

RESEARCH ARTICLE

Dihydroartemisinin suppresses ovalbumin-induced airway inflammation in a mouse allergic asthma model

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Abstract

Asthma is a complex disease characterized by reversible airway obstruction, airway hyper-responsiveness (AHR) and chronic inflammation of the airways. Dihydroartemisinin (DHA), a semi-synthetic derivative of artemisinin isolated from the traditional Chinese herb *Artemisia annua*, has been shown to possess antimalarial and antitumor activities, but whether it can be used in asthma treatment has not been investigated. In this study, we attempted to determine whether DHA regulates inflammatory mediators in the ovalbumin (OVA)-induced mouse asthma model. BALB/c mice were sensitized and challenged by OVA to induce chronic airway inflammation. The intragastrical administration of DHA at 30 mg/kg significantly decreased the number of infiltrating inflammatory cells, T-helper type 2 (Th2) cytokines, OVA-specific immunoglobulin E (IgE) and AHR. Treatment with DHA also attenuated OVA-induced mRNA expression of Muc5ac and chitinase 3-like protein 4 (Ym2) in lung tissues. In addition, lung histopathological studies revealed that DHA inhibited inflammatory cell infiltration and mucus hypersecretion. Then signal transduction studies showed that DHA significantly inhibited extracellular signal-regulated protein kinase (ERK), p38 mitogen-activated protein kinase phosphorylation. DHA also inhibited nuclear factor- κ B (NF- κ B) activation via the inhibition of phosphorylation of I κ B α . These findings provide new insight into the immunopharmacological role of DHA in terms of its effects in a mouse model of asthma.

Keywords

Asthma, dihydroartemisinin, MAPK, NF- κ B, Th2 cytokines

History

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Introduction

Allergic asthma is a chronic inflammatory disease characterized by the hypersecretion of mucus, obstruction and inflammation of the airways and enhanced bronchial reactivity to spasmogenic stimuli. The prevalence of asthma is rapidly increasing worldwide, especially in young children, and asthma has become a significant cause of morbidity and mortality in developing countries¹. Evidence has shown that these inflammatory responses are mediated by Th2 cells, together with mast cells, B cells and eosinophils, as well as a number of inflammatory cytokines².

The induction of cytokine expression and the recruitment and activation of inflammatory cells in asthma involve the increased transcription of inflammatory cytokine genes, which are regulated via transcription factors. Some signaling pathways, such as the Ras/ERK mitogen-activated protein kinase (MAPK) cascade, and the PKC/NF- κ B pathway are known to play important roles³. NF- κ B appears to be a critical transcription factor that regulates Th2 cell differentiation and

cytokine expression. Increased NF- κ B activity has been identified in airway samples from asthma patients⁴. In addition, MAPK signaling cascade has been shown to be important in the activation of various immune cells⁵. It has been reported that inhibition of the MAPK signaling pathway in lung inflammatory cells may have therapeutic potential in the treatment of allergic diseases such as asthma⁶. In addition to p38, the ERK pathway has also been implicated in the regulation of IL-5 and other cytokines⁷. ERK and JNK have been shown to be associated with IgE switching^{8,9}. Therefore NF- κ B and MAPK have emerged as promising molecular target for the treatment of asthma.

Dihydroartemisinin (DHA) is a semi-synthetic derivative of artemisinin that is isolated from the herb *Artemisia annua*. This traditional Chinese herb has been used as a remedy for chills and fevers for centuries in China¹⁰. Artemisinin derivatives, including DHA, have been used as antimalarial drugs, effective for both uncomplicated and severe malaria¹¹. In addition, these derivatives have been shown to possess antiviral¹², anticancer¹³ and anti-inflammatory^{14–16} activities. It has been reported that DHA ameliorates inflammatory disease by its reciprocal effects on Th and regulatory T cell function via modulating the mammalian target of rapamycin pathway¹⁷. In addition, artesunate attenuates experimental allergic asthma via inhibition of the phosphoinositide 3-kinase/Akt pathway¹⁸. There is also a US Patent

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Application US 2012/0015922 A1 by Wai Shiu Fred Wong, Singapore, titled: use of artemisinin derivatives for the treatment of asthma and chronic obstructive pulmonary disease (COPD). To date, the effects of DHA treatment on a non-infectious mouse airway inflammatory model have not been evaluated. In the study described here, we have employed a mouse airway inflammatory and bronchial asthma model to determine if there is an anti-inflammatory effect of orally administered DHA.

Materials and methods

Animals

Female BALB/c mice (6–8 weeks), which weighed 18–20 g, were purchased from the Center of Experimental Animals of Baiqiu Medical College of Jilin University (Jilin, China). All animals were housed in microisolator cages, and received food and water *ad libitum*. Laboratory temperature was $24 \pm 1^\circ\text{C}$, and relative humidity was 40–80%. Before experimentation, mice were housed for a minimum of one week to adapt them to the environment. All animal experiments were performed in accordance with the guide for the Care and Use of Laboratory Animals published by the US National Institute of Health.

