Echinacoside alleviates airway remodeling and inflammation in an ovalbumin-induced neonatal mouse model of asthma by modulating the SIRT1-NF-κB pathway

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Abstract

Purpose: Echinacoside (ECH) has been reported to have anti-inflammatory and anti-immune effects, and may be effective for treating asthma. This study aimed to investigate the effect of ECH on asthma.

Methods: A mouse model of asthma was established by ovalbumin (OVA) induction, and the effect of ECH on airway remodeling in mice was evaluated using the Periodic Acid-Schiff stain and enzyme-linked immunosorbent serologic assay (ELISA). Additionally, the effect of ECH on collagen deposition in asthmatic mice was assessed using Western blotting (WB) analysis, and response to airway inflammation was evaluated by ELISA. The signaling pathway regulated by ECH was also investigated using WB.

Results: Our findings demonstrated that ECH restored OVA-induced increase in mucin, immunoglobulin E, and respiratory resistance. ECH also alleviated OVA-induced collagen deposition, including collagen I, collagen III, alpha smooth muscle actin, and epithelial (E)-cadherin. Moreover, ECH restored the elevated levels of interleukin (IL)-13, IL-17, and the increased number of macrophages, eosinophils, lymphocytes, and neutrophils induced by OVA. ECH mainly exerted its regulatory effects by modulating the silent mating type information regulation 2 homolog 1 (Sirtuin 1/SIRT1)-nuclear factor kappa B (NF-κB) signaling pathway in the mouse models of asthma.

Conclusion: This study highlights the therapeutic potential of ECH for attenuating airway remodeling and inflammation in an OVA-induced neonatal mouse model of asthma through the modulation of SIRT1/NF-κB pathway.

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KEYWORDS
asthma; echinacoside; NF-κB; SIRT1

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Introduction

Bronchial asthma (or simply asthma) is a common noninfectious chronic disease that poses a serious threat to human health, affecting approximately 334 million people globally. Pediatric patients are the primary group affected by asthma. Airway remodeling, characterized by an increase in the volume of airway smooth muscles (ASM), is a key feature of persistent asthma. The increase in ASM volume is partly due to the overexpression of extracellular matrix (ECM) around ASM, leading to protein deposition and ASM hyperplasia. Hyperplasia and hypertrophy of smooth muscles not only thicken airway wall, causing airway stenosis and reducing the contractility of airway wall, but also increase the synthesis of ECM and exacerbate the fibrosis of tracheal wall.

Inflammatory responses also occur in asthma, and silent mating type information regulation 2 homolog 1 (Sirtuin 1/SIRT1) is a key gene that regulates numerous pathophysiological processes, including autoimmunity and apoptosis. A previous study showed that serum SIRT1 levels were positively correlated with serum immunoglobulin E (IgE) levels in asthmatic patients. SIRT1 activators inhibit inflammatory cell infiltration and cytokine production in asthmatic mice, whereas SIRT1 deletion exacerbates airway inflammation in a mouse model of allergic asthma. Therefore, it is important to identify a drug that reduces inflammatory response and lowers the expression of key asthma proteins, with minimal adverse events.

ECH can reverse myocardial remodeling, improve cardiac function, and inhibit fibrosis by regulating the expression of the SIRT1/forkhead box O3a (FOXO3a)/superoxide dismutase 2, mitochondrial (MnSOD) signaling axis. Moreover, ECH reduces osteoarthritis by up-regulating SIRT1. Despite these findings, the role of ECH in asthma remains unclear. Therefore, this study aimed to investigate the pharmacodynamic effect of ECH on asthma.

