Bixin Protects Mice Against Bronchial Asthma Though Modulating PI3K/Akt Pathway

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Research

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Abstract

**Background:** Accumulating evidence has implicated the potential of natural compounds in treatment of asthma. Bixin is a natural food coloring isolated from the seeds of *Bixa Orellana*, which possesses antitumor, anti-inflammatory and antioxidative properties. Nevertheless, its therapeutic effect in asthma has not been elucidated.

**Methods:** Acute and chronic asthma models of Balb/c mice were established by ovalbumin (OVA) sensitization. For the establishment of a glucocorticoids (GCs) resistant asthma model, Freund's Adjuvant (CFA) was injected intraperitoneally with OVA. After Bixin treatment, cells in Bronchoalveolar lavage fluid (BALF) were stained with Diff Quick staining and the levels of cytokines were measured by enzyme linked immunosorbent assay (ELISA). The levels of protein in cells and tissues were determined by immunoblotting and/or immunostaining with specific antibodies. The histological changes were determined by Hematoxylin and eosin (H&E), PAS and MASSON staining.

**Results:** Our present study demonstrated that administration of Bixin suppressed allergic airway inflammation and reversed GCs resistance, as well as alleviated airway remodeling and airway hyperresponsiveness (AHR) in asthmatic mice. *In vitro* studies showed that Bixin treatment could inhibit the development of epithelial-mesenchymal transition (EMT) mediated by transforming growth factor beta (TGF-β) signaling. Importantly, Bixin antagonized activation of phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) pathway both *in vitro* and *in vivo*.

**Conclusions:** Above all, our findings reveal that Bixin functions as a potent antagonist of PI3K/Akt signaling to protect against allergic asthma, highlighting a novel strategy for asthma treatment based on natural products.

**Background**

Asthma is one of the most prevalent chronic respiratory diseases, characterized by airway hyperresponsiveness (AHR), airway inflammation and remodeling [1]. Approximately 235 million people worldwide are estimated to be affected by asthma, leading to a significant healthcare and economic burden [2]. Glucocorticoids (GCs) are the cornerstone of asthma treatment based on their powerful anti-inflammatory properties. However, in some cases, patients develop resistance to GCs treatment and long-term therapy causes severe side effects [3]. Therefore, it is urgent and imperative to develop novel drugs for asthma prevention.

Chemical compounds derived from natural products have been widely used for treatment of human ailments from ancient times [4]. One of the most successful examples is Penicillin, which is originally isolated from the *Penicillium moulds* and used for treatment of bacterial infection [5]. Paclitaxel isolated from *Pacific yew*, as well as Vinblastine from *Catharanthus roseus*, were clinically used to treat a number of types of cancer [6, 7]. Various natural compounds exhibit potent activity against asthma with significant therapeutic potential, despite none of them has been currently approved in clinical
applications [8]. For instance, Zingerone is a major flavor component of ginger, which exhibits remarkable anti-oxidant capacities via activating Nrf2 signaling and thus protects mice from allergic asthma [9]. It has been reported the natural phenylpropanoid derivative Dehydrodieugenol targets STAT3/SOCS3 and MAPK pathways to counteract allergic airway inflammation in asthmatic mice [10]. Other natural compounds exert their anti-asthma activities via modulating a variety of cellular signaling including Wnt/β-catenin [11], TGF-β [12], NF-κB [13] and phosphatidylinositol 3kinase/protein kinase B (PI3K/Akt) pathways [14].

PI3K represents a group of kinases which convert phosphatidylinositol (3,4)-bisphosphate (PI3,4P) to phosphatidylinositol (3,4,5)-trisphosphate (PI3,4,5P), leading to translocation and phosphorylation of protein kinase B (PKB/Akt) [15, 16]. Studies have showed that PI3K/Akt pathway is broadly involved in a range of cellular process such as cell differentiation and proliferation, inflammation, metabolism and apoptosis [15, 17]. Accumulating evidence has uncovered the key role of PI3K/Akt pathway in immune response and remodeling in airway, driving the pathological changes of asthma [18]. Antagonizing the aberrant activation of PI3K/Akt pathway inhibits inflammatory cell infiltration into the lung, reduce airway remodeling and improve lung function in in vivo studies [19–21]. PI3Kδ or PI3Ky-knockout mice are resistant to Th2-driven asthma and demonstrate decreased production of IL-5 and IL-13 [22, 23]. Consequently, PI3K/Akt pathway is a potential therapeutic target for the treatment of asthma.

