Soluble Epoxide Hydrolase Inhibitor Attenuates Inflammation and Airway Hyperresponsiveness in Mice

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Abstract

Control of airway inflammation is critical in asthma treatment. Soluble epoxide hydrolase (sEH) has recently been demonstrated as a novel therapeutic target for treating inflammation, including lung inflammation. We hypothesized that pharmacological inhibition of sEH can modulate the inflammatory response in a murine ovalbumin (OVA) model of asthma. BALB/c mice were sensitized and exposed to OVA over 6 weeks. A sEH inhibitor (sEHI) was administered for 2 weeks. Respiratory system compliance, resistance, and forced exhaled nitric oxide were measured. Lung lavage cell counts were performed, and selected cytokines and chemokines in the lung lavage fluid were measured. A LC/MS/MS method was used to measure 87 regulatory lipids mediators in plasma, lung tissue homogenates, and lung lavage fluid. The pharmacological inhibition of sEH increased concentrations of the antiinflammatory epoxy eicosatrienoic acids and simultaneously decreased the concentrations of the proinflammatory dihydroxyeicosatrienoic acids and dihydroxyoctadecenoic acids. All monitored inflammatory markers, including FeNO levels, and total cell and eosinophil numbers in the lung lavage of OVA-exposed mice were reduced by sEHI. The type 2 T helper cell (Th2) cytokines (IL-4, IL-5) and chemokines (Eotaxin and RANTES) were dramatically reduced after sEHI administration. Resistance and dynamic lung compliance were also improved by sEHI. We demonstrated that sEHI administration attenuates allergic airway inflammation and airway responsiveness in a murine model. sEH may have potential as a novel therapeutic strategy for allergic asthma.

Keywords: soluble epoxide hydrolase; asthma; inflammation; lipid mediators; type 2 T helper cell cytokines

Clinical Relevance

We demonstrated that inhibition of soluble epoxide hydrolase attenuates allergic airway inflammation and airway responsiveness in a murine model. Therefore, sEH inhibitors may have potential as a novel therapeutic strategy for allergic asthma.

Three hundred million people worldwide suffer from episodic or persistent asthma (1). The cornerstones of treatment for persistent asthma are inhaled corticosteroids and β-agonist bronchodilators; however, a significant minority of patients with asthma does not respond well to these therapies (2). Thus, there are ongoing efforts to develop novel treatment strategies (3), such as specific antagonists of type 2 T helper cell (Th2) cytokines and mediators.
Epoxycosatrienic acids (EETs, or EpETrEs according to LIPIDMAPS nomenclature) are a class of important lipid mediators with critical physiological functions that include vasodilation, antiinflammation, antihypertension, organ protection, and analgesic effects (4). Specifically in lung health and lung disease, EETs are reported to affect lung epithelial ion transport (5–7), relax precontracted bronchi (8), reduce inflammation (9, 10), regulate endothelial permeability in the lung (11), and regulate pulmonary vascular pressures (12, 13). Thus, modulation of endogenous EETs is an attractive approach to potentially control the symptoms of asthma, which include chronic airway inflammation and airway hyperresponsiveness (AHR).

The soluble epoxide hydrolase (sEH) hydrolyzes these bioactive EETs to their corresponding diols, which are less beneficial and may be toxic. Using potent inhibitors of sEH to stabilize endogenous EETs (14–17), sEH has been recently demonstrated in animal models as a novel therapeutic target (4) for treating cardiovascular diseases (18–20), inflammation (21, 22), pain (23–25), and pulmonary diseases such as pulmonary hypertension (26, 27) and tobacco smoke–induced chronic obstructive pulmonary disease (9, 10).

