Impact of Epoxyeicosatrienoic Acids in Lung Ischemia–Reperfusion Injury

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ABSTRACT

Objective: Epoxyeicosatrienoic acids (EETs) are protective in both myocardial and brain ischemia, variously attributed to activation of K_{ATP} channels or blockade of adhesion molecule upregulation. In this study, we tested whether EETs would be protective in lung ischemia–reperfusion injury.

Methods: The filtration coefficient (K_f), a measure of endothelial permeability, and expression of the adhesion molecules vascular cell adhesion molecule (VCAM) and intercellular adhesion molecule (ICAM) were measured after 45 minutes ischemia and 30 minutes reperfusion in isolated rat lungs.

Results: K_f increased significantly after ischemia–reperfusion alone vs time controls, an effect dependent upon extracellular Ca^{2+} although not on the EET-regulated channel TRPV4. Inhibition of endogenous EET degradation or administration of exogenous 11,12- or 14,-15-EET at reperfusion significantly limited the permeability response to ischemia–reperfusion. The beneficial effect of 11,12-EET was not prevented by blockade of K_{ATP} channels nor by blockade of TRPV4. Finally, 11,12-EET-dependent alteration in adhesion molecules expression is unlikely to explain its beneficial effect, since the expression of the adhesion molecules VCAM and ICAM in lung after ischemia–reperfusion was similar to that in controls.

Conclusion: EETs are beneficial in the setting of lung ischemia–reperfusion, when administered at reperfusion. However, further study will be needed to elucidate the mechanism of action.
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KEY WORDS: calcium channels, sEH inhibitor, TRPV4, ATP-sensitive K+ channels, adhesion molecules

INTRODUCTION

Metabolism of arachidonic acid by P450 epoxygenases results in synthesis of a family of four epoxyeicosatrienoic acid (EET) regioisomers: 5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET. In heart and brain, EETs appear to act as protective mediators in the setting of ischemia and reperfusion. Either administration of exogenous EETs or blockade of EET degradation by inhibiting soluble epoxide hydrolase limit cerebral and myocardial ischemic injury and/or infarct size [7,9,27]. Furthermore, upregulation of a P450 epoxygenase enzyme in rat brain after transient ischemic preconditioning is associated with reduced infarct size resulting from carotid occlusion [1].

At present, we do not know whether EETs can modulate the acute lung injury response to global ischemia and reperfusion in the lung. Some EETs can display pro-inflammatory properties in lung. Both 5,6- and 14,15-EET increase endothelial permeability in rat lung via activation of the transient receptor potential channel TRPV4 [2,4]. Similarly, endogenous EETs mediate the permeability response to mechanical perturbation in mouse lung in a TRPV4-dependent fashion [10,12]. Thus, their beneficial impact in heart and brain does not necessarily predict outcomes after lung ischemia–reperfusion. Of the EET regioisomers, 11,12-EET appears to be the most likely candidate to provide

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therapeutic benefit in lung ischemia–reperfusion, because it has no impact on lung endothelial permeability [2]. Furthermore, 11,12-EET displays anti-inflammatory action in systemic endothelial cells by inhibiting TNFα-induced expression of the adhesion molecule vascular cell adhesion molecule 1 (VCAM-1), and to much less extent intercellular adhesion molecule (ICAM) [28]. While 11,12-EET stimulates neutrophil aggregation, it nonetheless decreases adherence of activated neutrophils to endothelium [29].

Taylor and colleagues have shown that the increase in endothelial permeability after ischemia–reperfusion in the rat lung can be attenuated by interfering with leukocyte-endothelial cell adhesion [23,25]. Furthermore, the increase in endothelial permeability induced by ischemia–reperfusion can be blocked by antibodies against TNFα [13]. Thus, plausibly administration of exogenous 11,12-EET could limit TNFα-induced upregulation of adhesion molecules leading to attenuation of acute lung injury after ischemia and reperfusion. Nonetheless, as ischemia–reperfusion may enhance endogenous EET synthesis [26], EETs could actually contribute to the increase in lung endothelial permeability [2,4]. To resolve these alternatives, in this study we tested whether ischemia–reperfusion injury in rat lung requires Ca^{2+} entry via TRPV4, whether endogenous and/or exogenous EETs would attenuate the increase in lung endothelial permeability induced by ischemia–reperfusion and finally whether upregulation of the adhesion molecules VCAM and ICAM could participate.

