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

Karthik Suresh, Laura Servinsky, Jose Reyes, Syeda Baksh, Clark Undem, Michael Caterina, David B. Pearse, and Larissa A. Shimoda

1Division of Pulmonary and Critical Care Medicine, Department of Medicine and 2Department of Neurosurgery, Johns Hopkins School of Medicine, Baltimore, Maryland

Submitted 7 August 2015; accepted in final form 7 October 2015

ARDS; calcium; lung injury; ROS; TRPV4

that requires the Src kinase Fyn. Cytoskeletal changes can also rapidly increase endothelial cell intracellular ROS. Indeed, 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).

Address for reprint requests and other correspondence: L. Shimoda, Johns Hopkins Asthma and Allergy Bldg., 5501 Hopkins Bayview Circle, Baltimore, MD 21224 (e-mail: lshimod1@jhmi.edu).

http://www.ajplung.org 1040-0605/15 Copyright © 2015 the American Physiological Society L1467
For example, Ca^{2+} entry via TRPV4 was recently shown to be critical in chlorine 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 may in fact be mechanistically linked; ROS increased [Ca^{2+}]_i in various tissues including the lung (40, 60), but the specific channel responsible for ROS-induced Ca^{2+} influx in the lung microvasculature is not known.

While TRPs clearly play a major role in endothelial cell responses to various stimuli, little is known regarding the mechanisms by which the function of these channels is regulated in LMVECs. In expression systems, TRP channels, including TRPV4, can be differentially regulated by phosphorylation (61, 63). The Src family kinase (SFK) family of kinases, 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 of a variety of kinases, including Src, has been shown to affect the activity of TRPC3 and TRPC6 (25, 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^-/-J) 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, prepped and incubating 25 μl of Biotin-Hybaid Dynabeads (Invitrogen) with 1 μg of biotinylated Griffonia simplicifolia lectin (EY Labs) for 30 min at room temperature. After incubation, the beads were washed with isolation buffer, the cells centrifuged, resuspended in complete growth media, and grown to confluence. Human lung microvascular endothelial cells (HLMVECs) were purchased from Lonza; cells were grown on gelatinized cultureware with Lonza endothelial growth media-2 (EGM-2) supplemented with 1% penicillin/streptomycin and an extra 5% FBS. All experiments were performed on cells at passage 5–6. To ensure that cells did not undergo transdifferentiation in vitro, both HLMVEC and MLMVECs were stained for endothelial (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, 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 HLMVEC, 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 MgSO4, 1.18 KH2PO4, 25 NaHCO3, 2.5 CaCl2, and 10 glucose gassed with 16% O2 and 5% CO2 at 37°C. For Ca^{2+}-free experiments, CaCl2 was omitted and 1 mM EGTA added to the solution. Ratiometric 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 (F340,F380), 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 F340/F380 measured in calibration solutions with Ca^{2+} concentrations of 0–1,350 nM (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 (phosphorysine) 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 instructions. After 4 h, complete media (EGM-2, Lonza) was added. An additional 24 h, the media was changed, and the cells were harvested for protein analysis or used for Ca^{2+} experiments at 48 h.

**H_{2}O_{2} electrode.** H_{2}O_{2} concentrations were measured with an electrode system (Apollo Free Radical Analyzer, World Precision Instruments). Following calibration, an H_{2}O_{2}-sensing electrode (ISO-HPO-2) was inserted into vials containing warmed Krebs solution as well as Krebs solution with various concentrations of H_{2}O_{2} (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 [Ca^{2+}]i measurements, data were collected from up to 30 cells and the values were averaged to obtain a single value for each experiment. Change in [Ca^{2+}]i, was computed by subtracting the average basal [Ca^{2+}]i, determined from 1 min of data collected immediately prior to challenge with agonists, from the peak [Ca^{2+}]i, 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 H_{2}O_{2} sensor data. A P value of <0.05 was accepted as statistically significant.

