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The anti-inflammatory effects of soluble epoxide hydrolase inhibitors are independent of leukocyte recruitment [☆]

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ABSTRACT

Excess leukocyte recruitment to the lung plays a central role in the development or exacerbation of several lung inflammatory diseases including chronic obstructive pulmonary disease. Epoxyeicosatrienoic acids (EETs) are cytochrome P-450 metabolites of arachidonic acid reported to have multiple biological functions, including blocking of leukocyte recruitment to inflamed endothelium in cell culture through reduction of adhesion molecule expression. Inhibition of the EET regulatory enzyme, soluble epoxide hydrolase (sEH) also has been reported to have anti-inflammatory effects *in vivo* including reduced leukocyte recruitment to the lung. We tested the hypothesis that the *in vivo* anti-inflammatory effects of sEH inhibitors act through the same mechanisms as the *in vitro* anti-inflammatory effects of EETs in a rat model of acute inflammation following exposure to tobacco smoke. Contrary to previously published data, we found that sEH inhibition did not reduce tobacco smoke-induced leukocyte recruitment to the lung. Furthermore, sEH inhibition did not reduce tobacco smoke-induced adhesion molecule expression in the lung vasculature. Similarly, concentrations of EETs greater than or equal to their reported effective dose did not reduce TNF α induced expression of the adhesion molecules. These results suggest that the anti-inflammatory effects of sEH inhibitors are independent of leukocyte recruitment and EETs do not reduce the adhesion molecules responsible for leukocyte recruitment *in vitro*. This demonstrates that the widely held belief that sEH inhibition prevents leukocyte recruitment via EET prevention of adhesion molecule expression is not consistently reproducible.

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1. Introduction

Excess leukocyte recruitment to the lung plays a central role in the development or exacerbation of several lung inflammatory

Abbreviations: sEH, soluble epoxide hydrolase; EETs, epoxyeicosatrienoic acids; SH, spontaneously hypertensive; TSP, total suspended particulates; BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; HLMVEC, human lung microvascular endothelial cells; HUVEC, human umbilical vein endothelial cells; sEHI, sEH inhibition; CI, confidence intervals; TS, tobacco smoke; LPS, lipopolysaccharide; LTX, leukotoxins; StARD1, acute neurosteroid producing gene.

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diseases that have high mortality rates, including acute respiratory distress syndrome, cystic fibrosis, pulmonary fibrosis, respiratory distress syndrome in premature infants, chronic obstructive pulmonary disease [1,2]. Recent research suggests that inhibition of soluble epoxide hydrolase (sEH) may change or reduce several components of inflammation, such as pain, leukocyte recruitment and mortality related to acute systemic inflammation during septic shock [3–11]. sEH inhibitors are receiving increased attention as potentially important anti-inflammatory pharmaceuticals that could be novel in their action on inflammation and beneficial for disease.

It has been suggested that the anti-inflammatory effects of sEH inhibitors are facilitated through several mechanisms. There is evidence that sEH inhibitors act by stabilizing epoxyeicosatrienoic acids (EETs), which are cytochrome P-450 metabolites of arachidonic acid. *In vitro* and *ex vivo* studies have shown that EETs have multiple biological functions including regulating vascular tone [12] and inflammation [13,14] that are thought to be controlled by the conversion of EETs to their less active corresponding diols

by sEH [15]. Specifically, EETs have been proposed to reduce NF κ B signaling leading to reduction in adhesion molecule expression and, thus, leukocyte recruitment [13]. Additionally, EETs may reduce COX2 expression that reduces prostaglandins [14,16].

In this study we investigated whether the anti-inflammatory effects of sEH inhibition are facilitated by reducing leukocyte recruitment via stabilizing EETs. A tobacco smoke-induced model of acute lung inflammation in spontaneously hypertensive (SH) rats was used to test sEH inhibitor reduction of leukocyte recruitment to the lung [17–21]. We tested whether sEH inhibition reduced leukocyte recruitment to the lung through EET reduction of adhesion molecule expression.

2. Methods

2.1. Animals

Twelve-week-old male spontaneously hypertensive (SH) rats were purchased from Charles River Laboratories (Portage, MI). All animals were handled according to the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health, and all procedures were performed under the supervision of the University Animal Care and Use Committee, IACUC approved protocol number 07-12922 (University of California, Davis).