MATERIALS AND METHODS

Animal protocols for this study were approved by the Animal Care and Use Committee of the University of South Alabama, and adhered to the Guide for the Care of Use of Laboratory Animals (Department of Health and Human Services).

Isolated Lung Preparation and Assessment of Endothelial Injury

The isolated rat lung preparation and measurement of endothelial permeability in our hands have been previously described [2,4]. Lungs isolated from anesthetized rats (pentobarbital sodium, 50 mg/kg, i.p.) were perfused at a constant flow (∼0.03 mL/g body weight, pH 7.4, 37°C) with 4% albumin buffer (in mmol/L: 116 NaCl, 5 KCl, 0.8 $MgSO_4$,

1.0 NaH_2PO4, and 5.6 glucose) containing physiologic or low $CaCl_2$ (2.2 or 0.02 mM, respectively). Pulmonary artery (P_a), venous (P_v), and end-expiratory airway (P_{aw}) pressures and lung weight were recorded (Astromed polygraph, model 7400). P_v was adjusted to establish a baseline isogravimetric state under zone III conditions, with P_{aw} of 3 cmH$_2$O. Total pulmonary vascular resistance (R_t) was calculated. Pulmonary endothelial permeability was assessed using the filtration coefficient (K_f), by measuring the rate of lung weight gain 13–15 minutes after increasing P_v by 8–10 cmH$_2$O. This rate was divided by the concomitant increment in capillary pressure (P_c) and then normalized to 1 g dry lung wt. P_c was measured by the double occlusion technique [35].

Experimental Protocols

In the first series of experiments, perfusate contained physiologic Ca^{2+}. Responses in time controls ($n = 7$) were compared with those in lungs challenged with ischemia–reperfusion. During the ischemic period (45 minutes), lungs were not perfused or ventilated. K_f and hemodynamic measurements were repeated after 30 minutes of reperfusion. These ischemia–reperfusion experiments were interspersed between other groups described below and results in the cumulative group presented ($n = 15$).

We determined whether entry of extracellular Ca^{2+} was involved in the lung endothelial permeability response to ischemia–perfusion. Lungs perfused with low Ca^{2+} were challenged with 45 ($n = 4$) or 60 ($n = 4$) minutes ischemia followed by 30 minutes reperfusion. These results were compared with those in a low Ca^{2+} time control group ($n = 5$). We have previously shown that lung endothelial permeability is stable with this low Ca^{2+} concentration (0.02 mM), and further, that this concentration effectively prevents permeability responses to activation of store-operated channels or TRPV4 [3,4]. As EETs promote endothelial Ca^{2+} entry via TRPV4 and result in Ca^{2+} entry-dependent acute lung injury [4,37], we ruled out participation of TRPV4 in ischemia–reperfusion injury by pretreating a group of lungs with the TRPV antagonist ruthenium red prior to 45 minutes ischemia followed by 30 minutes reperfusion (1 μM, $n = 3$). In this latter group and all remaining experiments, buffer containing physiological Ca^{2+} was used.

In the next series of experiments, we addressed the impact of 11,12-EET on the permeability response to 45 minutes ischemia followed by reperfusion, and evaluated potential mechanisms by which 11,12-EET might act. A group of lungs was pretreated with 1-adamantanyl-3-{5-[2-(2-ethoxyethoxy)ethoxy]pentyl]}urea (AEPU, 100 μM, $n = 6$), also known as sEHI 950 [16]. Inhibition of soluble epoxide hydrolase should prevent degradation of endogenous EETs [30] prior to ischemia–reperfusion. AEPU is well adapted to perfusion studies such as these due to its relatively high water solubility (120 μg/mL), low melting point leading to rapid dissolution (mp 78.5–79°C), and relatively low lipophilicity (log P 2.8). In a parallel series of experiments, we determined whether administration of exogenous 11,12-EET (300 nM) could attenuate the permeability responses to ischemia–reperfusion: lungs were treated with 11,12-EET either prior to ischemia ($n = 5$) or at reperfusion ($n = 6$). To determine whether the impact of 11,12-EET was specific to this regioisomer, 14,15-EET (300 nM) was added at reperfusion in a separate group of lungs ($n = 4$). As blockade of K_{ATP} channels prevents EET-induced protection against ischemia–reperfusion injury in the heart [9], a group of lungs was pretreated with the K_{ATP} channel inhibitor glibenclamide (10 μM, $n = 5$) and 11,12-EET administered at reperfusion. While most EET isomers activate TRPV4 and increase lung endothelial permeability [4], we considered it unlikely that any beneficial effect of 11,12-EET would be due to TRPV4-mediated Ca^{2+} entry. Nonetheless, to rule out any participation of this cation channel, we pretreated another group of lungs with the broad TRPV antagonist ruthenium red (1 μM), then administered 11,12-EET at reperfusion ($n = 5$).

