Role of EETs in regulation of endothelial permeability in rat lung

Diego F. Alvarez, Eli-Anne B. Gjerde, and Mary I. Townsley

Role of EETs in regulation of endothelial permeability in rat lung. Am J Physiol Lung Cell Mol Physiol 286: L445–L451, 2004. First published October 24, 2003; 10.1152/ajplung.00150.2003.—This study tested the hypothesis that epoxygenase metabolite(s) of arachidonic acid (EETs) derived from arachidonic acid via \( \text{P-450} \) epoxygenases are soluble factors linking depletion of endoplasmic reticulum \( \text{Ca}^{2+} \) stores and store-dependent regulation of endothelial cell (EC) permeability in rat lung. EC permeability was measured via the capillary filtration coefficient (\( K_{\text{f,c}} \)) in isolated, perfused rat lungs. 14,15-EET and 5,6-EET increased EC permeability, a response that was significantly different from that of 8,9-EET, 11,12-EET, and vehicle control. The permeability response to 14,15-EET was not significantly attenuated by the nongenotoxic \( \text{Ca}^{2+} \) channel blocker \( \text{Gd}^{3+} \). In lungs perfused with low [\( \text{Ca}^{2+} \)], 14,15-EET tended to increase EC permeability, although a significant increase in \( K_{\text{f,c}} \) was observed only following \( \text{Ca}^{2+} \) add-back. As positive control, we showed that the 3.7-fold increase in \( K_{\text{f,c}} \) evoked by thapsigargin (TG), a known activator of store depletion-induced \( \text{Ca}^{2+} \) entry, was blocked by both \( \text{Gd}^{3+} \) and low [\( \text{Ca}^{2+} \)] buffer. Nonetheless, the permeability response to TG could not be blocked by the phospholipase A\(_2\) inhibitors mepacrine or methyl arachidonyl fluorophosphonate or the \( \text{P-450} \) epoxygenase inhibitors 17-octadecenoic acid (17-ODYA) and clotrimazole to inhibit \( \text{P-450} \) epoxygenases (18), an observation that supports the notion that EETs play an important role in regulation of EC permeability. However, there is no evidence at this point that this regulatory mechanism is an obligate participant in regulation of EC permeability regardless of the source or the injury paradigm. Because cytochrome \( \text{P-450} \) epoxygenase metabolites are known to be expressed in peripheral rat lung (32), we sought to evaluate their role in capacitative \( \text{Ca}^{2+} \) entry and increased EC permeability in this species. The specific goal of this study was to test the hypothesis that EETs are involved in the store-dependent regulation of EC permeability in rat lung.

METHODS

Isolated Lung Preparation

The isolated lung preparation has been previously described (5, 23, 42). Adult male CD\(_40\) rats (\( n = 113, 368 \pm 10 \) g, mean \( \pm \) SE) were anesthetized with pentobarbital sodium (50 mg/kg ip). After tracheotomy, mechanical ventilation was initiated with room air at 50–70 strokes per min (6 ml/kg body wt). The thorax was opened via a subdiaphragmatic incision, and then 5,000 units of heparin were injected into the left ventricle and allowed to circulate for 5 min. A plastic cannula (PE 240) connected to a reservoir was advanced into the pulmonary artery via an incision in the right ventricular free wall, and the cannula was secured with 3-0 silk; low flow of perfusate was maintained. Next, a plastic cannula was advanced into the left atrium via an incision in the apex of the left ventricle. Both cannulas were secured by umbilical tapes tied around the ventricles. The lung and heart were removed en bloc and suspended from a calibrated force transducer. The lung was ventilated (5% \( \text{CO}_2 \), 30% \( \text{O}_2 \), balance \( \text{N}_2 \)), perfused at constant flow (38°C) with Earle’s buffer salt solution containing 4% albumin (unless indicated) and pH adjusted to 7.4; total circulating volume was \( \approx \)45 ml. Initially in all experiments, an isotropic state was established with the lung perfused under zone 3 conditions. Venous pressure (\( P_v \)) was set to 4 cmH\(_2\)O and positive involving soluble mediators has not been ruled out. Among the candidates proposed for the latter are arachidonic acid (AA) metabolites generated via cytochrome \( \text{P-450} \) epoxygenases (epoxygenase metabolites in regulation of \( \text{Ca}^{2+} \) entry and EC permeability in this study was to test the hypothesis that EETs are involved in the store-dependent regulation of EC permeability in rat lung.

