

RESEARCH ARTICLE

Attenuation of allergic airway inflammation in a murine model of asthma by Licochalcone A

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Abstract

Context: Licochalcone A (Lico A) is a major and biogenetically characteristic chalcone isolated from the root of Xinjiang liquorice, *Glycyrrhiza inflata*.

Objective: We focused on investigating whether Lico A possesses distinct anti-inflammatory activity on a non-infectious mouse model of asthma, and we aimed to elucidate its involvement with the mitogen-activated protein kinases pathway.

Methods: BALB/c mice that were sensitized and challenged to ovalbumin (OVA) were treated with Lico A (50 mg/kg) 1 h before they were challenged with OVA.

Results: Our study demonstrated that Lico A may effectively inhibit the increase in T-helper type 2 cytokines, such as interleukin (IL)-4, IL-5 and IL-13 in bronchoalveolar lavage fluid, and reduced serum levels of OVA-specific IgE and IgG. Furthermore, Lico A substantially inhibited OVA-induced eosinophilia in lung tissue and mucus hyper-secretion by goblet cells in the airway. Meanwhile, pretreatment with Lico A resulted in a significant reduction in mRNA expression of acidic mammalian chitinase, chitinase 3-like protein 4 (Ym2), E-selectin, Muc5ac, CCL11 and CCR3 in lung tissues and airway hyper-responsiveness to methacholine.

Conclusions: These findings suggest that Lico A may effectively delay the progression of airway inflammation and could be used as a therapy for patients with allergic airway inflammation.

Keywords

Asthma, CCL11, CCR3, IgE, Lico A, Muc5ac, Ym2

History

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Introduction

Bronchial asthma is recognized as a chronic type of airway inflammation with an associated airway inflammation, mucus hyper-secretion, airway hyper-responsiveness (AHR) and elevated IgE levels^{1,2}. Numerous pro-inflammatory cytokines, chemotactic cytokines (chemokines) and adhesion molecules are involved in asthma. Inhaled corticosteroids (ICS) have been used in asthma to prevent and suppress inflammation³. However, it is estimated that 5–10% of patients with difficult-to-treat asthma are refractory to the current therapies, and long-term use of ICS has been associated with side effects⁴.

It has been shown that the prevalence of asthma is rapidly increasing worldwide. Dexamethasone, we used in our study as a positive control, is a glucocorticoid which is always used on clinical infection. While, Chinese Materia Medica seem to be an alternative to treat asthma. Licochalcone A (Lico A) is a major and biogenetically characteristic chalcone isolated from the root of Xinjiang liquorice, *Glycyrrhiza inflata*⁵ which

possesses anti-tumor activities⁶ can modify the Bcl-2 level⁷. It also proves to be a specific inhibitor for Stat3 and would be employed for the treatment of various diseases caused by disorders of the Jak/Stat pathway⁸.

The mitogen-activated protein kinases (MAPKs) consist of three main protein kinase families: the extracellular signal-regulated protein kinases (ERKs), the c-Jun N-terminal kinases (JNKs) and the p38 family of kinases. It has been reported that inhibition of the MAPK signaling pathway in lung inflammatory cells (e.g. mast cells) may have therapeutic potential in the treatment of allergic diseases such as asthma⁹. Our previous study demonstrated that Lico A portend to be a novel anti-inflammation component in acute lung injury induced by lipopolysaccharide¹⁰. Based on studies investigating the effect of Lico A, however, no available study has been done in a mouse model of allergic airway inflammation, so we investigated the effects of Lico A on ovalbumin (OVA)-induced Th2-mediated allergic airway inflammation *in vivo* a mouse asthma model and explored the anti-inflammatory mechanism of it.

Methods

Animals

Female BALB/c mice, weighing approximately 16–18 g, were purchased from the Center of Experimental Animals of

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Baiquien Medical College of Jilin University (Jilin, China). Mice were housed for 2–3 days to adapt them to the environment before experimentation. The mice were housed in micro-isolator cages and received food and water ad libitum. The laboratory temperature was $24 \pm 1^\circ\text{C}$, and relative humidity was 40–80%. All animal experiments were performed in accordance with the guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Reagent

The IL-4, IL-5 and IL-13 enzyme-linked immunosorbent assay (ELISA) kits were purchased from Biolegend (CA). OVAs (Grade V) were purchased from Sigma-Aldrich (St. Louis, MO). Lico A (purity >98%, Figure 1) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Phospho-specific antibodies for ERK1/2, p38 and JNK as well as antibodies against ERK, p38 and JNK proteins were obtained from Cell Signaling Technologies (Beverly, MA). Peroxidase-conjugated Affinipure goat anti-mouse IgG (H + L) and peroxidase-conjugated Affinipure goat anti-rabbit IgG (H + L) were purchased from PTG (Chicago, IL). The ELISA kits for IgE, IgG and IgG1 were purchased from R&D (Anniston, AL). The purity of all chemical reagents was of analytical grade.