VCAM and ICAM Expression

Immunohistochemistry was used to document expression of VCAM and ICAM in rat lung. Lungs were perfusion-fixed with 4% paraformaldehyde upon isolation (controls, $n = 3$) or after baseline measurements and subsequent 45 minutes of ischemia and 30 minutes of reperfusion ($n = 3$). In parallel time control experiments, lungs were perfused for a similar period of time after baseline measurements (75 minutes), but without ischemia or reperfusion ($n = 3$). A final group of lungs was fixed by airway instillation of paraformaldehyde immediately at the conclusion of the ischemic period

($n = 3$), to insure that the vasculature did not experience any period of reperfusion. Sections (5 μm) were cut from paraffin-embedded lung blocks and placed on poly-L-lysine coated slides for immunohistochemistry. Following overnight incubation with goat anti-rat polyclonal primary antibodies targeting VCAM or ICAM (1 : 50, 4°C), slides were treated for 1 hour with horseradish peroxidase-conjugated secondary antibodies (1 : 200 or 1 : 500, respectively). Diaminobenzidine was used for color development, while hematoxylin was used for counterstaining. Images were collected via light microscopy (20×).

Volume fractions for VCAM and ICAM in the alveolar septal wall were determined using a morphometric point counting method. Images of immunostained lung sections were visualized in Paint Shop Pro with a grid overlay. A total of 8–12 images per lung from three lungs in each treatment group were analyzed. VCAM or ICAM volume fractions were calculated for each lung as the ratio of positively stained points relative to total points landing on the alveolar septal wall. The volume fraction data were then averaged for each treatment group.

Drugs

11,12- and 14,15-EET were obtained from Biomol (Plymouth Meeting, PA, USA). Buffer reagents were obtained from Fisher Scientific (Pittsburgh, PA, USA). Anti-VCAM and secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-ICAM antibodies were obtained from R & D Systems, Inc. (Minneapolis, MN, USA). All other reagents and drugs were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Statistics

Data are presented as mean ± SE. Statistical comparisons between groups were performed by using an unpaired t-test or one-way ANOVA with Tukey's *post hoc* test. P values < 0.05 were considered statistically significant.

RESULTS

When lungs were perfused with physiologic buffer, 45 minutes of ischemia followed by 30 minutes reperfusion led to a 7.8-fold increase in K_f (Figure 1). These data are compared with
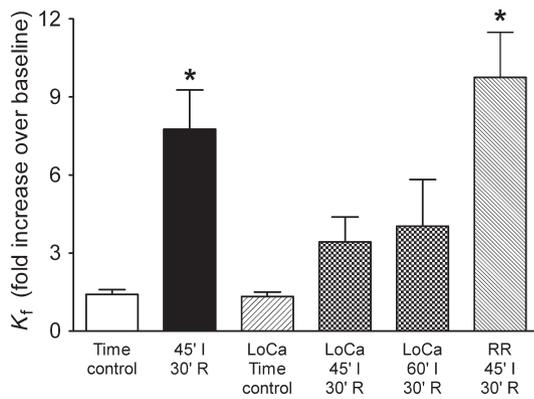
Figure 1. Dependence of ischemia–reperfusion injury on extracellular Ca^{2+}. In rat lung, 45 minutes ischemia (45′ I) followed by 30 minutes reperfusion (30′ R) increased the filtration coefficient (K_f) nearly eightfold ($n = 15$) compared with that in time controls ($n = 7$). Although low Ca^{2+} perfusate (LoCa) had no impact in time control lungs ($n = 5$), this perfusate did blunt the permeability response to 45 ($n = 4$) or 60 minutes ($n = 4$) ischemia followed by reperfusion. In contrast, the TRPV antagonist ruthenium red (RR) had no effect. *$P < 0.05$ vs control (one-way ANOVA). Thus, while the permeability response to ischemia–reperfusion in rat lung requires Ca^{2+} entry, the Ca^{2+} channel TRPV4 does not appear to play a role.

