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Hydrogen peroxide-induced calcium influx in lung microvascular endothelial cells involves TRPV4

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Suresh K, Servinsky L, Reyes J, Baksh S, Undem C, Caterina M, Pearse DB, Shimoda LA. Hydrogen peroxide-induced calcium influx in lung microvascular endothelial cells involves TRPV4. Am J Physiol Lung Cell Mol Physiol 309:L1467–L1477, 2015. First published October 9, 2015; doi:10.1152/ajplung.00275.2015.—In acute respiratory distress syndrome, both reactive oxygen species (ROS) and increased intracellular calcium ([Ca2+]i) are thought to play important roles in promoting endothelial paracellular permeability, but the mechanisms linking ROS and [Ca2+]i in microvascular endothelial cells are not known. In this study, we assessed the effect of hydrogen peroxide (H2O2) on [Ca2+]i, in mouse and human lung microvascular endothelial cells (MLMVEC and HLMVEC, respectively). We found that in both MLMVECs and HLMVECs, exogenously applied H2O2 increased [Ca2+]i through Ca2+ influx and that pharmacologic inhibition of the calcium channel transient receptor potential vanilloid 4 (TRPV4) attenuated the H2O2-induced Ca2+ influx. Additionally, knockdown of TRPV4 in HLMVEC also attenuated calcium influx following H2O2 challenge. Administration of H2O2 or TRPV4 agonists decreased transmembrane electrical resistance (TER), suggesting increased barrier permeability. To explore the regulatory mechanisms underlying TRPV4 activation by ROS, we examined H2O2-induced Ca2+ influx in MLMVECs and HLMVECs with either genetic deletion, silencing, or pharmacologic inhibition of Fyn, a Src family kinase. In both MLMVECs derived from mice deficient for Fyn and HLMVECs treated with either siRNA targeted to Fyn or the Src family kinase inhibitor SU-6656 for 24 or 48 h, the H2O2-induced Ca2+ influx was attenuated. Treatment with SU-6656 decreased the levels of phosphorylated, but not total, TRPV4 protein and had no effect on TRPV4 response to the external agonist, GSK1016790A. In conclusion, our data suggest that application of exogenous H2O2 increases [Ca2+]i, and decreases TER in microvascular endothelial cells via activation of TRPV4 through a mechanism that requires the Src kinase Fyn.

ARDS; calcium; lung injury; ROS; TRPV4

ACUTE RESPIRATORY DISTRESS SYNDROME (ARDS) is a common condition that affects ~200,000 patients every year (43) with no effective pharmacologic therapies. Loss of endothelial barrier function and formation of paracellular gaps in the lung microvascular endothelium are key features in the pathobiology of ARDS (62). The lung microvascular endothelium is composed of a specific phenotype of lung endothelial cells that are present in the alveolar capillaries and are known to be distinct from the endothelial cells of the larger conduit vessels (i.e., macrovascular endothelium) with regards to various signaling pathways and expression of membrane channels (13, 21, 31, 51). Two mediators known to be involved in lung microvascular endothelial cell (LMVEC) barrier disruption in ARDS include reactive oxygen species (ROS) and increased intracellular calcium concentration ([Ca2+]i).

Increased ROS are thought to play a major role in the development of endothelial barrier dysfunction in ARDS. In particular, high levels of oxidative stress are present in ARDS, and are associated with higher mortality (41, 62). Experimentally, increasing ROS by treatment with oxidants induces edema formation in isolated perfused lungs (6, 52). Similarly, application of external oxidants or induction of intracellular ROS production worsens barrier function in vitro (5, 40, 67). Conversely, protective effects were reported with augmentation of endothelial antioxidant mechanisms (8, 49, 50). ROS, such as H2O2, serve as signaling molecules at low levels and are maintained at these levels through various intracellular and extracellular antioxidant mechanisms (9). However, ROS levels can rise quickly because of increased production from intracellular sources such as NAPDH oxidase and mitochondria. Additionally, release of ROS by adherent neutrophils can also rapidly increase endothelial cell intracellular ROS. Indeed, ROS derived from circulating inflammatory cells have been shown to induce lung injury (47), and neutrophil-derived H2O2 is thought to play a major role in endothelial injury (59). Despite these known associations between ROS and endothelial barrier dysfunction, the mechanisms by which exogenous ROS, and H2O2 in particular, induce formation of paracellular gaps in the microvascular endothelium are incompletely understood.

In addition to increased ROS, elevated [Ca2+]i via influx through membrane channels has also been shown to be a key contributor to endothelial hyperpermeability (9, 62). Intracellular Ca2+ is required for activation of the contractile apparatus in endothelial cells, leading to cell retraction and disruption of the endothelial cell-cell junctions (18). Several members of the transient receptor potential (TRP) family of Ca2+ channels, including canonical TRPs (TRPCs) and vanilloid TRPs (TRPVs), have been implicated in various models of lung injury, including ischemia-reperfusion lung injury (65), stretch-induced lung injury (22), and heart failure models of pulmonary edema (54). TRPV4 in particular has been implicated in various models of lung injury and is thought to play a key role in lung epithelial and endothelial barrier function (37).
For example, Ca\(^{2+}\) entry via TRPV4 was recently shown to be critical in chloride inhalation and gastric acid aspiration models of lung injury (7). Additionally, a critical role for members of the TRPC family in regulating Ca\(^{2+}\) influx in the setting of various injurious stimuli (such as thrombin and LPS) in both pulmonary artery and mouse lung microvascular endothelial cells (MLMVECs) has been established (2, 34, 53, 55, 56). ROS and increased [Ca\(^{2+}\]\(_i\)]) in MLMVECs has been established, including TRPV4, can be differentially regulated by phosphorylation (61, 63). The Src family kinase (SFK) family of tyrosine kinases contains several members, including Src, Fyn, Yes, and Lyn, that are involved in a variety of cell signaling events (42, 44). Recently, the role of SFKs in Ca\(^{2+}\) channel regulation has been under investigation. For instance, TRPV4 activity was modulated by SFKs in HEK cells (64, 70) while phosphorylation by a variety of kinases, including Src, has been shown to affect the activity of TRPC3 and TRPC6 (25, 27, 29). Previous work established that Fyn is expressed in LMVECs (5), but the role of Fyn, or other SFKs, in regulating lung TRPs has not been determined.