2.2. Tobacco smoke exposure

Groups of four or six SH rats each were exposed to filtered air or to tobacco smoke, at a concentration of approximately 80–90 mg/m³ total suspended particulates (TSP), for 6 h/day for 3 days. Whole body exposure to cigarette smoke was done using a TE10 smoke exposure system [22] that combusts 3R4F research cigarettes (Tobacco and Health Research Institute, University of Kentucky, KY) with a 35 ml puff volume of 2 seconds duration, once each minute (Federal Trade Commission smoking standard).

2.3. Delivery of sEH inhibitor

Soluble epoxide hydrolase inhibitors were delivered in a variety of concentrations and by a variety of different methods. In studies one and two, 10 mg/kg/day and 5 mg/kg/day ADUA-be 12-(3-adamantan-1-yl-ureido)-dodecanoic acid butyl ester) was delivered via daily subcutaneous injection, respectively. In studies three and four, 1.9 mg/kg/day of sEH inhibitor 1709(1-(1-methanesulfonyl-piperidin-4-yl)-3-(4-trifluoromethoxy-phenyl)-urea) and 4.1 mg/kg/day of sEH inhibitor 1471(trans-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid) was delivered in drinking water, respectively.

2.4. Tissue preparation

SH rats were anesthetized with an overdose of sodium pentobarbital either 1–3 h or 18–20 h following the final day of exposure. Plasma was drawn for oxy-lipid analysis. The trachea was cannulated, the left lung bronchus tied, and the right lung lavaged with Ca²⁺/Mg²⁺-free Hank's buffered salt solution (HBSS) bronchoalveolar lavage (BAL) fluid (BALF) was collected in tubes and kept on ice prior to processing. For histology, the suture on the left lung bronchus was released, and the lung was inflated with 4% paraformaldehyde at 30 cm water pressure for 1 h, followed by storage of the inflation-fixed lung immersed in fixative.

2.5. BALF analysis

The BALF was centrifuged at 250g for 10 min at 4 °C and the cell pellet was resuspended in Ca²⁺/Mg²⁺-free HBSS (17). Total cell number was determined using a hemocytometer. Cytospin slides (Shandon, Pittsburgh, PA) were prepared using aliquots of cell suspension that were then stained with Hema 3 (Fisher Scientific, Pittsburgh, PA). Cell differentials in BALF were assessed by counting macrophages, neutrophils, lymphocytes, and eosinophils on cytocentrifuge slides using light microscopy (500 cells counted per sample). Eosinophils are not reported since they made up less than 0.01% of the total cells and were too few for statistical analysis.

2.6. Histology

Histology was performed using cross-sectional lung tissue slices containing the first and second intrapulmonary airway generations from rats exposed to filtered air or tobacco smoke. Five micron thick sections were cut from paraffin-embedded tissue blocks on a microtome. Sections were placed on glass slides and baked overnight at 37 °C. Sections were subsequently deparaffinized in toluene and hydrated through a graded series of alcohol. For hematoxylin and eosin staining, sections were stained with the following American Master Tech Scientific materials: Harris Hematoxylin, Differentiating Solution, Bluing Solution, and Eosin Y Stain. Sections were then dehydrated in ethanols and mounted with Clear Mount (American Tech Master Scientific, Inc., Lodi, CA).

2.7. Immunohistochemistry

Immunohistochemistry was performed using transverse lung tissue slices containing the first and second intrapulmonary airway generations from rats exposed to filtered air or tobacco smoke. Sections were deparaffinized in toluene and hydrated through a graded series of alcohol. Antigen retrieval by decloaker (123 °C for 2 min and 83 °C for 10 s, Decloaking Chamber, Biocare Medical) in EDTA (pH 8, IHC Select). Incubation with primary antibodies was for one hour for all sections. Goat anti-rat ICAM (0.01 μ g/ml), VCAM (0.1 μ g/ml), and E-selectin (0.1 μ g/ml) antibodies were used as primary antibodies. For antibody visualization, the following was used; biotinylated anti-Goat IgG (Vector BA5000) with HRP Streptavidin (Zymed 50-209Z, South San Francisco, CA) and Liquid DAB + Substrate (Dako K3468). Sections were then hematoxylin counterstained. A semi-quantitative immunohistochemical (IHC) was used to score adhesion molecule staining. A score of zero was given to the rat with the least staining and a score of five was given to the rat with the most prominent staining, based on both the frequency and intensity of staining in the blood vessels at each location within the lung.

