Transient Receptor Potential Vanilloid 4–Mediated Disruption of the Alveolar Septal Barrier

A Novel Mechanism of Acute Lung Injury

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Abstract—Disruption of the alveolar septal barrier leads to acute lung injury, patchy alveolar flooding, and hypoxemia. Although calcium entry into endothelial cells is critical for loss of barrier integrity, the cation channels involved in this process have not been identified. We hypothesized that activation of the vanilloid transient receptor potential channel TRPV4 disrupts the alveolar septal barrier. Expression of TRPV4 was confirmed via immunohistochemistry in the alveolar septal wall in human, rat, and mouse lung. In isolated rat lung, the TRPV4 activators 4α-phorbol-12,13-didecanoate and 5,6- or 14,15-epoxyeicosatrienoic acid, as well as thapsigargin, a known activator of calcium entry via store-operated channels, all increased lung endothelial permeability as assessed by measurement of the filtration coefficient, in a dose- and calcium-entry-dependent manner. The TRPV antagonist ruthenium red blocked the permeability response to the TRPV4 agonists, but not to thapsigargin. Light and electron microscopy of rat and mouse lung revealed that TRPV4 agonists preferentially produced blebs or breaks in the endothelial and epithelial layers of the alveolar septal wall, whereas thapsigargin disrupted interendothelial junctions in extralveolar vessels. The permeability response to 4α-phorbol-12,13-didecanoate was absent in TRPV4−/− mice, whereas the response to thapsigargin remained unchanged. Collectively, these findings implicate TRPV4 in disruption of the alveolar septal barrier and suggest its participation in the pathogenesis of acute lung injury. (Circ Res. 2006;99:988-995.)

Key Words: permeability ■ TRP channels ■ TRPV4 ■ acute lung injury

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cute lung injury and its more severe form the adult respiratory distress syndrome are characterized by disruption of the alveolar septal barrier, leading to patchy alveolar flooding and hypoxemia. Effective clinical treatments are limited and mortality remains high. Such endothelial barrier disruption is often dependent on entry of Ca2+ from the extracellular space. For example, in the intact rat lung, thapsigargin-induced store depletion and Ca2+ entry via store-operated channels increase endothelial permeability. Store depletion targets extralveolar vessel endothelium, although the septal microvasculature appears to be spared. Although endothelial cells derived from extralveolar pulmonary arteries and septal microvessels are phenotypically distinct and both could potentially be targeted in acute lung injury, disruption of the septal barrier is more likely to promote alveolar flooding and impair gas exchange than disruption in extralveolar vessels. Importantly, Ca2+ entry pathways involved in regulation of barrier integrity in the alveolar septal compartment have not been elucidated.

The consensus of data suggests that TRPC1 and TRPC4, members of the canonical subfamily of transient receptor potential (TRP) channels, comprise subunits of store-operated Ca2+ channels in lung endothelium. In the isolated rat lung, the permeability response to thrombin-induced store depletion was attenuated in TRPC4−/− mice. Furthermore, loss of the permeability response to thapsigargin-induced store depletion in rat lung after heart failure was associated with downregulation of TRPC1 and TRPC4, channels which are normally expressed in extralveolar endothelium. Retention of the permeability response to 14,15-epoxyeicosatrienoic acid (14,15-EET), a P450 epoxygenase-derived arachidonic acid metabolite, in this heart failure model was intriguing because the permeability responses to both thapsigargin-induced store depletion and to 14,15-EET are dependent on Ca2+ entry. This striking observation drives home the point that EETs must target Ca2+ entry pathways in lung endothelium distinct from store-operated channels.

We propose that Ca2+ entry via TRPV4, a member of the vanilloid subfamily of TRP channels, contributes critically to regulation of endothelial permeability and barrier integrity in the lung. This hypothesis is based on several observations: 5,6-EET and 8,9-EET have been linked to activation of TRPV4 and subsequent Ca2+ entry in aortic
endothelial cells, 25, 26 5, 6-EET and 14, 15-EET increase endothelial permeability in rat and canine lung, 6, 27 and the permeability response to 14, 15-EET, at least, is dependent on entry of extracellular Ca 2+ . In the current study, we tested the hypothesis that Ca 2+ entry via TRPV4 regulates lung endothelial permeability and barrier integrity. Furthermore, we tested whether Ca 2+ entry via TRPV4 and that occurring through store-operated channels evoke barrier disruption in spatially distinct compartments of the pulmonary vasculature.