In the present study, we hypothesized that pharmacological inhibition of sEH can modulate the inflammatory response in a well-established murine ovalbumin (OVA)–induced asthma model. We found that administration of an sEH inhibitor (sEHI; t-TUCB) reduced total inflammatory cell infiltration into the airway and lung and inhibited OVA–induced influx of eosinophils. The profiling of regulatory lipid mediators shows that t-TUCB administration not only increased the antiinflammatory lipid mediators (EETs) but also increased other antiinflammatory mediators, such as 17-hydroxydocosahexaenoic acid, and decreased proinflammatory lipid mediators, including dihydroxyoctadecenoic acids (DHOMEs) and LTB4, in plasma, lung tissue, and lavage. Stabilization of the antiinflammatory epoxide lipid mediators through pharmacological inhibition of sEH decreased production of Th2 cytokines at the protein and mRNA levels after OVA induction. Furthermore, compliance and resistance of the respiratory system were improved after sEHI administration. These findings support the hypothesis that sEH is a potential target to treat asthma.

Materials and Methods

Animals

Pathogen-free male BALB/c mice, aged 8 to 10 weeks, were purchased from Charles River Laboratory (Wilmington, MA). All mice were maintained in a HEPA-filtered laminar flow cage rack with a 12-hour light/dark cycle and allowed free access to food and water. Figure 1A shows the animal protocol. All procedures with mice were performed in accordance with an IACUC-approved protocol.

Drug Solutions and Exposure of Mice to OVA Aerosol

The sEHI trans-4-{4-[3-(4-trifluoromethoxyphenyl)-ureido]-cyclohexyloxy}-benzoic acid (t-TUCB) was synthesized as previously described (17). t-TUCB was dissolved in 0.05% (v/v) Tween-80 water solution. This solution (1 or 3 mg/kg) was administered subcutaneously to the mice every day for 14 days. On the last day, the drugs were administered 30 minutes before OVA aerosol exposure. The exposure procedures of OVA have been described in detail previously (28).

Lung Compliance and Resistance Measurements

Dynamic lung compliance and respiratory system resistance were simultaneously measured with a whole body plethysmograph for restrained animals (Buxco Inc., Troy, NY) 1 to 3 hours after termination of the final OVA exposure. Further details are provided in the online supplement.

Measurement of Exhaled NO

A 5-minute sample of exhaled gases was collected from the cannulated mice through the ventilator exhalation port immediately after insertion of the mouse into a plethysmograph as previously described (29).

Cytokine and Chemokine Assays

The concentrations of selected cytokines and chemokines (Eotaxin, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, RANTES, and TNF-α) from the bronchoalveolar lavage fluid (BALF) supernatant were measured with commercially available multiplex immunoassays according to the manufacturer’s instructions (Millipore, St. Charles, MO).

Measurement of t-TUCB Concentration

Blood (10 μl) was diluted with 0.1% aqueous EDTA (50 μl) and mixed
vigorously. Samples were then extracted using 200 µl of ethyl acetate twice and dried by Speedvac (Thermo Scientific, Waltham, MA). The residue was reconstituted to 50 µl of internal standard solution and measured by LC/MS/MS.

**Regulatory Lipid Mediator Profiling**

Profiles of regulatory mediators were measured using the LC/MS/MS method as described previously (30). Aliquots of plasma (250 µl), BAL supernatant (2 ml), or lung tissue (∼100 mg) were used for the measurements, respectively. Further details are provided in the online supplement.

**Quantitative Real-Time Reverse Transcription PCR**

Total RNA was isolated from lung tissue using Trizol and a Quick-RNA Mini prep isolation kit (Zymo Research, Irvine, CA). cDNA synthesis and the RT-PCR process are described in the online supplement.

**Statistical Analysis**

Data were presented as means ± SEM. Data were analyzed using unpaired values compared by two-tailed Student’s t test or one-way or two-way ANOVA with Tukey’s post test where appropriate, using the Prism 5.0 software package (GraphPad, Inc., San Diego, CA), with statistical significance defined as \( P \leq 0.05 \).