Sensitization and challenge with ovalbumin and treatment

Mice were divided into the following four groups ($n=8$): negative control group, ovalbumin (OVA)+DHA group, OVA+dexamethasone (DEX) group and OVA group. Mice were sensitized with OVA (Grade V; Sigma-Aldrich, St. Louis, MO) adsorbed in 100 µg/ml of Imject Alum (Pierce, Rockford, IL) by intraperitoneally (i.p.) injection on day 0 and day 14. On days 25–27, the mice were anesthetized and intranasally challenged with OVA (100 µg) in PBS (50 µl). DHA at 30 mg/kg was administered intragastrically 1 h before OVA challenge. The single dose of DHA was chosen based on previous studies^{19–21}. DHA was resolved in saline with 0.1% dimethyl sulfoxide (DMSO) and then sterile filtered before used. DEX at 2 mg/kg was administered i.p., as a positive control. Phosphate-buffered saline (PBS) was used in place of DHA and DEX for the OVA group. DHA was purchased from the National Institutes for Food and Drug Control (Beijing, China, purity >98%).

Collection of blood and bronchoalveolar lavage fluid

Twenty-four hours after the last inhalational exposure, the mice were anesthetized and bled via the brachial plexus for collection of blood samples used to estimate IgE production. The bronchoalveolar lavage fluid (BALF) was collected by cannulating the upper part of the trachea and then infusing PBS in three aliquots (0.3, 0.3, 0.4 ml) for a total volume of 0.9 ml. The lavage fluid was centrifuged, and the supernatants were stored at -70°C until cytokine assays were performed. Cell pellets were resuspended in 1 ml of PBS and used for total and differential cell counts. The total cell number in the BALF was counted in a hematology counter (Sysmex SF 3000, Sysmex Co., Kobe, Japan). Eosinophils, neutrophils and macrophages were counted in the BALF using cytopins

(centrifuged preparations) stained with the Kwik-Diff staining set (Thermo Fisher Scientific Inc., Pittsburgh, PA). A minimum of 200 cells were counted per slide.

Determination of airway hyper-responsiveness

Twenty-four hours after the last inhalational exposure, the mice were anesthetized, and a tracheotomy was performed as described²². The internal jugular vein was cannulated and connected to a microsyringe for intravenous methacholine (Mch) administration. Airway resistance (RI, which refers to the pressure driving respiration divided by the flow) and lung compliance (C_{dyn}, which 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) in response to increasing concentrations of Mch were recorded using the Flexivent system (SCIREQ, Montreal, Canada) as described²². The results are expressed as the percentage of the respective basal values in response to PBS.

Determination of serum OVA-specific IgE and BALF cytokines

Serum samples were stored at -70°C after centrifugation (3000 rpm, 10 min). OVA-specific IgE levels were measured using enzyme-linked immunosorbent assays (ELISA). The levels of interleukin-4 (IL-4), IL-5 and IL-13 in BALF were determined by specific mouse IL-4, IL-5 and IL-13 ELISA kits (BioLegend, Inc., Camino Santa Fe, Suite E, San Diego, CA).

Histopathologic evaluation

At 24 h after the last OVA challenge, lung tissues were removed by dissection and fixed in 10% paraformaldehyde. Lung tissues were sectioned, embedded in paraffin and cut into 3-µm sections. Tissue sections were then stained with hematoxylin and eosin (H&E) for general morphology²³ and with alcian blue-periodic acid-Schiff (AB-PAS) for the identification of goblet cells in the epithelium²⁴.

RNA preparation and quantitative real-time polymerase chain reaction

The mice were euthanized at 24 h after the last OVA challenge, total RNA was isolated from lungs (five mice in each group) using the TRIzol reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instruction. Reverse transcription was carried out using the first-strand cDNA synthesis kit (Bioer, Hangzhou, China). Real-time polymerase chain reaction (PCR) was performed on cDNA samples using the SYBY Green system (Bio-Rad; Richmond, CA). Primers used were: Muc5ac, sense 5'GTGTCGCCGG AGAAAGTTGGT3', and antisense 5'GTCCTGTTGAGCCT GGCCTGTG3'; Ym2, sense 5'TCCACTTTGAACCACATTC CAAGGC3' and antisense 5'CGAGAGACTGAGACAGTTC AGGGA3. Cycling conditions were 1 cycle at 94°C for 3 min, then 94°C for 1 min, 61°C for 30 s and 72°C for 1 min for 30 cycles. Analysis used the sequence detection software supplied with the instrument. β -actin serves as the internal control. The relative expression of the target gene versus β -actin was determined using " $2^{-\Delta\text{CT}}$ ", where ΔCT represents

the CT (threshold cycle) value of the target gene minus the CT value of β -actin.

