

Effects of Baicalin on Airway Remodeling in Asthmatic Mice

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Key words

- baicalin
- airway remodeling
- TGF- β_1
- VEGF
- IL-13
- ERK

Abstract

Airway remodeling is an important characteristic of asthma, linking inflammation with airway hyperresponsiveness. Baicalin, a major active component, was isolated from *Radix Scutellariae*. Many studies show that baicalin has anti-inflammatory, anti-bacterial, and anti-allergic effects. Here we investigate the influence of baicalin on asthmatic airway remodeling and the mechanism underlining the anti-remodeling effect *in vivo*.

Asthmatic airway remodeling mice model was established by ovalbumin exposure. Seventy female BALB/c mice were randomly assigned to seven experimental groups: blank, ovalbumin, hexadecadrol, control, and baicalin (25 mg/kg, 50 mg/kg, 100 mg/kg) groups. Pulmonary function was measured using a whole-body plethysmograph in conscious and unrestrained mice. The lung pathology was observed and measured. The production of cytokines in bronchoalveolar lavage fluid and serum was measured using enzyme-labeled immunosorbent assay kits, and the expression levels of transforming growth factor- β_1 and vascular endothelial growth factor were detected by immunohistochemistry. The protein expression levels of transforming growth factor- β_1 , vascular endothelial growth factor, extracellular signal-regulated kinase, and p21ras were measured using Western blot. The results show that ovalbumin exposure significantly increased the expression of interleukin-13 in BALF and serum, and transforming growth factor- β_1 , vascular endothelial growth factor, extracellular signal-regulated

kinase and p21ras expressions in the lungs. Baicalin attenuated the effects of ovalbumin significantly.

It can be concluded that baicalin has significant anti-remodeling effect on ovalbumin-induced asthmatic airway remodeling mice model by decreasing expression of transforming growth factor- β_1 , interleukin-13, and vascular endothelial growth factor and inhibiting the activation of the extracellular signal-regulated kinase pathway.

Abbreviations

AHR:	airway hyperresponsiveness
AIOD:	average integral optical density
ASMC:	airway smooth muscle cells
BALF:	bronchoalveolar lavage fluid
ELISA:	enzyme-labeled immunosorbent assay
ERK:	extracellular signal-regulated kinase
GINA:	global initiative for asthma
HE	hematoxylin and eosin
IHC:	immunohistochemistry
IL-13:	interleukin-13
N:	amount of ASMC nucleus
OVA:	ovalbumin
Pbm:	bronchial basement membrane perimeter
Penh:	enhanced pause
TGF- β_1 :	transforming growth factor- β_1
VEGF:	vascular endothelial growth factor
Wai:	inner airway area
Wam:	smooth muscle area

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Introduction

Bronchial asthma is a serious and widespread disease affecting the world-wide population. It is now estimated that as many as 300 million people of all ages and all ethnic backgrounds suffer from

asthma [1]. The burden of this disease on governments, health care systems, families, and patients is increasing. As a result, strengthening clinical and basic research on bronchial asthma is the interest of the global community. Bronchial asthma in GINA reports is defined as chronic inflamma-

tory disease of the airway involving different cell types and cell components, influenced by genetic and environmental factors and having a strong correlation with AHR. Asthma is a disease characterized by episodic airflow obstruction that is at least partially reversible [2].

There are many causes of asthma, but the precise mechanism is not clear. In recent years, a number of pathological studies have shown that not only is inflammation of the airways and AHR apparent, but pathological modifications of the bronchial airways structure also occurred. These structural changes are termed as airway remodeling [3]. Remodeling refers to structural changes that occur in organs and tissues as a result of disease. Airway remodeling refers to the changes that occur in and around the trachea, bronchi, and bronchioles specifically [4].

Airway remodeling is evident as the following: subepithelial fibrosis, increased airway smooth muscle, increased vascularization of the airway wall, goblet cell, and submucosal gland hyperplasia [5,6].

Airway remodeling is believed to start as a repair process in response to airway injuries resulting from sustained inflammation or mechanical stretch [7,8]. Additionally, recent data suggests that the extent of airway remodeling is associated with progression of airway hyperresponsiveness and the severity of asthma. The consequences of airway remodeling are increased AHR, fixed airflow obstruction, and irreversible loss of lung function. Airway remodeling has been recognized as an important characteristic of asthma, and therefore understanding its molecular mechanism and looking for a drug to modify it is of great importance to asthma treatment [9].

In addition to the role of inflammatory cells in remodeling, structural cells, including ASMC, epithelial cells, and fibroblasts may participate in increasing inflammation and remodeling through the release of cytokines, chemokines, and extracellular matrix proteins [7]. Many studies on airway remodeling in asthma have focused on the cytokines, chemokines, and growth factors involved in this process. Several mediators of remodeling have been identified including TGF- β , IL-13, and VEGF [10].

An increase in airway smooth muscle mass is one of the best-known features of asthmatic remodeling. Recently, it has been shown that the ERK signaling pathway plays an important role in the proliferation of ASMC [11]; its inhibition reduces ASMC proliferation and the extent of remodeling.