**Results**

**sEHI Was Successfully Delivered and Well Engaged**

After 14 days of subcutaneous injection of t-TUCB, concentrations of t-TUCB in blood (Figure 2A) reached 55.6 ± 13.2 nM (1 mg/kg) and 213 ± 38 nM (3 mg/kg), which are 42.8 and 164 times higher, respectively, than the IC\(_{50}\) of t-TUCB in mouse on the murine recombinant sEH (1.3 nM) using trans-diphenylpropene oxide as substrate (17). As a result of enzyme inhibition, the concentrations of 14,15-EpETrE, an endogenous substrate of sEH, increased by 3- to 3.6-fold from 0.78 nM to 2.56 or 2.86 nM with the administration of 1 or 3 mg/kg t-TUCB compared with the vehicle control (Figure 2B). Increased concentrations of 14,15-EpETrE after exposure to t-TUCB confirmed the efficacy of t-TUCB as an sEHI in this study.

**sEHI Administration Increased Antiinflammatory Mediator Concentrations and Decreased Proinflammatory Mediator Concentrations**

We analyzed the regulatory lipid mediators from BALF, plasma, and lung tissue homogenate using LC/MS/MS. Figure 3 shows the results presented as heatmaps, including a simplified arachidonic acid cascade listing the major lipid mediators (Figure 3A) and the significantly changed regulatory lipid mediators after t-TUCB administration (Figures 3B–3D). In general, for the P450 and sEHI pathways, sEHI administration increased epoxides in plasma and BALF and decreased diols in BALF and lung homogenates. In plasma, sEHI administration increased some COX and LOX metabolites (11-HETE, 9-HETE, 5-HETE, and 5-HEPE). The low dose of sEHI significantly reduced the proinflammatory mediators 6-keto-PGF\(_{1\alpha}\) (the metabolite and surrogate of prostacyclin-PGI\(_2\)) and LT\(_{B_2}\). In BALF, sEHI administration increased LOX metabolites, including 11-HETE, 9-HETE, 5-HETE, 15-HEPE, 17HD0HE, and 8-HETE. In lung homogenates, sEHI administration increased the COX metabolites 6-keto-PGF\(_{1\alpha}\), TX\(_B_2\), PGF\(_{2\alpha}\), PGE\(_3\), PGJ\(_2\), and 11-HETE and increased the LOX metabolites, including 15-HETE, 15(s) HET\(_E_2\), 15-HEPE, 17-hydroxy docosahexaenoic acid, and 8-HETE.

**sEHI Administration Reduced Th2 Cytokines and Chemokines**

Several inflammatory cytokines were induced after OVA exposure (Figure 4 and Figure E1). After sEHI administration, IL-4 and IL-5 in lung lavage fluid decreased to almost the baseline level of the control animals (Figures 4A and 4B). By contrast, there were no clear trends for Th1 and innate immune cytokines assayed (Figure E1). Because of the methodological issues
involved in IL-13 detection, we could not make firm conclusions regarding the involvement of IL-13 in this model system. The chemokine eotaxin was induced after OVA exposure, and its levels were blunted by inhibition of sEH (Figure 4C). These data suggest that inhibition of sEH could reduce the Th2-specific cytokines and chemokines, which are important in eosinophil trafficking, recruitment, and maturation in airways. Lung expression of IL-4 and IL-5 also showed that RNA levels of these Th2 cytokines were down-regulated by sEHI administration (Figures 4D and 4E).

sEHI Administration Reduced Inflammatory Cell Infiltration in Lung Tissues and Lavage Fluid

Sensitization and exposure of mice to OVA induced significant inflammatory cell infiltration into the airway (Figure 5A). The total cell count in BALF reached approximately 2.6 × 10^6 cells/ml. t-TUCB administration at 1 mg/kg dose decreased the number of inflammatory cells infiltrated into the BALF. Furthermore, 3 mg/kg t-TUCB significantly reduced the total cell number in BALF to approximately 46.2%. ANOVA showed that there was significant reduction of total live cell numbers after sEHI administration (P = 0.04).