Western blot analysis of MAPK and NF- κ B

Lung tissues were harvested at 24 h after the last OVA challenge and the lung tissues were homogenized and incubated in lysis buffer containing protease and phosphatase inhibitors (Roche, Basel, Switzerland) to obtain extracts of lung protein. The homogenates, which contained 40 μ g of protein, were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 3% bovine serum albumin (BSA) (3 g BSA was dissolved in 100 ml Tris-Tween-buffered saline) for 1 h at 37°C and incubated with specific antibodies against ERK, p38 and JNK, and phospho-specific antibodies to ERK, p38, JNK, NF- κ B p65 and I κ B (Beverly, MA) in 3% BSA. With the use of peroxidase-conjugated secondary anti-mouse and anti-rabbit antibodies, the bound antibodies were detected by SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific Inc., Rockford, IL). β -actin was detected as an internal control of protein loading.

Statistical analysis

All values were expressed as the mean $\pm$ standard error of the mean (SEM). Differences between mean values of normally distributed data were assessed by the one-way analysis of variance (Dunnett's *t*-test) and the Student's *t*-test. Differences were considered significant if $p < 0.05$.

Results

DHA suppresses inflammatory cells and reduces Th2 cytokines

To examine the inhibitory effect of DHA on asthma, we induced asthma in BALB/c mice using OVA and evaluated the number of inflammatory cells and cytokines in the BALF. As shown in Figure 1(a), OVA sensitization and challenge induced a significant elevation in the levels of IL-4, IL-5 and IL-13 compared to control mice. As shown in Figure 1(b), OVA challenge induced higher levels of total cells, eosinophils, neutrophils and macrophages compared to the control group. However, DHA (30 mg/kg) and DEX (2 mg/kg) significantly decreased the number of inflammatory cells and cytokines.

DHA decreases the level of OVA-specific IgE in serum

To determine the effect of DHA on OVA-specific IgE release in OVA-challenged mice, an ELISA was performed on serum samples. As shown in Figure 2(a), OVA-specific IgE levels in serum were dramatically elevated in OVA-challenged mice compared to PBS-challenged mice. OVA-challenged mice pretreated with DHA (30 mg/kg) and DEX (2 mg/kg) had reduced OVA-specific IgE levels compared to untreated, OVA-challenged mice.

DHA inhibits OVA-challenge inflammatory gene expression

OVA-challenged group markedly upregulated lung mRNA levels of Muc5ac, which is essential for mucus

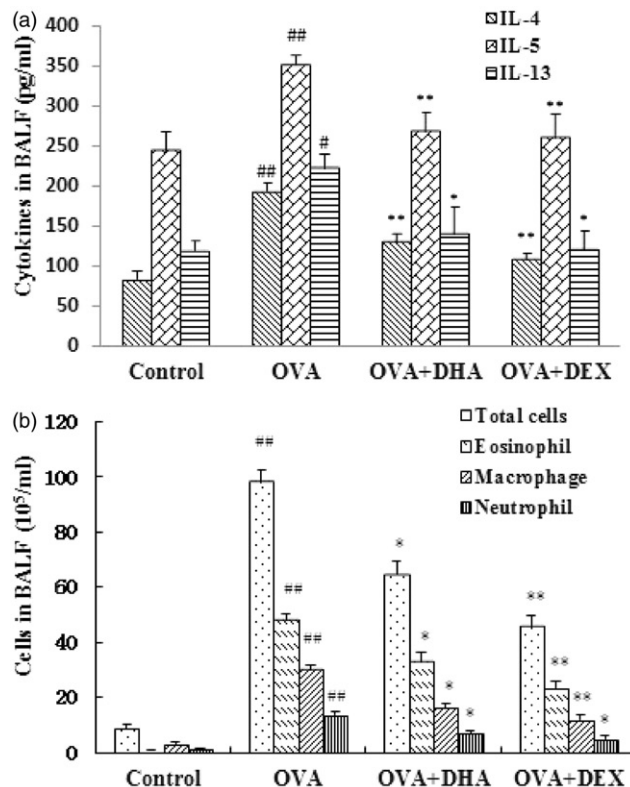


Figure 1. Effect of DHA (30 mg/kg) and DEX (2 mg/kg) on the secretion of IL-4, IL-5, and IL-13 (a) and the recruitment of inflammatory cells (b) in BALF. The lavage fluid was centrifuged and the supernatants were analyzed via ELISA. The cell pellets were resuspended in 1 ml of PBS and applied to a slide by cytopspin to obtain differential cell counts. The values represent the mean $\pm$ SEM of three independent experiments. # $p < 0.05$, ## $p < 0.01$ versus control group, * $p < 0.05$, ** $p < 0.01$ versus OVA.

hypersecretion, and of chitinase Ym2, which has recently been shown to play critical roles in airway inflammation and remodeling. As shown in Figure 2(b) and (c), pretreatment with DHA (30 mg/kg) and DEX (2 mg/kg) led to suppression of Muc5ac and Ym2.