Antigen sensitization, challenge and Lico A treatment

Groups of mice ($n=6$) receiving the following treatments were studied: (1) sham-sensitization plus challenge with phosphate buffered saline (PBS); (2) sensitization plus challenge with OVA and (3) sensitization plus challenge with OVA and treated with Lico A. Groups of six animals were used for each experimental condition. Mice were sensitized with OVA (20 μg) adsorbed in Imject Alum (100 $\mu\text{g}/\text{mL}$, Pierce, Rockford, IL) by intraperitoneal application on days 0 and 14. On days 25–27, mice were again anesthetized, intranasally challenged with OVA (100 μg) in PBS (50 μL). The negative controls were sham-sensitized and challenged with PBS plus dimethyl sulfoxide (DMSO, 0.1% (v/v)) following the same protocol as Lico A. Lico A (50 mg/kg) or dexamethasone (2 mg/kg) was prepared by dissolving in DMSO and final concentrations of DMSO were adjusted to 0.1% (v/v), and intraperitoneal application at 1 h before all OVA challenges on days 25–27.

BALF and serum collection

Mice were anesthetized 24 h after the last OVA challenge and were bled via the brachial plexus to collect the blood samples that were used to estimate IgE, IgG and IgG1 production. Ice-cold PBS (0.5 mL) was instilled twice into the lungs, and BAL

fluid was collected. Total cell counts were performed using a hemocytometer. The fluid recovered from each sample was centrifuged (4°C , 3000 rpm, 10 min) to pellet the cells, and the supernatant was kept at -70°C until it was used for cytokine measurements. The cell pellets were re-suspended in PBS to stain and count the total number of cells using the Wright–Giemsa staining method. At least 200 cells were counted per slide.

Cytokine levels in lung tissues

The concentrations of cytokine IL-4, IL-5 and IL-13 in the supernatants of the bronchoalveolar lavage fluid (BALF) were measured by sandwich enzyme-linked immunosorbent assay using commercially available reagents according to the manufacturer's instructions.

Serum immunoglobulin production

To further evaluate whether Lico A could modify an ongoing OVA-specific Th2 response *in vivo*, serum levels of total IgE, IgG and IgG1 were determined using ELISA. The analysis was carried out using mouse-specific anti-IgE-antibody, anti-IgG-antibody and anti-IgG1-antibody. Briefly, microplate wells were coated with 1% OVA in coating buffer (0.05 M sodium carbonate–bicarbonate, pH 9.6) overnight at 4°C . The wells were then incubated with blocking buffer (1% BSA in PBS, pH 7.2) at room temperature for 1 h and washed. Then, the diluted serum samples were introduced to the microplate, which were then incubated at room temperature for 2 h, washed, and incubated with Biotin anti-mouse IgE, IgG and IgG1. The samples were followed by the addition of extravidin-peroxidase at room temperature for 30 min and 3,3',5,5'-tetramethylbenzidine substrate for 15 min. The enzymatic reaction was stopped with 2 M H_2SO_4 , and the absorbance was read at 450 nm. Units are reported as the optical density at 450 nm.

Determination of AHR

Mice were anesthetized 24 h after the last OVA challenge, and tracheotomy was performed as described¹¹. The internal jugular vein was cannulated and connected to a microsyringe for intravenous methacholine administration. Airway resistance (RI) and lung compliance (Cdyn) in response to increasing concentrations of methacholine were recorded using a whole-body plethysmograph chamber (Buxco, Sharon, CT) as described by Le et al.¹². RI is defined as the pressure driving respiration divided by flow. Cdyn refers to the distensibility of the lung and is defined as the change in volume of the lung produced by a change in pressure across the lung. Results are expressed as the percentage of the respective basal values.

Histological examination

Histopathologic evaluation was performed 24 h after the last OVA challenge on mice which were not subjected to BALF. Left lung was removed by dissection and fixed in 4% paraformaldehyde. Lung tissues were sectioned, embedded in paraffin and cut at 3 μm . Tissue sections were then stained with hematoxylin and eosin (H&E) for general morphology

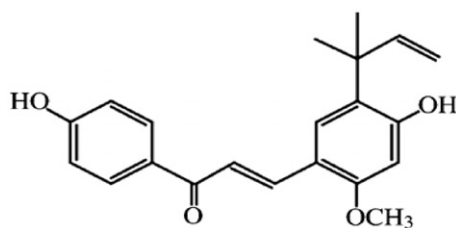


Figure 1. Chemical structure of Lico A.

andalcian blue-periodic acid-Schiff (AB-PAS) for the identification of goblet cells in the epithelium. Quantitative analysis was performed blinded as described¹¹.