Figure 5B shows the differential cell counts determined by the Hem-3 stain set. After OVA exposure, the eosinophil is the dominant inflammatory cell type in BALF, comprising up to 75% of the total inflammatory cell infiltrate. After sEHI administration, the percentage of eosinophils was reduced to 65%. t-TUCB at a dose of 3 mg/kg significantly reduced eosinophil infiltration into lung lavage from 1.63 × 10^6 to 7.05 × 10^5. ANOVA shows that there is significant reduction of total live cell numbers after sEHI administration (P = 0.04).

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Figure 4. t-TUCB administration dramatically decreased Th2 cytokines IL-4 and IL-5 (A and B) and chemokine (Eotaxin) (C) production in BALF and down-regulated the gene expression of these Th2 cytokines (D and E) in lung homogenates. *Significant difference from the OVA+vehicle group (n = 5 in all groups, except n = 4 in nonimmunized + Vehicle group).
Figure 5. (A) t-TUCB administration reduced the infiltrated inflammatory cells (cells/ml) in BALF. (B) Differential cell counts by Hema-3 stain shows that soluble epoxide hydrolase inhibitor (sEHI) reduced the number of eosinophil (cells/ml) in BALF. (C) t-TUCB administration reduced the inflammatory marker FeNO. (D–G) Hematoxylin and eosin stain results of lung tissues shows that sEHI reduces the infiltration of inflammatory cells into lung tissues. Shown are results from the air control group (D), the vehicle control group after OVA exposure (E), the 1 mg/kg t-TUCB–treated group (F), and the 3 mg/kg t-TUCB–treated group (G). *Significant difference from the OVA+vehicle group.
increased from 5.43 to 15.1 ppb after OVA exposure (Figure 5C). Treatment with 1 and 3 mg/kg of t-TUCB decreased the induction of this inflammatory biomarker to 3.68 and 6.57 ppb, respectively. ANOVA with Bonferroni post test shows that there is significant reduction of FeNO after sEHI administration (P = 0.0006). In lung tissues, there was marked inflammatory cell influx in the peribronchiolar space after OVA exposure, as shown in the hematoxylin and eosin stain result (Figures 5D and 5E), which is consistent with the lavage data (Figures 5A and 5B). After inhibition of sEH, inflammatory cells in the lung tissue were reduced in a dose-dependent manner (Figures 5F and 5G). These figures show that sEHI administration reduced the lung inflammatory cell infiltration, which is consistent with the inflammatory cell results in the BALF cell counts.

Our results reveal severe eosinophil-dominant inflammation in the airway and alveolar of the mice after OVA exposure. sEHI administration reduced this inflammation, as shown in total live cell number, inflammatory cell differentiation, hematoxylin and eosin–stained lung tissue, and FeNO.

Figure 6. Airway hyperreactivity (A) and Dynamic lung compliance (B) results show that t-TUCB administration rescues OVA-induced asthmatic airway hyperresponsiveness (n = 4 for OVA+Vehicle group and the OVA+3 mg/kg t-TUCB group; n = 3 for the nonimmunized+Vehicle group and the OVA+1 mg/kg t-TUCB group).

Discussion

We demonstrated that administration of an sEHI markedly attenuates the allergic airway inflammation and AHR caused by exposing mice to OVA. Specifically, the sEHI reduced the total inflammatory cell number by 50%. Compared with two clinically available compounds (montelukast and dexamethasone) tested in the similar animal model, sEHI had a greater effect on reduction of the inflammatory cells infiltration than montelukast and dexamethasone did (40 and 28%) (31). In addition, sEHI reduced IL-5 levels to 12.5% of those mice treated with OVA and vehicle. Taken together, these findings indicate that sEHI is a promising potential candidate drug to treat allergic asthma.