Address for reprint requests and other correspondence: M. I. Townsley, Dept. of Physiology, MSB 3074, Univ. of So. Alabama, Mobile, AL 36688 (E-mail: mtownsley@usouthal.edu).

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end-expiratory pressure to 3 cmH2O. Arterial pressure (Pp, Pa, and lung weight were continuously recorded on an Astromed polygraph (model 7400).

Evaluation of EC Permeability and Hemodynamics

The capillary filtration coefficient (Kec) was measured as an index of pulmonary EC permeability (5, 42). Kec was determined by dividing the rate of lung weight gain measured 13–15 min after increasing Pp by 7–10 cmH2O by the resultant increment in capillary pressure (Pc) (43) and normalizing the result per gram dry lung weight. This rate of weight gain was corrected for any nonsorgramiometric rate of weight gain during the period just preceding the Kec maneuver. Pp was measured by the double occlusion technique (41). Kec measurements were made at baseline and at specific time points (30–90 min) after treatment, as described under experimental protocols. Using measures of Pp, Pa, and Pv, we determined not only total vascular resistance (RR) but also arterial (RA) and venous (RV) vascular resistance (41, 43).

Experimental Protocols

Time controls. To demonstrate the stability of the isolated lung preparation in our hands, lungs were isolated and perfused as described and flow increased to the maximal isotropic flow in zone 3. Baseline Kec and hemodynamics were measured initially, and then the lungs were subsequently perfused at a constant flow for ~3 h before final measurements of Kec and hemodynamics were repeated (n = 6).

Effect of EETs on lung EC permeability and hemodynamics. The epoxygenase metabolites of AA are synthesized as a family of four regioisomers, including 5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET (32). EETs have been proposed as mediators of acute lung injury (2), and Ca2+ blocks Ca2+ entry. After the baseline measurements, lungs were pretreated with vehicle (50 nM, n = 5–8) was added as a bolus to the venous reservoir following the baseline measurements. Final hemodynamics and Kec were measured after 60 min.

Requirement for Ca2+ entry. EETs have been associated with Ca2+ entry in aortic EC (16, 21) and Ca2+ entry in EC often results in increased permeability (17, 39). Thus in these protocols we tested whether the observed increase in Kec following 14,15-EET involved Ca2+ entry. After the baseline measurements, lungs were pretreated with gadolinium chloride (Gd3+), a lanthanide mineral that nonspecifically inhibits cation channels and capacitative Ca2+ entry (44). Because of the ability of Gd3+ to bind to albumin (3), lungs used for these experiments were perfused throughout with 1% albumin-3% clinical grade dextran, a combination that does not alter Kec in the lung (30). The lungs were treated for 30 min with vehicle (50 μL H2O, n = 8) or Gd3+ (30 μM, n = 5) followed by administration of 14,15-EET (3 μM). Final measures of hemodynamics and Kec were made 60 min later.

Thapsigargin (TG), an alkaldoid derivative that blocks Ca2+ reuptake into the endoplasmic reticulum and evokes capacitative Ca2+ entry (40), was used as a positive control (4, 17). After baseline measurements, lungs were pretreated with vehicle (n = 6) or Gd3+ (n = 5) for 30 min before addition of TG (150 nM). After 60 min, the final measurements of hemodynamics and Kec were made. To further address the requirement for Ca2+ entry in 14,15-EET-induced lung injury, we perfused lungs with a bicarbonate-buffered physiological salt solution (in mM: 116.0 NaCl, 5.2 KCl, 0.9 MgSO4, 1.0 NaHPO4, and 8.3 d-glucose) containing 4% bovine serum albumin and either physiological (2.2 mM) or low (0.02 mM) CaCl2 (4). After measurement of baseline hemodynamics and Kec, lungs were treated with 14,15-EET (3 μM, n = 5) for 45 min before a second Kec was made. Subsequently, CaCl2 was added to the perfusate to achieve a 2.2 mM circulating concentration, and the final Kec was measured 15 min later. In parallel experiments, we evaluated the effect of low Ca2+ and Ca2+ add-back on the permeability response to TG (150 nM, n = 5), as a positive control.

