Original Research

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Hirudin relieves airway mucus hypersecretion and inflammation in Ovalbumin-Induced Asthma

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Abstract

Asthma is a chronic inflammatory disease of the airways caused by multiple allergens or other factors, involving multiple cells and cellular components, Currently, the main drugs used to treat asthma include glucocorticoids, β-2 agonists, leukotriene receptor antagonists, theophyllines, anticholinergics, mast cell stabilizers, and cytokine monoclonal antibodies. Hirudin is an active ingredient extracted from the leeches and its salivary glands. However, there is no relevant articles explaining the relationship between hirudin and Asthma. For the present study, we hypothesized that hirudin ameliorates the pathology in the OVA-induced mouse asthma model by its anti-inflammatory effects and alleviates the vicious airway remodeling. The lung tissues and HE staining was performed, we therefore found hirudin ameliorates the pathology of OVA-induced asthma. To further detect the treatment of hirudin on asthma, we then performed AB-PAS staining and western blot, and found that hirudin ameliorates airway mucus hypersecretion. The results of Immunocytochemistry, Toluidine blue staining and western blot also proofed that hirudin ameliorates airway inflammation. In conclusion, hirudin ameliorates the pathology in the OVA-induced mouse asthma model by its anti-inflammatory effects and alleviates the vicious airway remodeling, which is potentially amenable to therapeutic manipulation for clinical application.

Keywords: Asthma, Hirudin, airway inflammation, airway mucus hypersecretion

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Introduction

Asthma, also known as bronchial asthma, is a chronic inflammatory disease of the airways caused by multiple allergens or other factors, involving multiple cells and cellular components, and is often accompanied by airway hyperresponsiveness and airway remodeling, thus contributing to recurrent and persistent wheezing, shortness of breath, dyspnea, chest tightness, and coughing [1-3]. Currently, approximately 300 million people worldwide, or 3.7% of the world's population, suffer from asthma, and 250,000 of these deaths occur each year[3, 4]. Comorbidity with other diseases significantly increases the risk of death in asthma patients, and in recent years, the incidence of asthma has been trending toward a younger age [5]. The pathogenesis of asthma includes airway inflammation, airway hyperresponsiveness (AHR), and airway remodeling. Airway inflammation is the basic pathogenesis of asthma and is the basis for airway hyperresponsiveness and airway remodeling [6]. In the pathogenesis of asthma, many inflammatory factors such as interleukin-4 (IL-4), interleukin-10 (IL-10), and interleukin-17 (IL-17) regulate and induce each other with a variety of inflammatory cells to promote the development of inflammation [7, 8]. Airway hyperresponsiveness is a fundamental feature of asthma and is characterized by excessive or premature airway constriction resulting in bronchial narrowing [9, 10]. Airway remodeling refers to a series of structural changes in the airways resulting from recurrent asthma exacerbation, including mucinization of epithelial cells, thickening of the reticular basement membrane, hypertrophy and hyperplasia of airway smooth muscle, degeneration and hyperplasia of cupped cells, and increased production of airway blood vessels and lymphatic vessels [11, 12]. Airway remodeling leads to structural changes in the bronchi, resulting in poor airflow and increased airway resistance, which increases airway hyperresponsiveness and affects the nor-mal physiological function of the lungs[13] . Also, the mechanism by which airway remodeling occurs is related to the release of inflammatory factors and growth factors by certain cells in the airways[14]. Currently, the main drugs used to treat asthma include glucocorticoids, β-2 agonists, leukotriene receptor antagonists, theophyllines, anticholinergics, mast cell stabilizers, and cytokine monoclonal antibodies. Of these drugs, the first four are the majority used. However, long-term use of glucocorticoids may lead to metabolic disorders and decreased resistance of the body; long-term use of β-2 agonists and leukotriene receptor antagonists often leads to the development of drug resistance in the body and a significant decrease in drug efficacy [15, 16]. In the patients suffered from asthma, chronic inflammation can con-tribute to deleterious airway remodeling which is characterized by subepithelial fibrosis and an increase in smooth muscle mass, airway wall thickness, and mucus gland density[17]. Therefore, to prevent airway inflammation is crucial in asthma treatment.