We and others have previously shown the antiinflammatory effects of...
sEHs in different disease models (9, 21, 22, 32). The mechanism is believed to be through stabilizing of the antiinflammatory EETs, which regulate NF-κB translocation (33), or through reducing production of proinflammatory diols, including DHOMEs (34) and dihydroxy eicosatetraenoic acids (DHETs) (35). Here, our data show that the concentration of sEH in the plasma significantly altered the circulating EET and DHET levels present in plasma, BALF, and lung homogenates (Figures 3C and 3D). Node (33) reported that EETs (EpETrEs) can reduce endothelial cell VCAM-1 expression in response to TNF-α, IL-1α, and LPS. At the same time, sEH administration significantly reduced proinflammatory DHETs in BALF and lung homogenate (Figures 3C and 3D). The DHETs were reported as essential for monocyte chemotaxis to MCP-1 (35). In particular, sEH reduced the DHOMEs, metabolites of leukotxin, and iso-leukotxin. It was found that these DHOMEs are more toxic than EpOMEs and are associated with multiple organ failure and adult respiratory distress syndrome (34). Taken together, these findings indicate that the effect of sEHI on the concentrations of EETs and DHETs may contribute to the antiinflammatory effects of sEH in this murine asthmatic model.

In this study, we also observed that additional lipid mediators were affected upon sEHI administration. The proinflammatory lipid mediator LTB4 was decreased in the plasma after low-dose sEHI administration. It was reported that LTB4 participates in the allergic sensitization process in animal models (36). Therefore, the effect of inhibition of sEH might also benefit from a reduction of proinflammatory LTB4.

Another lipid mediator, 17HDoHE, which is a precursor to resolvins and possesses biological activity that inhibits TNF-α-induced IL-1β expression (37), was increased in lavage and lung homogenates after sEHI administration. Resolvins have been reported to promote the resolution of the allergic airways response (38).

Our findings add to those reported elsewhere by describing the effects of sEH inhibition on allergic airway inflammation. Previous studies (10, 21) have demonstrated that sEHI can reduce the inflammatory cytokines IL-1β, IL-6, and INF-γ. In the present study, we found that sEHI reduced Th2 cytokines and chemokines, which are known to play major roles in the asthmatic immune response (39). Specifically, the pronounced effect of sEHI on IL-5 and eotaxin-1, a key cytokine and a chemokine responsible for the release of eosinophil from the bone marrow and homing of eosinophil to the lung, is rather intriguing. Indeed, mepolizumab, a monoclonal antibody against IL-5 used for the treatment of severe asthma, is garnering significant attention in the clinical realm (40). The inhibition of sEH may be an alternative strategy for decreasing IL-5 levels in concert with other key mediators of lung inflammation.

There are reports indicating that some of these regulatory lipid mediators, such as EETs, have direct function on the bronchi (6, 41). We observed that sEHI administration increased EETs in the BALF (Figure 5C), which may directly rescue airflow hyperreactivity. However, inflammation may also play a role in regulating lung compliance and resistance. The antiinflammatory effects of sEHI might have contributed to the improvement of lung function after sEHI administration in this acute model of asthma. In addition, lipid mediators such as HETEs are reported to have effects on the airways (42). Alterations in the levels of various lipid mediators may explain why treatment with 3 mg/kg of sEHI did not show improved rescue (in comparison to treatment with 1 mg/kg) of the resistance induced by 2.0 mg/ml of methacholine (Figure 6B). Direct pulmonary administration of a sEH would provide additional evidence on how sEH regulates lung compliance and resistance. We have not developed an effective system for administering sEH directly to the lung.