Western blot analysis

Tissues were harvested 24 h after the last OVA challenge and frozen in liquid nitrogen immediately until homogenization. Samples were homogenized in radio immuno precipitation assay buffer and lysed for 30 min on ice. Total protein fractionation was performed using a cell lysis buffer for western blot and IP (Beyotime Institute of Biotechnology; China) according to the manufacturer's protocol. Protein concentration was assayed using the Bio-Rad protein kit, and equal amounts of protein were loaded into wells on a 10% sodium dodecyl sulfate-polyacrylamide gel. Subsequently, proteins were transferred onto polyvinylidene difluoride membranes, blocked overnight with 5% (wt/vol) non-fat dry milk, and probed according to the method described by Towbin et al.¹³ with specific antibodies against phospho-specific antibodies for ERK1/2, p38 and JNK as well as antibodies against ERK, p38 and JNK in 5% (wt/vol) BSA dissolved in tween tris buffer saline. With the use of a peroxidase-conjugated secondary anti-mouse or anti-rabbit antibody, bound antibodies were detected by ECL plus (GE Healthcare Buckinghamshire, UK).

RNA preparation and quantitative reverse transcription PCR (RT-PCR)

Total RNA was isolated 24 h after the last OVA challenge (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For each RT-PCR, 2 µg of total RNA was used to synthesize cDNA using SuperScript II Reverse Transcriptase (Invitrogen). The cDNA sequence of β-actin was used as an internal control to assess reverse transcription efficiency. The primers used were as follows: E-selectin sense: 5'-CCCTTCCACAGAACCTACCA-3', antisense: 5'-TCAGCAGACATTGCTTCACC-3'; chitinase 3-like protein 4 (Ym2) sense: 5'-TCCACTTTGAACCACATTCCAAGGC-3', antisense: 5'-CGAGAGACTGAGACAGTTCAGGGA-3'; acidic mammalian chitinase (AMCase) sense: 5'-TGGACACACCTTCATCCTGA-3', antisense: 5'-CCTCAGTGGCTCCACTTC

TC-3'; Muc5ac sense: 5'-GTGTCGGCCGGAGAAAGTTG GT-3', antisense: 5'-GTCCTGTTGAGCCTGGCCTGTG-3'; ccl11 sense: 5'-AAACCATAAACAAACCTCCTC-3', antisense: CAATAATCCCACATCTCCTT-3'; ccr3 sense: 5'-TC TGCTGAGATGTCCCAATA-3', antisense: 5'-TCACCAACA AAGGCGTAG-3'.

Statistical analysis

All values are expressed as the mean ± standard error of the mean (SEM). Differences between the mean values of normally distributed data were assessed with a one-way analysis of variance (ANOVA, Dunnett's *t* test) and a two-tailed Student's *t* test. Statistical significance was accepted at $p < 0.05$ or $p < 0.01$.

Results

Lico A reduces OVA-induced BALF T helper type 2 cytokine levels

Th2 cytokine levels in the bronchoalveolar lavage were measured by a sandwich ELISA. The concentrations of IL-4, IL-5 and IL-13 were increased in OVA-immunized samples compared with control mice (Figure 2). Treatment with Lico A caused a reduction in the levels of Th2-associated cytokines compared with OVA-immunized mice (Figure 2). ($\#p < 0.05$, $\##p < 0.01$ versus control group mice, $*p < 0.05$, $**p < 0.01$ versus OVA-challenged mice).

Lico A reduces OVA-induced serum levels of OVA-specific IgE, IgG and IgG1

OVA-induced serum levels of OVA-specific IgE, IgG and IgG1 were analyzed by a sandwich enzyme-linked immunosorbent assay. OVA-immunized mice treated with a vehicle had high levels of serum anti-OVA IgE, IgG and IgG1 antibodies compared with control mice (Figure 3). A significant reduction in OVA-specific IgE and IgG antibodies was observed in mice treated with Lico A (Figure 3) ($\#p < 0.05$, $\##p < 0.01$ versus control group mice, $*p < 0.05$, $**p < 0.01$ versus OVA-challenged mice).

Figure 2. Effect of Lico A on the secretion of IL-4, IL-5 and IL-13. The lavage fluid was centrifuged, and the supernatants were measured by ELISA. The values represent the mean ± SEM of three independent experiments. DXM = Dexamethasone. ($\#p < 0.05$, $\##p < 0.01$ versus control group mice, $*p < 0.05$, $**p < 0.01$ versus OVA-challenged mice.)