Bixin is a liposoluble di-apocarotenoid obtained from Bixa orellana seeds, which is traditionally used for infectious and inflammatory diseases in Mexico and South America [24]. It is also a food additive and colorant approved by Food and Drug Administration (FDA). In recent years, studies have demonstrated that Bixin exhibits multiple pharmaceutical properties including anti-tumor [25], antioxidant [26], anti-inflammatory [27] and neuroprotective effects [28]. In this study, our results indicated that Bixin functions as a potent PI3K/Akt antagonist to alleviate allergic inflammation, restore sensitivity and prevent airway remodeling in asthmatic mice, highlighting its potential value in asthma prevention.

**Materials And Methods**

**Chemicals**

Bixin (> 97% purity) purchased from Med Chem Express (MCE, USA) was dissolved in DMSO or coin oil according to the experiments purpose. Aluminum hydroxide adjuvant was purchased from Imject® Alum, Pierce (USA). Ovalbumin (OVA) and Complete Freund's Adjuvant (CFA) was purchased from Sigma-Aldrich (China). TGF-β1 was obtained from RD System (Minneapolis, MN).

**Cell culture**

The mouse airway epithelial cell line MLE-12 cells were procured from the China Cell Collection Center (Beijing, China) and maintained in Dulbecco's Modified Eagle medium (DMEM, Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco) in a humidified atmosphere of 5% CO₂ at 37°C. The culture medium was replenished every three days.
Animals

Six-week-old Balb/c female mice were supplied by the Liaoning Changsheng Biotechnology Co., Ltd (Liaoning, China). All animals were raised in enclosures of uniform temperatures (24°C ± 2°C) and humidity (45%). All animal experimental procedures were approved from the Ethics Committee of Medical Experiment Animals in the College of Basic Medicine of Jilin University.

Establishment of an acute asthma model

An acute murine asthma model was established as described before [9]. Briefly, 1mg aluminum hydroxide and 20 µg OVA were dissolved in 200 µl sterile phosphate-buffered saline (PBS). The solution was injected intraperitoneally (i.p.) to mice for sensitization on day 0, 7, and 14. The same volume of PBS only was administered in the same manner into control mice. From day 21 to 23, the mice were subjected to nebulized OVA (1% OVA in PBS) for 30min daily. As controls, mice were injected and nebulized with PBS. For the treatment, Bixin (50mg/kg or 100mg/kg, dissolved in corn oil) was given via i/p. injection daily from day 18 to day 23. All animals were sacrificed on day 25 after anesthetization. The experimental procedure is outlined in Fig. 1A.

Establishment of a chronic asthma model

Experimental and control group mice were sensitized following the same protocol used to establish acute asthma models. From day 17 to 58, mice were treated thrice a week with 30min of nebulized OVA (1% OVA in PBS) [29]. Mice of the control group were given nebulized PBS alone. Bixin was given from day 45 till day 58 and mice were sacrificed on day 59. The experimental procedure is outlined in Fig. 4A.

Establishment of a GCs-resistant asthma model

To establish a GCs-resistant asthma model [30], mice were injected subcutaneously on day 0 with CFA emulsified OVA. From days 21–23, mice were exposed to aerosolized OVA (1% OVA in PBS) for 30min daily. Mice were treated with Bixin (100mg/kg), dexamethasone (5 mg/kg i.p. injection) or both from day 18 till day 23 and mice were sacrificed on day 25. The experimental procedure is outlined in Fig. 3A.