2.8. In vitro cytometry

Human lung microvascular endothelial cells (HLMVEC) and human umbilical vein endothelial cells (HUVEC) were purchased at Passage 4 (Cascade Biologics, UK) after ~13 cell divisions from initial isolation, expanded to 80–90% confluence, and then seeded onto six-well, tissue-culture plates (Becton Dickinson, San Jose, CA, USA) between Passages 5 and 6 (16–19 total cell divisions) with or without serum (Table 1). To determine whether a range of concentration of EETs could reduce adhesion molecule expression in cell culture, the following two stock solutions of mixed EET free acids from were used in this study: (1) a 25:50:7:18 ratio of 14(15), 11(12), 8(9), 5(6) EET, and (2) a 35:50:5:10 ratio of 14(15), 11(12), 8(9), 5(6) EET. Additionally, 11,12 EET from Cayman Chemicals (Ann Arbor, MI), was purchased. Cells were treated with

Table 1

Adhesion molecule expression was tested under various conditions.

Condition #	N=	Cell type	Adhesion molecules tested			Stimulation	EET concentration	EET (source)	Serum
			Eselectin	ICAM	VCAM				
1	2	HLMVEC	X	X	X	0.05 ng/ml IL-1 β	1.0–0.01 μ M EETs	Mixed EETs #1 (Hammock)	Yes
2	1	HLMVEC	X	X	X	0.1 ng/ml TNF α	1.0–0.01 μ M EETs	Mixed EETs #1 (Hammock)	Yes
3	3	HLMVEC	X	X	X	0.1 ng/ml TNF α	100–1.0 μ M EETs	Mixed EETs #2 (Hammock)	Yes
4	3	HLMVEC	X	X	X	0.01 ng/ml TNF α	0.1–0.001 μ M EETs	11,12 EET (Cayman)	Yes
5	3	HLMVEC	X	X	X	0.1 ng/ml TNF α	0.1–0.001 μ M EETs	11,12 EET (Cayman)	Yes
6	2	HLMVEC	X	X	X	0.1 ng/ml TNF α	0.1–0.001 μ M EETs	11,12 EET (Cayman)	No
7	3	HUVEC	X	X	X	0.1 ng/ml TNF α	2.0–0.1 μ M EETs	Mixed EETs #1 (Hammock)	Yes

various doses of EETs immediately before TNF α stimulation for 4 h. Adhesion molecule expression was measured by flow cytometry. Cell surface expression of ICAM, VCAM and E-selectin was assessed using specific fluorochrome-labeled antibodies to ICAM, VCAM and E-selectin or isotype controls that were incubated with the HAEC and HUVEC for 30 min. After washing and detachment with PBS–EDTA, cells were analyzed by flow cytometry and expressed as percent cytokine stimulated mean fluorescent intensity/ 1×10^5 cells.

2.9. Statistical analysis

Leukocyte recruitment outcomes (total leukocytes and percent neutrophils) were assessed in two-way ANOVA models (with fixed effects for study and treatment condition) in order to estimate the pooled TS + sEH inhibition (sEHI) vs. TS contrast and to test the one-sided null hypothesis that adding sEH inhibitors to TS reduces mean levels of these outcomes by at least 20%. Hence, non-inferiority p -values <0.05 indicate that sEHI is statistically significantly neutral (within a non-inferiority margin of 20%) with respect to leukocyte recruitment. The 20% non-inferiority margin was based on our judgment of what constitutes clinically significant reductions in leukocyte recruitment. For decision makers with alternative judgments, the one-sided lower 95% confidence intervals (CI) for these contrasts are also reported.

To determine if EETs reduced adhesion molecule expression in cell culture, we used a regression model to predict the one sided 95% CI at the previously reported effective dose. Although all EETs may have anti-inflammatory properties [13,14] the effective dose for the reduction of adhesion molecule expression has only been established for the 11,12 EET [13]. Therefore, we performed linear regression of 11,12 EET and adhesion molecule expression reporting the one sided lower 95% CI for the predicted value at the previously reported effective concentration of 11,12 EET.

To determine whether sEH was effectively inhibited in each study, the effects of sEH inhibitors on the epoxide diol ratio analyzed by two-way ANOVA (with fixed effects for smoke and sEH inhibitor) and the p value for the effect of sEH inhibitor is reported for each study.