Based on these data, we sought to understand the relationship between ROS and increased [Ca\(^{2+}\]\(_i\)]) in LMVECs and to determine whether inhibition of Ca\(^{2+}\) influx pathways could affect H\(_2\)O\(_2\)-induced barrier dysfunction in LMVECs. We hypothesized that H\(_2\)O\(_2\) would increase [Ca\(^{2+}\)]\(_i\), by activating a membrane-bound Ca\(^{2+}\) channel and that inhibiting H\(_2\)O\(_2\)-induced Ca\(^{2+}\) influx would attenuate H\(_2\)O\(_2\)-induced endothelial permeability in vitro. Using fluorescence microscopy, measurement of endothelial resistance in monolayers, genetic deletion and silencing approaches, and pharmacological inhibitors, we determined the role of TRPV4 and the Src kinase Fyn in regulating H\(_2\)O\(_2\)-induced Ca\(^{2+}\) influx in rodent and human LMVECs.

**METHODS**

All procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of The Johns Hopkins University School of Medicine.

Isolation and culture of LMVEC. Adult (8–10 wk), wild-type (WT) C57/B6 and Fyn-deficient (Fyn\(^{-/-}\); strain B6.129S7-Fyn\(^{-/-}\)) male mice were purchased from Jackson labs. Mice were killed by cervical dislocation, and the lungs were quickly removed by dissection and immersed in DMEM (Gibco). Complete media for MLMVEC was prepared by using 400 ml DMEM with 20% FBS, 0.1% nonessential amino acids, 0.1% antibiotics/antimycotics, and endothelial cell growth supplement (Millipore). Peripheral mouse lung tissue was obtained by dissection, then minced and digested by incubation with 5% Type IA Collagenase (Sigma) for 10 min. The digested cell solution was strained through a 70-μm mesh and washed with complete media, then centrifuged at 2,000 rpm for 10 min. The pellet was resuspended in 1 ml of complete media and the cell suspension incubated for 30 min with CD31-conjugated beads (Invitrogen). The supernatant was removed, and cells adherent to the conjugated beads were washed with isolation buffer (Invitrogen), centrifuged, and the pellet resuspended and plated on a T75 flask. After reaching confluence, cells were trypsinized and incubated with beads conjugated with Griffonia simplicifolia lectin, preincubating 25 μl of Biotin-11-N-Hydrazide Dynabeads (Invitrogen) with 1 μg of biotinylated Griffonia simplicifolia lectin and smooth muscle (smooth muscle specific α-actin) markers at each passage prior to experiments. Only cells that were positive for Griffonia simplicifolia lectin and did not express smooth muscle specific α-actin, were used for experiments. For MLMVECs, experiments were conducted on cells isolated from at least three different mice; for HLMVECs, cells from three different donors were used.

Intracellular Ca\(^{2+}\) measurements. Cells were grown to 50–60% confluence on glass coverslips and loaded with 5 μM of Fura-2 AM (Molecular Probes) for 1 h at 37°C prior to being placed in a temperature-controlled (37°C) laminar flow chamber on the stage of an inverted microscope and perfused with modified Krebs solution containing (in mM) 118 NaCl, 4.7 KCl, 0.57 MgSO\(_4\), 1.18 KH\(_2\)PO\(_4\), 25 NaHCO\(_3\), 2.5 CaCl\(_2\), and 10 glucose gassed with 16% O\(_2\) and 5% CO\(_2\) at 37°C. For Ca\(^{2+}\)-free experiments, CaCl\(_2\) was omitted and 1 mM EGTA added to the solution. Ratioometric measurement of Fura-2 fluorescence was performed by using a collimated light beam from a xenon arc lamp filtered at 340 and 380 nm and focused onto the endothelial cells via a ×20 fluorescence objective. After excitation at 340 and 380 nm (F\(_{340}/F\(_{380}\)) light emitted from the cells was returned through the objective, and detected at 510 nm by an imaging camera. An electronic shutter was used to minimize photo bleaching. The outflow rate of the flow chamber was set to 0.5 ml/min. Reservoirs containing Krebs solution as well as various drugs were connected via a manifold to a single inlet which connected to the inflow port of the chamber. At the beginning of each experiment, cells were perfused for 15 min to allow for establishment of stable baseline. Drugs (dissolved in Krebs) were added to the reservoirs prior to the experiment, thus allowing for quick switches between baseline, H\(_2\)O\(_2\), and drug containing solutions. Hydrogen peroxide (Sigma) was freshly prepared in warmed Krebs prior to each experiment. For experiments involving drug pretreatment, cells were either incubated with drug in the media (1–48 h) or perfused with drug in Krebs for 10–30 min prior to exposure to H\(_2\)O\(_2\). Intracellular calcium ([Ca\(^{2+}\)]\(_i\)) was estimated from F\(_{340}/F\(_{380}\) measured in calibration solutions with Ca\(^{2+}\) concentrations of 0–1,350 mM (Molecular Probes, Eugene, OR).

