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STIM1 Controls Endothelial Barrier Function Independently of Orai1 and Ca²⁺ Entry

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Abstract

Endothelial barrier function is critical for tissue fluid homeostasis and its disruption contributes to various pathologies, including inflammation and sepsis. Thrombin is an endogenous agonist that impairs endothelial barrier function. Here, we showed that the thrombin-induced decrease in transendothelial electric resistance of cultured human endothelial cells required the endoplasmic reticulum-localized, calcium-sensing protein STIM1, but was independent of Ca²⁺ entry across the plasma membrane and the Ca²⁺ release-activated Ca²⁺ channel protein Orai1, which is the target of STIM1 in the store-operated calcium entry pathway. We found that STIM1 coupled the thrombin receptor to activation of the guanosine triphosphatase RhoA, stimulation of myosin light chain phosphorylation, formation of actin stress fibers, and loss of cell-cell adhesion. Thus, STIM1 functions in pathways that are dependent and independent of Ca²⁺ entry.

INTRODUCTION

The vascular endothelium forms a tightly regulated barrier between the bloodstream and the interstitial space in tissues. Thrombin is a vascular mediator that acts on a class of G protein-coupled receptors (GPCRs), the protease-activated receptors (PARs) (1). In endothelial cells, the protease-activated receptor-1 (PAR-1) can couple to G_i, G_q, and G_{12/13} and disrupt the endothelial barrier (2, 3). Activation of Rho guanosine triphosphatase (GTPase) downstream of PAR-1 stimulation plays a central role in controlling the actin cytoskeletal rearrangements necessary for increased endothelial permeability (4, 5). The Rho guanine

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nucleotide exchange factors (GEFs) link $G_{\alpha_{12/13}}$ and Rho activation during PAR-1 signaling (6–9). However, a second pathway from GPCR activation to endothelial barrier function has been extrapolated from contractility studies in muscle, in which GPCR activation stimulates Ca^{2+} entry across the plasma membrane through the store-operated Ca^{2+} entry (SOCE) pathway and this Ca^{2+} entry signal (not Ca^{2+} release from internal stores) activate myosin light chain kinase (MLCK) (10). In endothelial cells, activation of this pathway would result in MLCK-mediated phosphorylation of MLC, formation of actin stress fibers, and endothelial “contractility,” which would result in loss of intercellular barrier function (11–14). Another possible pathway through which PAR-1 may couple to Ca^{2+} signals is through activation of transient receptor potential channels (TRP) (15).

SOCE is a ubiquitous receptor-evoked Ca^{2+} entry pathway in non-excitabile cells whereby the depletion of inositol 1,4,5-trisphosphate (IP_3)-sensitive endoplasmic reticulum (ER) Ca^{2+} stores stimulates Ca^{2+} entry across the plasma membrane (16, 17). Biophysically, SOCE is mediated through the Ca^{2+} release-activated Ca^{2+} (CRAC) current (18), a highly Ca^{2+} -selective channel (19, 20). Stromal interacting molecule 1 (STIM1) and Orai1 are the molecular components of SOCE and CRAC (21–24). STIM1 is the Ca^{2+} sensor, present mainly in the ER membrane, which senses the decrease in ER Ca^{2+} concentrations, aggregates into puncta, and redistributes into discrete regions of close ER-plasma membrane contacts to activate the plasma membrane-localized Orai1 channel, which mediates CRAC (25–27). STIM1 gates Orai1 channels through direct protein-protein interactions involving binding of a C-terminal 100 amino acid domain of STIM1 [called SOAR for STIM/Orai-activating region(28)] to the C- and N-termini of Orai1(28, 29).

Earlier studies, prior to discovery of STIM1/Orai1 proteins, proposed that SOCE in endothelial cells involves either one of the TRPC isoforms, TRPC1 or TRPC4, or a heteromultimeric channel composed of TRPC1 and TRPC4 (13, 15, 30–35). However, TRPC encode non-selective cation channels and their involvement in SOCE remains highly controversial (25, 36). In human umbilical vein and pulmonary artery endothelial cells (HUVECs and HPAECs, respectively), thrombin activates SOCE and CRAC mediated by STIM1 and Orai1, independently of TRPC1 and TRPC4 (37). Here, we showed that another primary endothelial cell type, human dermal microvascular endothelial cells (HDMECs) display STIM1- and Orai1-mediated SOCE and CRAC in response to either agonist stimulation with thrombin or passive store depletion. We further showed that thrombin-mediated decrease in trans-endothelial electric resistance (TER), an indication of endothelial barrier disruption, in HUVECs and HDMECs required STIM1 independently of Orai1, MLCK, and Ca^{2+} entry across the plasma membrane. Although knockdown of TRPC1, TRPC4, or TRPC6 inhibited thrombin-mediated disruption of endothelial barrier function, the role of STIM1 in endothelial barrier function was not due to STIM1-mediated interaction with TRPC channels. Rather, STIM1 was required for RhoA activation, MLC phosphorylation, and formation of actin stress fibers.

RESULTS

STIM1, but not Orai1, is required for thrombin-mediated disruption of endothelial barrier function

To investigate the role of STIM1 and Orai1 in thrombin-mediated disruption of endothelial barrier function we knocked down STIM1 or Orai1 in HUVEC with specific siRNA (Fig. 1A). STIM1 knockdown substantially inhibited thrombin-mediated loss of HUVEC barrier function measured as the change in TER using Electric Cell-substrate Impedance Sensing (ECIS)(38) (Fig. 1B). Two additional siRNA against STIM1 (#3 and #6) resulted in STIM1 knockdown of varying degrees (fig. S1A) and the ability to influence the HUVEC response to thrombin correlated with the effectiveness of knockdown (fig. S1B). However, knockdown of Orai1 failed to affect HUVEC TER in response to thrombin (Fig. 1B). Previous studies reported lack of correlation between receptor-regulated Ca^{2+} signals and loss of endothelial barrier function in response to thrombin (39, 40): Whereas 5 nM thrombin caused maximal decrease in TER, 100 nM thrombin was required to cause a maximal Ca^{2+} signal. Therefore, we tested whether thrombin-mediated decrease in TER might display a different requirement for STIM1 or Orai1 at high concentrations of thrombin. Even in the presence of 100 nM thrombin, only STIM1 siRNA but not Orai1 siRNA impaired the thrombin-induced decrease in TER (Fig. 1C).