Materials and Methods

Animals

Protocols were approved by our Institutional Animal Care and Use Committee, conforming to the NIH Guide for the Care and Use of Laboratory Animals. Adult male CD rats (n = 139, Charles River) or 8- to 10-week-old TRPV4 wild-type mice (TRPV4+/+) and null littermates (TRPV4−/−) of either sex (n = 39) were anesthetized with sodium pentobarbital (50 mg/kg body weight IP). Lungs were isolated for ex vivo perfusion as previously described. 6

TRPV4 Expression

Human lung resection specimens (n = 3), obtained under a protocol approved by the Institutional Review Board, were fixed by immersion in 10% formalin or 100% ethanol. Rat and mouse lungs (n = 2 to 3 in each group) were perfusion fixed with 4% paraformaldehyde. Sections (5 μm) were processed for immunohistochemistry using a rabbit anti-TRPV4 polyclonal antibody (Alomone), stained with diaminobenzidine and counterstained with hematoxylin. Western blots were prepared from lysates (40 μg of total protein) of rat pulmonary artery and microvascular endothelium and TRPV4 detected using enhanced chemiluminescence. Total RNA from mouse lung was reverse transcribed, then PCR performed using primers designed to amplify the pore-loop region of TRPV4 (see the online data supplement, available at http://circres.ahajournals.org).

Microscopic Assessment of Acute Lung Injury

Light microscopy and transmission electron microscopy (EM) were used to evaluate structural changes in glutaraldehyde-fixed lung, 19 after treatment with vehicle (910 μL of ethanol), 4αPDD (3 or 10 μmol/L in rat or mouse lung, respectively), 14, 15-EET (3 μmol/L, rat lung only), or thapsigargin (150 μmol/L) for 60 minutes (n = 3 per group). Using 1-μm thick sections, extraalveolar vessel cuffed and alveolar flooding were evaluated. Cuff frequency and the cuff volume (Vc) fraction of total wall volume (Vw/Vc) was determined, the latter using a point-counting strategy. 28 Point-counting was used to determine alveolar fluid volume (Vaf) fraction in the alveolar space (Vaf/Va). Thin sections (80 nm) from the same blocks were examined via transmission EM. Junctional discontinuities (gaps), blebs, or breaks in septal capillaries were enumerated. Means for cuff frequency, volume fractions and blebs or breaks per capillary were determined separately for each lung, then overall descriptive statistics derived for each group.

Isolated Lung and Assessment of Endothelial Permeability

Both rat and mouse lungs were perfused at constant flow with buffer (in mmol/L: 116.0 NaCl, 5.2 KCl, 0.9 MgSO4, 1.0 NaHPO4, 2.2 NaHCO3, 8.3 d-glucose) containing 4% BSA and either physiological (2.2 mmol/L) or low (0.02 mmol/L) CaCl2 at pH 7.4 (38°C). Hemodynamics and the filtration coefficient (Kf) were measured as previously described, 6, 19, 30, 31 using zone 3 conditions. Kf was calculated as the rate of weight gain obtained from 15 to 15 minutes after a 7 to 10 cmH2O increase in pulmonary venous pressure, normalized per g lung dry weight. Kf, the product of specific endothelial permeability and surface area for exchange, is a sensitive measure of lung endothelial permeability when surface area is fully recruited. 32

Protocols

In rat lungs perfused with physiological [Ca 2+ ], Kf and hemodynamics were measured at baseline and 60 minutes after treatment with the TRPV4 agonist 4α-phorbol-12, 13-didecanoate (4αPDD) (1 to 10 μmol/L, n = 3 per dose) or the TRPV1 agonist 4α-phorbol-12, 13-didecanoate-20 homovanillate (4αPDDHV) (10 μmol/L, n = 4). Using 0.02 mmol/L [Ca 2+ ] perfusate, Kf and hemodynamics were measured at baseline and 45 minutes after treatment (n = 5 per group) with 4αPDD (3 μmol/L), 5, 6-EET and 14, 15-EET (3 μmol/L, Biomed, or thapsigargin (150 μmol/L), with or without the TRPV antagonist ruthenium red (1 μmol/L). Subsequently, CaCl2 was added to achieve physiological [Ca 2+ ] (Ca 2+ add-back), and measurements repeated 15 minutes later. Lungs isolated from TRPV4+/+ mice or wild-type littermates (n = 4 to 5 per group) were perfused with buffer containing 4% albumin and physiological [Ca 2+ ]. Kf and hemodynamics were measured baseline and 60 minutes after treatment with vehicle (50 μL DMSO), 4αPDD (10 μmol/L), or thapsigargin (150 μmol/L). Vehicle controls in rat lungs (n = 5 each) included ethanol (910 μL or 2%) or DMSO (50 μL or 0.1%); DMSO was also evaluated in lungs from wild-type mice (n = 4). All drugs were added to the perfusate; final circulating concentrations are noted.

Statistical Analysis

Quantitative data are presented as mean±SEM. Group means were compared using a paired t test (2 tailed) or ANOVA, as appropriate; the Newman–Keuls multiple comparison test was used to identify specific differences. Probability values of <0.05 were considered statistically significant.

Results

Baseline Parameters in Isolated Rat and Mouse Lung

Total pulmonary vascular resistance, the distribution of vascular resistance and baseline Kf were similar in rat and mouse lungs (Table 1) and were not impacted by the choice of perfusate [Ca 2+ ]. There were no differences with respect to baseline measurements between TRPV4+/+ and TRPV4−/− mice.