Western blotting. Cell lysates were prepared in T-PER (Life Biotechnologies) lysis buffer containing a protease inhibitor cocktail tablet (Boehringer) and phosphatase inhibitor cocktail solutions 2 and 3 (Sigma). Protein concentrations were determined using a BCA Protein Assay kit (Pierce). Equal amounts of protein (20 μg) were loaded into each well of an 8% gel and subjected to electrophoresis. Following separation, proteins were transferred to nitrocellulose membranes (iBlot, Life Biotechnologies), which were then blocked with blocking buffer (5% BSA in TBS-T) and incubated with primary antibody (anti-TRPV4 1:500, Alomone, or anti-phospho-tyrosine 1:3,000, Cell Signaling) at either room temperature for 1 h (phosphorytrosine) or overnight at 4°C (TRPV4). Membranes were then washed and incubated with secondary antibodies (goat anti-mouse 1:3,000 or goat anti-rabbit 1:3,000, Bio-Rad) for 1 h. Bands were visualized by enhanced chemiluminescence. Membranes were then stripped and reprobed with anti-GAPDH horseradish peroxidase-conjugated antibody (1:25,000, Bio-rad). Densitometry was performed to quantify the
amount of protein of interest, and this value was normalized to the housekeeping protein by using ImageJ software.

**Electrical cell impedance sensing.** Cells were seeded on gold electrodes in 0.5-ml electrode wells (Applied BioPhysics) and grown to confluence. Following measurement of baseline transmembrane electrical resistance (TER), the media was changed to serum-free basal media (EBM, Lonza), followed by a period of equilibration (1 h). Subsequently, agonists or diluent were added directly to wells. Data were analyzed by using electrical cell impedance sensing (ECIS) ZTheta system (Applied Biophysics).

**Small interference RNA transfection.** TRPV4 small interference RNA (siTRPV4) and control nontarget siRNA (siNT) were designed and synthesized by Dharmacon. HLMVEC were transfected with 100 nM of siRNA for 4 h in serum- and antibiotic-free EBM (Lonza) by using GeneSilencer (Genlantis) according to the manufacturer’s instruction. After 4 h, complete media (EGM-2, Lonza) was added. After an additional 24 h, the media was changed, and the cells were harvested for protein analysis or used for Ca²⁺ experiments at 48 h.

**H₂O₂ electrode.** H₂O₂ concentrations were measured with an electrode system (Apollo Free Radical Analyzer, World Precision Instruments). Following calibration, an H₂O₂-sensing electrode (ISO-HPO-2) was inserted into vials containing warmed Krebs solution as well as Krebs solution with various concentrations of H₂O₂ (250-1,000 μM). Measurements were taken at subsequent time points, and the solutions were maintained at 38°C with a heater during the course of the experiment.

**Data analysis/statistics.** All values are expressed as means ± SE. For [Ca²⁺], measurements, data were collected from up to 30 cells and the values were averaged to obtain a single value for each experiment. Change in [Ca²⁺], was computed by subtracting the average basal [Ca²⁺], determined from 1 min of data collected immediately prior to challenge with agonists, from the peak [Ca²⁺] measured in the first 5 min after beginning agonist challenge. For ECIS data, resistance measurements (R) from each ECIS well were normalized to the resistance value at the beginning of the experiment (R₀). Data were compared by unpaired Student’s t-test or by one-way ANOVA with a Holms-Sidak post hoc test to determine differences between groups. A repeated measures two-way ANOVA was used to analyze H₂O₂ sensor data. A P value of <0.05 was accepted as statistically significant.

**RESULTS**

**Effect of H₂O₂ on [Ca²⁺]i in MLMVECs and HLMVECs.** Since many of our experiments utilized H₂O₂ prepared in Krebs buffer solution, we measured the stability of H₂O₂ in this solution. Using an electrode-based ROS sensor, we measured changes in current at a fixed voltage when the electrode was placed in solutions containing 250 μM, 500 μM, and 1 mM H₂O₂. The concentration of [H₂O₂] in each of the solutions decreased within the first 30 min but subsequently remained stable up to 3 h (Fig. 1). To determine the effects of H₂O₂ on LMVECs, [Ca²⁺], was measured in semiconfluent (50–60%) MLMVECs and HLMVECs exposed to increasing concentrations of H₂O₂. In MLMVEC, no significant change in [Ca²⁺], was observed in response to 250 μM H₂O₂ (Fig. 2A). When a higher concentration of H₂O₂ was applied (1 mM H₂O₂), a large rise in [Ca²⁺], was observed within 120 s that was sustained for the duration of the exposure (Fig. 2, B and C). To determine whether a change in [Ca²⁺], following H₂O₂ exposure was a species-specific phenomenon, the effect of H₂O₂ on HLMVECs was also tested. Consistent with the results obtained in MLMVECs, H₂O₂ increased [Ca²⁺], in HLMVECs, although these cells appeared much more sensitive to H₂O₂ in that concentrations as low as 250 μM increased [Ca²⁺], (Fig. 2D). At a concentration of 1 mM, H₂O₂ produced a steep increase in HLMVEC [Ca²⁺], that was similar in magnitude to that observed at this concentration in MLMVECs. Unlike the MLMVECs, however, the peak change in [Ca²⁺], induced by H₂O₂ in HLMVECs was followed by a small reduction to a sustained plateau that was still significantly greater than baseline (Fig. 2, E and F).

**Identifying the source of Ca²⁺ in H₂O₂-induced responses.** To determine whether the H₂O₂-induced rise in [Ca²⁺], was due to Ca²⁺ release from the endoplasmic reticulum or influx

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**Fig. 1.** Traces of HPO electrode current at 450 mV poise voltage when placed in warmed Krebs solution containing 250 μM, 500 μM, and 1 mM H₂O₂ (n = 4–5 experiments/group). *Significant difference from control.