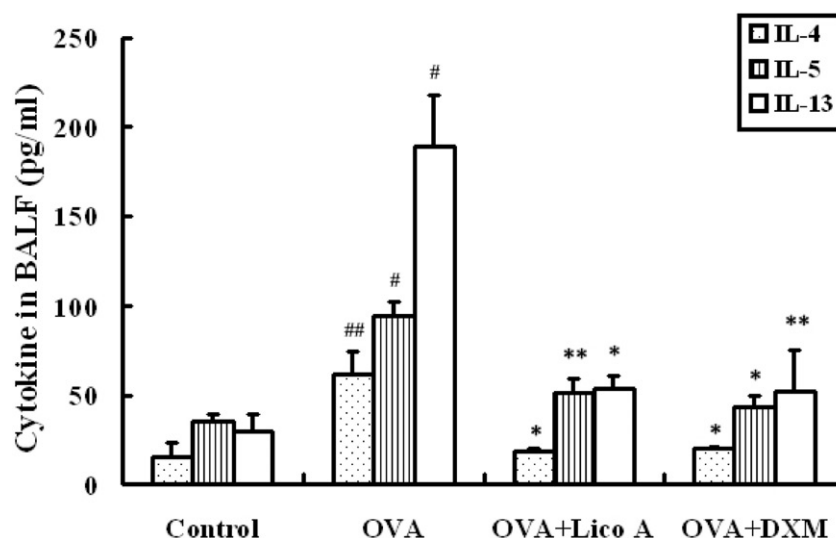


Figure 3. Effect of Lico A on OVA-specific IgE, IgG and IgG1 in serum. OVA-specific IgE, IgG and IgG1 levels in the serum were measured by ELISA. Results (mean $\pm$ SEM) are expressed as optical density values and are representative of at least three independent experiments. DXM = Dexamethasone. ($\#p < 0.05$, $\#\#p < 0.01$ versus control group mice, $*p < 0.05$, $**p < 0.01$ versus OVA-challenged mice.)

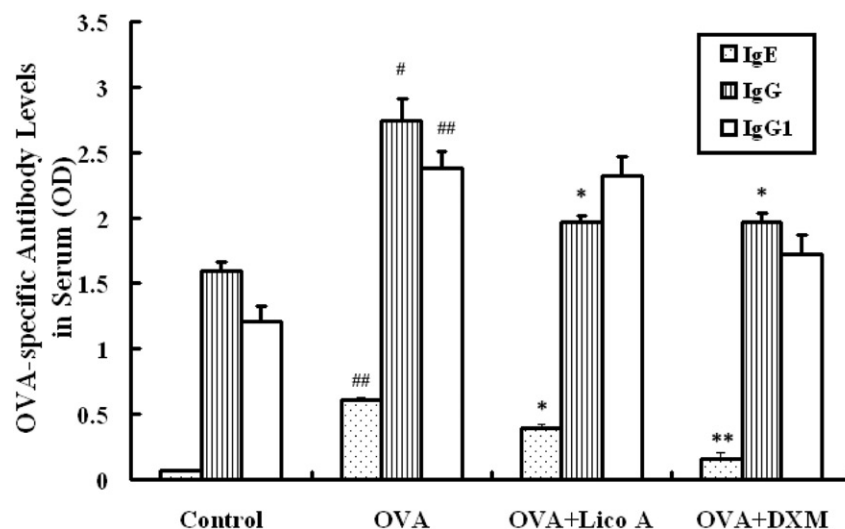
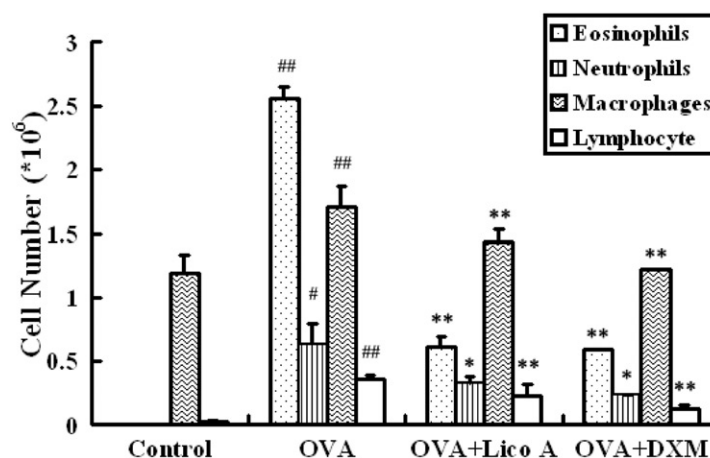


Figure 4. Effect of Lico A on the recruitment of inflammatory cell in BALF. The lavage fluid was centrifuged, and the cell pellets were re-suspended and applied to a slide by cytopinning to obtain differential cell counts by staining with a modified Giemsa method. The values represent the means $\pm$ SEM of three independent experiments. DXM = Dexamethasone. ($\#p < 0.05$, $\#\#p < 0.01$ versus control group mice, $*p < 0.05$, $**p < 0.01$ versus OVA-challenged mice.)



Lico A reduces OVA-induced BALF inflammatory cell recruitment

The total cell counts and differential cell counts in the BALF were evaluated 24 h after the last OVA challenge. As shown in Figure 4, OVA-immunized mice treated with a vehicle had higher levels of eosinophils, neutrophils, lymphocyte and macrophages compared with the control group. However, Lico A significantly decreased the number of eosinophils, neutrophils, lymphocyte and macrophages (Figure 4) ($\#p < 0.05$, $\#\#p < 0.01$ versus control group mice, $*p < 0.05$, $**p < 0.01$ versus OVA-challenged mice).

Lico A affects on OVA-induced AHR

To investigate the effect of Lico A on AHR in response to increasing concentrations of methacholine, we measured both RI and Cdyn in mechanically ventilated mice. OVA-challenged mice developed AHR, as was typically reflected by a high RI and low Cdyn (Figure 5). Lico A treatment dramatically reduced RI and restored Cdyn in OVA-challenged mice in response to methacholine (Figure 5) ($\#\#p < 0.01$ versus control group mice, $**p < 0.01$ versus OVA-challenged mice).