Expression of TRPV4 in Lung:

Functional Consequences

TRPV4 was expressed in the septal compartment of lungs from humans, rats and mice (Figure 1A through 1C, respectively), as well as in bronchiolar epithelium (not shown). TRPV4 was also expressed in smooth muscle in human and rat extraalveolar vessels. Although TRPV4 expression in cultured rat pulmonary artery endothelium was similar to that in microvascular endothelium (Figure 1D), TRPV4 was not consistently expressed in extraalveolar vessel endothelium in intact lung (Figure 1A through 1C). In rat lung, 1, 5, and 10 μmol/L 4αPDD increased Kf by 1.7-, 4.2-, and 5.6-fold, respectively (Figure 1E), supporting the notion that TRPV4 may play a role in regulating endothelial permeability. In contrast, the TRPV1 agonist 4αPDDHV had no impact on endothelial permeability despite use of a >EC 100 dose. 33 To determine whether activation of TRPV4 increased permeability in a Ca 2+ entry-dependent fashion, Kf was reevaluated using the low Ca 2+/Ca 2+ add-back strategy. The Ca 2+ concentration chosen for the low Ca 2+ segment of these experiments was based on the lowest concentration that allowed stable Kf for at least 1 hour (see the online data supplement). Ca 2+ add-back provides a normal inward Ca 2+ gradient, and if Ca 2+...
permeant channels have been activated by the treatment, Ca2+ entry results and endothelial permeability increases. The ≈3-fold increase in Kf induced by 4aPDD was clearly dependent on Ca2+ entry (Figure 2A) and was blocked by ruthenium red (Figure 2B), which potently blocks TRPV4 by binding to an extracellular domain on the channel.24,33 We confirmed that the permeability response to 14,15-EET and thapsigargin in rat lung is dose dependent (see the online data supplement) and Ca2+ entry dependent (Figure 2A) and documented a similar pattern for 5,6-EET (online data supplement and Figure 2A). At 0.02 mmol/L [Ca2+], both 5,6-EET and 14,15-EET evoked a small increase in permeability, although Ca2+ add-back was required for the development of the normal permeability response to the EETs.6 In the absence of Ca2+ add-back, the increase in Kf induced by 14,15-EET in 0.02 mmol/L [Ca2+] (0.010±0.003 to 0.024±0.003 mL/min per cm H2O per gram dry weight, P=0.0007, n=5) was transient and Kf returned to baseline within 15 minutes (P=0.007). Ruthenium red (Figure 2B) blocked the Ca2+ entry-dependent component of the permeability response to 5,6- and 14,15-EET but had no impact on the Ca2+ entry-dependent permeability response to thapsigargin. In the absence of treatment, 0.02 mmol/L [Ca2+] had no impact on Kf with or without Ca2+ add-back (see the online data supplement).  

### TABLE 1. Baseline Hemodynamics and Permeability in Isolated Rat and Mouse Lung

<table>
<thead>
<tr>
<th></th>
<th>Rat Lung</th>
<th>Mouse Lung</th>
</tr>
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<tbody>
<tr>
<td>Perfusate [Ca2+] mmol/L</td>
<td>2.2</td>
<td>2.2</td>
</tr>
<tr>
<td>N</td>
<td>51</td>
<td>76</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>344±6</td>
<td>377±5</td>
</tr>
<tr>
<td>Q, mL/min/g body weight</td>
<td>0.041±0.001</td>
<td>0.037±0.001</td>
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<tr>
<td>Q, L/min/100 g PLW</td>
<td>1.05±0.03</td>
<td>0.93±0.02</td>
</tr>
<tr>
<td>Pa, cm H2O</td>
<td>17.0±0.1</td>
<td>14.8±0.2</td>
</tr>
<tr>
<td>Pc, cm H2O</td>
<td>10.2±0.1</td>
<td>9.0±0.1</td>
</tr>
<tr>
<td>Pv (cm H2O)</td>
<td>4.0±0.0</td>
<td>4.0±0.0</td>
</tr>
<tr>
<td>Ra, cm H2O/L/min/100 g PLW</td>
<td>6.8±0.2</td>
<td>6.4±0.2</td>
</tr>
<tr>
<td>Rv, cm H2O/L/min/100 g PLW</td>
<td>6.1±0.2</td>
<td>5.5±0.2</td>
</tr>
<tr>
<td>Rt, cm H2O/L/min/100 g PLW</td>
<td>12.9±0.4</td>
<td>11.9±0.3</td>
</tr>
<tr>
<td>Kf, mL/min/cm H2O/g dry weight</td>
<td>0.0102±0.0003</td>
<td>0.0106±0.0004</td>
</tr>
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</table>

Q indicates perfusate flow; PLW, predicted lung wet weight55,56; Pa, pulmonary artery pressure; Pc, pulmonary capillary pressure; Pv, pulmonary venous pressure; Ra, arterial vascular resistance; Rv, venous vascular resistance; Rt, total vascular resistance.