To confirm that the differential effects of STIM1 knockdown were not the result of differences in the abilities of the monolayers to establish a barrier, we monitored the effect of siRNA against STIM1 and Orai1 on barrier establishment of cells plated on ECIS plates. Cells in which STIM1 or Orai1 were knocked down established the same maximal TER in a similar period of time as did cells with the control siRNA (fig. S2A). Furthermore, control non-targeting siRNA did not render endothelial monolayers more susceptible to changes in TER because vehicle addition to monolayers failed to cause changes in TER, whereas thrombin produced the typical drop in TER (fig. S2B).

We previously showed that STIM1 and Orai1 mediate SOCE and CRAC currents in HUVECs independently of TRPC1 and TRPC4 (37). However, the involvement of Orai1 in SOCE may be unique to HUVECs and other endothelial cell types, including mouse microvascular endothelial cells, might mediate SOCE through STIM1-dependent regulation of TRPC4 channels (41). Therefore, we investigated the involvement of STIM1 and Orai1 in SOCE and CRAC currents in another endothelial cell type, primary HDMECs. SiRNA against either STIM1 or Orai1 caused significant and specific knockdown of their respective proteins in HDMECs (Fig. 2A) and essentially abrogated SOCE activated by thrombin (Fig. 2B). We measured CRAC in HDMECs activated by passive store depletion with the calcium chelator BAPTA dialyzed through the patch pipette in both Ca^{2+} -containing and divalent-free (DVF) bath solutions (Fig. 2C). In DVF bath solutions, Na^+ ions become the charge carrier. The first DVF switch that was performed right after whole-cell break-in, at the time when CRAC development was negligible, was used to gauge background currents before store depletion. BAPTA dialysis produced small, slowly developing inward Ca^{2+} current in control cells that was fully activated within 4–5 minutes. This current was potently amplified by replacing the Ca^{2+} -containing bath solution with a DVF solution and was

blocked by lanthanides (Gd^{3+}). This CRAC current was absent in the HDMECs in which STIM1 or Orai1 was knocked down (Fig. 2C). The current/voltage (I/V) curves for siRNA control, siRNA STIM1, and siRNA Orai1 based on the traces shown in Fig. 2C showed an inwardly rectifying current with positive reversal potential, which is indicative of high Ca^{2+} selectivity (Fig. 2D). Analyses on CRAC measured at -100 mV in DVF bath solutions, conditions in which the current is carried by Na^+ ions instead of Ca^{2+} ions, from HDMECs transfected with control non-targeting siRNA, siRNA STIM1, or siRNA Orai1 confirmed the requirement of STIM1 and Orai1 in mediating this current (Fig. 2E). Although either STIM1 or Orai1 knockdown inhibited SOCE and CRAC in HDMECs, only STIM1 knockdown inhibited thrombin-mediated decrease in TER, suggesting that thrombin-induced change in endothelial barrier function may not require Orai1 or involve SOCE (fig. S3).

Thrombin-mediated disruption of endothelial barrier function is independent of Ca^{2+} entry

The data suggested that Orai1 did not participate in the control of thrombin-induced decrease in TER and questioned the need for Ca^{2+} entry in this process. One could suggest that Orai1 knockdown was not 100% and that even a small amount of Ca^{2+} entering the endothelial cells might be sufficient to drive the decrease in TER. Removal or chelation of Ca^{2+} from the outside milieu as a means to assess the role of Ca^{2+} entry in endothelial barrier function is not practical, because Ca^{2+} ions are required for cell adhesion and maintenance of endothelial monolayer integrity and results obtained from such protocol would be difficult to interpret. Therefore, we used an approach in which Ca^{2+} entry in response to thrombin was completely abrogated in HDMECs by physiological Na^+ -based bath solutions containing 2 mM Ca^{2+} and 10 μ M Gd^{3+} , which inhibits Ca^{2+} entry including that through SOCE triggered by thapsigargin (an inhibitor of the ER-localized calcium ATPase) or thrombin in endothelial cells (37). Pre-incubation of cells with 10 μ M Gd^{3+} inhibited Ca^{2+} entry in response to thrombin with no effect on Ca^{2+} release. Ca^{2+} release was measured in physiological Na^+ -based bath solutions nominally free of Ca^{2+} , and Ca^{2+} entry was measured in the same cells with the same solutions subsequently supplemented with 2 mM Ca^{2+} (Fig. 3A; fig. S4). We measured the TER of the HDMEC monolayer under basal conditions and in response to thrombin in parallel from the same population of cells and in the same conditions and found that the cells exhibited a reversible decrease in TER in response to thrombin. Under conditions in which Ca^{2+} entry was inhibited by Gd^{3+} in HDMECs, the decrease in TER produced by thrombin was similar to the decrease in the absence of Gd^{3+} (Fig. 3B), strongly arguing that the thrombin-induced decrease in TER was independent of Ca^{2+} entry and thus SOCE.

Because experiments with thapsigargin to disrupt endothelial cell monolayers were interpreted as evidence that SOCE is important for decreasing endothelial barrier function (32), we measured the TER of HUVECs and HDMECs exposed to thapsigargin and incubated in Na^+ -based bath solutions containing 2 mM Ca^{2+} in the absence or presence of 10 μ M Gd^{3+} to block SOCE. The effects of thapsigargin on TER were distinct from those of the physiological agonist, thrombin. Initially, thapsigargin enhanced TER, followed by a steady decrease in TER (Fig. 3C), which occurred in the presence or absence of Gd^{3+} . To decipher the response to thapsigargin with increased time resolution, we measured TER at shorter intervals and found that thapsigargin triggered an initial, immediate, and sharp

decrease in TER, which was followed by a phase during which TER increased, in agreement with Cioffi *et al.* (fig. S5A). This early transient decrease in TER that occurred in response to thapsigargin was insensitive to 10 μ M Gd^{3+} (fig. S5B), suggesting that it is independent of SOCE and Ca^{2+} entry.