outcomes in experiments designed to evaluate a role for Ca^{2+} entry (Figure 1). Ischemia–reperfusion had little impact on lung endothelial permeability when a low Ca^{2+} perfusate was utilized, regardless of whether the lungs were ischemic for 45 or 60 minutes. These data suggest that the permeability response to ischemia–reperfusion is significantly dependent upon extracellular Ca^{2+}. Although ischemia–reperfusion and TRPV4 activation both target the lung microcirculation and both require Ca^{2+} entry [4,15], blockade of Ca^{2+} entry with the TRPV antagonist ruthenium red did not prevent the permeability response to ischemia–reperfusion Final K_f remained near baseline in time control experiments utilizing either the physiologic or low Ca^{2+} buffers. A summary of hemodynamics and K_f during baseline for all isolated lung experiments (Table 1) shows that these measures were not significantly impacted by lowering perfusate Ca^{2+} to 0.02 mM.

The next series of experiments evaluated whether EETs could protect barrier integrity in the setting of lung ischemia–reperfusion. Pretreatment with the soluble epoxide hydrolase inhibitor AEPU significantly attenuated the permeability response to

Table 1. Baseline Hemodynamics and Permeability in Isolated Rat Lungs

	Perfusate $[Ca^{2+}]$, mM	
	1.8	0.02
n	56	13
Body weight, g	371 ± 8	417 ± 16
Q, mL/min	13.2 ± 0.2	13.7 ± 0.4
P_a, cmH$_2$O	14.4 ± 0.4	14.7 ± 0.6
P_c, cmH$_2$O	9.5 ± 0.2	9.6 ± 0.4
P_v, cmH$_2$O	4.2 ± 0.1	4.0 ± 0.2
R_t, cmH$_2$O/L/min	0.77 ± 0.03	0.77 ± 0.05
K_f, mL/min/ cmH$_2$O/g dry wt	0.0087 ± 0.0004	0.0085 ± 0.0009

Q, perfusate flow; P_a, pulmonary artery pressure; P_c, pulmonary capillary pressure; P_v, pulmonary venous pressure; Rt, total vascular resistance; K_f, filtration coefficient.
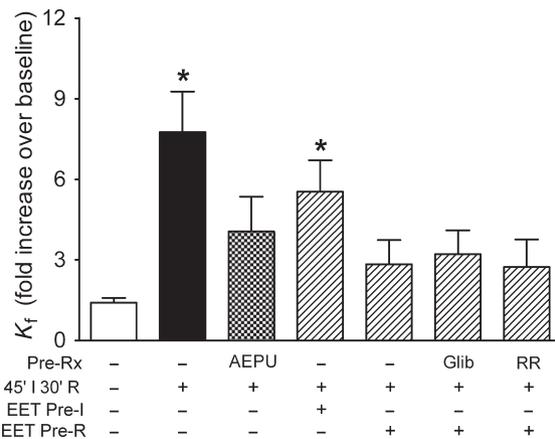

Figure 2. Endogenous and exogenous EETs protect against ischemia–reperfusion in rat lung. Results in controls and lungs challenged with 45′ ischemia–30′ reperfusion (IR, from Figure 1) are repeated here for ease of comparison. Inhibition of soluble epoxide hydrolase (AEPU) limited the permeability response to ischemia–reperfusion, supporting a protective role for endogenous EETs. Although 11,12-EET did not have marked impact when given prior to ischemia (Pre I), it was effective given at reperfusion (Pre R). Neither glibenclamide (Glib) nor ruthenium red (RR) prevented the protective effect of 11,12-EET in this model. *$P < 0.05$ vs control (one-way ANOVA).

ischemia–reperfusion (Figure 2). K_f only increased 4.1-fold after ischemia–reperfusion (not significantly different from control), suggesting that endogenous EETs could protect the lung to some extent against ischemia–reperfusion injury. Although not shown, blockade of P450 epoxygenases

with PPOH to limit EET synthesis was not protective. Several lungs failed immediately upon reperfusion, with fulminant alveolar flooding. In the remaining five lungs, mean K_f increased sevenfold on average compared with baseline. Administration of 11,12-EET prior to ischemia had minimal impact on the permeability response (Figure 2). However, when 11,12-EET was added at reperfusion, K_f only increased 2.8-fold. Although not shown in Figure 2, addition of 14,15-EET at reperfusion had a similar protective effect: K_f increased 2.4 ± 0.8-fold after ischemia–reperfusion. Protection afforded by addition of 11,12-EET at reperfusion could not be prevented by pretreatment of lungs with the K_{ATP} channel antagonist glibenclamide: K_f increased 3.2-fold from baseline in this group after ischemia–reperfusion. Similarly, use of ruthenium red to block Ca^{2+} entry via TRPV4 did not prevent the beneficial effect of 11,12-EET: K_f increased 2.7-fold.