Figure 1. Activation of TRPV4 expressed in lung increases endothelial permeability. In human (A), rat (B), and mouse (C) lung, TRPV4 was expressed in the alveolar septal compartment (left panels) and bronchial epithelium (not shown). TRPV4 expression in vascular smooth muscle in extraalveolar vessels (right panels) was observed in human and rat lung, whereas little was seen in mouse lung. Western blotting (D) showed similar TRPV4 expression in rat microvascular (MV) and pulmonary artery (PA) endothelium (TRPV4/β-actin band density was 1.1 in both groups). However, TRPV4 was not consistently expressed in extraalveolar vessel endothelium in intact lung (A through C). The TRPV4 agonist 4aPDD (E) increased the filtration coefficient Kf in isolated rat lung (P=0.021) in a dose-dependent fashion (P=0.002); *P<0.05 vs 1 or 5 μmol/L. The TRPV1 agonist 4aPDDHV had no effect (P=0.201, paired t test).
Activated potassium channels (BKCa), providing an increased driving gradient for Ca\(^{2+}\) entry.34,35 However, blockade of BKCa channels did not alter the EET-induced permeability response (see the online data supplement).

**Compartmentalization of Barrier Disruption**

Results of the morphometric analysis from light microscopy (Figure 3) and transmission EM (Figure 4) are shown in Table 2. 4aPDD and thapsigargin resulted in frequent extraalveolar blebs and/or breaks, whereas thapsigargin-induced store depletion targeted extraalveolar vessels. The TRPV4 agonists typically caused endothelial blebs and/or breaks, whereas thapsigargin induced formation of gaps at endothelial cell junctions. These data suggest that distinct endothelial compartments, and possibly distinct subcellular compartments of lung endothelial cells, must be targeted subsequent to Ca\(^{2+}\) entry via these TRP channels. The observation that the Ca\(^{2+}\)-entry-dependent component of the permeability response to 5,6- and 14,15-EET, as well as the TRPV4 agonist 4aPDD, was ablated by pretreatment of rat lung with ruthenium red suggests an important role for TRPV4 in regulation of lung endothelial permeability. This notion is corroborated by the loss of the permeability response to the TRPV4 agonist 4aPDD in lungs from thapsigargin). Furthermore, the TRPV4 agonists disrupted type I alveolar epithelial cells, manifested as separation of the epithelium from the basal lamina. 4aPDD increased V\(_{\text{a}}\)/V\(_{\text{va}}\) compared with control and thapsigargin, in both rat and mouse lung (P=0.003 and 0.011, respectively). Whereas 14,15-EET caused significant septal injury, the increase in V\(_{\text{a}}\)/V\(_{\text{va}}\) was more variable, and as a result the 4-group ANOVA was not significant. In contrast, thapsigargin primarily targeted interendothelial junctional complexes in extraalveolar vessels, leading to formation of gaps; the septal capillary network was spared, and V\(_{\text{a}}\)/V\(_{\text{va}}\) did not increase. Together, these data support the notion that activation of TRPV4 (via 4aPDD or EETs) and activation of store-operated channels (via thapsigargin) promote barrier disruption in discrete vascular compartments of the lung even though these agents cause similar increases in total lung K\(_{\text{r}}\) in a Ca\(^{2+}\)-entry-dependent fashion.

The 4aPDD and EET-mediated permeability responses were inhibited with ruthenium red, a pleiotropic antagonist that blocks TRPV4. Although activation of TRPV1 had no impact on endothelial permeability in rat lung, it was possible that ruthenium red blocked other TRPV isoforms, contributing to its impact on the permeability response. Thus, we used mice genetically engineered for a targeted deletion of exon 12 in the trpv4 gene,28 encoding the transmembrane domains 5 and 6, which includes the predicted pore loop region of the channel (TRPV4\(^{-/-}\)). Genotyping (see the online data supplement) and PCR (Figure 5A) were used to document expression of TRPV4 in wild-type mice. Whereas 3 \(\mu\)mol/L 4aPDD had no impact on permeability in TRPV4\(^{-/-}\) mice, 10 \(\mu\)mol/L 4aPDD increased K\(_{\text{r}}\) 2.8-fold (Figure 5B); this dose did not alter K\(_{\text{r}}\) in lungs from TRPV4\(^{-/-}\) littermates. Thapsigargin increased K\(_{\text{r}}\) by 3-fold in both TRPV4\(^{-/-}\) and TRPV4\(^{+/-}\) mice. These results confirm that activation of TRPV4 increases endothelial permeability in the mouse lung.