Years of clinical researches show that *Scutellaria baicalensis* can protect the lung tissue and reduce the degree of asthma. Baicalin, a major active component isolated from plants of the *Scutellaria baicalensis* family, has also been studied in detail. Results of these studies showed that baicalin has anti-inflammatory, anti-bacterial, and anti-allergic effects [12,13], but few articles have studied the effectiveness of baicalin treatment on asthmatic remodeling.

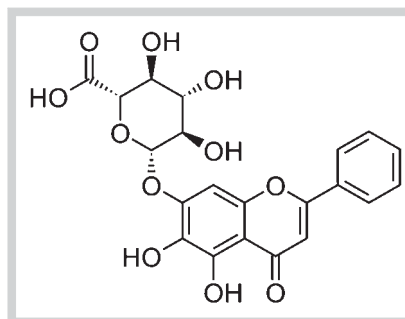


Fig. 1 Chemical structure of baicalin.

Materials and Methods

Baicalin

Baicalin was purchased from Chengdu Mansite Biotechnology Co. Ltd. (purity>99.8%; molecular formula: $C_{21}H_{18}O_{11}$; molecular weight: 446.4). Its chemical structure is shown in **Fig. 1**. Taking into consideration the content of baicalin in *Scutellaria baicalensis* and the conversion coefficient of drug dosage between human and mice, we figured out the baicalin dosage for mice.

Animal studies

All experimental procedures (including seventy BALB/c mice and 60 days experimental period) were conducted in conformity with institutional guidelines of the Fudan University, Shanghai, China, which conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Laboratory animal numbers: SCXK (Shanghai 2008-0016); animal licenses: SYXK (Shanghai 2010-0099); animal certification numbers: 2008001606181.

Four- to six-week-old female BALB/c mice weighing 18–22 g were obtained from the Department of Laboratory Animal Science, School of Medicine, Fudan University. The animals were housed under specific pathogen-free conditions in a temperature- and humidity-controlled environment and given *ad libitum* access to water and food. Mice were housed for 7 days for acclimation before experiments. The whole protocol was approved by the Institutional Animal Care and Use Committee of the Fudan University. Mice were randomly assigned to 7 experimental groups ($n = 10$ per group).

Experimental groups were as follows. Group A, blank group, did not receive any treatment. Group B, OVA group, was sensitized and challenged with OVA (grade V, Sigma) according to the protocol described previously [14]. Briefly, on day 0, mice were intraperitoneally injected with 100 μ g of OVA precipitated with 1 mg of aluminum hydroxide gel in 0.2 mL saline. This was repeated on day 8 for sensitization. On day 15, each animal was placed into an individual chamber and inhaled 1% OVA to challenge them for 42 consecutive days, every time for 30 minutes, to replicate asthmatic remodeling (**Fig. 2**).

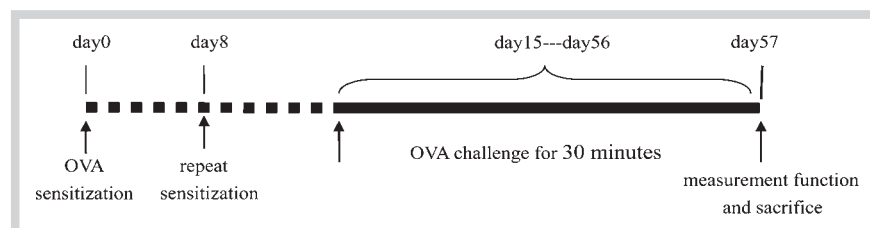


Fig. 2 Protocol and measurements for the mice model of asthmatic remodeling.

Groups C, D, and E were baicalin low-, moderate-, and high-dose groups. Mice in these groups were sensitized and challenged as described in the OVA group above, to simulate asthmatic remodeling. Simultaneously from day 15 to 42, they were administered (gavage) with 25, 50, and 100 mg/kg baicalin solution in 0.3 mL one hour before OVA challenge, respectively. Baicalin solution was dissolved in DMSO (Sigma). The concentration of DMSO in the solution was always < 1% vol. to prevent possible toxicity. Group F was the hexadecadrol group. Mice in this group were administered 1 mg/kg hexadecadrol (Shandong Xinhua Pharmaceutical Co., Ltd.; purity $\geq$ 97%). The process of treatment was the same as in group C. Group G was the control group. Animals were placed into the same apparatus as described in the OVA group and administered normal saline instead of monomer.

Measurement of pulmonary function

The technique for the non-invasive respiratory function measurements used in this study has been described previously [15]. Pulmonary function was measured by whole-body plethysmography (Buxco's unrestrained WBP systems) in conscious and unrestrained mice. Subjects can move freely in the chambers for minimum stress and be studied under the most natural conditions. Buxco's WBP systems include the most modern plethysmograph chambers, utilizing their patented pneumotachographs for the quietest baselines available. The WBP application involves measuring a "box flow" which is the net (or sum) of nasal and thoracic flows. Actual flows are calculated in the software, taking into account temperature, humidity, and pressure.

The systems are often specified for asthma studies using the Penh index of airway hyper-reactivity as an indicator of changes in airway resistance, and original studies reporting the use of Penh showed that it correlated closely with airway resistance.