All other data was analyzed by one-way ANOVA with Bonferroni corrections for multiple comparisons.

3. Results

3.1. sEH inhibition does not reduce tobacco smoke-induced leukocyte recruitment to the lung

Leukocyte recruitment to the lung was determined by measuring the total leukocytes and percent neutrophils recovered in the BALF, which is historically the most sensitive measurement of inflammation in this model. In four experiments (see methods for experimental conditions), animals treated with sEH inhibitors did not show a reduction in total leukocytes or percent neutrophils recovered in the BALF (Fig. 1A, B). The combination of all

experiments yielded the most accurate estimate possible. Compared to tobacco smoke (TS) exposure alone, TS exposure plus sEH inhibition increased the geometric mean total leukocyte by 21%, ratio = 1.21 with a 95% 1-sided CI ≥ 0.92 (an 8% decrease) and had a non-inferiority p value of 0.006, indicating that sEH inhibition did not reduce leukocytes recruited to the lung by at least 20% (Fig. 1C). sEH inhibition increased the TS-only mean (51.6) of the % neutrophils by 0.13 with a 95% 1-sided CI ≥ -8.42 (a 16% decrease) and had a non-inferiority p value of 0.023, indicating that sEH inhibition did not reduce the percent neutrophils recruited to the lung by at least 20% of the TS-only mean (Fig. 1D).

3.2. sEH inhibitors do not reduce tobacco smoke induced adhesion molecule expression in the bronchial wall blood vessels in vivo

The anti-inflammatory effects of sEH inhibition has been proposed to be due to the stabilization of EETs, which then blocks adhesion molecule expression necessary for leukocyte recruitment. Therefore, we tested whether sEH inhibition reduced adhesion molecule expression (E selectin, ICAM, VCAM). sEH inhibition did not change TS-induced protein expression of E selectin, ICAM, and VCAM adhesion molecules as seen by immunohistochemistry (Fig. 2A) and as determined by blinded ranking of immunohistochemical staining intensities (Fig. 2B). ICAM is constitutively expressed in type 1 aveolar epithelial cells [23]. This expression is unrelated to leukocyte recruitment and is not altered by smoke exposure [24].

3.3. sEH inhibitors stabilize sEH substrates in vivo

To determine whether sEH was effectively inhibited, the ratio of its key substrates (including EETs) to its products was measured. Subcutaneous injection of 5–10 mg/kg/day of ADUA-BE before smoke exposure or drinking water delivery of sEH inhibitors 1709 or 1471 at 1.9 mg/kg/day and 4.1 mg/kg/day, respectively, significantly increased the ratio of the sEH substrates (epoxides) to their products (diols) in the plasma, confirming inhibition of sEH in all four studies (Fig. 3).

3.4. EETs do not reduce cytokine induced adhesion molecule expression at physiological relevant concentrations

To test the possibility that sEH inhibition did not increase EETs to a concentration high enough to have an anti-inflammatory effect in the lung, we tested whether a range of concentration of EETs could reduce adhesion molecule expression in two cell lines, human lung microvascular endothelial cells (HLMVEC) and human umbilical vein endothelial cells (HUVEC) (Table 1). We found concentrations of EETs greater than or equal to their previously reported effective dose did not reduce cytokine stimulated expression of the adhesion molecules, E-selectin, ICAM, and VCAM (Fig. 4). In a dose dependent investigation of this effect, only 10 μ M mixed EETs, which contained 250 times the previously reported