Degradation of TG-induced lung injury on EETs. EETs have been postulated to act as soluble factors involved in capacitative Ca2+ entry (1, 13). Thus we tested whether TG-induced EC injury in rat lung was dependent on EET synthesis, as previously shown for canine lung (18). First, the effect of TG alone was documented (150 nM, n = 12). Then, we evaluated whether EETs play a role in the response to TG by blocking sequential steps in EET synthesis and metabolism, starting with inhibition of the rate of lung weight gain measured 13–15 min after increasing Pp by 7–10 cmH2O by the resultant increment in capillary pressure (Pc) (43) and normalizing the result per gram dry lung weight. This rate of weight gain was corrected for any nonsorgramiometric rate of weight gain during the period just preceding the Kec maneuver. Pp was measured by the double occlusion technique (41). Kec measurements were made at baseline and at specific time points (30–90 min) after treatment, as described under experimental protocols. Using measures of Pp, Pa, and Pv, we determined not only total vascular resistance (RR) but also arterial (RA) and venous (RV) vascular resistance (41, 43).

Drugs

EETs were obtained from Biomol. MAfF and PPOH were obtained from Cayman Chemical. KCl, CaCl2, and NaHPO4 were obtained from Fisher. All other reagents were purchased from Sigma Chemical. TG was dissolved in DMSO, aliquoted, and stored protected from light at −20°C. Mepacrine and Gd3+ were dissolved in H2O and stored at room temperature. DCU was dissolved in H2O and stored protected from light at 4°C. 17-ODYA and ibuprofen were dissolved in 90% ethanol and stored at room temperature. Drugs were added as a bolus to the venous reservoir; concentrations noted are final concentrations in the perfusate. Bolus volumes for EETs (or EET vehicle) were 0.91 ml. For all other drugs, the bolus volume was <100 μl.

Statistics

Data are presented as means ± SE. Statistical comparisons between groups were done using one-way or two-way analysis of variance (ANOVA) with repeated measures followed by Tukey’s or Bonferroni’s post hoc t-test, respectively, as appropriate. P values < 0.05 were considered statistically significant.

RESULTS

The stability of the isolated rat lung in our hands was confirmed by time control experiments. Using a perfusate flow of 21.8 ± 0.7 ml/min (~0.06 ml/g body wt) and Pp, set to 4.0 cmH2O, baseline Pa and Pv averaged 21.3 ± 0.4 and 13.3 ± 0.3 cmH2O, respectively, in this group, and Rb averaged 0.79 ± 0.03 cmH2O·ml−1·min−1. None of these hemodynamic parameters was altered by 3-h perfusion, even at this flow rate. The baseline Kec was not different from the final Kec measured after 3 h of constant flow perfusion (0.0071 ± 0.0010 vs. 0.0081 ± 0.0010 ml/min−1·cmH2O−1·g dry weight−1, respectively). In the remaining protocols, lungs were perfused at 14–16 ml/min with either albumin-normal [Ca2+], albumin-dextran, or albumin-low [Ca2+] solution. When these groups overall were compared, the albumin-dextran perfusate was found to result in significant pulmonary hypertension, as evidenced by...
increases in $R_t$ (1.44 ± 0.05 vs. 1.17 ± 0.03 cmH$_2$O·ml$^{-1}$·min$^{-1}$, $P < 0.05$), $P_a$ (27.2 ± 0.5 vs. 19.8 ± 0.3 cmH$_2$O, $P < 0.05$), and $P_c$ (18.0 ± 0.3 vs. 12.7 ± 0.2 cmH$_2$O, $P < 0.05$) compared with lungs perfused with albumin-normal [Ca$^{2+}$]. In contrast, the low [Ca$^{2+}$] buffer decreased $R_t$ (0.78 ± 0.02 vs. 1.17 ± 0.03 cmH$_2$O·ml$^{-1}$·min$^{-1}$, $P < 0.05$), $P_a$ (15.2 ± 0.2 vs. 19.8 ± 0.3 cmH$_2$O, $P < 0.05$), and $P_c$ (9.0 ± 0.1 vs. 12.7 ± 0.2 cmH$_2$O, $P < 0.05$), compared with that in lungs perfused with physiological [Ca$^{2+}$]. Nonetheless, as shown in Table 1, there were no differences in the baseline $K_{f,c}$ in the lungs perfused with albumin-dextran or albumin-low [Ca$^{2+}$] compared with those perfused with albumin-normal [Ca$^{2+}$].