**Discussion**

This study provides the first evidence of a functional role for TRPV4 in lung endothelium, implicating TRPV4 in Ca\(^{2+}\)-entry-dependent regulation of endothelial permeability and barrier integrity in the alveolar septal network. Despite similar impact on K\(_{\text{r}}\), activation of TRPV4 and store-operated channels led to injury in distinct vascular compartments. Activation of TRPV4 by 4aPDD and 14,15-EET preferentially targeted the alveolar septal microvessels, whereas thapsigargin-induced store depletion targeted extraalveolar vessels. The TRPV4 agonists typically caused endothelial blebs and/or breaks, whereas thapsigargin induced formation of gaps at endothelial cell junctions. These data suggest that distinct endothelial compartments, and possibly distinct subcellular compartments of lung endothelial cells, must be targeted subsequent to Ca\(^{2+}\) entry via these TRP channels. The observation that the Ca\(^{2+}\)-entry-dependent component of the permeability response to 5,6- and 14,15-EET, as well as the TRPV4 agonist 4aPDD, was ablated by pretreatment of rat lung with ruthenium red suggests an important role for TRPV4 in regulation of lung endothelial permeability. This notion is corroborated by the loss of the permeability response to the TRPV4 agonist 4aPDD in lungs from thapsigargin. Furthermore, the TRPV4 agonists disrupted type I alveolar epithelial cells, manifested as separation of the epithelium from the basal lamina. 4aPDD increased V\(_{\text{a}}\)/V\(_{\text{va}}\) compared with control and thapsigargin, in both rat and mouse lung (P=0.003 and 0.011, respectively). Whereas 14,15-EET caused significant septal injury, the increase in V\(_{\text{a}}\)/V\(_{\text{va}}\) was more variable, and as a result the 4-group ANOVA was not significant. In contrast, thapsigargin primarily targeted interendothelial junctional complexes in extraalveolar vessels, leading to formation of gaps; the septal capillary network was spared, and V\(_{\text{a}}\)/V\(_{\text{va}}\) did not increase. Together, these data support the notion that activation of TRPV4 (via 4aPDD or EETs) and activation of store-operated channels (via thapsigargin) promote barrier disruption in discrete vascular compartments of the lung even though these agents cause similar increases in total lung K\(_{\text{r}}\) in a Ca\(^{2+}\)-entry-dependent fashion.

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TRPV4−/− mice. In light of the critical vulnerability of the alveolar septal barrier in acute lung injury,2,3 our findings in the rodent models of lung injury and the finding of TRPV4 expression in human alveolar septum lead us to hypothesize that TRPV4 is likely to play a role in the development of acute lung injury and the acute respiratory distress syndrome in humans.

TRP Channels and Permeability

Ca2+ entry into endothelial cells occurs via activation of selective Ca2+ channels or nonselective cation channels, such as those encoded by the large superfamily of TRP channel proteins. Much of the focus in lung endothelium has been on the TRPC subfamily. Endothelial cells express mRNA and protein for TRP channels, including TRPC1, TRPC3, TRPC4, TRPC6, and TRPC7.14,36,37 In rat lung, TRPC1, TRPC3, TRPC4, and TRPC6/7 protein is expressed in endothelium of extraalveolar vessels. Although mRNA for several members of the TRPV subfamily, including TRPV4, are expressed in human pulmonary artery endothelium,36 only a subset of TRP proteins may actually play a physiological role in regulation of lung endothelial permeability. Two studies have addressed a role for TRPC channels in regulation of permeability in the intact lung. Tiruppathi et al reported a partial loss of the permeability response to thrombin in TRPC4−/− mice,18 and we recently found that in an aortocaval fistula model of heart failure, endothelial TRPC1 and TRPC4 expression in extraalveolar vessels is downregulated and the
permeability response to thapsigargin is lost.19 Although Ca\(^{2+}\) entry can occur via receptor-operated TRP channels (TRPC3, TRPC6, and TRPC7),38 and these channels are indeed expressed in rat lung endothelium, activation of these channels with a diacylglycerol analog had no impact on endothelial permeability in rat lung.19 A role in regulation of lung endothelial permeability has not been explored for TRP proteins outside the canonical TRP family. Aside from thrombin and thapsigargin, we do not know whether the Ca\(^{2+}\) entry-dependent increases in endothelial permeability are attributable to gating of TRP channels, nor which TRP channels are involved. Our previous work evaluating the impact of EETs, P450 epoxygenase derivatives of arachidonic acid, on lung endothelial permeability in normal rats and rats with chronic heart failure6,19 supports the notion of heterogeneity in Ca\(^{2+}\)-dependent regulation of endothelial permeability in lung. P450 epoxygenases metabolize arachidonic acid to form four regioisomers: 5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET.39 EETs are released from human lung after inflammatory challenge,40 suggesting their potential involvement in acute lung injury. In canine lung, blockade of EET synthesis attenuates the pulmonary edema and hypoxemia resulting from ethchlorvynol.41 Furthermore, exogenous 5,6-EET and 14,15-EET increased endothelial permeability in canine and rat lung,6,27 indicating that Ca\(^{2+}\) entry-dependent manner. The notion that EETs must target Ca\(^{2+}\) permeant channels distinct from TRPC1/TRPC4 is based on the following evidence: (1) the permeability response to both EETs and thapsigargin in rat lungs requires Ca\(^{2+}\) entry; (2) the response to 14,15-EET was retained after experimentally induced heart failure, although that to store depletion was lost; and (3) TRPC1 and TRPC4 expression were downregulated in extraalveolar vessels from rats with heart failure,6,19 supporting the notion of heterogeneous Ca\(^{2+}\) entry-dependent regulation of endothelial permeability.

