HHS Public Access

Author manuscript

Vascul Pharmacol. Author manuscript; available in PMC 2023 June 18.

Published in final edited form as:

Vascul Pharmacol. 2022 August ; 145: 107088. doi:10.1016/j.vph.2022.107088.

Apoptosis signal-regulating kinase-1 regulates thrombin-induced endothelial permeability

Hemant Giri,

Amit K. Srivastava,

Ulhas P. Naik*

Cardeza Center for Hemostasis, Thrombosis, and Vascular Biology, Cardeza, Foundation for Hematologic Research, Department of Medicine, Division of Hematology, Sydney Kimmel Medical College, Thomas Jefferson University, Philadelphia, USA

Abstract

Thrombin-induced endothelial permeability is associated with various pathological conditions. Apoptosis signal- regulating kinase-1 (ASK1), one of the upstream MAP3K, has been reported to be an important regulator of endothelial stress and apoptosis. Despite this, its role in endothelial permeability is unknown. The aim of this study was to determine the role of ASK1 in thrombin-induced endothelial permeability. To do so, a live cell monitoring system and transwell assay were used to evaluate *in vitro* endothelial permeability, while a Miles assay was used for *in vivo* permeability. Immunofluorescence and western blotting were used to visualize integrity of the junctions and phosphorylation of various proteins, respectively. We observed that *in vivo* thrombin-induced vascular permeability was attenuated in $Ask1^{-/-}$ mice. Pretreatment of human primary endothelial cells (ECs) with GS-4997 (ASK1 inhibitor) and deficiency of ASK1 in primary mouse lung ECs significantly attenuated the thrombin-induced endothelial permeability. Furthermore, in the presence of GS-4997, the following were also significantly reduced: thrombin-induced para-cellular gap formation, VE-cadherin proteolysis, and dislocation of VE-cadherin, JAM-A, and ZO1 from the junctions. Inhibition of ASK1 restored peripheral location of F-actin, similar to that induced by *sphingosine-1-phosphate*. These results suggest a unique role for ASK1 in regulating thrombin-induced endothelial permeability.

Keywords

Endothelial permeability; Thrombin; Apoptosis signal-regulating kinase-1 (ASK1); Junction proteins

*Corresponding author at: Cardeza Center for Hemostasis, Thrombosis, and Vascular Biology, Thomas Jefferson University, Jefferson Alumni Hall, 1020 Locust Street, Suite 394, Philadelphia, PA, 19107, USA. ulhas.naik@jefferson.edu (U.P. Naik).

Author statement

HG: Methodology, Data curation, Formal analysis, Project administration, Writing - original draft. AKS: Resources, Software, Supervision, Validation, Visualization, Writing - review & editing. UPN: Conceptualization, Funding acquisition, Investigation, Supervision, Validation, Writing - review & editing.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vph.2022.107088.

Declaration of Competing Interest

The authors have no conflict of interest to declare.