**Effect of EETs on EC Permeability and Hemodynamics**

To address whether EETs affect $K_{f,c}$ in the isolated rat lung, we tested each of the four regioisomers. EETs had a heterogeneous effect on EC permeability. The results are shown in Fig. 1, where the paired permeability responses measured at baseline and after treatment with each of the four EET regioisomers are compared. There was a significant increase in EC permeability with 5,6-EET (2.6-fold) and 14,15-EET (4.2-fold) when the final $K_{f,c}$ was compared relative to baseline. These responses were significantly different than those resulting from 8,9-EET, 11,12-EET, or vehicle control, based on two-way ANOVA with repeated measures. Although there were no differences in baseline hemodynamics between groups, treatment of the lungs with 11,12- and 14,15-EET resulted in a mild increase in $R_t$. 11,12-EET increased $R_t$ from 1.51 ± 0.14 to 1.66 ± 0.15 cmH$_2$O·ml$^{-1}$·min$^{-1}$ (baseline vs. final, $P < 0.05$), whereas 14,15-EET increased $R_t$ from 1.51 ± 0.14 to 1.66 ± 0.15 cmH$_2$O·ml$^{-1}$·min$^{-1}$ (baseline vs. final, $P < 0.05$). We did not observe this response in any other group (two-way ANOVA with repeated measures). Furthermore, the increase in $R_t$ (baseline vs. final) primarily resulted from increased resistance in the arterial pulmonary vascular compartment for 11,12-EET ($R_a$ increased from 0.65 ± 0.13 to 0.81 ± 0.21 cmH$_2$O·ml$^{-1}$·min$^{-1}$), whereas the venous compartment was primarily targeted by 14,15-EET ($R_c$ increased from 0.87 ± 0.12 to 0.99 ± 0.14 cmH$_2$O·ml$^{-1}$·min$^{-1}$); $P < 0.05$ as compared by paired $t$-test.

**Table 1. Baseline measurements in isolated rat lung**

<table>
<thead>
<tr>
<th>Perfusate</th>
<th>4% Albumin Normal [Ca$^{2+}$]</th>
<th>1% Albumin</th>
<th>3% Dextran</th>
<th>4% Albumin Low [Ca$^{2+}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>$n$</td>
<td>73</td>
<td>24</td>
<td>10</td>
<td>24</td>
</tr>
<tr>
<td>Q ($\mu$l/min)</td>
<td>15.3 ± 0.6</td>
<td>16.2 ± 0.5</td>
<td>14.5 ± 0.2</td>
<td>15.2 ± 0.2</td>
</tr>
<tr>
<td>$P_a$ (cmH$_2$O)</td>
<td>19.8 ± 0.3</td>
<td>27.2 ± 0.5</td>
<td>15.2 ± 0.2</td>
<td>9.0 ± 0.1</td>
</tr>
<tr>
<td>$P_c$ (cmH$_2$O)</td>
<td>4.1 ± 0.1</td>
<td>4.2 ± 0.1</td>
<td>4.1 ± 0.0</td>
<td>9.0 ± 0.1</td>
</tr>
<tr>
<td>$R_t$ (cmH$_2$O)</td>
<td>1.17 ± 0.03</td>
<td>1.44 ± 0.05</td>
<td>0.78 ± 0.02</td>
<td>0.008 ± 0.0008</td>
</tr>
<tr>
<td>$K_{f,c}$ (mM)</td>
<td>0.0082 ± 0.0004</td>
<td>0.0088 ± 0.0005</td>
<td>0.0090 ± 0.0008</td>
<td></td>
</tr>
</tbody>
</table>