Hirudin is an active ingredient extracted from the leeches and its salivary glands[18] . It is a small protein that consists of 65-66 amino acids. It is reported that Hirudin is the most potent natural specific thrombin inhibitor found to date[19, 20]. It has a strong inhibitory effect on thrombin with anticoagulant, antithrombotic, antitumor, and anti-fibrosis functions. Recently, hirudin has been found exerts anti-inflammatory effects through p38 MAPK/ NF-κB path-way and protect against kidney damage in a diabetic nephropathy rat [21]. Hirudin was also can inhibited the oxidative stress and the expression of fibrosis-related factors to ease the myocardial fibroblasts [22] and inflammatory response in immuneglobulin A nephropathy (IgAN) [21].

However, there is no relevant articles explaining the relationship between hirudin and Asthma.

For the present study, we hypothesized that Hirudin ameliorates the pathology in the OVA-induced mouse asthma model by its anti-inflammatory effects and alleviates the vicious airway remodeling, which is potentially amenable to therapeutic manipulation for clinical application.

Material and methods

Model establishment

Female BALB/c mice (5 weeks, 20±2 g) were purchased from Kunming University. Animal experiments were approved by Experimental Animal Welfare and Ethics Committee, Kunming University. And conducted in accordance with the "Guidelines for in vivo experimental research reports in animal experiments" (ARRIVE Guidelines). Laboratory animals underwent all operations under anesthesia and every effort was made to minimize pain and death. BALB/c mice were housed in a pathogen-free animal care facility with free access to water and food. Mice were divided into 4 groups, control group (n = 6), Asthma group (n = 6), Asthma treated with Hirudin group (Asthma + Hirudin group, n = 6), Asthma treated with positive drug group (Asthma + PC group, n = 6). In control group, mice were treated with phosphate-buffered saline (PBS). To induce asthma model, mice were treated with ovalbumin (OV) as shown in Figure 1 (Sigma Aldrich, USA)[23]. Mice were sensitized on days 1 and 14 with intra-peritoneal injections of 200 μL containing 20 μg OVA and 2 mg aluminum hydroxide (USA). Then 5% OVA inhalation was performed daily for 25 min from day 21 to 48. Hirudin (2.4 $mg \cdot kg^{-1} \cdot d^{-1}$) and dexamethasone (1.0 $mg \cdot kg^{-1} \cdot d^{-1}$) were garaged to the mice 30 min before OVA in-halation from day 42-48. At day 49, the mice were euthanized by cervical dislocation and lung tissues were harvested.

Hematoxylin & eosin (HE) staining, AB-PAS staining and Toluidine blue staining.

Lung tissue samples were fixed with 4% polyformaldehyde and embedded with paraffin. Then, the tissue samples were cut into slices. After being dried, the tissue slices were deparaffinated using dimethylbenzene and dehydrated with water-free 95% and 80% ethanol. The slices were washed with phosphate buffer saline (PBS) (PH 7.4). Subsequently, the slices were immersed in the hematoxylin and hydrochloride alcohol and washed with running water. Afterwards, slices were dehydrated with ethanol at gradient degree, and then transparented with dimethylbenzene. The tissue slices were observed using the microscope. AB-PAS [24] staining and Toluidine Blue [25] staining was performed as described.