In this study, neither COX-2 nor 5-LOX in lung homogenates was significantly suppressed by sEHI administration in OVA-exposed mice (Figure E2). These findings suggest, at least in lung homogenates, that the major effects of sEHI are unlikely due to the NF-κB pathway.

sEHI administration increased the antiinflammatory mediators systemically and in the airways, as indicated by the lipid mediator levels from the lavage, while simultaneously decreasing proinflammatory mediators in the lung tissues and airways. These changes in lipid mediators influenced the reduction and down-regulation of Th2 cytokines and chemokine expression in the airways. The reduction of these Th2 cytokines and chemokines further decreased the recruitment of inflammatory cells infiltration into the lungs and airways. The reduction in overall lung inflammation and the increase of EETs in airway contributes to the alleviation of AHR.

The data are consistent with the sEH (EPHX2) being primarily responsible for the conversion of fatty acid epoxides to the corresponding diols in the lung and with t-TUCB inhibiting this catalytic activity. The activity of mEH (EPHX1) on this substrate is low, as are pulmonary levels of the mEH. We have found no evidence for catalytic activity of EH3 (EPHX3) or EH4 in the lung. Although there is no evidence of inhibition of EPHX1, -3, or -4 or an unknown enzyme by t-TUCB, we cannot exclude the possibility of off-target effects.

Among the limitations of this study is that we used only prophylactic and not therapeutic treatment. Long-term pulmonary inflammation, including asthma, leads to chronic changes in the lung. However, our short-term model did not assess the effects of sEHI on fibrotic biomarkers because this would require a longer-term exposure to inflammation, particularly one that is associated with chronic diseases involving the use of multiple drugs with different mechanisms of action. Future studies need to address the possible beneficial and detrimental effects of long-term sEH use.

Author disclosures are available with the text of this article at www.atsjournals.org.


Supplementary Files

Soluble Epoxide Hydrolase Inhibitor Attenuates Inflammation and Airway Hyperresponsiveness in Mice

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SUPPLEMENTARY MATERIALS AND METHODS

Lung compliance and resistance measurements

The dynamic lung compliance (Cdyn) and resistance of the respiratory system (Rst) was measured with a plethysmograph for restrained animals (Buxco Inc., Troy, NY) 1–3 hours after termination of the final (6th) OVA exposure. Mice were deeply anesthetized and sedated with medetomidine, 0.5 mg/kg (Domitor, Orion Pharma, Finland), and tiletamine/zolpidem, 50 mg/kg (Telazol, Fort Dodge Laboratories, Fort Dodge, IA) and surgically cannulated and ventilated at 7–8 cc/kg using a mouse ventilator (MiniVent, Harvard Apparatus, Cambridge, MA) for the duration of the procedure. Cdyn (mL/cm of H2O) and Rst (cm of H2O*sec/mL) measurements were made at baseline and immediately following serial 3 minute nebulizations of saline and methacholine (0.5, 1.0 and 2.0 mg/ml), with 2-minute recovery periods allowed after each exposure.

Lung Tissue Processing

Immediately after lung physiology measurements, mice were killed with an overdose of Beuthanasia-D. After blood collection, lungs were lavaged twice with 1 ml of PBS (pH 7.4), and then centrifuged at 2,500 rpm on a tabletop centrifuge for 10 minutes. The total lavage live cell number and differential cell counts were determined.

Mice had their lungs fixed or immediately frozen and stored at -80 °C. Lungs were fixed for histological evaluation with 1% paraformaldehyde. After fixation and paraffin embedding, the lungs were stained with hematoxylin and eosin to qualitatively assess peribronchial inflammation. Half of right lungs were immersed in RNA Later solution (Life Technology, Carlsbad, CA) for the followed RT-PCR measurements.
Airway Inflammatory Cell Populations

Total cell counts in the lung lavage fluid were determined using a hemocytometer and trypan blue exclusion; 100 μL of the remaining cell suspension was processed onto slides using a cytocentrifuge at 1650 rpm for 7 minutes for determination of the cell differential counts. Slides were air-dried, stained with a Hema3 stain set as described in the manufacturer's instructions (Fisher Scientific, Kalamazoo, MI).