Data are means ± SE. $n$, Number of isolated lungs; Q, perfusate flow (ml/min); $P_a$, pulmonary artery pressure (cmH$_2$O); $P_c$, pulmonary capillary pressure (cmH$_2$O); $P_r$, pulmonary venous pressure (cmH$_2$O); $R_t$, total pulmonary vascular resistance (cmH$_2$O·ml$^{-1}$·min$^{-1}$); $R_{f,c}$, capillary filtration coefficient (ml·min$^{-1}$·cmH$_2$O·g dry wt$^{-1}$); $\ast P < 0.05$ vs. that in 4% albumin-normal [Ca$^{2+}$] perfused lungs.

**Role of Extracellular Ca$^{2+}$ in the Permeability Response to EETs**

To determine whether the observed EET-induced increase in EC permeability was due to Ca$^{2+}$ entry, we evaluated the permeability response to 14,15-EET in the absence and presence of the nonselective cation channel blocker Gd$_3$+, and compared it with the TG response. There was no difference in the baseline $K_{f,c}$ in either group compared with lungs perfused with albumin alone (see Table 1). However, the response to 14,15-EET in the absence of Gd$_3$+ was significantly less ($P < 0.05$) in the albumin-dextran perfused lungs (2.5-fold increase in $K_{f,c}$) compared with the response in lungs perfused with albumin alone (4.2-fold, $P < 0.05$), whereas the response to TG was unchanged. The permeability response to TG was significantly attenuated in the presence of 30 nM Gd$_3$+, as shown in Fig. 2 ($P < 0.05$, two-way ANOVA), confirming that Ca$^{2+}$ entry is required for the EC injury induced by TG. In contrast, the tendency for the response to 14,15-EET to be reduced by Gd$_3$+ was not statistically significant ($P = 0.068$), suggesting that parallel mechanisms independent of Ca$^{2+}$ may be involved. To more clearly elucidate a definitive role for Ca$^{2+}$ in the EET-induced lung injury, we utilized a perfusate with minimal [Ca$^{2+}$] (4). A dose-response curve was first generated to determine the lower limit for perfusate [Ca$^{2+}$], which allowed stability of the lung for 2 h, as measured by $K_{f,c}$. These data show that $K_{f,c}$ remained stable with a perfusate [Ca$^{2+}$] as low as 0.02 mM (0.0142 ± 0.0010 vs. 0.0194 ± 0.0008 ml·min$^{-1}$·cmH$_2$O·g dry wt$^{-1}$, baseline vs. final respectively, $n = 3$). Although baseline $K_{f,c}$ remained normal at 0.01 mM [Ca$^{2+}$], final $K_{f,c}$ was elevated (data not shown). Thus we chose to set the low [Ca$^{2+}$] at 0.02 mM for further study. The results (Fig. 3) show that in lungs treated with 14,15-EET, $K_{f,c}$ tended to increase despite the low [Ca$^{2+}$] ($P = 0.09$). Nonetheless, a significant increase in $K_{f,c}$ was elicited upon Ca$^{2+}$ add-back ($P < 0.05$ compared with baseline, as analyzed by one-way ANOVA). In contrast, TG-induced lung injury was prevented in the low [Ca$^{2+}$] buffer, whereas addi-
also may contribute. The role of endogenous EETs in regulation of lung EC permeability is unclear. P-450 epoxygenases are known to be expressed in the lung. Specifically in rat lung, expression of epoxygenases from the CYP2C, 2B, 2J, and 1A families has been demonstrated (32). The EET dose chosen for this study (3 \mu M) is similar to the circulating concentration found in perfused human lung ex vivo, after challenge with Ca\textsuperscript{2+} ionophore (20). Although 5,6- and 14,15-EET clearly increase permeability, our results suggest that these P-450 metabolites are not involved in the link between store depletion and Ca\textsuperscript{2+} entry in rat lung EC.