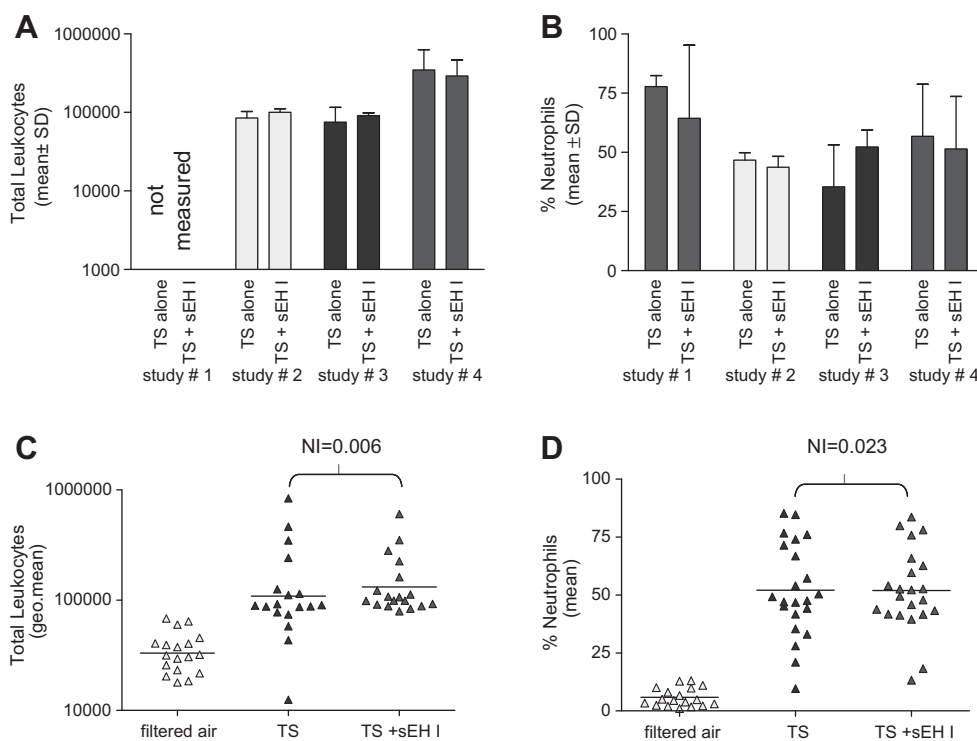


Fig. 1. sEH inhibition does not reduce the percent neutrophils or total leukocytes recovered in the BALF of SH rats exposed to tobacco smoke (TS) (see methods for experimental conditions). (A) Total leukocytes recovered in the BALF. (B) Percent neutrophils recovered in the BALF. (C) Total leukocytes recovered in the BALF from all studies combined. (D) Percent neutrophils recovered in the BALF from all studies combined. NI = non-inferiority *p*-value (for a non-inferiority margin of 20%).

IC₅₀ of 11,12 EET, reduced VCAM expression. However, this concentration appeared to have a slightly toxic effect as indicated by an increase in the number of floating cells (data not shown). Although all EETs may have anti-inflammatory properties [13,14], the effective dose for the reduction of adhesion molecule expression has only been established for the 11,12 EET [13]. Therefore, we performed linear regression of 11,12 EET and adhesion molecule expression reporting the one sided lower 95% CI for the predicted value at the previously reported effective concentration of 11,12 EET (E-selectin 0.1 μ m, ICAM 0.1 μ m and VCAM 0.02 μ m [13]). 11,12 EET did not inhibit as previously reported: E-selectin, 103.1% with a 95% 1-sided CI \geq 98.0% (Fig. 4D), ICAM 101.6% with a 95% 1-sided CI \geq 96.0% (Fig. 4E), and VCAM 101.1% with a 95% 1-sided CI \geq 95.0% (Fig. 4F) as compared to cytokine stimulated controls.

4. Discussion

sEH inhibitors have been reported to have various anti-inflammatory effects. This study tested whether the anti-inflammatory effects are facilitated by reducing leukocyte recruitment to the lung through EET stabilization. Utilizing a rat model of tobacco smoke-induced lung inflammation, we successfully inhibited sEH by delivery of inhibitors via subcutaneous injection or in the drinking water. Despite inhibition of sEH in these animals, tobacco smoke-induced leukocyte infiltration into the lung was not reduced. Additionally, sEH inhibition was not found to alter tobacco smoke induced adhesion molecule expression in the lung vasculature. In further support of these findings we found that the proposed underlying mechanism of EET reduction of leukocyte adhesion molecule was also not reproducible. EETs did not reduce adhesion molecule expression in human lung microvascular

endothelial cells and human umbilical vein endothelial cells at previously reported effective doses.

These results indicate that sEH inhibitors reduce inflammation independent of effects on leukocyte recruitment, and EETs do not reduce leukocyte adhesion molecule expression in cell culture. This contradicts the previously published papers by Smith et al. [18] and Node et al. [13]. We find that our previous findings published in Smith et al. are not reproducible. We do not know why this experiment was not reproducible. One possibility is simply that this current work is much more rigorous than the previously published paper. Smith et al. only had an N of 4, and this work tested more than five times that number of animals over the course of four studies. Although we successfully inhibited sEH in each study, tobacco smoke-induced leukocyte recruitment to the lung was not reduced. We can be 95% confident that sEH inhibition does not reduce leukocyte recruitment by >8%; a sample result that would be extraordinarily unlikely if the previously reported 40% reduction were the true population effect.