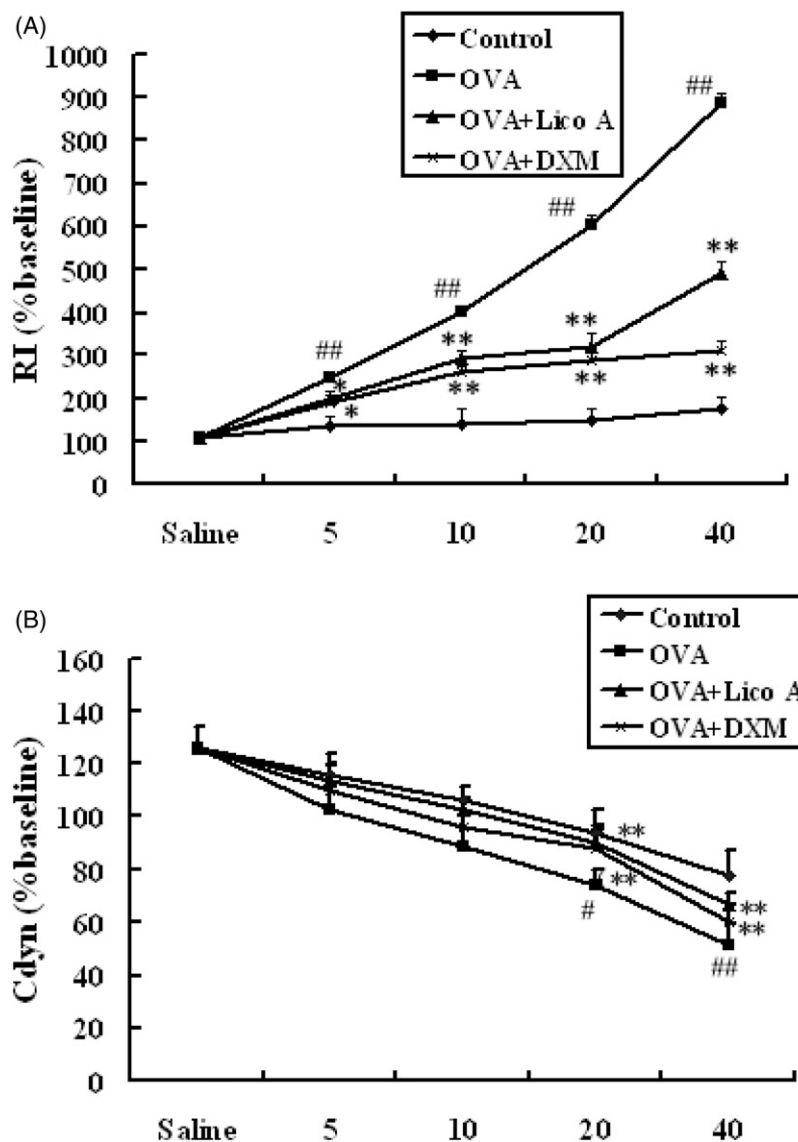
Lico A affects on OVA-induced histopathological changes in lung

Using H&E staining, inflammatory cell infiltration in peribronchial and perivascular areas was observed in OVA-challenged mice (Figure 6). Treatment with Lico A markedly reduced the degree of inflammatory cell infiltration in the peribronchial and perivascular areas. To evaluate the goblet cell hyperplasia, we stained lung sections with an AB-PAS stain to evaluate OVA-challenged mice-induced airway goblet cell hyperplasia and mucus production. Lico A treatment dramatically reduced mucus secretion and epithelial cell disruption (Figure 6).

Lico A affects on OVA-induced inflammatory gene expression

OVA aerosol challenge markedly upregulated lung mRNA levels of adhesion molecule E-selectin, which is pivotal for pulmonary recruitment of inflammatory cells, such as eosinophils and lymphocytes^{14,15}. Ym1, Ym2 and AMCase are chitinase proteins, has recently been found to be markedly elevated in allergic airway inflammation and to play a role in the pathogenesis of asthma^{16,17}. Muc5ac, which is essential

Figure 5. Effect of Lico A on OVA-induced AHR in mice. AHR was assessed by percentage change from the baseline level of (A) lung RI ($n=6$ mice per treatment group) and (B) dynamic compliance (Cdyn, $n=6$ mice per treatment group). Mch = Methacholine, DXM = dexamethasone. ($\#p<0.05$, $\#\#p<0.01$ versus control group mice, $*p<0.05$, $**p<0.01$ versus OVA-challenged mice.)



for mucus hyper-secretion¹⁸. Pretreatment with Lico A (50 mg/kg) demonstrated strong suppression of Ym-2, AMCase, Muc5ac, E-selectin, CCL11 and CCR3 in the allergic airways (Figure 7).