Western Blot

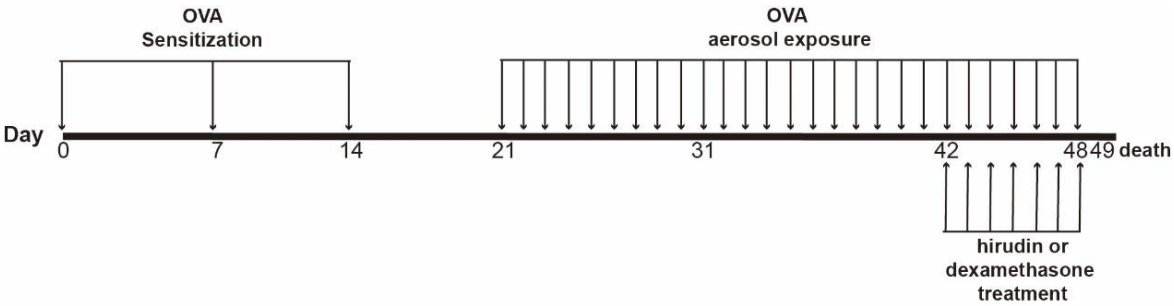

Figure 1 The procedure of OVA model establishment

Total proteins were extracted using a Total Protein Extraction Kit following the instructions. BCA Protein Assay Kit was used to detect the protein concentration. The total protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked using 5% skimmed milk at room temperature for 2 h. Primary antibodies shown in Table.1 were incubated overnight at 4°C and the secondary antibodies were incubated for 1h at room temperature. Enhanced chemilumine-scence (ECL) assay was used and captured through Imager. The optical density of the bands was analyzed using NIH Image J software.

Immunocytochemistry

The paraffin sections that had been prepared at 14 and 42 days after the model had been created were then conventionally baked, dewaxed, and hydrated with their antigen repaired. Following this, the sections were treated with 5% hydrogen peroxide for 20 minutes and sealed by 5% normal goat serum for one hour. Rabbit anti-mouse monoclonal antibody to IL-5 and IL-33 were added (1:2000, 1:4000; Abcam Inc., Cambridge, Massachusetts) for incubation at 4°C overnight, followed by goat anti-rabbit immunoglobulin G (IgG; 1:200; Sigma–Aldrich Chemical Company, St. Louis, Missouri) at 37°C for one hour and diaminobenzidine (DAB) solution (Sigma–Aldrich Chemical Company), followed by incubation for four to five minutes at room temperature with the avoidance of light. In addition, the specimens were redyed by haematoxylin (Shanghai Bogoo Biotechnology Inc., Shanghai, China) for 2.5 minutes, conventionally dehydrated for transparency and then mounted by neutral balsam. The samples were then observed under a light microscope (Olympus microscope with BX51 device imaging system) and were photographed with brown reaction products representing antigen localization. The known positive film was regarded as the positive control and the first antibody was replaced by phosphate buffered saline (PBS), which served as the NC. The positive depth and number of positive staining was estimated in order to determine the expression intensity of IL-5 and IL-33.

Statistical analysis

Data were expressed as mean ± Standard Error of Mean (SEM), and analyzed with Graph-Pad Prism 5.0 software (GraphPad Software, San Diego, CA). Statistical analyses were perfor-med using one-way ANOVA, followed by Turkey's post-test. Differences with $P < 0.05$ were considered statistically significant.

