Online Supplement: Alvarez *et al.* TRPV4-mediated disruption of the alveolar septal barrier: A novel mechanism of acute lung injury

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 [1]. 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 [1].

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 [Ca^{2+}] for the low Ca^{2+}/Ca^{2+} add-back strategy in isolated rat lungs was assessed by determining the lowest [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 [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 Ca^{2+}/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 Ca^{2+} (0.02 mmol/L) perfusate, with or without Ca^{2+} add-back to 2.2 mmol/L.

To determine whether EET-induced activation of large conductance Ca^{2+}-activated potassium channels (BK_{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_{Ca} inhibitors charybdotoxin (100 nmol/L) and apamin (300 nmol/L) [2], using the low Ca^{2+}/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 Ca^{2+} add-back.

RESULTS

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

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 K_f 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 BK_{Ca} channels, thus providing an increased driving gradient for Ca^{2+} entry [3, 4], we found that blockade of BK_{Ca} channels did not alter the permeability response to 14,15-EET (Online Figure 5).

REFERENCES

1. Liedtke W, Friedman JM. Abnormal osmotic regulation in trpv4$^{-/-}$ mice. *Proc Natl Acad Sci U S A* 2003;100:13698-13703.
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3. Harder DR, Campbell WB, Roman RJ. Role of cytochrome P-450 enzymes and metabolites of arachidonic acid in the control of vascular tone. *J Vasc Res* 1995;32:79-92.
4. Roman RJ. P-450 metabolites of arachidonic acid in the control of cardiovascular function. *Physiol Rev* 2002;82:131-185.

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 (K_f) 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 K_f (bottom panel). The tendency for K_f to increase in 0.01 mmol/L L [Ca^{2+}] was reversed with Ca^{2+} add-back.

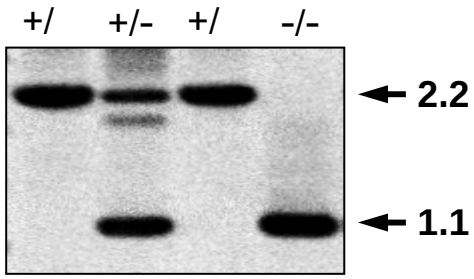
Online Figure 3. Dose-dependent effect of 5,6- and 14,15-EET on endothelial permeability in isolated rat lung. In separate groups of lungs, the filtration coefficient (K_f) was measured at baseline and 60 min after treatment with 0.5-5 μmol/L 5,6- or 14,15-EET (n=2-3 per dose). Both EET regioisomers 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 (K_f) 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 Ca^{2+}.

Online Figure 1.



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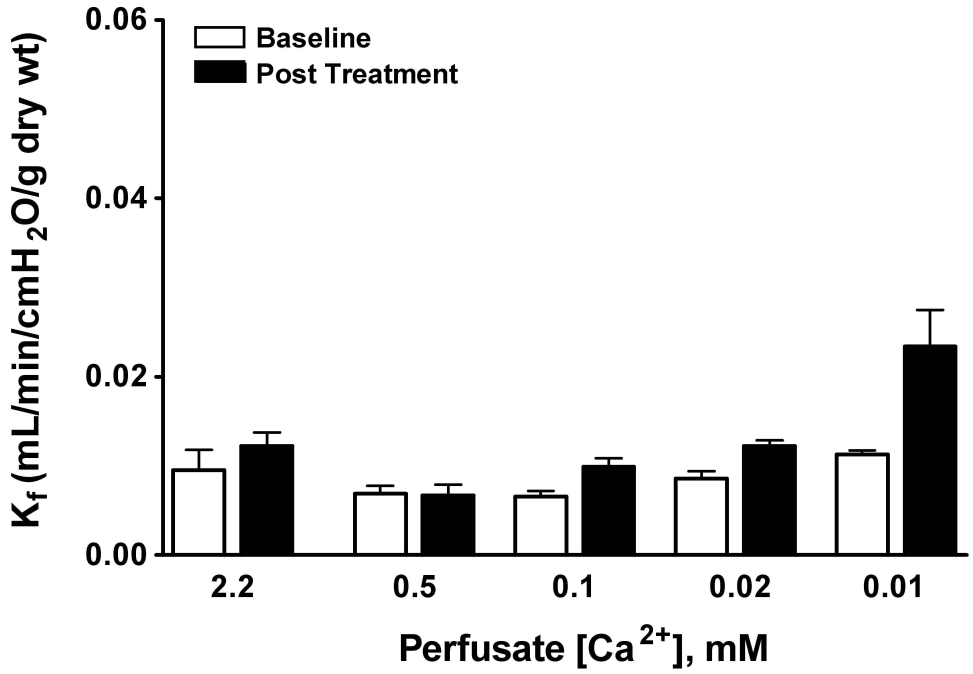

