High Vascular Pressure–Induced Lung Injury Requires P450 Epoxygenase–Dependent Activation of TRPV4

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High vascular pressure targets the lung septal network, causing acute lung injury. While calcium entry in septal endothelium has been implicated, the channel involved is not known. This study tested the hypothesis that the vanilloid transient receptor potential channel, TRPV4, is a critical participant in the permeability response to high vascular pressure. Isolated lungs from TRPV4^{+/+} or TRPV4^{-/-} mice were studied at baseline or during high pressure challenge. Permeability was assessed via the filtration coefficient. Endothelial calcium transients were assessed using epifluorescence microscopy of the lung subpleural network. Light microscopy and point counting were used to determine the alveolar fluid volume fraction, a measure of alveolar flooding. Baseline permeability, calcium intensity, and alveolar flooding were no different in TRPV4^{+/+} versus TRPV4^{-/-} lungs. In TRPV4^{+/+} lungs, the high pressure–induced permeability response was significantly attenuated by low calcium perfusate, the TRPV antagonist ruthenium red, the phospholipase A₂ inhibitor methyl arachidonyl fluorophosphate, or the P450 epoxygenase inhibitor propargyloxyphenyl hexanoic acid. Similarly, the high pressure–induced calcium transient in TRPV4^{+/+} lungs was attenuated with ruthenium red or the epoxygenase inhibitor. High vascular pressure increased the alveolar fluid volume fraction compared with control. In lungs from TRPV4^{-/-} mice, permeability, calcium intensity, and alveolar fluid volume fraction were not increased. These data support a role for P450-derived epoxyeicosatrienoic acid–dependent regulation of calcium entry via TRPV4 in the permeability response to high vascular pressure.

Keywords: epoxyeicosatrienoic acid; capillary permeability; respiratory distress syndrome; TRPV cation channels

In lung, high vascular pressure (HiPv) exceeding a threshold of 30 to 50 cm H₂O increases endothelial permeability (1–4). While mechanical stress failure of the alveolar septal barrier can occur at higher pressures, leading to overt alveolar flooding (5), the molecular mechanisms underlying the early HiPv-induced increase in endothelial permeability are not well understood. Kuebler and colleagues (6) have reported that moderate HiPv promotes Ca²⁺ entry into lung endothelium, and acute lung injury is often dependent upon such Ca²⁺ transients (7–10). Although HiPv-induced lung injury could plausibly be dependent upon Ca²⁺ entry, this has not been experimentally confirmed nor has a candidate channel been identified.

The Ca²⁺ permeable channel TRPV4, a member of the vanilloid subfamily of transient receptor potential (TRP) chan-

CLINICAL RELEVANCE

This work supports a critical role for TRPV4 in high vascular pressure–induced lung injury and alveolar flooding. These findings are relevant to clinical acute lung injury, which is well recognized to be localized to the alveolar septal compartment.

nels, is expressed in the alveolar septal compartment (8). The notion that TRPV4 might subserve HiPv-induced Ca²⁺ entry in lung endothelium is based on the observation that the channel can be activated by mechanical stress, such as hypotonic cell swelling or shear stress (11–13). *In vitro* studies have shown that activation of TRPV4 with mechanical stress requires hydrolysis of membrane phospholipids via phospholipase A₂ (PLA₂) and subsequent arachidonic acid metabolism by cytochrome P450 epoxygenases to form epoxyeicosatrienoic acids or EETs (12, 14, 15). TRPV4 heterologously expressed in HEK-293 cells or the endogenous TRPV4 in aortic endothelium can be activated by EETs, promoting Ca²⁺ entry (16). Further, we have shown that EETs or direct activation of TRPV4 with 4 α -phorbol didecanoate (4 α PDD) lead to Ca²⁺ entry-dependent acute lung injury, disruption of the lung septal barrier, and alveolar flooding (8).