Lico A affects on MAP kinase phosphorylation

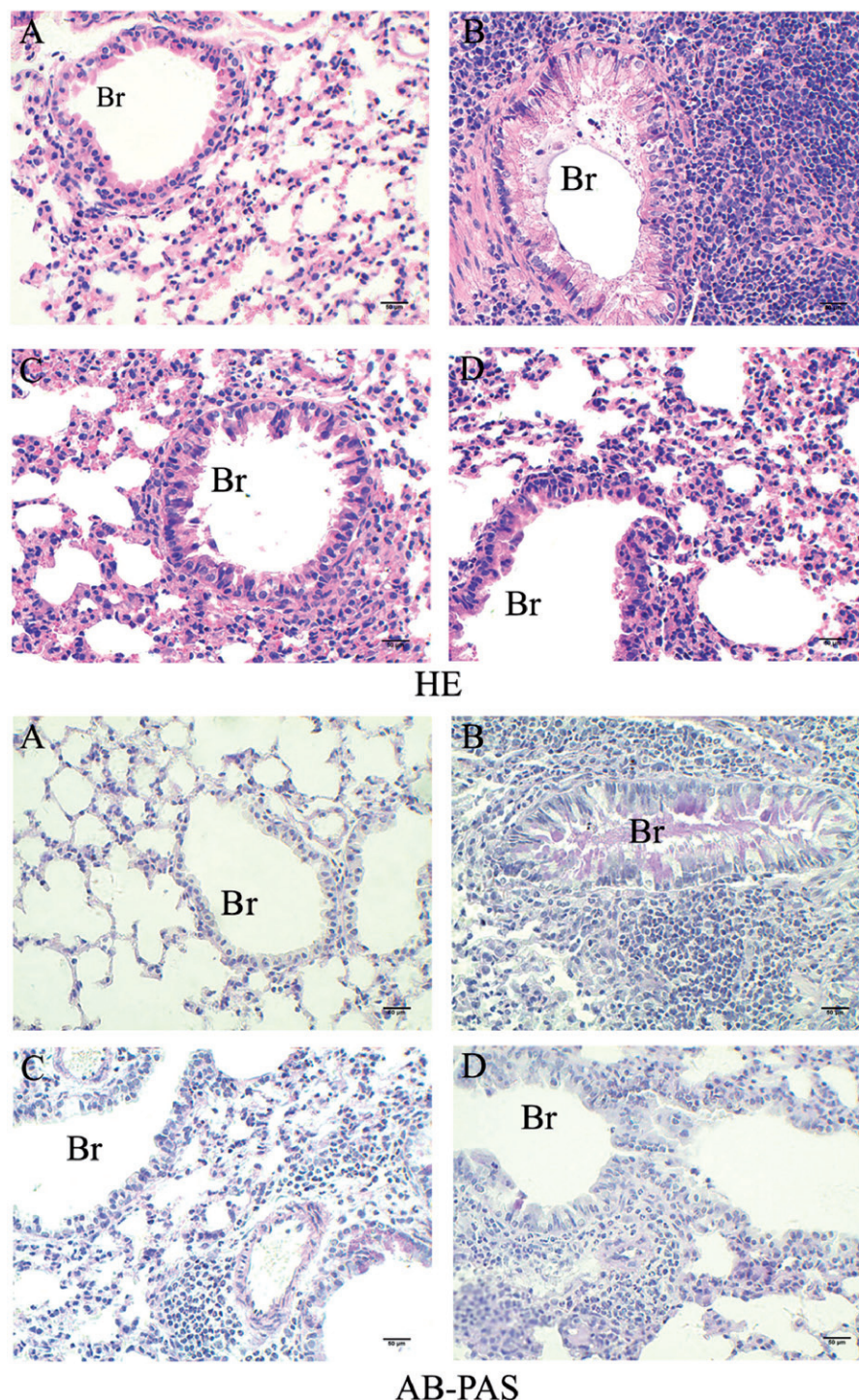
To verify that the anti-inflammatory effects of Lico A in OVA-challenged mice were mediated by the inhibition of MAPK. The MAPK molecules are among the important signaling pathways that control the synthesis and release of pro-inflammatory mediators by activated macrophages during the inflammatory response. Our data showed that OVA stimulation rapidly induced the phosphorylation of ERK, p38 and JNK in mice ($\#p<0.05$, $\#\#p<0.01$ versus control group mice). Lico A (50 mg/kg) significantly suppressed the ratio of p-ERK/ERK and p-P38/P38 ($*p<0.05$, $**p<0.01$ versus OVA-challenged mice) (Figure 8). However, there was no significant change in the ratio of p-JNK/JNK between OVA-challenged mice and the group treated with Lico A (Figure 8).

Discussion

The airway inflammation in asthma is typically eosinophilic with increased expression of IgE and Th2 cytokines. Numerous studies have shown that eosinophil recruitment is induced by the application of OVA in asthmatic mouse models^{19,20}. OVA induce allergic airway disease in mice and have proven useful in investigating the mechanisms underlying airway inflammation. In this study, we developed the OVA-induced an asthmatic mouse model in mice to investigate Lico A's influence on allergic airway inflammation *in vivo*.

