MEDICAL SCIENCE MONITOR

ANIMAL STUDY

e-ISSN 1643-3750
© Med Sci Monit, 2019; 25: 1656-1662
DOI: 10.12659/MSM.915427

Received: 2019.01.29
Accepted: 2019.02.20
Published: 2019.03.04

Bulleyaconitine A Effectively Relieves Allergic Lung Inflammation in a Murine Asthmatic Model

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Source of support: This study was supported by the National Natural Science Foundation of China (31470014, 81172790, and 81671586), the Key Project of the Natural Science Foundation of Anhui Provincial Educational Department (KJ2018A0263, KJ2016A736, and KJ2016A739), the Outstanding Young People Abroad Study Tour Project in Anhui Province (gxfx2017071), and the Academic and Technical Leader Project of Wannan Medical College (010202041703)

Background: Bulleyaconitine A (BLA) has been widely used as analgesic against chronic inflammatory pain in China. However, its potential therapeutic role in asthma remains unclear. The purpose of this study was to investigate the effect of BLA on airway inflammation in mice with allergic asthma.

Material/Methods: Specific-pathogen-free (SPF) female Balb/c mice were randomly divided into the following 6 groups: (1) Control group (NC), (2) Asthma group (AS), (3) BLA-L group, (4) BLA-M group, (5) BLA-H group, and (6) Dexamethasone group. An asthma mouse model was established by administration of ovalbumin (OVA) and mice were sacrificed within 24 h after the last challenge. Enzyme-linked immunosorbent assay (ELISA) method was used to determine the relative expression levels of IgE and IgG in mouse serum. In addition, bronchoalveolar lavage fluid (BALF) was collected and IL-4, TNF-α, and MCP-1 levels were determined by ELISA. Furthermore, eosinophils, lymphocytes, and macrophages in BALF were classified and analyzed, and inflammatory cell infiltration in the airways of mice was determined by hematoxylin-eosin (HE) staining. The expression of NF-κB1 and PKC-δ in mouse lung tissue was determined by Western blot analysis.

Results: The levels of serum IgE and IgG in BLA- or Dex- treated mice were significantly reduced compared to those in the asthma (AS) group ($P<0.01$), whereas the levels of cytokines IL-4, TNF-α, and MCP-1 were significantly decreased ($P<0.01$). HE-staining showed that BLA significantly reduced inflammatory cell infiltration and mucus secretion in lung tissue. Moreover, BLA inhibited the expression of NF-κB1 and PKC-δ via the NF-κB signaling pathway in the lung.

Conclusions: Our data show that BLA activates PKC-δ/NF-κB to reduce airway inflammation in allergic asthma mice.

MeSH Keywords: Asthma • Hypersensitivity • Inflammation • NF-kappa B p50 Subunit • Protein Kinase C-delta

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/915427

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Background

Allergic asthma is a serious global health problem with an increasing prevalence in many countries, and affects patients from childhood into adulthood [1]. Exposure to airborne allergens contributes to asthma severity [2,3]. Although short-term inhaled corticosteroids and long-acting β-agonists can effectively relieve clinical symptoms in patients with acute asthma, up to 30% of treated patients remain uncontrolled, symptomatic, or both, and will potentially suffer from exacerbated symptoms and poorer clinical outcomes [3–6]. The precise underlying mechanism of asthma is not well-established.

