Requirement for Ca$^{2+}$ signaling in the mechanism of thrombin-induced increase in endothelial permeability

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Sandoval, Raudel, Asrar B. Malik, Tabassum Naqvi, Dolly Mehta, and Chinnaswamy Tiruppathi. Requirement for Ca$^{2+}$ signaling in the mechanism of thrombin-induced increase in endothelial permeability. Am J Physiol Lung Cell Mol Physiol 280: L239–L247, 2001.—We compared the thrombin-activated responses in human umbilical vein endothelial cells (HUVECs) and a HUVEC-derived cell line, ECV304. Thrombin induced a 40–50% decrease in transendothelial monolayer electrical resistance and a twofold increase in $^{125}$I-albumin permeability in HUVECs, whereas it failed to alter the endothelial barrier function in ECV304 cells. Thrombin produced a brisk intracellular Ca$^{2+}$ concentration transient and phosphorylation of 20-kDa myosin light chain in HUVECs but not in ECV304 cells. Thrombin-induced phosphoinositide hydrolysis was comparable in ECV304 cells and HUVECs, indicating the activation of thrombin receptors in both cell types. La$^{3+}$ reduced both the thrombin-induced decrease in endothelial monolayer electrical resistance and the increase in $^{125}$I-albumin permeability in HUVECs. Because the absence of Ca$^{2+}$ signaling could explain the impairment in the permeability response in ECV304 cells, we studied the effect of increasing intracellular Ca$^{2+}$ concentration in ECV304 cells with thapsigargin. Exposure of ECV304 cells to thapsigargin caused decreased endothelial monolayer electrical resistance and increased $^{125}$I-albumin permeability. These results indicate that Ca$^{2+}$ influx and activation of Ca$^{2+}$-dependent signaling pathways are important determinants of the thrombin-induced increase in endothelial permeability.

human umbilical vein endothelial cells; ECV304 cells; cadherins; calcium signaling

ELLIS ET AL. (4), Nguyen et al. (21), and others (6) have shown that thrombin-induced activation of proteinase-activated receptor-1 (PAR-1) on the endothelial cell surface increases endothelial permeability. This response is associated in a sequential manner with the generation of inositol 1,4,5-trisphosphate [Ins(1,4,5)P$_3$], a rise in intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$), and protein kinase C (PKC) activation (15–19). The increase in [Ca$^{2+}$]$_i$ leads to activation of Ca$^{2+}$/calmodulin-dependent myosin light chain kinase (MLCK) (5, 9, 20, 33), which promotes actin-myosin interaction by phosphorylation of the 20-kDa MLC (MLC$_{20}$) (5, 9, 20, 33). Activation of the monomeric GTPase Rho also contributes to MLC$_{20}$ phosphorylation in endothelial cells and thus is involved in the mechanism of cell retraction (29, 30). In addition to endothelial cell contraction-retraction, increased endothelial permeability induced by thrombin and histamine may result from the disruption of the vascular endothelial (VE)-cadherin junctional complex (22, 24, 32) by the activation of the Ca$^{2+}$-dependent PKC isoform PKC-€ (22, 24).

Winter et al. (32) identified differences in the histamine response in human umbilical vein endothelial cells (HUVECs) and the spontaneously transformed HUVEC line ECV304 (12, 26). They showed that VE-cadherin expression was absent in ECV304 cells and that histamine failed to decrease endothelial monolayer resistance (a measure of endothelial cell retraction) in ECV304 cells compared with HUVECs (32). Because ECV304 cells may provide important clues into the mechanism of regulation of endothelial permeability, in the present study, we addressed the differences between ECV304 cells and HUVECs by comparing the thrombin-activated responses in the two cell types. Thrombin challenge in ECV304 cells failed to increase $[\mathrm{Ca}^{2+}]_i$, and had no effect on endothelial monolayer resistance and on MLC$_{20}$ phosphorylation. In contrast, thrombin elicited an increase in $[\mathrm{Ca}^{2+}]_i$, an ~50% decrease in endothelial monolayer electrical resistance, and MLC$_{20}$ phosphorylation in HUVECs. Interestingly, the Ca$^{2+}$ influx and increased $[\mathrm{Ca}^{2+}]_i$ induced with thapsigargin in ECV304 cells resulted in decreased transendothelial monolayer electrical resistance and increased $^{125}$I-albumin permeability. Comparison of ECV304 cells and HUVECs revealed that Ca$^{2+}$ influx and activation of Ca$^{2+}$-dependent signaling pathways are important determinants of the thrombin-induced increase in endothelial permeability.