Based on these observations, this study was devised to test the hypothesis that HiPv increases lung endothelial permeability by promoting Ca²⁺ entry via TRPV4 and that this mechanically induced injury process required EET synthesis. To test this hypothesis, lung endothelial permeability, Ca²⁺ transients in subpleural septal endothelium, and alveolar flooding were assessed in wild-type mice and TRPV4^{-/-} littermates.

MATERIALS AND METHODS

Animals

Male or female wild-type (TRPV4^{+/+}) and null (TRPV4^{-/-}) littermate mice were used at 8 to 10 weeks of age (17). Animal protocols were approved by the Institutional Animal Care and Use Committee of the University of South Alabama, adhering to the NIH guidelines for the care and use of laboratory animals.

Isolated Lung Preparation

Lungs isolated from anesthetized mice were cannulated, perfused, and ventilated as described (8). Perfusates included 4% bovine serum albumin (BSA) or 1% BSA/3% dextran in Earle's buffer (2.2 mM Ca²⁺), or 4% BSA in a low Ca²⁺ buffer (0.02 mM Ca²⁺). Lungs were flushed with sufficient perfusate at isolation to ensure that the nominal Ca²⁺ concentrations were reached, that is, that mixing with residual blood was not an issue. We have previously shown that the low Ca²⁺ buffer is completely effective in preventing a permeability response to direct activation of store-operated TRP channels or TRPV4 (8). The filtration coefficient (K_f) was measured as an index of pulmonary endothelial permeability (3, 8), in each of several experimental groups:

1. Pressure threshold for the permeability to HiPv. Since TRPV4 is activated at temperatures exceeding 27°C (18), we rationalized

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that if TRPV4 was involved in the permeability response to HiPv, then the threshold for HiPv-induced injury should be temperature dependent. Thus, we compared the pressure threshold for a HiPv-induced permeability increase at perfusate temperatures of 26 ($n = 7$) and 33 ($n = 9$) °C. In each lung, paired measurements of K_f were made at baseline and during 15 minutes of challenge with Pv ranging between 25 and 37 cm H₂O. Subsequent experiments used a HiPv of 30 cm H₂O and perfusate temperatures of 33°C.

- 2. Requirement for Ca²⁺ entry via TRPV4. In wild-type mice, the permeability response to HiPv was evaluated using a low Ca²⁺/Ca²⁺ addback protocol (7, 8) to identify whether the permeability increase was dependent upon extracellular Ca²⁺ ($n = 5$) and using ruthenium red to block TRPV channels ($n = 4$). HiPv challenge was also evaluated in lungs from TRPV4^{-/-} mice ($n = 7$).
- 3. Requirement for P450-mediated arachidonic acid derivatives in the permeability response to HiPv. In lungs from TRPV4^{+/+} mice, the permeability response to HiPv was evaluated after inhibition of phospholipase (PLA₂) with methyl arachidonyl fluorophosphonate (MAFP, 2.5 μM, $n = 4$) or P450 epoxigenases with propargyloxyphenyl hexanoic acid (PPOH, 50 μM, $n = 6$).

HiPv-Induced Ca²⁺ Response in Subpleural Microvessels

Endothelium in isolated lungs was loaded with the Ca²⁺-sensitive fluorescent indicator Fluo 4-AM (3 μM), then the vasculature flushed with fresh buffer. After transfer to the microscope stage, perfusion at constant flow (1.5 ml/min, at 33°C) was continued. During excitation of the lung surface (490 ± 5 nm excitation, 535 ± 13 nm emission), images were acquired from the same area every minute for 5 minutes at baseline (Pv of 5 cm H₂O) and at HiPv (30 cm H₂O), and analyzed using MetaMorph software (Molecular Devices, Sunnyvale, CA). Five groups were evaluated: TRPV4^{+/+} controls ($n = 6$), TRPV4^{+/+} treated with ruthenium red (3 μM, $n = 3$) or PPOH (50 μM, $n = 3$), TRPV4^{-/-} controls ($n = 3$), and TRPV4^{-/-} ($n = 3$) treated with gadolinium chloride (GdCl₃, 30 μM).