**Fig. 2.** Representative traces (A and B) and bar graph (C) showing mean ± SE change in intracellular Ca²⁺ ([Ca²⁺]i) from baseline in mouse lung microvascular endothelial cells (MLMVEC) exposed to 250 μM and 1 mM H₂O₂ in Krebs buffer (n = 4–6 experiments, 25–30 cells/experiment). Representative traces (D and E) and bar graph (F) showing mean ± SE in human lung microvascular endothelial cells (HLMVEC) exposed to increasing doses of H₂O₂ (n = 6–12 experiments, 20–40 cells/experiment for all groups). *Significant difference from 250 μM; #significant difference between peak and plateau Δ[Ca²⁺].
through plasma membrane channels, MLMVECs and HLMVECs were perfused with Ca\(^{2+}\)-free solution supplemented with EGTA (1 mM) to chelate any residual extracellular Ca\(^{2+}\) in the system while being challenged with H\(_2\)O\(_2\). In MLMVECs, the H\(_2\)O\(_2\)-induced rise in [Ca\(^{2+}\)]\(_i\) was largely absent when extracellular Ca\(^{2+}\) was removed (Fig. 3, A and B). Similarly, in HLMVECs the change in [Ca\(^{2+}\)]\(_i\) observed in response to application of H\(_2\)O\(_2\) was greatly reduced in the absence of extracellular Ca\(^{2+}\) (Fig. 3, C and D). To verify that perfusion with EGTA containing Ca\(^{2+}\) absent when extracellular Ca\(^{2+}\) was removed (Fig. 3, A and B). In MLMVECs treated with cyclopiazonic acid (CPA; 10 \(\mu\)M) after being perfused with normal or Ca\(^{2+}\)-free Krebs. The CPA-induced increase in [Ca\(^{2+}\)]\(_i\), indicative of store release, was similar in cells perfused with normal or Ca\(^{2+}\)-free media (Fig. 3E).

The next step was to determine the nature of the channel involved in H\(_2\)O\(_2\)-induced Ca\(^{2+}\) influx. The ability of H\(_2\)O\(_2\) to increase [Ca\(^{2+}\)]\(_i\) was measured in MLMVECs pretreated with the following Ca\(^{2+}\) channel blockers: SKF96365 (SKF; 30 \(\mu\)M), a nonspecific inhibitor of nonselective cation channels (NSCC); mibefradil (Mib; 10 \(\mu\)M), to inhibit T-type Ca\(^{2+}\) channels; or ruthenium red (RuR; 20 \(\mu\)M), an inhibitor of TRPV4 as well as other cation channels. There was no significant change in baseline [Ca\(^{2+}\)]\(_i\) with any drug treatment (Table 1). Neither SKF nor Mib prevented the increase in [Ca\(^{2+}\)]\(_i\) after H\(_2\)O\(_2\) exposure (Fig. 4, A and B). In contrast, pretreatment with RuR significantly attenuated the H\(_2\)O\(_2\)-induced rise in [Ca\(^{2+}\)]\(_i\) (Fig. 4, C and D).

Effect of TRPV4 agonism on HLMVECs. To directly evaluate the potential role of TRPV4 in the H\(_2\)O\(_2\)-induced increase in [Ca\(^{2+}\)]\(_i\), that we observed, we first assayed the expression of TRPV4 protein and studied the effect of TRPV4 agonism on endothelial [Ca\(^{2+}\)]\(_i\), and barrier function. Immunoblot analysis revealed that TRPV4 protein was clearly expressed in human and rat endothelial cells from conduit pulmonary arteries and in

![Figure 3](http://www.ajplung.org/download/10.1152/ajplung.00275.2015)

**Fig. 3.** Representative trace and bar graph showing mean ± SE change in [Ca\(^{2+}\)]\(_i\) in MLMVEC (A and B) and HLMVEC (C and D) exposed to H\(_2\)O\(_2\) while perfused with Krebs buffer containing 0 mM Ca\(^{2+}\) (\(n = 5–9\) experiments, 20–40 cells/experiment for all groups). Mean traces (E) and bar graph (F) showing mean ± SE change in [Ca\(^{2+}\)]\(_i\) in HLMVEC exposed to cyclopiazonic acid (10 \(\mu\)M) in regular and Ca\(^{2+}\)-free Krebs solutions (\(n = 3\) experiments, 20–40 cells/experiment for all groups). *Significant difference from 2.5 mM Ca\(^{2+}\) group.

![Figure 4](http://www.ajplung.org/download/10.1152/ajplung.00275.2015)

**Fig. 4.** Representative tracings showing change in [Ca\(^{2+}\)]\(_i\) following pretreatment with SKF96365 (SKF; 30 \(\mu\)M) (A), mibefradil (Mib; 10 \(\mu\)M) (B), and ruthenium red (RuR; 20 \(\mu\)M) (C). D: bar graph showing mean change ± SE in [Ca\(^{2+}\)]\(_i\) following H\(_2\)O\(_2\) in cells pretreated with SKF, Mib, or RuR. *Significant difference from control; \(n = 6\) experiments each, 25–30 cells/experiment.

<table>
<thead>
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<th>Condition</th>
<th>Baseline [Ca(^{2+})] (\pm) SE</th>
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<tr>
<td>WT</td>
<td>171 ± 13</td>
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<td>261 ± 40</td>
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<tr>
<td>WT + Mib</td>
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<tr>
<td>WT + RuR</td>
<td>179 ± 33</td>
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<tr>
<td>Fyn(^{-/-})</td>
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<td>siFyn</td>
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MLMVEC, mouse lung microvascular endothelial cells; WT, wild-type; SKF, SKF-96365; Mib, mibefradil; RuR, ruthenium red; HLMVEC, human LMVEC; HC, HC-067047; SU, SU-6656; siNT, nontarget small interference RNA; siTRPV4, transient receptor potential vanilloid 4 siRNA; siFyn, Fyn siRNA.

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**Table 1. Baseline [Ca\(^{2+}\)]\(_i\),**
rat, mouse, and human lung microvascular endothelial cells (Fig. 5A). To determine whether TRPV4 was functional in these cells, we exposed HLMVEC to the specific TRPV4 agonist, GSK1016790A (GSK; 100 nM). Application of GSK induced a rapid, large increase in \([\text{Ca}^{2+}]_i\) (830 ± 330 nM; n = 4; Fig. 5B). To further understand the functional consequence of TRPV4-mediated \([\text{Ca}^{2+}]_i\) influx, we performed ECIS studies on confluent monolayers of HLMVECs exposed to GSK. Control cells treated with vehicle displayed no significant change in TER; however, TER transiently decreased in cells treated with GSK, followed by recovery to near baseline levels. These results suggest that \([\text{Ca}^{2+}]_i\) influx via TRPV4 reduces barrier function in HLMVEC (Fig. 5, C and D).