Increasing evidence has shown that $CD4^+$ helper T cells play pivotal roles in various immunological diseases, including allergic asthma [7]. Naïve precursors of $CD4^+$ T cells that are stimulated by antigens undergo functional differentiation into effector subsets of helper T cells, which is determined by the cytokine environment [8,9]. For example, interleukin (IL)-12 promotes Th1 differentiation and interferon (IFN)-γ signaling, and in turn maintains Th1 development [10]. IL-4 has as a key functional role during Th2 polarization, and IL-2 regulates Th2 differentiation by activating IL-4 and IL-13 receptors [11–13]. Increasing evidence has demonstrated that a Th1/Th2 imbalance plays a crucial role during asthma attacks [14]. Activated Th2 cells secrete cytokines such as IL-4, IL-5, and IL-13 (responsible for eosinophil activation), and produce IgE (necessary for allergic inflammation) [15,16]. In addition, previous studies have shown that hedgehog (Hh) signaling-dependent transcription in T cells drives Th2 cell differentiation and exacerbates allergic disease [17, 18]. Interferon (IFN)-inducible transmembrane (IFITM) proteins were also found to promote Th2 transcriptional programs and differentiation, and exacerbate allergic asthma inflammation [19]. However, Wnt-1-driven activation of the canonical, but not the non-canonical, Wnt signaling pathway attenuates allergen-specific Th2 cell activation and airway inflammation [20].

Macrophages are the most abundant immune cells in the lung [21], and play a vital role in asthmatic inflammation induced by airborne allergens [22,23]. In previous studies, it has been shown that macrophages are M1 and M2 phenotypes according to their functional spectrum [24], which display inflammatory and anti-inflammatory functions, respectively [25]. M1 macrophages, induced by IFN-γ and lipopolysaccharide (LPS), drive inflammation in response to intracellular pathogens [26]. Conversely, M2 macrophages, induced by IL-4 and IL-13, are involved in anti-inflammatory responses [27,28]. Previous studies have suggested that increased M2 polarization and activation is involved in allergic asthma [29,30].

Bulleyaconitine A (BLA) is an "aconitine-like" diterpenoid alkaloid that was initially isolated from *Aconitum bulleyanum* plants in 1980 [31]. In China, BLA has been widely used since 1985 as analgesic against chronic inflammatory pain resulting from rheumatic, rheumatoid arthritis, and scapulohumeral periarthritis [31,32]. However, its potential therapeutic role in asthma remains unclear. In the present study, we assessed the anti-inflammatory role of BLA in a murine asthma model to provide an experimental basis for its potential effect in treating allergic asthma.

Material and Methods

Animals

We purchased 6–8-week-old female BALB/c mice (18~22 g) from Elmet Technology Co. of Suzhou Industrial Park (Suzhou, China) (License No: SCXK 2014-0007). Mice were supplied food and water ad libitum and were housed under specific-pathogen-free (SPF) conditions. All experimental procedures were approved by the Animal Research Ethics Committee of Wannan Medical College (Wuhu, China).

Establishing mouse asthmatic models

Mouse asthmatic models were established as previously described [33] and were appropriately modified. Briefly, 60 female BALB/c mice were randomly divided to 6 groups (n=10 per group): a PBS group (NC), an asthma group (AS), a low-dose BLA group (BLA-L), a middle-dose BLA group (BLA-M), a high-dose BLA group (BLA-H), and a dexamethasone group (Dex). Mice were intraperitoneally (i.p.) injected with 10 μg ovalbumin (OVA) (cat. no. A5253, Sigma, St. Louis, MO, USA), dissolved in 200 μl PBS, with 2% (W/V) $Al(OH)_3$ (cat. no. 239186, Sigma, St. Louis, MO, USA) on days 0, 7, and 14. Mice in the control group (NC) were given PBS. On day 21, mice were aerosolized with OVA (0.5 μg/ml) for 30 min at a time for 7 consecutive days. Mice in the NC group were given PBS. Mice in BLA-L, BLA-M, and BLA-H groups received an i.p. injection of BLA at doses of 0.16 mg/kg, 0.32 mg/kg, and 0.48 mg/kg, respectively. Treatment with BLA was given daily at 30 min before the aerosol inhalation on days 25 to 27 for enhancing immunity. Mice in the PBS group (NC) received both i.p. injections and nebulization with PBS. Mice in the asthmatic group (AS) did not receive any treatment. Mice in the Dex group received an i.p. injection of Dex at a dose of 1 mg/kg.

