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Butylphthalide ameliorates airway inflammation and mucus hypersecretion *via* NF-κB in a murine asthma model

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ABSTRACT

Butylphthalide (NBP) is a phthalide compound contained in *Angelicae Sinensis Radix* which is one of the most widely used traditional Chinese medicines. This study aims to explore the therapeutic effect of NBP on airway inflammation, mucus hypersecretion and their possible mechanism in asthma mice. BALB/c mice were sensitized and challenged with ovalbumin (OVA) for establishment of asthma model and then treated with NBP during day 22–77. The pulmonary function of the mice was determined, and the pathology of lung tissue and goblet cell hyperplasia were observed through analyzing inflammation scores and goblet cell percentage, respectively. Cytokine IL-4, IL-8, IL-13 and tumor necrosis factor-alpha (TNF-α) in bronchoalveolar lavage fluid (BALF) and total immunogloblin E (T-IgE) and OVA-specific IgE in serum were examined by enzyme-linked immunosorbent assay (ELISA). The expressions of Mucin 5AC (Muc5ac) and nuclear transcription factor-kappa B (NF-κB) in lung tissues were evaluated by immunohistochemistry, western blot and real-time polymerase chain reaction (RT-PCR). The results show that 50 mg/kg NBP significantly reduced OVA-induced increase in inflammation scoring, goblet cell percentage and mucus secretion of airway tissue, and improved the pulmonary function. NBP could also decrease IL-4, IL-8 IL-13, and TNF-α in BALF and T-IgE and OVA-specific IgE in serum. The expression of Muc5ac and NF-κB in lung tissue was significantly down-regulated after NBP treatment. This study suggested that NBP may effectively inhibit airway inflammation and mucus hypersecretion in asthma by modulating NF-κB activation.

1. Introduction

Bronchial asthma (asthma) is one of the most common airway inflammatory diseases worldwide [1]. Recently, the morbidity and mortality of asthma are on the rise due to the aggravation of environmental pollution and the increase of allergic population [2]. Airway inflammation is the pathological basis of asthma. The excessively secreted airway mucus that carries a large number of inflammatory substances stays in airway and provides the prerequisites for responsiveness and restructure of respiratory tract, eventually leading to a decrease in pulmonary function. It has been reported that the core mechanism of airway inflammation and mucus hypersecretion in asthma is T helper 2 (Th2) cell dominant response and abnormal activation of NF-κB signaling pathway. Th2 cells and their specific cytokines, such as IL-4 and IL-13, are widely involved in the pathological process of asthma. NF-κB enters the nucleus and is directly involved in airway inflammation and mucus hypersecretion by regulating gene transcription and protein synthesis. Through activating NF-κB, IL-13 and TNF-α promote the high expression of Muc5ac, which is the main source of airway mucous viscoelasticity [3].

At present, the common anti-asthmatic drugs, including bronchodilator, anti-inflammatory anti-asthmatic drugs and anti-allergic anti-asthmatic drugs, have not yet effectively inhibited mucus hypersecretion [4]. More importantly, under the interaction of genetic and environmental factors, asthma is a heterogeneous disease, which has different classification in phenotype and endotype [5]. And especially in the treatment of neutrophil asthma, hormone-resistant asthma and asthma airway remodeling, the commonly used antiasthmatic drugs (including glucocorticoids) are not very effective [6,7]. Therefore, it is of urgency to develop new and efficient drugs for the treatment of asthma.

Angelica sinensis (Oliv.) Diels mainly distributes in East Asia, and its

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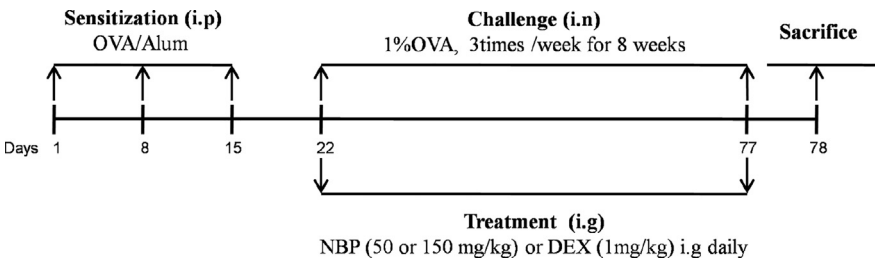

Fig. 1. Experimental protocols for the OVA-induced model and treatment with NBP (50 and 150 mg/kg) or DEX (1 mg/kg). Mice were sensitized with OVA and Alum on days 1, 8 and 15 and then challenged with 1% OVA for 30 min each time, 3 times per week for 8 weeks. The control group and the NBP group were sham-sensitized and challenged with PBS instead of OVA. Starting with the challenge, the mice in each group were given drugs at 20 ml/kg by intragastric administration once a day for eight weeks. There drugs were replaced by 0.05% Tween-80 in the control group and the OVA group.

dried root (*Angelicae Sinensis Radix*) is one of the most important Chinese medicines. As recorded in "Shen Nong Ben Cao Jing", *Angelicae Sinensis Radix* has the efficacy of treating cough and asthma. It can be not only used alone for the treatment of cough and pertussis but also compatible with other traditional Chinese medicines for the treatment of respiratory diseases such as asthma. Butylphthalide (NBP) is a phthalide extracted from *Angelicae Sinensis Radix* and can also be synthesized artificially currently. Owing to the effects on protecting nerve cells, antioxidation and improving brain circulation, NBP is clinically used for treatment of ischemic stroke [8]. Previous studies have reported that NBP can suppress the inflammatory response caused by cerebral ischemia, which is related to the inhibition of NF-κB signaling pathway [9,10]. It is also shown that NBP has antitussive, expectorant and anti-inflammatory effects in mice [11]. However, the effects of NBP on airway inflammation, goblet cell hyperplasia and mucus hypersecretion in the asthmatic process and their mechanisms are still unknown.

In this study, we measured the effect of NBP on airway inflammation and mucus hypersecretion in the mice model of asthma and investigated its possible molecular mechanism. We hypothesized that NBP might attenuate mucus hypersecretion of asthma airway by restraining goblet cell hyperplasia with inhibiting NF-κB pathway. Finally, we found that NBP could relieve airway inflammation, goblet cell hyperplasia and mucus hypersecretion, and the inhibition of NF-κB is one of their mechanisms. As a drug for the treatment of cerebral ischemia, this study might provide potential application prospects for NBP as a drug to treat asthma, especially neutrophil asthma.

2. Materials and methods

2.1. Regents

OVA (Grade V) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Aluminum hydroxide (Alum) was obtained from Thermo (Rockford, Illinois, USA). The IL-4, IL-8, IL-13, TNF-α and Muc5ac ELISA kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). The T-IgE and OVA-specific IgE ELISA kits were purchased from Cayman Chemical Co. (Ann Arbor, Michigan, USA). Rabbit monoclonal antibodies p-p65, p65, p-IκB, IκB, Muc5ac and GAPDH were purchased from GeneTex Inc. (Southern California, USA).

2.2. Animals

Female specific pathogen-free (SPF) BALB/c mice, weighting 18–20 g, were provided by the Lanzhou Veterinary Institute of Chinese Academy of Agricultural Sciences (Certificate: SCXK (Gan) 2015-0001; Gansu, China), and housed in Laboratory Animal Center (Gansu University of Chinese Medicine, China). The laboratory temperature and the relative humidity were 22 ± 2 °C and 50 ± 5%, respectively. The mice were free to feed with normal diets in simulating artificial condition of the 12 h light/dark cycle. All animal experiments were performed in accordance with experimental animal practices of Gansu University of Chinese Medicine and the whole experiment was approved by the experimental animal ethics committee of the Gansu University of Chinese Medicine.

2.3. NBP

NBP (Fig. S1, molecular formula: $C_{12}H_{14}O_2$, molecular weight: 190.24) was obtained from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). The purity of NBP was 98.8% based on HPLC analysis. NBP (15 mg/ml) was dissolved in Tween-80 solution as emulsion and freshly diluted to the indicated concentration with purified water before use. Tween-80 concentration in test conditions was < 0.05% (w/v) and all configured emulsions were stored at 2–8 °C.

2.4. Establishment of asthma model and treatment

Female BALB/c mice were copied the asthma model by ovalbumin (OVA) sensitization and challenge as described in the literatures [12,13]. The protocols for 6 groups are summarized in Fig. 1. Briefly, mice were sensitized with 200 μl sensitized solution (phosphate buffer saline (PBS) suspension containing 20 μg OVA and 2 mg aluminum hydroxide, pH = 7.3) by intraperitoneal injection on day 1, 8 and 15. From the 22nd day the mice breathed 1% OVA ultrasound atomized by the GYD-CHT systemic exposure/nasal exposure inhalation system (CH Technologies, USA), and the challenge lasted 30 min each time, 3 times per week for 8 weeks. Mice were randomly divided into 6 groups and 10 mice in each group: vehicle mice group (Control; PBS only), mice treated with NBP group (NBP; 150 mg/kg), OVA-induced mice group (OVA), OVA-induced mice treated with dexamethasone (DEX) group (OVA + DEX; 1 mg/kg; the positive control group) [14], OVA-induced mice treated with low dose NBP group (OVA + NBP50; 50 mg/kg) and OVA-induced mice treated with high dose NBP group (OVA + NBP150; 150 mg/kg) [15]. The control group and the NBP group were sensitized and challenged with PBS instead of OVA. Starting with the challenge, the mice in each group were given NBP or DEX at 20 ml/kg by intragastric administration once a day for eight weeks. There drugs were replaced by 0.05% Tween-80 in the control group and the OVA group.