Assessment of airway hyperresponsiveness (AHR)

Non-invasive whole-body plethysmography (Model PLY 3211; Buxco, Sharon, CT, USA) was used to measure AHR at 24h after the last challenge. In brief, conscious and spontaneously breathing mouse was placed in a chamber for measurement of pressure/time waves by the connected sensor and related computer data acquisition system. The degree of AHR was presented as Enhanced Pause (Penh) values. The mice were treated with different concentrations of nebulized methacholine (2.5, 5, 10, 25 and 50mg/ml; Sigma-Aldrich), and Penh values were recorded with each dose.

Bronchoalveolar lavage uid (BALF)
After euthanasia, mice trachea was exposed via blunt dissection. The trachea was incised and a small-caliber tube was placed into the airway. Sterile ice-cold PBS (1ml) was then used to lavage the lungs twice. BALF cells were pelleted by centrifugation and the supernatant was stored in -80°C. The cell pellets were resuspended with cold PBS, followed by spun onto glass slides for Diff Quick staining (Soledad Bao, Beijing, China).

**Cytokine quantification**

Protein levels of interleukin-5 (IL-5), IL-13, IL-1β, IL-6, IL-17A, interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) were measured by enzyme linked immunosorbent assay (ELISA) using commercial kits (Elabscience, Wuhan, Hubei, China) according to the instruction manual.

**Collagen content measurement**

Lung tissue collagen content from the various experimental groups were quantified via a Sircol assay kit (Biocolor, Carrickfergus) in accordance with manufacturer protocols.

**Hematoxylin and eosin (H&E), PAS and MASSON staining**

Briefly, the left lungs of sacrificed mice were harvested and fixed with 4% formaldehyde, followed by embedded in paraffin. The lung tissue was cut into 4-µm sections, followed by H&E, PAS and MASSON staining in compliance to standard manufacturer protocols (Soledad Bao, Beijing, China).

**Immunohistochemistry**

The lung paraffin sections warmed overnight in a 60°C oven. Sections were dewaxed, hydrated and boiled in 1 × sodium citrate (PH = 6) and cooled to room temperature for antigen retrieval. After blocking endogenous peroxidases and nonspecific staining, the slides were incubated sequentially with p65 (Proteintech), E-cadherin, N-cadherin and α-SMA (Abclonal) overnight in a wet box at 4°C. The following morning, a secondary antibody (Abclonal, China) labelled with biotin was used to incubate the slides. Lastly, 3,3′-diaminobenzidine (DAB) chromogen solution was used to stain the section before they were subjected to conventional dehydration, xylene clearing and fixing.

**Antibodies and Immunoblotting**

Total proteins of the cells or tissues under different conditions were lysed using a 1×RIPA Kit (Beyotime, Shanghai, China). A bicinchoninic acid assay (BCA) kit (Thermo Scientific, Rockford, USA) was utilized to ensure equal protein loading. 40µg of protein was electrophoresed using a 10% SDS-PAGE gel before being blotted onto nitrocellulose membranes. Membranes were blocked for an hour with 5% milk powder and incubated overnight with primary antibodies at 4°C. Primary antibodies against p65, p-IκBα, IκBα (Proteintech), E-Cadherin, N-Cadherin, α-SMA, p-PI3K, PI3K, p-Akt, AKT, p-mTOR, mTOR p-SMAD2, p-SMAD3, SMAD2, SMAD3 and GAPDH (Abclonal) were used. The next day, membranes were rinsed thrice with PBST and incubated at room temperature with secondary antibodies conjugated HRP at room
temperature. Samples were then exposed to an ECL reagent (Beyotime, Shanghai, China) after rinsing them thrice with PBST.

Statistical analysis

The GraphPad Prism 6 (La Jolla, CA) was used to carry out data analysis. The one-way or two-way analysis of variance (ANOVA) followed by Turkey’s post hoc test or Dunnett-t post hoc test was used to analyze all results. Statistical significance was designated to results with p-values of less than 0.05.