Evaluating antibodies in sera and cytokines in bronchoalveolar lavage fluid (BALF)

Mice were anesthetized with isoflurane (Cat no. 04537, Sigma, St. Louis, MO, USA) after the final nebulization at 24 h. Then, mouse lungs were injected with 0.3, 0.3, or 0.4 ml of sterilized PBS, respectively, and bronchoalveolar lavage fluid (BALF)

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was collected. A total of 1.0 ml of BALF was collected from each mouse. Subsequently, BALF was centrifuged at 3000×*g* and 4°C for 10 min, and the upper layer of fluid was collected and stored at –80°C for subsequent analysis. Blood samples of mice were obtained from the orbital cavity and centrifuged at 4000×*g* and 4°C for 5 min, and stored at –80°C for future experiments. ELISA was used to determine the IL-4, IL-10, TNF-α, and MCP-1 levels in BALF. Serum antibodies of total IgE and IgG were also determined by the above-mentioned approach (R&D Systems, Minneapolis, MN, USA).

Determination of cell classification in BALF

The cell pellet obtained from BALF was resuspended in 0.5 ml of PBS, then the total number of cells in BALF was determined using a modified Neubauer counter (Shanghai Qiujing Biochemical Reagent Instrument Co., Shanghai, China), and adjusted to a concentration of 1.0×10^4 cells/ml. A total of 100 µl of cell suspension was added to the cytospin smear device (Shandon™ Cytospin™ 4, Thermo Scientific, Runcorn, UK), centrifuged at 1200 rpm for 10 min at 4°C, and BALF cells were directly plated on the slide by centrifugation. The slides were immediately dried with cold air, fixed in absolute ethanol for 30 min, and subjected to Wright's staining. A total of 200 cells were counted using a 40× optical microscope (IX71, Olympus, Tokyo, Japan), then cell sorting was performed, and the number of eosinophils, lymphocytes, and macrophages were determined.

Histopathological examination of mouse lung tissue

Lung tissue sections were fixed overnight in 4% neutral paraformaldehyde, and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin (HE). Based on the scoring system described previously [34], we used a microscope to evaluate changes in inflammatory cell recruitment and assessed the inflammatory changes of lung based on the extent of eosinophil infiltration, epithelial damage, and pulmonary edema.

Western blot (WB) analysis

We collected 20 µg of total proteins from lung extracts, separated by 10% SDS-PAGE, and transferred onto a pure nitrocellulose blotting membrane (BioTrace™, PALL Life Sciences, Shanghai, China). Membranes were blocked in blocking buffer at room temperature for at least 1 h. After washing in PBS 3 times, membranes were incubated for 2 h at 37°C with primary antibodies in blocking buffer, including anti-NF-κB1 (cat. no. D220134, Sangon Biotech, Shanghai, China) and anti-PKC-δ (cat. no. 9616s, Cell Signaling Technology, Shanghai, China) rabbit polyclonal antibodies 2000-fold diluted in PBS. Next, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (cat. no. AQ132P, Sigma-Aldrich Co., St. Louis, MO, USA) in PBS at a 2000-fold dilution at 37°C for 1 h, and washed with blocking buffer 3 times at room temperature. Transferred proteins were visualized by using a horseradish catalase DAB color kit (cat. no. C520017, Sangon Biotech, Shanghai, China). The intensity of immunoreactivity bands was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

SPSS software (version 18.0, Chicago, IL, USA) was used for statistical analysis. Data are expressed as the mean ± standard deviation (SD), and one-way analysis of variance was used for comparison. Least significant difference-t (LSD-t) and Thamhane's T2 methods were used for comparison between groups, and $P<0.05$ was considered statistically significant.