2.5. Pulmonary function

Pulmonary function was measured 24 h before the first sensitization and 24 h after the last challenge, respectively. After debugging the WPB PLT - UNR - duration - 2 type animal lung function detection system (EMKA, France), a mouse was put into a closed system. Under the condition of time and event pattern, the pulmonary function parameters, including enhanced pause (Penh) and peak expiratory flow (PEF), were detected through the whole-body plethysmography after the mouse was quiet. Data were analyzed by *emka Technologies iox* software (EMKA, France).

2.6. Specimen collection

After measuring pulmonary function, mice were anesthetized by 1% pentobarbital sodium and blood was collected from posterior orbital vein. The coagulated blood was centrifuged at 3000 rpm for 10 min,

and serum was separated and stored at −70 °C. BALF was extracted after the mice were sacrificed. The upper lobe of the right lung was taken for the analysis of lung histopathology, goblet cell hyperplasia and the expression of Muc5ac and NF-κB. The remaining lung tissue was rinsed with saline and stored at −70 °C to determine the expression of proteins and genes.

2.7. Collecting and processing of BALF

The sacrificed mice were supinely fixed, and the right bronchus was ligated after trachea was exposed. Connected with 2 ml syringes, the 22G blunt needle was inserted into the trachea about 0.5 cm between the second and third tracheal cartilage rings and fixed with surgical wire. The trachea was irrigated with 0.8 ml physiological saline (2–8 °C) for 3 times, and the average recovery rate of lavage fluid was about 80%. The specific method of irrigation is: 0.8 ml PBS lavage solution was absorbed with 2 ml needle tube, while only 0.2–0.3 ml PBS was pushed forward in each lavage. After the lung lobes were gently rubbed for about 20 s, the 0.2–0.3 ml PBS was collected back. The irrigation was repeated for 5 times, and finally all the lavage fluid was tried to be absorbed. After centrifuged at 3000 rpm for 10 min, the supernatant of BALF was collected and stored at −70 °C for further determination. 100 μl cell sediment of remaining BALF was assayed for inflammatory cells immediately after shaking in red blood cell lysis buffer, and another 50 μl cell sediment was smeared, dried and stained for counting the total number of inflammatory cells and the number of eosinophils, neutrophils, macrophages and lymphocytes under the microscope (Olympus, Japan).

2.8. Determination of cytokines, Muc5ac and IgE

According to the manufacturer's instructions of the ELISA kits, the supernatant of BALF was added to the corresponding reaction wells and incubated with biotinylated antibodies. After washing, horseradish peroxidase labeled avidin was added into reaction wells. Through cleaning and coloring, the optical density (OD) value of each well was measured at 450 nm. Finally, the levels of IL-4, IL-8, IL-13, TNF-α and Muc5ac in the BALF were determined. The total IgE (T-IgE) and OVA-specific IgE in serum were determined with ELISA as well.

2.9. Morphometric analysis of lung tissue

The upper lobe of the right lung was fixed with 10% paraformaldehyde, embedded in paraffin, and cut into pieces about 4–5 μm thickness. The pieces were stained with hematoxylin and eosin (H&E) to observe pulmonary histopathology. Double-blind method was used to analyze the degree of inflammatory cell infiltration, bronchiolic epithelial injury, and edema in the bronchiole, vascular and surrounding tissues. The scoring criteria are listed as follows: 0 points = bronchiolic and vascular tissue without obvious staining, 1 point = bronchiolic or vascular tissue was stained slightly, 2 points = bronchiolic or vascular tissue was stained obviously, 3 points = bronchiolic and vascular tissue were stained obviously, and 4 points = entirely bronchiolic and vascular tissue were stained deeply [16]. Meanwhile, after finding ten complete bronchioles with the internal diameter range from 100 to 150 μm, the basement membrane perimeter (Pbm), the area of airway wall (WAt) and smooth muscle (WAm) were determined by morphologic analysis system (Taimeng Tech., Chengdu, China). Then WAt/Pbm and WAm/Pbm were used to estimate airway remodeling [17].

The goblet cell in right upper lung was observed using periodic acid Schiff (PAS) staining. Ten bronchioles with an internal diameter of 100–150 μm in each slide were found and PAS-positive cells (goblet cells) numbers and epithelial cells numbers on them were counted under the optical microscope (Olympus, Japan). The results are expressed as goblet cell percentage, which is calculated from the number of goblet cells each bronchus divided by the total number of epithelial cells per bronchus.

2.10. Immunohistochemical analysis

The pieces of the right upper lung were dewaxed, hydrated and exposed to H_2O_2 to block endogenous peroxidase. After repairing antigen by citric acid microwave repair liquid, primary antibody against Muc5ac or NF-κB p65 was added. After incubation overnight, the slides were incubated with corresponding horseradish peroxidase-labeled secondary antibody, stained with DAB and mild restained by hematoxylin for observing the expression characteristics of Muc5ac and NF-κB p65. The average OD value, measured by BI-2000 medical image analysis system (Taimeng Tech., Chengdu, China), was used to estimate the expressions of Muc5ac and NF-κB p65.

2.11. Analysis of Muc5ac and NF-κB gene expression

In order to analyze the gene expression of Muc5ac and NF-κB, the primer sequences of Muc5ac and NF-κB were synthesized by TaKaRa (Japan) (table S1). Using the Trizol reagent kit and following its instruction, the total RNA was extracted from lung tissue of mice. After determination of the concentration and purity, the RNA was translated into cDNA utilizing GoScript™ Reverse Transcription System Reagent Kit (Promega Corporation, USA). Ten microlitre reaction system was established on QuantStudio5 fluorescent quantitative RT-PCR instrument (Bio-Rad, USA) and β-Actin gene was used as the internal reference gene. RT-PCR conditions were as follows: 1 cycle of pre-denaturation at 95 °C for 2 min followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min and elongation at 72 °C for 20 s. Detecting the fluorescence intensity of target gene (Muc5ac and NF-κB) and internal reference gene (β-Actin) expression, the relative expression of target genes was calculated by the way of $2^{-\Delta\Delta Ct}$ method.

2.12. Western blot

The lung tissues of each group and lysate were placed in labeled EP tubes to crack tissue. After centrifuging tissue lysate (13,000 rpm, 15 min), the protein concentrations of supernatants were detected with BCA protein quantitative method. These protein samples, which were added to the protein loading buffer, were separated by 10% SDS-polyacrylamide gel (concentrated gel: 60 V, 30 min; separating gel: 120 V, 1 h), and transferred to the polyvinylidene fluoride (PVDF) membranes under the constant pressure of 120 V for 90 min. After blocking with 5% non-fat dry milk for 2 h at 37 °C, these membranes were exposed to monoclonal antibody against p-p65, p65, p-IκB, IκB or Muc5ac (1:500) overnight at 4 °C. The PVDF membranes were washed three times with TBST and then incubated with horseradish peroxidase-labeled secondary anti-IgG (1:5000) for 2 h at 37 °C. Appropriate amount of ECL luminescence reagent was used to show the protein bands which were analyzed with Chemi DOC XRS + gel imaging analysis system (Bio Rad Laboratories, USA). The gray values of the target bands and the internal reference bands were measured by Image Lab Version 4.0 software (Bio Rad Laboratories, USA). The relative expressions of p-p65, p65, p-IκB, IκB and Muc5ac in lung tissues were analyzed by the ratio of the gray value of target protein to that of GAPDH.

2.13. Statistical analysis

The experimental data was expressed as mean ± standard deviation (SD). The differences between groups were analyzed by one-way analysis of variance (ANOVA) and Dunn's post-test for post-hoc t testing was used. Statistical data were analyzed with SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA) and a P-value of < 0.05 was considered to be statistically significant in all cases. After converting the data into histogram by OriginPro (version 8.0 for Windows,

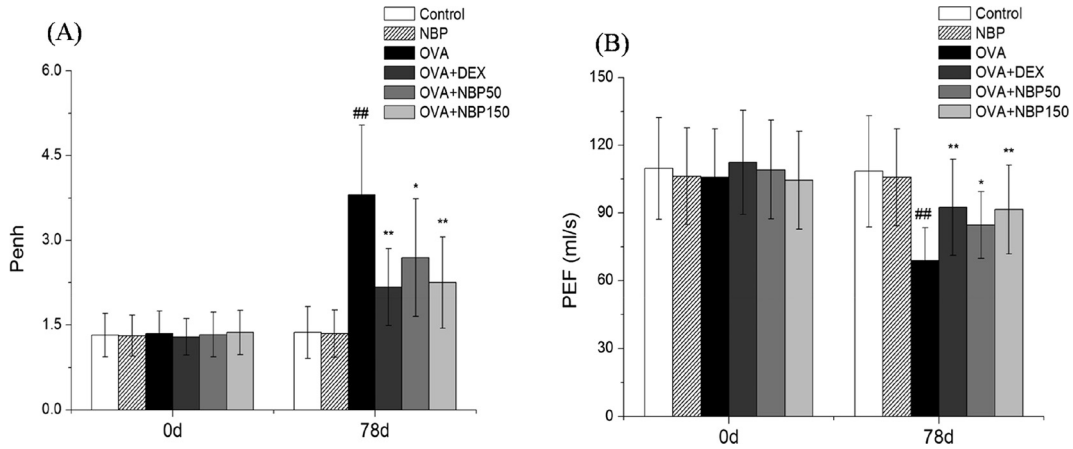

Fig. 2. Effect of NBP on the pulmonary function in OVA-induced mice. (A) Penh (B) PEF were detected by the animal lung function detection system 24 h before the first sensitization and 24 h after the last challenge. The values are shown as the mean ± SD ($n = 10$). $^{\#\#}P < 0.01$ *vs* control; $^{*}P < 0.05$ *vs* OVA; $^{**}P < 0.01$ *vs* OVA.