Following the respiratory function measurements, the mice were anaesthetized with 1% sodium pentobarbitone (wt/vol) at a dose of 50 mg/kg by intraperitoneal injection, then blood and tissues were obtained for analysis.

BALF preparation

Mice were anaesthetized, and a tracheal cannula was inserted via a midcervical incision; the right lung was ligated and the airway of each mice was lavaged 3 times with 1 mL of PBS. Then 1 mL was collected and immediately centrifuged. The supernatant was collected and stored at -80°C .

Histopathological evaluation

The lungs were removed with one section fixed in 10% neutral-buffered formalin, embedded in paraffin, cut into 4 μm sections and stained with HE, another was stored at -80°C .

The histological slides with HE stain were read by light microscope at high power (200 $\times$). Histopathological assessment in the bronchus was performed in a blind fashion. The specific method was as follows: 3 bronchus were randomly chosen per slide, with photos taken of sections that are circular with diameter about 200–400 μm and major/minor axis $\leq$ 1.2.

To utilize Image-pro Plus medical image analysis system, parameters including Pbm, Wam, Wai, and N were standardized using Pbm and recorded.

Immunohistochemistry assay

IHC stain was performed using a two-step EnVision/HRP technique according to the manufacturer's instruction. Polyclonal rabbit antihuman TGF- β_1 and VEGF antibodies (dilution 1:100)

were obtained from Santa Cruz Biotechnology; the omission of primary antibodies was used as the negative control. For three slides, cytoplasm stained with brown was scored as positive. The expression of TGF- β_1 and VEGF was quantitatively evaluated using Olympus Cx31 microscope with Image-pro Plus medical image analysis system. The digital images were captured using a digital camera (Canon A640). The positive area and OD of TGF- β_1 and VEGF positive cells were determined by measuring three randomly selected microscopic fields (25 $\times$ 10) for each slide. The IHC index was defined as average integral optical density (AIOD) (AIOD = positive area $\times$ OD/total area).

Measurement of IL-13 in BALF and serum

After treatment, serum was separated by centrifuging at 1200 g for 15 min at 4°C . Aliquot serum was stored at -80°C for use. The concentrations of IL-13 in BALF and serum were determined using ELISA kits (obtained from R&D), according to the manufacturer's instructions.

Western blotting analysis

Proteins were extracted from each lung tissue sample using the cell lysis buffer kit with the following addition to the kit made just prior to use: PMSF (1 mM). The protein concentrations were determined using a modified BCA assay; the supernatant was stored at -80°C for analysis.

After sample preparation, proteins were separated by 13% SDS-PAGE and then transferred to the polyvinylidene fluoride membranes. The membranes were blocked in a PBS-Tween buffer with 5% nonfat milk at room temperature for 2 h. The membranes were then probed using antibodies specific for antihuman TGF- β_1 (1:1000 Santa Cruz), antihuman VEGF (1:1000 Santa Cruz), antihuman ERK (1:1000 Santa Cruz), antihuman p21ras (1:1000 Santa Cruz), or antihuman GAPDH (1:1000 Santa Cruz) in the blocking buffer overnight at 4°C , with continuous shaking. The membranes were then washed with TBS-T and subsequently incubated with secondary antibodies conjugated to horseradish peroxidase (1:1000) for 2 h. The staining was visualized using ECL reagents. The densitometric evaluation was carried out using UN-SCAN-IT gel analysis software (version 6), and the relative quantity of an individual protein was expressed as the ratio of the gray scale respective to that of GAPDH.

Statistical analysis

Data were from three independent experiments and expressed as mean $\pm$ SEM. Statistical analyses were performed by the Student's t-test for paired data and the one-way analysis of variance (ANOVA) for differences between treatment groups. All analyses were undertaken using statistic software SPSS 16.0. A p-value less than 0.05 was considered statistically significant.

Results



Whole-body barometric plethysmography was used to noninvasively assess baseline lung function in mice. Airway responsiveness to methacholine was measured at 24 h after last airway challenge with OVA. Penh, an index of bronchoconstriction, was measured at baseline and after sequential delivery of increasing concentrations of methacholine (3.125–50 mg/mL).

The OVA group exhibited substantially enhanced bronchial responsiveness to inhaled methacholine compared with the blank group (● Fig. 3A). However, baicalin high- and moderate-dose