1. Introduction

Endothelial barrier integrity is essential for maintaining homeostasis and organ function [1]. Disruption of the endothelial barrier has been involved in the genesis and/or progression of various pathological conditions, including COVID-19, acute lung injury, acute respiratory distress syndrome, chronic bowel disease, cancer, sepsis, trauma, ischemic stroke, multi-organ ischemia, and multi-organ failure [2–8]. The altered barrier function in these conditions is often linked to the release of soluble mediators from resident cells and the interaction of these mediators with receptors expressed on the surface of ECs. Interaction of mediators with ECs disrupts endothelial barrier function either by altering the expression of adhesive proteins in the inter-endothelial junctions, by altering the organization of the cytoskeleton, or both [9]. Thrombin, a pro-coagulant serine protease, is one of the mediators that plays a significant role in the regulation of endothelial permeability [10]. Thrombin is generated in the tissues in response to infection or injury [11,12]. On ECs, thrombin activates the PAR1 receptor by proteolytic cleavage and initiates a series of events: an increase in intracellular Ca^{2+}, Rho/ROCK signaling, stress fiber formation, and myosin light chain (MLC) kinase activation [13]. The phosphorylation of MLC stimulates stress fiber formation and triggers actomyosin contraction to result in junctional proteins disarray and EC hyperpermeability [11,14]. Mitogen-activated protein (MAP) kinases play a key role in downstream-of-thrombin signaling and endothelial physiology, including permeability [15]. Various MAP kinases such as Erk1/2, c-Jun N-terminal Kinase (JNK), and p38 are reported to be involved in thrombin-induced increase in endothelial permeability [16,17], but no information is available on the role of upstream kinases in altered endothelial permeability. Apoptosis signal-regulating kinase-1 (ASK1), one of the upstream MAP3K, is an important regulator of differentiation, inflammation, apoptosis, and endothelial stress [18,19]. There is no report, however, on the role of ASK1 in regulating endothelial permeability. In this study, we proposed and tested the hypothesis that ASK1 regulates thrombin-induced increase in endothelial permeability, and that genetic ablation or pharmacological inhibition of ASK1 attenuate the altered endothelial permeability.

2. Materials and methods

2.1. Animals

ASK1 knockout ($Ask1^{-/-}$) mice in the F10 generation on a C57BL/6 J background [20] were provided by Prof. Hidenori Ichijo from Tokyo Medical and Dental University, Japan. $Ask1^{-/-}$ mice aged 8–12 weeks and sex-matched wild-type (WT) C57BL/6 J were used for *in vivo* experiments. Animals were maintained in a normal day-night cycle (12/12) with free access to food and water. All animal procedures were approved and conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Thomas Jefferson University.

2.2. Miles assay for vascular permeability measurement in vivo

To study the effect of genetic ablation of *ASK1* on vascular permeability *in vivo*, isoflurane-anesthetized $Ask1^{-/-}$ mice ($n = 4$) and WT mice ($n = 3$) were retro-orbitally injected with 2% of Evans blue (80 μl). To stimulate vascular permeability, after 10 min, 20 μl of thrombin

(2 U/ml) in phosphate buffered saline (PBS) was given by intradermal injection into the right hind limb pad of the animal. The left hind limb pad was injected with PBS only (20 μl). Thereafter, mice were photographed and euthanized by cervical dislocation under anesthesia. Vascular permeability was analyzed by removing the skin area injected with thrombin or PBS *via* biopsy punch and extracting the Evans blue dye with formamide at 55 °C for 18 h. The amount of vascular leakage was quantitated by measuring absorbance at 610 nm on a spectrophotometer (Perkin Elmer).

2.3. Human endothelial cell culture and treatment

To study the role of ASK1 in macro, micro, and immortalized human ECs, primary human umbilical vein endothelial cells (HUVECs) and human microvascular endothelial cells (HMVECs) were purchased from Invitrogen (Carlsbad, CA) and maintained in endothelial basal medium with growth factor kit (Lonza, Walkersville, MD). Telomerase immortalized microvascular ECs of the dermis (TIME) were purchased from ATCC (Manassas, VA) and maintained in vascular cell basal medium with growth factor kit (ATCC, Manassas, VA). Attachment factor (GIBCO, Gaithersburg, MD) was used to coat the cell-culture dish for adhesion of the cells, and they were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C. The culture medium was replaced every 1–2 days; cells at 85–90% confluence were passaged at a ratio of 1:3 confluence. The cells were used between passages two and five in all experiments. To detect the effect of various inhibitors, cells were serum starved with basal EBM containing 1% fetal bovine serum (FBS) for 4 h and treated with 10 μM of GS-4997 (ASK1 inhibitor), SB203680 (p38 inhibitor), SP600125 (JNK1/2/3 inhibitor), Y7632 (ROCK inhibitor), BAPTA-AM (selective calcium chelator), and Vorapaxar (VPX; PAR1 receptor inhibitor) for 30 min. Thereafter, cells were stimulated with thrombin (1 to 2 U/ml).

