



Full length article

Inhibition of soluble epoxide hydrolase attenuates airway remodeling in a chronic asthma model



Jun-xia Jiang^{a,c,1}, Yan Guan^{a,c,1}, Hui-juan Shen^{b,c}, Yong-liang Jia^c, Jian Shen^c, Lin-hui Zhang^{a,c}, Qi Liu^c, Yi-liang Zhu^d, Qiang-min Xie^{a,c,*}

^a Children's Hospital, Zhejiang University School of Medicine, Hangzhou, 310052, China

^b The Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, 310009, China

^c Zhejiang Respiratory Drugs Research Laboratory of State Food and Drug Administration of China, Zhejiang University School of Medicine, Hangzhou, 310058, China

^d Hangzhou Medical College, Hangzhou, 310053, China

ARTICLE INFO

Keywords:

Soluble epoxide hydrolase

AUDA

Asthma

Airway remodeling

Airway hyperresponsiveness

Epoxycyclo-oxygenase

ABSTRACT

Airway remodeling in asthma is difficult to treat because of its complex pathophysiology that involves proinflammatory cytokines, as well as the arachidonic acid cytochrome P-450 (CYP) pathway; however, it has received little attention. In this study, we assessed the efficacy of a soluble epoxide hydrolase (sEH) on airway remodeling in a mouse model of chronic asthma. The expression of sEH and CYP2J2 and the level of 14,15-epoxyeicosatrienoic acid (14,15-EET), airway remodeling and hyperresponsiveness (AHR) were analyzed to determine the level of sEH inhibition. AUDA, a sEH inhibitor, was given daily for 9 weeks orally, which significantly increased the level of 14,15-EET by inhibiting the expression of sEH and increasing the expression of CYP2J2 in lung tissues. The inhibition of sEH reduced the expression of remodeling-related molecular markers, such as interleukin (IL)-13, IL-17, matrix metalloproteinase 9, N-cadherin, α -smooth muscle actin (α -SMA), S100A4, Twist, epithelial goblet cell metaplasia, and collagen deposition in bronchoalveolar lavage fluid (BAL fluid) and lung tissues. Moreover, remodeling-related eosinophil accumulation in the BAL fluid and infiltration into the lung tissue were improved by AUDA. Finally, AUDA alleviated AHR, which is a functional indicator of airway remodeling. The effect of AUDA on airway remodeling was related to the downregulation of extracellular-regulated protein kinases (Erk1/2), c-Jun N-terminal kinases (JNK) and signal transducer and activator of transcription 3 (STAT3). To our knowledge, this is the first report to demonstrate that inhibition of sEH exerts significant protective effects on airway remodeling in asthma.

1. Introduction

Asthma is a chronic respiratory inflammatory disease, and its incidence has increased significantly in the past two decades, placing a huge burden on society (Sagani and Lloyd, 2015; Global Initiative for Asthma (GINA), 2019). Airway obstruction with a narrowed airway lumen diameter is a hallmark feature of asthma. The main cause of pathological changes in the airway is chronic inflammation of the airway wall, accompanied by plasma extravasation, edema, and infiltration of inflammatory cells such as mast cells, eosinophils, neutrophils, macrophages and lymphocytes (Sagani and Lloyd, 2015). Clinical features of asthma are dyspnea caused by airway hyperresponsiveness (AHR) and bronchoconstriction, which can be reversed with bronchodilator therapy. However, bronchodilator therapy does

not always relieve dyspnea in some severe patients. Therefore, other factors involved in this persistent airflow obstruction should be considered (Dunican et al., 2018). One of the key mechanisms is airway remodeling, which is a summary term that includes pathologies such as goblet cell metaplasia (GCM), epithelial-to-mesenchymal transition (EMT), excessive subepithelial collagen deposition, airway smooth muscle hyperplasia, and increased vascularity (Fehrenbach et al., 2017; Carpaij et al., 2019). Although most think that airway remodeling as an abnormal response to chronic airway inflammation, recent evidence suggests that both processes can occur simultaneously, especially in asthmatic children (Malmström et al., 2017; Tillie-Leblond et al., 2008). The effect of asthma therapy on airway remodeling has not been widely studied and there are no specific drugs. As an anti-inflammatory drug, glucocorticoid has been a first-line drug for asthma treatment because

* Corresponding author. Zhejiang University School of Medicine, #866 Yuhangtang Rd., Hangzhou, 310058, Zhejiang Province, China.

E-mail address: xieqm@zju.edu.cn (Q.-m. Xie).

¹ These authors contributed equally to this work.

of its action on inflammatory cells and structural cells, as well as in airway remodeling (Hoshino et al., 1999; Roth et al., 2004). Early interventions to prevent remodeling may possibly help prevent the development of asthma but much remains to be studied about this possibility (Hirota and Martin, 2013). Although we still do not know how efficiently prevent airway remodeling, there is an urgent need to explore the mechanisms and identify reliable drugs for the prevention and treatment of the airway remodeling processes that underly asthma pathology.

The metabolites of cytochrome P450 (CYP) epoxygenases and epoxyeicosatrienoic acids (EETs) modulate many physiological functions, including inflammation. Regulation of EETs occurs primarily via release, biosynthesis and enzymatic transformation by the soluble epoxide hydrolase (sEH) (Wagner et al., 2017). The Hammock lab reported for the first time that administration of the sEH inhibitor t-TUCB attenuates allergic airway inflammation and airway responsiveness in a murine model (Yang et al., 2015). In our previous research, we reported that a sEH inhibitor AUDA, could treat pulmonary fibrosis. AUDA can reduce bleomycin-induced lung fibrosis in mice, including inhibiting pulmonary inflammation, expression of proinflammatory cytokines and EMT-related factors, as well as improve declining lung function (Dong et al., 2017). However, there is little information on whether inhibition of sEH can be used to inhibit airway remodeling, and the possible mechanisms remain yet to be established. Molecular studies suggest that the mitogen-activated protein kinases (MAPK) family (Pu et al., 2019; Schuliga, 2015) and signal transducer and activator of transcription 3 (STAT3) (Vale, 2016), in addition to other pathways, play pivotal roles in regulating allergic airway remodeling and inflammation in asthma under various contexts.

In the present study, we evaluated the effects and the possible mechanisms of inhibition of sEH on airway remodeling in a chronic asthmatic model. We found that AUDA inhibited airway hyperresponsiveness (AHR) and remodeling by inhibiting sEH, and activating expression of CYP2J2, and decreasing the phosphorylation of Erk1/2, JNK and STAT3 signaling pathways. These results demonstrated that the inhibition of sEH may be a potential therapeutic target for airway remodeling.

2. Materials and methods

2.1. Reagents and antibodies

Ovalbumin (OVA) and methacholine (Mch) were purchased from Sigma-Aldrich (St. Louis, MO). AUDA was purchased from the Cayman Chemical Company (Ann Arbor MI). Primary antibodies against GAPDH (Santa Cruz Biotechnology, CA); sEH (Abcam, Cambridge, MA, USA), p-Erk1/2, Erk1/2, p-JNK, JNK, p-p38, p38, p-STAT3, and STAT3 (Cell Signaling Technology, Danvers, MA) were used in the immunoblotting analysis. The enzyme-linked immunosorbent assay (ELISA) kits for mouse IL-13, IL-17, matrix metalloproteinase 9 (MMP-9) and 14, 15-

EET/DHET were purchased from eBioscience (San Diego, CA) and R&D (Detroit, MI, USA).

2.2. Animals

All animal experiments were carried out strictly in accordance with international ethical guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals. Female ICR mice (weighing 20 ± 2 g) were purchased from Shanghai Slac Laboratory Animal Co Ltd (NO. SCXK, 2012-0002). Mice were housed for 7 days before experiments to adapt themselves to the environment. Four to five mice were housed in isolated ventilated cages, kept at 45–65% humidity, at 20–23°C and a 12 h light/12 h dark cycle. Water and standard mouse food were provided *ad libitum*. All of the animal procedures and experiments conducted in this study were approved by the Animal Ethics Committee of the Zhejiang University (Permit No. ZJU20160274). All the animals received humane care in accordance with the guide prepared by the Committee on the Care and Use of Laboratory Animals of Zhejiang University.

To investigate the impact of sEH on airway remodeling, ICR mice were randomly divided into four groups, including a control group ($n = 8$), an OVA model group ($n = 10$), an OVA + AUDA (3 mg/kg) group ($n = 10$), and an OVA + AUDA (10 mg/kg) group ($n = 10$). The AUDA dose design in the experiment was based on our previous study (Dong et al., 2017). The study did not include a positive control group because there were no specific or approved drugs for airway remodeling.