Results

Hirudin ameliorates the pathology of OVA-induced asthma

The asthma model was conducted as shown in figure 1. Specifically, on days 0, 7 and 14, the mice were injected intraperitoneally with 200 μL of sensitizing solution (containing 50 μg of OVA and 2 mg of aluminum hydroxide), and from day 21, the mice were stimulated by daily inhalation with 5% OVA solution in a nebulizer inhalation chamber for 30 min each time for 4 weeks. From day 42-48, hirudin and dexamethasone solution were administered by gavage at 30 min before daily inhalation. After mice were euthanized, we first harvested the lung tissues from mice. In normal mice, the lung tissue was uniformly ruddy, without swelling and congestion, hemorrhage, and fluid exudation, while in asthmatic mice, the lung volume was significantly increased and several areas on the lung surface were swollen and whitish, with a map-like appearance, also, irregularly shaped dark red congested areas were observed. While, the lung volume of hirudin-treated and dexamethasone-treated mice was reduced compared with that of the asthmatic group, and some of the whitish areas tended to be red and moist, the irregularly shaped dark red congested areas were smaller compared with that of the asthmatic group (Figure 2A). Also, result of HE staining showed that, in normal mice, the bronchial wall and alveolar wall were intact, without obvious cup cells and mucus secretion, only a small amount of inflammatory cell infiltration was seen around the bronchus and pulmonary vessels, without obvious collagen deposition and basement membrane thickening, while in asthmatic mice, the bronchial epithelium was incomplete, with a small amount of detachment, proliferation of airway epithelial cup cells, high mucus secretion on the air surface, thickening of the wall, narrowing of the lumen or even complete occlusion, thickening of the reticular basement membrane and smooth base layer, excessive collagen deposition, and a large number of inflammatory cells, mainly lymphocytes, in the bronchial wall and perivascular area (Figure 2B). The treatment of hirudin and dexamethasone obviously alleviated the inflammatory cell infiltration and vascular smooth muscle proliferation in lung tissue. Therefore, hirudin ameliorates the pathology of OVA-induced asthma.

Hirudin ameliorates airway mucus hypersecretion

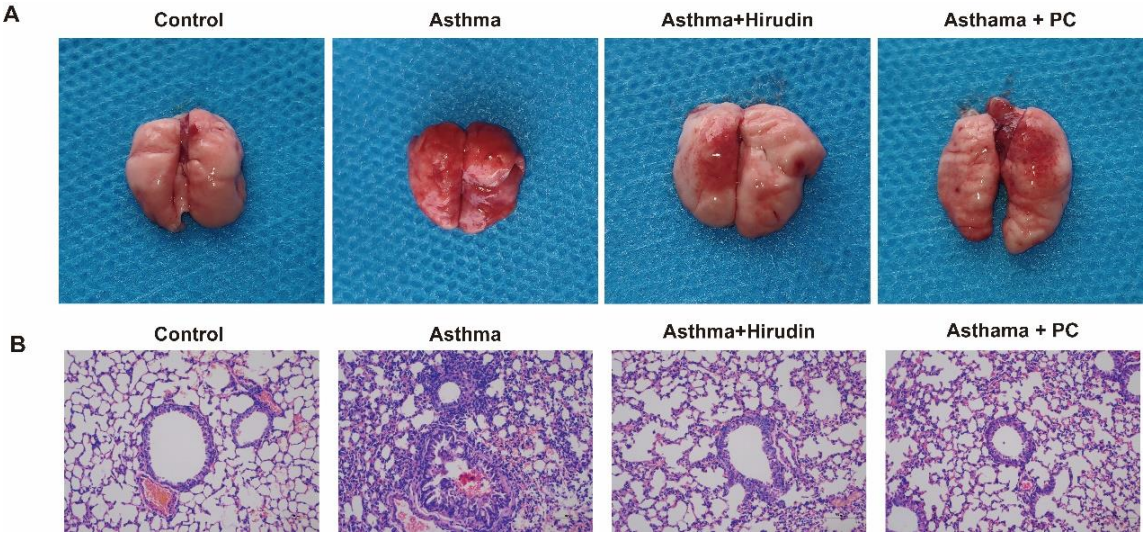

Figure 2. Hirudin ameliorates the pathology of OVA-induced asthma. Lung tissue (A) and HE staining of lung tissue (B) in different group.