DHA decreases airway hyper-responsiveness in OVA-induced mouse asthma

Airway hyper-responsiveness (AHR) was assessed by measuring RI and Cdyn in response to increasing doses of Mch administration in mechanically ventilated mice. OVA-challenged mice developed AHR, typically reflected by high RI and low Cdyn values. DHA (30 mg/kg) and DEX (2 mg/kg) treatment dramatically reduced RI (Figure 3a) and restored Cdyn (Figure 3b) in OVA-challenged mice in response to Mch.

DHA attenuates airway inflammation, goblet cell hyperplasia and mucus production

To evaluate the effect of DHA on airway inflammation, airway goblet cell hyperplasia and mucus production, we stained lung sections with H&E and AB-PAS stain. As shown in Figure 4, inflammatory cell infiltration into the peribronchial and perivascular areas (H&E staining), and goblet cell hyperplasia (AB-PAS staining) were observed in OVA-challenged mice. DHA (30 mg/kg) and DEX (2 mg/kg)

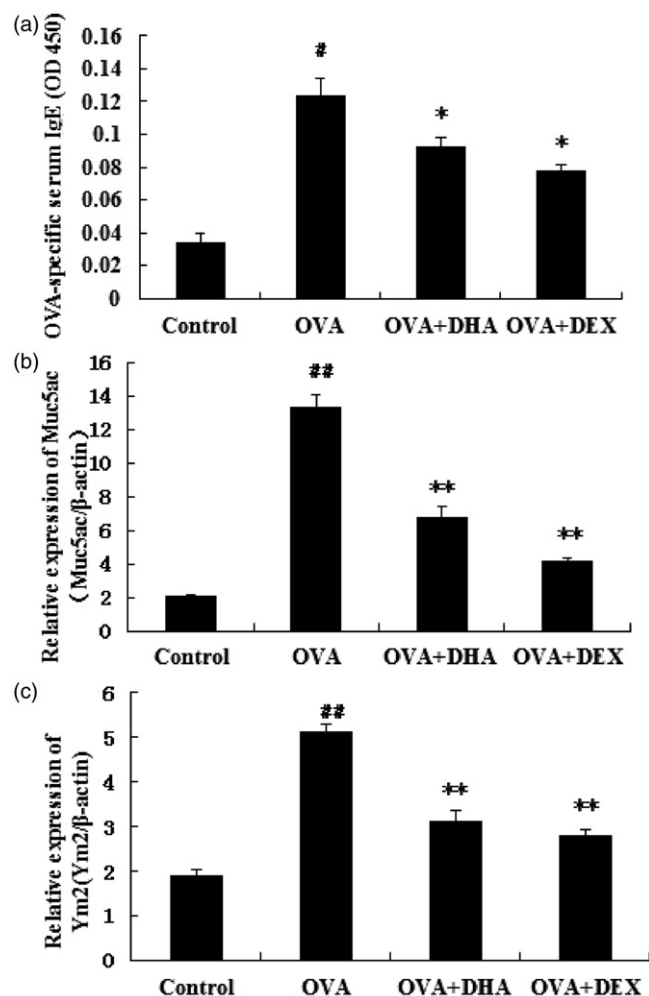


Figure 2. Effect of DHA (30 mg/kg) and DEX (2 mg/kg) on OVA-specific IgE in serum and expression of Muc5ac and Ym2. OVA-specific IgE levels in the serum were measured by ELISA (a). The mRNA in whole lung extracts was measured by real-time PCR (b, c). Treatment with DHA reduced IgE and mRNA expression in OVA-induced mouse. The values represent the mean $\pm$ SEM of five animals in each group. # p < 0.05, ## p < 0.01 versus control group, * p < 0.05, ** p < 0.01 versus OVA.

treatment reduced mucus secretion, goblet cell hyperplasia and epithelial cell disruption.

DHA inhibits MAPK and NF- κ B pathway activation

To evaluate the effect of DHA on MAPK and NF- κ B activation in a mouse asthma model, we homogenized the lung tissue of mice to obtain total and nuclear proteins. To determine whether the therapeutic effect of DHA on OVA-challenge asthma occurs through MAPK and NF- κ B inhibition, we examined the levels of phosphorylated ERK, JNK, p38, I κ B- α and nuclear p65 as well as the levels of ERK, JNK and p38 in the lung tissues of mice subjected to OVA challenge. As shown in Figure 5, the phosphorylation levels of the ERK and p38 isoforms decreased dramatically in DHA-treated and DEX-treated to OVA-challenged mice; however, there was no change in the phosphorylation levels of the JNK isoforms. As shown in Figure 6(a) and (b), DHA suppressed phosphorylation of I κ B- α in lung parenchyma, and attenuated nuclear localization of p-NF- κ B p65 in lung tissues. These results demonstrated that DHA may inhibit