2.3. Chronic asthmatic model and treatment procedure

The procedure for antigen sensitization in mice is based on our previous methods. (Guan et al., 2018; Cao et al., 2011). In brief, 2 mg OVA (Grade V) was absorbed in 10 mg of an aluminium hydroxide gel adjuvant in 1.0 ml saline. Each mouse was intraperitoneally (i.p.) injected by with 0.5 ml, and subcutaneously injected (s.c.) with 0.05 ml/site at the four footpads, neck, back, and two groin regions, for a total of 10 sites on day 0. Normal control mice were injected with only the aluminium hydroxide gel adjuvant in saline following the same protocol. To enhance sensitization, 2 mg of ovalbumin in 0.5 ml of the 1% aluminum hydroxide gel solution was again injected by i.p. on the 14th day. Then, starting on day 21, mice were exposed to 10 mg/ml OVA by aerosol for 30 min daily until day 28. After day 28, mice were exposed to 10 mg/ml OVA three days a week for another eight weeks. OVA was replaced with normal saline in the control group during sensitization and challenge. The mice were i.g. administered 3 or 10 mg/kg AUDA 30 min before OVA challenge, while the mice in the control and the model groups were instead given the same volume of vehicle (0.5% CMC-Na). The OVA sensitization, challenge, and drug treatment procedure is shown in Fig. 1.

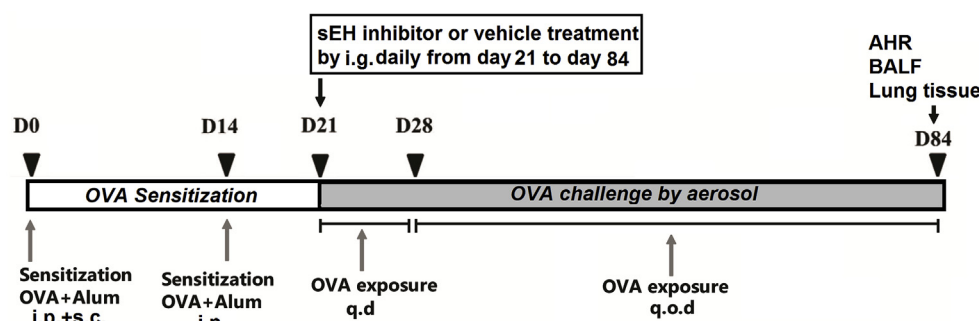


Fig. 1. Chronic asthma model and treatment procedure. The mice were sensitized with ovalbumin (OVA) and aluminum hydroxide gel by intraperitoneal (i.p.) and subcutaneous (s.c.) injection on days 0 and 14, and OVA (10 mg/ml) challenge was performed by aerosol for 30 min per day (q.d) from day 21 to day 28. The mice continue to inhale OVA for 30 min every other day (q.o.d) from day 29 to day 84. The control mice were sensitized and challenged only with only saline. The sEH inhibitor (AUDA) or vehicle (0.5% CMC-Na) were given by intragastric administration at

30 min before each OVA challenge daily from day 21 to day 84. Airway hyperresponsiveness (AHR), bronchoalveolar lavage fluid (BAL fluid) collection were performed and lung tissues were collected 60 min after the final antigen challenge on day 84.

Table 1

Primer sequences used in the present study.

Genes	Primer Sequences (5'-3')	
IL-13	Sense:	CCTCTGACCCTTAAGGAGCTTAT
	Antisense:	CGTTGCACAGGGGAGTCT
IL-17	Sense:	GAGAAGATGCTGGTGGGTGT
	Antisense:	TTTCATTGTGGAGGGCAGAC
MMP-9	Sense:	GTATGGTCGTGGCTCTAAGC
	Antisense:	AAAACCTCTTGGTCTGCGG
N-Cadherin	Sense:	CGGTGCCATCATTGCCATCCT
	Antisense:	AGTCATAGTCCTGGTCTTCTCTCCT
Twist	Sense:	TACGCCCTTCTCCGTCTGG
	Antisense:	CTAGTGGGACGCGGAC
β -actin	Sense:	GGCTGTATCCCTCCATC
	Antisense:	ATGCCATGTTCAATGGGGTA

2.4. Determination of airway hyperresponsiveness (AHR)

To assay lung function and evaluate AHR in unrestrained conscious mice, a whole-body plethysmography (WBP) and associated software (Buxco Electronics, Troy, NY) were used. The principle of the measurement method has been reported before. Its main parameter, enhanced pause (Penh), is the total lung airflow during the respiratory cycle, which can represent airway resistance (Hamelmann et al., 1997; Hoymann, 2007). Each mouse was placed in a measurement chamber for 10 min before AHR evaluation. Saline or methacholine (3.125, 6.25, 12.5, 25 and 50 mg/ml) were aerosolized via a chamber inlet for 90 s. Respiratory function was recorded for 3 min after each nebulization of the various methacholine concentrations. The change in percentage of airway resistance was expressed as an increased percentage of the Penh value.

2.5. Collection of bronchoalveolar lavage fluid (BAL fluid) and cell counts

To collect BAL fluid, mice were anesthetized with isoflurane, the

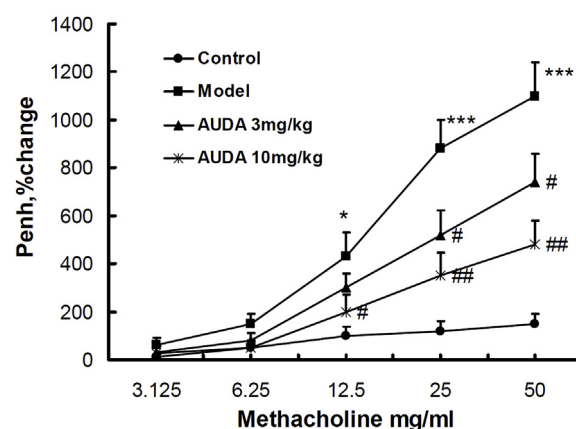


Fig. 3. Effects of sEH inhibition on airway hyperresponsiveness. Lung function of was measured 24 h after the last challenge following treatment with AUDA at doses of 3 mg/kg and 10 mg/kg. AHR to methacholine (3.125–50 mg/ml) was induced by administration via inhalation. Airway resistance was measured as Penh values. The results were expressed as the percentage change in Penh value above baseline. The data are represent as the means $\pm$ S.D. ($n = 8-10$ for each group). * $P < 0.05$, *** $P < 0.001$ vs. control. # $P < 0.05$, ## $P < 0.01$ vs. model.

trachea was intubation, and the lungs were lavaged three times with 0.5 ml PBS containing 1% BSA and 5000 IU/L heparin. The total number of cells in the BAL fluid was counted using a Neubauer chamber. The BAL fluid was centrifuged at 250 g at 4°C for 10 min. The supernatant was stored at -80°C for analysis by ELISA. The centrifugal sediment was smeared on a glass slide and stained with Wright-Giemsa. Two hundred cells from the BAL fluid were counted under a light microscope, and cell differentials were determined by counting the number of eosinophils, macrophages and lymphocytes. The total number of each cell type was determined by multiplying the percentage