Results

Bixin reduces allergic inflammation in a murine model of acute asthma

To evaluate the anti-inflammatory effects of Bixin in asthma, we established an OVA-induced acute asthma murine model, followed by treatment with different doses of Bixin (Fig. 1A). As indicated in Fig. 1B, OVA sensitization induced an obvious infiltration of mixed inflammatory cells, mainly eosinophils, which was significantly reversed by Bixin treatment. Consistent with this observation, the protein levels of inflammatory cytokines including IL-5, IL-13, IL-6 and IL-1β were upregulated in mice BALF in response to OVA stimulation, while Bixin treatment remarkably decreased the release of these cytokines in a dose-dependent manner (Fig. 1C). These results suggest Bixin treatment effectively reduced the airway allergic inflammation in acute asthmatic mice.

Nuclear factor kappa B (NF-κB) is a key transcription factor in mediating inflammatory response, which is thought to play a pivotal role in allergic inflammation of asthma [31]. By stained with a p65 specific antibody, an enhanced nuclear p65 staining within the epithelium and submucosal compartments was observed in the lungs of asthmatic mice, whereas treatment with Bixin blocked the nuclear translocation of p65 (Fig. 1D). Besides, the levels of p-κBα and p65 in the lung tissues of OVA-induced asthmatic mice were decreased following Bixin treatment (Fig. 1E). Taken together, Bixin treatment attenuated airway inflammation in OVA-stimulated mice models of acute asthma.

Bixin protects mice from acute asthma

HE staining was further performed to evaluate the pathological features in the lungs of acute asthmatic mice with or without Bixin treatment. As shown in Fig. 2A, inflammatory cells extensively infiltrated into the blood vessels, alveolar ducts and alveoli, and the thickness of tracheal smooth muscle remarkably increased in the lungs of asthmatic mice. Pretreatment of Bixin obviously attenuated such pathological changes in a dose dependent manner (Fig. 2A). Goblet cell hyperplasia and submucosal hypertrophy in airway significantly contribute to a less effective mucociliary clearance, resulting in the formation of a mucus plug, which aggravates airway obstruction in asthmatic patients [32]. By PAS staining, we observed OVA stimulation induced marked goblet cells hyperplasia and mucus plugging in the airways, which were ameliorated by pretreatment of Bixin (Fig. 2A). Bronchial hyperresponsiveness is a hallmark of asthmatics, which is well reproduced in OVA sensitized mice. While challenge with increasing levels of methacholine led to increases in Penh values in asthmatic mice, Bixin administration dose-dependently
reduced the airway hyperresponsiveness caused by allergy sensitization (Fig. 2B). These findings suggest Bixin treatment protects against pathological lesions and symptoms in asthmatic mice.

**Bixin restores steroids sensitivity in a GCs-resistant asthma model**

GCs are the first-line therapy for asthma. However, a few asthmatic patients with non-allergic inflammation driven by neutrophils rather than T helper 2 cells, represent partial or complete resistance to glucocorticoid treatment [33]. Herein, a GCs-resistant asthma model was established to evaluate the therapeutic effects of Bixin (Fig. 3A). Administration of Dex has no detectable effects on the infiltration of inflammatory cells in the GCs resistant asthma challenged by OVA, whereas Bixin treatment slightly reduced the number of inflammatory cells in BALF (Fig. 3B). Importantly, combined administration of Bixin and Dex significantly inhibited inflammatory cells infiltration into airway (Fig. 3B). Expectedly, combined administration of Bixin and Dex also decreased the levels of IL-17, IL-6, IFN-γ and TNF-α in BALF of GCs resistant asthmatic mice, which showed no response to the Dex treatment (Fig. 3C). In addition, the results of H&E staining showed that treatment with Dex and Bixin, rather than Dex alone, remarkably alleviated the pathological lesions in GCs-resistant asthmatic mice (Fig. 3D). These results demonstrated that combined treatment of Bixin and Dex obviously attenuated airway inflammation in GCs resistant asthma model, indicating Bixin treatment restores steroids sensitivity.