LC/MS/MS analysis for 𝑡-TUCB

The liquid chromatography system used for analysis was an Agilent 1200 SL liquid chromatography series (Agilent Corporation, Palo Alto, CA). The autosampler was kept at 4 °C. Liquid chromatography was performed on an ACQUITY UPLC BEH C18, 1.7 μm, 2.1× 50 mm column (Waters, Milford, MA). Mobile phase A was water with 0.1% glacial acetic acid. Mobile phase B consisted of acetonitrile with 0.1% glacial acetic acid. Gradient elution was performed at a flow rate of 400 μL/min. The gradient began with 50% B and reached 95% B at 3 min. After holding at 95% for 0.5 min, the composition of mobile phase went back to 50% at 3.5 min for another 0.5 min. The column was connected to a 4000 QTrap tandem mass spectrometer (Applied Biosystems Instrument Corporation, Foster City, CA) equipped with an electrospray source (Turbo V). The instrument was operated in negative MRM mode. Individual analyte standards were infused into the mass spectrometer and MRM transitions and source parameters optimized for 𝑡-TUCB. The MRM transition for 𝑡-TUCB is 437.2/137.1; the transition for CUDA is 339.2/214.3.

Extraction Protocol for Plasma Oxylinip Profile
The Oasis cartridges (Waters,) were washed with ethyl acetate and methanol, and equilibrated to the initial condition with the solution containing H₂O and methanol. Diluted supernatant was loaded on the cartridge and washed with SPE solution (H₂O with 5% methanol with 0.1% acetic acid) twice. The cartridges were dried by vacuum and analytes were eluted with 0.5 mL of methanol and 1.5 mL of ethyl acetate to tubes with 6 µL of trapping solution (30% glycerol in methanol). The elutes were dried by vacuum (SpeedVac) and reconstitute with internal solution (200 nM CUDA methanol solution).

**Extraction Protocol for BALF Oxylipin Profile**

Ten microLiters of antioxidant solution (0.2 mg/mL of BHT and EDTA) were added to 2 mL BAL fluid supernatant followed by the addition of 10 µL of surrogate solutions, in which 9 deuterated internal standards were included. BAL fluids were then loaded on the Oasis cartridges as mentioned above. The followed SPE protocol is same with the plasma extraction protocol described above.

**Extraction Protocol for Lung Homogenates Oxylipin Profile**

Ten microLiters of anti-oxidant solution were added to 100mg of lung tissue followed by the addition of 10 µL of surrogate solution. After addition of 400 µL of ice-cold methanol with 0.1 % of acetic acid and 0.1% of BHT, the lung tissues were stored at -80°C freezer for 30 min. Then, the lung tissue samples were homogenized using Mixer Mill MM301 (Retsch, Haan, Germany) at 30 Hz for 10 min. The homogenates were stored at -80°C freezer overnight. After centrifuged at 10,000 rpm for 10 min, the supernatant were collected and the remaining pellets were washed with 100 µL of ice-
cold methanol with 0.1% of acetic acid and 0.1% of BHT and centrifuged again. The
supernatants of each sample were combined and diluted with 2 mL of H₂O and load onto
SPE cartridges. Then the SPE protocol is the same as the one for plasma extraction
protocol.