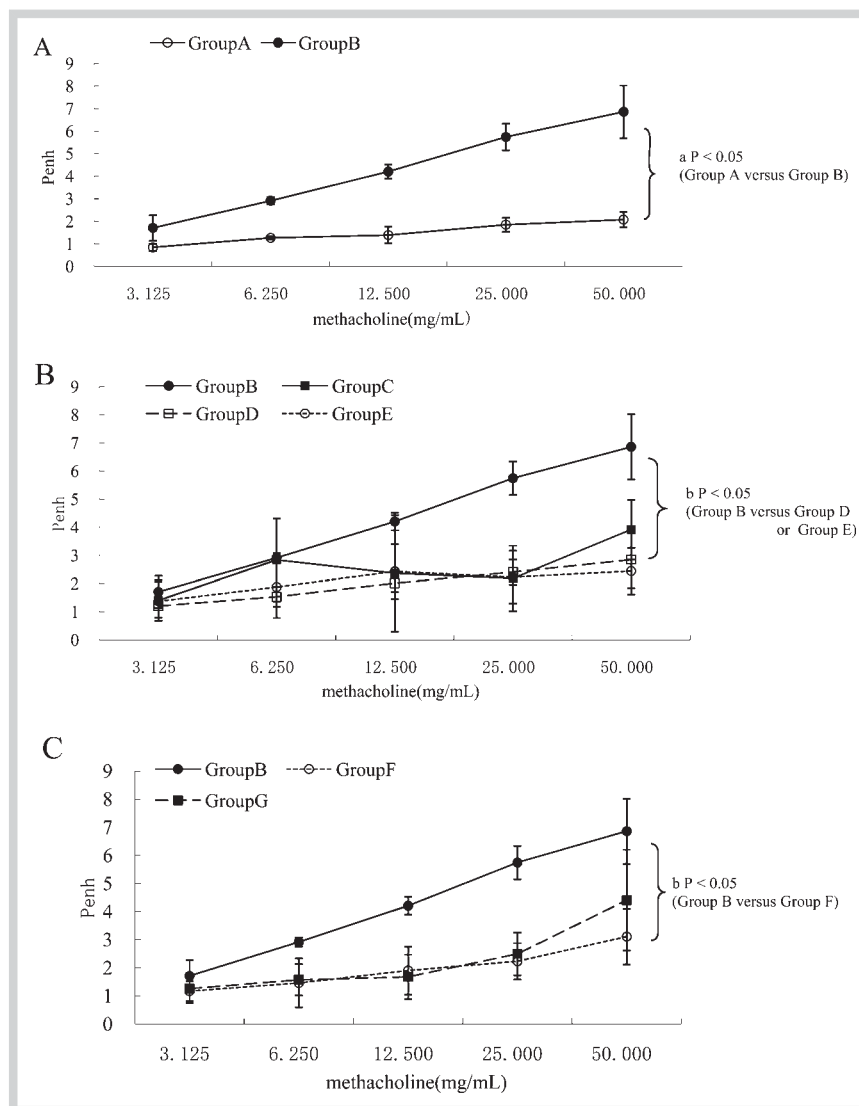


Fig. 3 Responsiveness to inhaled methacholine was measured using whole-body barometric plethysmography in awake, unrestrained mice. Group size: $n = 10$. Group A: blank group; Group B: OVA group; Groups C, D, E: baicalin low-, moderate-, and high-dose group; Group F: hexadecadrol group; Group G: control group. **A** Group A versus Group B ($^a p < 0.05$); **B** Group D and Group E versus Group B ($^b p < 0.05$); **C** Group F versus Group B ($^b p < 0.05$).

group mice exhibited dramatically reduced bronchial responsiveness to inhaled methacholine compared with the OVA group, while there was no significant difference in methacholine responsiveness between the baicalin low group and the OVA group (● Fig. 3B).

Compared with the OVA group, bronchial responsiveness to inhaled methacholine in the hexadecadrol group mice was significantly reduced, and bronchial responsiveness of the control group had no obvious increase (● Fig. 3C).

According to histological pictures of lung tissue, we found that the alveolar wall was thin and smooth and the alveolar space had no exudate and inflammatory cell infiltration in the histology of the lung tissue of the blank group. The histology of the lung tissue of the OVA group at higher magnification showed narrowed tracheal lumen, thickened bronchial mucosa, and ASM. Using Image-pro Plus medical image analysis, we measured three parameters including Wam/Pbm, Wai/Pbm, and N. Measurement results of Wai/Pbm displayed that OVA group exhibited a substantial increase compared with the blank group, whereas the airway area in the baicalin high-dose group had significantly decreased as compared to the OVA group (● Fig. 4A). In addition, there were significant differences in Wam/Pbm between baicalin moderate and high groups, and OVA group (● Fig. 4B). Measurement re-

sults of N showed that the baicalin high-dose group had a significant reduction as compared to the OVA group (● Fig. 4C). The hexadecadrol and control groups showed no significant improvement compared with the OVA group. Histological pictures of lung tissue are shown in ● Fig. 4D.

In each group, there was universal cytoplasmic TGF- β_1 positive immunostaining, and scattered nuclear TGF- β_1 positive immunostaining was found. The percentage of TGF- β_1 immunostaining positive area and IOD are given in ● Fig. 5A and B in detail. Compared with the blank group, the percentages of positive area and IOD in the OVA group increased markedly. The percentage of positive area and IOD in baicalin low, moderate, and high groups was significantly lower in comparison with the OVA group. In addition, in the hexadecadrol group there was a significant reduction compared with the OVA group.