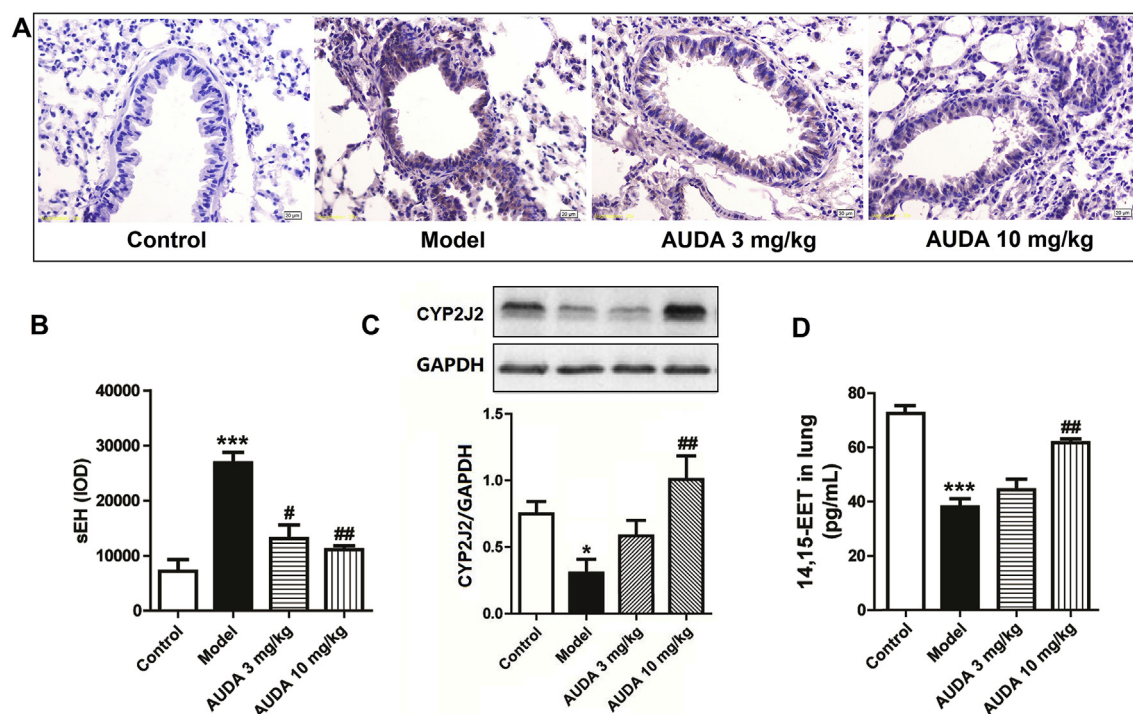


Fig. 2. Effects of sEH inhibition on the expression of soluble epoxide hydrolase and CYP2J2, and the levels of 14,15-EET. Soluble epoxide hydrolase (sEH) and CYP2J2 protein expression were assessed by immunohistochemistry (A and B, $n = 8-10$ for each group) and Western blot (C, $n = 6$ for each group), and the 14,15-EET levels were assessed by ELISA (D, $n = 8-10$). All measurements were performed 24 h after the last challenge following treatment with the sEH inhibitor AUDA at doses 3 mg/kg and 10 mg/kg. The data represent means $\pm$ S.D. * $P < 0.05$, *** $P < 0.001$ vs. control. # $P < 0.05$, ## $P < 0.01$ vs. model. Scale bar, 20 μm .

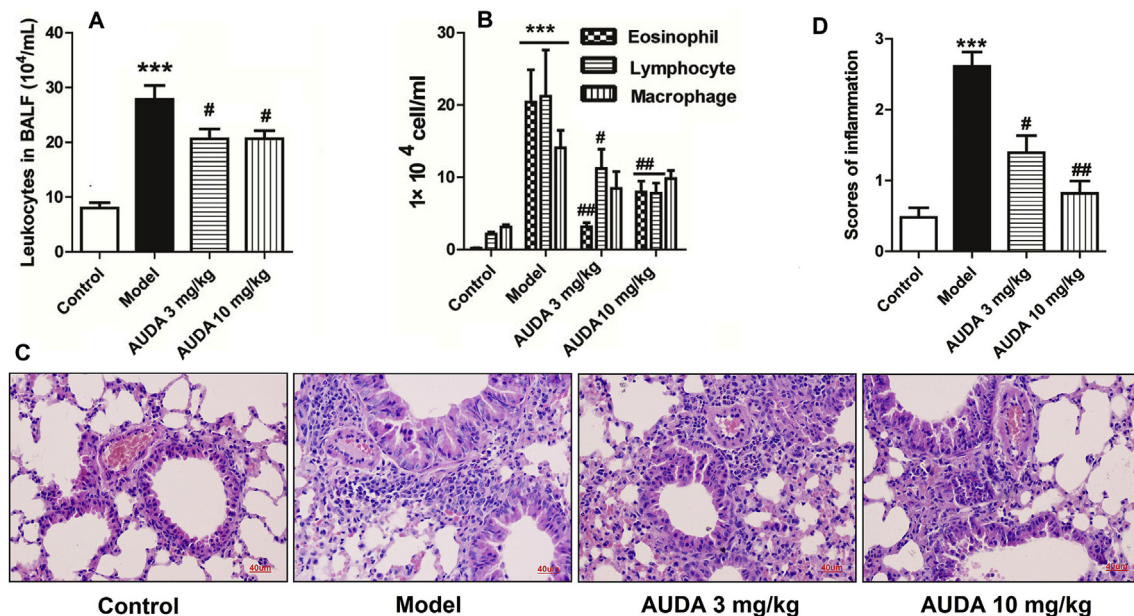


Fig. 4. Effects of SEH inhibition on airway inflammation. (A) The total number of inflammatory cells in the BAL fluid was counted, (B) Differential cell counts in the BAL fluid were determined by Wright-Giemsa staining and cell classification was performed on a minimum of 200 cells to classify eosinophils, neutrophils, lymphocytes and macrophages 24 h after the final antigen challenge. (C) Lung sections were stained with H&E to measure the inflammatory cells infiltrate in the peribronchiolar space. (D) Inflammatory cell infiltration was graded based on the severity of inflammation (bar graph). The data are represented as the means $\pm$ S.D. ($n = 8-10$ for each group). *** $P < 0.001$ vs. control. # $P < 0.05$, ## $P < 0.01$ vs. model. Scale bar, 40 μm .

of each cell type by the total number of cells.

2.6. Histopathological and immunohistochemical examination

Samples of the left lung tissue were fixed in 10% formalin, and were then dehydrated by in increasing concentration of ethanol. After paraffin-embedding, the lung tissue sample was sectioned into 3–4 μm slices. Lung sections were prepared for routine hematoxylin-eosin (H&E), periodic acid-Schiff (PAS) and Masson's trichrome stains. H&E staining was used to evaluate inflammatory cell infiltration using light microscopy, based on the peribronchial cell counts and the severity of inflammatory cells infiltration. A 5-point scoring system was used as described previously (Guan et al., 2018; Cao et al., 2011). Goblet cell hyperplasia was observed with PAS staining. The percentage of PAS staining-positive cells in the airway epithelium was quantified (Guan et al., 2018; Cao et al., 2011). Collagen deposition around the bronchial airway was observed by Masson's trichrome staining. The severity of collagen deposition was evaluated using the Image Pro 6.1 software system according to the positive staining area. The criterion for grading the severity of airway remodeling, including the thickness of the airway smooth muscles, collagen deposition, and immunohistochemistry analysis of airway remodeling markers including N-Cadherin, α -SMA, S100A4 and MMP-9. Under $200\times$ magnification, five fields of each section were randomly selected and photographed for semiquantitative analysis. Immunohistochemistry analysis was performed according to the specification of the Streptavidin-Biotin Complex kit (Boster Bio-engineering Ltd. Co., Wuhan, China); lung tissue slides were incubated at 4°C overnight with primary antibodies against SEH, N-Cadherin, α -SMA, MMP-9 and S100A4 (1:200 dilution). Then 3,3'-Diaminobenzidine (DAB) and the DP2-BSW software (Olympus, Tokyo, Japan) were used to visualize and measure the immunocomplexes. The staining was quantified using Image Pro 6.1 software. All the analyses were performed in a blind fashion.

2.7. Measurement of 14, 15-EET, IL-13, IL-17 and MMP-9 by ELISA

Lung tissues were collected 24 h after the last challenge. 14, 15-

epoxyeicosatrienoic acid (14, 15-EET) levels in the supernatant of the lung tissue homogenate were measured by ELISA according to the manual. The BAL fluid were collected 24 h after the last challenge. The concentrations of IL-13, IL-17 and MMP-9 in the BAL fluid were detected and analyzed using ELISA kits using with paired and matched antibodies according to the manufacturer's instructions. The color absorbance of the samples at 450 nm was determined using a Bio-Rad microplate reader. 14,15-EET, IL-13, IL-17 and MMP-9 concentrations were calculated by generating a standard curve using standard proteins and analyzed using curve expert 1.3 software.

2.8. RNA assay

RNA assays were carried out based on our previous report (Jiang et al., 2017). In brief, total RNA was extracted from lung homogenates according to the TRIzol Reagent (Takara Biotechnology, Dalian, China) manufacturer's instructions. A total of 4 μg of oligo-dTs were used to generate the first-strand cDNA from the total RNA through reverse transcription reactions. The PCR primers (Shanghai Bioengineering Ltd, China) were checked against the basic local alignment search tool for selectivity. The real-time PCR cycling instrument, the constitution of the PCR mixture, and the PCR reaction program were exactly the same as our previous report (Jiang et al., 2017). The mRNA levels were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method (relative) (Livak and Schmittgen, 2001). The primer sequences are shown in Table 1.