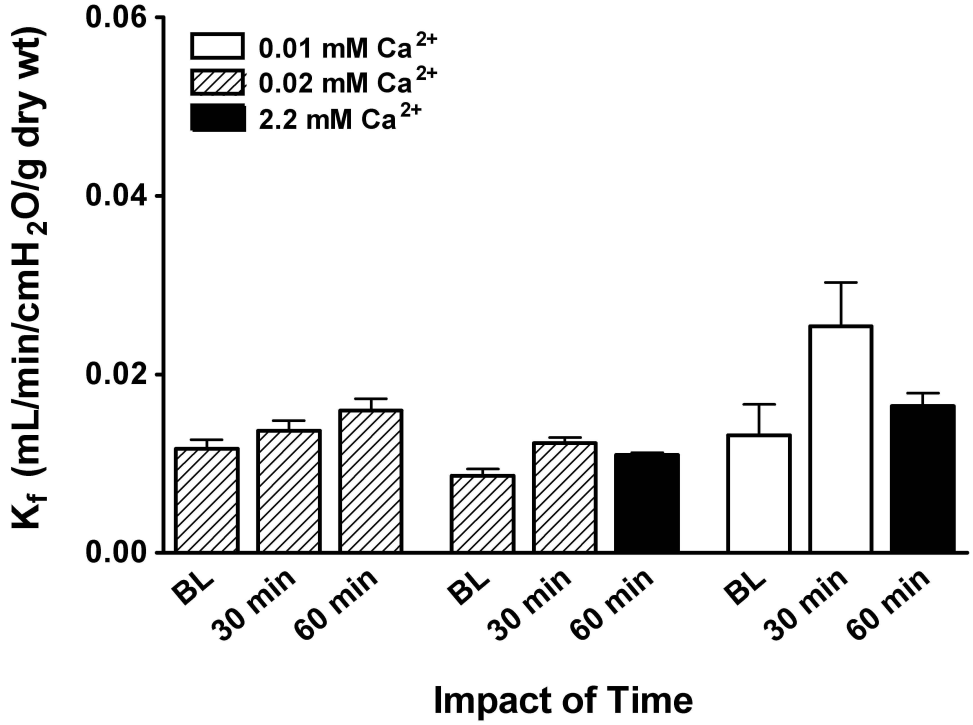

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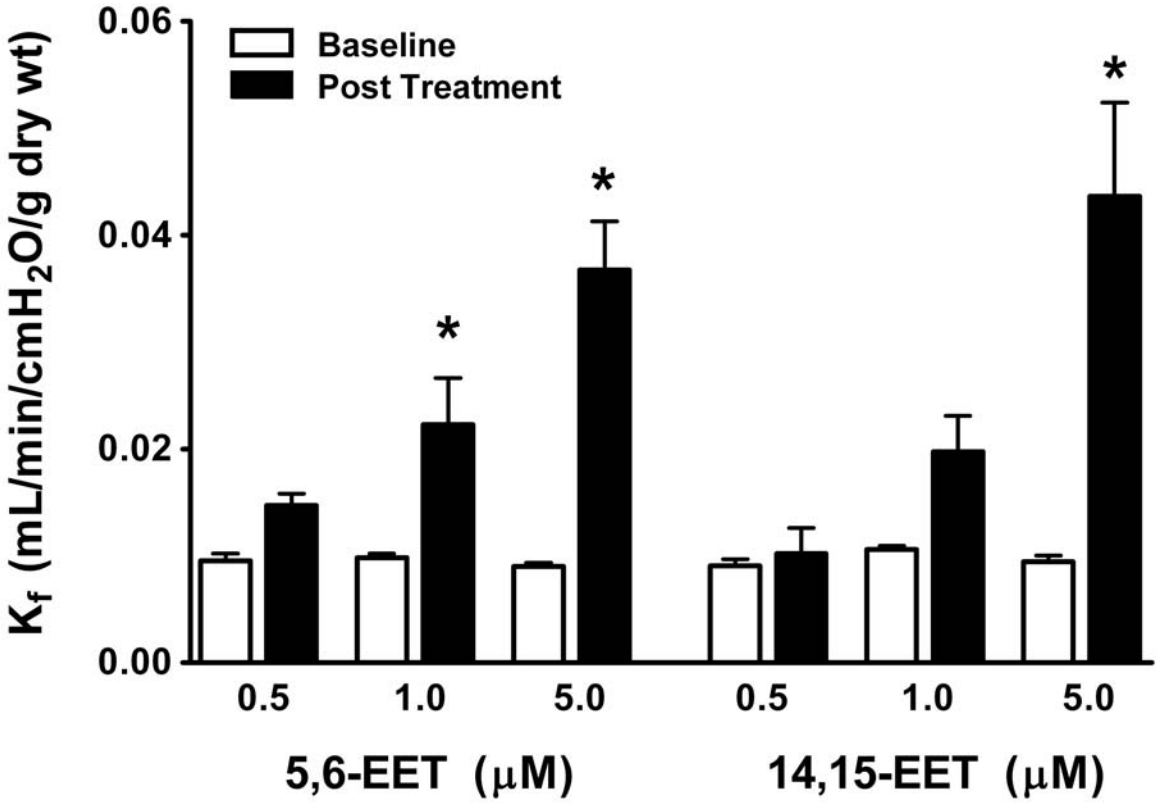