**Bixin alleviates airway inflammation in a chronic asthma model.**

Asthma is a chronic respiratory disease with characteristics of persistent airway inflammation and airway remodeling [34]. However, the acute asthma model only reproduces acute exacerbation of asthma, which cannot reflect the developmental features of asthma. The value of Bixin in asthma treatment was also assessed in a chronic asthma murine model with long term OVA exposure (Fig. 4A). The results showed that administration of Bixin decreased the inflammatory cells infiltration into the airway (Fig. 4B), as well as the levels of cytokines in the BALF (Fig. 4C), indicating Bixin treatment effectively attenuated the chronic airway inflammation in asthma.

**Bixin reverses airway remodeling in chronic asthmatic mice**

Chronic airway inflammation is accompanied with pathological structural changes and collagen fiber deposition in the airway of asthmatics. The results of histological analysis showed that airway remodeling features including severe goblet cell hyperplasia, submucosal hypertrophy, smooth muscle thickening and fibrosis were observed in the experimental chronic asthma model (Fig. 5A). Bixin treatment played a protective role by reducing the degree of pathological lesions (Fig. 5A). Additionally, Bixin treatment decreased the elevated deposition of collagen I in the lungs of chronic asthmatic mice (Fig. 5B), as well as reduced the increased Penh value in a dose dependent manner (Fig. 5C). These findings demonstrate in addition to reducing airway chronic inflammation, Bixin also prevents the airway remodeling in asthma.

**Bixin prevents epithelial-mesenchymal transition (EMT).**
EMT plays an essential role in airway remodeling in asthma [35]. Compared with normal mice, the lungs of chronic asthmatic mice were stained with a lower epithelial marker E-Cadherin and higher mesenchymal markers, N-Cadherin and α-SMA, confirming the occurrence of EMT in the chronic asthma model (Fig. 6A). Remarkably, Bixin treatment suppressed the development of EMT in chronic asthma (Fig. 6A), which was validated by the followed immunoblotting analysis detecting the protein levels of E-Cadherin, N-Cadherin and α-SMA in the lungs (Fig. 6B).

TGF-β1 is a potent inducer of EMT, the levels of which is elevated in lungs of asthma patients [36]. We further explored the anti-EMT properties of Bixin in the normal mice airway epithelial cell line MLE12 cells stimulated with TGF-β1. While stimulation of TGF-β1 caused MLE12 cells transform from a typical multilateral paving stone-like appearance to a spindle shape, Bixin treatment partially reversed the TGF-β1 mediated transition in cells morphology (Fig. 6C). By immunoblotting analysis, we also found Bixin treatment inhibited TGF-β1 induced downregulation of E-Cadherin, as well as the upregulated levels of N-Cadherin and α-SMA in MLE12 cells (Fig. 6D). Collectively, we demonstrated that Bixin treatment restrained EMT progression \textit{in vitro} and \textit{in vivo}.

\textbf{Bixin inhibits PI3K/Akt pathway activation}

Recently studies have uncovered the key role of PI3K/Akt pathway in mediated inflammation, steroids resistance and airway remodeling in asthma [18]. Thus, we hypothesized Bixin protects against asthma through modulation of PI3K/Akt pathway. Increased levels of p-PI3K, p-Akt and p-mTOR were observed in the lungs of the three types of asthma models (Fig. 7A-C), confirming the notion that PI3K/Akt pathway is intimately involved in development of asthma. Bixin treatment significantly reduced the levels of p-PI3K, p-Akt and p-mTOR, without affecting their non-phosphorylated form (Fig. 7A-C). TGF-β1 stimulation induced marked upregulation of p-PI3K, p-Akt and p-mTOR in MLE12 cells, which were decreased by Bixin treatment (Fig. 7D). Intriguingly, Bixin treatment has no effects on the levels of phosphorylated SMAD2 and SMAD3 in TGF-β1-stimulated MLE12 cells (Fig. 7D). These findings indicate Bixin functions as a potent inhibitor suppressing PI3K/Akt pathway activation.