We can see universal cytoplasmic VEGF positive immunostaining in each group. The analytic data of the percentage of VEGF immunostaining positive area and IOD in detail are given in ● Fig. 6A and B. The percentage of positive area and IOD in the OVA group was markedly higher compared with the blank group. There was a sensible difference in the percentage of positive area and IOD between baicalin moderate and high group and OVA group. The hexadecadrol group showed no dramatic reduction compared

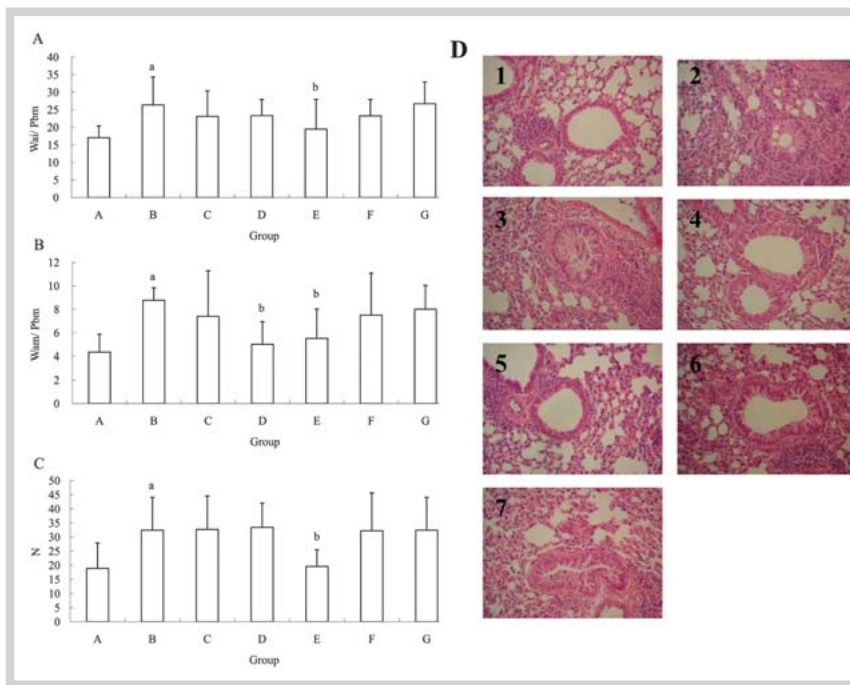


Fig. 4 A Wai/Pbm measurement; B Wam/Pbm measurement; C N measurement. Results are expressed as means $\pm$ SEM (group size: $n = 10$). Group A: blank group; Group B: OVA group; Groups C, D, E: baicalin low-, moderate-, and high-dose group; Group F: hexadecadrol group; Group G: control group. ^a $p < 0.05$ versus blank group; ^b $p < 0.05$ versus OVA group. **D** Micrographs of lung tissue (HE staining) were observed under a microscope (200 $\times$). (1) Lung from blank group; (2) lung from OVA group; (3), (4), (5) lung from 25 mg, 50 mg, and 100 mg baicalin group, respectively; (6) lung from hexadecadrol group; (7) lung from control group. (Color figures available online only.)

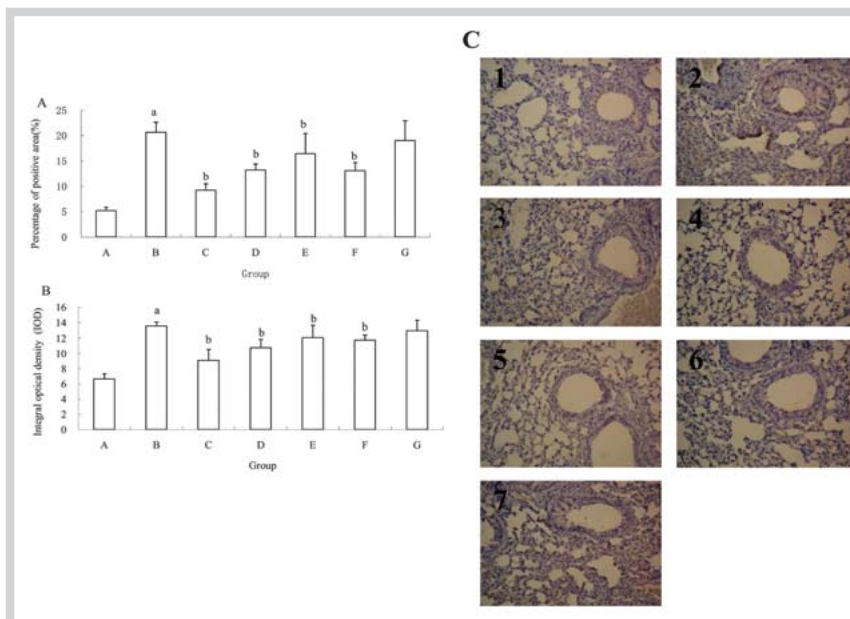


Fig. 5 Immunostaining of TGF- β_1 . **A** Percentage of positive area; **B** IOD. Results are expressed as means $\pm$ SEM (group size: $n = 10$). Group A: blank group; Group B: OVA group; Groups C, D, E: baicalin low-, moderate-, and high-dose group; Group F: hexadecadrol group; Group G: control group. ^a $p < 0.05$ versus blank group; ^b $p < 0.05$ versus OVA group. **C** Micrographs of TGF- β_1 immunostaining were observed under a microscope (200 $\times$). (1) Lung from blank group; (2) lung from OVA group; (3), (4), (5) lung from 25 mg, 50 mg, and 100 mg baicalin group, respectively; (6) lung from hexadecadrol group; (7) lung from control group. (Color figures available online only.)

with the OVA group. The photomicrographs of TGF- β_1 and VEGF immunostaining are shown in **Fig. 5C** and **6C**.