2.9. Western blot assay

The lung samples were lysed in an immunoprecipitation assay (IPA) buffer (Biyuntian Biotechnology, Haimen, China) containing 1% PMSF (Haixin Biotechnology, Hangzhou, China) and 1% protease inhibitors and then denatured. The BCA Protein Assay Kit (CWbiotech, Beijing, China) was used to determine the total protein concentration. SDS-polyacrylamide gels separated equal quantities of 30 μg protein samples. The proteins were then transferred to 0.45 μm nitrocellulose membranes. The membranes were blocked with 5% fat-free milk and incubated with antibodies specific for CYP2J2 (polyclonal antibody,

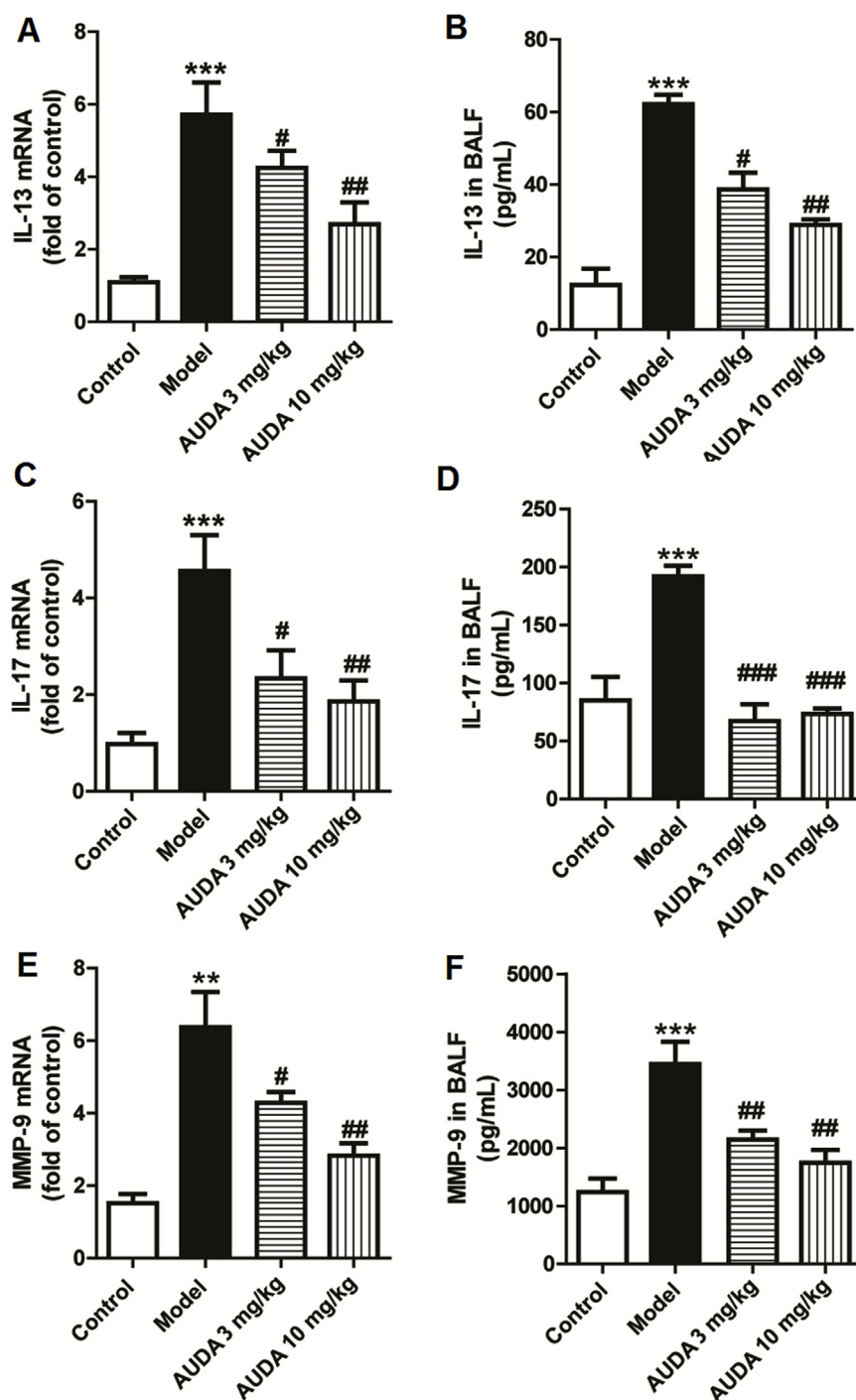


Fig. 5. Effects of sEH inhibition on expression of IL-13, IL-17 and MMP-9 in lung tissues. IL-13, IL-17 and MMP-9 mRNA and protein in lung tissues were measured by qRT-PCR (A, C, E) and ELISA (B, D, F). The data are represented as the means $\pm$ S.D. ($n = 8-10$ for each group). * $P < 0.05$, *** $P < 0.001$ vs. control. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. model.

1:1000, Invitrogen, USA), p-Erk1/2, Erk1/2, p-JNK, JNK, p-p38, p38, p-STAT3, and STAT3 (1:1000, Cell Signaling Technology, Beverly, MA, USA) or GAPDH (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), at 4 °C overnight. Afterwards, the blots were incubated with goat anti-rabbit 800 antibodies (1:5000) for 2 h at 26 °C. A two-color infrared imaging system (Odyssey; LI-COR, Lincoln, NE, USA) was used to visualize the immunoreactive bands.

2.10. Statistical analysis

Data analysis was carried out with GraphPad Prism V5.0 software (GraphPad Prism, CA, USA). SPSS software (version 16.0; SPSS Inc., Chicago, IL, USA) was used for statistical tests. One-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls test was used to analyze differences between the mean values of multiple groups. The data are presented as the means $\pm$ S.E.M. Statistical significance was set at $P < 0.05$.

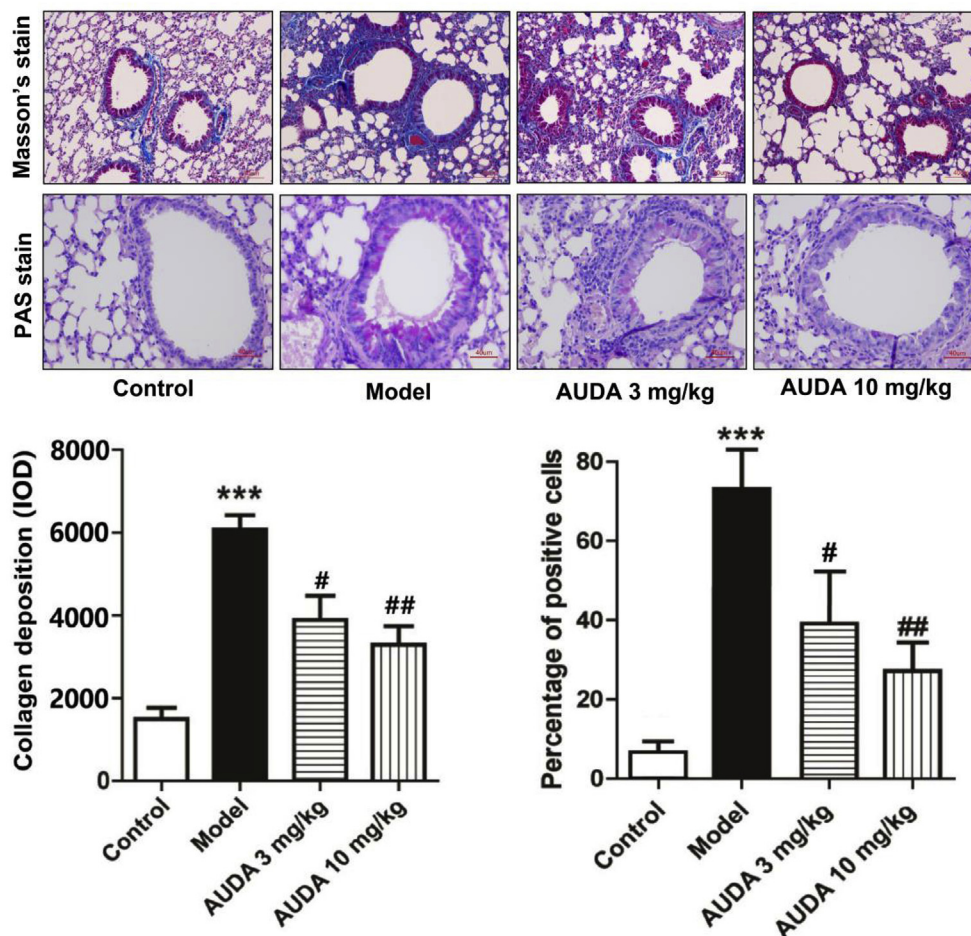


Fig. 6. Effects of sEH inhibition on collagen deposition and goblet cell hyperplasia. Lung sections were stained with Masson's trichrome stain for measurement of the sub-epithelial deposition of collagen and fibrosis. Masson's trichrome staining analysis of collagen deposition was calculated (bar graph). Lung sections were stained with PAS to assess goblet cell hyperplasia. PAS-positive and PAS-negative epithelial cells were counted, and the percentage of PAS-positive cells per bronchiole was calculated (histogram). The data are represented as the means $\pm$ S.D. ($n = 8-10$ for each group), *** $P < 0.001$ vs. control. # $P < 0.05$, ## $P < 0.01$ vs. model. Scale bar, 40 μ m.