One question that may arise when considering the variable response to the EET regioisomers is whether the lung preparation per se is stable. We found that rat lungs could be perfused at a higher flow (0.04–0.06 ml-min\textsuperscript{-1}g body wt\textsuperscript{-1}) compared with that typically used (0.03 ml-min\textsuperscript{-1}g body wt\textsuperscript{-1}) (4, 19), while still retaining an isogravimetric state at baseline. As a result, \(P_A\) and \(P_F\) were higher than those described at lower flow but were similar to those reported in vivo (34). In the time control experiments, we demonstrated that even a flow rate of 0.06 ml-min\textsuperscript{-1}g body wt\textsuperscript{-1} did not compromise the lung, as evidenced by maintenance of a constant \(K_{fc}\) during the 3-h experiment. A benefit of higher flow is better recruitment of exchange area in the lung (35, 42).

Given the stability of the preparation, we believe that the differing responses to the EET regioisomers was not due to deterioration in the lung preparation.

Our results showed heterogeneity in the effect of EET regioisomers on pulmonary EC permeability. Regiospecificity with respect to other actions of EETs is well recognized. EETs may relax or contract airway or vascular smooth muscle, depending on the tissue, isomer, and concentration. EETs have been found to exert vasoactive effects on the pulmonary vasculature in vitro and in vivo studies. In isolated canine

\section*{Involvement of EETs in Capacitative Ca\textsuperscript{2+} Entry}

To evaluate the potential involvement of the P-450-mediated AA metabolism in the TG-induced increase in EC permeability, we used inhibitors to target several steps in the AA cascade. As shown in Fig. 4, TG increased EC permeability 3.7-fold \((P < 0.05)\), a response that was not blocked by pretreatment of the lung with the PLA\textsubscript{2} inhibitors mepacrine or MAFP. In contrast to our observation in the canine lung, inhibition of P-450 activity with either 17-ODYA or PPOH did not alter the TG-induced permeability response in the rat lung. Similarly, inhibition of EET metabolism with ibuprofen and DCU had no effect on the TG-induced increase in EC permeability. A further analysis showed no significant difference between groups, except for the mepacrine pretreatment, which enhanced the response to TG \((P < 0.05)\), two-way ANOVA with repeated measures). Collectively, these results provide support for the notion that P-450 epoxygenase metabolites are not involved in the link between TG-induced store depletion and increased EC permeability in the rat lung. None of these inhibitors had any effect on \(K_{fc}\) in the absence of TG (data not shown).

\section*{DISCUSSION}

This study provides the first evidence that EETs regulate EC permeability in the rat lung in a regiospecific manner. Though the mechanism by which EETs increase permeability appears to involve Ca\textsuperscript{2+} entry, a parallel Ca\textsuperscript{2+}-independent pathway...
lung, 5,6-EET induced vasodilation that was localized to the arterial compartment (36). In vitro studies have shown contraction of pulmonary artery rings induced by all of the four EET regioisomers in rabbits (51) and rats (49). More recently, 5,6-EET was found to produce dilation of extralobar pulmonary artery rings, whereas vasoconstriction was observed in intrapulmonary arteries in isolated rabbit lung (37). Although not the primary focus of our study, our results indicate that there was a mild increase in the total resistance of pulmonary vessels following treatment with 11,12- and 14,15-EET, but not the other two regioisomers. Although these results support the notion that specific EETs are vasoactive, there are clearly regioisomer- and species-dependent differences in the magnitude, targeted vascular compartment, and directionality of that vasoactive effect. One concern that is often raised is whether increases in vascular resistance affect the measure of EC permeability. Previous studies have shown that K_{ec} is unresponsive to even fourfold increases in R_{t} induced by vasoconstrictors (29). Furthermore, an impact of vasoconstriction can be ruled out in our studies since 11,12- and 14,15-EET both increased R_{t}, yet only 14,15-EET altered EC permeability.