Results

Assessment of the anti-inflammatory feasibility of BLA

To confirm the anti-inflammatory activity of BLA in the asthmatic mouse model, ELISA was applied to determine the IL-4, TNF-α, and MCP-1 levels in BALF, and total IgE and IgG antibodies in serum. Our data show that compared with mice in the NC group (169.07±2.60 pg/ml), BLA-treated mice produced a lower level of IL-4 (459.54±49.45 pg/ml) compared to mice in the AS group (339.47±2.52 pg/ml), BLA-L group (298.82±3.86 pg/ml), BLA-M group (277.89±7.53 pg/ml), and BLA-H group (277.81±5.98 pg/ml) ($P<0.01$, Figure 1A). Moreover, mice treated with the middle or high dose of BLA produced a lower level of IL-4 compared to mice in the BLA-L group ($P<0.01$, Figure 1A). However, no significant differences in IL-4 were observed between mice in BLA-M, BLA-H, and Dex (276.67±4.36 pg/ml) groups ($P>0.05$). Levels of TNF-α and MCP-1 were similar to that of IL-4 ($P<0.01$, Figure 1B, 1C). However, the MCP-1 level in mice in the BLA-H group was increased compared with that of mice in the BLA-M group (Figure 1C).

The antibody levels of total IgE and IgG in serum indicated that IgE levels in BLA-L (44.38±3.56 ng/ml), BLA-M (32.48±2.62 ng/ml), BLA-H (24.54±2.11 ng/ml), and Dex (25.48±1.30 ng/ml) groups were notably lower than in the AS group (64.16±5.91 ng/ml, P<0.01) (Figure 2A). Moreover, IgE levels showed a dose-dependent decrease in BLA-L, BLA-M, and BLA-H groups, but no significant differences were observed between BLA-H and Dex groups.

Total serum IgG levels were significantly different for the BLA-L group (25.41±1.19 ng/ml), BLA-M group (18.66±0.78 ng/ml), BLA-H group (17.70±0.63 ng/ml), and Dex group (15.72±0.46 ng/ml) compared with the AS group (34.38±2.08 ng/ml, $P<0.01$) (Figure 2B). However, the serum IgG level in the Dex group

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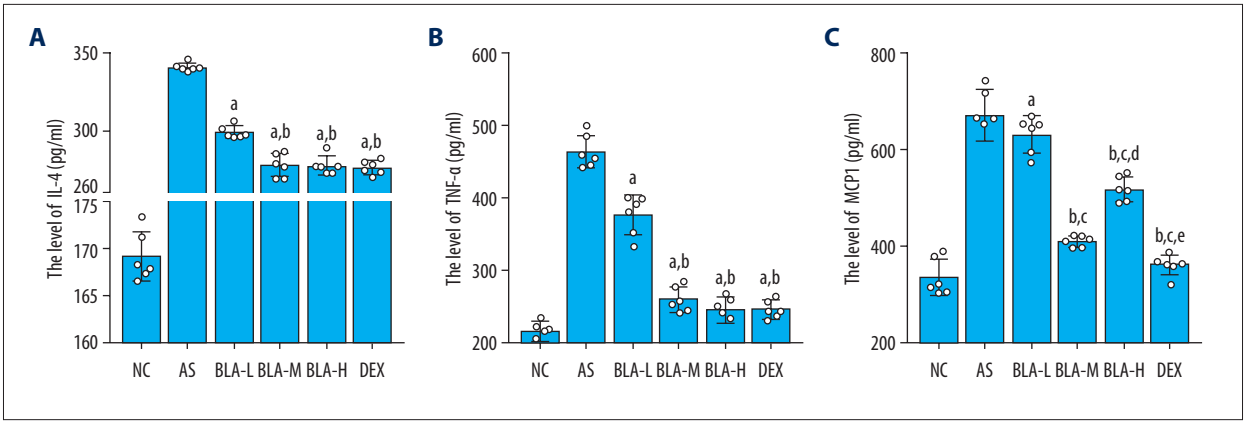

Figure 1. The levels of IL-4 (**A**), TNF-α (**B**) and MCP-1 (**C**) in bronchoalveolar lavage fluid (BALF) after treatment with bulleyaconitine A (BLA). The PBS group represented the negative control (NC), the asthma group (AS) represented the control group, BLA-L, BLA-M and BLA-H represented experimental groups, and the dexamethasone (Dex) group served as a positive control. a – when compared with the AS group, $P<0.01$; b – compared with BLA-L group, $P<0.01$; c – when compared with the BLA-M group, $P<0.01$; d – when compared with the BLA-M group, $P<0.01$; e – when compared with the BLA-M group, $P<0.05$.