3. Results

3.1. Inhibition of sEH reduces sEH expression, and increases CYP2J2 expression and 14,15-EETs level

To verify CYP450 pathway changes in the chronic asthma model, the expression of sEH and CYP2J2 and levels of 14,15-EETs were observed by immunohistochemical assay, Western blot and ELISA. We observed an increase in protein expression of sEH and the decrease in CYP2J2 expression and 14,15-EETs levels in the model group after sensitization and challenge with OVA, which was reversed by 10 mg/kg AUDA. Treatment with 3 mg/kg AUDA significantly reduced the expression of sEH but did not affect the expression of CYP2J2 or the levels of 14,15-EETs (Fig. 2A–D).

3.2. Inhibition of sEH reduces methacholine-induced AHR

To investigate the effect of AUDA on AHR, the airway resistance (Penh value) in response to methacholine (3.125–50 mg/ml) inhalation was measured using a whole-body plethysmography. OVA-challenged mice had remarkably increased Penh values compared to the saline-challenged mice starting with 12.5 mg/ml to 50 mg/ml of methacholine ($P < 0.05$, $P < 0.001$), which was significantly reduced by both doses of AUDA (3 mg/kg and 10 mg/kg; $P < 0.05-0.01$) (Fig. 3).

3.3. Inhibition of sEH prevents infiltration of inflammatory cells into lung tissues

The inflammatory cells in BAL fluid were measured among the different groups. As shown in Fig. 4A–B, the numbers of total

leucocytes, eosinophils, lymphocytes and macrophages were significantly higher in the model group compared to the control group ($P < 0.001$). Treatment with AUDA at 3 or 10 mg/kg significantly inhibited the numbers of total leucocytes, eosinophils and lymphocytes, but not macrophages, compared to the model group ($P < 0.05-0.01$).

Histological examination of H&E-stained lung sections showed a marked infiltration of eosinophils and lymphocytes cells in the peribronchiolar space in the model group, compared to control group (Fig. 4C). The model group displayed typically characteristics including a significant infiltration of eosinophils and lymphocytes in the peribronchiolar space as shown in the H&E-stained lung sections (Fig. 4C). AUDA significantly reduced the inflammatory cell infiltration (Fig. 4D).

3.4. Inhibition of sEH suppresses IL-13, IL-17 and MMP-9 mRNA and protein expression

As shown in Fig. 5, IL-13, IL-17 and MMP-9 mRNA expression in the lung tissues were significantly elevated in the model group compared to the control group ($P < 0.01$ to $P < 0.001$). AUDA at 3 or 10 mg/kg markedly reduced the mRNA expression of IL-13, IL-17 and MMP-9 ($P < 0.05$ to $P < 0.001$) (Fig. 5A, C, 5E). Further studies found that IL-13, IL-17 and MMP-9 changes at the protein levels were consistent with the observed changes in the mRNA expression (Fig. 5B, D, 5F).

3.5. Inhibition of sEH decreases airway remodeling-related markers

To evaluate the effects of AUDA on OVA-induced collagen deposition around the bronchus and goblet cell hyperplasia in the airway epithelium, collagen deposition and goblet cell hyperplasia were measured by Masson's straining and of PAS straining, respectively. As

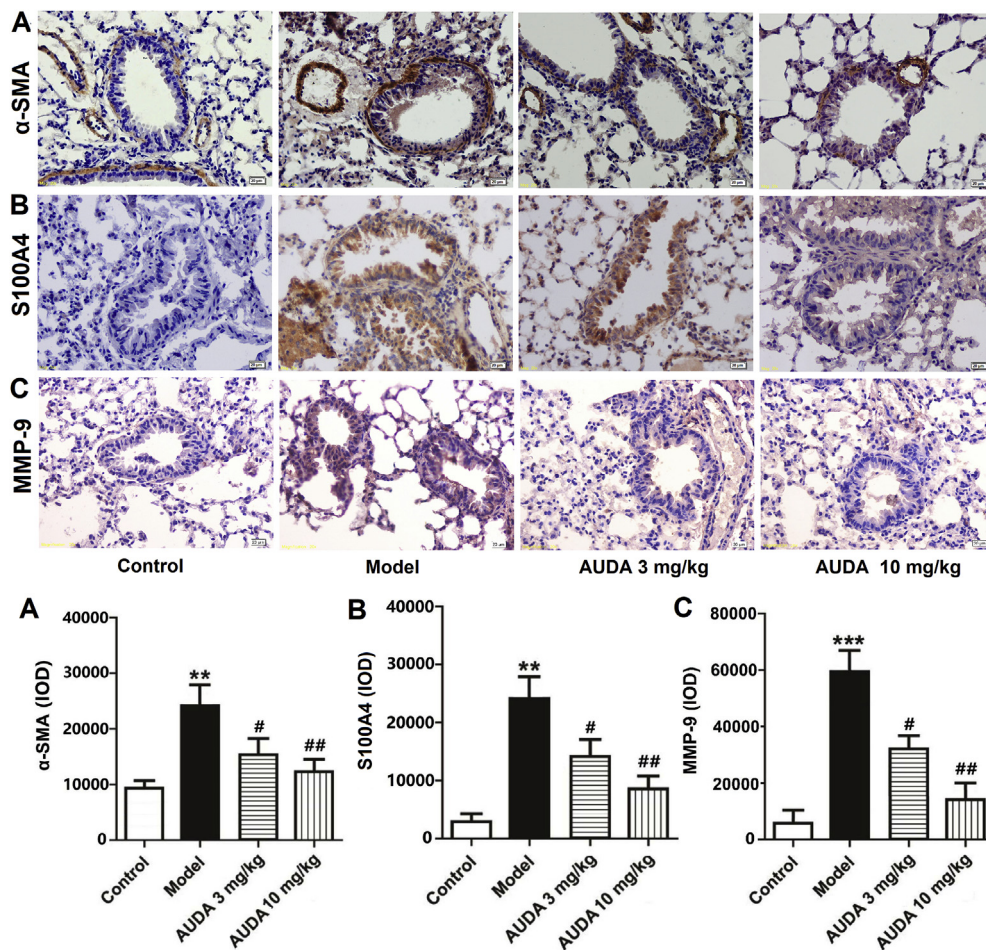


Fig. 7. Effects of sEH inhibition on the overexpression of α -SMA, S100A4 and MMP-9. In the representative pictures, paraffin sections were prepared and stained by immunohistochemistry to evaluate the expression of the α -SMA (A), S100A4 (B) and MMP-9 (C) proteins in lung tissues. A semiquantitative analysis was performed. The data are represented as the means $\pm$ S.D. ($n = 8-10$ for each group). ** $P < 0.01$, *** $P < 0.01$ vs. control. # $P < 0.05$, ## $P < 0.01$ vs. model. Scale bar, 20 μ m.

shown in Fig. 6, thicker-walled alveoli with small diameters, thickened septa and an accumulations of collagen deposition (blue staining) around the bronchi emerged in the model group, which was significantly different from the control group ($P < 0.001$). AUDA at 3 mg/kg and 10 mg/kg decreased these pathological changes in a dose-dependent fashion ($P < 0.05$ to $P < 0.001$). Similarly, the percentage of goblet cell positively stained by PAS in the model group was significantly increased to that in the control group ($P < 0.001$), but this increase was significantly weakened by AUDA at 3 mg/kg and 10 mg/kg ($P < 0.05$ to $P < 0.001$).

To investigate the effects of AUDA on OVA-induced the protein expression of EMT-related markers, immunohistochemistry was employed to investigate the expression of α -SMA, S100A4 and MMP-9 protein in the lung tissues. As shown in Fig. 7, A-C, the expression of the α -SMA, S100A4 and MMP-9 proteins in the model group was significantly higher than that in the control group. In particular, the increase in α -SMA expression and vascular smooth muscle, and these changes were markedly reduced by AUDA treatment. In addition, the N-cadherin and Twist mRNA expression and protein levels were examined using qPCR and Western Blot, respectively. As shown in Fig. 8A-D, we found an increase in both N-cadherin and Twist mRNA and proteins, and their protein levels were consistent with their changes in the mRNA expression. However, treatment with AUDA 3 or 10 mg/kg significantly inhibited the changes in N-cadherin and Twist mRNA and protein in the lung tissues compared to the model group.

3.6. Inhibition of sEH downregulates the activation of MAPKs and STAT3 signaling

Next, we analyzed the MAPKs and STAT3 signaling proteins associated with molecular mechanism of AUDA in airway remodeling by Western blotting. As shown in Fig. 9A, B, D, the phosphorylated Erk1/2, JNK and phosphorylated STAT3 protein were significantly increased in the model group compared to the control group. These changes were inverted by AUDA treatment. However, AUDA had no effect on p38 (Fig. 9C).