STIM1 functions independently of EB1 and FAK in the thrombin-induced decrease in TER

Because STIM1 and the plus-end microtubule-binding protein EB1 interact (42), we investigated if the STIM1-EB1 interaction contributed to STIM1-mediated loss of TER in response to thrombin. We performed “erase and replace” experiments in which endogenous STIM1 was depleted from the HUVECs by siRNA followed by rescue with either wild-type STIM1 or STIM1 with a mutation in the EB1 binding domain (EB1 Mut.) that cannot interact with EB1 and thus cannot bind microtubules (42). Both versions of STIM1 rescued the thrombin-induced decrease in TER (Fig. 3D), which indicates that the interaction of STIM1 with EB1 was not essential for disruption of endothelial barrier function. STIM1 knockdown led to a significant increase in basal phosphorylation of focal adhesion kinase (FAK) and paxillin in HUVECs (fig. S6), Thrombin stimulation of HUVECs knocked down of STIM1 caused further increase in FAK and paxillin phosphorylation (fig. S6A). However, quantification of data from 4 independent experiments revealed that thrombin-mediated increase in paxillin phosphorylation in STIM1 knocked down cells was marginal compared to control (fig. S6B).

STIM1 functions independently of TRPC channels in the thrombin-induced decrease in TER

TRPC1 (32, 35), TRPC4 (30, 31, 33, 34), and TRPC6 (43–46) channels have been proposed to play a role in endothelial barrier function and STIM1 stimulates TRPC channels through a mechanism involving electrostatic interactions between 2 positively charged residues in the STIM1 C-terminus (Lys⁶⁸⁴, Lys⁶⁸⁵) and 2 negatively charged residues in TRPC1 (Asp⁶³⁹, Asp⁶⁴⁰) (47). Individual knockdown of TRPC1, TRPC4, or TRPC6 significantly inhibited the thrombin-mediated decrease in TER in HUVECs, with each TRPC4 and TRPC6 knockdown showing a greater effect than TRPC1 knockdown (Fig. 4A), which correlated with the different efficiencies of TRPC isoform knockdown (fig. S7).

To determine whether the role of STIM1 in thrombin-mediated disruption of endothelial barrier function was mediated through activation of TRPC channels, we used an enhanced yellow fluorescent protein (eYFP)-tagged K684E, K685E mutant STIM1 (KK/EE) that does not support electrostatic interactions with TRPC channels but does activate Orai1 channels (47). Overexpression of this STIM1 KK/EE in either HUVECs (Fig. 4B, C) or HDMECs (Fig. 4D, E) enhanced Ca^{2+} entry upon stimulation with either thrombin or thapsigargin, confirming that SOCE in endothelial cells is mediated through STIM1 activation of Orai1 channels. Erase and replace experiments in HUVECs showed that STIM1 KK/EE rescued the thrombin-mediated decrease in TER in a manner similar to that of wild-type eYFP-STIM1 (Fig. 5A, B). Thus both TRPC channels and STIM1 contributed to the control of endothelial barrier function, but the effects of STIM1 on TER involve other downstream targets in addition to TRPC channels.

STIM1 contributes to the thrombin-induced decrease in TER through RhoA, but not MLCK

The thrombin-induced decrease in TER in endothelial cell monolayers requires cytoskeletal rearrangements, including the rearrangement of the actin cytoskeleton from peripheral actin to stress fibers and phosphorylation of myosin light chain (MLC) to mediate contraction of the actin cytoskeleton. Depletion of STIM1 prevented the thrombin-mediated increase in stress fibers containing phosphorylated MLC in HUVECs (Fig. 6A), suggesting that STIM1 is required for thrombin-induced stress fiber formation. Control cells had more stress fibers and substantial disruption of VE-cadherin cell-cell junction in response to thrombin (Fig. 6A, white arrows in panels 2 and 4) compared to the STIM1-knockdown cells. Furthermore, control cells had a more substantial staining and colocalization of actin stress fibers and phosphorylated MLC compared to STIM1-knockdown cells (Fig. 6A, panels 6 and 8).

Phosphorylation of MLC can be mediated by the ROCK, a kinase activated by RhoA (48), or by MLCK, a kinase activated by calcium-calmodulin (49). Because SOCE was not required for thrombin-induced reduction in TER in endothelial cell monolayers, we hypothesized that MLCK would not be involved in this process, since MLCK is activated by Ca^{2+} signaling through Ca^{2+} /calmodulin. Knockdown of MLCK did not prevent the thrombin-mediated decrease in HUVEC TER (Fig. 6B). We used 2 independent siRNAs targeting MLCK (siMLCK#2 and siMLCK#3; fig. S8A). A third siRNA targeting MLCK (siMLCK#1; fig. S8A) was less efficient at reducing MLCK abundance and affected cell viability and, therefore, not used for TER experiments. Similar to HUVECs, we knocked down MLCK in HDMECs with 2 independent siRNAs (fig. S8B) and this failed to effect thrombin-induced decrease in TER in these endothelial cell monolayers (fig. S8B).

To determine if the thrombin-mediated reduction in TER that involved STIM1 was mediated by RhoA, which could influence MLC phosphorylation through ROCK, we measured RhoA activity in thrombin-treated HUVECs in which STIM1, Orai1, or MLCK were knocked down. Only STIM1 knockdown significantly reduced RhoA activity in response to thrombin (Fig. 6E), indicating that STIM1 functions as an upstream effector coupling the PAR1 receptor to RhoA during thrombin-mediated decrease in TER and disruption of endothelial barrier function.