Effect of TRPV4 inhibition on \(\text{H}_2\text{O}_2\)-induced \([\text{Ca}^{2+}]_i\) influx and barrier dysfunction. To assess whether blockade of TRPV4 alters ROS-induced \([\text{Ca}^{2+}]_i\) influx and paracellular permeability, HLMVEC were pretreated with RuR (20 \(\mu\)M) or the more specific TRPV4 inhibitor HC-067047 (1 \(\mu\)M) for 30 min and then exposed to 250 \(\mu\)M \(\text{H}_2\text{O}_2\). Similar to MLMVEC, treatment with RuR significantly attenuated the increase in \([\text{Ca}^{2+}]_i\) observed in response to \(\text{H}_2\text{O}_2\) (Fig. 6, A and B). HC treatment inhibited this response at least as well as RuR. Indeed, small rises in \([\text{Ca}^{2+}]_i\), occurring several minutes after initiation of ROS exposure, were observed with RuR but not HC treatment. Baseline \([\text{Ca}^{2+}]_i\) did not significantly change with HC or RuR treatment. With respect to barrier function, no significant change in TER was noted in control or vehicle-treated cells. Consistent with prior reports (40), we observed a decrease in TER with application of 250 \(\mu\)M \(\text{H}_2\text{O}_2\) (Fig. 6, C and D) consisting of a large initial decrease followed by transient recovery. The \(\text{H}_2\text{O}_2\)-induced decrease in TER was significantly attenuated following pretreatment with HC.

Role of Fyn in \(\text{H}_2\text{O}_2\)-induced TRPV4 activation. We next sought to understand the regulatory mechanisms underlying activation of TRPV4 following exposure to \(\text{H}_2\text{O}_2\). The Src family of kinases (SFK) has been previously implicated in the regulation of TRP channels. We hypothesized that \(\text{H}_2\text{O}_2\) might induce phosphorylation of TRPV4 via Fyn, thus activating the channel. To test this possibility, we measured \(\text{H}_2\text{O}_2\)-induced \([\text{Ca}^{2+}]_i\) influx in HLMVEC treated with SU-6656 (SU; 5 \(\mu\)M), an SFK inhibitor. No statistically significant difference in \(\text{H}_2\text{O}_2\)-induced \([\text{Ca}^{2+}]_i\) increase was observed in HLMVECs treated acutely (1 h) with SU (Fig. 7A). However, when HLMVECs were pretreated with SU for 24 h (Fig. 7B) or 48 h
(Fig. 7C), the increase in \([\text{Ca}^{2+}]_i\) induced by \(\text{H}_2\text{O}_2\) was significantly attenuated (Fig. 7D). We also measured \([\text{Ca}^{2+}]_i\) influx in MLMVECs isolated from mice deficient in Fyn (\(\text{Fyn}^{-/-}\) mice). In these cells, no difference in basal \([\text{Ca}^{2+}]_i\) was observed compared with MLMVEC isolated from WT mice; however, there was a complete lack of \([\text{Ca}^{2+}]_i\) influx following exposure to 1 mM \(\text{H}_2\text{O}_2\) (Fig. 8).

**Effect of TRPV4 and Fyn depletion on \(\text{H}_2\text{O}_2\)-induced \([\text{Ca}^{2+}]_i\) influx.** To further define the role of TRPV4 and Fyn in \(\text{H}_2\text{O}_2\)-induced \([\text{Ca}^{2+}]_i\) influx, we measured \([\text{Ca}^{2+}]_i\) in response to \(\text{H}_2\text{O}_2\) challenge in HLMVEC following TRPV4 and Fyn depletion with siRNA. At a concentration of 100 nM, siRNAs targeted against TRPV4 and Fyn (Dharmacon “Smart Pool”) produced a >70% reduction in TRPV4 and Fyn protein levels after 48 h (Fig. 9, A–D). Interestingly, the \(\text{H}_2\text{O}_2\)-induced \([\text{Ca}^{2+}]_i\) response in cells transfected with nontargeting siRNA (siNT) was higher than that in untransfected cells, though this increase did not reach statistical significance (\(P = 0.1\)). This effect may be related to the transfection process. Compared with siNT controls, \(\text{H}_2\text{O}_2\)-induced \([\text{Ca}^{2+}]_i\) influx was significantly attenuated in cells transfected with siTRPV4. Similar to pharmacologic inhibition and genetic absence, silencing of Fyn (siFyn) also significantly attenuated \(\text{H}_2\text{O}_2\)-induced \([\text{Ca}^{2+}]_i\) influx (Fig. 9, D and E).

**Phosphorylation of TRPV4 by Fyn and \([\text{Ca}^{2+}]_i\) responses.** Since loss of Fyn appeared to reduce \(\text{H}_2\text{O}_2\)-induced \([\text{Ca}^{2+}]_i\) responses, we investigated whether Fyn inhibition could alter TRPV4 activation by other stimuli or alter phosphorylation of TRPV4 protein. When HLMVECs were treated with SU for 48 h before exposure to GSK, the change in \([\text{Ca}^{2+}]_i\) was similar in magnitude to the response observed in untreated cells (Fig. 10, A and B). When immunoblots were probed for phosphotyrosine and TRPV4 (Fig. 10, C–E), HLMVECs that had been treated with SU (5 \(\mu\text{M}\) for 48 h) exhibited less phosphotyrosine signal at the molecular weight of TRPV4 (~100 kD), without any change in the total TRPV4 protein levels, suggesting that SU treatment reduced basal phosphorylation of TRPV4. We next measured whether \(\text{H}_2\text{O}_2\) caused acute changes in TRPV4 phosphorylation. Treatment with 250 \(\mu\text{M}\) \(\text{H}_2\text{O}_2\) produced no...
change in phosphorylated or total TRPV4, suggesting that ROS exposure does not activate TRPV4 via acute phosphorylation of the channel.