Finally, we evaluated adhesion molecule expression in lungs using immunohistochemistry (Figure 3) and quantitative morphometry (Figure 4). In controls, VCAM was expressed in bronchiolar epithelium, type II alveolar epithelium, and broadly across sections of the alveolar septal wall (Figure 3A). We interpret the latter result to mean predominantly positive expression in septal capillaries. In contrast, ICAM expression appeared to be limited to alveolar type I epithelium (Figure 3D). In lungs subjected to ischemia–reperfusion, VCAM
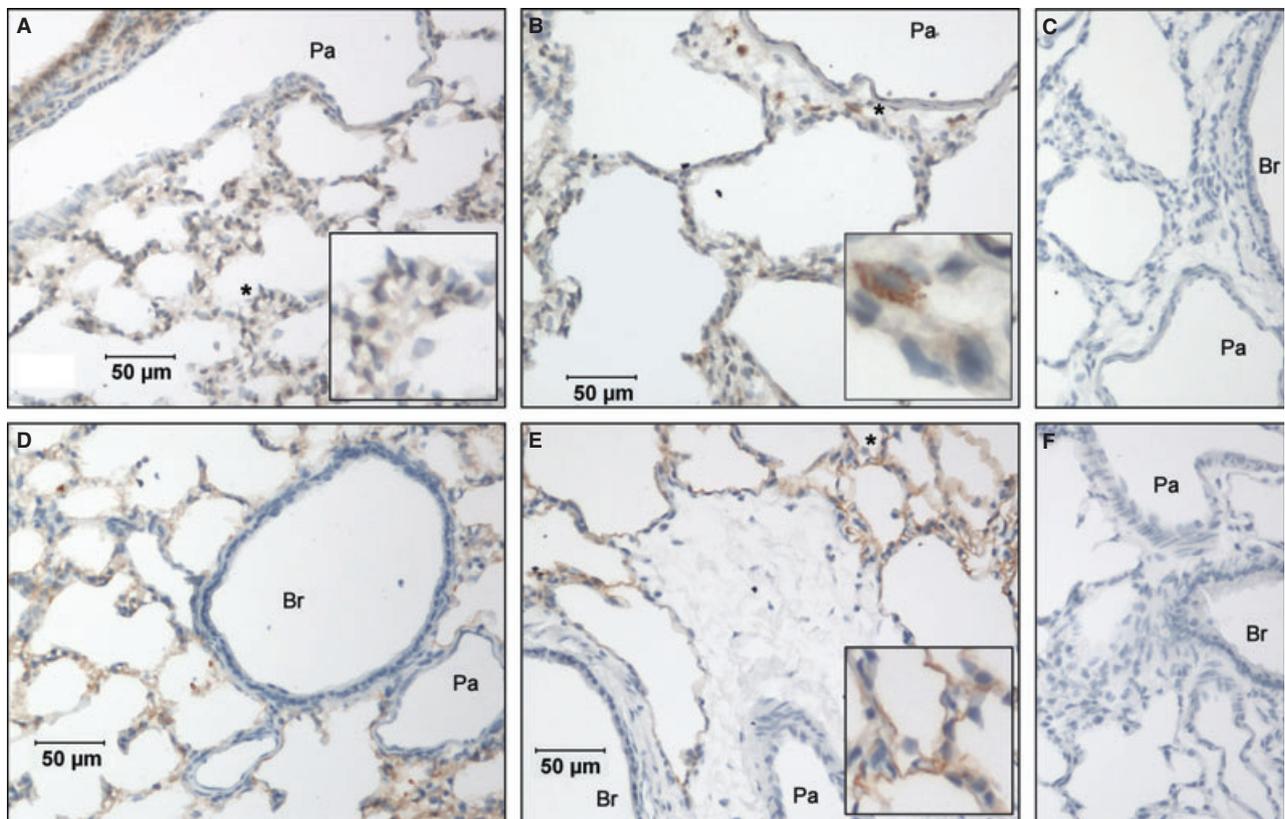
Figure 3. VCAM and ICAM expression in rat lung. In control lungs, VCAM was expressed predominantly in bronchiolar epithelium and type II alveolar epithelium, with some positive staining across sections of the alveolar septal wall (**A**). In lungs subjected to ischemia–reperfusion, the pattern of VCAM expression in airways and the alveolar septal wall did not change, although positive staining was occasionally observed in mononuclear cells situated around the septal wall, in perivascular/peribronchiolar interstitial cuffs, and in endothelium of small extra-alveolar vessels (**B**). In contrast to the pattern of VCAM staining, ICAM expression in controls appeared to be limited to alveolar type I epithelium (**D**), a pattern which did not change after ischemia–reperfusion (**E**). Sections of lung exposed to only secondary antibodies showed no positive staining at either the 1 : 200 (**C**) or 1 : 500 dilutions (**F**). Br, bronchiole; Pa, pulmonary arteriole; *areas enlarged in insets. Additional images for these two groups, as well as representative images from lungs exposed only to ischemia or in time control lungs, are available in Figures S1 and S2.

