 24 h postfinal OVA challenge. The numbers of different inflammatory cells in these BALF samples were then counted. Relative to control rats ($36.5 \times 10^8$ cells/L), asthmatic model rats exhibited a 4-fold increase in the total number of cells in these BALF samples, while ligustrazine treatment ($80 \text{mg/kg}$) was associated with a significant reduction in these cell numbers, although theophylline and dexamethasone had an even more significant suppressive impact on inflammatory cell infiltration into the lungs (Figure 4(a)). Relative to the BALF from control rats which contained $0.25 \times 10^8$ neutrophils/L, $1.86 \times 10^8$ eosinophils/L, $31.18 \times 10^8$ lymphocytes/L, and $1.72 \times 10^8$ monocytes and macrophages/L, BALF samples from OVA-challenged model rats exhibited 130-, 15-, 2-, and 7-fold increases in the numbers of these cell types, respectively (Figure 4(b)).
Ligustrazine (80 mg/kg) treatment was associated with a significant reduction in neutrophil and eosinophil numbers in the BALF of these animals relative to samples from vehicle control-treated model animals (Figure 4(b)). This suggests that ligustrazine can inhibit the influx of inflammatory cells into the lungs in response to allergen sensitization and subsequent challenge in this rat model system.

### 3.4. Ligustrazine Attenuates Airway Inflammation

Samples of lung tissue were collected from all rats at 24 h following the final OVA challenge. Analysis of these tissue samples revealed that asthma model animals exhibited significant increases in inflammatory cell infiltration (Figure 5(b)), with eosinophil infiltration into the perivascular and peribronchial space (arrow) being particularly enhanced relative to control rats (Figure 5(a)). Ligustrazine (Figures 5(c) and 5(d)) or theophylline and dexamethasone (Figures 5(e) and 5(f)) treatments were, in turn, associated with a significant reduction in the OVA-induced eosinophil infiltration of the lungs of these model animals relative to vehicle control treatment.

### 3.5. Ligustrazine Suppresses PDE Expression at the mRNA Level in Asthmatic Model Rats

We next evaluated PDE gene expression via qPCR in lung tissue samples from our different rat treatment groups. We determined that OVA sensitization and challenge were associated with significant increases in PDE (Figure 6(a)) and PDE4 (Figure 6(b)) mRNA expression relative to control rats. When rats were treated with ligustrazine, however, they exhibited reduced lung PDE and PDE4 expression relative to vehicle control-treated model animals (Figures 6(a) and 6(b)). Theophylline and dexamethasone treatment also significantly inhibited the expression of these two PDEs in the lungs in response to OVA sensitization and challenge (Figures 6(a) and 6(b)).

### 3.6. Ligustrazine Suppresses PDE Expression at the Protein Level in Asthmatic Model Rats

Lastly, we evaluated PDE expression at the protein level in the lungs of these asthma model rats. We found that model animals exhibited significant increases in both PDE and PDE4 levels in the lungs following OVA challenge relative to control animals (Figures 7(a) and 7(b)). In contrast, ligustrazine treatment was associated with a significant reduction in the levels of both of these PDEs in the lungs of model rats relative to vehicle control treatment (Figures 7(a) and 7(b)). Both theophylline and dexamethasone significantly decreased PDE and PDE4 protein levels in the lungs of these animals relative to vehicle control treatment (Figures 7(a) and 7(b)). These findings were also validated via densitometric analyses (Figure 7(b)). Together, our findings indicate that ligustrazine can attenuate allergic responses via a mechanism linked to reduced PDE and PDE4 expression.