Morphometric Analysis of Alveolar Flooding by Light Microscopy

After baseline measurements, wild-type and TRPV4^{-/-} lungs were challenged with a Pv of 15 or 30 cm H₂O for 15 minutes ($n = 2-4$ per group) before fixation by vascular perfusion, as described (8). Thick sections (1 μm) from post-fixed and embedded lung blocks were stained with toluidine blue and examined by light microscopy. Alveolar flooding was assessed using a point counting method (8), and alveolar fluid volume reported as a fraction of total alveolar space (V_{af}/V_{as}).

Statistics

Data are presented as mean ± SE. Statistical comparisons between groups were performed using ANOVA with repeated measures and Tukey's *post hoc t* test. *P* values < 0.05 were considered statistically significant.

Additional details regarding materials and methods are available in the online supplement.

RESULTS

Pulmonary hemodynamics, including total pulmonary vascular resistance, were similar in lungs from TRPV4^{+/+} and TRPV4^{-/-} mice (Table 1). Further, these measures were not altered by the choice of perfusate [Ca²⁺]. There were no differences with respect to baseline K_f between TRPV4^{+/+} or TRPV4^{-/-} mice. Lowering perfusate temperature to 26°C affected neither the baseline endothelial permeability nor hemodynamics.

We first needed to document the pressure threshold for the HiPv-induced increase in endothelial permeability in mouse lung. Results are shown in Figure 1. At a perfusate temperature of 26°C, K_f remained near baseline until lungs were challenged with Pv exceeding 30 cm H₂O. Above this threshold, K_f increased in a pressure-dependent fashion. Although there were

TABLE 1. BASELINE MEASUREMENTS IN ISOLATED MOUSE LUNGS PERFUSED WITH 4% ALBUMIN

n	Perfusate		
	Normal [Ca ²⁺]		Low [Ca ²⁺]
	TRPV4 ^{+/+} 23	TRPV4 ^{-/-} 10	TRPV4 ^{+/+} 5
BW, g	23 ± 0.5	23.5 ± 0.8	23.7 ± 1.2
Q, ml/min	1.2 ± 0	1.2 ± 0	1.2 ± 0
Pa, cm H ₂ O	11.7 ± 0.3	10.8 ± 0.4	11.3 ± 0.2
Pc, cm H ₂ O	10.2 ± 0.2	9.5 ± 0.3	10.5 ± 0.3
Pv, cm H ₂ O	6.5 ± 0.1	6.4 ± 0.1	6.9 ± 0.2
Rt, cm H ₂ O/ml/min	4.0 ± 0.3	3.7 ± 0.4	3.5 ± 0.2
K _f , ml/min/cm H ₂ O/g dry wt	0.0102 ± 0.0005	0.0099 ± 0.0005	0.0118 ± 0.0003

Definition of abbreviations: BW, body weight; K_f, filtration coefficient; Pa, pulmonary artery pressure; Pc, pulmonary capillary pressure; Pv, pulmonary venous pressure; Q, perfusate flow; Rt, total pulmonary vascular resistance.

Data are means ± SE. Normal [Ca²⁺], 2.2mM Ca²⁺; low [Ca²⁺], 0.02 mM Ca²⁺.

no significant differences in baseline K_f as a function of perfusate temperature, increasing the temperature to 33°C resulted in a left-shift in the pressure-response curve. Since at 33°C, Pv ranging from 28 to 34 cm H₂O reliably increased K_f , we used Pv in this range as the test HiPv and a perfusate temperature of 33°C for the remaining experiments.