DISCUSSION

In this study, we demonstrated that application of H$_2$O$_2$ increased [Ca$^{2+}$]$_i$ levels in LMVECs through a mechanism that involves the Ca$^{2+}$ channel, TRPV4. Furthermore, our data suggest that the Src kinase family member Fyn participates in basal phosphorylation of TRPV4 and that this phosphorylation may be necessary for H$_2$O$_2$-induced Ca$^{2+}$ influx. Lastly, we showed that both TRPV4 agonism and exogenous ROS worsen barrier function in vitro, and that these effects are attenuated by inhibition of TRPV4.

We have previously shown that exogenous provision of ROS using H$_2$O$_2$ dissolved in a solution free of serum results in rapid uptake and a sustained increase in intracellular H$_2$O$_2$ in MLMVEC (49). It would appear that this holds true for HLMVECs as well, as we show that both murine and human LMVECs respond to H$_2$O$_2$ with changes in [Ca$^{2+}$]$_i$, and that in HLMVECs the changes in [Ca$^{2+}$]$_i$ are associated with changes in TER. Given the propensity of H$_2$O$_2$ to undergo rapid degradation in solution in the presence of trace metals and other solutes, we measured [H$_2$O$_2$] in the buffer solution used for our Ca$^{2+}$ experiments by using an H$_2$O$_2$ electrode technique previously shown by us and others to provide reliable and robust measurement of [H$_2$O$_2$] (33, 49). Our data reveal an initial decrease in [H$_2$O$_2$], followed by stable [H$_2$O$_2$] for several hours. Since we typically allow solutions containing H$_2$O$_2$ to be warmed and equilibrated for 30–45 min prior to the start of experiments, these data suggest that H$_2$O$_2$ levels were stable in our experimental solutions over the time course (1–2 h) of our experiments, ensuring that the lack of response seen in some of our experimental conditions was not due to degradation of H$_2$O$_2$.

Though extracellular delivery of H$_2$O$_2$ was stable, it is known that large differences exist between extracellular ROS and intracellular ROS because of a variety of factors, including diffusive properties of H$_2$O$_2$ itself and antioxidant capacity of various cell types (15, 26). Though the exact intracellular concentration of H$_2$O$_2$ is not known, we do not believe that it was at a level that induced cell death. In our Ca$^{2+}$ experiments, a short washout period was included at the end of H$_2$O$_2$ challenge, and during this time period we observed recovery of [Ca$^{2+}$]$_i$ toward baseline levels (data not shown). Likewise, in our ECIS measurements of TER in HLMVEC treated with 250 µM of H$_2$O$_2$, some recovery (although not to baseline) was noted following H$_2$O$_2$ exposure. Moreover, cells tracked during Ca$^{2+}$ imaging did not detach from cultureware or lose Fura-2 signal during the experiment, suggesting that cells remained viable for the duration of the experiment. Finally, our measurement of H$_2$O$_2$ in buffer solution revealed that the actual concentration of H$_2$O$_2$ delivered to cells in vitro was likely a stable lower dose than the initial concentration; thus it is unlikely that the changes observed in our experiments were due to nonspecific toxic effects of H$_2$O$_2$.

Our Ca$^{2+}$ results are consistent with other reports where H$_2$O$_2$ has been shown to increase [Ca$^{2+}$]$_i$ in various vascular beds (1, 36, 38, 58, 66), including bovine, human, and mouse lung endothelial cells. In bovine LMVEC (40) and human pulmonary artery endothelial cells (35), H$_2$O$_2$ increased [Ca$^{2+}$]$_i$ and decreased TER similar to our current findings in HLMVEC. While both HLMVECs and MLMVECs responded to H$_2$O$_2$ with an increase in [Ca$^{2+}$]$_i$, there is a clear difference in sensitivity. Unlike HLMVECs, which exhibit substantial increases in [Ca$^{2+}$]$_i$ and barrier disruption (as measured by ECIS) at concentrations of H$_2$O$_2$ as low as 250 µM (40), reductions in MLMVEC barrier function required very high concentrations of H$_2$O$_2$ (5). The difference in sensitivity to H$_2$O$_2$ with respect to barrier disruption in human and murine cells was reflected in the difference in [Ca$^{2+}$]$_i$ responses to H$_2$O$_2$ with MLMVECs exhibiting little increase in [Ca$^{2+}$]; at concentrations lower than 1 mM H$_2$O$_2$, the same concentration needed for barrier disruption. The exact reason for the varied sensitivity is unclear, but it may be explained by species-specific differences in antioxidant capacity (17). The difference in H$_2$O$_2$ responsiveness between mouse and human LMVECs served as a rationale for performing our inhibitor experiments in both cell types and validating our MLMVEC observations in more clinically relevant human cell cultures.

In HLMVEC, the profile of the Ca$^{2+}$ response differed based on H$_2$O$_2$ dose. While the highest concentration of H$_2$O$_2$ tested produced a large peak followed by a significantly lower but still elevated plateau, exposure to H$_2$O$_2$ at lower doses (i.e., 250 µM) produced an increase in [Ca$^{2+}$]$_i$ that was reproduc-
ibly stable around 100–150 nM with no significant differences between the peak and plateau [Ca\(^{2+}\)]. In MLMVEC, 1 mM of H\(_2\)O\(_2\) produced sustained elevations in [Ca\(^{2+}\)], without a peak or plateau, similar to the sustained rise in [Ca\(^{2+}\)] seen in bovine lung microvascular endothelial cells (40). The reason for the differences in profile is unclear, but may reflect additional influx, efflux, or release mechanisms. In both MLMVECs and HLMVECs, the H\(_2\)O\(_2\)-induced increase in [Ca\(^{2+}\)] was significantly attenuated when extracellular Ca\(^{2+}\) was removed, suggesting that Ca\(^{2+}\) influx is the primary determinant of H\(_2\)O\(_2\)-induced Ca\(^{2+}\) influx. However, the possibility of store depletion and store-operated entry playing a role in our H\(_2\)O\(_2\)-induced Ca\(^{2+}\) influx cannot be excluded based on our data. Indeed, small, late rises in [Ca\(^{2+}\)] were reliably observed even in Ca\(^{2+}\)-free experiments as well as in other experimental conditions (including TRPV4 and Fyn knockdown), perhaps corresponding to emptying of ER stores in these cells because of either prolonged absence of extracellular Ca\(^{2+}\) or a later H\(_2\)O\(_2\)-induced signaling mechanism not dependent on TRPV4. One concern when perfusing cells with Ca\(^{2+}\)-free media containing EGTA is depletion of stores, but brisk store release was noted when HLMVEC were exposed to CPA following perfusion in Ca\(^{2+}\)-free Krebs with EGTA, indicating that stores were still intact. Further experiments will be required to fully elucidate the source of this residual [Ca\(^{2+}\)]; response, the possible role of store-operated Ca\(^{2+}\) entry, and whether these mechanisms play a role in H\(_2\)O\(_2\)-induced permeability.