**Quantitative Real-time Reverse Transcription–PCR (RT-PCR)**

Total RNA was isolated from tissue using Trizol and a Quick-RNA Mini prep isolation
kit (Zymo Research, Irvine, CA), and cDNA synthesis and RT-PCR process are fully
described in the supplementary files. was done as previously described (1). Quantitative
detection of β-actin and differentially expressed genes was performed with LightCycler
LC480 (Roche, Indianapolis, IN) using the Fast SYBR Green Master Mix (Life
Technologies, Grand Island, NY) according to the manufacturer’s instructions. DNA-free
total RNA (1.0 μg) was reverse-transcribed using 4 U Omniscript reverse transcriptase
(RT; Qiagen) and 1 μg oligo(dT)₁₅ in a final volume of 40 μl. The primers for each gene
were designed on the basis of the respective cDNA or mRNA sequences using OLIGO
primer analysis software provided by Steve Rozen and the Whitehead Institute/MIT
Center for Genome Research (2). So, that the targets were 100–200 bp in length. The
following primer sequences were used: mouse β-actin (forward primer, 5’-
AGCCATGTACGTAGCCATCC-3’; reverse primer, 5’-CTCTCAGCTGTGGTGGTGA-3’),
mouse IL-4 (forward primer, 5’-TCAACCCCAGCTAGTTGTC-3’; reverse primer, 5’-
TGTTCTTCGGCTGTGAGG-5’), mouse IL-5 (forward primer, 5’-GAAGTGTGGCGAGGAGAC-3’; reverse primer, 5’-
GCACAGTTTTGTGGGTGTTT-3’) and mouse IL-13 (forward primer:5’-
CAGCTCCCTGGTTCTCTCAC-3'); reverse primer: 5’-CCACACTCCCATACCATGCTG-3’). PCR amplification was carried out in a total volume of 20 μl containing 2 μl cDNA, 10 μl 2 × Fast SYBR Green Master Mix, and 0.2 μM of each primer. The PCR cycling conditions were 95°C for 30 sec followed by 40 cycles of 94°C for 3 sec, and 60°C for 30 sec. Detection of the fluorescent product was performed at the end of the 72°C extension period. Negative controls were concomitantly run to confirm that the samples were not cross-contaminated. A sample with DNase- and RNase-free water instead of RNA was concomitantly examined for each of the reaction units described above. To confirm the amplification specificity, the PCR products were subjected to melting curve analysis.

**Western blot method**

The lung tissue were solicited in a homogenization buffer containing 0.1% SDS, protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and 574 μM phenylmethanesulfonyl fluoride (PMSF) in PBS. Homogenates were centrifuged at 10,000 rpm for 20 minutes and the resulting supernatant was stored at -80 °C until further use.

Antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA) unless otherwise stated. Total protein concentration of homogenate samples was determined using the Micro BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Samples containing 20 μg of protein were incubated at 65 °C for 15 minutes, electroporated under reducing conditions, and transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked in 5% dry milk in PBS for 1 hour at 25 °C, then incubated in
0.4 μg/ml of goat, anti-mouse Arg1, 0.4 μg/ml of goat, anti-mouse Arg2, or 0.4 μg/ml of rabbit anti-human α-actinin overnight at 4 °C, then incubated for 1 hour at 25 °C in 40 ng/ml of horseradish peroxidase (HRP)-conjugated donkey anti-goat IgG (Pierce Biotechnology, Rockford, IL) or 40 ng/ml of horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (Pierce Biotechnology, Rockford, IL). Bands were visualized using the Immobilon western chemiluminescent HRP substrate kit (Millipore, Billerica, MA) and images captured using Image Reader LAS-3000 version 2.1 (FUJIFILM, Cypress, CA).

References


SUPPLEMENTARY FIGURE LEGENDS

Figure E1. The inflammatory cytokine panel as well as the IL-4, IL-5, eotaxin shown in Figure 4 responded differently to the OVA exposure and administration of sEHI.

Figure E2. COX2 (A) and 5-LOX (B) induced by OVA challenge but were not reduced by administration of sEHI.
Supplementary Table E1. The concentrations of lipid mediators in Plasma, BALF and lung homogenates. The units in plasma and BALF are in nmol/L. The unit in lung homogenates is in pmol/g tissue.

Supplementary Table E2. The epoxides to diols ratios supported the engagement of sEHI in plasma, BALF and lung homogenates.

This table is in Excel format. It can be accessed from this issue’s table of contents online at www.atsjournal.org.

Figure E1.
Figure E2.

(A)

(B)