METHODS

Materials. Human α-thrombin was obtained from Enzyme Research Laboratories (South Bend, IN). Thapsigargin was obtained from Calbiochem-Novabiochem (La Jolla, CA). Me-
dium 199 and Hanks’ balanced salt solution (HBSS) were obtained from Gibco BRL (Life Technologies, Grand Island, NY). Fetal bovine serum (FBS) was obtained from HyClone (Logan, UT). Endothelial cell growth medium (EGM-2) was obtained from Clonetics (San Diego, CA). Electrodes for endothelial monolayer electrical resistance measurements were obtained from Applied Biophysics (Troy, NY). Transwell cell culture chamber inserts were obtained from Corning Costar (Cambridge, MA). Fura 2-AM and Alexa 488 goat anti-mouse IgG were obtained from Molecular Probes (Eugene, OR). VE-cadherin and N-cadherin monoclonal antibodies (Mabs) were obtained from Transduction Laboratories (Lexington, KY). PAR-1 MAb was obtained from Immunotech (Westbrook, ME). Secondary goat anti-mouse and goat anti-rabbit antibodies (Abs) conjugated to horseradish peroxidase (HRP) were obtained from Kirkegaard and Perry Laboratories (Gaithersburg, MD). All other reagents and chemicals were obtained from Sigma (St. Louis, MO).

**Cell culture.** ECV304 cells obtained from American Type Culture Collection were grown in medium 199 supplemented with 10% FBS. HUVECs obtained from Venous Endothelial Cells (Rensselaer, NY) were grown in EGM-2 supplemented with 10% FBS. HUVECs between passages 4 and 8 were used for all described experiments.

**Transendothelial electrical resistance measurement.** Endothelial cell retraction was measured by the method described by Tiruppathi et al. (27). HUVECs and ECV304 cells were seeded on a gelatin-coated gold electrode (5.0 × 10^-4 cm²) and grown to confluence. The small electrode and the larger counter electrode were connected to a phase-sensitive lock-in amplifier. A constant current of 1 μA was supplied by a 1-V, 4,000-Hz AC signal connected serially to a 1-MΩ resistor between the small electrode and the larger counter electrode. The voltage between the small electrode and the large counter electrode was monitored by a lock-in amplifier, stored, and processed by a personal computer. The same computer controlled the output of the amplifier and switched the measurement to different electrodes in the course of an experiment. Before the experiment, confluent endothelial monolayers were incubated with growth medium containing 0.1% FBS for 2 h and were then used for measuring the thrombin-induced change in endothelial monolayer electrical resistance. The data are presented as the change in resistive portions of the impedance normalized to its value at time 0 as previously described (4, 27).

**Transendothelial 125I-albumin permeability.** Permeability of 125I-albumin across endothelial cell monolayers was determined with Costar Transwell units (14, 21). This system measures transendothelial flux (luminal to abluminal) of tracer macromolecules in the absence of hydrostatic and oncotic pressure gradients. The system consists of luminal and abluminal compartments separated by a polycarbonate filter (0.4-μm pore size, 6.5-mm diameter). The luminal (upper) side of filters was coated with gelatin. Cells were seeded at 1 × 10^4 cells/filter and grown for 3–5 days to attain confluence. Endothelial monolayers were washed and incubated in 10 mM HEPES-DMEM, pH 7.4, before the experiment. Both the luminal and abluminal compartments contained 5 mg/ml of BSA in 10 mM HEPES-DMEM, pH 7.4, at volumes of 0.2 and 1.0 ml, respectively. Tracer 125I-albumin (5 × 10^6 counts·min⁻¹·ml⁻¹) was added in the upper compartment, and 0.05-ml samples from the lower compartment were collected at 10-min intervals for 60 min for determination of the transendothelial clearance rate of 125I-albumin as previously described (14, 21).