It is clear from previous work that ROS can increase [Ca\(^{2+}\)] in endothelial cells (20, 23, 35, 40); however, the key channels responsible for H\(_2\)O\(_2\)-induced Ca\(^{2+}\) influx in LMVECs were unclear. Our initial inhibitor studies performed in MLMVEC served to determine the category of channels that may be responsible for H\(_2\)O\(_2\)-induced Ca\(^{2+}\) influx in LMVECs. Neither SFK96365-sensitive channels nor T-type Ca\(^{2+}\) channels appear to be important players in the H\(_2\)O\(_2\)-induced [Ca\(^{2+}\)] response, as pharmacologic inhibition of these channels had no significant effect. In contrast, RuR diminished H\(_2\)O\(_2\)-induced Ca\(^{2+}\) influx in MLMVEC, suggesting TRPV4 or some other RuR-sensitive channel as the channel responsible in this cell type. To our knowledge a role for TRPV4 in oxidant-induced Ca\(^{2+}\) influx has not been previously described. Although TRPV4 has been studied extensively in MLMVEC, less is known about TRPV4 localization and function in HLMVEC. The presence of a large Ca\(^{2+}\) response to GSK in HLMVEC functionally confirmed our immunoblot data, demonstrating presence of TRPV4 in these cells. Additionally, RuR also inhibited the H\(_2\)O\(_2\)-induced Ca\(^{2+}\) influx in HLMVEC. However, it should be noted that RuR also inhibits other cation channels, and has been reported to both activate and inhibit sarcoplasmic reticulum and mitochondrial channels (16, 57, 71). To more firmly establish the role of TRPV4 in H\(_2\)O\(_2\)-induced Ca\(^{2+}\) influx in HLMVECs, we inhibited TRPV4 with HC-067047, a putative specific inhibitor of TRPV4 previously shown to have minimal off-target effects (19, 69). Coupled with experiments in which TRPV4 was genetically depleted, our data suggest that TRPV4 plays a key role in mediating the acute Ca\(^{2+}\) response to H\(_2\)O\(_2\) in the lung microvasculature.

The fact that TRPV4 appeared to be the major channel responsible for the H\(_2\)O\(_2\)-induced increase in [Ca\(^{2+}\)], in human microvascular endothelial cells was somewhat surprising, as prior research had implicated members of the TRPM family (TRPM2 and TRPM4) as redox-sensitive Ca\(^{2+}\) channels that respond to increases in intracellular H\(_2\)O\(_2\) (23, 46). Several explanations exist for this variation in findings. A major difference between the studies demonstrating a role for TRPM2 and our current work is the location in the lung from which cells were derived. It is now widely recognized that there is regional heterogeneity within the pulmonary endothelium, and that LMVECs are phenotypically distinct from other cell types such as pulmonary conduit or systemic endothelial cells (11, 14, 21, 51). Our results suggest that this heterogeneity may extend to the channels and/or mechanisms by which H\(_2\)O\(_2\) induces barrier disruption, with TRPM2 playing a primary role in proximal, and TRPV4 a larger role, in microvascular endothelial cells. The type of Ca\(^{2+}\) entry (i.e., store-operated entry vs. receptor-operated Ca\(^{2+}\) influx) as well as the specific Ca\(^{2+}\) channel responsible is likely stimulus dependent; thus our findings of TRPV4 involvement are most applicable to physiologic states where ROS are delivered to the endothelium from exogenous sources, such as circulating inflammatory cells. Furthermore, it is possible that more than one Ca\(^{2+}\) channel is responsible for the change in [Ca\(^{2+}\)], seen in our experiments. For example, TRP channels can form heterotramers, and thus it is possible that the observed Ca\(^{2+}\) influx occurs because of functional coupling of TRPV4 with other TRP family members (i.e., TRPM2, TRPC1, and TRPC6) as well as non-TRP channels such as Ca\(^{2+}\)-activated K\(^{+}\) channels (30, 39). Lastly, Ca\(^{2+}\) responses in endothelial cells may be temporally heterogeneous; H\(_2\)O\(_2\)-induced Ca\(^{2+}\) influx in LMVECs may involve multiple channels depending on the dose of the stimulus and the time point studied. Thus the endothelial Ca\(^{2+}\) response to ROS may involve additional Ca\(^{2+}\) channels at later time points and/or sequential activation of multiple channels.