OriginLab Corporation, Northampton, Massachusetts, USA), the histogram and the picture are joined together to form a complete graph and exported in the image file.

3. Results

3.1. NBP ameliorated pulmonary function of the OVA-mediated mice

All the mice survived during the establishment of asthma model and treatment. During OVA challenge, upper respiratory symptoms, such as runny nose and scratching nose, gradually appeared in the OVA group. Under the intervention of drugs, the above respiratory symptoms were alleviated to varying degrees. The decline of pulmonary function is one of the main characteristics of asthma [18]. As shown in Fig. 2, before the experiment (Day 0), there was no obvious difference in the pulmonary function among groups. After 77 days of OVA replicating asthma model, the Penh increased, while the PEF decreased compared with those of the control group (Fig. 2A–B; $n = 10$; $P < 0.01$). Under the treatment with NBP or DEX on asthma mice, the above abnormal changes were significantly alleviated ($P < 0.05$).

3.2. NBP inhibited OVA-mediated inflammation and inflammatory cells infiltration in lungs

Asthma is an inflammatory disease of the airways, and the inflammatory cell infiltration and inflammatory reaction in the lung tissue can directly reflect the state of an illness [19]. According to the H &E staining (Fig. 3A), there was rare inflammation in the lung tissues of the control group. However, a large number of inflammatory cells could be observed in the lung tissues of the OVA group, besides swelling and shedding of bronchiolic epithelial cells, thickening of basilar membrane, and blur of bronchiolic wall structure. The above pathological changes were visibly ameliorated after the treatment of NBP or DEX, and the semi-quantitative results indicated that NBP and DEX could significantly reduce the inflammation scores, WAt/Pbm and WAm/Pbm (Fig. 3 B-C; $n = 10$; $P < 0.05$). Correspondingly, the number of total inflammatory cells, macrophages, eosinophils, lymphocytes and neutrophils in BALF of different dosage groups of NBP and DEX was significantly decreased compared with those of the OVA group (Fig. 3D; $P < 0.01$). Meanwhile, DEX showed stronger inhibitory effect on the total inflammatory cells, macrophages, eosinophils and lymphocytes than NBP, but NBP demonstrated significantly stronger inhibition on neutrophils ($P < 0.05$).

3.3. NBP reduced the production of cytokines in BALF and IgE in serum

Cellular immunity involved by cytokines and humoral immunity involved by antibodies directly participate in the airway inflammatory response of asthma [20]. After OVA sensitization and challenge, the levels of IL-4, IL-8, IL-13 and TNF-α in BALF of the OVA group increased significantly compared with those in the control group (Fig. 4A–D; $n = 10$; $P < 0.01$). After intragastric administration of different dosages of NBP or DEX, the secreted levels of IL-4, IL-8, IL-13 and TNF-α in BALF were dramatically declined ($P < 0.01$). Meantime, T-IgE and OVA-specific IgE in serum were significantly decreased in the different dosage groups of NBP (Fig. 4E–F; $n = 10$; $P < 0.01$). Although DEX had stronger inhibitory effect on IL-13, TNF-α, T-IgE and OVA-specific IgE than low dose NBP, NBP showed stronger inhibitory effect on IL-8 (Fig. 4B–F; $n = 10$; $P < 0.05$).

3.4. NBP restrained goblet cell hyperplasia in airway epithelium

Goblet cell hyperplasia is one of the manifestations of bronchiolic remodeling, induced by which mucus hypersecretion has become a pathological feature of asthma [17]. The PAS staining showed that the acidic mucin was presented indigo granules, and mainly distributed in the goblet cells and the inner wall of the airway. The color of the OVA group was obviously deeper than the control group, and the color of drug therapy groups was shallower to different degrees than that of the OVA group (Fig. 5A). The percentage of goblet cells in bronchiolic epithelium significantly increased in the OVA group, while this percentage fell obviously after treating with different dosage of NBP (Fig. 5B; $n = 10$; $P < 0.01$).

3.5. NBP inhibited Muc5ac high expression in lung tissue induced by OVA

Muc5ac secreted by goblet cells is the main mucin in the airway mucus and could be stained with gray-black clumps through immunohistochemical method. The secretion of Muc5ac in the OVA group was extended to the airway and markedly enhanced compared with that in the control group (Figs. 6A & S2A). However, these trends were reversed in the NBP low and high dose groups, and the average OD value, Muc5ac in BALF and the density of Muc5ac were obviously decreased (Figs. 6B–C & S2B; $n = 10$; $P < 0.01$). The data analysis of Muc5ac gene expression also confirmed the above statement (Fig. 6D; $n = 10$; $P < 0.01$).

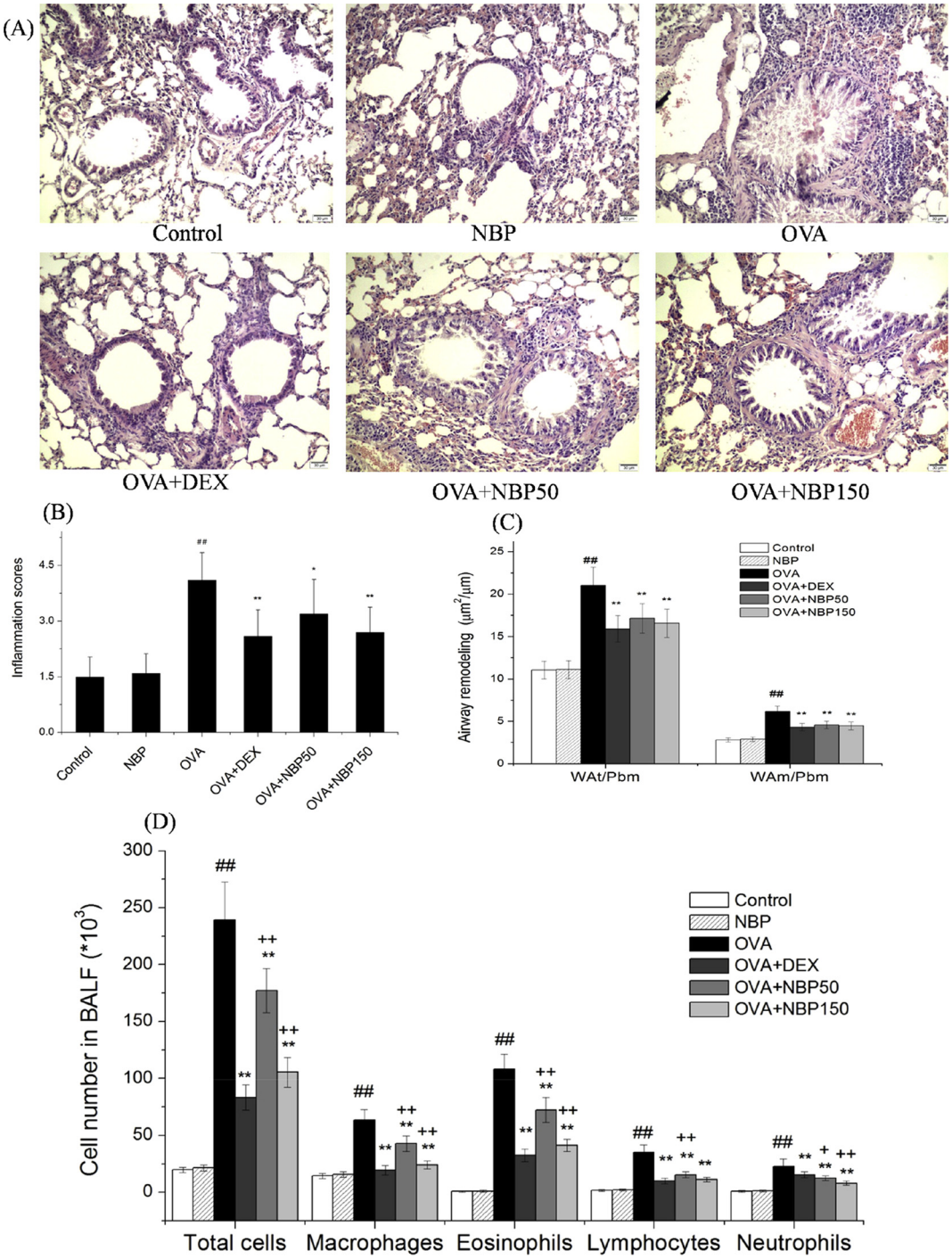

Fig. 3. Effects of NBP on inflammation in lung tissue, airway remodeling and inflammatory cells in BALF of OVA-induced mice. (A) Representative pictures of HE-stained lung tissue of six groups (scale bar = 30 μm). (B) Inflammation scores in lung sections. (C) Airway remodeling. (D) Number of inflammatory cells in BALF. The values are shown as the mean ± SD ($n = 10$). $^{\#\#}P < 0.01$ *vs* control; $^{*}P < 0.05$ *vs* OVA; $^{**}P < 0.01$ *vs* OVA; $^{+}P < 0.05$ *vs* OVA + DXM, $^{++}P < 0.01$ *vs* OVA + DXM.

3.6. NBP inhibited NF-κB and its mRNA expression in lung tissue induced by OVA

NF-κB is widely involved in the regulation of airway inflammation and mucus hypersecretion in asthma [21]. The results of western blot and immunohistochemical staining indicated that the expressions of p-p65, p65, p-IκB and IκB augmented significantly in the OVA group compared with the control group (Fig. 7A, C–F & S3A–B; $n = 10$; $P < 0.01$). After treatment with different doses of NBP, those protein expressions in the lung tissues were significantly decreased ($P < 0.01$), and these results were consistent with the NF-κB mRNA expression (Fig. 7B; $P < 0.01$). DEX had a stronger inhibitory effect on the expression of NF-κB and its gene than low dose NBP, but high dose NBP has a stronger inhibitory effect on the expression of p-p65 and p-IκB than DEX (Fig. 7A–F; $P < 0.01$), and these results suggested that NBP could inhibit the activation of NF-κB in a dose-dependent manner.