DISCUSSION

In a previous study (37), we reported that STIM1 and Orai1 mediate SOCE and CRAC currents in HUVECs. In this study, we showed that SOCE and CRAC currents in HDMECs were also mediated by STIM1 and Orai1, indicating that the involvement of STIM1 and Orai1 in SOCE is not unique to HUVECs (41). Using HUVEC and HDMEC monolayers and ECIS, we found that STIM1 and Orai1 did not function together in thrombin-mediated disruption of endothelial barrier function. STIM1 was required for thrombin-mediated decrease in TER of HUVECs and HDMECs; whereas Orai1 was not. The independence of the thrombin-induced reduction in TER on Orai1, along with the independence of the thrombin TER response on Ca^{2+} entry, indicated that SOCE was not involved in mediating disruption of endothelial barrier function in response to the physiological agonist thrombin. These results are in agreement with previous studies indicating that the thrombin-induced disruption in endothelial barrier function is independent of G_q -mediated Ca^{2+} signals (39).

However, the SOCE-independent nature of the thrombin response contrasts with the conclusions of studies that used thapsigargin, which activates SOCE, to disrupt endothelial barrier function (32, 50).

To explore this discrepancy, we used ECIS to monitor the nature and kinetics of the change in TER in endothelial monolayers exposed to thapsigargin stimulation and found that the effect on TER of thapsigargin was different from that of the physiological agonist thrombin. Whereas thrombin caused a substantial decrease in TER that was reversible within 2–3 hours, thapsigargin triggered more complex effects: Thapsigargin caused a sharp, low amplitude, and brief (lasting less than 15 minutes) decrease in TER, followed by an increase in TER within 1 hour, and then a slow, decrease in TER and loss of monolayer integrity. Thapsigargin is an inhibitor of the ER calcium ATPase, which not only disrupts calcium signaling, but also ER function, and the loss of monolayer integrity likely reflects the toxic nature of long-term exposure to thapsigargin. We found that the response of endothelial monolayers to thapsigargin was the same when Ca^{2+} entry was blocked with low concentrations of lanthanides, indicating that the thapsigargin-induced changes in TER were independent of Ca^{2+} entry and SOCE.

TRPC1, TRPC4, and TRPC6 channels have all been implicated in control of endothelial barrier function (30–35, 43–46). Our data showing that TRPC1, TRPC4, or TRPC6 knockdown inhibited thrombin-induced decrease in TER are in agreement with these studies and highlight the importance of TRPC channels in endothelial barrier function. However, we found that TRPC channel-mediated disruption of endothelial barrier function was independent of Ca^{2+} entry and that the STIM1-mediated reduction in HUVEC or HDMEC TER that occurred in response to thrombin was independent of the STIM1 and TRPC interaction. Indeed, introduction into either HUVECs or HDMECs of a mutant version of STIM1 that cannot interact with TRPC channels but can interact with Orai1 (47) failed to inhibit and, instead, potentiated SOCE in response to thapsigargin and thrombin. These data indicated that SOCE in endothelial cells is mediated by CRAC channels composed of STIM1 and Orai1, independently of TRPC channels, and that TRPC channels were not responsible for the STIM1-mediated response.

In vitro Fluorescence resonance energy transfer (FRET), coimmunoprecipitation assays, and electrophysiological studies in pulmonary artery endothelial cells in which Orai1 was knocked down indicated that Orai1 interacts with TRPC1/4 channels in endothelial cells to confer their Ca^{2+} selectivity (32). However, the electrophysiological data (32) are difficult to interpret because the conditions used would have caused store depletion, which would activate SOCE through Orai1 and TRPC channels in response to Ca^{2+} released from the endoplasmic reticulum or Ca^{2+} entry through Orai1 (36). The effect of Orai1 knockdown on the whole-cell currents is consistent with activation of several conductances that would be detected under the conditions used. The finding that at high Ca^{2+} concentrations the current is increased only when Orai1 is present can also be explained by Ca^{2+} block of Orai1 at low external Ca^{2+} concentrations (51, 52). Therefore, the data in Cioffi *et al.* (32) are fully compatible with the presence of two independent, but closely-associated, channels in endothelial cells: a store depletion-activated Ca^{2+} -selective channel composed of Orai1 and a Ca^{2+} -activated non-selective channel composed of TRPC. Indeed, both Orai1 and TRPC

are activated by phospholipase C (PLC)-coupled agonists (53, 54) and local Ca^{2+} entry through Orai1 stimulates TRPC1 and TRPC5 channel activity (55, 56).

How TRPC channels couple to endothelial barrier function remains a puzzle. TRPC channels may contribute to a Ca^{2+} signal, may function as adaptor proteins, or may trigger membrane depolarization by mediating Na^+ entry. Membrane depolarization activates RhoA and disrupts barrier function (57). Indeed, we identified activation of RhoA as a potential mechanism for the STIM1-mediated contribution to thrombin-mediated disruption of endothelial barrier function. Consistent with ROCK as a kinase that phosphorylates MLC (48), we found that STIM1 knockdown caused increased basal phosphorylation of FAK and paxillin, and inhibited RhoA activity, MLC phosphorylation, and actin stress fiber formation in response to thrombin, providing a mechanism for STIM1 action.

Unexpectedly, we found that knockdown of MLCK failed to affect the reduction in TER that occurred in response to thrombin. Despite effective MLCK knockdown, the residual amounts of MLCK in our cells might be sufficient to decrease TER in response to thrombin or the studies previously implicating MLCK may have alternative interpretations. Earlier work that implicated MLCK in endothelial barrier function was based on the use of pharmacological inhibitors, such as ML7 and ML9, or peptide inhibitors (49, 58, 59), and the results might reflect the nonspecific nature of MLCK inhibitors and peptides used in these studies. Interestingly, ML9 efficiently blocks STIM1 movement and reorganization in response to store depletion, and this effect is independent of MLCK inhibition (60). Several studies have implicated MLCK as contributing to disruption of endothelial barrier function. Introduction of constitutively active MLCK into coronary venular endothelial cells increased phosphorylation of MLC and increased transendothelial flux of albumin across endothelial monolayers (58). Mice with an endothelial-specific knockout of MLCK (MLCK210) have reduced microvascular hyperpermeability in models of atherosclerosis or in response to severe burns (61, 62). However, another study with endothelial-specific MLCK-knockout mice found that the increase of microvascular endothelial permeability in response to lipopolysaccharides was not dependent on MLCK (63), in agreement with our study here. The reasons for these discrepancies are unclear, but it seems likely that the involvement of MLCK in regulating endothelial permeability depends on the stimulation or endothelial bed considered.