Our data demonstrate not only that TRPV4 is important for H\(_2\)O\(_2\)-mediated Ca\(^{2+}\) responses and permeability, but also that activation of TRPV4 is sufficient to induce barrier disruption in HLMVECs. The decrease in TER induced by TRPV4 agonists was qualitatively and quantitatively similar in profile to that induced by exogenous H\(_2\)O\(_2\). Taken with our ECIS experiments showing that TRPV4 inhibition attenuated H\(_2\)O\(_2\)-induced decreases in TER in HLMVEC, these data suggest that TRPV4-mediated Ca\(^{2+}\) influx modulates the pulmonary microvascular barrier, at least in vitro. One caveat in comparing the [Ca\(^{2+}\)] and TER experiments is that since serum can quench H\(_2\)O\(_2\) (12), we used serum-free media during our ECIS experiments. Unfortunately, HC-067047 did not dissolve easily in our media buffer, resulting in the need to use a higher dose for the ECIS compared with [Ca\(^{2+}\)]; experiments. Thus an off-target effect of HC-067047 cannot be entirely ruled out. Additionally, since endothelial monolayers lack smooth muscle cell interactions, laminar flow, and the presence of circulating immune cells, in vivo experiments will be required to determine the role of TRPV4 in oxidant-induced increases in microvascular endothelial permeability in the intact lung.

TRPV4 is activated by multiple stimuli such as heat, osmolarity, stretch, and internal cell signaling molecules, such as the arachidonic acid metabolite epoxyeicosatrienoic acid, or EET (4, 48, 63, 68). In our study, [Ca\(^{2+}\)], measurements were performed in a temperature-controlled chamber with laminar flow, and measurements were obtained after a period of perfusion with Krebs buffer solution. The exact experimental
conditions are relevant given TRPV4 is known to have varying stimulus sensitivity based on temperature (22) and endothelial cells are known to undergo flow-adaptive changes in cytosolic Ca\(^{2+}\) in response to tonic shear stress from lammar flow. In addition, site-specific regulation of TRPV4 has been previously shown in transfected cell systems (61, 64); the responsiveness of TRPV4 to certain stimuli can be experimentally abolished by site-based mutagenesis of various phosphorylation sites (61, 63). For instance, mutation of the putative SFK phosphorylation site Y110 renders TRPV4 resistant to activation by internal cell signals and shear stress (64). However, the exact SFK involved in regulating TRPV4 function in the endothelium is not known.

In neuronal tissues, the SFK member, Lyn, was found to participate in Ca\(^{2+}\) signaling by phosphorylating TRPV4 (3). On the other hand, we have previously shown that genetic deletion of Fyn attenuated ROS-induced barrier dysfunction in MLMVECs (5). These observations, along with our current data showing significant loss of phosphorylation in response to H\(_2\)O\(_2\) in Fyn\(^{-/-}\) MLMVECs, suggested that Fyn inhibition would attenuate H\(_2\)O\(_2\)-induced Ca\(^{2+}\) influx in HLMVECs. Surprisingly, short-term pretreatment of HLMVECs with SFK inhibitor SU-6656 did not significantly alter H\(_2\)O\(_2\)-induced Ca\(^{2+}\) influx. Moreover, no increase in TRPV4 phosphorylation was observed upon challenge with H\(_2\)O\(_2\), indicating that acute phosphorylation of TRPV4 by Fyn was not required for direct activation of TRPV4 by H\(_2\)O\(_2\). However, TRPV4 protein turnover has been reported to occur slowly (24), and thus led us to speculate that the differential effect of genetic deletion and acute inhibition of Fyn might be due to baseline Fyn-dependent phosphorylation of TRPV4 being necessary, but not sufficient, to induce Ca\(^{2+}\) influx. In this case, we reasoned that activation of TRPV4 by H\(_2\)O\(_2\) would require both basal phosphorylation and subsequent activation of TRPV4 via another mechanism. Indeed, inhibition of Fyn for a longer period of time (48 h) reduced phosphorylation, but not total membrane expression, of TRPV4 and attenuated H\(_2\)O\(_2\)-induced Ca\(^{2+}\) influx.

One limitation of the current data is that it is not possible to fully elucidate whether Fyn phosphorylates TRPV4 directly or if an intermediate kinase is involved; further investigations focused on delineating the physical interactions between TRPV4 and Fyn or the possible role of kinases downstream of Fyn will be useful in this regard. Interestingly, exogenous agonism of TRPV4 was not altered by prolonged SFK inhibition; this channel behavior is in concordance with several prior studies that have suggested that SFK-mediated phosphorylation of TRPV4 is necessary for activation by some stimuli but not exogenous agonists (3, 64, 70). While off-target effects of SFK inhibition of TRPV4 and Fyn or the possible role of kinases downstream of TRPV4 is necessary for activation by some stimuli but not exogenous agonists (3, 64, 70), the responsiveness of TRPV4 to certain stimuli can be experimentally abolished by site-based mutagenesis of various phosphorylation sites (61, 63). For instance, mutation of the putative SFK phosphorylation site Y110 renders TRPV4 resistant to activation by internal cell signals and shear stress (64). Therefore, the exact SFK involved in regulating TRPV4 function in the endothelium is not known.

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The exact mechanism by which H\(_2\)O\(_2\) activates TRPV4 is still under investigation. One possibility is that ROS activates Ca\(^{2+}\)-independent phospholipase A\(_2\) (32), leading to production of arachidonic acid (AA) metabolites which are known to directly activate TRPV4 (63). Though our data suggest a role for membrane-bound Fyn and basal phosphorylation of TRPV4 in transduction of ROS signaling, prior work has shown that Fyn also functions in a protective role through effects on focal adhesion kinase phosphorylation in response to thrombin-induced endothelial injury (45).

In conclusion, our data suggest that TRPV4 plays a key role in the early rise in [Ca\(^{2+}\)]\(_i\) in mouse and human LMVECs following exposure to H\(_2\)O\(_2\), and that inhibiting TRPV4-mediated Ca\(^{2+}\) influx attenuates barrier dysfunction in response to H\(_2\)O\(_2\) in vitro. Furthermore, our data suggest a novel role for Fyn in regulating the function of TRPV4 through phosphorylation. Although these results provide insight into some of the mechanisms that regulate Ca\(^{2+}\) signaling in vitro, further work is needed to determine whether other TRP channels also contribute to H\(_2\)O\(_2\)-induced Ca\(^{2+}\) influx and the role of TRPV4 in oxidant injury and barrier function in vivo.

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