4. Discussion

NBP has been reported to relieve tracheal smooth muscle

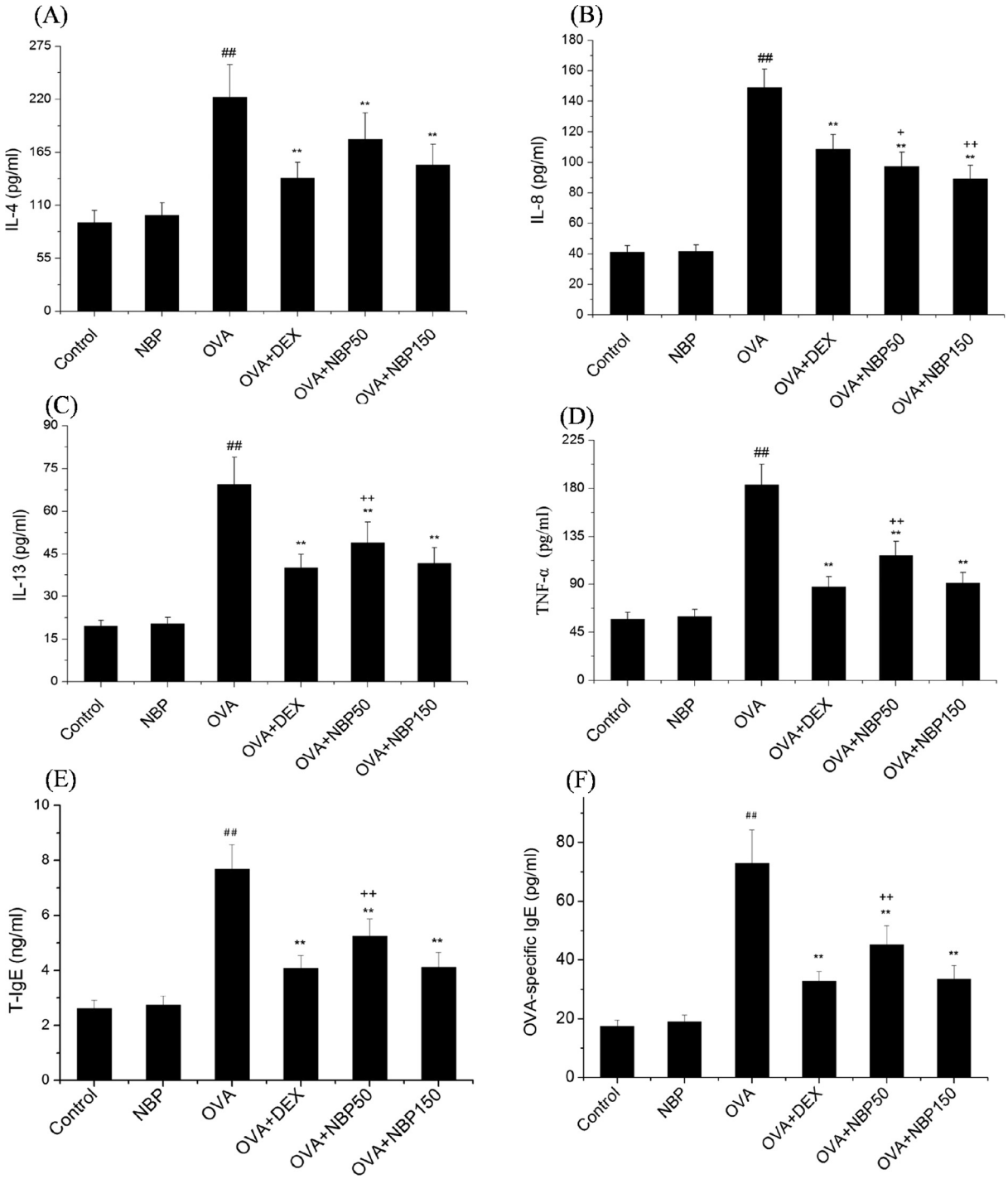

Fig. 4. Effects of NBP on IL-4, IL-8, IL-13 and TNF-α in BALF and T-IgE and OVA-specific IgE in serum. (A) IL-4, (B) IL-8, (C) IL-13 and (D) TNF-α in BALF and (E) T-IgE and (F) OVA-specific IgE in serum were measured by ELISA. The values are shown as the mean ± SD ($n = 10$). $^{\#\#}P < 0.01$ *vs* control; $^{**}P < 0.01$ *vs* OVA; $^{+}P < 0.05$ *vs* OVA + DXM, $^{++}P < 0.01$ *vs* OVA + DXM.

contraction and improve asthmatic symptoms in guinea pig [15]. In the present study, airway inflammatory response, goblet cell hyperplasia and mucus hypersecretion appeared in the asthmatic mice after sensitization and challenging with OVA. Meanwhile, the mice exhibited the asthmatic features, such as runny nose and scratching nose, as well as the decline of pulmonary function. Notably, the NBP-treated mice displayed significant improvement on the above asthmatic parameters. Moreover, NBP could inhibit the expression of NF-κB and decrease Th2 immune-response advantage in lung tissue.

Asthma is an airway inflammatory disease, and the most basic pathological change of asthma is airway inflammation which causes mucus hypersecretion and airway remodeling. The airway inflammation caused by asthma induces infiltration of a large number of inflammatory cells and the subsequent release of inflammatory substances, which give rise to airway hyperemia, mucus hypersecretion, epithelial cells swollen and shedding. A large number of respiratory mucus and exfoliated tissues can cause airway stenosis [22]. Airway inflammation causes hyperblastosis and eventually leads to a decline in pulmonary function, and the noninvasive pulmonary function index in waking state can reflect the pulmonary function objectively [23,24]. In

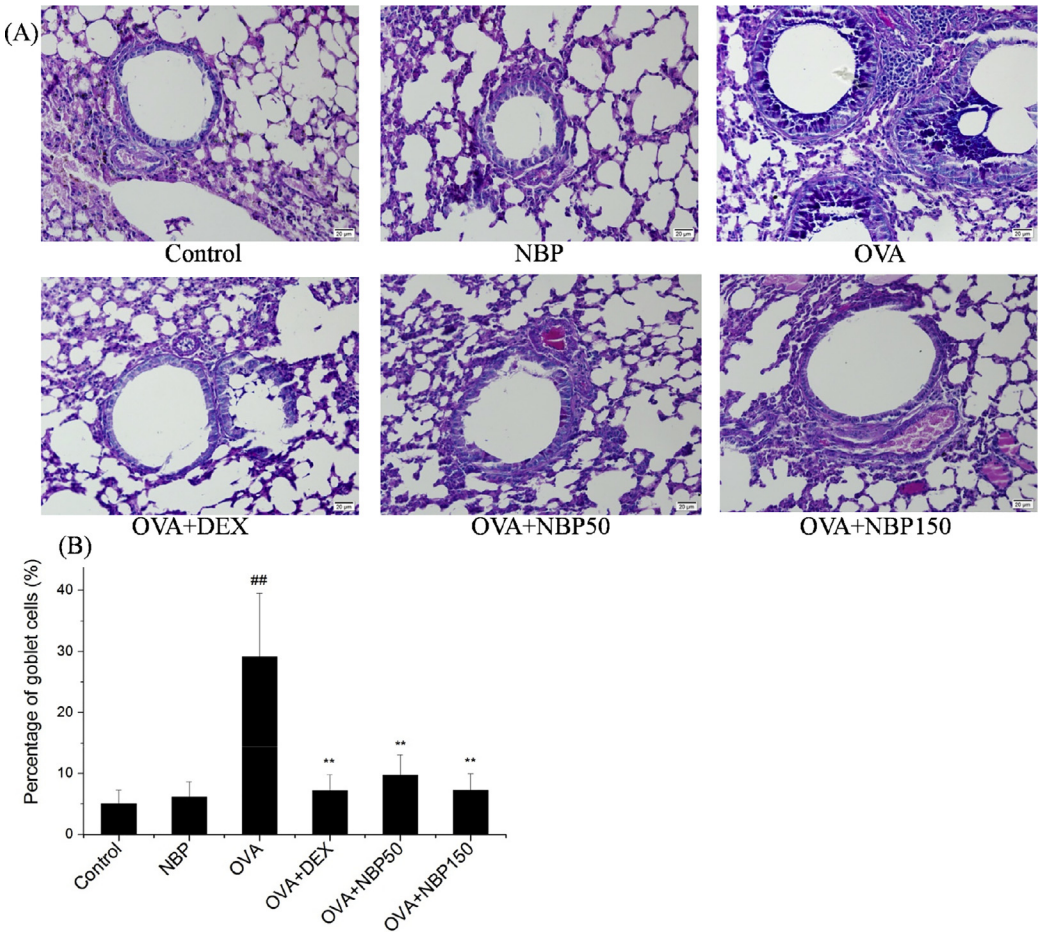

Fig. 5. Effect of NBP on goblet cell in lung tissue by periodic acid-Schiff staining (PAS staining). (A) Representative pictures of PAS-stained lung tissue of six groups (scale bar = 20 μm). (B) The percentage of goblet cells in bronchiolic epithelium. The values are shown as the mean ± SD (n = 10). $^{\#\#}P < 0.01$ *vs* control; $^{**}P < 0.01$ *vs* OVA.

our research, asthmatic mice gradually developed respiratory symptoms, such as runny nose and grasping nose. At the stage of challenge, some of the mice had asthmatic symptoms — irritability, dyspnea and abdominal breathing, for example. On the basis of alleviating respiratory symptoms, NBP could increase pulmonary function and relieve asthma symptoms. The detection of pulmonary histopathology and BALF showed that NBP could improve the pulmonary histopathology of asthma, relieve airway remodeling, inhibit inflammatory cell infiltration in lung tissue and reduce the total number of inflammatory cells and their classification in BALF. And there was a good correlation between inflammation scores in lung sections and Penh in lung function (r = 0.8908).