Figure 2 summarizes the results for paired baseline and HiPv K_f measurements in wild-type lungs. K_f increased 5.3-fold with HiPv in this group ($P < 0.05$). To determine a role for extracellular Ca²⁺ in this response, we used a low Ca²⁺/Ca²⁺ add-back paradigm (7, 8). There was no difference in the baseline K_f when lungs were perfused with normal Ca²⁺ or low Ca²⁺ buffer. However, the HiPv-induced increase in K_f was prevented in low Ca²⁺ (0.02 mM) perfusate. The addition of Ca²⁺ to achieve a physiologic concentration of 2.2 mM was required to restore the HiPv-induced increase in K_f ($P < 0.01$ versus baseline and low Ca²⁺). In separate lungs from wild-type mice, blockade of TRPV channels with ruthenium red significantly attenuated the permeability response to HiPv ($P < 0.001$). Further, specific knockout of TRPV4 also blunted the permeability response to HiPv ($P < 0.001$). These results support the notion that Ca²⁺ entry via TRPV4 plays an important role in HiPv-induced increase in lung endothelial permeability.

Figure 1. Effect of temperature on the lung endothelial permeability response to high venous pressure (Pv). The filtration coefficient (K_f) was evaluated in isolated lungs from wild-type mice after a step change in Pv from approximately 5 to 15 cm H₂O. Pv was returned to baseline and lungs allowed to

recover for 30 minutes. K_f was measured again 15 minutes after Pv was increased to a pressure in the range from 25 to 37 cm H₂O. Permeability remained near baseline when lungs were challenged with Pv less than 30 cm H₂O, while above this break point, permeability increased in a pressure-dependent fashion. Increasing perfusate temperature to 33°C, that is, a temperature within the activation range for TRPV4, resulted in a left shift in this pressure-response curve. Subsequent experiments used Pvs ranging from 28 to 33 cm H₂O and perfusate temperatures of 33°C.

Figure 2. Effect of Ca^{2+} entry on the permeability response to high venous pressure (HiPv) in mouse lung. To document the requirement for Ca^{2+} entry in the permeability response to HiPv, paired measurements of the K_f were made in the isolated mouse lung at baseline (BL) and after treatment with Pv ranging from 28 to 33 cm H_2O (HiPv). The subset of responses to this HiPv challenge in wild-type controls (from Figure 1, 33°C) are repeated here for comparison (open bars). In separate lungs from wild-type mice, a low $\text{Ca}^{2+}/\text{Ca}^{2+}$ addback protocol was used to document that the permeability response to HiPv required Ca^{2+} entry (shaded bars). Blockade of TRPV channels with ruthenium red significantly attenuated the permeability response to HiPv (hatched bars). Similarly, specific knockout of TRPV4 (cross-hatched bars) also blunted the permeability response to HiPv. Results are shown as mean $\pm$ SE. * $P < 0.05$ versus BL; # $P < 0.05$ versus wild-type control group.

To evaluate the potential involvement of the P450-mediated arachidonic acid metabolism in the HiPv-induced permeability response, we used pharmacologic inhibitors to target several steps in this cascade. As shown in Figure 3, the HiPv-induced increase in K_f in wild-type lungs (repeated from Figure 2 for comparison) was significantly attenuated by pretreatment with PLA_2 inhibitor MAFP ($P < 0.001$) or the P450 epoxygenase inhibitor PPOH ($P < 0.001$). These results provide support for the notion that P450 epoxygenase metabolites are involved in the permeability response to HiPv.