Eosinophils are identified as the major effector cells underlying allergic airway inflammation¹⁴. CCL11 (Eotaxin) is a potent eosinophil chemoattractant, the major receptor for CCL11 is the CC chemokine receptor 3 (CCR3). The migration of eosinophils is mostly dependent on signaling of several chemokines acting through CCR3. Numerous studies showed that CCR3 antagonists are important effectors for the treatment of allergic diseases²¹⁻²⁴. Our present findings showed that Lico A prevented inflammatory cell

Figure 6. Effect of Lico A on airway inflammation, mucus production. Representative H&E and AB-PAS stained sections of lung from: (A) PBS-challenged mice; (B) OVA-challenged mice; (C) OVA-challenged mice treated with Lico A; (D) OVA-challenged mice treated with dexamethasone. Br = bronchi, scale bars = 50 μ m.



infiltration in airway. Furthermore, Lico A can reduce CCL11 and CCR3 mRNA expression in the lungs of OVA-challenged mice.

The T helper 2 (Th2)-type cytokines play a central role and controls the allergic response²⁵. Many studies report that IL-4-deficient mice have an attenuated allergic response elicited by antigen challenge compared with wild-type mice^{26–28}. IL-5 is a well-known mediator involved in the eosinophil growth, differentiation, mobilization, recruitment^{29,30} and survival recruitment of eosinophils into the airways in response to antigen challenge³¹. IL-13 is known to be an important factor in eosinophilic inflammation, mucus hyper-secretion and

AHR^{32–34}. Our data demonstrated that Lico A inhibited OVA-induced AHR resulting in inhaled methacholine. Meanwhile, eosinophilia aggregation into tissue was also obstructed by Lico A. Therefore, the inhibition of AHR and eosinophilia aggregation into lungs by Lico A may be associated with the reduction of IL-5 and IL-13 production.

Furthermore, among Th2-type cytokines, IL-13 is a potent inducer of mucus secretion and goblet cell hyperplasia. Blocking IL-13 significantly inhibits mucus overproduction in a murine model of asthma³⁵. The major mucin component of airway mucus secretion is Muc5ac in asthma³⁶. Cumulative evidence indicates that IL-5 plays an important role in goblet

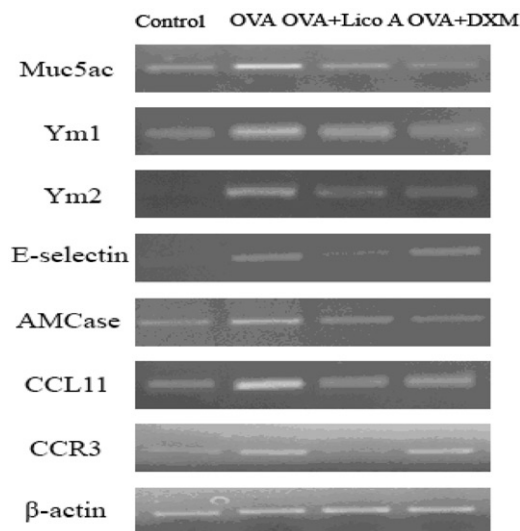


Figure 7. Effect of Lico A on the mRNA levels of adhesion molecule E-selectin, Ym1, Ym2, AMCase, Muc5ac, CCL11 and CCR3. Three independent experiments were performed. DXM = Dexamethasone.

cell hyperplasia and mucin Muc5ac gene and protein expression in mice³⁷. We observed a substantial drop in Muc5ac mRNA by Lico A in OVA-challenged lung tissues. As such, the marked decrease in mucus production in the lungs of Lico A-treated mice may be attributable to a significant reduction of IL-5 and IL-13 levels.

AMCase is a 50 kDa protein, which seems to be associated with asthma and allergic ocular pathologies in human and in mouse asthma models^{16,38,39}. AMCase has also been proposed as a potential therapeutic target in TH2-mediated inflammation⁴⁰. Ym1, Ym2 and AMCase are all chitinase proteins, which play an important role in asthma^{16,40}. Our data show that Lico A markedly down regulated AMCase and Ym2 mRNA expression in the lungs of OVA-challenged mice. These may be a consequence of the major drop in Th2-type cytokine levels in the airways with Lico A treatment.

In addition, our data demonstrated that Lico A inhibited OVA-induced AHR resulting in inhaled methacholine. Meanwhile, eosinophilia aggregation into tissue was also obstructed by Lico A. Airway inflammation is a major contributing factor to the pathogenesis and pathobiology of allergic asthma. Treatment strategies aimed at normalizing surrogates of airway inflammation (e.g. sputum eosinophils