Mice IL-13 levels were quantitated in BALF and serum using ELISA's specific for each cytokine. They are shown in **Fig. 7**. Levels of IL-13 increased markedly after OVA stimulation both in BALF and serum, whereas baicalin treatment can inhibit the cytokines release. It may also be seen that baicalin can inhibit cytokines release while a certain drug concentration prevails. Results of ELISA showed that cytokines levels did not significantly decrease after hexadecadrol intervention.

To verify the IHC, we further examined the expression of TGF- β_1 and VEGF using Western blots. Compared with the blank group, the expression levels of TGF- β_1 and VEGF were significantly increased in the asthmatic remodeling mice. However, in a certain

concentration in the baicalin groups, the expression levels of TGF- β_1 and VEGF significantly decreased. They are shown in **Fig. 8**.

The expression levels of ERK and p21ras were substantially increased after OVA exposure, whereas the expression levels of ERK and p21ras significantly decreased in the baicalin group. They are shown in **Fig. 9**.

Discussion

The concept of airway remodeling in asthma was first proposed in 1922 by Huber and Koessler in cases of fatal asthma, but its importance did not arouse enough attention. Recently, researches

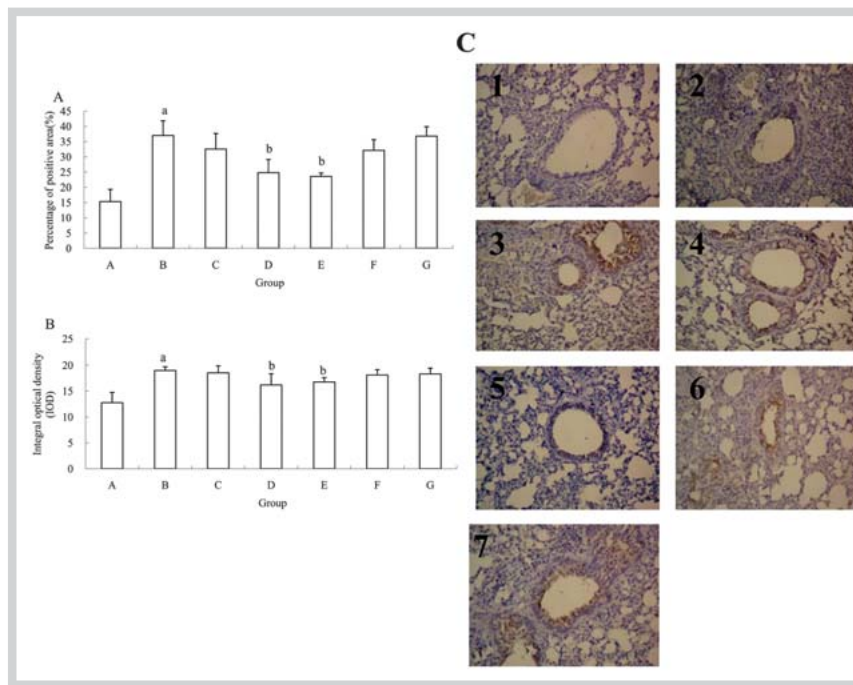


Fig. 6 Immunostaining of VEGF. **A** Percentage of positive area; **B** IOD. Results are expressed as means $\pm$ SEM (group size: $n = 10$). Group A: blank group; Group B: OVA group; Group C, D, E: baicalin low-, moderate-, and high-dose group; Group F: hexadecadrol group; Group G: control group. ^a $p < 0.05$ versus blank group; ^b $p < 0.05$ versus OVA group. **C** Micrographs of VEGF immunostaining were observed under a microscope (200 $\times$). (1) Lung from blank group; (2) lung from OVA group; (3), (4), (5) lung from 25 mg, 50 mg, and 100 mg baicalin group, respectively; (6) lung from hexadecadrol group; (7) lung from control group. (Color figures available online only.)

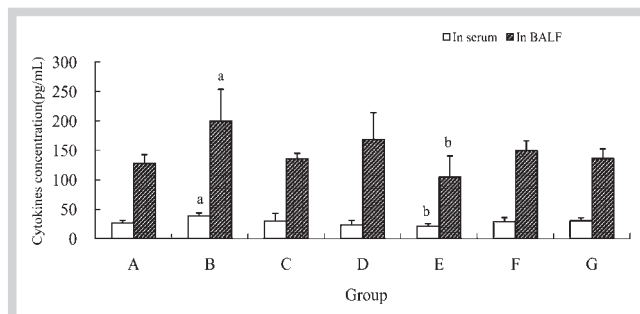


Fig. 7 Cytokines concentration. Results are expressed as means $\pm$ SEM (group size: $n = 10$). Group A: blank group; Group B: OVA group; Group C, D, E: baicalin low-, moderate-, and high-dose group; Group F: hexadecadrol group; Group G: control group. ^a $p < 0.05$ versus blank group; ^b $p < 0.05$ versus OVA group.

have shown that different degrees of airway remodeling occurred in the majority of patients with bronchial asthma [16]. These modifications are the pathological results of irreversible airflow obstruction, persistent airway hyperresponsiveness, and refractory asthma. Furthermore asthmatic airway remodeling has also been associated with persistent and progressive pulmonary function damage [17].