\textbf{Discussion}

Airway inflammation plays a central role in the pathogenesis and development of asthma, which is mainly driven by the T helper type 2 (Th2) response [37]. The activated Th2 cells secrete cytokines such as IL-4, IL-5, IL-9 and IL-13 to promote co-stimulatory interactions with B cells, which is essential to the maturation of B cells and the production of IgE [37, 38]. The Th2 response is also required for the maturation of eosinophils and their influx into the airway [38]. When challenge by subsequent allergens, IgE-coated mast cells and recruited eosinophils release active mediators to promote smooth muscle contraction, vascular permeability and mucus production, resulting in reversible lower airway obstruction in asthmatic individuals termed as acute asthma attack [39]. Studies have demonstrated Bixin shows an immunomodulatory role of in domestic animals [40, 41]. In this study, we found that treatment with Bixin significantly reduced both the levels of Th2 cytokines and the inflammatory cells infiltration in airway of
an acute asthma murine model (Fig. 1B and 1C), which well reproduces the features of acute asthma attack in human.

Besides the episodes of acute inflammatory response, an airway chronic inflammation often persistently presents, even in the absence of allergen stimulus [34]. In the chronic asthma models, we can observe administration of Bixin attenuates the chronic inflammatory response in the airway of asthmatic mice (Fig. 3B and 3C). The active mediators released by recruited inflammatory cells contribute to AHR in asthmatics [42] and thus it is expected that Bixin improves the AHR in the both of the two asthma models through reducing the airway inflammation, indicating Bixin protects mice against allergic asthma base on its anti-inflammatory capacities.

Chronic inflammation is usually accompanied by airway remodeling in asthma [34]. Despite pathogenesis of airway remodeling remains elusive, the chronic inflammatory response in airway is the main force driving the processes of remodeling [43]. The infiltrating inflammatory cells, predominantly eosinophils and mast cells release hyperactive enzymes such as major basic proteins (MBPs), neurotoxin, peroxidase and cationic protein, leading to lung tissue damage in asthma, which triggers aberrant repair pathways [34]. Due to the potent anti-inflammatory activity, it is not surprising that Bixin reduced the airway remodeling extent by inhibition of goblet cell hyperplasia, smooth muscle thickening and collagen deposition in the chronic asthmatic mice (Fig. 5A). In addition to the impairment on structural cells of the airways, inflammatory cells-secreted cytokines, especially TGF-β, induces airway remodeling related EMT processes [34, 44]. Bixin treatment increased the levels of E-Cadherin, as well as decreased the levels of N-Cadherin and SMA in the lungs of chronic asthmatic mice, indicating this treatment retarded EMT progression (Fig. 6A). Besides, Bixin treatment also obviously ameliorated TGF-β1 induced EMT in vitro (Fig. 6B). These findings suggest the antagonism effects of Bixin against airway remodeling is not only attributed to its anti-inflammatory activity, but also dependent on its anti-EMT capacity.