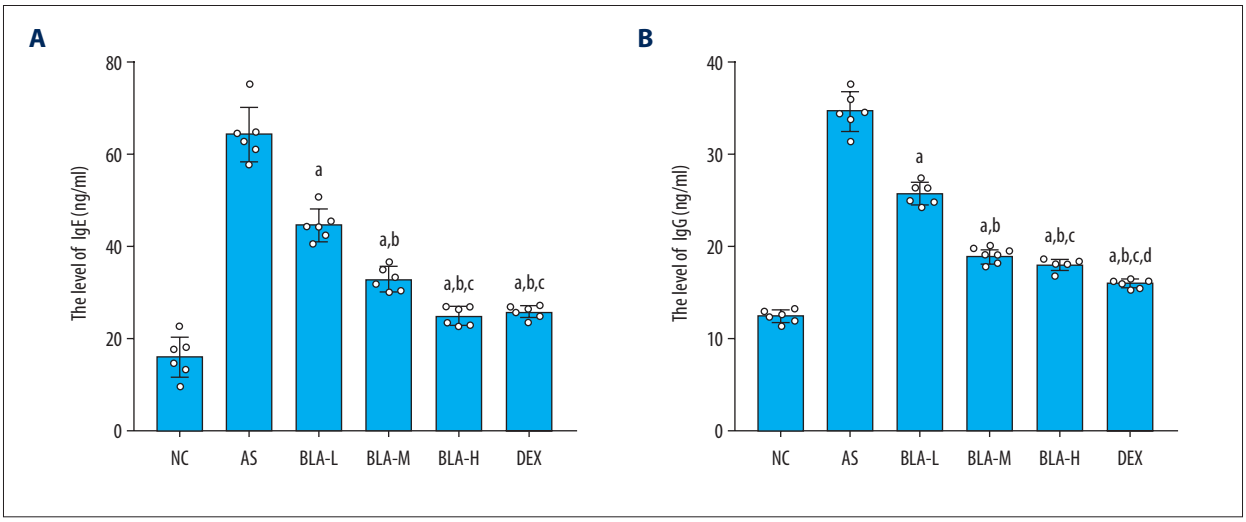

Figure 2. The levels of serum IgE (**A**) and IgG (**B**) antibodies after treatment with bulleyaconitine A (BLA). The PBS group was the negative control (NC), the asthma group (AS) was the control group, BLA-L, BLA-M, and BLA-H represented experimental groups, and the dexamethasone (Dex) group served as the positive control group. a – when compared with the AS group, $P<0.01$; b – when compared with the BLA-L group, $P<0.01$; c – when compared with the BLA-M group, $P<0.01$; d – when compared with the BLA-M group, $P<0.05$.

was significantly lower than in AS, BLA-L, BLA-M, and BLA-H groups (Figure 2B).

To further understand the effect of BLA on the differentiation of leukocytes, we determined the classification of leukocytes in BALF. The results are shown in Figure 3A. The number of eosinophils in the NC group, the AS group, the BLA-L group, the BLA-M group, the BLA-H group, and the Dex group were 0.39±0.03, 5.58±0.22, 3.05±0.17, 1.87±0.17, 1.46±0.16, and (1.29±0.20)×10^4/ml, respectively. The number of eosinophils in BLA-treated groups was markedly lower than in the asthma (AS) group ($P<0.01$, Figure 3A). Moreover, Dex treatment significantly decreased the number of eosinophils in mice in the Dex group compared to the BLA-H group. The number of macrophages was similar to that of eosinophils ($P<0.01$, Figure 3C).