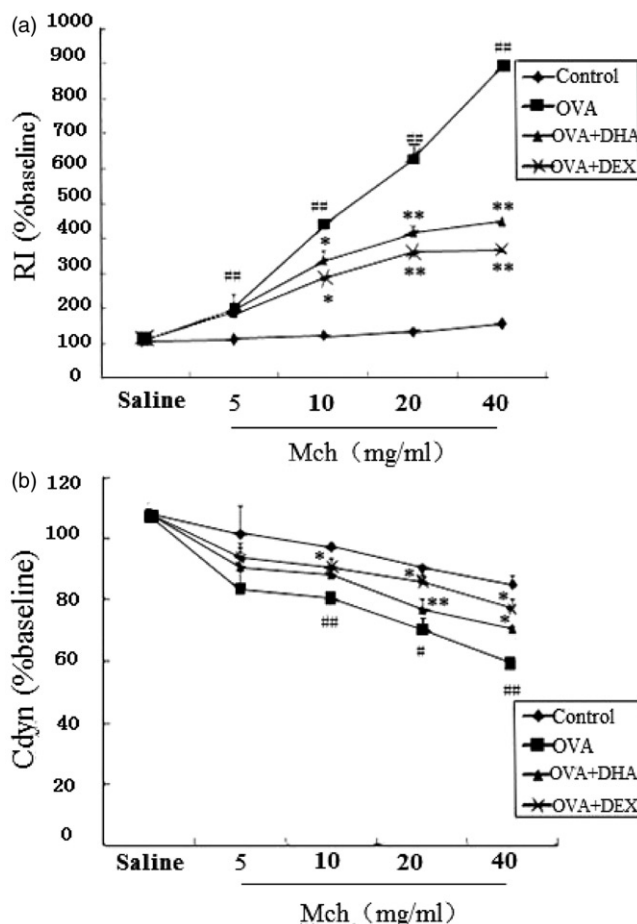


Figure 3. Effects of DHA (30 mg/kg) and DEX (2 mg/kg) on OVA-induced AHR in mice. AHR was assessed by percentage change from the baseline level of (a) lung resistance (RI, n = 10 mice per treatment group) and (b) dynamic compliance (Cdyn, n = 10 mice per treatment group). # p < 0.05, ## p < 0.01 versus control group, * p < 0.05, ** p < 0.01 versus OVA.

OVA-challenged asthma via inhibition of ERK, p38 and NF- κ B.

Discussion

Allergic asthma is characterized by reversible airway obstruction in response to allergens, chronic eosinophilic airway inflammation and non-specific AHR. Despite important advances in diagnosis and treatment, asthma remains one of the most prevalent chronic respiratory disorders, affecting 7–10% of the global population²⁵. Corticosteroid treatment remains the first preference for treatment of allergic disease; however, these therapies are not always completely effective and are associated with side effects and steroid resistance. Due to such limitations, the development of new treatments represents a major goal for both the pharmaceutical industry and academic researchers.

NF- κ B is detected in most cell types and has been shown to have a critical function in immune and inflammatory responses, including asthma²⁶. Indeed, activation of NF- κ B in bronchial epithelial cells and immune cells is highly associated with the development of asthma²⁷, and several NF- κ B-targeting strategies, such as small molecule inhibitors and proteasome inhibitors, are effective in experimental asthma models^{28,29}. In our study, we demonstrated that