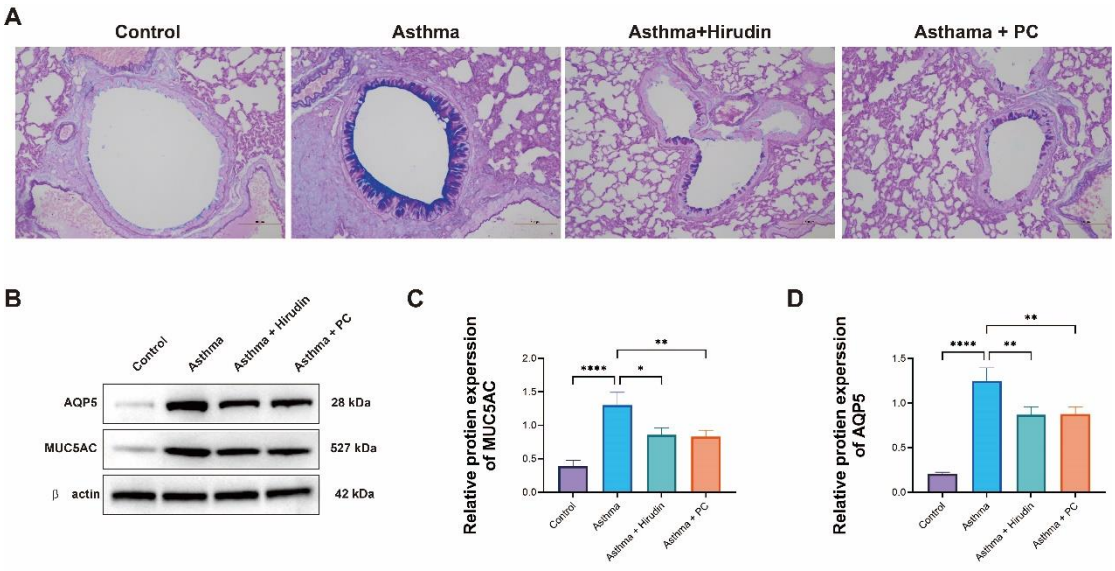

Figure 3. Hirudin ameliorates airway mucus hypersecretion. (A)AB-PAS staining (B) western blot detection of mucoprotein MUC5AC and water channel protein AQP5. Error bars represent SD. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$.

Then, to find out the effect of hirudin on airway mucus hypersecretion, we conducted the AB-PAS staining (Figure 3A). The results showed that, the mucus production and epithelial goblet cell hyperplasia on bronchial was obviously increased compared with control group, while the treatment relieved these pathologies. Also, the results of western blot showed that the expression of mucoprotein MUC5AC and water channel protein AQP5 increased in asthmatic mice, while the treatment of hirudin and dexamethasone reversed the results (Figure 3B). It is obvious that hirudin ameliorates airway mucus hypersecretion.

Hirudin ameliorates airway inflammation

To detect the airway inflammation, we preformed immunohistochemistry to detect the location and expression of IL-5 and IL-33 (Figure 4A). Both IL-5 and IL-33 were primarily located at extracellular matrix of bronchial epithelium. The expression of IL-5 and IL-33 was significantly increased in OVA-induced asthma model, while the expression was supperssed by hirudin or dexamethasone treatment. To observe the aggregation of mast cell, toluidine blue was performed (Figure 4B). The results showed that, the aggregation of mast cell was increased in in OVA-induced asthma model, while the aggregation was suppressed by hirudin or dexamethasone treatment. Also, the expression of inflammatory cytokines IL-1, IL-6, TNF- α showed the same trend (Figure 4C-F). Which indicated that hirudin ameliorates airway inflammation.

Discussion

In this manuscript, the lung tissues and HE staining was performed, we therefore found hirudin ameliorates the pathology of OVA-induced asthma. To further detect the treatment of hirudin on asthma, we then performed AB-PAS staining and western blot, and found that hirudin ameliorates airway mucus hypersecretion. The results of Immuno-cytochemistry, Toluidine blue staining and western blot also proofed that hirudin ameliorates airway inflammation.