Figure 3B shows that lymphocytes in the NC group, the AS group, the BLA-L group, the BLA-M group, the BLA-H group, and the Dex group were 0.22±0.03, 0.47±0.05, 0.52±0.07, 0.35±0.04, 0.30±0.03, and 0.39±0.04×10^4/ml, respectively. When compared with the AS group, the number of lymphocytes in the BLA-M group, BLA-H group, and the Dex group decreased ($P<0.05$, Figure 3B). However, the number of lymphocytes in the BLA-L group was not significantly different from that in the AS group ($P>0.05$, Figure 3B).

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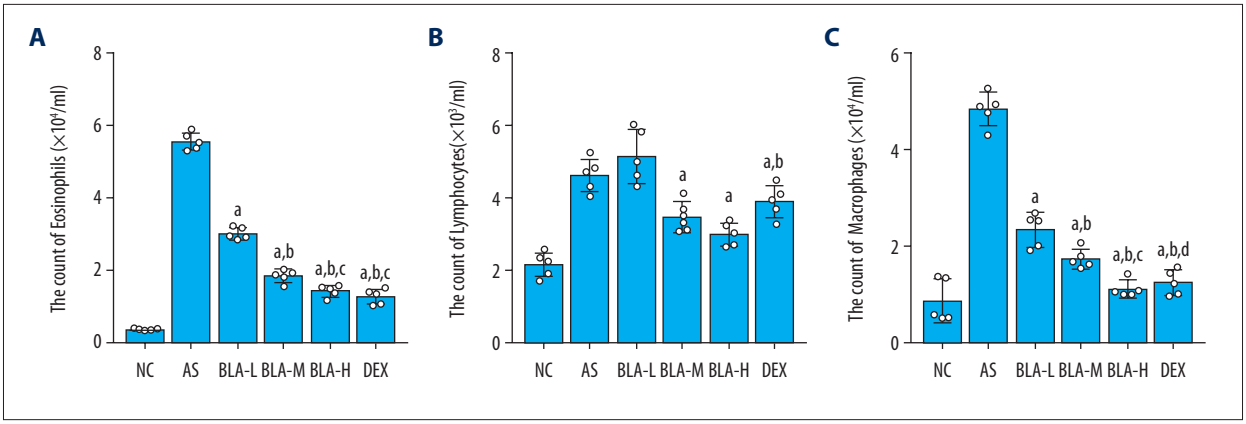

Figure 3. The number of eosinophils (**A**), lymphocytes (**B**), and macrophages (**C**) in bronchoalveolar lavage fluid (BALF) after treatment with bulleyaconitine A (BLA). The NC group was the negative control, the asthma group (AS) was the control group, BLA-L, BLA-M, and BLA-H were experimental groups, and the dexamethasone (Dex) group served as the positive control. a – when compared with the AS group, $P<0.01$; b – when compared with the BLA-L group, $P<0.01$; c – when compared with the BLA-M group, $P<0.01$; d – when compared with the BLA-M group, $P<0.05$.