Additionally, in this study we demonstrate that EETs at or above the previously reported effective dose do not inhibit leukocyte adhesion molecules in endothelial cells from two different vascular beds. This shows that the proposed mechanism by which leukocyte recruitment is blocked by sEH inhibition is also not reproducible and further supports the conclusion that the anti-inflammatory effects of sEH inhibitors are independent of leukocyte recruitment.

The model systems used in this work are limited to conclusions about leukocyte recruitment and therefore, we cannot make conclusions on the effects of sEH inhibitors on other aspects of inflammation. However, we have used these same sEH inhibitors in multiple studies to block systemic inflammation in lipopolysaccharide (LPS) injected mice [4,6–8,10,11], suggesting that the anti-inflammatory effects of sEH inhibition may be limited to systemic inflammation that may be achieved by affecting non-EET

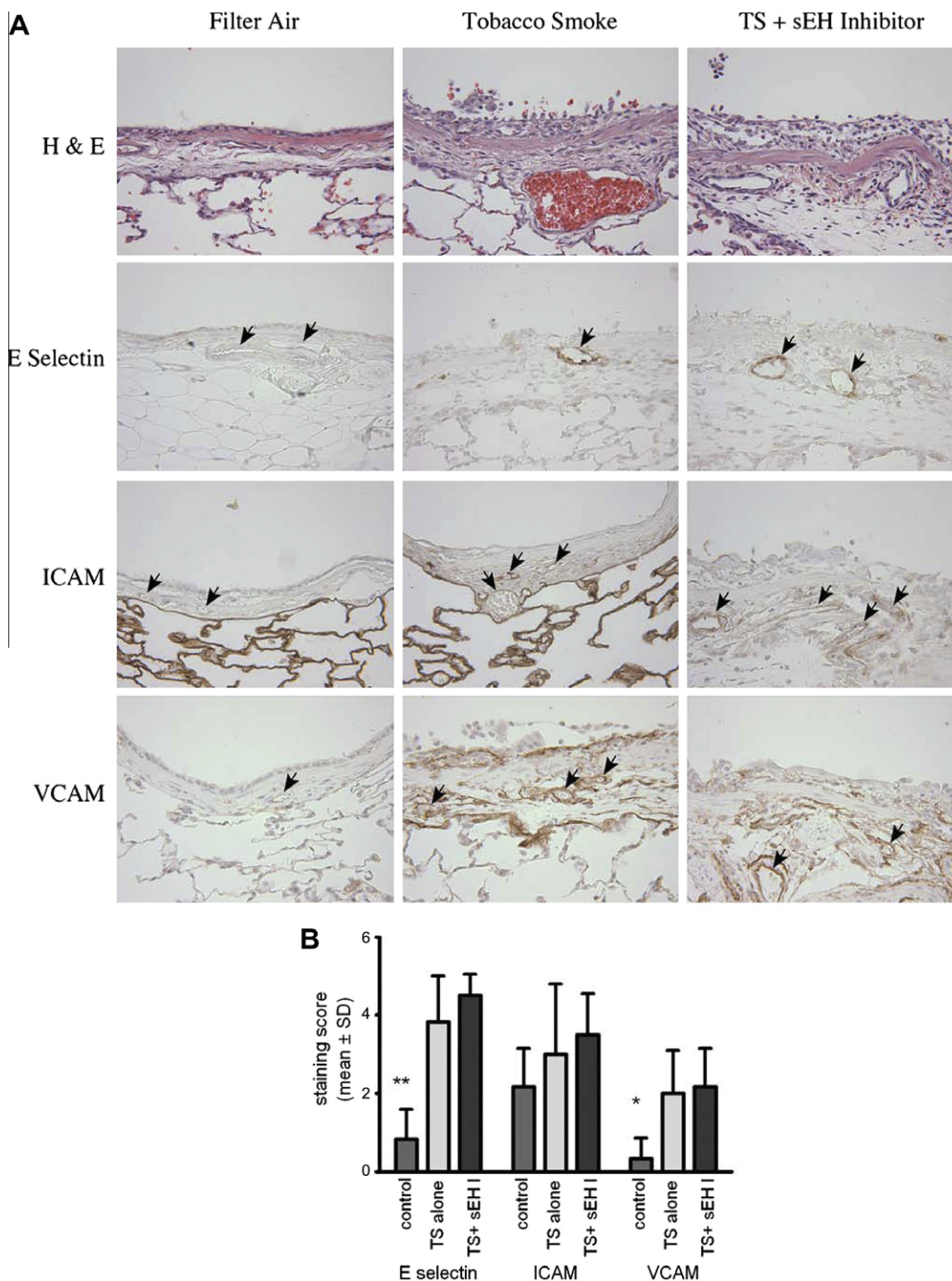


Fig. 2. sEH inhibitors did not reduce tobacco smoke induced damage to the bronchial epithelium or adhesion molecule expression in the bronchial wall blood vessels (arrows). (A) Sections of the bronchial wall from tobacco smoke (TS) exposed SH rats with or without sEH inhibitor (sEHI) treatment stained with H&E or immunohistochemical (IHC) staining for E-selectin, ICAM, and VCAM. (B) Blinded ranking of IHC staining intensities for E-selectin, ICAM and VCAM. * $p < 0.05$, ** $p < 0.01$ as compared to tobacco smoke alone.