Airway mucus hypersecretion is an important pathophysiological and clinical manifestation of chronic airway inflammatory diseases, which can cause airway bacterial colonization, airflow limitation and ventilation

dysfunction in asthma patients, thus making it difficult to control their symptoms[26, 27]. Airway mucus is composed of mucin (MUC), complex sugar and water secreted by airway epithelium, with mucin as its main component. research shows that the mechanism of MUCSAC secretion involves several signaling pathways, including G protein-coupled receptor-mediated, tyrosine protein kinase (TPK)-mediated, and non-receptor tyrosine kinase mediated, among

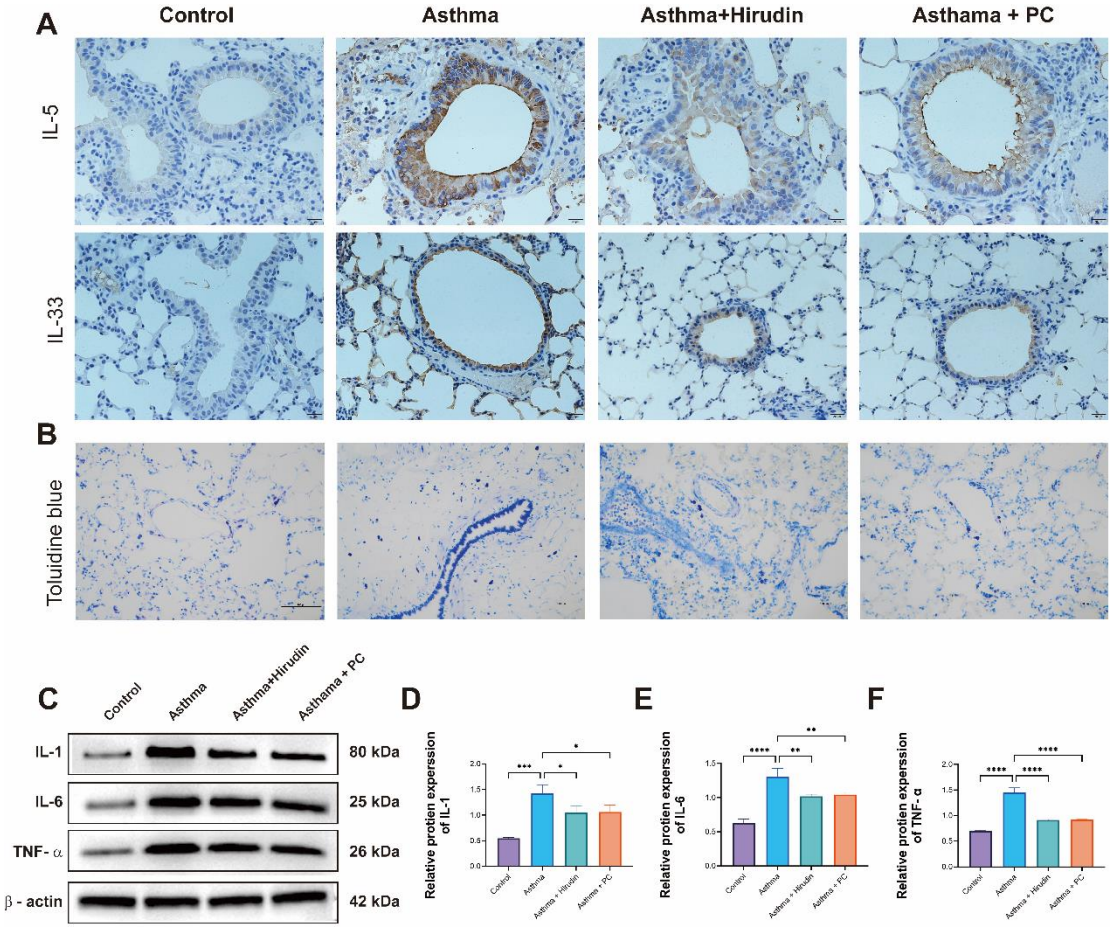

Figure 4. Hirudin ameliorates airway inflammation. (A)Immunocytochemistry of IL-5 and IL-33. (B) Toluidine blue staining (C-F) The expression of inflammatory cytokines IL-1, IL-6, TNF- α waws detected via western blot. Error bars represent SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