There is a minor pool of STIM1 at the plasma membrane (~10–15% of total STIM1) that was proposed to play a role in the activation of the store-independent arachidonate-regulated Ca^{2+} (ARC) channels (64). Our data show that the effects of STIM1 on TER responses to thrombin could be rescued by eYFP-tagged STIM1 that does not reach the plasma membrane (65), suggesting the STIM1 in the ER is likely mediating the effects on RhoA and TER in response to thrombin. An important question that future studies should address is how does STIM1 couple PAR1 receptor to RhoA activation? Erase and replace ECIS experiments with STIM truncation mutants may determine the region of STIM1 necessary for the reduction in TER in response to thrombin. This region of STIM1 could be subsequently used to identify additional binding partners and signaling molecules using affinity purification or yeast two hybrid studies. This knowledge will likely reveal STIM1

partners and shed light on the molecular mechanisms controlling endothelial barrier function.

MATERIALS AND METHODS

Reagents

The BCA Protein Assay Kit, Super Signal West Pico, West Femto and Restore Western Blot Stripping Buffer were from Pierce Biotechnology, Inc. (Rockford, IL). The Quickchange® II Site- Directed Mutagenesis kit was purchased from Stratagene (La Jolla, CA). iQ™ SYBR® Green Supermix was from Bio-Rad (Hercules, CA) and RNeasy Mini Kit and QIA Shredder were from Qiagen (Valencia, CA). Gd³⁺ was purchased from Acros Organics; thapsigargin was from Calbiochem. Na-methanesulfonate and Cs-methanesulfonate, and thrombin were purchased from Sigma (St. Louis, MO, USA). Cs-BAPTA was from Invitrogen (Eugene, OR, USA). siRNA sequences for MLCK were obtained from Invitrogen and all other siRNA sequences were from Dharmacon. Specific primers for STIM1, the STIM1 EB1 mutant, with point mutations Ile⁶⁴⁴ to Asn and Pro⁶⁴⁵ to Asn, and MLCK were synthesized by Integrated DNA Technologies. All other chemical products were obtained from Fisher Scientific (Pittsburgh, PA, USA) unless specified otherwise. Antibodies used for Western blots and immunofluorescence are listed in table S1, which contains source, species, and dilutions used.

Cell culture

HUVECs were purchased from Cascade Biologicals (Invitrogen, Carlsbad, CA). HUVECs were grown in EBM-2 Bullet Kit (CC-3156 & CC-4176) (Lonza Walkersville, Inc., Walkersville, MD) containing 2% fetal bovine serum (FBS). HDMECs were isolated from neonatal foreskins (obtained from the maternity ward of the Albany Medical Center Hospital with IRB approval) by incubating the obtained cells with magnetic beads coated with an antibody to CD31 (DYNAL) as described previously(66). Cells were assessed for VE-cadherin and lectin binding to confirm their endothelial origin and then frozen in liquid nitrogen. HDMECs were grown in EGM-2MV BulletKit (CC-3156 & CC-4147) (Lonza Walkersville, Inc.) containing 5% FBS. For all experiments, HUVECs and HDMECs were between passages 2 and 6.

TER experiments

Changes in TER, which is a measure of endothelial barrier integrity of HUVEC and HDMEC monolayers, was determined using electric cell-substrate impedance sensing apparatus (ECIS, Applied BioPhysics Inc, Troy NY), as described in detail previously (38). Briefly, HUVECs or HDMECs (1.2×10^5 cells) transfected with either non-targeting control siRNA, siRNA targeting specific proteins, or STIM1 plasmids or lentiviruses were seeded onto ECIS 10E culture ware ($0.8 \text{ cm}^2/\text{well}$) pre-coated with 0.2% gelatin, and the cells were incubated for 3 days. The cells were serum-starved for 4h. The electrical impedance across the monolayer was measured at 1 V, 4000 Hz with current flowing through 10 small gold electrodes per well plus one large counter electrode, using the culture medium as the source of electrolytes. Once resistances were relatively constant, stimuli (thrombin, thapsigargin) were added directly to the wells. Impedance was monitored by the lock-in amplifier, stored,

and resistance and capacitance were calculated with the manufacturer's software. For comparing experimental conditions, data were normalized to the mean resistance of each condition once the monolayers had a constant resistance immediately prior to stimuli addition.

For ECIS experiments using pre-incubation with 10 μM Gd^{3+} and the corresponding controls, the Na^+ -based medium was adapted from Waheed *et al.* (57), with a slight modification. The composition was as follows: 130 mM NaCl, 3 mM KCl, 1 mM MgCl_2 , 2 mM CaCl_2 , 5 mM glucose, and 20 mM Na-HEPES pH 7.4.