EETs have been shown to promote Ca^{2+} entry in a number of cell types, including systemic EC (13, 16, 29). The mechanism by which these P-450-derived lipids promote Ca^{2+} entry may be multifactorial and tissue dependent. We have preliminary evidence supporting the notion that 14,15-EET promotes Ca^{2+} entry in rat lung microvascular EC (11). Although there is ample evidence that EETs promote Ca^{2+} entry in systemic EC, there have been no other reports in which their effect on Ca^{2+} entry in pulmonary EC has been studied. Because EETs were demonstrated in the present study to increase pulmonary EC permeability and since Ca^{2+} entry in EC often results in increased EC permeability (4, 9, 17, 26), it was reasonable to postulate that EET-induced Ca^{2+} entry explained the resultant increase in K_{ec} observed in this study. We used two different approaches to address this hypothesis: the lanthanide mineral Gd^{3+}, used as a nonspecific cation channel blocker, and a low [Ca^{2+}]_i perfusate, used to directly test the requirement for Ca^{2+} entry. Gd^{3+} blocks store depletion-induced Ca^{2+} entry via transient receptor potential channels, which have been characterized as putative store-operated channels (2, 14, 25). Gd^{3+} is difficult to use in vivo since it binds avidly to albumin (3), which may limit its efficacy with respect to blockade of Ca^{2+} channels. By using a combination of 1% albumin with 3% dextran, we could minimize binding of Gd^{3+} to albumin yet maintain adequate colloid in the perfusate and normal baseline permeability (30). The observation that 14,15-EET was less effective in lungs perfused with the albumin-dextran mixture was surprising, particularly since the response to TG was unchanged. This could potentially be due to increased EET metabolism, based on the decreased albumin binding capacity in this perfusate. To identify the optimal Gd^{3+} concentration, we completed a preliminary dose-response relationship, using the degree of inhibition of the TG-induced increase in K_{ec} with Gd^{3+} pretreatment as our endpoint. The TG-induced permeability response was inhibited by 60% with 27 μM Gd^{3+}, a response that was maintained up to 50 μM Gd^{3+} (data not shown). We were not able to test higher doses due to limitations in Gd^{3+} solubility. On the basis of these results, we chose 30 μM Gd^{3+} for the subsequent studies. Pretreatment of the rat lung with 30 μM Gd^{3+} did not prevent 14,15-EET-induced increase in K_{ec} but blocked TG-induced lung injury. As we
could not achieve 100% inhibition of the permeability response induced by 14,15-EET with 30 μM Gd3+. We cannot conclude from this experiment that the permeability response to the eicosanoid in rat lung is due to Ca2+ entry. We sought a more definitive answer by using a low-Ca2+ buffer. Chetham et al. (4) previously reported that a low-Ca2+ buffer prevented TG-induced EC injury in rat lung. In our hands, a dose-response curve revealed that 20 μM was the lowest [Ca2+]i that allowed stability of the lung, as evidenced by stable Kf.e during a 2-h period. As expected, the permeability response to TG was blocked in the presence of a low-Ca2+ buffer. Importantly, we showed that readdition of Ca2+ to the perfusate to achieve physiological levels (2.2 mM) resulted in TG-induced lung injury in the same lungs. Similarly to TG, 14,15-EET increased EC permeability following readdition of Ca2+, supporting the notion that EETs promote Ca2+ entry in rat lung EC and that this contributes to the EET-induced increase in permeability. However, there was a tendency for Kf.e to increase following EET administration, even in the low [Ca2+]i perfusate, a response not significantly different from that seen after Ca2+ add-back. These data suggest that 14,15-EET also impacts a Ca2+-independent pathway involved in the regulation of EC permeability.