**Measurement of Ins(1,4,5)P_3 generation.** Thrombin-induced Ins(1,4,5)P_3 generation was measured by using a Bio-trak d-nmioy-Ins(1,4,5)P_3-[3H] assay kit from Amersham Pharmacia Biotech (Piscataway, NJ). Endothelial cells grown to confluence on 60-mm culture dishes were washed two times with medium containing 0.1% FBS and incubated for 2 h at 37°C in similar medium. After incubation, the cells were stimulated with 10 nM thrombin for different time intervals, and the reaction was stopped by the addition of ice-cold 15% (vol/vol) trichloroacetic acid. The endothelial cells were scraped off the dishes and then centrifuged for 15 min at 2,000 g. The supernatant was washed three times with 10 volumes of water-saturated diethyl ether and neutralized by titration to pH 7.5 with NaHCO_3. Total Ins(1,4,5)P_3 generation was determined following the protocol described by Amersham Pharmacia Biotech.

**[Ca<sup>2+</sup>]i measurement.** [Ca<sup>2+</sup>]i in single cells was measured with fura 2-AM as previously described (28). Cells were grown on 25-mm-diameter glass coverslips coated with 0.1% gelatin to confluence. The cells were washed two times with HBSS and loaded with 3 μM fura 2-AM for 30 min at 37°C. The cells were then washed twice with HBSS and imaged with a Zeiss Axiovert S100 inverted microscope and a F-Fluar ×40, 1.3-numerical aperture oil-immersion objective. Regions of interest on individual cells were marked and excited at 334 and 380 nm, with emission at 520 nm. The increases in the 334- to 380-nm excitation ratio as a function of [Ca<sup>2+</sup>]i, was captured at 5-s intervals. At the end of each experiment, 5 μM ionomycin was added to obtain fluorescence of Ca<sup>2+</sup>-saturated fura 2 (maximal [Ca<sup>2+</sup>]i) and 10 mM EGTA was added to obtain fluorescence of free fura 2 (minimal [Ca<sup>2+</sup>]i). The [Ca<sup>2+</sup>]i was calculated based on a dissociation constant of 225 nM, with a two-point fit curve.

**MLC<sub>20</sub> phosphorylation.** Phosphorylation of MLC<sub>20</sub> was determined by urea-PAGE as previously described (5). Endothelial cells grown to confluence on 100-mm dishes were washed two times with DMEM containing 0.1% FBS and 10 mM HEPES, pH 7.4, followed by incubation for 2 h at 37°C. After treatment with thrombin for 2 min, the reaction was stopped by the addition of ice-cold 10% trichloroacetic acid and 10 mM diethiothreitol. Endothelial cells were scraped off and centrifuged at 3,000 rpm for 5 min. Cell pellets were washed three times with diethyl ether and suspended in 40 μl of 6.7 M urea sample buffer. Extracted proteins were separated by glycerol-urea-PAGE and transferred to nitrocellulose membrane. Phosphorylated and nonphosphorylated MLCs were detected by incubating the membrane with anti-MLC<sub>20</sub> Ab followed by incubation with HRP-conjugated goat anti-rabbit IgG for visualization by enhanced chemiluminescence. Phosphorylated and nonphosphorylated bands of MLCs on nitrocellulose membranes were localized as previously described (5).

**Immunoblotting.** Endothelial cells grown to confluence on 100-mm culture dishes were washed two times with ice-cold PBS and lysed with lysis buffer (1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 10 mM Tris·HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, pH 8.0, 0.2 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride, and 2 μg/ml of aprotinin) for 30 min at 4°C. Insoluble material was removed by centrifugation for 10 min at 14,000 rpm. Lysates were resolved by SDS-PAGE on a 7.5% separating gel under reducing conditions and subsequently transferred to a nitrocellulose membrane. Membranes were incubated in blocking buffer (5% nonfat dry milk in 10 mM Tris·HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20) for 60 min at room temperature. After being blocked, the membranes were incubated with the indicated Abs (diluted in blocking buffer) for 2 h at
room temperature. After a brief wash, the membranes were incubated for 2 h at room temperature with HRP-conjugated goat anti-mouse Ab. Protein bands were detected by enhanced chemiluminescence.