Immunofluorescence assays

HUVECs were transfected with control and STIM1 siRNAs. Cells (1.2×10^5 cells/well) were seeded on Biocoat 8-well glass culture slides (BD Falcon) pre-coated with 0.2 % gelatin (Acros Organics). Two days later, cells were serum starved in EBM2 supplemented only with 0.3% FBS for 16 h before adding 10 nM thrombin for 5 minutes. The cells were fixed for 30 min with 4% paraformaldehyde and processed for immunofluorescence with antibodies recognizing VE-cadherin or phosphorylated MLC at Thr¹⁸ and Ser¹⁹, (67)] (see table S1 for details) and with Alexa 594- or Alexa-647-conjugated secondary antibodies. F-actin was stained with either Alexa-488- or Alexa-594-conjugated phalloidin and nuclei were stained with DAPI. Images were obtained using a Zeiss Axio Observer Z1 inverted microscope with the Apotome module and Zeiss AxioVision software.

siRNA transfections

Several different siRNAs per target gene were initially assessed for their ability to reduce mRNA abundance using quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR; the primers used for each target mRNA and the different siRNA sequences used in the study are listed in table S2. SiRNA sequences that induced significant decreases in their target mRNA (over 70%) without effecting other mRNAs were used and knockdown efficiency was confirmed by Western blotting. All transfections in HUVECs and HDMECs were performed using the Nucleofector device II (Amata Biosystems, Gaithersburg, MD) with program #A-034 and M-003, respectively, according to the manufacturer's instructions. Green fluorescent protein (GFP, 0.5 μg) was co-transfected with siRNA for identification of successfully transfected cells and to serve as a control for YFP- or GFP-tagged plasmid constructs used in transfections. Efficiency of siRNA and plasmid transfections typically exceeded 95% and 75%, respectively.

Production of lentiviruses

Precision LentiORF individual clone for STIM1 (STIM1 pLOC, Accession number DQ895509) was from Open Biosystems. This lentiviral vector has nuclear localized TurboGFP. In this system, because of a 2a self cleaving peptide, both STIM1 and TurboGFP are translated as two distinct proteins. This lentiviral STIM1 ORF and STIM1 (EB1 mut were used to produce lentiviruses. Viral particles were generated using standard protocols. PolyJet was used as a transfection reagent (SignaGen) to transfect HEK293FT cells (Invitrogen). Briefly, the lentiviral constructs pCMV-VSVG, pCMV-dR8.2 and either STIM1 pLOC or STIM1 pLOC STIM1 EB1 mut were cotransfected into a flask of 95%

confluent HEK293FT cells. Cell culture media with viral particles were collected at 48h and 72h after transfection and was concentrated using Amicon Ultra-15 filter (Millipore, Billerica, Massachusetts) by centrifugation and stored at -80°C . For in vitro infections, cells were typically assayed 7 days post-infection.

HUVECs were infected at 50% confluence in EBM-2 supplemented with 5% heat-inactivated FBS. Media was changed to growth media and the infected cells were allowed to grow to confluence before trypsinization and reseeding for experiments. Infection efficiency was evaluated by visualization of nuclear GFP in live cells.

Erase and replace assays

HUVECs (1×10^6) were electroporated with either control siRNA or STIM1 siRNA (20 μg) and incubated for 72 hours. Cells were then detached and electroporated again with siRNA along with plasmids encoding human STIM1 or STIM1KK/EE mutant. Cells were allowed to recover for an additional 48 hours after which, the cells were serum starved using MCDB 131 media (12 hr) (Gibco, Invitrogen). Cells were stimulated with thrombin (5 nM) and TER was measured using ECIS. Cells were plated in parallel dishes to verify protein production from the STIM1 plasmids by Western blots or by fluorescence microscopy (for eYFP-STIM1 plasmids). For lentiviral transfections, HUVECs (1×10^6) were electroporated with either control siRNA or STIM1 siRNA (20 μg) and seeded $< 50\%$ confluence in 6-well dishes. On the following day, these cells were infected with lentiviral particles encoding either STIM1 or STIM1 EB1 mut and allowed to recover for 3 days. The presence of nuclear TurboGFP served as a gauge of the efficiency of infection. On the 4th day, the cells were electroporated again with either control or STIM1 siRNAs and plated onto the ECIS plates. Cells were allowed to recover for an additional 48 hours after which the cells were serum starved using MCDB 131 media (12 hr) (Gibco, Invitrogen). After serum starvation, cells were treated with thrombin (5 nM) and TER was measured. Cells were plated in parallel dishes to verify protein expression of STIM1 constructs by Western blots and fluorescence microscopy.

Calcium imaging

HUVECs and HDMECs were cultured on 30-mm glass coverslips for performing Ca^{2+} imaging as previously described (37). Coverslips with cells attached were mounted in a Teflon chamber. Cells were incubated (37°C , 25–30 min) in culture medium containing 4 μM Fura-2/AM. Upon completion of incubation, cells were washed 3 times and bathed in HEPES-buffered saline solution (140 mM NaCl, 1.13 mM MgCl_2 , 4.7 mM KCl, 2 mM CaCl_2 , 10 mM D-glucose, and 10 mM HEPES; pH 7.4) for at least 5 min before Ca^{2+} measurements were made. For Ca^{2+} measurements, cells were stimulated by either thrombin or thapsigargin at the concentrations indicated in the a nominally Ca^{2+} -free solution and Ca^{2+} was restored to the extracellular milieu after the Ca^{2+} release phase was complete. This protocol separates detection of intracellular Ca^{2+} release from Ca^{2+} entry. A digital fluorescence imaging system (InCyt Im2; Intracellular Imaging Inc., Cincinnati, OH) was used for measurement of Fura2 fluorescence signal and fluorescence images of several cells were recorded and analyzed simultaneously. The 340/380 nm ratio images were obtained on pixel-by-pixel basis. Figures showing Ca^{2+} traces are an average from several cells attached

on one coverslip and are representative of several independent recordings as mentioned in the figure legends. For Ca^{2+} measurements depicted in Fig. 3, the bath solutions used were the Na^{+} -based solutions (with and without 2 mM Ca^{2+} ; see composition under “ECIS section” above) instead of regular HBSS.