substrates: a possible class of substrates is the linoleic acid epoxides, also referred to as leukotoxins (LTX). During robust systemic inflammation, activated neutrophils release LTX, which is converted by sEH to the cytotoxic LTX-diol. Thus, the prevention of death and anorexia seen in the murine LPS models following inhibition of sEH may be the result of blocking the production of LTX diol [25–30]. Other mechanisms by which sEH inhibitors may produce anti-inflammatory effects independent of effects on

leukocyte recruitment are through the regulation of the acute neurosteroid producing gene (StARD1) [5] or shunting the arachidonic acid pathway towards an anti-inflammatory profile.

The results from this study indicate that the anti-inflammatory effects of sEH inhibitors are independent of leukocyte recruitment, and EETs do not reduce adhesion molecules responsible for leukocyte recruitment *in vitro*. This demonstrates that the widely held belief that sEH inhibition prevents leukocyte recruitment via EET

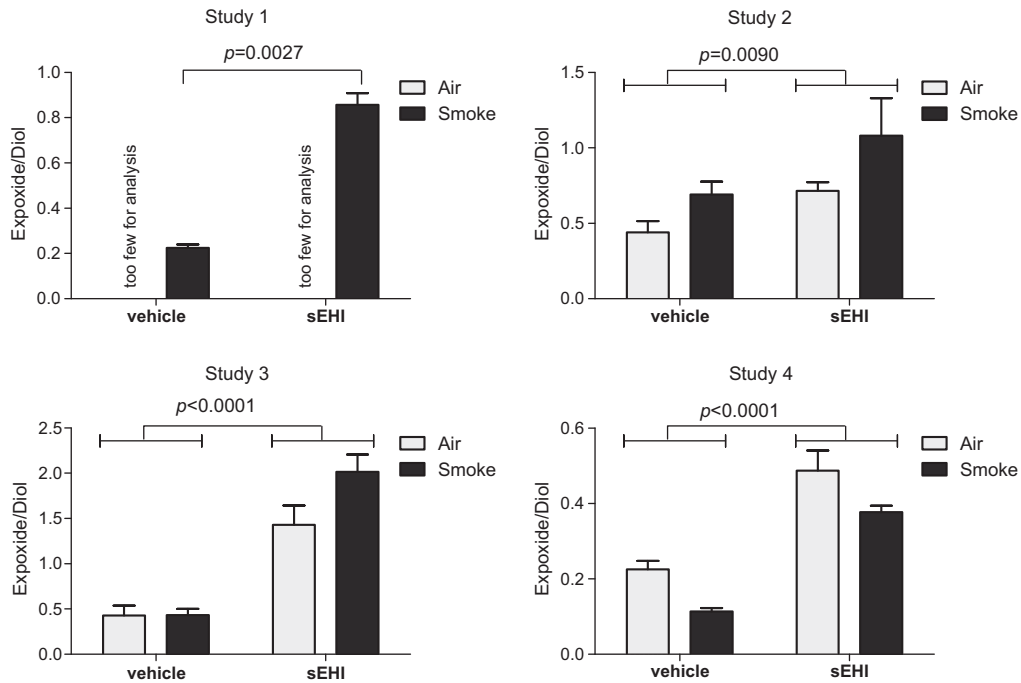


Fig. 3. Various inhibitors of sEH stabilize sEH substrates in the plasma of SH rats. Ratio of the six major sEH substrates, linoleic acid epoxides and arachidonic acid epoxides (EETs) to their corresponding diols in each study. *p* Value for the effect of sEH inhibitor treatment on the epoxide to diol ratio by two way ANOVA except for study one where a *t*-test was used.