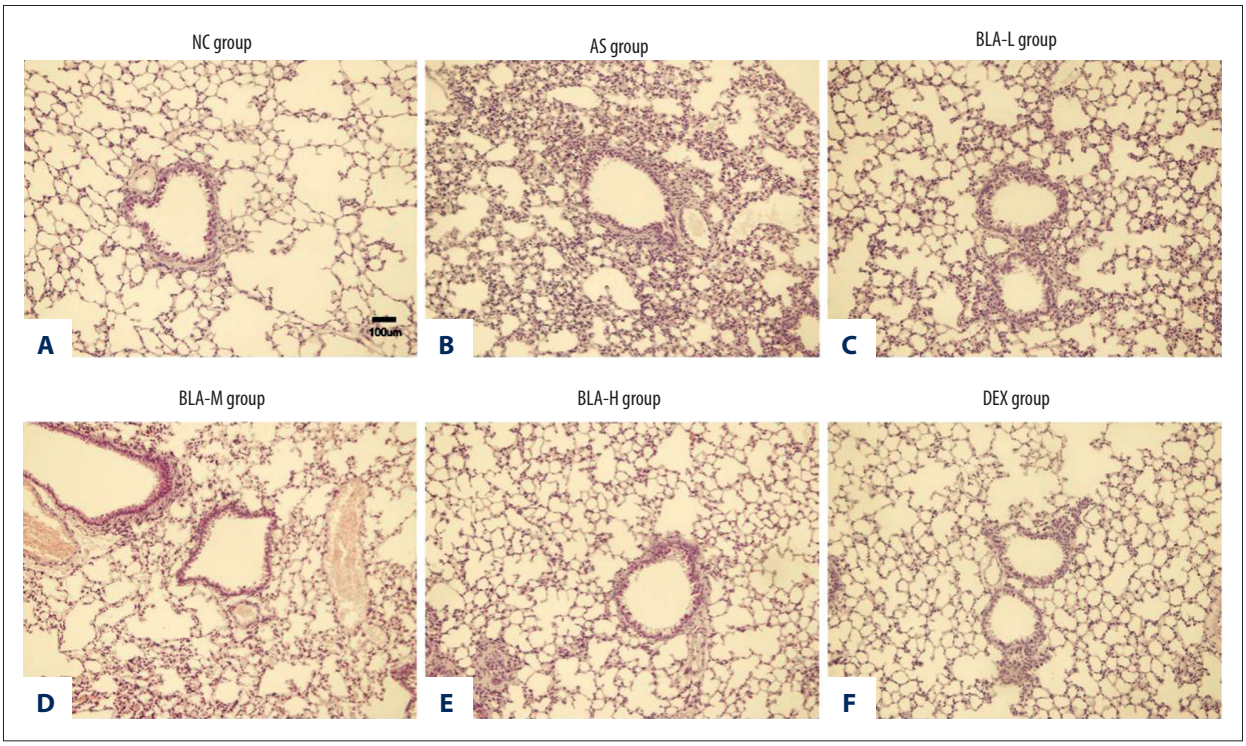

Figure 4. Histopathological changes in mouse lung (HE-staining, ×100). (**A**) In the asthma mice sensitized with OVA, severe pulmonary inflammation (**B**) was observed in the adjacent airway of different sizes, while lung inflammation, goblet cell proliferation, and mucous production were notably reduced in mice treated with BLA-L, BLA-M, BLA-H, and dexamethasone (Dex) (**C–F**).

Pathological changes in lung tissue

Histological examination of lung tissue from mice in the AS group showed intensive inflammatory infiltration that predominantly consisted of eosinophils (Figure 4B). Epithelial cells were overtly hypertrophied. Also notable were signs of epithelial shedding, smooth muscle destruction, and goblet cell hyperplasia. By comparison, mice in the NC group showed normal lung structure and normal morphology of the respiratory epithelium. No inflammatory cells were observed in peribronchial and perivascular regions (Figure 4A). BLA-L, BLA-M, BLA-H, and Dex-treated mice showed alleviated symptoms (Figure 4C–4F) when compared with mice in the AS group.

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Figure 5. The expression of NF-κB1 and PKC-δ in lung after treatment with bulleyaconitine A (BLA). The PBS group was the negative control (NC), the asthma group (AS) was the control group, BLA-L, BLA-M, and BLA-H were experimental groups, and the dexamethasone (Dex) group served as the positive control group.

BLA downregulates the expression of NF-κB1 and PKC-δ in lung tissue

To investigate the potential mechanism of the anti-inflammatory effects of BLA on allergic asthma, we analyzed the effect of BLA on NF-κB signaling using Western blot analysis. As shown in Figure 5, the levels of NF-κB1 and PKC-δ in mice in the AS group were induced after OVA stimulation. In addition, the expression of NF-κB1 and PKC-δ was significantly decreased after treatment with BLA.