Western blotting

Cells were transfected with either the control nontargeting siRNA or STIM1, Orai1, or MLCK-specific siRNAs. Three days after transfection, cells were rinsed and lysed on ice in standard RIPA lysis buffer [50 mM Tris-HCl (pH 8), 150 mM NaCl, 1% Triton X-100, 0.2 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate), 2 mM phenylmethylsulfonyl fluoride (PMSF), 10% protease inhibitor cocktail (Roche), 10% phosphatase inhibitor cocktail (Roche)], and protein concentrations were determined using the BCA Assay Reagent (Pierce). Lysates (50–200 μg) were subjected to SDS-PAGE electrophoresis and transferred to Immuno-Blot PVDF membranes (Bio-Rad). Membranes were blocked (5% nonfat milk in TBS-Tween, 4°C overnight), incubated with antibodies (5% nonfat Milk in TBS Tween, 4°C overnight), washed, and then incubated with horseradish peroxidase (HRP)-linked secondary antibodies (2% nonfat milk, TBS-Tween for 1 hr). Bound antibodies were detected by enhanced chemiluminescence using Super Signal West Pico or Femto reagents (Pierce). Signal intensity was measured with a Fuji LAS4000 Imaging Station. Membranes were then stripped and reprobed with an antibody against β actin to verify equal loading and densitometric analysis was performed using Image J software. For phosphorylated FAK and phosphorylated paxillin experiments, cells were serum starved (6 hr), then placed on plate warmer (37°C) and stimulated with HBSS (with 2 mM Ca^{2+} and 10 mM HEPES) followed by thrombin addition (10 nM). The dishes were then transferred to ice and lysed in RIPA lysis buffer (0.2 ml lysis buffer per 35-mm dish) after various time points. Lysates were collected into ice-cold 1.5-ml tubes and cleared by centrifugation at 14,000 rpm in a microfuge at 4°C for 10 min and snap frozen in liquid nitrogen and stored at -80°C until use.

RhoA activity assays

RhoA activity was measured using Rho G-LISA Activation Assays Biochem Kit™ (Cytoskeleton Inc., Denver CO), according to the manufacturer's recommendations. HUVECs were electroporated with siRNAs, seeded at confluence and grown for 3 more days to form mature confluent monolayers in 35-mm dishes. Monolayers were washed twice at room temperature with EBM-2 medium and incubated (4 hr) before stimulation in HBSS (containing 2 mM Ca^{2+} and 10 mM HEPES) with thrombin (5 nM). After 5 min, solutions were aspirated and cell lysis buffer (4°C) was added to culture dishes placed on ice. Cell lysates were centrifuged (14,000 rpm 4°C, 2 min in a microfuge) and an aliquot was removed for determination of protein concentration by BCA Protein Assay Kit (Pierce). Following adjustment for protein concentration, cell lysates were snap frozen and stored at -80°C . After thawing, 50 μl of lysate was added to the wells of plates coated with RhoA binding domain peptides. Additional wells were filled with lysis buffer or constitutively active RhoA as negative and positive controls, respectively. Plates were placed immediately on an orbital shaker (400 rpm, 4°C, 30 min), washed twice with HBSS, and antigen-presenting buffer (200 μl) was added to each well (2 min, at room temperature). After three

washes, diluted RhoA primary antibody (50 μ l) was added (45 min with shaking). Following 3 washes, secondary HRP-labeled antibody (50 μ l, diluted 1:100) was added to each well (45 min), followed by 3 washes and HRP detection reagent (37°C, 15 min). Finally, HRP stop buffer (50 μ l) was added and the signal measured immediately at 490 nm using a microplate spectrophotometer.

Whole-cell patch clamp experiments

Whole-cell patch clamp recordings were carried out using an Axopatch 200B and Digidata 1440A (Axon Instruments) as previously published (68–70). Clampfit 10.1 software was used for data analysis. Pipettes were pulled from borosilicate glass capillaries (World Precision Instruments, Inc.) with a P-97 flaming/brown micropipette puller (Sutter Instrument Company) and polished with DMF1000 (World Precision Instruments, Inc.) to a resistance of 2–4 M Ω when filled with pipette solutions. HDMECs were electroporated with STIM1, Orai1, or control siRNAs 3 days before recordings. Cells were seeded on round coverslips 36 h before experiments. Immediately before the experiments, cells were washed with bath solution. Only cells with tight seals (>16 G Ω) were selected to break in. Cells were maintained at a 0 mV holding potential during experiments and subjected to voltage ramps from +150 to –140 mV lasting 250 ms every 2 s. “Reverse” ramps were designed to inhibit Na⁺ channels present in these cells. 3 μ M nimodipine was added to the bath solution to generally stabilize membrane patches and reach better seals. High MgCl₂ (8 mM) was included in the patch pipette to inhibit TRPM7 currents.

Bath Solution—135 mM Na-methanesulfonate, 10 mM CsCl, 1.2 mM MgSO₄, 10 mM HEPES, 20 mM CaCl₂, and 10 mM glucose (pH was adjusted to 7.4 with NaOH).

Pipette Solution—145 mM Cs-methanesulfonate, 20 mM Cs-1,2-*bis*-(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (Cs-BAPTA), 8 mM MgCl₂, and 10 mM HEPES (pH adjusted to 7.2 with CsOH).

Divalent-free (DVF) bath solution—155 mM Na-methanesulfonate, 10 mM HEDTA, 1 mM EDTA, and 10 mM HEPES (pH 7.4, adjusted with NaOH).

Statistical analysis

Data are expressed as means $\pm$ SE, and statistical analysis using One-way ANOVA was done with Origin 8.5 software (OriginLab, Northampton, MA). Throughout the text, figures and figure legends *, ** and *** indicates p values < 0.05, 0.01 and 0.001 respectively. Differences were considered significant when P < 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. STIM1, but not Orai1, knockdown inhibits thrombin-induced decrease in TER of HUVECs

(A) Western blots show specific knockdown of STIM1 and Orai1 with siRNA. Graph shows the efficiency of knockdown quantified from independent transfections (STIM1, n=10; Orai1, n=6). (B) Effect of STIM1 (n=10) or Orai1 (n=6) knockdown on the thrombin-induced decrease in TER. (C) Effects of STIM1 or Orai1 knockdown on the decrease in TER induced by a high concentration of thrombin (n=3). The “n” number indicates ECIS experiments from independent transfections; cells from each transfection were assayed at least in triplicate. ** indicates p values < 0.01.