4. Discussion

Severe asthma is characterized by airway remodeling and inflammatory responses associated with aberrant metabolism of arachidonic acid. Clinical studies in the asthmatic patients have demonstrated that EETs levels are decreased by oxidative stress and sEH activity. Inhibitors of sEH increased EETs that mediated antiproliferative actions, suggesting a new therapeutic approach for severe asthma (Ono et al., 2014). In experimental studies, inhibitors of sEH have been shown to reduce lung injury caused by lipopolysaccharide (Zhou et al., 2017; Tao et al., 2016), bleomycin-induced pulmonary fibrosis (Dong et al., 2017), asthma caused by OVA (Yang et al., 2015), and COPD induced by cigarette smoke (Wang et al., 2012; Smith et al., 2005). EETs are the downstream metabolites of CYP2J or CYP2C and can be hydrolyzed by sEH into the less active dihydroxyeicosatrienoic acids (Shahabi et al.,

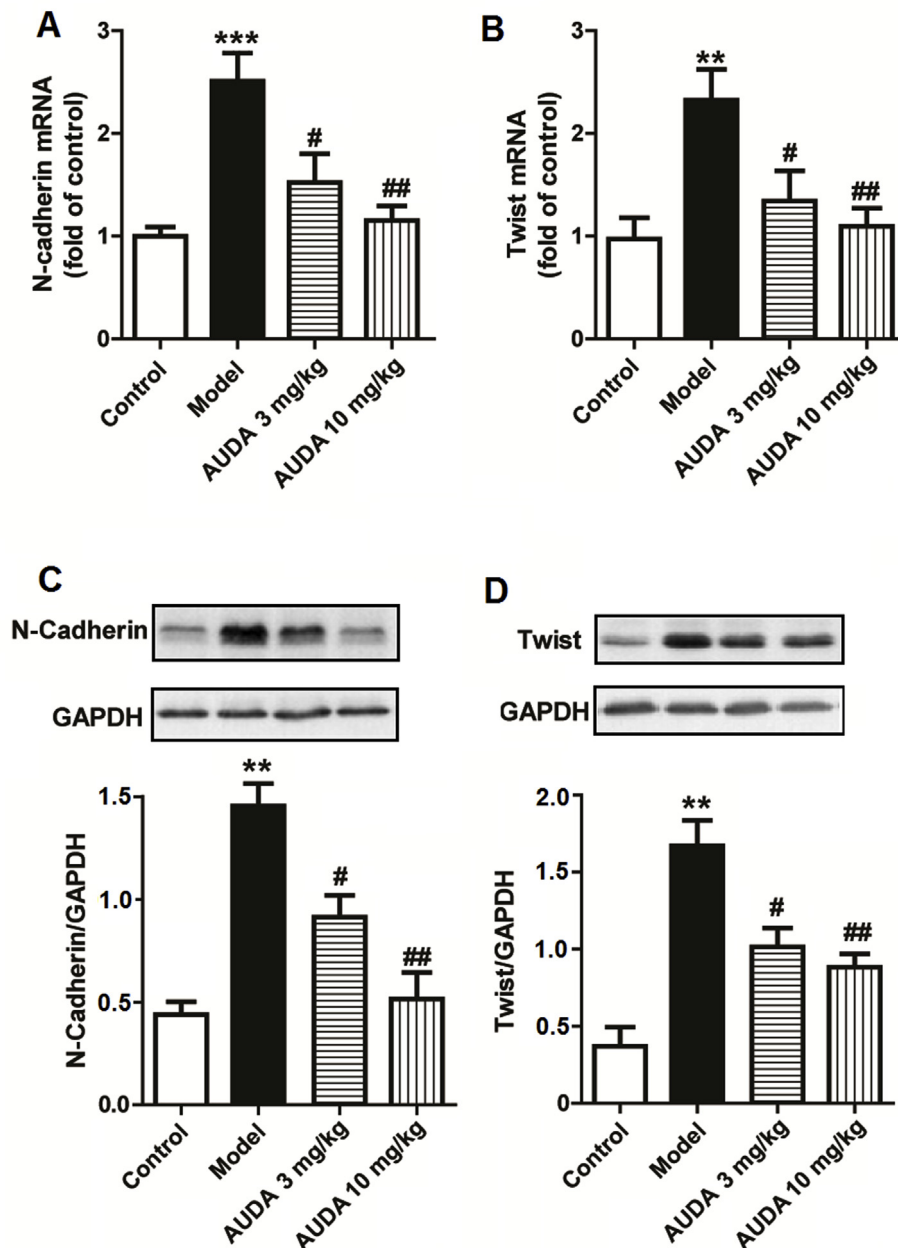


Fig. 8. Effects of sEH inhibition on the expression of N-cadherin and Twist. The expression of N-cadherin and Twist mRNA and protein were by evaluated by qRT-PCR (A and B, $n = 6$ for each group) and Western blot (C and D, $n = 6$ for each group). The data are represented as the means $\pm$ S.D. ** $P < 0.01$, *** $P < 0.001$ vs. control. # $P < 0.05$, ## $P < 0.01$ vs. model.

2014). In this study, we found that CYP2J2 protein expression and 14,15-EETs level in lung tissues were significantly suppressed in the OVA mouse model, while the AUDA-treated group had significantly increased CYP2J2 expression and 14,15-EETs level. Moreover, the asthmatic group had increased sEH protein expression, which was reversed by AUDA. These results demonstrate that the CYP pathway is involved in the airway remodeling process that is triggered in the chronic asthma model. This outcome is in agreement with previous reports demonstrating that the sEH inhibitor t-TUCB attenuates inflammation and airway responsiveness by inhibiting sEH and increasing EETs levels in mice (Yang et al., 2015).

Airway remodeling in asthma is thought to be the result of chronic and persistent inflammation including the irreversible destruction of airway tissue and chronic tissue repair. Thus, chronic inflammation has been described as a major driver of most aspects of airway remodeling

(Zhou-Suckow et al., 2017). The main basis for this inflammatory theory is that glucocorticoid therapy in asthma patients can not only reduce airway inflammation, but also significantly slows down airway remodeling (Hoshino et al., 1999; Roth et al., 2004). Infiltrating inflammatory cells and damaged airway structure cells create a large number of cytokines, enzymes, metabolites and growth factors that result in airway remodeling. In this study, we found that the number of total leucocytes, eosinophils and lymphocytes in the BAL fluid were increased after OVA challenge and that AUDA dose-dependently inhibited inflammatory cell infiltration into the airways. The histological examination of lung tissues also demonstrated similar results. Thus, the role of AUDA in inhibiting airway remodeling is at least partially related to its anti-inflammatory effects. Further anti-inflammatory evidence could also be obtained from the effect that AUDA had in reducing the mRNA and protein expression of the proinflammatory cytokines IL-