Asthmatic pathogenesis is quite complex and involves various types of immune cells and cytokines, and it is generally believed that Th2 cell plays a key role in regulating immune function and inflammatory response level through the release of its characteristic cytokines, such as IL-4 and IL-13. Airway inflammation in asthma is mainly caused by eosinophils, and IL-4 can promote eosinophils production and enlist eosinophil to airway epithelium [25]. IL-13 is widely involved in multiple links in asthma, including eosinophil recruitment, goblet cell hyperplasia, mucus hypersecretion and decline of pulmonary function [26,27], and therefore IL-13 determines the severity of life-threatening paroxysmal asthma [28]. On the other hand, neutrophils are widely involved in airway inflammation in non-eosinophilic asthma. Under the chemotaxis of IL-8, neutrophils aggregate into airway tissues and are activated to participate in the immune inflammatory response of asthma. Research finds that abnormal elevation of neutrophils is an important inflammatory phenotype associated with asthma severity, steroid resistance and airway remodeling [29]. According to the current research, NBP could restrain exorbitant release of IL-4 and IL-13 into the airway. These findings are accordant with the result gained from the pathological analysis of lung tissue and inflammatory cells in BALF and suggested that NBP could prevent airway inflammation by reducing the secretion of Th2-related cytokines. Meanwhile, the effect of NBP on IL-8 in BALF of asthmatic mice is more obvious than that of glucocorticoid, which is consistent with the effect of NBP on neutrophils in BALF, suggesting that NBP is more effective for non-eosinophilic asthma with mainly neutrophils.

It has been reported that nuclear transcription factor kappa B (NF-κB) plays an important role in the asthmatic pathogenesis by regulating the expression of inflammatory genes and the synthesis of active substances [30]. For example, under the stimulation of TNF-α, the activated NF-κB enters Th2 nucleus or monocytes/macrophages nucleus, combines with the recognition site of specific κB in the promoter or the enhanced sequence of the gene and promotes gene transcription and protein expression such as IL-4 IL-13 or IL-8 [31,32]. Murine experiment shows that TNF-α is widely involved in airway inflammation in asthma. And previous studies also showed that NBP could decrease NF-κB expression in astrocytes. In order to further investigate the involvement of NF-κB in the anti-asthmatic mechanism of NBP, the expression of NF-κB in lung tissue was measured by immunohistochemistry, western blot and RT-PCR. The results indicated that the abnormal activation of NF-κB in lung tissue was significantly inhibited by NBP, and there was a good correlation between TNF-α and NF-κB (r = 0.8954). These results suggested that NBP had a therapeutic effect on OVA-induced asthma through inhibiting NF-κB signal pathway.

Mucus on the surface of the airway is a liquid gel that protects the airway epithelium from damage by humidifying, heating and cleaning air [33]. However, mucus hypersecretion causes the accumulation of

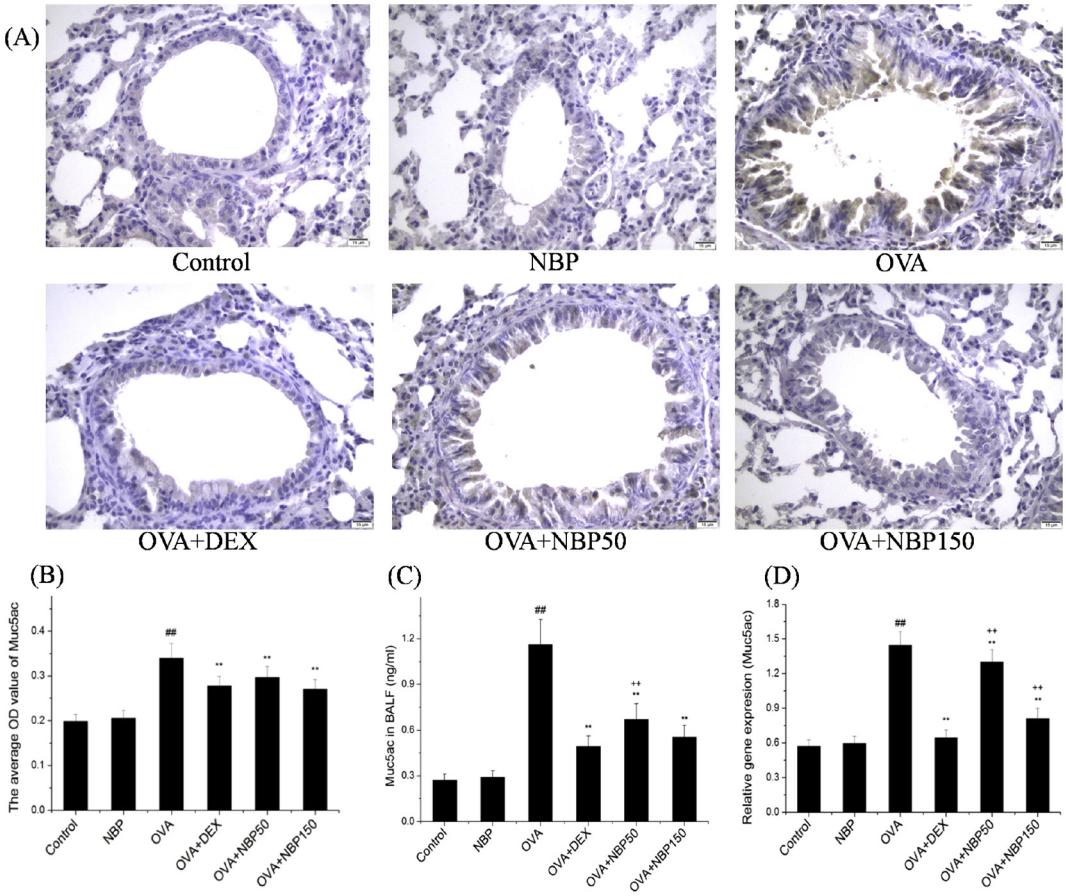

Fig. 6. Effect of NBP on Muc5ac in OVA-induced mice. (A) Representative pictures of lung tissue slides (scale bar = 15 μm) of six groups. (B) The average OD value of Muc5ac in lung tissue slides. (C) The concentrations of Muc5ac in BALF. (D) Muc5ac gene expression in lung tissues. The values are shown as the mean ± SD (n = 10). $^{\#\#}P < 0.01$ *vs* control; $^{**}P < 0.01$ *vs* OVA; $^{++}P < 0.01$ *vs* OVA + DXM.

mucus or even the obstruction of the airways, thereby leading to a decline in pulmonary function and a significant increase in morbidity and mortality of asthmatic patients [34]. The viscoelasticity of airway mucus is mainly derived from mucin, especially the Muc5ac, which is mainly secreted by goblet cells [35]. Hence, goblet cell hyperplasia is the pathological basis of mucus hypersecretion and may contribute to asthma deaths [36]. Our results also showed that the goblet cells in the asthmatic airway increased significantly, and synthesis of Muc5ac changed correspondingly.

Research has shown that mucin hypersecretion is closely related to IL-13 and TNF-α. Blockading IL-13 will inhibit mucin expression, and CD4 T cells and IL-9 can stimulate mucus production only through IL-13 [37]. Under the stimulation of IL-13, Th2 and neutrophils infiltrate into the airway epithelium and then release TNF-α which regulates mucus hypersecretion by NF-κB [38,39]. Through activating IκB kinase, TNF-α promotes nuclear translocation of NF-κB, which promotes mucin synthesis [40,41]. The animal model of asthma confirms that NF-κB plays a key role in the synthesis of mucin in goblet cells [42]. Our results also showed that there was a good correlation between MUC5AC level in BALF and NF-κB level in lung tissue (r = 0.8979). And NBP could significantly decrease OVA-induced goblet cell hyperplasia and mucus hypersecretion and one of mechanisms is to inhibit NF-κB through depressing TNF-α and IL-13.

Drugs treating mucus hypersecretion in asthma include glucocorticoid, *N*-acetylcysteine, ambroxol and hypertonic saline. Owing to more clinical contraindications, treatment of asthma, especially airway mucus hypersecretion, requires more drugs for choice [43]. NBP is a drug developed for clinical treatment of ischemic stroke in 2005, and has fewer adverse reactions at the usual dose (0.8 g) of oral treatment for ischemic stroke [44]. In this study, there was no difference between the control group and the mice treated with NBP alone (the NBP group), though NBP had obvious effects on asthmatic mice. Meanwhile, although the adverse reaction of high dose NBP was not observed, low dose NBP (minimum effective dose) has already demonstrated obvious therapeutic effects and hence was considered as the therapeutic dose in this work to minimize the drug administration. According to the results of the low dose NBP (50 mg/kg) in treating asthma mice, it should be safer under the clinical treatment of asthma with NBP calculated by body surface area method, because the common clinical dosage (0.4 g) for treating asthma is only 50% for treating ischemic stroke [45]. However, this experiment only studied the therapeutic effect of NBP on asthmatic mice. It needs further study whether NBP has the above effect on other animals, especially on asthmatic patients.