**Immunofluorescence.** Endothelial cells were seeded at a density of $1 \times 10^4$ on glass coverslips coated with 0.1% gelatin and grown to confluence. The cells were washed twice with HBSS and fixed with 4% paraformaldehyde for 20 min at room temperature. After fixation, the cells were washed three times for 5 min each with 100 mM glycine in HBSS and three times for 10 min each with HBSS alone. After a wash, the cells were preincubated with blocking solution (5% goat serum, 0.2% BSA, and 0.01% NaN₃ in HBSS) for 60 min at room temperature. The cells were incubated with the indicated Abs diluted to a concentration of $2 \mu g/ml$ in blocking solution for 2 h at room temperature.

After three washes with HBSS, Alexa 488 goat anti-mouse IgG (secondary Ab) was added, and the cells were incubated for 2 h at room temperature. After three washes with HBSS, the coverslips were mounted onto glass slides with the Pro-Long Antifade mounting medium kit (Molecular Probes). Cell surface fluorescence was visualized with digital imaging fluorescence microscopy as previously described (4).

**Stable expression of VE-cadherin in ECV304 cells.** The full-length human VE-cadherin cDNA cloned into the mammalian expression vector pC DNA 3.0 was from Dr. P. Kouklis (University of Illinois at Chicago, Chicago IL). Briefly, before the day of transfection, ECV304 cells were seeded at a density of $2 \times 10^5$ on 60-mm culture dishes. The following day, the cells were transfected with 1 $\mu g$ of DNA with the Effectene transfection reagent from QIAGEN (Valencia, CA). After 24 h, the medium was removed and replaced with fresh growth medium. Three days after transfection, cells were grown in growth medium containing G-418 (300 mg/ml). After 2 wk, the surviving cells were isolated, and the clones expanded in the presence of G-418 were used for experiments.

**Statistical analysis.** Statistical comparisons were made with a two-tailed Student’s t-test. Experimental values are reported as means ± SE. Differences in mean values between two or more groups were measured by one-way analysis of variance with Bonferroni correction. The values were considered significant at $P < 0.05$.

**RESULTS**

**Differential endothelial permeability responses and MLC$_{20}$ phosphorylation in HUVECs and ECV304 cells.** We studied the effects of thrombin on endothelial cell monolayer electrical resistance (a measure of endothelial cell retraction) and transendothelial 125I-albumin permeability in ECV304 cells and HUVECs. The addition of 10 nM thrombin failed to induce a decrease in endothelial monolayer resistance in ECV304 cell monolayers (Fig. 1A); in contrast, 10 nM thrombin produced an ~50% decrease in electrical resistance in HUVECs (Fig. 1A). Increasing the thrombin concentration from 10 to 100 nM also had no effect on endothelial

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![Fig. 1](http://ajplung.physiology.org/)

**Fig. 1.** A: effect of thrombin on transendothelial monolayer electrical resistance. Human umbilical vein endothelial cells (HUVECs) and ECV304 cells were grown to confluence on gold electrodes. Cells were washed 2 times, incubated with growth medium containing 0.1% fetal bovine serum (FBS) for 2 h, and challenged with 10 nM thrombin (arrow). See METHODS for details. Experiments were repeated 4–6 times with similar results. Data from a representative experiment are shown.

B: effect of thrombin on transendothelial 125I-albumin permeability. Transendothelial clearance rate shown was determined over 1 h after treatment with 10 nM thrombin. Experiment was repeated 4 times in triplicate. Values are means ± SE. * $P < 0.05$ compared with respective control.