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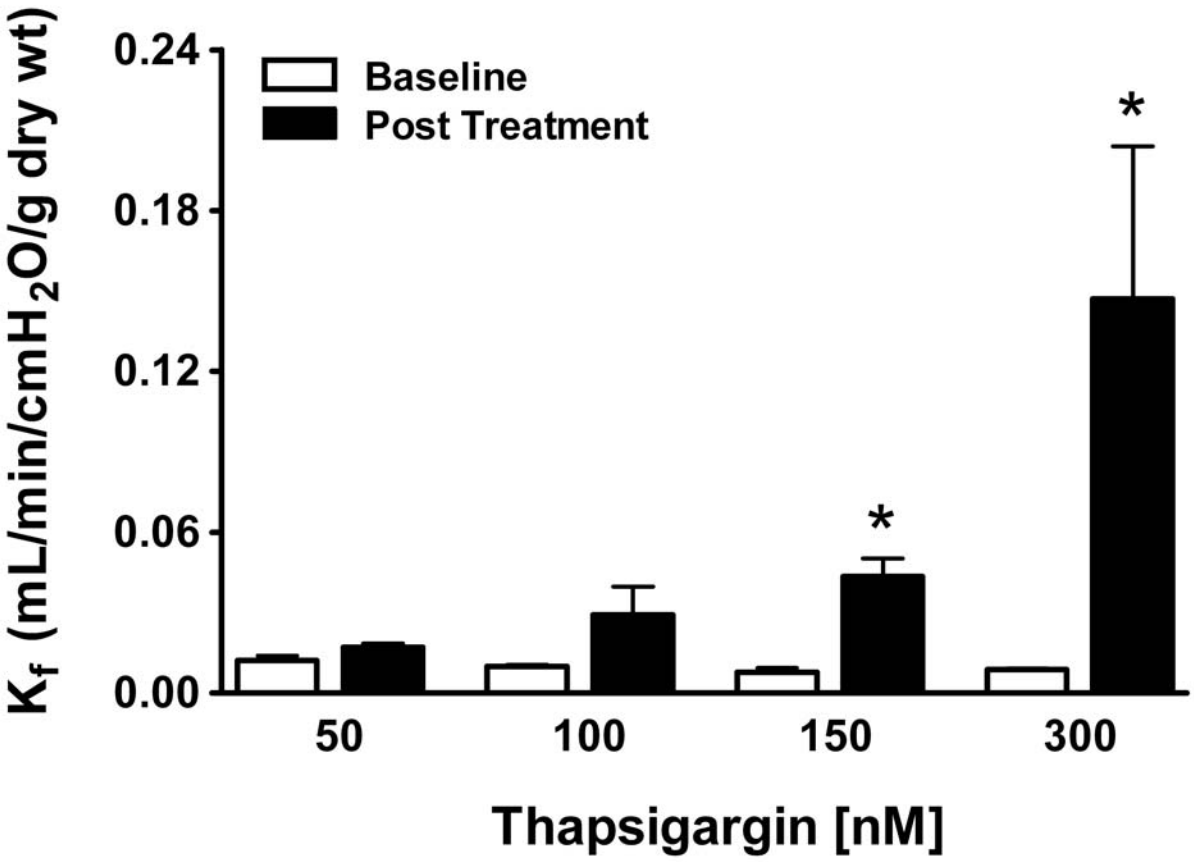

Online Figure 5.