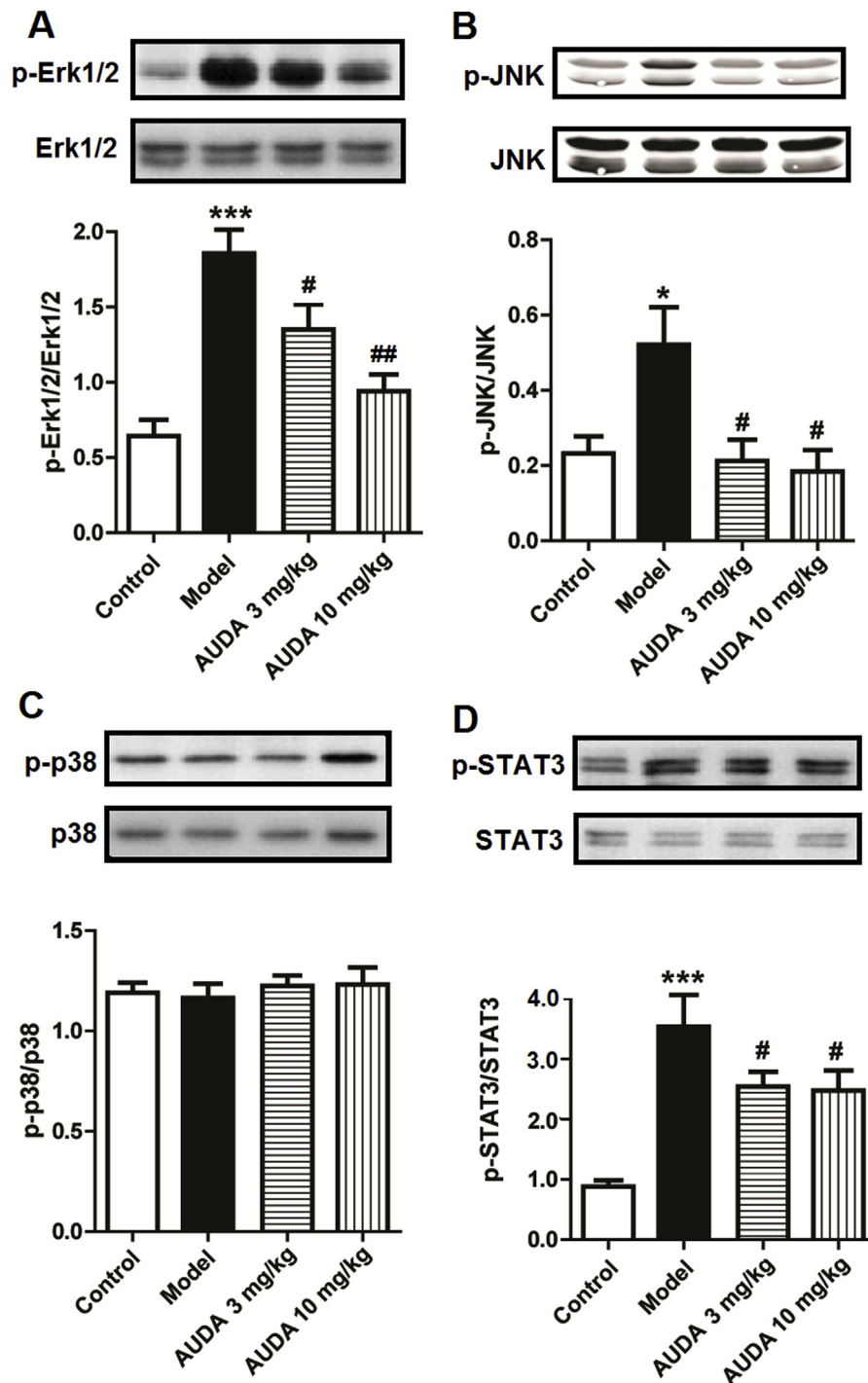


Fig. 9. Effects of sEH inhibition on the activation of the Erk1/2, JNK MAPK and STAT3. Mouse lung tissues from different treatment groups were harvested and the phosphorylation of Erk1/2 (A), JNK (B), p38 (C) and STAT3 (D) was measured by Western blot. The data are represented as the means $\pm$ S.D. ($n = 6$ for each group). ^{*} $P < 0.05$, ^{***} $P < 0.001$ vs. control. [#] $P < 0.05$, ^{##} $P < 0.01$ vs. model.

13 and IL-17.

In this study, we focused on both IL-13 and IL-17 because unlike IL-4 and IL-5, these cytokines act on structural cells in the airway (Ingram and Kraft, 2012; Malavia et al., 2008). IL-13 can induce expression of mucus proteins and mucus metaplasia in airway epithelial cells and submucosal glands in mice (Guan et al., 2018; Therien et al., 2008). It also plays a key role in human goblet cell hyperplasia (Wang et al., 2017). In addition, IL-13 induces the release of the profibrotic TGF- β by epithelial cells (Malavia et al., 2008). However, whether Th17 cells directly involved in airway remodeling is debatable. The loss of

Th17 cells leads to reduced airway remodeling, with reduced collagen fibers staining and α -SMA in a chronic asthma model (Zhao et al., 2013). In another chronic model, neutralization of IL-17 cells and IL-17a were found to increase with the increase in peribronchovascular density. Neutralization of IL-17 eliminated these signs of airway vascular remodeling (Lu et al., 2015). Therefore, in this study, the IL-13 and IL-17 cytokines were measured. The results showed that AUDA had an inhibitory effect on both proinflammatory factors, inhibiting both cytokines.

Increasing evidence suggests that the involvement of the EMT in the

bronchial remodeling of asthmatic patients (Ijaz et al., 2014; Post et al., 2018; Fischer et al., 2016; Johnson et al., 2011). In particular, the epithelium is increasingly gaining significant importance considering its potential as a source and target of inflammatory mediators, extracellular matrix components and growth factors. Our studies showed inflammation, airway remodeling and AHR after long-term exposure to antigen in a chronic asthma model over a period of 12 weeks. Airway remodeling or EMT-related markers such as the expression of N-cadherin, α -SMA, S100A4, and Twist were increased after exposure to antigen. This is increase in agreement with previous reports in a murine transgenic model in which airway epithelial cells express the lac-Z reporter gene. After 5 days of exposure to house dust mite allergens, epithelial cells underwent EMT, coexpressed the S100A4 protein and accumulated in the smooth muscle. Other epithelial cells coexpressing vimentin were found in the subepithelial region. These results suggest that the EMT is implicated in the airway remodeling associated with asthma (Pu et al., 2019; Fischer et al., 2016; Johnson et al., 2011). In addition, in this study, the indicators associated with airway remodeling such as bronchial epithelial goblet cell metaplasia, collagen deposition, and overexpression of the α -SMA, S100A4 and MMP-9 proteins, were also observed by histological and immunohistochemical examinations. The change in AHR pulmonary function as a comprehensive marker of airway remodeling is one of the clinic manifestations observed in the chronic asthma model. Our results showed that AUDA decreased EMT-related markers, such as goblet cell metaplasia, collagen deposition, and overexpression of N-cadherin, α -SMA, S100A4, Twist, and MMP-9, thereby improving AHR.

To further dissect the molecular mechanisms underlying the effects of sEH inhibition on airway remodeling and inflammation, we focused on the MAPK and STAT3 pathways. The MAPK family is fundamental in regulating multiple cell functions such as cytokine expression, proliferation, and apoptosis. MAPK and STAT3 activation in the airways drives a majority of allergen-induced inflammation and is a significant contributor to the structural remodeling of the airway wall (Pu et al., 2019; Zhang et al., 2013, 2019). Increased activation of MAPK has been demonstrated in the lungs after allergen challenge, as well as in airway epithelial cells, lung fibroblast and airway smooth muscle cells. Therefore, MAPK has emerged as a promising molecular target for the treatment of asthma (Zhang et al., 2013). The results in this study indicated that lung tissues in the chronic asthma model had significantly increased the phosphorylation of the Erk1/2 and JNK proteins whereas AUDA markedly suppressed Erk1/2 and JNK activity in this OVA-induced model of asthma. However, AUDA had no effects on the phosphorylation of p38. STAT3 has been shown previously to regulate the allergic response in asthma. In particular, epithelial STAT3 was identified as a critical regulator of allergen-induced inflammation and AHR in a murine model of asthma (Simeone-Penney et al., 2007). Here, we observed the clear phosphorylation of STAT3 in response to OVA, while AUDA inhibited the phosphorylation of STAT3 in the OVA-induced allergy model of asthma. These findings suggested that the therapeutic effects of sEH inhibition on airway remodeling are correlated with MAPK and STAT3 signaling.

5. Conclusion

We present the first study demonstrating that the inhibition of sEH effectively reduces airway remodeling, inflammation and AHR by inhibiting sEH and increasing EETs levels in the chronic asthma model. In addition, AUDA was associated with regulation of Erk1/2, JNK and STAT3 phosphorylation. These findings provide effective evidences that the inhibition of sEH may be a potent molecular target for airway remodeling.

Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (81872876, 81573439 and 81603117).