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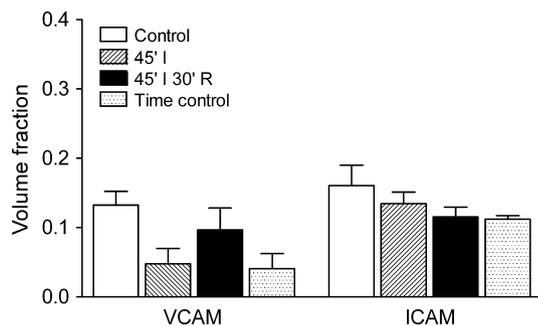
Figure 4. VCAM and ICAM volume fractions in the alveolar septal wall. A morphometric approach was used to quantitatively assess volume fractions for adhesion molecule expression in the alveolar septal wall (see text for details). Neither the VCAM nor ICAM volume fraction differed among the four experimental groups: control lungs fixed after isolation, lungs exposed to ischemia alone or ischemia–reperfusion, and time controls (one-way ANOVA).

expression in airways and the alveolar septal wall did not change. However, positive staining was occasionally observed in mononuclear cells situated around the septal wall or in perivascular/peribronchiolar interstitial cuffs and in extra-alveolar vessel endothelium (Figure 3B). There was no significant change in the VCAM volume fraction in the alveolar septal wall (Figure 4) in lungs subjected to ischemia, ischemia–reperfusion, or time controls. Similarly, the pattern of ICAM staining in lung after ischemia–reperfusion (Figure 3E) did not change after ischemia–reperfusion, with expression remaining limited to type I alveolar epithelium. The volume fraction for ICAM in the alveolar septal wall did not vary significantly between the four experimental groups (Figure 4). Sections of lung exposed to only secondary antibodies showed no positive staining at either the 1 : 200 (Figure 3C) or 1 : 500 dilutions (Figure 3F). Additional images for all four experimental groups can be found in Figures S1 and S2.

DISCUSSION

Results of this study confirm the significant impact of ischemia–reperfusion on lung endothelial permeability. In our hands, K_f increased nearly eightfold after ischemia–reperfusion. As alveolar capillaries and post-capillary venules are the target of this injury, substantial alveolar flooding ensues [5,15,31]. Despite the magnitude of this insult, we show for the first time that blockade of EET degra-

dation or administration of low-dose exogenous EETs provide significant protection against the increased lung endothelial permeability resulting from ischemia–reperfusion. However, the mechanism of this protection remains elusive. Neither ischemia alone nor ischemia–reperfusion was accompanied by up-regulation of VCAM or ICAM in the alveolar septal wall within the time frame of this study, compared with controls. Furthermore, while ischemia–reperfusion injury requires Ca^{2+} entry from the extracellular fluid, the protective effect of 11,12-EET could not be abrogated by blockade of TRPV4 channels. Finally, although protection afforded by EETs in other vascular beds can be mediated via activation of K_{ATP} channels, blockade of these channels did not prevent the EET-dependent attenuation of the ischemia–reperfusion permeability response in rat lung.