C: effect of thrombin on 20-kDa myosin light chain (MLC$_{20}$) phosphorylation in HUVECs and ECV304 cells. Confluent HUVEC and ECV304 cell monolayers were stimulated with 10 nM thrombin for 2 and 5 min; total cell lysates were extracted for myosin light chains (MLCs) and separated by glycerol-urea-PAGE. Human endothelium expressed both smooth muscle and nonmuscle MLC isoforms (7). Multiple bands on the gels observed in nonphosphorylated (Non-Phos), monophosphorylated (Mono-Phos), or diphosphorylated (Di-Phos) isoforms were due to the expression of these MLC isoforms. See METHODS for details. Experiment was repeated 3 times. Data from a representative experiment are shown. C, control.
monolayer electrical resistance in ECV304 cells (data not shown). Thrombin failed to increase transendothelial $^{125}$I-albumin flux in ECV304 cells, whereas it produced a twofold increase in $^{125}$I-albumin flux in HUVECs (Fig. 1B).

We measured MLC$_{20}$ phosphorylation in ECV304 cells and HUVECs to address whether the difference in the permeability response was associated with a differential MLC$_{20}$ in the two cell types. Thrombin challenge increased MLC$_{20}$ phosphorylation in HUVECs, whereas it failed to induce MLC$_{20}$ phosphorylation in ECV304 cells (Fig. 1C).

**Thrombin-activated Ins(1,4,5)P$_3$ generation and Ca$^{2+}$ signaling.** We measured thrombin-induced phosphoinositide hydrolysis and the increase in [Ca$^{2+}$]$_i$ to assess activation of Ca$^{2+}$-regulated signaling pathways. Thrombin-induced Ins(1,4,5)P$_3$ generation in ECV304 cells was similar to that in HUVECs (Fig. 2A); in contrast, thrombin failed to increase [Ca$^{2+}$]$_i$ in ECV304 cells compared with HUVECs (Fig. 2B). Thrombin elicited a peak [Ca$^{2+}$]$_i$ increase of 900 ± 45 nM in HUVECs, whereas in ECV304 cells, thrombin produced a peak response of 100 ± 8 nM. Basal [Ca$^{2+}$]$_i$ levels in both cell types were similar.

Because the thrombin-induced increase in endothelial permeability is the result of endothelial surface activation of PAR-1 (4, 6), we assessed the level of PAR-1 expression in ECV304 cells. Immunofluorescence analysis revealed that cell surface PAR-1 expression was abundant and normal in ECV304 cells (Fig. 3A). Thrombin exposure induced the internalization of PAR-1 in a time-dependent manner (Fig. 3B and C) similar to results observed in HUVECs (data not shown). In both cell types, cell surface anti-PAR-1 MAb immunostaining decreased within 60 min of thrombin exposure (Fig. 3C), indicating that PAR-1 cleavage by thrombin caused internalization of PAR-1. However, within 3 h after thrombin exposure, cell surface PAR-1 expression was restored in ECV304 cells (Fig. 3D) similar to our results in HUVECs (data not shown). Thapsigargin failed to induce MLC$_{20}$ phosphorylation in ECV304 cells as well as in HUVECs (data not shown).

**Thrombin-induced increase in endothelial permeability in ECV304 cells cannot be restored by expression of VE-cadherin.** Because the absence of endothelial cell-specific VE-cadherin in ECV304 cells (32) may explain the failure of thrombin to activate the permeability response in these cells, we addressed the possible contribution of VE-cadherin. Immunoblotting and immunofluorescence analysis showed that ECV304 cells did not express VE-cadherin, whereas HUVECs expressed VE-cadherin (Fig. 4). However, ECV304 cells abundantly expressed the N-cadherin isoform at
cell-cell junctions (Fig. 4B), which can substitute for the function of VE-cadherin at adherens junctions in endothelial cells (2, 3, 32).