Methods

Animals

Newborn male C57BL/6 mice were purchased from Beijing Weitong Lihua Experimental Animal Technology Co. Ltd. (Beijing, China) and kept in an animal room. The animals had free access to food and water, and the room temperature was maintained at 22°C. To establish a mouse model of asthma, mice were intraperitoneally (i.p.) injected with 10 µg of ovalbumin (OVA; A8041; Solarbio, Beijing, China) on postnatal day (P) 5 and 10, followed by a challenge with 3% aerosol OVA for 10 min every day from P18 to P20. The control group was administered with phosphate-buffered saline (PBS). On P21, the mice were subjected to a physiological assessment of airway function using the FlexiVent device. The left/right main bronchus was subsequently ligated, and the right/left lung was washed thrice with 500-µL sterile saline using endotracheal tube. Bronchoalveolar lavage fluid (BALF) was collected by centrifugation to separate cell pellets. ECH (HY-N0020; MCE, Shanghai, China) was used as a therapeutic drug in this experiment. The following experimental groups were established: control group, OVA group, OVA + ECH (10 mg/kg) group, and OVA + ECH (20 mg/kg) group. Ethical approval was obtained from the Ethics Committee of Yongchuan Hospital of Chongqing Medical University. Complete experimental process with animals is shown in Figure 1.

Histology, staining, and quantification

Serial sagittal paraffin-embedded sections (5 µm) were generated from the middle of the left lung lobe as described previously by Aven et al., followed by rehydration and PBS washing. Mucin was stained using periodic acid-Schiff (PAS; C0142M; Beyotime, Shanghai, China) whereas ASM was immunolabeled using anti-alpha smooth muscle actin (αSMA) antibody (1:100, 251813; Abbiotec, CA, USA) and visualized with 3,3′-diaminobenzidine (DAB). Medium-size airways were imaged using a brightfield microscope (TL4000 BFDF; Leica, Wetzlar, Germany) at 40× magnification. To quantify αSMA density, the observed αSMA-positive area was divided by the length of the basement membrane using the Image J software. In all, three mice and 12 airways were quantified and averaged for each treatment group.

Enzyme-linked immunosorbent serologic assay (ELISA)

Blood and BALF were collected from mice on P21. OVA-specific IgE levels in the serum were measured using an ELISA kit (SEKM-0095; Solarbio, Beijing, China). The levels of IL-13 and IL-17 in BALF were also measured using corresponding ELISA kits (SEKM-0014, SEKM-0018; Solarbio, Beijing, China).

Physiological Measures of Airway Responsiveness

Airway resistance was measured using FlexiVent as described by Yocum et al. Briefly, mice were anesthetized (pentobarbital 50 mg/kg i.p.) and ventilated through...
The role of echinacoside in regulating asthma

tracheotomy (tidal volume, 10 mg/kg, 150 breaths/min, 3 cmH₂O positive end expiratory pressure) using an 18-gauge cannula. Airway resistance was measured during a graded nebulized methacholine challenge (50% duty cycle, 0-50 mg/mL). Airway resistance (respiratory system resistance [RSR]) was compared between groups using two-way ANOVA with matched comparisons and a Bonferroni post-test to compare resistance at each methacholine concentration.

BALF cell count

Bronchoalveolar lavage fluid was collected by rinsing the lungs of mice with 1 mL of pre-cooled PBS for 30 s. Different cell types were then counted in BALF using a hemocytometer. Briefly, after centrifugation of BALF, cell pellet was resuspended in 200 μL of cold PBS and spun on histological slides using a cytospin. Slides were then mounted and stained with Hema3 stain (Biodee, Beijing, China). Approximately 200 cells were counted per BALF sample to determine and compare the relative abundance of different immune cell types.