### Table 2. Extraalveolar Cuffs and Alveolar Septal Barrier Disruption

<table>
<thead>
<tr>
<th></th>
<th>Extraalveolar Vessels (n)</th>
<th>Percentage of Extraalveolar Vessels With Capsules</th>
<th>Cuff Volume Fraction (Vc/Vw)</th>
<th>Septal Capillaries (n)</th>
<th>Blebs, Breaks (n Per Capillary)</th>
<th>Alveolar Fluid Volume Fraction (Vaf/Vas)</th>
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<tbody>
<tr>
<td>Control</td>
<td>231</td>
<td>6.0±3.6</td>
<td>0.158±0.054</td>
<td>367</td>
<td>0.15±0.05</td>
<td>0.008±0.008</td>
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<tr>
<td>4αPDD</td>
<td>129</td>
<td>29.0±2.6†</td>
<td>0.320±0.118</td>
<td>223</td>
<td>1.34±0.25†</td>
<td>0.055±0.007</td>
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<tr>
<td>14,15-EET</td>
<td>219</td>
<td>8.3±5.2</td>
<td>0.237±0.081</td>
<td>470</td>
<td>1.29±0.36†</td>
<td>0.172±0.157</td>
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<tr>
<td>Thapsigargin</td>
<td>190</td>
<td>36.2±7.0†</td>
<td>0.458±0.089</td>
<td>397</td>
<td>0.33±0.08</td>
<td>0.003±0.003</td>
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<td>Isolated mouse lung (TRPV4(^{+/−}))</td>
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<tr>
<td>Control</td>
<td>434</td>
<td>13.8±0.2†</td>
<td>0.157±0.049</td>
<td>528</td>
<td>0.16±0.03</td>
<td>0.012±0.003</td>
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<tr>
<td>4αPDD</td>
<td>454</td>
<td>17.1±4.9†</td>
<td>0.254±0.068</td>
<td>408</td>
<td>1.49±0.42†</td>
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<td>Thapsigargin</td>
<td>348</td>
<td>21.4±7.3†</td>
<td>0.202±0.025</td>
<td>403</td>
<td>0.26±0.15†</td>
<td>0.015±0.009</td>
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</table>

Data are mean±SEM (3 lungs per group). Injury in the extraalveolar compartment was not quantified, because at this magnification the entire vessel perimeter is not visualized. *P<0.05 vs control, †P<0.05 vs 14,15-EET, ‡P<0.05 vs thapsigargin.

**Figure 5.** Activation of TRPV4 increases endothelial permeability in mouse lung. A, Using primers designed to amplify across the pore-loop region of TRPV4 mRNA, the predicted 612-bp product was observed in wild-type and heterozygote mice but not in null animals. B, In isolated mouse lung, we showed that the TRPV4 agonist 4αPDD (10 μmol/L) increased endothelial permeability (K\(_t\)) in only TRPV4\(^{+/−}\) mice (P=0.036, paired t test). In contrast, activation of store-operated channels via thapsigargin (150 nmol/L) increased permeability in both wild-type and null mice (P=0.032 and 0.008, respectively, paired t test). These results confirm the role of TRPV4 in mediating acute lung injury. *P<0.05 vs baseline (paired t test).

**Functional Role for TRPV4 Channels in the Alveolar Septal Network**

TRPV4, a channel originally described as activated by hypotonicity,20,23,42 appears to participate in detection of changes in extracellular fluid osmolality.28 Although TRPV channels are appreciated for their role in sensory transduction,43–46 nonsensory functions are now recognized.21,47–50 Work from Nilius and colleagues55,26,51 suggests that TRPV4 likely plays a significant role in endothelial Ca\(^{2+}\) signaling. Both 4αPDD and 5,6-EET activate TRPV4 heterologously expressed in HEK-293 cells as well as endogenous TRPV4 in aortic endothelial cells, promoting Ca\(^{2+}\) entry which was inhibited by ruthenium red.26 Furthermore, EET-induced Ca\(^{2+}\) entry in aortic endothelial cells was diminished in endothelium derived from TRPV4\(^{−/−}\) mice.25 Collectively,
these data suggested that TRPV4 could play a role in Ca\(^{2+}\) entry-dependent regulation of endothelial permeability. Our results provide a functional role for TRPV4 expressed in the lung alveolar septal network. The TRPV4 agonist 4aPDD increased endothelial permeability in rat lung in a Ca\(^{2+}\) entry-dependent fashion. This response, and the Ca\(^{2+}\) entry-dependent permeability response to 5,6- and 14,15-EET, could be blocked with ruthenium red. Furthermore, the permeability response to 4aPDD observed in TRPV4\(^{-/-}\) mice was lacking in TRPV4\(^{+/+}\) littermates.