To test whether the absence of VE-cadherin contributed to the defect in thrombin-induced permeability response in ECV304 cells, we stably expressed VE-cadherin (see METHODS for details). VE-cadherin expression was evident at cell-cell junctions after transfection of ECV304 cells with the VE-cadherin plasmid (Fig. 5B). The pattern of expression was similar to VE-cadherin immunostaining in HUVECs (Fig. 4B compared with Fig. 5B). However, thrombin challenge had no effect on either endothelial monolayer electrical resistance or transendothelial $^{125}$I-albumin flux in the VE-cadherin-transfected ECV304 cells (Fig. 5C and D). Thrombin also failed to induce an increase in $[\text{Ca}^{2+}]_i$ in the VE-cadherin-transduced ECV304 cells (data not shown).

Increased $^{125}$I-albumin permeability in ECV304 cells in response to $\text{Ca}^{2+}$ influx induced by thapsigargin. Because the failure of thrombin to increase endothelial permeability in ECV304 cells may be due to an impaired increase in $[\text{Ca}^{2+}]_i$ [see Thrombin-activated Ins(1,4,5)P$_3$ generation and Ca$^{2+}$ signaling], we studied the effects of thapsigargin (which is known to promote an increase in $[\text{Ca}^{2+}]_i$ by depleting intracellular store Ca$^{2+}$ and causing capacitative Ca$^{2+}$ entry in cells (11, 19, 25)) on the Ca$^{2+}$ transients and barrier function. Results indicated that thapsigargin increased $[\text{Ca}^{2+}]_i$ in both ECV304 cells and HUVECs (Fig. 6A). Thapsigargin also induced a 40–50% decrease in endothelial monolayer electrical resistance in ECV304 cells that was comparable to that in HUVECs (Fig. 6B). Finally, thapsigargin produced two- to threefold in-
creases in transendothelial $^{125}\text{I}$-albumin flux in both cell types (Fig. 6C).

Lanthanum chloride inhibits thrombin-induced $\text{Ca}^{2+}$ influx and endothelial cell retraction. Because a defect in the $[\text{Ca}^{2+}]_i$ increase in ECV304 cells was identified as a critical factor responsible for the impaired permeability response, we studied the effect of the $\text{Ca}^{2+}$ channel blocker lanthanum chloride [which inhibits capacitative $\text{Ca}^{2+}$ entry in cells (23)] on thrombin-induced $\text{Ca}^{2+}$ entry and endothelial monolayer electrical resistance in control HUVECs. The thrombin-induced increase in $[\text{Ca}^{2+}]_i$ was associated with both intracellular store $\text{Ca}^{2+}$ depletion and extracellular $\text{Ca}^{2+}$ influx (i.e., capacitative $\text{Ca}^{2+}$ entry) (16, 24). Lanthanum chloride (2.5 mM) inhibited the thrombin-induced plateau phase $[\text{Ca}^{2+}]_i$ (which accounts for extracellular $\text{Ca}^{2+}$ influx) in HUVECs without significantly affecting the initial peak increase in $[\text{Ca}^{2+}]_i$. (Fig. 5).
A), which is due to the release of Ca$^{2+}$ from intracellular stores (19, 23). This response was similar to that observed with thrombin in the absence of extracellular Ca$^{2+}$ (data not shown). Exposure of HUVECs to a higher concentration of lanthanum chloride (5 mM) blocked the thrombin-induced increase in [Ca$^{2+}$]$_i$ (data not shown). Lanthanum chloride at 2.5 mM concentration also inhibited ~50% of the thrombin-induced decrease in endothelial cell monolayer electrical resistance (Fig. 7B). These results implicate the importance of Ca$^{2+}$ influx in the mechanism of thrombin-induced increase in endothelial permeability.

DISCUSSION

In the present study, we compared thrombin-activated responses in ECV304 cells (a transformed cell line derived from the human umbilical vein) (12, 26) and in primary HUVECs. We showed that thrombin produced an ~50% decrease in transendothelial monolayer electrical resistance and a twofold increase in $^{125}$I-albumin permeability in HUVECs, whereas it failed to decrease endothelial monolayer resistance and increase $^{125}$I-albumin permeability in ECV304 cells. Thrombin exposure elicited an increase in [Ca$^{2+}$]$_i$ and phosphorylation of MLC$_{20}$ in HUVECs, but it failed to induce either response in ECV304 cells. Surprisingly, the thrombin-induced phosphoinositide hydrolysis in ECV304 cells was comparable to that in HUVECs. We also showed that cell surface PAR-1 was present in both cells and was internalized in a similar manner and kinetics after thrombin exposure. Thus the differential permeability response to thrombin in ECV304 cells and HUVECs may be ascribed to a defect in Ca$^{2+}$ influx in ECV304 cells and the failure to activate the Ca$^{2+}$-dependent signaling machinery required to increase endothelial permeability.