Western Blotting (WB) Analysis

Mice lung tissues were lysed using radioimmunoprecipitation assay (RIPA) buffer to determine protein concentration. A total of 20-μg protein was mixed with loading buffer and boiled for 5 min at 95°C to denature protein samples. Then, protein samples were loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to electrophoresis using 300-mA electricity for 60-90 min. Then protein samples were transferred onto a polyvinylidene fluoride (PVDF) membrane. The membranes were blocked with 5% non-fat dry milk for 2 h, followed by overnight incubation at 4°C with collagen I (Cat. No. 14695-1-AP, 1:1000; Proteintech, CA, USA), collagen III (Cat. No. 22734-1-AP, 1:1000; Proteintech, CA, USA), αSMA (Cat. No. 14395-1-AP, 1:1000; Proteintech, CA, USA), epithelial (E)-cadherin (Cat. No. 20874-1-AP, 1:1000; Proteintech, CA, USA), SIRT1 (Cat. No. 13161-1-AP, 1:1000; Proteintech, CA, USA), p65 (Cat. No. 10745-1-AP, 1:1000; Proteintech, CA, USA), phosphorylated (p)-p65 (Cat. No. 66535-1-lg, 1:1000; Proteintech, CA, USA), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Cat. No. 60004-1-lg, 1:1000; Proteintech, CA, USA). Next day, the membranes were incubated with specific horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000; Binoway, Beijing, China). Finally, the membranes were incubated with freshly prepared enhanced chemiluminescence (ECL) solution to detect protein signals. Image J was used to analyze blot images.

Statistical Analysis

All data analysis was performed using the GraphPad software. Data were subjected to normality and variance homogeneity tests prior to performing comparisons between experimental groups. Student’s t-test or one-way ANOVA was used if the data were distributed normally and variance was homogeneous, otherwise Wilcoxon signed-rank test was used. P > 0.05 was considered as statistically significant.

Results

Echinacoside relieves OVA-induced airway remodeling in neonatal mice

In order to evaluate the efficacy of ECH, a mouse model of asthma was established using neonatal mice. The mice airway structures started to mature on P21. Apparent asthmatic features were observed in neonatal mice challenged with OVA, compared with those treated with PBS. The main asthmatic manifestation included mucin overexpression, while mice treated with ECH showed a decrease of mucin; treatment with 20-mg/kg ECH showed more pronounced effect than 10-mg/kg ECH (Figure 2A). Furthermore, increased level of serum IgE, which was related to OVA treatment, was significantly reduced by ECH treatment, and the reduction effect by 20-mg/kg ECH was more prominent than that of 10-mg/kg ECH. Respiratory resistance was observed through FlexiVent, particularly reactivity to methacholine in the airway. OVA treatment significantly increased respiratory resistance, while ECH alleviated the

![Figure 2](image-url)

Figure 2 Effect of ECH on airway mucin, IgE expression, and respiratory resistance of mice treated with OVA. (A) Mucin expression in each group. (B) Histograms show IgE expression in each experimental group. (C) Respiratory resistance is expressed by the reaction rate of airway substances with methacholine. N = 3, **P < 0.01 vs control group, &P < 0.01 vs OVA group.
same. In line with other parameters studied as described above, treatment with 20-mg/kg ECH exhibited better effect than 10-mg/kg ECH (Figure 2B).

**Echinacoside reduces collagen deposition**

Airway remodeling, a key feature of persistent asthma, is characterized by abundant extracellular matrix deposition, including collagen.22 The expression levels of collagen I, collagen III, and αSMA after OVA induction were detected by WB. The results showed that, compared with the control group, OVA induced an increase in collagen I, collagen III, and αSMA but reduced the expression of E-cadherin. It was observed that ECH relieved the increase of collagen or reduced the expression of E-cadherin by OVA, and the effect of 20-mg/kg ECH was stronger, compared with that of 10-mg/kg ECH (Figure 3).

**Echinacoside relieves OVA-induced airway inflammation in neonatal mice**

To detect the effect of ECH on OVA-induced inflammatory response, ELISA was performed. As shown in Figure 4A, compared with the control group, OVA significantly increased the levels of interleukin (IL)-13 and IL-17, while ECH moderated this increase. The effect of 20-mg/kg ECH was greater than that of 10-mg/kg ECH. Further, detection of the number of cells in BALF revealed that, as shown in Figure 4B, compared with the control group, OVA treatment significantly increased the total cell number and the number of macrophages, eosinophils, lymphocytes, and neutrophils. ECH consistently moderated this rising trend, and the effect of 20-mg/kg ECH was greater than that of 10-mg/kg ECH. Taken together, our findings showed that ECH attenuated OVA-induced airway inflammation in neonatal mice.