References

- Cao, R., Dong, X.W., Jiang, J.X., Yan, X.F., He, J.S., Deng, Y.M., et al., 2011. M₃ muscarinic receptor antagonist bencycloquidinium bromide attenuates allergic airway inflammation, hyperresponsiveness and remodeling in mice. *Eur. J. Pharmacol.* 655, 83–90.
- Carpaiz, O.A., Burgess, J.K., Kerstjens, H.A.M., Nawijn, M.C., van den Berge, M., 2019. A review on the pathophysiology of asthma remission. *Pharmacol. Ther.* 201, 8–24.
- Dong, X.W., Jia, Y., Ge, L.T., Jiang, J.X., Shen, J., et al., 2017. Soluble epoxide hydrolase inhibitor AUDA decreases bleomycin-induced pulmonary toxicity in mice by inhibiting the p38/Smad3 pathways. *Toxicology* 389, 31–41.
- Dunican, E.M., Elicker, B.M., Gierada, D.S., Nagle, S.K., Schiebler, M.L., Newell, J.D., et al., 2018. Mucus plugs in patients with asthma linked to eosinophilia and airflow obstruction. *J. Clin. Investig.* 128, 997–1009.
- Fehrenbach, H., Wagner, C., Wegmann, M., 2017. Airway remodeling in asthma: what really matters. *Cell Tissue Res.* 367, 551–569.
- Fischer, K.D., Hall, S.C., Agrawal, D.K., 2016. Vitamin D Supplementation reduces induction of epithelial-mesenchymal transition in allergen sensitized and challenged mice. *PLoS One* 11, e0149180.
- Global Initiative for Asthma (GINA), 2019. Global strategy for asthma management and prevention. 2019. GINA website. <http://ginasthma.org>.
- Guan, Y., Zhu, J.P., Shen, J., Jia, Y.L., Jin, Y.C., Dong, X.W., et al., 2018. Salvianolic acid B improves airway hyperresponsiveness by inhibiting MUC5AC overproduction associated with Erk1/2/p38 signaling. *Eur. J. Pharmacol.* 824, 30–39.
- Hamelmann, E., Schwarze, J., Takeda, K., Oshiba, A., Larsen, G.L., Irvin, C.G., et al., 1997. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am. J. Respir. Crit. Care Med.* 156, 766–775.
- Hirota, N., Martin, J.G., 2013. Mechanisms of airway remodeling. *Chest* 144, 1026–1032.
- Hoshino, M., Takahashi, M., Takai, Y., Sim, J., 1999. Inhaled corticosteroids decrease subepithelial collagen deposition by modulation of the balance between matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 expression in asthma. *J. Allergy Clin. Immunol.* 104, 356–363.
- Hoymann, H.G., 2007. Invasive and noninvasive lung function measurements in rodents. *J. Pharmacol. Toxicol. Methods* 55, 16–26.
- Ijaz, T., Pazdrak, K., Kalita, M., Konig, R., Choudhary, S., Tian, B., et al., 2014. Systems biology approaches to understanding epithelial mesenchymal transition (EMT) in mucosal remodeling and signaling in asthma. *World Allergy Organ. J.* 7, 13.
- Ingram, J.L., Kraft, M., 2012. IL-13 in asthma and allergic disease: asthma phenotypes and targeted therapies. *J. Allergy Clin. Immunol.* 130, 829–842 quiz 843–4.
- Jiang, J.X., Zhang, S.J., Shen, H.J., Guan, Y., Liu, Q., Zhao, W., et al., 2017. Rac1 signaling regulates cigarette smoke-induced inflammation in the lung via the Erk1/2 MAPK and STAT3 pathways. *Biochim. Biophys. Acta Mol. Basis Dis.* 1863, 1778–1788.
- Johnson, J.R., Roos, A., Berg, T., Nord, M., Fuxe, J., 2011. Chronic respiratory aero-allergen exposure in mice induces epithelial mesenchymal transition in the large airways. *PLoS One* 6, e16175.
- Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) Method. *Methods* 25, 402–408.
- Lu, S., Li, H., Gao, R., Gao, X., Xu, F., Wang, Q., et al., 2015. IL-17A, but not IL-17F, is indispensable for airway vascular remodeling induced by exaggerated Th17 cell responses in prolonged ovalbumin-challenged mice. *J. Immunol.* 194, 3557–3566.
- Malavia, N.K., Mih, J.D., Raub, C.B., Dinh, B.T., George, S.C., 2008. IL-13 induces a bronchial epithelial phenotype that is profibrotic. *Respir. Res.* 9, 27.
- Malmström, K., Lohi, J., Sajantila, A., Jahnsen, F.L., Kajosaari, M., Sarna, S., et al., 2017. Immunohistology and remodeling in fatal pediatric and adolescent asthma. *Respir. Res.* 18, 94.
- Ono, E., Dutilleul, S., Kazani, S., Wechsler, M.E., Yang, J., Hammock, B.D., et al., 2014. Lipoxin generation is related to soluble epoxide hydrolase activity in severe asthma. *Am. J. Respir. Crit. Care Med.* 190, 886–897.
- Post, S., Heijink, I.H., Hesse, L., Koo, H.K., Shaheen, F., Fouadi, M., et al., 2018. Characterization of a lung epithelium specific E-cadherin knock-out model: implications for obstructive lung pathology. *Sci. Rep.* 8, 13275.
- Pu, Q., Zhao, Y., Sun, Y., Huang, T., Lin, P., Zhou, C., et al., 2019. TRPC1 intensifies house dust mite-induced airway remodeling by facilitating epithelial-to-mesenchymal transition and STAT3/NF- κ B signaling. *FASEB J.* 33, 1074–1085.
- Roth, M., Johnson, P.R., Borger, P., Bihl, M.P., Rüdiger, J.J., King, G.G., et al., 2004. Dysfunctional interaction of C/EBP α and the glucocorticoid receptor in asthmatic bronchial smooth-muscle cells. *N. Engl. J. Med.* 351, 560–574.
- Sagiani, S., Lloyd, C.M., 2015. Novel concepts in airway inflammation and remodelling in asthma. *Eur. Respir. J.* 46, 1796–1804.
- Schuliga, M., 2015. NF-kappaB signaling in chronic inflammatory airway disease. *Biomolecules* 5, 1266–1283.
- Shahabi, P., Siest, G., Meyer, U.A., Visvikis-Siest, S., 2014. Human cytochrome P450 epoxigenases: variability in expression and role in inflammation-related disorders. *Pharmacol. Ther.* 144, 134–161.

- Simeone-Penney, M.C., Severgnini, M., Tu, P., Homer, R.J., Mariani, T.J., Cohn, L., Simon, A.R., 2007. Airway epithelial STAT3 is required for allergic inflammation in a murine model of asthma. *J. Immunol.* 178, 6191–6199.
- Smith, K.R., Pinkerton, K.E., Watanabe, T., Pedersen, T.L., Ma, S.J., Hammock, B.D., 2005. Attenuation of tobacco smoke-induced lung inflammation by treatment with a soluble epoxide hydrolase inhibitor. *Proc. Natl. Acad. Sci. U. S. A.* 102, 2186–2191.
- Tao, W., Li, P.S., Yang, L.Q., Ma, Y.B., 2016. Effects of a soluble epoxide hydrolase inhibitor on lipopolysaccharide-induced acute lung injury in mice. *PLoS One* 11, e0160359.
- Therien, A.G., Bernier, V., Weicker, S., Tawa, P., Falgoutyret, J.P., Mathieu, M.C., et al., 2008. Adenovirus IL-13-induced airway disease in mice: a corticosteroid-resistant model of severe asthma. *Am. J. Respir. Cell Mol. Biol.* 39, 26–35.
- Tillie-Leblond, I., de Blic, J., Jaubert, F., Wallaert, B., Scheinmann, P., Gosset, P., 2008. Airway remodeling is correlated with obstruction in children with severe asthma. *Allergy* 63, 533–541.
- Vale, K., 2016. Targeting the JAK-STAT pathway in the treatment of 'Th2-high' severe asthma. *Future Med. Chem.* 8, 405–419.
- Wagner, K.M., McReynolds, C.B., Schmidt, W.K., Hammock, B.D., 2017. Soluble epoxide hydrolase as a therapeutic target for pain, inflammatory and neurodegenerative diseases. *Pharmacol. Ther.* 180, 62–76.
- Wang, L., Yang, J., Guo, L., Uyeminami, D., Dong, H., Hammock, B.D., et al., 2012. Use of a soluble epoxide hydrolase inhibitor in smoke-induced chronic obstructive pulmonary disease. *Am. J. Respir. Cell Mol. Biol.* 46, 614–622.
- Wang, X., Li, Y., Luo, D., Wang, X., Zhang, Y., Liu, Z., et al., 2017. Lyn regulates mucus secretion and MUC5AC via the STAT6 signaling pathway during allergic airway inflammation. *Sci. Rep.* 7, 42675.
- Yang, J., Bratt, J., Franzi, L., Liu, J.Y., Zhang, G., Zeki, A.A., et al., 2015. Soluble epoxide hydrolase inhibitor attenuates inflammation and airway hyperresponsiveness in mice. *Am. J. Respir. Cell Mol. Biol.* 52, 46–55.
- Zhang, Y., Cardell, L.O., Edvinsson, L., Xu, C.B., 2013. MAPK/NF- κ B-dependent upregulation of kinin receptors mediates airway hyperreactivity: a new perspective for the treatment. *Pharmacol. Res.* 71, 9–18.
- Zhang, Y., Li, S., Huang, S., Cao, L., Liu, T., Zhao, J., et al., 2019. IL33/ST2 contributes to airway remodeling via p-JNK MAPK/STAT3 signaling pathway in OVA-induced allergic airway inflammation in mice. *Exp. Lung Res.* 45, 65–75.
- Zhao, J., Lloyd, C.M., Noble, A., 2013. Th17 responses in chronic allergic airway inflammation abrogate regulatory T-cell-mediated tolerance and contribute to airway remodeling. *Mucosal Immunol.* 6, 335–346.
- Zhou-Suckow, Z., Duerr, J., Hagner, M., Agrawal, R., Mall, M.A., 2017. Airway mucus, inflammation and remodeling: emerging links in the pathogenesis of chronic lung diseases. *Cell Tissue Res.* 367, 537–550.
- Zhou, Y., Liu, T., Duan, J.X., Li, P., Sun, G.Y., Liu, Y.P., et al., 2017. Soluble epoxide hydrolase inhibitor attenuates lipopolysaccharide-induced acute lung injury and improves survival in mice. *Shock* 47, 638–645.