Based on the ability of the soluble epoxide hydrolase inhibitor AEPU to limit the increase in K_f induced by ischemia–reperfusion, we conclude that endogenous EETs indeed are protective in this setting. While the impact of ischemia–reperfusion on EET synthesis and release in lung has not been determined, EETs are released into canine coronary venous blood with ischemia–reperfusion [26]. The beneficial impact of the soluble epoxide hydrolase inhibitor supports the notion that EETs are synthesized and released from rat lung in our experiments. In both rat and human lung, 14,15-EET is the predominant EET regioisomer although 11,12-EET also makes a major contribution [38]. The pool may reflect steady state synthesis as well as EETs esterified into endothelial plasma membrane phospholipids [36]. The relative proportions of 14,15- and 11,12-EET in the endogenous pool of EETs in lung may not necessarily predict the predominant EET released with ischemia–reperfusion. In perfused human lung, challenge with the Ca^{2+} ionophore A23187 led to rapid release of 11,12-EET, but little 14,15-EET, into the perfusate [17]. Although micromolar concentrations of most EET regioisomers increase permeability in rat lung [2], blockade of EET synthesis with PPOH did not attenuate ischemia–reperfusion induced endothelial injury. This finding, along with the observation that AEPU-induced blockade of EET degradation was protective, suggests that on balance, endogenous EETs limit the increase in lung endothelial permeability induced by ischemia–reperfusion. Nonetheless, these experiments did not elucidate the mechanism underlying the protective affect. To

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that end, we utilized exogenous EETs and assessed a role for adhesion molecules and channels implicated in other experimental models.

The involvement of leukocytes (including lymphocytes, neutrophils, and macrophages) in lung injury subsequent to ischemia–reperfusion has been well documented [6,24]. Furthermore, TNFα and up-regulation of adhesion molecules subsequent to ischemia–reperfusion have been implicated in lung ischemia–reperfusion injury. TNFα levels rise early after ischemia–reperfusion [8]. This cytokine is known to upregulate neutrophil adhesion to endothelial cells in an ICAM-dependent fashion [11,18]. Taylor and colleagues provided convincing evidence that both the leukocyte retention and the increased permeability response to ischemia–reperfusion were ameliorated by immunoneutralization of ICAM [23] or TNFα [13]. Alternatively, the early injury response to ischemia–reperfusion in rat lung may require lymphocyte retention [25] and up-regulation of VCAM [34].

Given this background and the observation that 11,12-EET inhibited TNFα-induced VCAM, and to less an extent ICAM, expression in endothelial cells [28], 11,12-EET could plausibly have been protective in the setting of lung ischemia–reperfusion due to down-regulation of adhesion molecule expression. However, in the current study neither VCAM nor ICAM expression was significantly altered after ischemia–reperfusion compared with that in freshly isolated or time control lungs, despite a substantial ischemia–reperfusion-induced increase in endothelial permeability. Several possibilities may explain this result. First, lungs were perfused for a relatively brief period following ischemia. TNFα-dependent ICAM up-regulation, for example, may not be evident until 2–4 hours after TNFα application [11,22]. More likely, even brief flush of the lung vasculature, as in the control group (Figure 3 and Figure S1), evokes surface expression of adhesion molecules. The surface expression of VCAM and ICAM observed in controls may explain the retention of leukocytes in isolated lung in the absence of injury [23,23]. These data suggest that some trigger aside from adhesion molecule expression *per se* must be required to elicit endothelial injury after ischemia–reperfusion. Furthermore, while treatment of lungs with 11,12-EET at reperfusion clearly limited ischemia–reperfusion injury, this protective effect is unlikely to be due to inhibition of TNFα-mediated up-regulation of VCAM or ICAM expression. Further evidence supporting this

conclusion is derived from the often disparate effects of EETs. Most pertinent to this study, Node et al. [28] reported that 11,12-EET prevented the impact of TNFα on VCAM expression in endothelial cells and on mononuclear cell adhesion to carotid artery endothelium. In contrast, they found that 14,15-EET did not have similar anti-inflammatory effects. Nonetheless, in the rat lung 11,12- and 14,15-EET limited the impact of ischemia–reperfusion on endothelial permeability to a similar extent. Collectively, these observations suggest that a role for adhesion molecules in ischemia–reperfusion injury is complex. Furthermore, our data suggest that EETs are not likely providing benefit due to altered endothelial expression of adhesion molecules.