**RESULTS**

**Effect of H_{2}O_{2} on [Ca^{2+}]i in MLMVECs and HLMVECs.** Since many of our experiments utilized H_{2}O_{2} prepared in Krebs buffer solution, we measured the stability of H_{2}O_{2} 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 H_{2}O_{2}. The concentration of [H_{2}O_{2}] i 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 H_{2}O_{2} on MLMVECs, [Ca^{2+}]i, was measured in semiconfluent (50–60%) MLMVECs and HLMVECs exposed to increasing concentrations of H_{2}O_{2}. In MLMVEC, no significant change in [Ca^{2+}]i, was observed in response to 250 μM H_{2}O_{2} (Fig. 2A). When a higher concentration of H_{2}O_{2} was applied (1 mM H_{2}O_{2}), a large rise in [Ca^{2+}]i, 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 [Ca^{2+}]i, following H_{2}O_{2} exposure was a species-specific phenomenon, the effect of H_{2}O_{2} on HLMVECs was also tested. Consistent with the results obtained in MLMVECs, H_{2}O_{2} increased [Ca^{2+}]i, in HLMVECs, although these cells appeared much more sensitive to H_{2}O_{2} in that concentrations as low as 250 μM increased [Ca^{2+}]i, (Fig. 2D). At a concentration of 1 mM, H_{2}O_{2} produced a steep increase in HLMVEC [Ca^{2+}]i, that was similar in magnitude to that observed at this concentration in MLMVECs. Unlike the MLMVECs, however, the peak change in [Ca^{2+}]i, induced by H_{2}O_{2} 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 Ca^{2+} in H_{2}O_{2}-induced responses.** To determine whether the H_{2}O_{2}-induced rise in [Ca^{2+}]i, was due to Ca^{2+} release from the endoplasmic reticulum or influx...
through plasma membrane channels, MLMVECs and HLMVECs were perfused with Ca\textsuperscript{2+}-free solution supplemented with EGTA (1 mM) to chelate any residual extracellular Ca\textsuperscript{2+} in the system while being challenged with H\textsubscript{2}O\textsubscript{2}. In MLMVECs, the H\textsubscript{2}O\textsubscript{2}-induced rise in [Ca\textsuperscript{2+}]\textsubscript{i} was largely absent when extracellular Ca\textsuperscript{2+} was removed (Fig. 3, A and B). Similarly, in HLMVECs the change in [Ca\textsuperscript{2+}]\textsubscript{i} observed in response to application of H\textsubscript{2}O\textsubscript{2} was greatly reduced in the absence of extracellular Ca\textsuperscript{2+} (Fig. 3, C and D). To verify that perfusion with EGTA containing Ca\textsuperscript{2+}-free media did not lead to depletion of Ca\textsuperscript{2+}-stores, we measured [Ca\textsuperscript{2+}]\textsubscript{i} in HLMVECs treated with cyclopiazonic acid (CPA; 10 \mu M) after being perfused with normal or Ca\textsuperscript{2+}-free Krebs. The CPA-induced increase in [Ca\textsuperscript{2+}]\textsubscript{i}, indicative of store release, was similar in cells perfused with normal or Ca\textsuperscript{2+}-free media (Fig. 3E).

The next step was to determine the nature of the channel involved in H\textsubscript{2}O\textsubscript{2}-induced Ca\textsuperscript{2+} influx. The ability of H\textsubscript{2}O\textsubscript{2} to increase [Ca\textsuperscript{2+}]\textsubscript{i} was measured in MLMVECs pretreated with the following Ca\textsuperscript{2+} channel blockers: SKF96365 (SKF; 30 \mu M), a nonspecific inhibitor of nonselective cation channels (NSCC); mibebradil (Mib; 10 \mu M), to inhibit T-type Ca\textsuperscript{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\textsuperscript{2+}]\textsubscript{i} with any drug treatment (Table 1). Neither SKF nor Mib prevented the increase in [Ca\textsuperscript{2+}]\textsubscript{i} after H\textsubscript{2}O\textsubscript{2} exposure (Fig. 4, A and B). In contrast, pretreatment with RuR significantly attenuated the H\textsubscript{2}O\textsubscript{2}-induced increase in [Ca\textsuperscript{2+}]\textsubscript{i} (Fig. 4, C and D).