Searching for effective medication to prevent and treat asthmatic airway remodeling has become a new direction for research and a new strategy for treatment of asthma [9, 18]. But at present, the treatment of airway remodeling is ineffective. Corticosteroids effect on airway remodeling is still disputable [19] and the effect of leukotriene inhibitors has also been investigated [20]. Some studies suggested it may reverse some changes of pathogenesis such as ASM thickening. However, further investigations are required to confirm the effect of leukotriene inhibitors on tissue remodeling. Chinese medicine offers multi-channel, multi-target, and multi-link overall conditioning benefits and a good prospect, compared with modern medicine. In recent years, studies have

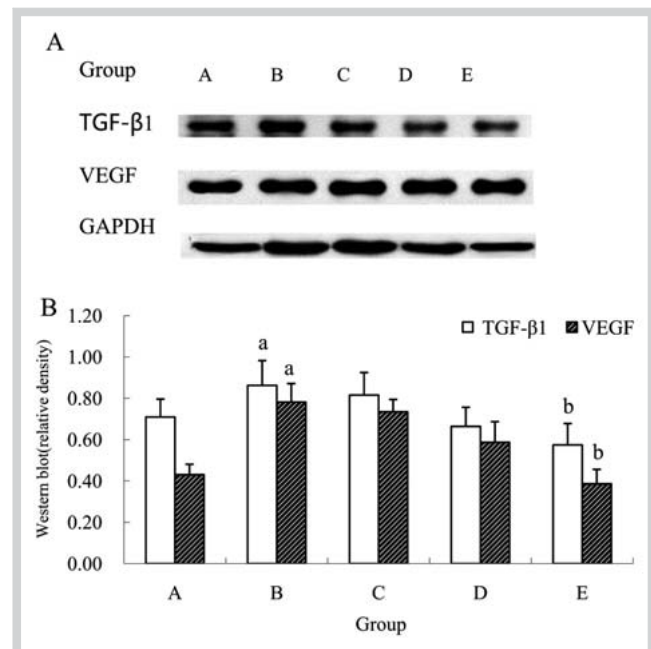


Fig. 8 **A** A representative Western blot visualizing TGF- β 1 and VEGF expression levels. GAPDH was used to demonstrate equal loading. **B** The densitometric quantification of individual protein is expressed as the fold change compared to GAPDH. Data are presented as the means $\pm$ SEM of triplicate experiments, and similar results were seen in all experiments. ^a $p < 0.05$ versus blank group; ^b $p < 0.05$ versus OVA group.

begun to pay attention to the role of Chinese medicine in airway remodeling.

Animal models of asthma have been very helpful in exploring and clarifying the mechanisms of the disease. Mouse models of allergen exposure designed to mimic asthma have been used extensively to study airway remodeling [21]. The various components of airway remodeling described in human asthma have been suc-

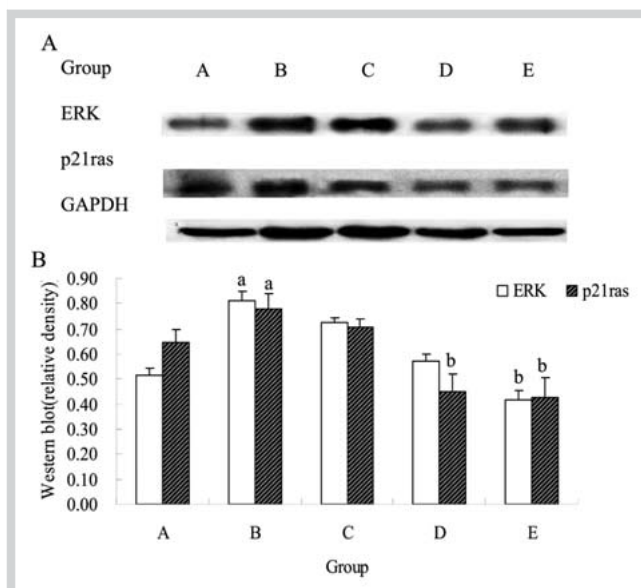


Fig. 9 A A representative Western blot visualizing the ERK and p21ras expression levels. GAPDH was used to demonstrate equal loading. B Densitometric quantification of individual protein is expressed as the fold change compared to GAPDH. Data are presented as the means $\pm$ SEM of triplicate experiments, and similar results were seen in all experiments. ^a $p < 0.05$ versus blank group; ^b $p < 0.05$ versus OVA group.

successfully reproduced in mice animal models [22]. Experimental airway remodeling in animals should be an essential tool for treatment discovery in the near future. OVA exposure remains a common method used to model asthma in mice [23]. In this study, mice were exposure to OVA for 30 minutes (every day) consecutively for 42 days to stimulate airway remodeling. Our asthmatic airway remodeling mice model showed characteristic pathological changes of airway remodeling in the trachea and bronchi. These pathological changes in mice are consistent with human asthma airway remodeling. Penh of the OVA group exhibited substantially enhanced bronchial responsiveness to inhaled methacholine compared with the blank group. It indicated that OVA exposure induced a significant increase of Penh. In addition, pathological pictures of lung tissue were shown to have characteristic tracheal lumen narrowing, bronchial mucosa, and ASM thickening. Measured results of three critical parameters of evaluating airway remodeling suggested that the OVA group showed significantly increased remodeling compared with the blank group. These findings confirmed that our asthmatic mice model of airway remodeling was successful.