Next, we turned to investigation of whether the effect of 11,12-EET was due to activation of critical channels in lung endothelium known to be targets for EETs, such as Ca^{2+} channels. We have shown that low micromolar doses of 5,6- and 14,15-EET increase lung endothelial permeability via a Ca^{2+} entry- and TRPV4-dependent mechanism [2,4]. As a result of EET-dependent TRPV4 activation, the endothelial barrier in the lung alveolar septum is disrupted and alveolar flooding ensues. While submicromolar concentrations of EETs do not impact lung endothelial permeability (On-line data supplement to Ref. 4), concentrations in this range can still promote Ca^{2+} entry into endothelial cells in a dose- and TRPV4-dependent manner [37]. Ischemia–reperfusion targets the septal microvascular barrier [5,15], although whether Ca^{2+} entry is required for the injury is controversial. Chetham and colleagues [5] concluded that ischemia–reperfusion injury was independent of Ca^{2+} entry, because K_f increased after ischemia–reperfusion when a low (10 μM) Ca^{2+} perfusate was used to limit Ca^{2+} entry. Alvarez et al. subsequently reported that lung endothelial permeability increased over time when perfusate Ca^{2+} concentrations lower than 20 μM were used. At 20 μM Ca^{2+}, however, barrier integrity is stable and the permeability response to activation of TRPV4 is completely abrogated [4]. Using this latter low Ca^{2+} paradigm, we found that the lung endothelial permeability response to ischemia–reperfusion does require extracellular Ca^{2+} (Figure 1). However, TRPV4 is not likely to be involved in either the development of ischemia–reperfusion injury or the protection afforded by EETs. Although blockade of EET degradation by target-

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ing soluble epoxide hydrolase attenuated the permeability response to ischemia–reperfusion, the TRPV antagonist ruthenium red failed to either attenuate the permeability response to ischemia–reperfusion or to block 11,12-EET-induced protection against injury.

One final mechanism considered for the 11,12-EET-mediated protection against ischemia–reperfusion injury involved K_{ATP} channels. EETs are known to activate K_{ATP} channels [19–21] localized to either the plasmalemmal or mitochondrial membranes in smooth muscle and cardiac myocytes. In the heart, blockade of sarcolemmal and/or mitochondrial K_{ATP} channels abrogates EET-induced protection against the myocardial dysfunction observed after ischemia and reperfusion [9,21, 32,33]. However, in our hands, blockade of K_{ATP} channels with glibenclamide, at a dose which should block both plasmalemmal and mitochondrial K_{ATP} channels, did not prevent 11,12-EET-mediated protection against ischemia–reperfusion. Furthermore, although our results showed that endogenous EETs could play a protective effect, Khimenko et al. previously reported that blockade of K_{ATP} channels did not prevent ischemia–reperfusion injury in rat lung [14]. Collectively these data rule out a role for K_{ATP} channels in the EET-mediated protection.

In conclusion, this study has demonstrated a clear protective benefit of endogenous and exogenous EETs against lung ischemia–reperfusion injury. While EETs do limit the increase in lung endothelial permeability resulting from ischemia–reperfusion, mechanisms commonly attributed to the benefit of EETs in coronary and brain ischemia–reperfusion do not appear to play a role in lung. As ischemia–reperfusion failed to up-regulate expression of either VCAM or ICAM within a time frame which includes a substantial increase in endothelial permeability, we have ruled out alterations in expression of the adhesion molecules as a potential mechanism. Finally, while EETs are known to regulate K_{ATP} or TRPV4 channels, neither action underlies the beneficial role of 11,12-EET in lung ischemia–reperfusion injury. Understanding the protective role of EETs in the setting of lung ischemia–reperfusion will require further study.

ACKNOWLEDGEMENTS

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article (http://www.microcirculationjournal.com, click on View content online):

Figure S1. VCAM and ICAM expression in rat lung. VCAM (**A-D**) and ICAM (**E-H**) expression was evaulated in each of four experimental groups: control lungs fixed after isolation (**A** and **E**), lungs exposed to ischemia alone (**B** and **E**) or ischemia–reperfusion (**C** and **F**), and time controls (**D** and **H**). All images were captured at 20× (scale bar shown in **F**). As noted in the text, VCAM expression was observed in bronchiolar epithelium, type II alveolar epithelium, and across sections of the alveolar septal wall. ICAM expression appeared to be limited to alveolar type I epithelium. Neither the pattern of VCAM nor ICAM expression changed markedly among the four experimental groups, although occasionally VCAM expression was observed in small extra-alveolar vessels after ischemia or ischemia–reperfusion (*). Br, bronchiole; Pa, pulmonary arteriole.

Figure S2. VCAM and ICAM expression in the alveolar septal wall. Enlarged images showing the pattern of VCAM (**A**) and ICAM (**B**) expression in the alveolar septal wall. Arrowheads (in **B**) denote ICAM staining lining the alveolar face of the septal wall.

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