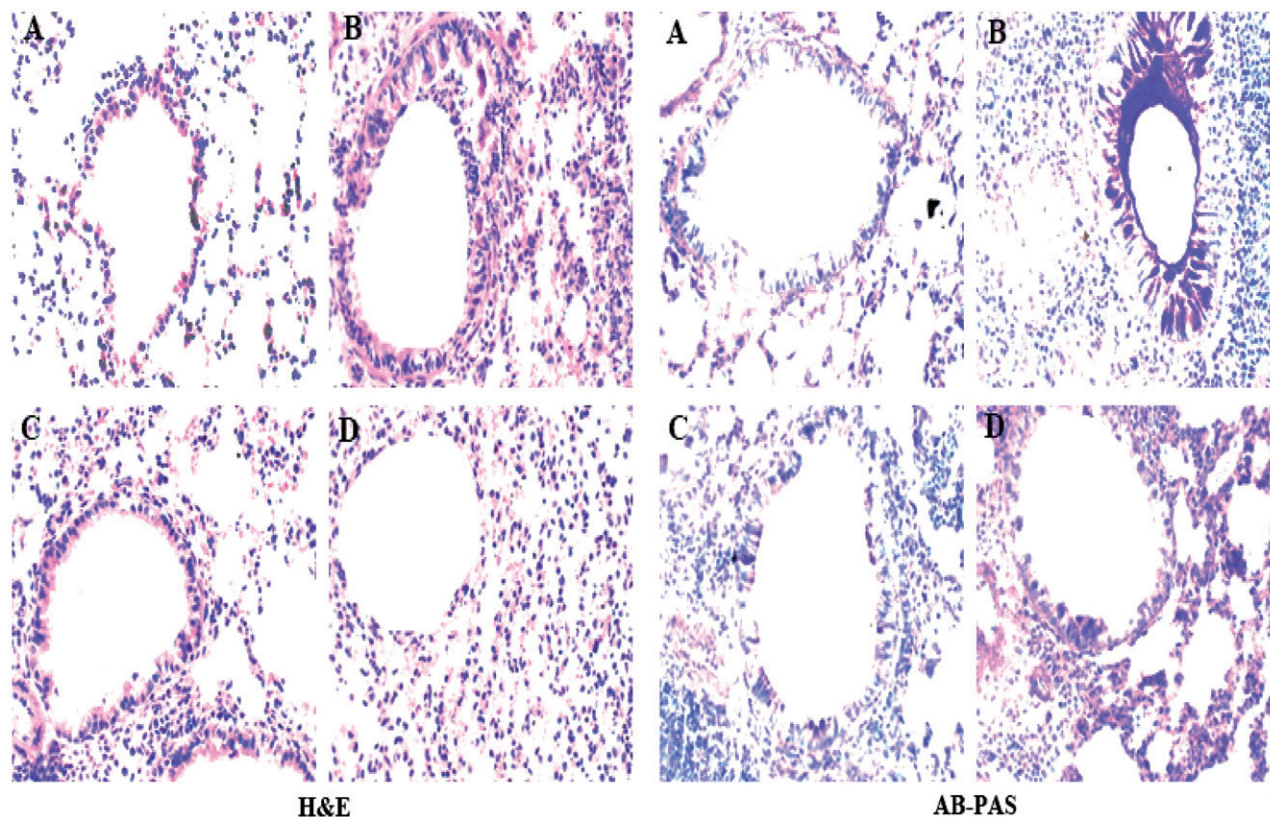


Figure 4. Effect of DHA (30 mg/kg) and DEX (2 mg/kg) on airway inflammation and mucus production. Representative H&E and AB-PAS stained sections of lung from PBS-challenged mice (A); and OVA-challenged mice (B); OVA-challenged mice treated with DHA (C); OVA-challenged mice treated with DEX (D). The panel is magnified 400 $\times$.

OVA-induced p-NF- κ B p65 translocation from the cytoplasm to the nucleus was strongly inhibited by DHA and that phosphorylation of I κ B α , which are required for p65 activation, were downregulated in DHA-treated mice *in vivo*. Muc5ac gene expression is dependent on the transcriptional activity of NF- κ B³⁰. We observed a substantial decrease in Muc5ac mRNA expression in the OVA-challenged lungs of DHA-treated mice. Taken together, our results suggest that DHA may suppress OVA-induced asthma by inhibiting NF- κ B activity. In addition, it has already been demonstrated that pharmacological inhibitor of ERK phosphorylation attenuates allergen-induced airway reactions⁶. The expression of Th2 cytokine genes has previously been reported to be blocked by p38 MAPK activation³¹. Therefore, MAPK is also a key mediator of allergic diseases. In trying to understand the mechanisms by which DHA regulates Th2 cytokines, we investigated the effect of DHA on the MAPK. Western blot analysis showed that DHA can inhibit the activation of p38 and ERK. Our results suggest that DHA may attenuate OVA-induced asthma by inhibiting p38 MAPK and ERK pathways, which might have therapeutic benefit in suppressing allergic inflammation.

The initial indication for cytokine involvement in the pathogenesis of asthma came from studies performed in the early 1990s, which showed that allergic asthma is associated with Th2 cytokine expression³². These Th2 cytokines induce inflammatory responses, such as airway infiltration and eosinophil activation, IgE production and mucus secretion. IL-4 and IL-13 play critical roles in IgE switching within B cells, in eosinophil infiltration into the

airway and in mucus hypersecretion, whereas IL-5 is essential for the activation and survival of eosinophils and the development of AHR³³. In knockout murine models, these cytokines have been implicated in sensitization and allergic responsiveness to increase in the IgE level and in eosinophilia in the airways. Because elevated IgE levels have been reported to be important in the development of asthmatic responses, we measured the IgE levels³⁴. In our study, DHA treatment significantly decreased the concentrations of IL-4, IL-5, IL-13 and IgE in the OVA-challenged mouse model. Therefore, it is possible that DHA may attenuate OVA-induced asthma by reducing Th2 cytokines and IgE levels.

Several lines of evidence suggest that inflammatory mediators released during allergic inflammation play a pivotal role in causing asthma symptoms such as AHR, airway inflammation, goblet cell hyperplasia, airway luminal narrowing and subepithelial fibrosis^{3,35}. Our results show that DHA significantly inhibited OVA-induced AHR in response to increasing concentrations of Mch, the degree of inflammatory cell infiltration in the peribronchial and perivascular areas and goblet cell hyperplasia. Thus, the observed reduction of AHR, airway inflammation and goblet cell hyperplasia may be attributed to the inhibition of Th2 cytokine production, eosinophils and serum IgE levels.