Transmission EM documented that both 4aPDD and 14,15-EET caused disruption of the alveolar epithelium, in addition to an impact on the septal endothelial barrier, which very likely contributed to the increase in K\(_e\) and alveolar flooding induced by these TRPV4 agonists. TRPV4 has previously been demonstrated to be expressed in respiratory epithelial cells,\(^52^-^54\) although its functional role in bronchiolar or alveolar epithelium in the intact lung has not been clarified. Our results suggest that an exploration of the signaling cascade linking Ca\(^{2+}\) to detachment of those cells from the basement membrane would be informative.

In summary, this work provides critical evidence of a functional role for TRPV4 in regulation of barrier integrity in the lung alveolar septal network. Although Ca\(^{2+}\) entry via store-operated TRP channels causes gap formation in extraalveolar vessels, the functional consequences of barrier disruption in this compartment are likely distinct from those resulting from TRPV4-dependent barrier disruption in the septal compartment. Activation of store-operated channels in extraalveolar endothelium leads to fluid accumulation in perivascular cuffs, although this likely has little impact on alveolar gas exchange. In contrast, disruption of the vulnerable alveolar septal barrier, such as that resulting from Ca\(^{2+}\) influx via TRPV4, leads to alveolar flooding and thus would be predicted to impair gas exchange. Indeed this is a hallmark of acute lung injury. The implication of this work for translational biomedical research is that TRPV4 is likely a novel molecular target for therapeutic intervention in acute lung injury.

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Disclosures

None.

References


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MATERIALS AND METHODS

Animals.
Genomic DNA was isolated from mouse tails harvested at weaning and used as a template for PCR-based genotyping. Forward and reverse primers were CATGAAATCTGACCTCTTGTCCCC and TTGTGTACTGTCTGCACACCAGGC, respectively. PCR products were resolved on a 0.8% agarose gel. A 2.1 kB band was observed in wild type TRPV4+/+ mice and a 1.1 kB band in TRPV4−/− mice; both bands were observed in heterozygotes. Results were confirmed by absence of an exon 12 amplicon, while the exon 15 amplicon was present.

TRPV4 Expression.
Human lung resection specimens (n=3), obtained under a protocol approved by the Institutional Review Board, were fixed by immersion in 10% formalin or 100% ethanol. Rat and mouse lungs (n=2-3 in each group) were perfusion-fixed with 4% paraformaldehyde. Sections (5 μm) were incubated overnight at 4 °C with a goat anti-TRPV4 polyclonal antibody (1:250, Alomone) and 1 h at room temperature with a horseradish peroxidase-conjugated secondary antibody (1:100, Santa Cruz Biotechnology). Sections were stained with diaminobenzidine and counterstained with hematoxylin. Western blots were prepared from lysates (40 μg total protein) of rat pulmonary artery and microvascular endothelium. TRPV4 was detected (1:200 primary antibody) using enhanced chemiluminescence. Blots were probed for β-actin (1:5000 primary antibody) as a loading control. Total RNA from mouse lung was reverse transcribed, then PCR performed (40 cycles) using forward (TCACGAAGAAATGCCCTGGAGTGA) and reverse (ACTGCAACTTCCAGATGTGCTTGC) primers designed to amplify nucleotides 1667-2278 of mouse TRPV4 (AF263522), coding for the pore-loop region, resulting in a 612 bp product in wild type or heterozygous mice. PCR products were resolved on a 2% agarose gel.

Protocols.
The optimal $[\text{Ca}^{2+}]$ for the low $\text{Ca}^{2+}/\text{Ca}^{2+}$ add-back strategy in isolated rat lungs was assessed by determining the lowest $[\text{Ca}^{2+}]$ which allowed a normal, stable $K_f$ for at least 1 hr. Separate groups of lungs ($n=2-3$ per group), were perfused upon isolation with buffer/albumin in which $[\text{Ca}^{2+}]$ was set at physiologic concentration (2.2 mmol/L) or one of several concentrations ranging to as low as 0.01 mmol/L. Paired measurements of $K_f$ were made at baseline and after 60 min perfusion. In separate experiments ($n=2-5$ per group), the stability of isolated rat lungs in the low $\text{Ca}^{2+}/\text{Ca}^{2+}$ add-back paradigm, in the absence of other treatment, was assessed. $K_f$ was measured at baseline and after 30 or 60 min perfusion with a low $\text{Ca}^{2+}$ (0.02 mmol/L) perfusate, with or without $\text{Ca}^{2+}$ add-back to 2.2 mmol/L.

To determine whether EET-induced activation of large conductance $\text{Ca}^{2+}$-activated potassium channels (BK$_{\text{Ca}}$) modulated the permeability response to EETs, we focused on 14,15-EET. The effect of 14,15-EET (3 µmol/L) on $K_f$ in rat lung was tested in the presence of the selective BK$_{\text{Ca}}$ inhibitors charybdotoxin (100 nmol/L) and apamin (300 nmol/L)₂, using the low $\text{Ca}^{2+}/\text{Ca}^{2+}$ add back protocol. $K_f$ was measured at baseline and then either vehicle (55 µL DMSO, $n=5$) or the combination of the two inhibitors ($n=5$) were added and allowed to circulate for 15 min before addition of 14,15-EET (3 µmol/L). A second $K_f$ was measured 30 min later, then again 15 min after $\text{Ca}^{2+}$ add-back.