In conclusion, the data from our study demonstrated that NBP played a crucial role in modulating the inhibition of airway inflammation, goblet cell hyperplasia and mucus hypersecretion in the presence of OVA through NF-κB signaling pathway that prevented Th2-dominated immune response. In particular, NBP has a better inhibitory effect on neutrophils and IL-8 than glucocorticoid. In addition, further research is currently in progress to confirm the precisely protective mechanisms of NBP on OVA-induced asthma. Therefore, NBP may be a promising therapeutic agent provided for asthmatic patients, especially for non-eosinophilic asthma with mainly neutrophils.

Declaration of competing interest

The authors declare that no financial or commercial conflict of interest exists in relation to this article.

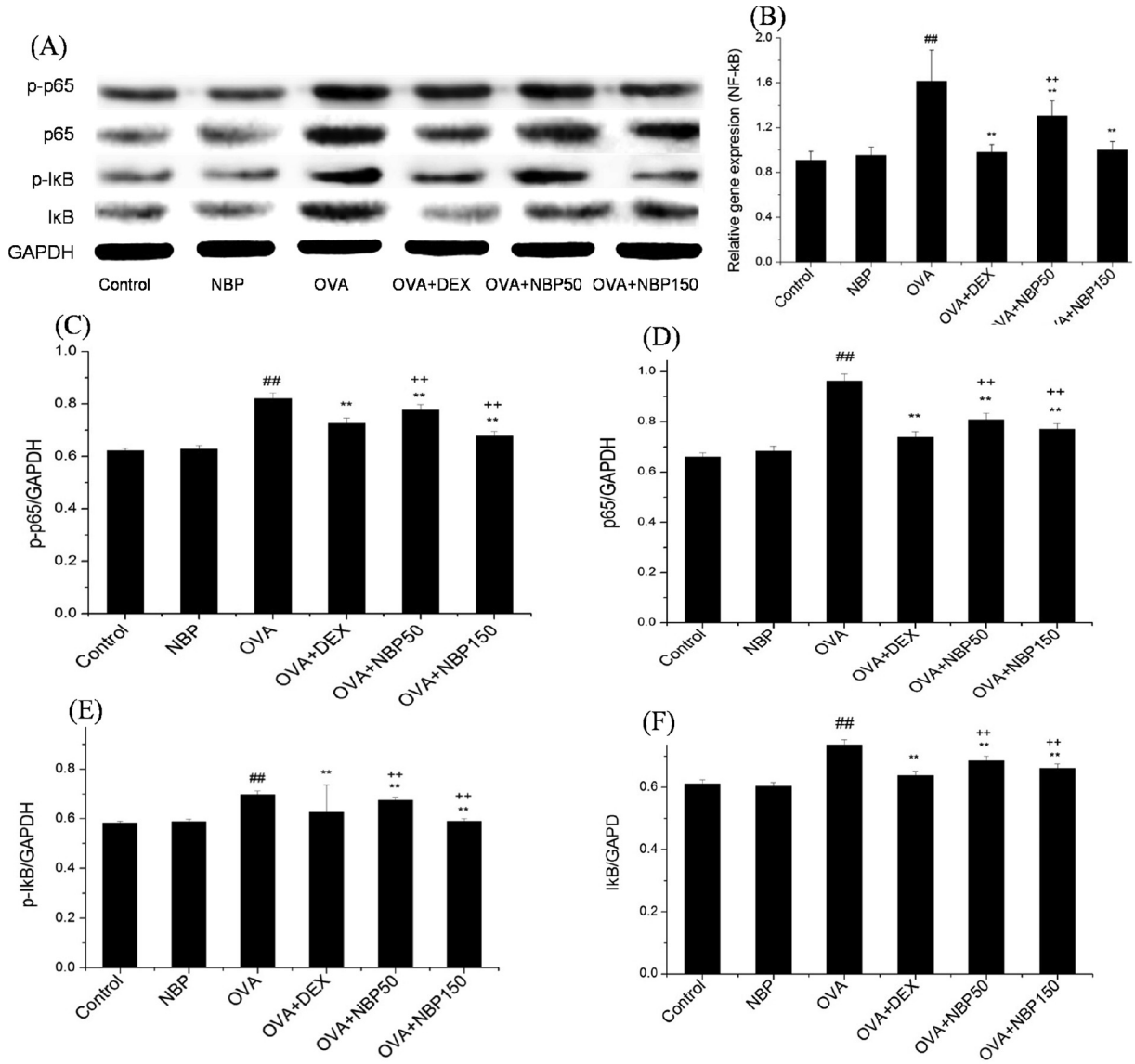

Fig. 7. Effect of NBP on NF-κB protein and gene expressions in lung tissue by western blot and RT-PCR. (A) Representative western blot pictures of p-p65, p65, p-IκB and IκB. (B) Gene expression levels of NF-κB in lung tissues were determined by RT-PCR. Protein expressions of (C) p-p65, (D) p65, (E) p-IκB and (F) IκB were quantified based on the density of the bands. GAPDH was used as an internal control. The values are shown as the mean ± SD ($n = 10$). $^{\#\#}P < 0.01$ *vs* control; $^{**}P < 0.01$ *vs* OVA; $^{++}P < 0.01$ *vs* OVA + DXM.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.105873.

References

[1] S.S. Braman, The gloal burden of asthma, Chest. 130 (2006) 4S–12S, https://doi.org/10.1378/chest.130.1-suppl.4S.

[2] G. Verlato, A. Corsico, S. Villani, I. Cerveri, E. Migliore, S. Accordini, A. Carolei, P. Piccioni, M. Bugiani, V. Lo Cascio, A. Marinoni, A. Poli, R. de Marco, Is the prevalence of adult asthma and allergic rhinitis still increasing? Results of an Italian study, J. Allergy Clin. Immunol. 111 (2003) 1232–1238, https://doi.org/10.1067/mai.2003.1484.

[3] U.B. Charith, Wijerathnea, C.S. Seo, J.W. Songa, H.S. Parka, O.S. Moonc, Y.S. Wonc, H.J. Kwona, H.Y. Sona, Isoimperatorin attenuates airway inflammation and mucus hypersecretion in an ovalbumin-induced murine model of asthma, Int. Immunopharmacol. 49 (2017) 67–76, https://doi.org/10.1016/j.intimp.2017.05.012.

[4] H. Xie, H. Zhang, K. Cao, P. He, H. Dai, S. He, Analysis of anti-asthmatic drug patents published in China between 2004 and 2013, Expert Opin Ther Pat 26 (2016) 363–376, https://doi.org/10.1517/13543776.2016.1136289.

[5] H. Piyadasa, A. Altieri, S. Basu, J. Schwartz, A.J. Halayko, N. Mookherjee, Biosignature for airway inflammation in a house dust mite-challenged murine model of allergic asthma, Biol. Open. 5 (2016) 112–121, https://doi.org/10.1242/bio.014464.

[6] Y. Le, F. Shi, Progress in targeted therapy of different asthma phenotypes, J. Biol.. 35 (2018) 81–84, http://doi.org/2095-1736(2018)02-0081-03.

[7] P.G. Woodruff, B. Modrek, D.F. Choy, G. Jia, A.R. Abbas, A. Ellwanger, L.L. Koth, J.R. Arron, J.V. Fahy, T-helper type 2-driven inflammation defines major sub-phenotypes of asthma, Am. J. Respir. Crit. Care Med. 180 (2009) 388–395, https://doi.org/10.1164/rccm.200903-0392OC.