The observation that the EET-induced increase in EC permeability is dependent on both Ca2+ entry and other Ca2+-independent mechanisms does not allow us to confirm the molecular target of these eicosanoids. For example, EETs could act via direct receptor-mediated activation of Ca2+-channels or activate a signaling cascade upstream of the Ca2+ entry pathway. EETs have been shown to evoke hyperpolarization in EC via activation of Ca2+-dependent potassium channels, an effect that would enhance the electrochemical gradient for Ca2+ entry (16). Although we have shown that potassium channel activation did not play a role in the canine lung (18), it remains possible that this is a critical step in rat lung EC. Potential targets that might plausibly be involved in the Ca2+-independent regulation of EC permeability by EETs include tyrosine kinases and the extracellular regulated protein kinases (ERK). Several reports provide evidence that EETs activate tyrosine kinases and thus downstream kinases such as MAPK and ERK in pig aortic and coronary artery EC (8, 15, 27). These observations are relevant, since ERK activation has been linked to Ca2+-dependent and -independent increases in pulmonary EC permeability (10, 45). Identification of the specific pathways involved in mediating EETs’ effect on EC permeability will require additional experiments beyond the scope of the present study.

Finally, EETs have been postulated to act as soluble factors involved in capacitative Ca2+ entry (1, 13). In bovine coronary artery and aortic EC, P-450 inhibitors blocked TG-induced Ca2+ entry, and 5,6-EET mimicked the effect of TG on Ca2+ entry (12, 13). In canine lung, P-450 inhibitors prevented acute lung injury following challenge with TG and limited edema formation following treatment with etcholvynol, suggesting that under some conditions, endogenous EET synthesis does participate in regulation of lung EC permeability (18, 38). On the basis of these studies and our current observation that the effect of 14,15-EET on EC permeability was in part dependent on Ca2+ entry, the hypothesis that EETs are soluble factors involved in capacitative Ca2+ entry in the rat lung was rational. However, these P-450 metabolites do not appear to mediate changes in EC permeability subsequent to store depletion in rat lung. Neither the nonspecific PLA2 inhibitor mepacrine or the more selective inhibitor MAFP was able to attenuate the TG-induced increase in EC permeability, suggesting that AA availability was not a factor in this process. Similarly, if metabolism of AA via P-450 eicosanoygenases was involved in regulating Ca2+ entry after TG, then 17-ODYA and PPOH should have blocked the TG-induced permeability response (24, 32). Again, we found no blunting of the TG-induced permeability response in lungs pretreated with these inhibitors. Thus in contrast to our previous observation in canine lung (18), there appears to be no role for P-450 eicosanoygenases in the response to TG in the rat lung. Although mepracrine enhanced the TG-induced permeability response, we believe that this is likely attributed to the relative nonspecificity of this inhibitor (6). Confirmation of our conclusion that EETs do not mediate the permeability response to TG was obtained by subsequent experiments that showed that inhibition of EET metabolism, achieved by concomitant treatment with the cyclooxygenase inhibitor ibuprofen and the soluble epoxide hydrolase inhibitor DCU, did not enhance the TG-induced increase in Kf.e. Thus despite the fact that 5,6- and 14,15-EET increase EC permeability, and 14,15-EET does so in a partially Ca2+-entry-dependent fashion in the rat lung, the mechanism linking store depletion and capacitative Ca2+ entry in rat pulmonary EC does not involve EETs.

In summary, we have demonstrated that EETs increase EC permeability in rat lung in a regiospecific and Ca2+-entry-dependent manner, although a Ca2+-independent pathway appears to be activated in parallel. On the basis of the observation that the response to TG does not involve EETs, we speculate that the increase in permeability evoked by EETs is independent of store depletion. Further work will be needed to establish a clear view of how these eicosanoygenase metabolites participate in regulation of endothelial barrier function. Specifically, the signaling mechanism(s) underlying the Ca2+-independent permeability response and the molecular identity of the Ca2+ entry modulated by EETs remain to be elucidated.

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