Discussion

Allergic asthma is a chronic inflammatory disease of the airways that is characterized by airway inflammation, increased IgE secretion, eosinophilia and eosinophil (EOS) infiltration, increased lymphocytes, and excessive mucus secretion [35,36]. An imbalance of Th1/Th2 cells is the main cause of allergic asthma [37]. In addition, macrophages are also involved in the development of airway inflammation in allergic asthma [38].

BLA is a diterpenoid alkaloid that is extracted from *Aconitum* [39]. Previous studies have shown that oral administration of BLA attenuates paclitaxel-induced neuropathic pain in C-fiber synapses in the spinal dorsal horn of rats [40], which relieves pain by preferentially blocking LTX-S sodium channels [41] and Nav channels [32] in the dorsal root ganglion neurons of rats with L5 spinal nerve ligation. Furthermore, BLA can directly stimulate the secretion of dynorphin from spinal microglia and participate in anti-allergic effects [42]. However, the anti-inflammatory effects of BLA in allergic asthma are still unknown.

Pulmonary macrophages induce inflammatory responses by secreting inflammatory factors such as IL-4, TNF-α, and MCP-1, which are essential in the pathogenesis of asthma [43]. Our data show that BLA significantly reduced serum total IgE and IgG levels, especially in the BLA-H group. Moreover, our data showed that the levels of IL-4, TNF-α, and MCP-1 in BALF were significantly downregulated in a BLA dose-dependent manner. MCP-1 is one of the members of the CC chemokine protein family that is secreted mainly by macrophages, and it is primarily involved in anti-inflammatory effects in the body [44]. In previous studies, it has been demonstrated that serum MCP-1 levels were significantly higher in asthma patients than in the normal population [44]. These findings were consistent with our results that MCP-1 expression in BALF was significantly higher in mice in the AS group when compared to mice in the NC group. BLA treatment significantly reduced the expression of MCP-1. We speculated that BLA may cause a decrease in the number of macrophages. We further analyzed and confirmed that BLA inhibited the expression of MCP-1 by reducing the number of macrophages in BALF. In addition, BLA treatment reduced the number of EOS and lymphocytes. The decline in the number of these cells helped to alleviate the symptoms of allergic asthma.

The transcription factor NF-κB is involved in regulating the expression of inflammation-related genes [45] and plays a key role in immunity, inflammation, and cell proliferation [46]. Stimulating the NF-κB signaling pathway promotes the increase of airway epithelial cytokines such as IL-4 and TNF-α during the pathogenesis of allergic asthma [47]. The expression of NF-κB is significantly elevated in patients with severe asthma [48]. Intraperitoneal injection of PKC-δ-specific inhibitors prevented airway phlegmonosis and airway hyperresponsiveness in an allergic asthma model mouse challenged by OVA [49], suggesting that activation of PKC-δ inhibited airway phlegmonosis and airway modelling [49]. To analyze the potential effect of BLA on the anti-inflammation of allergic asthma disease, NF-κB1 and PKC-δ in the NF-κB signaling pathway were selected and their levels were determined in the lung. The results showed that the expression of NF-κB1 and PKC-δ in BLA-treated mice was lower than in the AS group, indicating that BLA downregulated the expression of NF-κB1 and PKC-δ proteins in lung tissue via the NF-κB signaling pathway. The above results suggest that this reduction is an indirect consequence of the reduced inflammation. Therefore, subsequent studies require the use of NF-κB1- and PKC-δ-specific inhibitors and high-throughput techniques, such as RNA-seq or single cell sequencing to further clarify the exact anti-inflammatory mechanisms of BLA.

Conclusions

Our study demonstrates that BLA can alleviate OVA-induced allergic inflammation, and the mechanism might be involved in reducing the expression of PKC-δ/NF-κB1.

Conflict of Interest

None.

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