To verify that HiPv produced significant Ca^{2+} entry in lung subpleural microvascular endothelium, we turned to fluorescence microscopy. A representative image of a subpleural microvascular network from a wild-type lung and an area chosen for assessment (boxed) are shown in Figure 4A. Serial images of an assessment area illustrating the impact of HiPv in lung from wild-type and knockout mice are shown in Figure 4B. The Ca^{2+} response to HiPv, as determined by Fluo-4 fluorescence intensity, was reproducible in wild-type lung (Figure 4C). As shown in Figure 4D, there was no significant difference in fluorescence intensity at baseline among the five experimental groups studied. HiPv elicited a Ca^{2+} response in the lungs from wild-type mouse, an effect which was significantly attenuated by pretreatment with the P450 epoxygenase inhibitor PPOH or the TRPV channel blocker ruthenium red. Similarly, the response was attenuated in $\text{TRPV4}^{-/-}$ mice. Though there was a tendency for Ca^{2+} intensity to increase in these latter groups, HiPv-induced recruitment of Ca^{2+} entry pathways other than TRPV4 was considered unlikely, since the response to HiPv remained the same in knockout lungs pretreated with the nonspecific Ca^{2+} entry blocker GdCl_3 .

These data support the notion that the HiPv-induced permeability response is related to TRPV4. However, an increase in K_f does not elucidate the compartment of the lung targeted by the injury (8). Light microscopy (Figure 5) showed that HiPv

Figure 3. Requirement for P450-mediated arachidonic acid derivatives in the permeability response to HiPv. The filtration coefficient (K_f) was measured at baseline and during challenge with HiPv in lungs from wild-type mice (WT) after pretreatment with methyl arachidonyl fluorophosphonate (MAFP, 2.5 μM) to block PLA_2 -mediated arachidonic acid release or with the P450 epoxygenase inhibitor propargyloxyphenol hexanoic acid (PPOH, 50 μM) to block epoxygenase-mediated arachidonic acid (EET) synthesis from arachidonic acid. In both cases, the permeability response to HiPv was significantly attenuated compared with that in controls (* $P < 0.05$). These data support the requirement for EETs as intermediates in the signaling cascade linking HiPv and increased lung endothelial permeability. Results are shown as mean $\pm$ SE. * $P < 0.05$ versus BL; # $P < 0.05$ versus wild-type control group.

caused alveolar flooding in lungs from wild-type mice, compared with that in wild-type or $\text{TRPV4}^{-/-}$ controls or in the $\text{TRPV4}^{-/-}$ HiPv group. To quantify the degree of alveolar edema in each of these groups, we determined the alveolar fluid volume fraction ($V_{\text{af}}/V_{\text{as}}$). In wild-type control lungs fixed after one 15-minute baseline K_f at low venous pressure (15 cm H_2O), $V_{\text{af}}/V_{\text{as}}$ was 0.01 ± 0.01 , no different than that in previous reports (8). When the $V_{\text{af}}/V_{\text{as}}$ was reassessed in a separate group of wild-type control lungs after both baseline and final K_f measures at low venous pressure, $V_{\text{af}}/V_{\text{as}}$ was not significantly increased (0.07 ± 0.02). HiPv (30 cm H_2O) did increase $V_{\text{af}}/V_{\text{as}}$ significantly to 0.73 ± 0.08 in wild-type lung. In contrast, in lungs from $\text{TRPV4}^{-/-}$ mice, $V_{\text{af}}/V_{\text{as}}$ after HiPv (0.17 ± 0.08) was no different compared with that in low pressure controls (0.14 ± 0.07). Neither result in $\text{TRPV4}^{-/-}$ mice was significantly different from that in wild-type controls. These data confirmed that HiPv leads to TRPV4-dependent alveolar flooding.

DISCUSSION

In the present study, we provide the first evidence implicating TRPV4 in HiPv-induced lung injury. HiPv increased K_f , with a threshold of approximately 30 cm H_2O . This response in lung from wild-type mice was dependent upon temperature, PLA_2 activation, and P450 epoxygenase-mediated arachidonic acid metabolism. Further, the HiPv-induced increase in K_f was attenuated in low extracellular Ca^{2+} and by pharmacologic blockade or knockout of the TRPV4 channel. Fluorescence and light microscopy directly confirmed the impact of HiPv on Ca^{2+} entry and on alveolar flooding, as well as the dependence of these responses to HiPv on TRPV4.