In some cases, conventional drugs including steroids exhibit limited therapeutic efficacy in asthma by its high heterogeneity. Instead of eosinophils, the airway inflammation in refractory asthma is often associated with infiltrated neutrophils [33]. The levels of IL-17, IL-8 and TNF-α are elevated in the airway and serum of these asthmatic patients [45, 46], which has been confirmed in a CFA induced animal asthma models resistant to dexamethasone treatment (Fig. 3B-D) [47]. IL-17 has a powerful pro-inflammatory effect in activating airway epithelial cells, bronchial fibroblasts and smooth muscle cells to secrete IL-6, IL-8 and G-CSF, promoting the production of mature neutrophils [48, 49]. IL-17 can further promote the activation and recruitment of neutrophils through IL-8 and other pathways, thereby participating in the occurrence of neutrophilic asthma [48]. Bixin treatment significantly reduced the levels of IL-17, IL-6, IFN-γ and TNF-α, as well as ameliorated neutrophils recruitment in airway of GCs resistant asthmatic mice (Fig. 3B-D), suggesting the anti-inflammatory properties of Bixin is not limited to Th2 asthma but can be used for refractory asthma treatment.
The pharmacological mechanism of Bixin has not been fully elucidated yet. Previous studies reported that Bixin activated peroxisome proliferator-activated receptor gamma (PPARγ) and nuclear factor erythroid 2-related factor 2 (NRF2) signaling, thus playing a role in suppressing cancer cells proliferation [50], attenuating liver steatosis [51], inhibiting kidney fibrosis [52] and improving insulin sensitivity [53]. In addition to activation of NRF2 signaling, Bixin can also suppress TXNIP/NLRP3 inflammasome activity to reduced inflammatory response in experimental autoimmune encephalomyelitis [54]. In this study, we uncovered a new pharmacological mechanism of Bixin in regulating PI3K/Akt pathway. Administration of Bixin decreased the levels of p-PI3K, p-Akt and p-mTOR in the lungs of all the three types of asthma models (Fig. 7A-C). It is well established that PI3K/Akt pathway plays a pivotal role in airway inflammation of asthma. The activity of PI3K is required for the Th2 response [55] and recruitment of inflammatory cells into airways of asthmatics [56]. Additionally, blockade of PI3K effectively reduced the production of IFN-γ, IL-8 and IL-17 in immune cells from mice and asthmatic donors [57–59] and alleviates GCs resistance in refractory asthma [30, 60]. Thus, the antagonism effect of Bixin on PI3K/Akt pathway partially, if not all, contributed to its anti-inflammatory property in asthma. As a downstream signaling of TGF-β pathway, PI3K/Akt is deeply involved in the TGF-β mediated EMT [61], contributing to airway remodeling in asthma. Treatment with Bixin remarkably inhibits the TGF-β1 induced activation of PI3K/Akt in airway epithelial cells in vitro (Fig. 7D); yet Bixin has no effects on the phosphorylation of SMAD2/3 (Fig. 7D), suggesting anti-EMT ability of Bixin is specifically due to its impact on PI3K/Akt pathway.

**Conclusion**

In conclusion, our results demonstrate that Bixin play a protective role against asthma by its multiple pharmacological activities in anti-inflammation, anti-EMT and restoration of GCs sensitivity. We also reveal a novel regulatory mechanism of PI3K/Akt pathway by Bixin, providing a potential effective PI3K/Akt antagonist for treatment of asthma and other related diseases.

**Abbreviations**
**Ovalbumin**  
OVA

| Glucocorticoids | GCs |
| Complete Freund’s Adjuvant (CFA) | CFA |
| Bronchoalveolar lavage fluid | BALF |
| Enzyme linked immunosorbent assay | ELISA |
| Hematoxylin and eosin | H&E |
| Hyperresponsiveness | AHR |
| Epithelial-mesenchymal transition | EMT |
| Transforming growth factor beta | TGF-β |
| Phosphatidylinositol 3kinase | PI3K |
| Protein kinase B | PKB/Akt |
| Food and Drug Administration | FDA |
| Dulbecco’s Modified Eagle medium | DMEM |
| Bicinchoninic acid assay | BCA |
| Nuclear factor kappa B | NF-κB |
| Interleukin-5 | IL-5 |
| T helper type 2 | Th2 |
| Tumor necrosis factor alpha | TNF-α |
| Enhanced Pause | Penh |
| Major basic proteins | MBPs |
| Peroxisome proliferator-activated receptor gamma | PPARγ |
| Nuclear factor erythroid 2-related factor 2 | NRF2 |

**Declarations**

**Ethics approval**

All animal experimental procedures were approved from the Ethics Committee of Medical Experiment Animals in the College of Basic Medicine of Jilin University.

**Consent for publication**

Not applicable
Availability of data and material

All the data and materials are available in this research and all data generated or analyzed during this study are included in this article.

Competing interests

There are no financial or other issues that might lead to conflict of interest.

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Authors' contributions

LS (Lei Song), JL, DS and LP conceived the projects, YZ, HL, LS (Linzi Sun) and JJ performed the experiments. LS (Lei Song), JL, DS and LP analyzed data. YZ drafted the first version of the manuscript, and LS (Lei Song) revised the manuscript with input from all authors.