Baicalin 100 mg- and 50 mg-dose group mice exhibited dramatically reduced bronchial responsiveness to inhaled methacholine compared with the OVA group. The result of the three parameters measured indicated that the baicalin group had significantly improved as compared to the OVA group. It revealed that at certain concentrations baicalin had a pulmonary function protecting effect on mice with asthmatic airway remodeling.

A large number of mediators have been described in airways of asthmatics that could theoretically be relevant to airway remodeling. In many cases, the presence of these mediators correlate with the severity of airway remodeling and other clinical features [9]. TGF- β_1 is the most heavily studied mediator of tissue fibrosis and has been shown to play an important role in promoting fiber proliferation. TGF- β_1 promotes the differentiation of fibroblast

precursors to myofibroblast cells as well as triggers their proliferation [24]. TGF- β_1 also causes accumulation of extracellular matrix and formation of tissue fibrosis through activating Smad protein families. We have recently shown the role of TGF- β_1 in inducing smooth muscle proliferation and enhancing matrix production. Furthermore many substances including TGF- β_1 have been confirmed to promote ASM cell migration towards the epithelial layer, a mechanism that contributes to tissue remodeling [25]. All these diverse properties of TGF- β_1 support its role in airway remodeling.

From the immunohistochemistry data, TGF- β_1 increased significantly after OVA exposure. However, after baicalin treatment, TGF- β_1 levels in certain concentrations of baicalin groups significantly decreased compared with the OVA group. Similarly the results of Western blotting showed that the expression levels of TGF- β_1 significantly increased in the asthmatic mice, compared with the blank group. In baicalin groups, the expression levels of TGF- β_1 significantly lowered. Immunohistochemistry and Western blots of TGF- β_1 show its activation after OVA exposure, and that baicalin suppressed its expression.

In recent years, Th-2-derived cytokines play a key role in airway remodeling. These cytokines stimulate mast cells, promote leukocytosis and may also participate in the characteristic airway remodeling of asthma IL-13, one of Th-2 cytokines playing important roles in airway remodeling in asthma. IL-13 was shown to be a potent inducer of airway fibrosis. In addition, in the IL-13 over-expressed mice, IL-13 is closely related to increased mucus production and subepithelial fibrosis [26,27].

Mice IL-13 levels were measured in BALF and serum using ELISAs. The results all displayed that after OVA stimulation, levels of IL-13 increased markedly in both BALF and serum, whereas at certain concentrations baicalin can inhibit these cytokines release.

Analysis of TGF- β_1 and IL-13 data suggest that baicalin may inhibit fibrosis by means of decreasing expression of TGF- β_1 and IL-13, and this hints at the mechanism by which baicalin can alleviate airway remodeling in asthma.

Angiogenesis is a basic characteristic of airway remodeling. The increased vascularity may be due to many factors, in particular VEGF. In recent years, VEGF has been shown to be a multifunctional angiogenic regulator that stimulates epithelial cell proliferation and blood vessel formation. Asthmatics have higher VEGF levels, and levels correlate directly with disease activity and inversely with airway caliber and airway responsiveness. Studies on VEGF also suggest that it induces tissue remodeling and inflammation in transgenic mice [28,29].

From the immunohistochemistry and Western blots data, VEGF increased significantly after OVA exposure. However, VEGF in certain concentrations in the baicalin groups significantly decreased after baicalin treatment. This showed that baicalin may inhibit angiogenesis via decreasing expression levels of VEGF.

Thickening of the ASM layer is the most typical feature observed during airway remodelling in asthma. Proliferation has been suggested as one of the main causes of hyperplasia. The mechanisms regulating smooth muscle proliferation have been the subject of numerous reviews [30]. ERK activation appears to be the dominant signal transduction pathway for proliferation of ASM cells [11].

According to the analysis of the pathological picture, N of the baicalin treatment group decreased significantly compared with the OVA group. This suggests that baicalin can inhibit proliferation of ASM. Based on this, we considered whether baicalin can inhibit proliferation of ASM cells by suppressing activation of ERK path-

way. We tested ERK and p21ras, which are key cytokines in the ERK pathway. Western blot results show that after OVA exposure, the expression levels of ERK and p21ras were significantly increased, whereas the expression levels of ERK and p21ras significantly decreased in the baicalin group. This indicates that baicalin inhibit their expression and suppress activation of ERK pathway, leading to inhibition of the proliferation of ASM.

In conclusion, this study agrees with the hypothesis that baicalin can alleviate airway remodeling in asthma, and our results demonstrate that through decrease of the expression of TGF- β_1 , IL-13, and VEGF, and inhibition of the activation of the ERK pathway. In future experiments, further and more in-depth investigation of the mechanisms by which baicalin alleviate asthmatic airway remodeling should be carried out, for example, validation of these results *in vitro*, to better illustrate the mechanism by which baicalin has anti-remodeling effect through inhibiting the proliferation of ASMC.

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Conflict of Interest

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

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