Figure 2. STIM1 and Orai1 mediate SOCE and CRAC currents in HDMECs

(A) Western blots show knockdown of STIM1 and Orai1 in HDMECs. Graph shows the efficiency of knockdown quantified from 3 independent transfections. (B) Ca^{2+} imaging traces showing average from several cells assayed simultaneously from the same coverslip of each experimental condition (non-targeting control siRNA, n=11; siSTIM1, n=10; siOrai1, n=21). Quantification of Ca^{2+} entry in response to thrombin (plotted as the Fura2 ratio normalized to control) in cells transfected with the indicated siRNA is shown (control, n=6 coverslips, 105 cells; STIM1, n=4 coverslips, 53 cells; Orai1, n=7 coverslips, 164 cells). (C) Whole-cell CRAC currents elicited by store depletion with 20 mM BAPTA in the patch

pipette (pip) from HDMECs transfected with the indicated siRNA. Currents were measured in bath solutions containing 20 mM Ca^{2+} , divalent free solutions (DVF), or 5 μM Gd^{3+} as indicated. **(D)** I/V relationships of CRAC carried by Na^+ in DVF solutions obtained with voltage ramps (from -140 to $+150$ mV) from traces C, taken where indicated by the color-coded asterisks. **(E)** Graph shows the Na^+ CRAC current densities (pA/pF) measured at -100 mV from HDMECs transfected with either non-targeting control siRNA (n=4), STIM1 siRNA (n=5) or Orai1 siRNA (n=5). ** and *** indicate p values < 0.01 and 0.001 .

Figure 3. Ca^{2+} entry is not required for thrombin-induced decrease in TER

(A) Thrombin-induced SOCE in the presence or absence of the Ca^{2+} entry inhibitor Gd^{3+} (10 μM). HDMECs were preincubated with Gd^{3+} (or control) and Ca^{2+} release was assayed in the absence of extracellular Ca^{2+} , then Ca^{2+} entry was assayed in Na^+ -containing salt solutions supplemented with 2 mM Ca^{2+} . Quantification of calcium entry (plotted as the increase of Fura2 ratio normalized to control) in the control or presence of Gd^{3+} ($n = 3$ experiments and 63 cells for control and $n = 4$ experiments and 98 cells for Gd^{3+}) is shown. (B) The same HDMECs depicted in A were assayed using ECIS to determine TER response to thrombin on the same day using the same Na^+ -containing solutions supplemented with 2 mM Ca^{2+} and the same Gd^{3+} stock that blocked SOCE in the Ca^{2+} -imaging protocols. (C) The TER of HDMECs in response to thapsigargin was assayed in the absence and presence of Gd^{3+} under the same buffer conditions as used for the data shown in B. (D) Western blots from erase and replace experiments show control, knockdown of STIM1 and expression of either wild-type STIM1 or EB1 mut. STIM1 in HUVECs. The TER of HUVECs in response to thrombin was assayed in all four conditions shown. Data shown in B and C are representative of 6 independent experiments and data in D are representative of four independent experiments. *** indicates p values < 0.001 .

Figure 4. TRPC channels and TER in response to thrombin

(A) TER in response to thrombin in HUVECs in which the indicated siRNAs were expressed. Results are representative of three independent transfections (n=3). (B) The effect of the STIM1 KK/EE mutant on SOCE when expressed in HUVECs exposed to thapsigargin. (C) The effect of the STIM1 KK/EE mutant on SOCE when expressed in HUVECs exposed to thrombin. (D) The effect of the STIM1 KK/EE mutant on SOCE when expressed in HDMECs exposed to thapsigargin. (E) The effect of the STIM1 KK/EE mutant on SOCE when expressed in HDMECs exposed to thrombin. Quantification of the increase in calcium entry in B-E are from 3–5 independent experiments with a total of 17–99 cells.

Data are expressed as the change in the fluorescence ratio in response to thapsigargin (TG) or thrombin (Thr.), normalized to control (untransfected cells). ** and *** indicates p values < 0.01 and 0.001, respectively.

Figure 5. STIM1 controls TER in response to thrombin independently of TRPC channels
(A) Western blots from “erase and replace” experiments in HUVECs showing knockdown of endogenous STIM1 and expression of eYFP-tagged versions of STIM1: wild-type STIM1 (YFP-STIM1) or STIM1 KK/EE (KK/EE Mut.) **(B)** ECIS experiments from the same cells in A, show the TER responses to thrombin in the indicated cells. Data are representative of three independent experiments from independent transfections performed on separate days.

Figure 6. STIM1 controls thrombin-mediated decrease in TER through RhoA, but not MLCK
(A) Immunofluorescence of HUVEC monolayers transfected with either control non-targeting siRNA (panels 1,2,5,6) or STIM1 siRNA (panels 3,4,7,8). Nonstimulated HUVECs and cells stimulated with thrombin (10 nM for 5 min) were stained with either phalloidin (green) and an antibody recognizing VE cadherin (red; panels 1–4) or phalloidin (red) and di-phospho MLC Phospho-Threonine18, phospho-Serine19 MLC (ppMLC; green). White arrows point to areas of disrupted VE-cadherin junctions. Data are representative of three independent transfections performed on separate days. Quantification of disrupted VE-cadherin junctions was determined as average $\pm$ SE of disrupted sites per field and are 9 ± 3.7 in siControl versus 1 ± 0.4 in siSTIM1; $p<0.05$. **(B)** Effect of siRNA targeting MLCK on thrombin-induced reduction in TER (data are representative of 3 independent transfections performed on separate days). **(C)** RhoA activity was measured in HUVECs transfected with the indicated siRNA in the presence or absence of thrombin (10 nM for 5 min). Cells expressing constitutively active RhoA (Constit. RhoA) serve as a

positive control. Results are from three independent experiments representing three independent transfections performed on separate days. *, and **, indicates p values < 0.05, and 0.01.