[8] X.L. Wang, Z.Y. Wang, J. Yin, Y.H. Zhang, Advances in research on 3-n-

butylphthalide derivatives as natural anti-ischemic stroke drug, Progress in Pharmaceutical Sciences. 40 (2016) 89–95, doi:1001-5094(2016)02-0089-07.
[9] H.L. Xu, Y.P. Feng, Inhibitory effects of chiral 3-n-butylphthalide on inflammation following focal ischemic brain injury in rats, Acta Pharmacol. Sin. 21 (2000) 433–438, https://doi.org/10.1021/ar980063a.
[10] H.M. Wang, T. Zhang, J.K. Huang, X.J. Sun, 3-N-butylphthalide (NBP) attenuates the amyloid-beta-induced inflammatory responses in cultured astrocytes via the nuclear factor-kappaB signaling pathway, Cell. Physiol. Biochem. 32 (2013) 235–242, https://doi.org/10.1159/000350139.
[11] Z.W. Wang, X.Y. Fu, Y. Ren, Y.H. Wang, X.Y. Lin, R.Q. Wang, Antitussive, expectorant and anti-inflammatory effects of butylphthalide in mice, Chinese Journal of New Drugs. 26 (2017) 675–679, doi:1003-3734(2017)06-0675-05.
[12] Y.H. Kim, Y.J. Choi, E.J. Lee, M.K. Kang, S.H. Park, D.Y. Kim, H. Oh, S.J. Park, Y.H. Kang, Novel glutathione-containing dry-yeast extracts inhibit eosinophilia and mucus overproduction in a murine model of asthma, Nutr. Res. Pract. 11 (2017) 461–469, https://doi.org/10.4162/nrp.2017.11.6.461.
[13] Q.L. Yu, Z. Chen, Establishment of different experimental asthm a models in mice, Exp. Ther. Med. 15 (2018) 2492–2498, https://doi.org/10.3892/etm.2018.5721.
[14] Y. Wei, Q.L. Luo, J. Sun, M.X. Chen, F. Liu, J.C. Dong, Bu-Shen-Yi-Qi formulae suppress chronic airway inflammation and regulate Th17/Treg imbalance in the murine ovalbumin asthma model, J. Ethnopharmacol. 164 (2015) 368–377, https://doi.org/10.1016/j.jep.2015.01.016.
[15] Z.W. Wang, Y.H. Wang, Y. Ren, R.Q. Wang, X.Y. Lin, H.J. Duan, Preliminary pharmacodynamics study on antiasthmatic action of butylphthalide in guinea pig, Chinese. Journal. Of. Applied. Physiology. 33 (2017) 36–40, https://doi.org/10.12047/j.cjap.5463.2017.035.
[16] S. Underwood, M. Foster, D. Raeburn, S. Bottoms, J.A. Karlsson, Time-course of antigen-inducedair way inflammation in the guinea-pig and its relationship to airway hyperresponsiveness, Eur. Respir. J. 8 (1995) 2104–2113, https://doi.org/10.1183/109031936.95.08122104.
[17] C. Ming, Z.Q. Lv, W. Zang, L.J. Huang, X.L. Lin, J.T. Shi, Triptolide suppresses airway goblet cell hyperplasia and Muc5acexpression via NF-κB in a murine model of asthma, Mol. Immunol. 64 (2015) 99–105, https://doi.org/10.1016/j.molimm.2014.11.001.
[18] J. Liu, Y. Wei, Q. Luo, F. Xu, Z. Zhao, H. Zhang, L. Lu, J. Sun, F. Liu, X. Du, M. Li, K. Wei, J. Dong, Baicalin attenuates inflammation in mice with OVA-induced asthma by inhibiting NF-kappaB and suppressing CCR7/CCL19/CCL21, Int. J. Mol. Med. 38 (2016) 1541–1548, https://doi.org/10.3892/ijmm.2016.2743.
[19] U. Fujii, N. Miyahara, A. Taniguchi, N. Oda, D. Morichika, E. Murakami, H. Nakayama, K. Waseda, M. Kataoka, H. Kakuta, M. Tanimoto, A. Kanehiro, Effect of a retinoid X receptor partial agonist on airway inflammation and hyperresponsiveness a murine model of asthma, Respir. Res. 18 (2017) 1–10, https://doi.org/10.1186/s12931-017-0507-z.
[20] N.R. Shina, H.J. Kwonb, J.W. Koa, J.S. Kimc, I.C. Leeb, J.C. Kima, S.H. Kimd, I.S. Shina, S-allyl cysteine reduces eosinophilic airway inflammation and mucus overproduction on ovalbumin-induced allergic asthma model, Int. Immunopharmacol. 68 (2019) 124–130, https://doi.org/10.1016/j.intimp.2019.01.001.
[21] G. Yi, Z.Y. Liu, X.M. Fang, C.Y. Lin, J.Y. Pan, L. Shen, J.T. Chen, L.C. Chen, J.F. Liu, Abietic acid attenuates allergic airway inflammation in a mouse allergic asthma model, Int. Immunopharmacol. 38 (2016) 261–266, https://doi.org/10.1016/j.intimp.2016.05.029.
[22] J.J. Lee, D. Dimina, M.P. Macias, S.I. Ochkur, M.P. McGarry, K.R. O'Neill, C. Protheroe, R. Pero, T. Nguyen, S.A. Cormier, E. Lenkiewicz, D. Colbert, L. Rinaldi, S.J. Ackerman, C.G. Irvin, N.A. Lee, Defining a link with asthma in mice congenitally deficient in eosinophils, Science. 305 (2004) 1773–1776, https://doi.org/10.1126/science.1099472.
[23] S.L. Coleman, O.M. Shaw, Progress in the understanding of the pathology of allergic asthma and the potential of fruit proanthocyanidins as modulators of airway inflammation, Food Funct. 8 (2017) 4315–4324, https://doi.org/10.1039/c7fo00789b.
[24] H.L. Li, Q.B. Luo, J.J. He, L.P. Yang, Z.W. Yang, Effects of aloperine on pulmonary function, NF-Kb, TNF-α and IL-1β in experimental asthmatic mice, Pharmacology and Clinics of Chinese Materia Medica 32 (2016) 69–72, https://doi.org/10.13412/j.cnki.zyyl.2016.03.018.
[25] L. Chen, K.A. Grabowski, J.P. Xin, J. Coleman, Z. Huang, B. Espiritu, S. Alkan, H.B. Xie, Y. Zhu, F.A. White, J.J. Clancy, H. Huang, IL-4 induces differentiation and expansion of Th2 cytokine-producing eosinophils, J. Immunol. 172 (2004) 2059–2066, https://doi.org/10.1080/00397919508011401.
[26] H. Tanaka, M. Komai, K. Nagao, M. Ishizaki, D. Kajiwara, K. Takatsu, G. Delespesse, H. Nagai, Role of interleukin-5 and eosinophils in allergen-induced airway remodeling in mice, Am. J. Respir. Cell Mol. Biol. 31 (2004) 62–68, https://doi.org/10.1165/rcmb.2003-0305OC.
[27] A. Kibe, H. Inoue, S. Fukuyama, K. Machida, K. Matsumoto, H. Koto, T. Ikegami, H. Aizawa, N. Hara, Differential regulation by glucocorticoid of interleukin-13-induced eosinophilia, hyperresponsiveness, and goblet cell hyperplasia in mouse airways, Am. J. Respir. Crit. Care Med. 167 (2003) 50–56, https://doi.org/10.1164/rccm.2110084.
[28] J. Joseph-Bowen, N. de Klerk, P.G. Holt, P.D. Sly, Relationship of asthma, atopy, and bronchial responsiveness to serum eosinophil cationic proteins in early childhood, J. Allergy Clin. Immunol. 114 (2004) 1040–1045, https://doi.org/10.1016/j.jaci.2004.07.051.
[29] J. Yao, X. Zhao, Antagonism effects of Chinese medicine treatment of IL-8 and its relationship to asthmatic airway inflammation, SH. J. TCM Dec. 48 (2014) 94–97 (doi:1007-1334(2014)12-0094-03).
[30] Y. Shi, J. Dai, H. Liu, R.R. Li, P.L. Sun, Q. Du, L.L. Pang, Z. Chen, K.S. Yin, Naringenin inhibits allergen-induced airway inflammation and airway responsiveness and inhibits NF-kappaB activity in a murine model of asthma, Can. J. Physiol. Pharmacol. 87 (2009) 729–735, https://doi.org/10.1139/y09-065.
[31] J. Das, C.H. Chen, L. Yang, L. Cohn, P. Ray, A. Ray, A critical role for NF-kappa B in GATA3 expression and TH2 differentiation in allergic airway inflammation, Nat. Immunol. 2 (2001) 45–50, https://doi.org/10.1038/83158.
[32] S. Svenningsen, P. Nair, Asthma endotypes and an overview of targeted therapy for asthma, Front Med (Lausanne). 26 (2017) 154–158, http:/doi.org/10.3389/fmed.2017.00158.
[33] D.F. Rogers, Physiology of airway mucus secretion and pathophysiology of hypersecretion, Respir. Care 52 (2007) 1134–1146 discussion 1146-1149 https://doi.org/10.1097/01.PCC.0000282853.22148.49.
[34] A.E. Hesselink, D.A. van der Windt, B.W. Penninx, H.A. Wijnhoven, J.W. Twisk, L.M. Bouter, J.T. van Eijk, What predicts change in pulmonary function and quality of life in asthma or COPD? J. Asthma. 43 (2006) 513–519, https://doi.org/10.1080/02770900600856954.
[35] C.L. Ordonez, R. Khashayar, H.H. Wong, R. Ferrando, R. Wu, D.M. Hyde, J.A. Hotchkiss, Y. Zhang, A. Novikov, G. Dolganov, J.V. Fahy, Mild and moderate asthma is associated with airway goblet cell hyperplasia and abnormalities in mucin gene expression, Am. J. Respir. Crit. Care Med. 163 (2001) 517–523, https://doi.org/10.1164/ajrccm.163.2.2004039.
[36] T. Aikawa, S. Shimura, H. Sasaki, M. Ebina, T. Takishima, Marked goblet cell hyperplasia with mucus accumulation in the airways of patients who died of severe acute asthma attack, Chest 101 (1992) 916–921, https://doi.org/10.1378/chest.101.4.916.
[37] V. Steenwinckel, J. Louahed, C. Orabona, F. Huaux, G. Warnier, A. McKenzie, D. Lison, R. Levitt, J.C. Renauld, IL-13 mediates in vivo IL-9 activities on lung epithelial cells but not on hematopoietic cells, J. Immunol. 178 (2007) 3244–3251, https://doi.org/10.4049/jimmunol.178.5.3244.
[38] V. Baud, M. Karin, Signal transduction by tumor necrosis factor and its relatives, Trends Cell Biol. 11 (2001) 372–377, https://doi.org/10.1016/S0962-8924(01)02064-5.
[39] P.K. Jeffery, Differences and similarities between chronic obstructive pulmonary disease and asthma, Clin. Exp. Allergy 29 (1999) 14–26, https://doi.org/10.1046/j.1365-2222.1999.00004.x-i2.
[40] H. Hsu, H.B. Shu, M.G. Pan, D.V. Goeddel, TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways, Cell. 84 (1996) 299–308, https://doi.org/10.1016/S0092-8674(00)80984-8.
[41] J.M. Lora, D.M. Zhang, S.M. Liao, T. Burwell, A.M. King, P.A. Barker, L. Singh, M. Keaveney, J. Morgenstern, J.C. Gutiérrez-Ramos, A.J. Coyle, C.C. Fraser, Tumor necrosis factor-alpha triggers mucus production in airway epithelium through an I kappa B kinase beta-dependent mechanism, J. Biol. Chem. 280 (2005) 36510–36517, https://doi.org/10.1074/jbc.M507977200.
[42] C. Desmet, P. Gosset, B. Pajak, D. Cataldo, M. Bentires-Alj, P. Lekeux, F. Bureau, Selective blockade of NF-kappa B activity in airway immune cells inhibits the effector phase of experimental asthma, J. Immunol. 173 (2004) 5766–5775, https://doi.org/10.4049/jimmunol.173.9.5766.
[43] S. Matsukura, M. Kawaguchi, M. Adachi, The present situation and problems of asthma treatment, Nihon Yakurigaku Zasshi 131 (2008) 120–125, https://doi.org/10.1254/fpj.131.120.
[44] L.Y. Cui, Y.C. Zhu, S. Gao, J.M. Wang, B. Peng, J. Ni, L.X. Zhou, J. He, X.Q. Ma, Ninety-day administration of dl-3-n-butylphthalide for acute ischemic stroke: a randomized, double-blind trial, Chin. Med. J. 126 (2013) 3405–3410, https://doi.org/10.3760/cma.j.issn.0366-6999.20123240.
[45] J.H. Huang, X.H. Huang, Z.Y. Chen, Q.S. Zheng, R.Y. Sun, Dose conversion among different animals and healthy volunteers in pharmacological study, Chin J Clin Pharmacol Ther. 9 (2004) 1069–1072, doi:1009-2501(2004)09-1069-04.