\textbf{Effect of TRPV4 agonism on HLMVECs.} To directly evaluate the potential role of TRPV4 in the H\textsubscript{2}O\textsubscript{2}-induced increase in [Ca\textsuperscript{2+}]\textsubscript{i}, that we observed, we first assayed the expression of TRPV4 protein and studied the effect of TRPV4 agonism on endothelial [Ca\textsuperscript{2+}]\textsubscript{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

<table>
<thead>
<tr>
<th>Condition</th>
<th>Baseline [Ca\textsuperscript{2+}] \pm SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>171 \pm 13</td>
</tr>
<tr>
<td>WT + SKF</td>
<td>261 \pm 40</td>
</tr>
<tr>
<td>WT + Mib</td>
<td>137 \pm 8</td>
</tr>
<tr>
<td>WT + RuR</td>
<td>179 \pm 33</td>
</tr>
<tr>
<td>Fyn\textsuperscript{+/−}</td>
<td>160 \pm 8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Condition</th>
<th>Baseline [Ca\textsuperscript{2+}] \pm SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>90 \pm 7.4</td>
</tr>
<tr>
<td>RuR 1 h</td>
<td>123 \pm 15</td>
</tr>
<tr>
<td>SU 24 h</td>
<td>105 \pm 9</td>
</tr>
<tr>
<td>SU 48 h</td>
<td>95 \pm 5</td>
</tr>
<tr>
<td>siNT</td>
<td>73 \pm 4</td>
</tr>
<tr>
<td>siTRPV4</td>
<td>81 \pm 7</td>
</tr>
<tr>
<td>siFyn</td>
<td>67.7 \pm 10</td>
</tr>
</tbody>
</table>

MLMVEC, mouse lung microvascular endothelial cells; WT, wild-type; SKF, SKF-96365; Mib, mibebradil; RuR, ruthenium red; HLMVEC, human LMVEC; HC, HC-067047; SU, SU-6656; siNT, non-target small interference RNA; siTRPV4, transient receptor potential vanilloid 4 siRNA; siFyn, Fyn siRNA.

![Fig. 3. Representative trace and bar graph showing mean ± SE change in [Ca\textsuperscript{2+}]\textsubscript{i} in MLMVEC (A and B) and HLMVEC (C and D) exposed to H\textsubscript{2}O\textsubscript{2} while perfused with Krebs buffer containing 0 mM Ca\textsuperscript{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\textsuperscript{2+}]\textsubscript{i} in HLMVEC exposed to cyclopiazonic acid (10 \mu M) in regular and Ca\textsuperscript{2+}-free Krebs solutions (n = 3 experiments, 20–40 cells/experiment for all groups). *Significant difference from 2.5 mM Ca\textsuperscript{2+} group.](http://ajplung.physiology.org/)

![Fig. 4. Representative tracings showing change in [Ca\textsuperscript{2+}]\textsubscript{i} following pretreatment with SKF96365 (SKF; 30 \mu M) (A), mibebradil (Mib; 10 \mu M) (B), and ruthenium red (RuR; 20 \mu M) (C). D: bar graph showing mean change ± SE in [Ca\textsuperscript{2+}]\textsubscript{i} following H\textsubscript{2}O\textsubscript{2} in cells pretreated with SKF, Mib, or RuR. *Significant difference from control; n = 6 experiments each, 25–30 cells/ experiment.](http://ajplung.physiology.org/)
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 $[Ca^{2+}]_i$ (830 ± 330 nM; n = 4; Fig. 5B). To further understand the functional consequence of TRPV4-mediated $Ca^{2+}$ influx, we performed ECIS studies on confluent monolayers of HLMVECs exposed to GSK. Control cells treated with vehicle displayed no significant

decrease in TER; however, TER transiently decreased in cells treated with GSK, followed by recovery to near baseline levels. These results suggest that $Ca^{2+}$ influx via TRPV4 reduces barrier function in HLMVEC (Fig. 5, C and D).