2.4. Isolation and culture of mouse lung endothelial cells (MLECs)

Primary mouse ECs were isolated from lungs of twelve-week-old $Ask1^{-/-}$ and WT mice. Isolated lungs were minced and disintegrated using collagenase A (200 μg/ml) in presence of 250 mM of $CaCl_2$ and treated for 1 h at 37 °C, to ensure a single-cell suspension. The cells were then incubated with dyna-beads anti-rat (Invitrogen) magnetic beads conjugated to rat anti-mouse CD31 (BD Bioscience) PECAM-1 antibody for 1 h at 37 °C. The beads attached with cells were then washed and plated on 10 μg/ml VN-coated dishes. The cells were cultured in Dulbecco's modified essential medium (DMEM F12) 1:1 along with 20% FBS, 1% penicillin-streptomycin, 100 μg/ml heparin, and 50 μg/ml EC growth supplement (Invitrogen). ECs were characterized for the expression PECAM-1, an EC-specific antibody using flow cytometry. Confluent cells within three passages were used in the experiments.

2.5. xCELLigence Real-Time Cell Analysis (RTCA) of endothelial permeability in vitro

In vitro endothelial barrier function was assessed using a live cell monitoring system (xCELLigence). This system uses specialized plates covered in gold microsensors that sense impedance to electrical signals, which is recorded continuously in real-time. For the assay, the plate was incubated with 100 μl of growth medium for 10 min, and the background cell index (CI) value was measured. ECs were then seeded at a density of 30,000 cells/well and monitored for confluency. Once confluent as indicated by a plateau in CI curve, cells

were pre-treated with different inhibitors for 30 min followed by stimulation with thrombin (1 U/ml). The change in the value of CI of each well was automatically monitored by the xCELLigence system and recorded in a computer. To analyze results, cell index values were normalized (divided by their values at time of thrombin addition) as described previously [21]. Furthermore, all obtained normalized values were subtracted from normalized values of untreated cells. Final values were plotted as a baseline normalized cell index at various time points. Finally, a bar graph was prepared among various conditions for maximum change in CI values.

2.6. Transwell assay

The migratory response of ECs was measured by transwell assay. In brief, transwell filter polycarbonate inserts with 0.4 μm pore size and 12 mm diameter (Corning, New York) were coated with attachment factor (Thermo Fisher Scientific, Waltham, MA). HUVECs (3×10^4 cells/well) were seeded on the upper compartment and grown to confluence for 72h. Cells were treated in xCELLigence system, as mentioned, except fluorescein isothiocyanate (FITC)-dextran (40kDa, 1mg/ml;) was added before 5 min of thrombin treatment. From the lower compartment, 10 μl samples were taken after 20, 40, and 60min and diluted to 90 μl of basal medium. Finally, all diluted samples were read in a clear bottom fluorescence 96-well black plate at (excitation: 485nm; emission: 535nm) in a plate reader (Perkin Elmer). Control was set at 1 and the final graph plotted as fold change relative to the control.

2.7. Immunofluorescence microscopy

ECs were grown on a coverslip coated with attachment factor (Thermo Fisher Scientific, Waltham, MA). Once cells became 85–90% confluent, they were serum starved for 4 h and then treated with different inhibitors for 30 min before 2 units of thrombin were added. At the end, cells were fixed with 2% paraformaldehyde for 20 min and permeabilized with 0.1% triton-X-100 (Sigma-Aldrich, St. Louis, MO) for 5 min. Cells were blocked with 2% BSA in PBS and incubated with primary antibodies (anti–ZO-1, anti-VE-cadherin, anti-JAM-A, vinculin and anti-P-MLC antibody) in a blocking solution at 4 °C overnight. Th next day, after three washes with PBS, cells were incubated with corresponding secondary antibody for 1 h at room temperature. FITC-conjugated phalloidin was used to stain F-actin for 1 h at room temperature. After 8 washes with PBS, coverslips were mounted with Vecta-Shield (Hardset) mounting medium with DAPI (Vector Labs, Burlingame, CA). All images were captured with a Zeiss Microscope (Thornwood, NY) or EVOs microscope (Thermo Scientific, Philadelphia, PA). For quantitative analysis of thrombin-induced gap formation, the area covered by the cells was measured with ImageJ software at 5 randomly selected fields from three independent experiments.