Much of the earlier investigation into the impact of HiPv in lung presumed that this mechanical stress led to structural alterations in the endothelial barrier. This was thought to result in a stretched pore phenomenon, whereby HiPv elicited leak of fluid and protein into the lung interstitium and/or alveolar space (1, 2, 19). HiPv does indeed increase endothelial permeability

Figure 4. HiPv-induced Ca^{2+} responses in subpleural microvessels. The mouse lung endothelium was loaded by perfusion with the Ca^{2+} -sensitive fluorophore Fluo-4 AM for 20 minutes, then images acquired at 1-minute intervals at baseline (Pv 5 cm H_2O) or during HiPv challenge (30 cm H_2O). Representative pseudo-color images show a subpleural lung field (A) and selected capillary segments in wild-type $\text{TRPV4}^{-/-}$ evaluated over 5-minute control and HiPv periods (B). The fluorescence intensity scale is shown. The impact of HiPv on Ca^{2+} fluorescence intensity was reproducible in wild-type lung (C). Summary data (mean $\pm$ SE) for average pixel density in three to five vascular segments per lung (collected using Metamorph software), relative to that at Minute 1 of the baseline period, are compared for all treatment groups in D. The Ca^{2+} response to HiPv in wild-type (WT) lung was significantly attenuated by pretreatment with the P450 epoxygenase inhibitor PPOH or the TRPV channel blocker ruthenium red. Similarly, the response was attenuated in $\text{TRPV4}^{-/-}$ mice (KO). Though there was a tendency for Ca^{2+} intensity to increase in these latter groups, HiPv-induced recruitment of Ca^{2+} entry pathways other than TRPV4 was considered unlikely, since the response to HiPv remained the same in KO lungs pretreated with the nonspecific Ca^{2+} entry blocker gadolinium chloride (GdCl_3). * $P < 0.05$ for Pv = 30 versus Pv = 5 cm H_2O ; # $P < 0.05$ for all treatment groups versus wild-type control at this time point.

when Pv exceeds approximately 30 to 50 cm H_2O in mammalian lung (2–4, 20–22). Using vascular corrosion casting with low viscosity prepolymerized methyl methacrylate, we have shown that the alveolar septal barrier is the site of leak in the pulmonary circulation when HiPv increases permeability (23). Higher pressures can evoke frank stress failure, with disruption of the endothelial and/or epithelial layers of the alveolar septal barrier. West and colleagues (5) have suggested that the stress failure threshold is directly related to the thickness of the septal barrier. A thicker septal barrier, such as that in horse, apparently confers some mechanical advantage and the alveolar septal wall is able to withstand higher pressures—that is, the threshold is increased (5, 24). The relation between barrier thickness and the threshold for the permeability response to HiPv mimics the relation for stress failure. Thresholds for HiPv-induced increase in K_f do indeed increase (28, 31, and 37 cm H_2O ; current study and Refs. 4 and 22) as harmonic mean thickness of the blood gas barrier increases in mouse, rat, and dog (0.44, 0.39, and 0.66 μm) (24, 25), when a similar approach to measurement of K_f and similar duration of HiPv challenge are used. However, this relationship does not necessarily rule out participation from HiPv-induced activation of signaling, which initiates barrier dysfunction.

Mechanical stress, such as that induced by increased shear forces at the endothelial luminal surface, is well known to promote Ca^{2+} entry into the endothelium (26). However, since the early literature provided clear evidence for mechanical disruption of the septal barrier with HiPv, the notion that mechanical stress in lung may act in part by initiating intracellular signaling pathways which impact barrier function has only recently been investigated. Parker and Ivey (22) found that isoproterenol could protect against HiPv-induced acute lung injury, suggesting active regulation of barrier integrity could offset stresses induced by mechanical perturbation. Subsequently, they reported that another type of mechanically induced lung injury, that secondary to over-ventilation, increased K_f in a Ca^{2+} entry-dependent fashion (10). More recently, confocal microscopy was used to clearly document that HiPv promotes Ca^{2+} entry into subpleural septal endothelium via a gadolinium-sensitive channel in rat lung (6). An increase in left atrial pressure from 5 to 20 cm H_2O rapidly increased endothelial cell Ca^{2+} in a reversible fashion. Our data showed that increasing Pv from 5 to 30 cm H_2O produced an immediate increase in endothelial Ca^{2+} fluorescence, which recovered after vascular pressure was returned to baseline (data not shown). This data suggests the possibility for Ca^{2+} entry as a contributor to