RESULTS

Evaluation of the dose-dependent impact of lowering perfusate $[\text{Ca}^{2+}]$ in isolated rat lungs (Online Figure 2, top panel) showed that endothelial permeability remained stable down to 0.02 mmol/L $\text{Ca}^{2+}$. Although baseline $K_f$ was normal in 0.01 mmol/L $\text{Ca}^{2+}$, permeability tended to increase after 1 hr of perfusion. Thus, 0.02 mmol/L was chosen as the “low $\text{Ca}^{2+}$” concentration. Next, we tested the integrity of the endothelial barrier in isolated rat lung using the low $\text{Ca}^{2+}/\text{Ca}^{2+}$ add back protocol, without treatment (lower panel). Perfusion of the lung at 0.02 mmol/L $[\text{Ca}^{2+}]$ did not have a significant impact on
Kᵣ, nor did Ca²⁺ add-back to reestablish a physiological [Ca²⁺] have any effect in lungs perfused with this low [Ca²⁺].

We evaluated the permeability responses in rat lung to 5,6-EET, 14,15-EET (Online Figure 3) and thapsigargin (Online Figure 4). Both the EETs and thapsigargin increased Kᵣ in a dose-dependent fashion. Based on this information, we chose to use 3 μmol/L EETs and 150 nmol/L thapsigargin in subsequent studies. Higher doses were accompanied by significant pressor responses. Although EETs have been shown to activate BK⁶⁷ channels, thus providing an increased driving gradient for Ca²⁺ entry ³, ⁴, we found that blockade of BK⁶⁷ channels did not alter the permeability response to 14,15-EET (Online Figure 5).

REFERENCES

Online Figure Legends.

Online Figure 1. PCR-based genotyping in mice. A 2.1 kB band was observed in wild type TRPV4+/+ mice and a 1.1 kB band in TRPV4-/- mice; both bands were observed in heterozygotes.

Online Figure 2. Impact of low Ca^{2+} perfusate in isolated rat lung. Lowering perfusate [Ca^{2+}] had no significant effect on the filtration coefficient (Kf) down to a concentration of 0.02 mmol/L (top panel). In lungs perfused at 0.02 mmol/L [Ca^{2+}], Ca^{2+} add-back to 2.2 mmol/L had effect on Kf (bottom panel). The tendency for Kf to increase in 0.01 mmol/L [Ca^{2+}] was reversed with Ca^{2+} add-back.

Online Figure 3. Dose-dependent effect of 5,6- and 14,15-EET on endothelial permeability in isolated rat lung. In separate groups of lungs, the filtration coefficient (Kf) was measured at baseline and 60 min after treatment with 0.5-5 \( \mu \text{mol/L} \) 5,6- or 14,15-EET (n=2-3 per dose). Both EET regioisomers caused a dose-dependent increase in endothelial permeability. Higher doses were not evaluated due to vehicle-induced pressor responses. *p<0.05 vs. baseline.

Online Figure 4. Dose-dependent effect of thapsigargin on endothelial permeability in rat lung. The filtration coefficient (Kf) was measured at baseline and 60 min after treatment with thapsigargin, 50-300 nM thapsigargin (n=3 per dose). At 150 and 300 nmol/L, thapsigargin significantly increased endothelial permeability, though at 300 nmol/L the permeability response was accompanied by a significant pressure response. * p<0.05 vs. baseline.

Online Figure 5. BK_{Ca} channels do not modulate 14,15-EET-induced increases in endothelial permeability in rat lung. Pretreatment of the isolated rat lung with the selective BK_{Ca} inhibitors
charybdotoxin (CTX, 100 nM) and apamin (300 nM) had no significant impact on the permeability response to 14,15-EET (3 μmol/L) in rat lung. *p<0.05 vs. baseline; "p<0.05 vs agonist at low Ca²⁺.
Online Figure 1.

Online Supplement

TRPV4-mediated alveolar septal barrier disruption

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Online Figure 2.
Online Figure 3.
Online Figure 4.

**Graph Description:**

The graph illustrates the effect of Thapsigargin at different concentrations on a physiological parameter, likely related to alveolar septal barrier disruption. The parameter is measured in units of Kf (mL/min/cm H2O/g dry wt).

**Y-Axis:**
- The y-axis represents Kf in units of mL/min/cm H2O/g dry wt, ranging from 0.00 to 0.24.

**X-Axis:**
- The x-axis represents Thapsigargin concentrations in nM, ranging from 50 to 300 nM.

**Data Points:**
- For each concentration level, there are two bars indicating baseline and post-treatment conditions.
- Concentrations 150 and 300 nM show a significant increase in Kf values, marked with an asterisk (*).

**Legend:**
- Baseline (open bars)
- Post Treatment (filled bars)
Online Figure 5.