The increase in eosinophilic inflammation and the enhancement of AHR in the asthmatic individuals are regarded as the cardinal features of asthma³⁶. In addition, eosinophils, neutrophils and macrophages are also important mediators of the inflammatory response through their

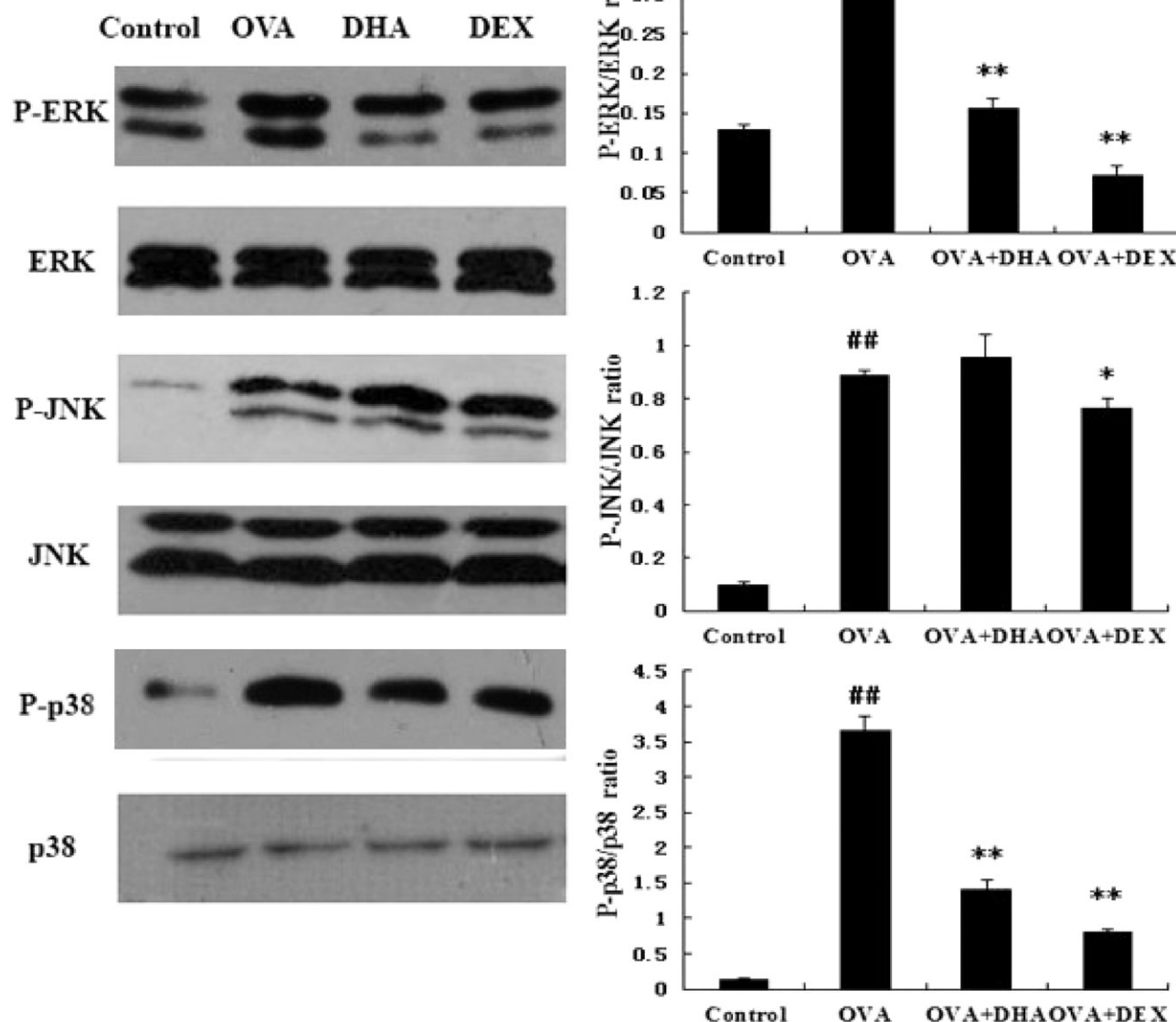


Figure 5. Effect of DHA (30 mg/kg) and DEX (2 mg/kg) on MAPK pathway activation. Detection of phosphorylated or total forms of the three MAPK molecules (ERK, p38 and JNK) by western blot. The values represent the mean $\pm$ SEM of three independent experiments. ^{##} $p < 0.01$ versus control group, ^{*} $p < 0.05$, ^{**} $p < 0.01$ versus OVA.

cytokines production. Our results showed that DHA treatment reduced the recruitment of total cell, neutrophil, macrophage and eosinophil. This observed effect of DHA treatment on eosinophils may be due to a reduction in lymphocyte populations or a decrease in the Th2 cytokines that are responsible for the recruitment of eosinophils into the lung during asthma. AHR, a useful marker of airway abnormality in asthma, has been used in epidemiological studies and is predictive of the subsequent course of disease³⁷. Our data demonstrate that DHA inhibited OVA-induced AHR in response to inhaled Mch. IL-13 has been established as an important mediator of AHR³¹. As such, the observed inhibition of AHR by DHA may be associated with the reduction in IL-13 production and tissue eosinophilia.