Figure 5. Role of TRPV4 in HiPv-induced alveolar flooding. Images (scale bar = 50 μ m) from wild-type (A and C) and TRPV4^{-/-} (B and D) lungs are shown, challenged with a control Pv of 15 cm H₂O (A and B) or HiPv (C and D). In wild-type lung, HiPv induced severe alveolar flooding, whereas HiPv had little impact in the TRPV4^{-/-} group. This was confirmed by measurement of the alveolar fluid volume fraction, V_{af}/V_{as} (see text for details).

signaling regulating endothelial barrier function. The attenuation of the HiPv permeability response in wild-type lungs when a low Ca²⁺ perfusate was used, along with the reappearance of the permeability response upon Ca²⁺ addback, supports this conclusion.

We considered a role for TRPV4 as the mechanosensitive cation channel activated by HiPv. This hypothesis developed from the observation that endothelial TRPV4 is activated by mechanical stresses, including hypotonic cell swelling and shear stress (11, 12, 18). Similarly, knockdown of TRPV4 expression in renal tubular epithelium with siRNA abolished hypotonicity-induced Ca²⁺ influx (13). We had previously reported that activation of TRPV4 led to Ca²⁺ entry-dependent injury and disruption of the alveolar septal barrier in both rat and mouse lung (8), the same compartment as that targeted by HiPv. The current study has provided clear-cut evidence that TRPV4 is a key element of signaling leading to HiPv-induced Ca²⁺ entry-dependent lung injury. Both the Ca²⁺ influx in subpleural endothelium and the permeability response in isolated lung observed in TRPV4^{+/+} lung challenged with HiPv were lacking after TRPV blockade or in TRPV4^{-/-} littermates. Since gadolinium did not cause further attenuation of the HiPv-induced Ca²⁺ response in TRPV4^{-/-} lung, we conclude that TRPV4 is the sole cation channel involved. Although TRPV4 was originally described as a channel activated by hypotonicity, it also appears

to participate in the Ca²⁺ entry-dependent regulation of lung endothelial permeability induced by the mechanical stress deriving from HiPv.

There are several potential mechanisms by which HiPv could regulate TRPV4, including direct mechanical gating or gating secondary to generation of some mechanically elicited second messenger. Force applied to the endothelium eliciting membrane stretch could directly activate the channel (13). The amino-terminal domain of TRPV4 includes three ankyrin repeats that appear to anchor the channel to the cytoskeleton (27). Deletion of these ankyrin repeats did abolish heat-induced activation of heterologously expressed TRPV4 (28). However, Liedtke and coworkers showed that this deletion only attenuated, but did not prevent, the activation by hypotonic stimuli (29). Their results suggested that direct perturbation of the membrane cytoskeleton was not absolutely essential for hypotonicity-induced gating of TRPV4. Alternatively, we considered whether HiPv activated TRPV4 indirectly, via a signaling pathway involving arachidonic acid metabolism. Arachidonic acid metabolites generated via P450 epoxygenases (such as 5,6-EET) activate TRPV4 in HEK-293 cells and in aortic endothelial cells, promoting Ca²⁺ entry (30). Both hypotonic cell swelling and shear stress increase cytosolic PLA₂ activity in endothelial cells and activate TRPV4, a process that requires EET synthesis (11, 12). Our previous work has shown that the increase in lung endo-

thelial permeability elicited by EETs requires extracellular Ca^{2+} and that TRPV4 mediates the EET-dependent permeability response (7, 8). In the current study we show that inhibition of PLA_2 or P450 epoxygenases limits the HiPv-induced Ca^{2+} transient in subpleural endothelium as well as the permeability response in isolated lung. Our results support the notion that HiPv activates TRPV4 by a signaling cascade that sequentially leads to activation of PLA_2 , PLA_2 -dependent release of arachidonic acid from membrane phospholipids, and cytochrome P450 epoxygenase-dependent synthesis of EETs from arachidonic acid, followed by activation of TRPV4.