Effect of TRPV4 inhibition on $H_2O_2$-induced $Ca^{2+}$ influx and barrier dysfunction. To assess whether blockade of TRPV4 alters $ROS$-induced $Ca^{2+}$ influx and paracellular permeability, HLMVEC were pretreated with RuR (20 μM) or the more specific TRPV4 inhibitor HC-067047 (1 μM) for 30 min and then exposed to 250 μM $H_2O_2$. Similar to MLMVEC, treatment with RuR significantly attenuated the increase in $[Ca^{2+}]_i$ observed in response to $H_2O_2$ (Fig. 6, A and B). HC treatment inhibited this response at least as well as RuR. Indeed, small rises in $[Ca^{2+}]_i$, occurring several minutes after initiation of ROS exposure, were observed with RuR but not HC treatment. Baseline $[Ca^{2+}]_i$ 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 μM $H_2O_2$ (Fig. 6, C and D) consisting of a large initial decrease followed by transient recovery. The $H_2O_2$-induced decrease in TER was significantly attenuated following pretreatment with HC.

Role of Fyn in $H_2O_2$-induced TRPV4 activation. We next sought to understand the regulatory mechanisms underlying activation of TRPV4 following exposure to $H_2O_2$. The Src family of kinases (SFK) has been previously implicated in the regulation of TRP channels. We hypothesized that $H_2O_2$ might induce phosphorylation of TRPV4 via Fyn, thus activating the channel. To test this possibility, we measured $H_2O_2$-induced $Ca^{2+}$ influx in HLMVEC treated with SU-6656 (SU; 5 μM), an SFK inhibitor. No statistically significant difference in $H_2O_2$-induced $[Ca^{2+}]_i$ 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
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\((Fyn^{-/-}\) 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 (\(~100\text{ kD}\)), 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 H2O2 increased [Ca2+]i levels in LMVECs through a mechanism that involves the Ca2+ 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 H2O2-induced Ca2+ 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 H2O2 dissolved in a solution free of serum results in rapid uptake and a sustained increase in intracellular H2O2 in MLVECs (49). It would appear that this holds true for HLMVECs as well, as we show that both murine and human LMVECs respond to H2O2 with changes in [Ca2+]i, and that in HLMVECs the changes in [Ca2+]i are associated with changes in TER. Given the propensity of H2O2 to undergo rapid degradation in solution in the presence of trace metals and other solutes, we measured [H2O2]i in the buffer solution used for our Ca2+ experiments by using an H2O2 electrode technique previously shown by us and others to provide reliable and robust measurement of [H2O2]i (33, 49). Our data reveal an initial decrease in [H2O2]i, followed by stable [H2O2]i for several hours. Since we typically allow solutions containing H2O2 to be warmed and equilibrated for 30–45 min prior to the start of experiments, these data suggest that H2O2 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 H2O2.

Though extracellular delivery of H2O2 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 H2O2 itself and antioxidant capacity of various cell types (15, 26). Though the exact intracellular concentration of H2O2 is not known, we do not believe that it was at a level that induced cell death. In our Ca2+ experiments, a short washout period was included at the end of H2O2 challenge, and during this time period we observed recovery of [Ca2+]i toward baseline levels (data not shown). Likewise, in our ECIS measurements of TER in HLMVEC treated with 250 μM of H2O2, some recovery (although not to baseline) was noted following H2O2 exposure. Moreover, cells tracked during Ca2+ 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 H2O2 in buffer solution revealed that the actual concentration of H2O2 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 H2O2.