Chitinase protein Ym2 has recently been found to be markedly elevated in allergic airway inflammation in human and in mouse asthma models³⁸. When given intratracheally,

IL-13 elevates Ym2 level in BALF from OVA-induced mice. Our results show that DHA markedly downregulated Ym2 mRNA expression in the lungs of OVA-challenged mice. These changes in expression may be a consequence of the large decreases in IL-4 and IL-13 levels in the airways after DHA treatment and may contribute to the diminished pulmonary eosinophilia.

In our study, we report here that the antimalarial agent DHA effectively reduced OVA-induced Th2 cytokines, inflammatory cell recruitment into BALF, pulmonary eosinophilia, OVA-specific IgE serum levels, mucus hypersecretion, goblet cell hyperplasia, expression of Muc5ac and Ym2, and AHR in a mouse asthma model, potentially via inhibition of the NF- κ B and MAPK signaling pathways. In conclusion, the results obtained from this study suggest that the DHA attenuates inflammation in an OVA-induced mouse model of allergic asthma. Although DHA sheds some light on this

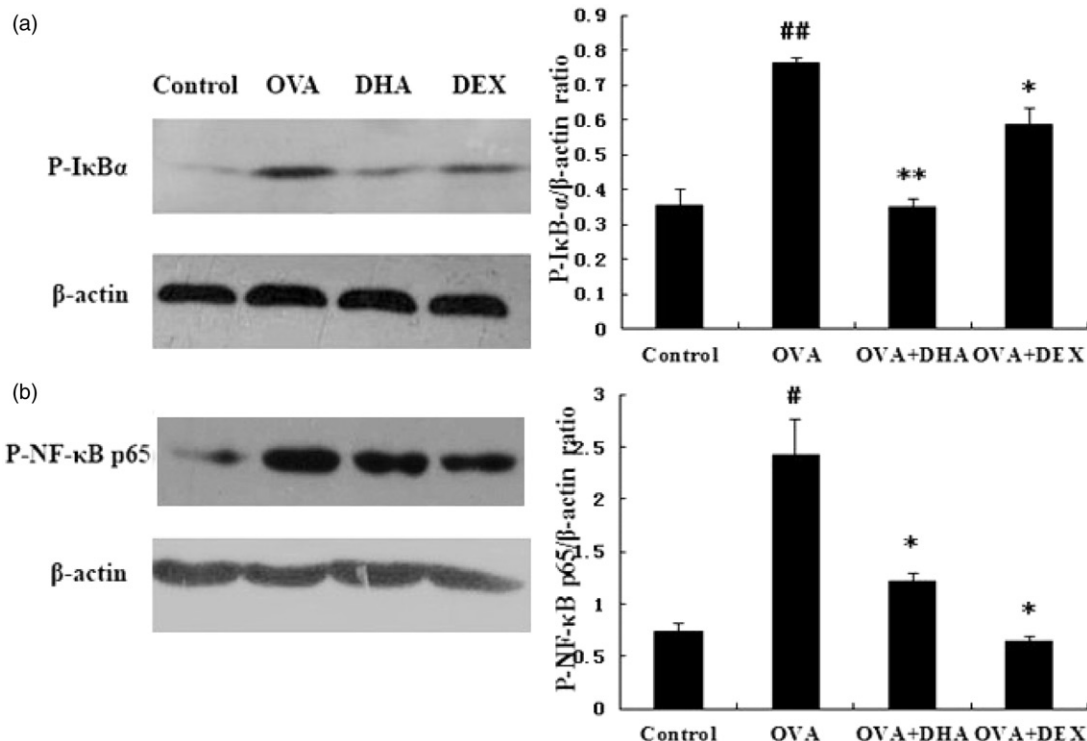


Figure 6. Effect of DHA (30 mg/kg) and DEX (2 mg/kg) on NF-κB pathway activation. (a) Detection of phosphorylated IκB-α and (b) detection of the P-NF-κB p65 subunit by western blot. The values represent the mean ± SEM of three independent experiments. [#] $p < 0.05$, ^{##} $p < 0.01$ versus control group, ^{*} $p < 0.05$, ^{**} $p < 0.01$ versus OVA.

issue, further and more comprehensive studies are still required before the full clinical application.

Declaration of interest

This work was supported by Natural Science Foundation of Jilin Province of China (No. 20101579) and National High-tech R&D Program of China (863 Program) (No. 2011AA10A214).

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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