**Echinacoside regulates the SIRT1/NF-κB pathway**

To further study the mechanism of ECH, the expression levels of SIRT1, p65, and p-p65 were detected using WB. The results shown in Figure 5 demonstrated that OVA significantly reduced the protein expression level of SIRT1, compared with the control group. However, ECH moderated this downward trend, and 20-mg/kg ECH restored the protein expression level of SIRT1 to the same level as that of the control group. In contrast, the phosphorylation level of p65 (p-p65) was significantly increased following the OVA treatment, but ECH relatively slowed down this rising trend as well. A dosage of 20-mg/kg ECH inhibited the phosphorylation level of p65 largely, compared with that of 10-mg/kg ECH.

**Discussion**

In this study, we successfully established a mouse model of asthma induced by OVA. This model demonstrated the key features of persistent asthma, such as overproduction of mucin, high expression of IgE, and increased respiratory resistance. These findings were consistent with results of the previous research,23–25 which supported the validity of our asthma mouse model. We demonstrated that ECH was effective in relieving the aforementioned features, indicating its therapeutic potential. This was consistent with the previous study conducted with a cocktail of four herbal medicines, DFSG (Eucommia ulmoides Oliv., Aconitum carmichaeli Debx., Cornus officinalis Sieb, and Lycium chinense Mill), which was found to reduce the expressions of IgE and mucin genes.26

Furthermore, this study found that ECH treatment reduced collagen deposition caused by OVA. This was in agreement with the previous study comprising cruciferous seeds, which caused a significant decrease in the expression levels of collagen I, collagen III, and αSMA, compared with that of the untreated control group.27 In addition, the expression of E-cadherin, which played a role in promoting the interaction of actin filaments in epithelial cells, was reduced in asthma, resulting in damaged epithelial barrier function.28 Andrographolide (AGP), a natural diterpene lactone, has been shown to restore E-cadherin expression in bronchial epithelial cells of Toluene diisocyanate (TDI)-exposed (asthmatic) mice.29 Our findings were in line with that of the existing studies and suggested a potential therapeutic effect of ECH.
The role of echinacoside in regulating asthma

Additionally, a study showed that gentiopicroside ameliorated OVA-induced airway inflammation in allergic asthmatic mice by modulating the SIRT1/NF-κB signaling pathway. All these studies pointed toward SIRT1/NF-κB pathway, indicating that it was a key signaling pathway activated in asthma, and targeting this pathway could be beneficial for the treatment of asthma. The results of this study also indicated ECH as a promising target for the treatment of asthma.

Limitation

Although the findings of this study supported the notion of ECH as a promising target for the treatment of asthma, there were some limitations. Classic biomarkers of asthma, such as periostin and sputum eosinophils, need to be investigated in the future research to validate our findings. Additionally, the highest dosage of ECH tested in this study was 20 mg/kg. Higher dosages may be necessary to test the minimum effective dosage of the drug. Finally, in vitro experiments should be included in the future study to verify further the findings of this study.
References

Both authors had read and approved the final manuscript. and reviewed the draft of the manuscript for publication. analyzed and interpreted the data. Yunbo Pan prepared experiment, and supervised the data collection. Yijun Liu in the present study are available from the corresponding author on reasonable request.

Competing interests

The authors stated that there was no conflict of interest to disclose.

Author Contributions

Yunbo Pan and Yijun Liu designed the study, completed the experiment, and supervised the data collection. Yijun Liu analyzed and interpreted the data. Yunbo Pan prepared and reviewed the draft of the manuscript for publication. Both authors had read and approved the final manuscript.

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