### 4. Discussion

Asthma is a potentially serious disease characterized by chronic lung inflammation and hyperreactivity, resulting in significant morbidity and mortality. Asthma prevalence has grown markedly in recent decades. At present, asthma is primarily treated using inhaled corticosteroids (ICS) and β2-adrenoceptor agonists, but these compounds often exhibit numerous side effects [26–28]. There is thus a need for the development of novel treatments for asthma that function more efficiently while inducing fewer adverse effects. In Chinese communities, TCM treatment strategies are commonly employed as an alternative approach to treating children that have asthma [29]. In order to better understand the therapeutic efficacy of TCM-based asthma treatments, we designed the present study focused on identifying the mechanistic basis for ligustrazine treatment efficacy in a rat model.
Ligustrazine is a vasoactive derivative of *Ligusticum Chuanxiong Hort* that has been shown to exhibit a range of antifibrotic, antioxidative, anti-inflammatory, and immunomodulatory activities [30]. It is commonly utilized for the treatment of cancer, cardiovascular disease, and cerebrovascular disease, and in asthma patients, it is often combined with glucocorticoids. A Chinese herbal monomer preparation composed of ligustrazine and herbal monomers has been found to significantly inhibit airway hyperresponsiveness [31]. Ligustrazine has also been previously shown to reduce eosinophil and neutrophil lung infiltration in a mouse model of asthma, with this effect being associated with changes in Th1/Th2 and Treg/Th17 cytokine profiles in these animals [32]. Ligustrazine has further been shown to promote endothelial cell production of nitric oxide, driving endothelium-dependent relaxation in the aortic rings of rats [33]. We, therefore, hypothesized that ligustrazine would function as a beneficial treatment for asthma. Consistent with this, we found that ligustrazine treatment (40 and 80 mg/kg) decreased airway hyperreactivity in a rat OVA-induced model of asthma via suppressing the inflammation associated with OVA sensitization and subsequent aerosolized OVA challenge. Our results are in line with prior work demonstrating that ligustrazine can inhibit OVA-induced airway hyperresponsiveness, eosinophilia, goblet cell hyperplasia, airway remodeling, and Th2 cytokine production [22, 31, 32, 34–36].

In addition, we found that OVA sensitization and challenge were associated with increased serum IgE levels in these rats, while ligustrazine and theophylline treatment was associated with significant decreases in these serum IgE levels. By analyzing the effect of ligustrazine on inflammatory cells in the lungs, we found that ligustrazine can inhibit the influx of inflammatory cells into the lungs to cope with allergen sensitization and subsequent challenge in this rat model system. Meanwhile, the analysis of these tissue...
samples from all rats at 24 h following the final OVA challenge revealed that ligustrazine or theophylline and dexamethasone treatments were associated with a significant decrease in this OVA-induced eosinophil infiltration of the lungs of these model animals relative to vehicle control treatment.

In individuals with asthma, pulmonary cAMP levels are reduced [21, 37], and cAMP-inducing reagents such as
theophylline, PDE4 inhibitors, and β₂ agonists can bolster lung function while inhibiting inflammation in animals and individuals with asthma [38–40]. β₂ agonists can promote cAMP-dependent MAP kinase phosphatase (MJP) upregulation, thereby promoting bronchodilation and suppressing inflammation. Previous work suggests that MKP-1 expression is controlled by cAMP, and PDE4 is the primary PDE necessary to break down cAMP in ASM cells in response to formoterol [41]. In rabbits, ligustrazine can induce corpus cavernosum smooth muscle relaxation via a mechanism independent of nitric oxide and the endothelium, but that is mediated by cAMP phosphodiesterase inhibition of cGMP phosphodiesterase [36]. This is similar to the PDE5 inhibitor sildenafil, which is effective for treating asthma and COPD (chronic obstructive pulmonary disease) in addition to being used as an erectile dysfunction drug [42, 43]. As it is associated with many side effects, however, the use of sildenafil remains controversial for the treatment of lung diseases [44].

Members of the PDE family of enzymes (PDE1-11) are capable of hydrolyzing cAMP and/or cGMP in biochemically and pharmacologically distinct manners. PDE inhibition can modulate many different inflammatory and immunological responses that are relevant in the context of asthma. PDE4 inhibitors, notably, have been shown to suppress airway hyperreactivity and inflammation in animal asthma models [45, 46]. Consistent with these results, PDE4 activation has been found to correlate with asthma-related inflammation, and PDE4 functions as a key mediator of asthma pathogenesis [47, 48]. Certain PDE4 inhibitors including roflumilast and cilomilast have been translated with success in phase II and III trials, with roflumilast (Daxas; Nycomed) having received marketing authorization from the European Commission as a maintenance treatment (Daxas; Nycomed) having received marketing authorization in it having fewer side effects than specific PDE4 inhibitors and potentially making it more beneficial for the treatment of asthma. Together, our findings indicate that ligustrazine can attenuate allergic responses via a mechanism linked to reduced PDE and PDE4 expression.

In summary, the results of this study demonstrate that ligustrazine pretreatment was able to markedly reduce airway hyperreactivity and inflammation in response to allergen stimulation in a rat asthma model system. The mechanism underlying this efficacy may be associated with the inhibition of lung PDE expression. Therefore, this study offers potential treatments for individuals affected by asthma that may yield better therapeutic outcomes.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Additional Points

Mini Abstract. Ligustrazine is capable of inhibiting PDE activity and improving lung function in a rat model of asthma, and these changes are associated with decreased PDE expression at the protein and mRNA levels.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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