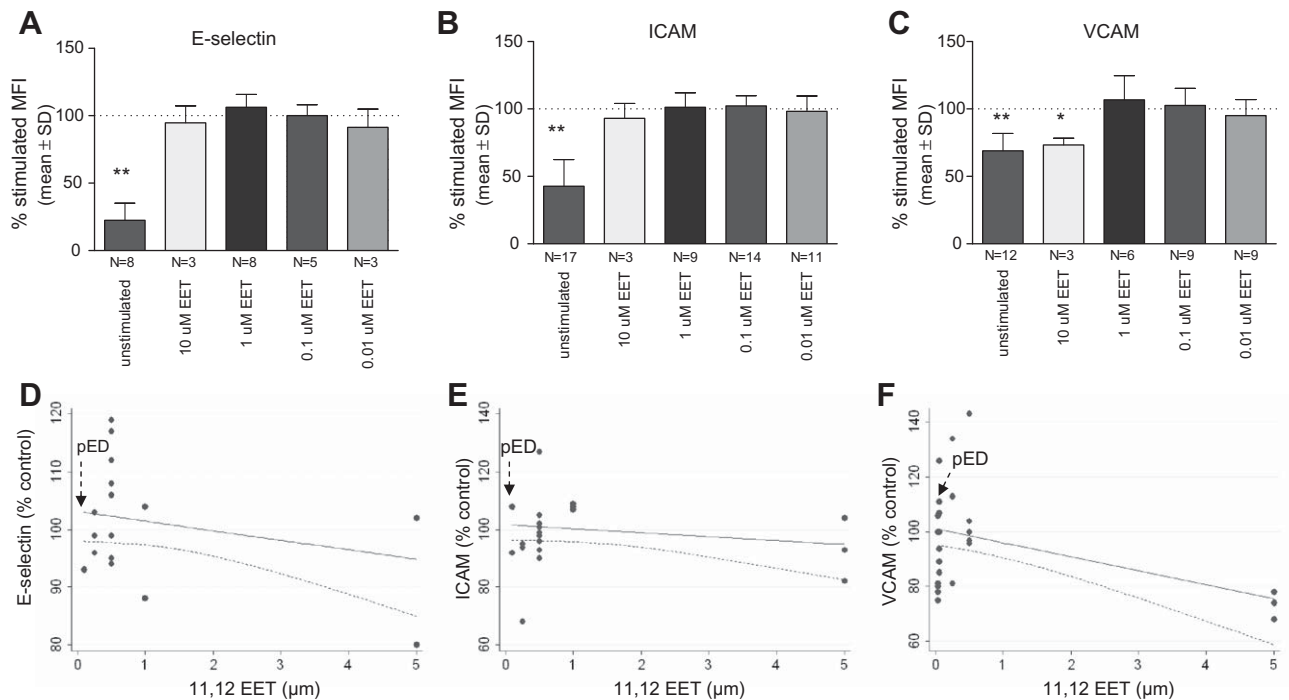


Fig. 4. EETs did not reduce cytokine stimulated adhesion molecule expression at the previously reported effective dose in cell culture. (A) E-selectin expression, (B) ICAM expression, (C) VCAM expression, and regression of the concentration of 11,12 EET with (D) E-selectin, (E) ICAM and (F) VCAM expression in human endothelial cells treated with concentrations above the previously reported effective dose. Dotted line represents the one way lower 95% CI. pED = previously reported effective dose. All data is standardized to the within plate cytokine stimulated control and reported as percent stimulated mean fluorescent intensity (MFI). **p* < 0.05, ****p* < 0.001 as compared to 100% of stimulated control.

prevention of adhesion molecule expression is not consistently reproducible. Further research is needed to understand why the effects of sEH inhibitors are not reproducible in an animal model of

acute tobacco smoke-induced lung injury, while sEHI effects in models of systemic inflammation [4,6–8,10,11], renal damage [31–34] and cardiovascular injury [35–39] are highly reproducible.

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