Our Ca2+ results are consistent with other reports where H2O2 has been shown to increase [Ca2+]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), H2O2 increased [Ca2+]i and decreased TER similar to our current findings in HLMVEC. While both HLMVECs and MLVECs responded to H2O2 with an increase in [Ca2+]i, there is a clear difference in sensitivity. Unlike HLMVECs, which exhibit substantial increases in [Ca2+]i and barrier disruption (as measured by ECIS) at concentrations of H2O2 as low as 250 μM (40), reductions in MLVEC barrier function required very high concentrations of H2O2 (5). The difference in sensitivity to H2O2 with respect to barrier disruption in human and murine cells was reflected in the difference in [Ca2+]i responses to H2O2, with MLVECs exhibiting little increase in [Ca2+]i; at concentrations lower than 1 mM H2O2, 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 H2O2 responsiveness between mouse and human LMVECs served as a rationale for performing our inhibitor experiments in both cell types and validating our MLVEC observations in more clinically relevant human cell cultures.

In HLMVEC, the profile of the Ca2+ response differed based on H2O2 dose. While the highest concentration of H2O2 tested produced a large peak followed by a significantly lower but still elevated plateau, exposure to H2O2 at lower doses (i.e., 250 μM) produced an increase in [Ca2+]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 MLMVEC 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 sarcoplastic 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 heterotrimers, 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, osmolality, 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 laminar 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 MLMEVC (5). These observations, along with our current data showing significant loss of Ca\(^{2+}\) influx in response to H\(_2\)O\(_2\) in Fyn\(^{-/-}\) MLMEVCs, suggested that Fyn inhibition would attenuate H\(_2\)O\(_2\)-induced Ca\(^{2+}\) influx in MLMEVCs. Surprisingly, short-term pretreatment of MLMECs 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 SU, which can target all SFK members, cannot be excluded, our hypothesis that Fyn is responsible for phosphorylation of TRPV4 is supported by the lack of H\(_2\)O\(_2\)-induced increases in [Ca\(^{2+}\)]\(_i\) in experiments performed in MLMEVCs from Fyn\(^{-/-}\) mice and MLMEVCs in which Fyn was silenced.

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). Since Fyn has been shown to translocate to various domains within the cell (10, 28, 45), we hypothesize that Fyn may function in both injurious or protective roles depending on the stimulus involved and specific cellular localization. Additionally, though our data suggest a role for basal Fyn activation, it is known that the activity of Fyn can be modulated by phosphorylation at Tyr residues 419 and 529 (10, 42). Thus it is entirely possible that stimulus-specific changes in Fyn phosphorylation may determine downstream effects of Fyn unrelated to our proposed effect on basal TRPV4 phosphorylation.

In the current study, we specifically focused on the early Ca\(^{2+}\) influx that occurs within 5–10 min of exposure to exogenous H\(_2\)O\(_2\) in LMVECs. That blockade of TRPV4 or Fyn significantly reduced the H\(_2\)O\(_2\)-induced change in TER suggests that Fyn-dependent activation of TRPV4 is an initiating event and likely a major factor governing the initial barrier response to oxidant injury. However, it is well known that ROS activates many signaling pathways, and it is likely that a myriad of other kinase and Ca\(^{2+}\) channel pathways, as well as calcium-independent mechanisms, may also play a role during both the time interval studied as well as at later time points. Further studies utilizing time-specific blockade of various channels, as well as studies utilizing inhibitors of non-Ca\(^{2+}\) dependent ROS pathways, will be required to further dissect the sequence of Ca\(^{2+}\) channels that are likely activated by ROS in endothelial cells and to more firmly establish the role of ROS-induced Ca\(^{2+}\) influx in the overall ROS response.

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.

GRANTS

Support for this study was provided by National Heart, Lung, and Blood Institute Grants F32HL-124930-02, T32HL-007534, R01HL-73859, and R01HL-075078.

DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES

2. Ahmmed GU, Mehta D, Vogel S, Holinstat M, Paria BC, Tiruppathi C, Malik AB. Protein kinase Calpha phosphorylates the TRPC1 channel