Collectively, our data suggest that TRPV4 is the cation channel that mediates the HiPv-induced increase in lung endothelial permeability. However, recent work by us and others has highlighted the potential for discrete targeting of extra-alveolar or septal microvascular endothelium in acute lung injury, an effect that can be masked when permeability is assessed by measurement of whole lung K_f (8, 9, 23). We previously reported that TRPV4 is expressed in the septal compartment of lungs from humans, rats, and mice, and that activation of TRPV4 via EETs results in disruption of the septal endothelial barrier and alveolar flooding in rat and mouse lung (8). In contrast, activation of store-operated cation channels in extra-alveolar endothelium had no impact on alveolar septal barrier integrity or the alveolar fluid volume fraction (8). In the current study, we found that the increase in $V_{\text{af}}/V_{\text{as}}$ observed in wild-type lung after HiPv was absent in $\text{TRPV4}^{-/-}$ lung. These data confirm previous reports that HiPv targets the alveolar septal compartment, evoking disruption of alveolar septal capillaries and alveolar flooding (5, 22–24, 31), and suggest that this is due to HiPv-induced activation of TRPV4.

There are several potential limitations or caveats which should be considered. First, one might argue that our findings in mouse lung may not predict responses to HiPv in lungs from larger mammals, particularly since edema accumulation was evident even in the control Pv experiments in the $\text{TRPV4}^{-/-}$ mice. However, pulmonary edema in and of itself does not impact the measure of K_f (32). As a case in point, our measures of baseline hemodynamics and K_f in the mouse lung (Table 1) are no different than those we have reported in rat, dog, or pig lung, when normalized for lung mass (4, 8, 33), and in our hands, these measures remain stable in control mouse lungs that are perfused for up to 3 hours (data not shown). Second, K_f tended to increase after HiPv, despite perfusion with low Ca^{2+} perfusate, pharmacologic blockade of TRPV4, or key steps in the signaling cascade regulating TRPV4, or deletion of TRPV4. We considered whether the apparent residual Ca^{2+} response to HiPv may be due to release of Ca^{2+} from intracellular stores. However, such release is not likely sufficient to explain the residual increase in K_f when TRPV4-mediated Ca^{2+} entry is blocked. Our conclusion is based on the observation that store depletion and the resultant store-operated Ca^{2+} entry does not produce inter-endothelial cell gap formation in pulmonary microvascular endothelium nor evoke alveolar flooding (8, 9). Further, since the Ca^{2+} response in $\text{TRPV4}^{-/-}$ lungs was not altered in the presence of gadolinium chloride, a nonspecific cation channel blocker, we suggest that the residual increase in K_f not attributed to TRPV4 is likely due to frank mechanically induced disruption of the alveolar septal barrier, that is, to stress failure (5).

We conclude that TRPV4 plays a critical role in eliciting a permeability response to HiPv in the mouse lung. Gating of TRPV4 during HiPv requires activation of PLA_2 , synthesis of EETs from arachidonic acid, and EET-dependent activation of TRPV4. This scenario may represent a conserved signaling pathway in vascular endothelium linking mechanical stress to

Ca^{2+} entry. In lung alveolar septal endothelium, TRPV4 activation evokes barrier disruption and alveolar flooding. These findings have clear relevance for the clinical setting, where acute lung injury is well recognized to be localized to this compartment (34).

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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