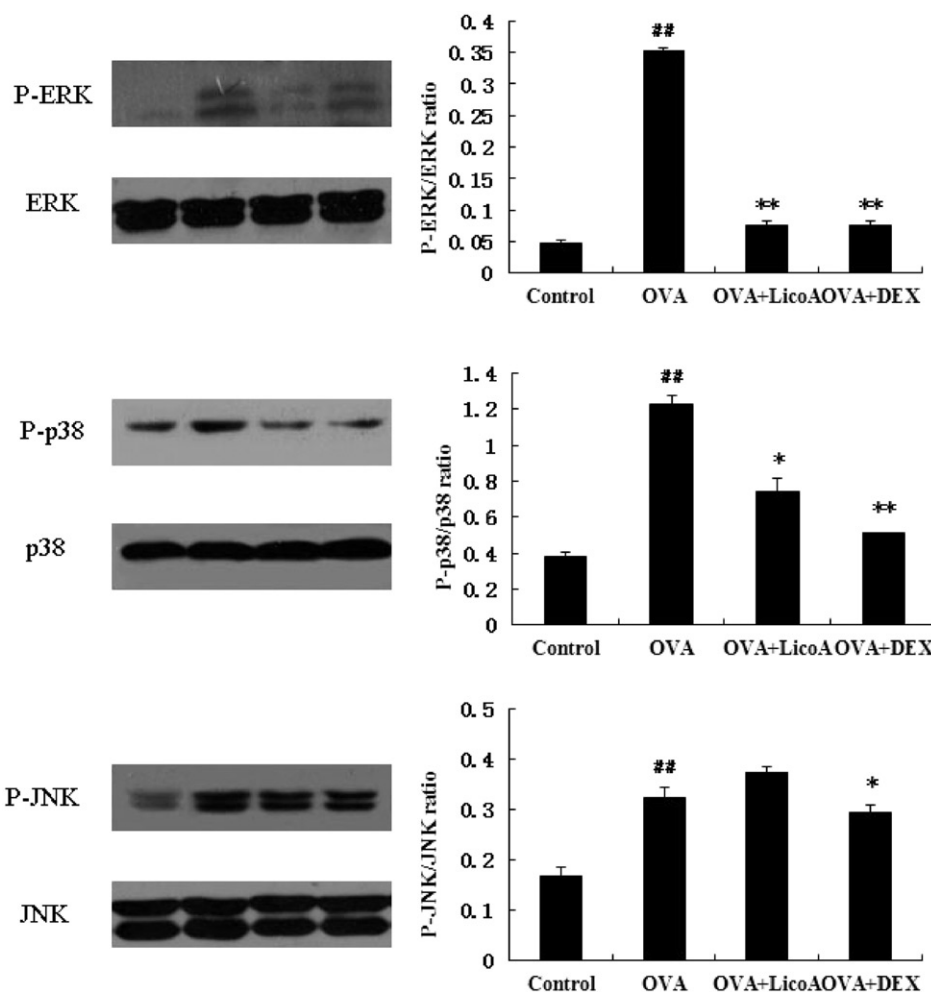


Figure 8. Effects of Lico A on OVA-induced phosphorylation of MAPK molecules in lungs. The cellular proteins from the lungs were used for the detection of phosphorylated or total forms of three MAPK molecules, ERK, p38 MAPK and JNK. Three independent experiments were performed, and the data shown indicate the mean ± SEM. (## $p < 0.01$ versus control group mice, ** $p < 0.01$ versus OVA-challenged mice.) ($p < 0.05$, ## $p < 0.01$ versus control group mice, * $p < 0.05$, ** $p < 0.01$ versus OVA-challenged mice.)

and AHR) have better outcomes than solely treating the symptoms or improving lung function^{41,42}. To confirm the mechanism of the reduction of Th2 cytokine and eosinophilia aggregation into the lungs, we evaluated the inhibitory effect of Lico A on the activation of MAPK in asthma mice.

The MAPK family includes three distinct stress-activated protein kinase pathways: p38, JNK and extracellular-regulating kinase (ERK). Inhibition of the ERK signaling pathway may have therapeutic potential for treatment of allergic airway inflammation⁴³. Western blot analysis showed that Lico A markedly attenuated OVA-induced tyrosine phosphorylation of ERK1/2 and p38. However, there was no significant change in phosphorylation of JNK between OVA-challenged mice and the group treated with Lico A. Our results showed that ERK and p38 signaling pathway may play a role in the anti-inflammatory property of Lico A in the asthma model. While more comprehensive studies are still required to clarify the anti-inflammatory mechanism of Lico A.

Conclusion

Our study demonstrated that Lico A may effectively inhibit the increase in Th2 cytokines, such as IL-4, IL-5 and IL-13 in BALF. In addition, the eosinophil count in BALF was significantly decreased after the treatment of Lico A compared with the OVA-challenged group. Histological studies demonstrated that Lico A substantially inhibited OVA-induced eosinophilia in lung tissue and mucus hypersecretion by goblet cells in the airway. Moreover, Lico A demonstrated strong suppression of Ym-2, AMCase, Muc5ac, E-selectin, CCL11 and CCR3 in the allergic airways. Our findings suggest that Lico A may effectively inhibit the ERK and p38 signaling pathway. So our study supports a novel therapeutic use of Lico A in patients with allergic airway inflammation, and sheds some light on the cure of other hyper-sensitivity disease. The effects of Lico A (50 mg/kg) were similar to those of DEX in some measured results. In any case, we thought that further and more comprehensive studies are still required before the full clinical application.

Declaration of interest

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