13 human MUC genes have been identified, namely MUC1, MUC2, MUC4, MUC5AC, MUC5B, MUC7, MUC8, MUC11, MUC13, MUC15, MUC19 and MUC20, according to the structural differences of mucins, they can be divided into two categories: secretory and membrane-bound, among which MUC2, MUC5AC, MUC5B genes are the main secretory mucin genes in the airway[28]. MUC5AC is the most important secretory mucin produced by goblet cells in the airway epithet-lium under pathological condition, basic which TPK-mediated signaling pathway has an important role in the secretion of MUC5AC[29, 30]. Meanwhile, more than 95% of airway mucus is water, which indicated the extremely vital of water channel proteins in airway mucus hypersecretion. Water channel proteins are small transmembrane proteins that are mainly involved in osmotic pressure-driven water transport [31]. There are four pulmonary-associated water channel proteins, namely AQP1, AQP3, AQP4, and AQP5, among which AQP1 is in pulmonary capillary endothelial cells, AQP3 and AQP4 are

in the basal surface of large and small airway mucosal epithelial cells, and AQP5 is in the luminal surface of alveolar type I cells (AT I) [32]. Previous studies [33] on the pulmonary physiological functions of aquaporins have focused on the formation and dissipation of pulmonary edema, airway humidification and other physiological processes directly related to water permeation across membranes, but many studies have found that AQPs in the lung are mainly involved in osmotically driven water transport and have little effect on water transport in most physiological and pathological conditions. In isolated lung perfusion experiments, the AQPs were found to be mainly involved in osmolarity-driven water transport [34]. In recent years, studies on AQPs have involved some new areas, such as AQPs and tumor cell migration and angiogenesis [35], and AQPs are involved in subcutaneous lipid metabolism with glycerol transport [36]. The abnormal expression of AQP is closely related to the imbalance of water-liquid metabolism and airway mucus hypersecretion in bronchial asthma[37], which is a new direction to study the pathogenesis of bronchial asthma in recent years. In this manuscript, we found that hirudin ameliorates airway mucus hypersecretion by decreasing the expression of MUC5AC and APQ5.

The recurrent attacks of bronchial asthma are due to immune dysregulation, and the activation of T lymphocytes into effector T cells by antigen is one of the key steps in the immune response to asthma[38]. CD4+ CD25+ and CD8+ CD28- T lymphocytes are a subset of regulatory T cells that are involved in autoimmune regulation and play an active and significant role in the maintenance of autoimmune tolerance [39]. CD4+ CD25+ cells play an important role in the maintenance of autoimmune tolerance[40]. They primarily inhibit effector T lymphocyte activation, proliferation, and kill toxicity through direct contact with other cells, thus exerting immune-modulatory effects. In recent years[41], CD8+ C28- cells have been shown to be another important regulatory cell that can achieve immunosuppressive functions by upregulating the expression of inhibitory receptors (e.g., ILT3, ILT4) on the surface of antigen-presenting cells. An important part of the pathogenesis of asthma is the deficiency of cytokines from Th1 cells and the increase of cytokines secreted by Th2 cells[42]. IL-5 [43-45] is secreted by Th2 cells, activates EOS progenitor cells in the bone marrow, causes peripheral blood and airway eosinophil aggregation, has been shown to be a key factor in the induction of eosinophil inflammation and airway hypertension in asthma, promotes the growth and differentiation of eosinophil precursor cells, activates airway eosinophils, and inhibits their apoptosis, enhances intracellular arachidonic acid metabolism, and produces large amounts of leukotriene C4. It also enhances intracellular arachidonic acid metabolism and produces large amounts of leukotriene C4, D4, E4 and other inflammatory mediators. In our manuscript, we found that, hirudin ameliorates airway inflammation by suppressing the expression of IL-5 and IL-33 and the aggressive of mast cells.

In conclusion, hirudin ameliorates the pathology in the OVA-induced mouse asthma model by its anti-inflammatory effects and alleviates the vicious airway remodeling, which is potentially amenable to therapeutic manipulation for clinical application.

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