Thrombin produces interendothelial gaps by the proteolytic cleavage of the PAR-1 NH$_2$ terminus between Arg$^{41}$ and Ser$^{42}$ to generate a new NH$_2$ terminus (4, 31). The new NH$_2$ terminus can thereby serve as a “tethered ligand” for PAR-1 (4, 31). Because of the proteolytic cleavage of PAR-1, the receptor is irreversibly activated, and it is subsequently internalized and degraded in lysosomes (4, 10). Thrombin-induced activation of cell surface PAR-1 also signals the translocation of the intracellular pool of PAR-1 to cell surface (4, 10). We assessed cell surface PAR-1 expression and thrombin-induced internalization of PAR-1 in ECV304 cells and HUVECs to determine whether possible differences in PAR-1 expression and activation could account for the aberrant permeability response observed in ECV304 cells. The results indicated that thrombin exposure promoted the internalization of PAR-1 and translocation of intracellular pool PAR-1 to the cell surface to the same degree in both ECV304 cells and HUVECs. Furthermore, thrombin-induced Ins(1,4,5)P$_3$ generation was similar in HUVECs and ECV304 cells, indicating that PAR-1 was activated in both cells.

Because thrombin failed to induce an increase [Ca$^{2+}$]$_i$ in ECV304 cells in the face of Ins(1,4,5)P$_3$ generation, a
possible explanation of our data is that dysregulation of Ca\(^{2+}\) homeostasis in ECV304 cells at the level of release of Ca\(^{2+}\) from intracellular stores and influx of Ca\(^{2+}\) by store-activated channels is responsible for the absence of the permeability response (1, 19). The increase in [Ca\(^{2+}\)]\(_i\) may be required for activation of the endothelial actin-myosin contractile machinery via activation of MLCK (5, 9, 20, 33) and Ca\(^{2+}\)-dependent PKC isoforms such as PKC-\(\alpha\) (24) that regulate the increase in endothelial permeability (22, 24).

Because the absence of Ca\(^{2+}\) signaling in ECV304 cells appears to be important in making these cells refractory to thrombin, we addressed the possibility of whether these cells could be “rescued” by treatment with thapsigargin, that is, the possibility that Ca\(^{2+}\) influx activated by another means could activate the increase in endothelial permeability in these cells. Thapsigargin (an endoplasmic reticulum Ca\(^{2+}\)-ATPase inhibitor) promotes intracellular store Ca\(^{2+}\) depletion and triggers capacitative Ca\(^{2+}\) entry in cells (11, 19, 25). We showed that thapsigargin resulted in decreased endothelial monolayer electrical resistance and increased 125I-albumin permeability in ECV304 cells, a response similar to that observed in HUVECs. However, the thapsigargin-induced increase in endothelial permeability was not associated with MLC\(_{20}\) phosphorylation (24). Because the ECV304 cell monolayer was capable of increasing permeability to 125I-albumin in response to an increase in [Ca\(^{2+}\)]\(_i\), the results further support the conclusion that the failure of thrombin to increase endothelial permeability in these cells is the result of impairment in Ca\(^{2+}\) signaling. Moreover, the results raise the interesting possibility that the rise in [Ca\(^{2+}\)]\(_i\) is alone not prerequisite for MLC\(_{20}\) phosphorylation, suggesting that thrombin-induced MLC\(_{20}\) phosphorylation may involve not only Ca\(^{2+}\)-activated signaling but also activation of Ca\(^{2+}\)-independent pathways such as p21-activated kinase (8).