Supplementary material

Butylphthalide ameliorates airway inflammation and mucus hypersecretion via NF-κB in a murine model with chronic asthma

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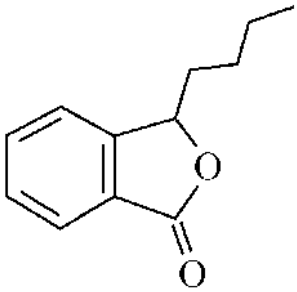

Fig. S1. The molecular structure of butylphthalide (NBP).

Table S1. Primer sequences of RT-PCR.

Gene	Primer	Sequences (5' to 3')	Size (bp)
Muc5ac	Forward	ACCACTTTCTCCTTCTCCACAC	150
	Reverse	AACAGGGCTCTTCACAGACAATA	
NF-κB p65	Forward	ATTGCTGTGCCTACCCGAAAC	158
	Reverse	TTTGAGATCTGCCCTGATGGTAA	
β-Actin	Forward	CATCCGTAAAGACCTCTATGCCAAC	218
	Reverse	ATGGAGCCACCGATCCACA	

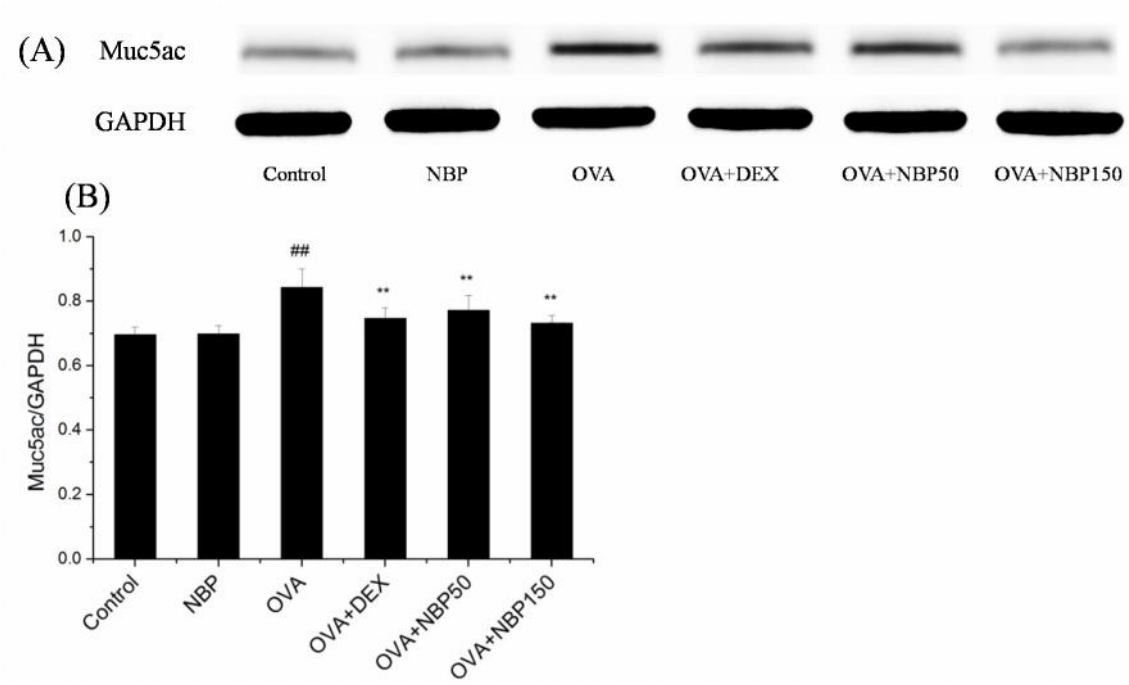

Fig. S2. Effect of NBP on Muc5ac expression in lung tissue by western blot. (A) Representative western blot pictures of Muc5ac. Protein expressions of (B) Muc5ac was quantified based on the density of the bands. GAPDH was used as an internal control. The values are shown as the mean ± SD (n = 10). $^{\#\#}P$ < 0.01 *vs* control; $^{**}P$ < 0.01 *vs* OVA.

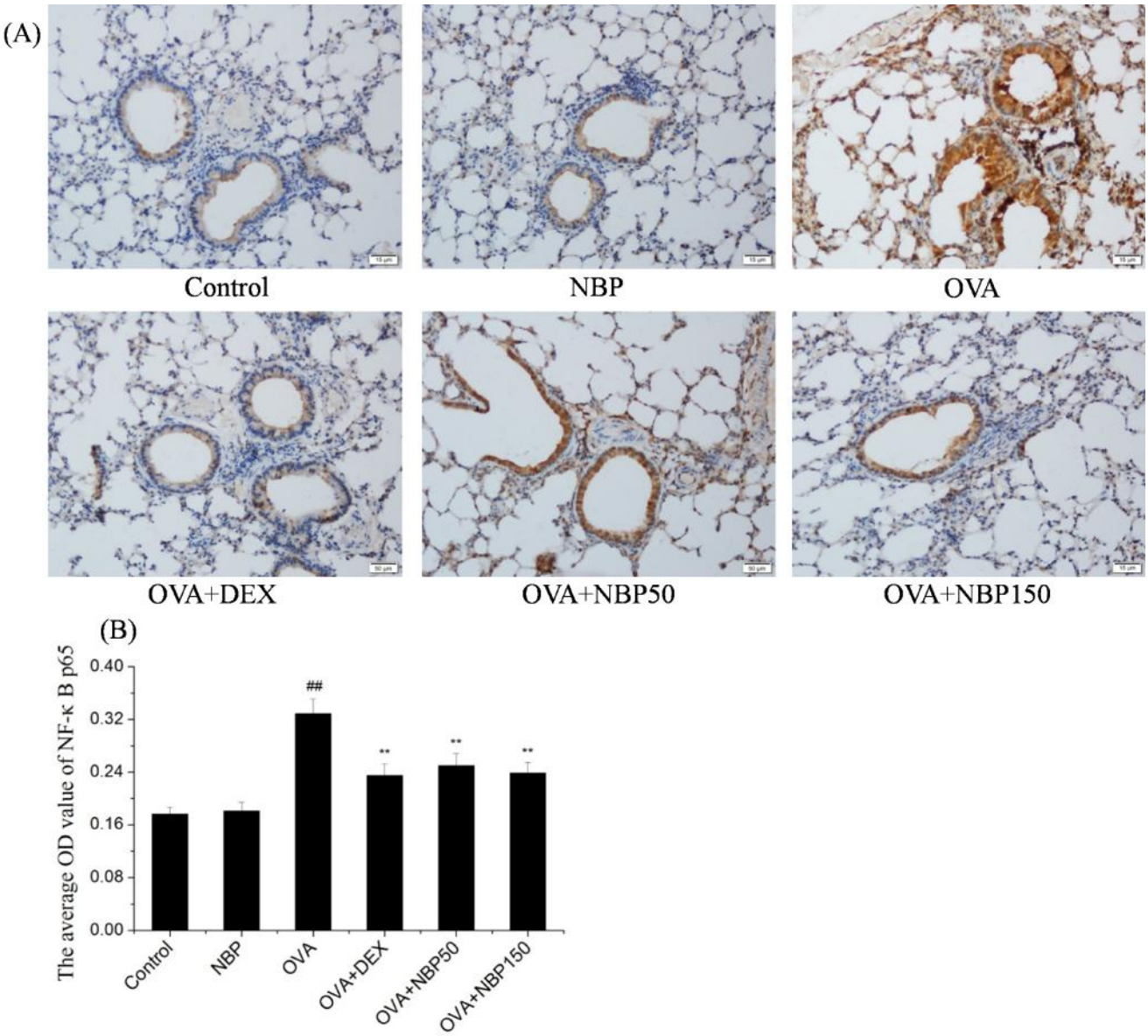

Fig. S3. Effect of NBP on NF-κB p65 in lung tissue by immunohistochemistry. (A) Representative pictures of lung tissue slides (scale bar = 15 μm) of six groups. (B) The average OD value of NF-κB p65 in lung tissue. The values are shown as the mean ± SD (n = 10). $^{\#\#}P < 0.01$ *vs* control; $^{**}P < 0.01$ *vs* OVA.