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**Figures**

(A) OVA OVA OVA + Bixin

Bixin lessens airway inflammation in acute asthmatic mice. A. Flowchart depicting protocols used for establishing a model of acute asthma and treatment regimens. B. Quantitative analysis of lymphocyte, eosinophil and neutrophil, as well as the total cells in BALF from mice. C. The levels of IL-5, IL-13, IL-6 and IL-1β in BALF of mice were determined by ELISA assay. D. The lung tissue was immunostained using a p65 specific antibody. E. The levels of p-IκBα, IκBα and p65 in the lung tissue were determined by
immunoblotting with corresponding antibodies. The data is depicted in terms of mean ± SEM. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.

**Figure 2**

Bixin alleviates lung lesions and AHR in acute asthmatic mice. A. The pathological lesions of lungs of asthmatic mice were evaluated by H&E staining (upper panel) and PAS staining (lower panel). Shown images represent results from one mouse in each group (n=6). B. Mice were exposed to indicated concentrations of methacholine and the analysis of Penh values were performed to represent the severity of AHR of mice. The data is depicted in terms of mean ± SEM. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.
**Figure 3**

Bixin sensitizes GCs resistant asthmatic mice to dexamethasone treatment. A. The flow diagram for establishing OVA/CFA-induced glucocorticoid-resistant asthma model and treatment regimens. B. Total cell count and classification in BALF were performed to evaluate the inflammatory cells infiltration. C. The levels of IL-17, IL-6, IFN-γ and TNF-α in BALF of mice were determined by ELISA assay. The data is depicted in terms of mean ± SEM. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.
Figure 4

Bixin alleviates airway inflammation in a chronic asthmatic murine model. A. Flowchart depicting protocols used for establishing a model of chronic asthma and treatment regimens. B. Total cell count and classification in BALF were performed to evaluate the inflammatory cells infiltration. C. The levels of IL-5 and IL-13 in BALF of mice were determined by ELISA assay. The data is depicted in terms of mean ± SEM. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.
Figure 5

Bixin attenuates the airway pathological lesions and AHR in the chronic asthmatic mice. A. The structural changes and collagen deposition in mice lungs were evaluated by H&E staining (top panel) and Masson staining (lower panel). Representative images are from one mouse in each group (n=6). B. The soluble collagen in lung tissues was quantified to evaluate the fibrosis in the airway of chronic asthmatic mice. C. The Penh values were determined in mice exposed to indicated concentrations of methacholine. The data is depicted in terms of mean ± SEM. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.
Figure 6

Bixin inhibits EMT development in vitro and in vivo. The levels of EMT-related proteins (E-cadherin, N-cadherin and α-SMA) in the lungs of chronic asthmatic mice were determined by immunostaining (A) and immunoblotting (B) with specific antibodies. Representative images are from one mouse in each group (n=6). C. MLE12 cells treated with indicated dose of Bixin were stimulated by TGF-β1 (10 ng/ml) for 72 hours and the images of cell morphology were taken by an Inverted microscope. D. The levels of E-cadherin, N-cadherin and α-SMA in MLE12 cells were determined by immunoblotting using specific antibodies. Each graph represents one of three independently performed experiments.
Bixin treatment induces suppression of PI3K/Akt pathway in vitro and in vivo. The levels of p-AKT, AKT, p-PI3K, PI3K, p-mTOR and mTOR in the lungs of acute asthmatic mice (A), GCs resistant asthmatic mice (B) and the chronic asthmatic mice (C) were determined by immunoblotting using specific antibodies. D. MLE12 cells treated with indicated dose of Bixin were stimulated with TGF-β1 (10 ng/ml) for 12 hours and the cell total proteins were isolated for detecting the levels of p-AKT, AKT, p-PI3K, PI3K, p-mTOR and mTOR, as well as the levels of p-SMAD2, SMAD2, p-SMAD3 and SMAD3. Each graph represents one of three independently performed experiments.