To address the importance of Ca\(^{2+}\) influx in signaling the thrombin-induced increase in endothelial permeability, we used the Ca\(^{2+}\) channel blocker lanthanum chloride (23). Lanthanum chloride prevented thrombin-induced Ca\(^{2+}\) entry in HUVECs and markedly reduced the thrombin-induced decrease in endothelial cell monolayer electrical resistance. These results indicate that Ca\(^{2+}\) influx is critical in the mechanism of thrombin-induced increase in endothelial permeability.

It is possible that the failure of thrombin to increase permeability in ECV304 cells is the result of loss of adherens junctional function because these cells lack VE-cadherin (32). Therefore, we assessed whether the thrombin-activated signals could also be rescued by the stable expression of VE-cadherin in ECV304 cells. VE-cadherin-transfected ECV304 cells exhibited VE-cadherin expression at cell-cell junctions; however, thrombin failed to increase [Ca\(^{2+}\)]\(_i\), induce MLC\(_{20}\) phosphorylation, and increase endothelial permeability in these cells. Although VE-cadherins are important in the regulation of endothelial barrier function in response to mediators such as thrombin and histamine in normal endothelial cells (2, 3, 22, 24), the role of VE-cadherin function is not likely to be revealed in the VE-cadherin-transduced ECV304 cells because of the defect in the upstream Ca\(^{2+}\) signaling.

The present results differ from the response to histamine in ECV304 cells (32). Histamine failed to decrease endothelial monolayer resistance in ECV304 cells, whereas it elicited an increase in [Ca\(^{2+}\)]\(_i\), that was comparable to that in HUVECs (32). Moreover, VE-cadherin expression in ECV304 cells restored the histamine-induced decrease in endothelial monolayer resistance (32). There may be various reasons for the different findings. First, the activation of Ca\(^{2+}\) signaling induced by the two mediators may differ in that histamine can directly activate plasmalemmal membrane receptor-activated Ca\(^{2+}\) channels, enabling the influx of Ca\(^{2+}\) (32); thus histamine would be capable of interfering with the cadherin-catenin assembly in the VE-cadherin-transduced ECV304 cells. Second, the histamine-induced endothelial permeability response differs from the thrombin response in that the latter requires activation of the actin-myosin cytoskeletal apparatus of endothelial cells (9), whereas histamine acts at sites of cadherin-mediated cell-cell interaction (32). Because the Ca\(^{2+}\) requirements for activation of actin-myosin cross bridging are greater (5, 9, 33), a defect in Ca\(^{2+}\) signaling would be expected to have a marked effect on thrombin-induced increase in endothelial permeability.

Even though the ECV304 cell monolayer lacked VE-cadherin, we observed that the basal transendothelial 125I-albumin fluxes in ECV304 cells and HUVECs were similar. This finding implies that adherens junctional integrity in ECV304 cells can be maintained by the expression of the N-cadherin isoform at cell-cell junctions as well as expression of desmosomal proteins that are characteristics of epithelial cell junctions (3, 13). Thus N-cadherin and desmosomal proteins can substitute for the loss of VE-cadherin in ECV304 cells (3, 13) and thereby maintain interendothelial junctional permeability.

In summary, the results indicate that thrombin failed to increase [Ca\(^{2+}\)]\(_i\), and had no effect on endothelial monolayer permeability and MLC\(_{20}\) phosphorylation in ECV304 cells. In contrast, thrombin elicited an increased [Ca\(^{2+}\)]\(_i\), increased endothelial permeability, and MLC\(_{20}\) phosphorylation in HUVECs. Increasing [Ca\(^{2+}\)]\(_i\) in ECV304 cells with thapsigargin, known to promote Ca\(^{2+}\) influx, resulted in an increase in 125I-albumin permeability and a decrease in monolayer electrical resistance. Moreover, inhibition of thrombin-elicited Ca\(^{2+}\) influx with lanthanum chloride significantly reduced thrombin-induced endothelial cell retraction. Thus Ca\(^{2+}\) influx and activation of Ca\(^{2+}\)-dependent signaling pathways are important determinants of the thrombin-induced loss of endothelial barrier function.

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