2.8. Western blotting

ECs were cultured to reach confluency and then were lysed by 1% Nonidet P-40 detergent containing lysis buffer. Cell lysates were denatured by adding 2× laemmli sample buffer (Bio-Rad, Hercules, California) and boiling the samples for 5 min. An equal amount of protein was resolved on SDS-PAGE and transferred to a PVDF membrane (Bio-Rad, Hercules, CA). Membranes were blocked with 3% BSA (Sigma-Aldrich, St. Louis, MO) and probed with respective primary antibodies overnight at 4 °C. The membranes were

washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature, followed by development using ECL reagent from Cell Signaling Technology (CST, Danvers, MA). The chemiluminescence was captured using a gel documentation system (Bio-Rad, Hercules, California).

2.9. Statistical analysis

Statistical analysis was performed using GraphPad software. To determine statistical significance, one-way ANOVA (mean ± standard error of the mean) was used; $p \leq 0.05$ was regarded as statistically significant. Each experiment was repeated independently at least 3 times.

2.10. Reagents and antibodies

The following reagents and antibodies were used: Human thrombin (Enzyme Research Laboratories, South Bend, IN); LPS (*E. coli* serotype 0111: B4) (Cell Signaling Technology, Beverly, MA); Phosphoserine 967 ASK1, P-p38, P-JNK1/2, JNK1/2, p38, PMLC2, MLC2 antibodies (Cell Signaling Technology, Beverly, MA); ASK1, Vinculin, VE-cadherin and ZO-1 antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA); JAM-A antibody (BD Biosciences, San Jose, CA); GS-4997 (ASK1 inhibitor) was a gift from Dr. Anton Simeonov (National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD); Y7632 (ROCK inhibitor; Millipore, Burlington, MA); SB203680 (p38 inhibitor) and SP600125 (JNK1/2 inhibitor; Calbiochem, MA); Vorapaxar (VPX, PAR1 receptor inhibitor) was a kind gift from Dr. Paul Bray (University of Utah Health Science Center, Salt Lake City, UT); BAPTA-AM and Evans blue (Sigma, St. Louis, MO); MnTMPyP (Santa Cruz, CA, USA); S1P (Avanti, INC).

3. Results

3.1. Deficiency of ASK1 attenuates thrombin-induced vascular permeability in mice

To evaluate the role of ASK1 vascular permeability *in vivo*, we performed a Miles assay using $Ask1^{-/-}$ mice and corresponding age-matched WT mice. We observed that local treatment of thrombin (2 units/ml) induced a robust vascular permeability in hind-limb foot-pad. Interestingly, in $Ask1^{-/-}$ mice, thrombin-induced permeability was significantly attenuated (Fig. 1Ai & B). This result suggests that thrombin-induced vascular permeability requires the presence of ASK1.

3.2. Inhibition of ASK1 or its deficiency attenuates thrombin-induced permeability in human primary endothelial cells

To determine the role of ASK1 in regulating endothelial permeability in human ECs, we treated HUVECs with GS-4997 (ASK1i), a specific ASK1 inhibitor developed by Gilead Bio-sciences Inc. [22]. Thrombin-induced endothelial permeability was measured using the xCELLigence system and FITC dextran transwell permeability assay [23]. We found that thrombin (1 U/ml) robustly induced endothelial permeability, measured as decrease in EC impedance (expressed as CI). Addition of thrombin induced a maximum permeability within 10–20 min which returned to basal levels within 1 h. This decrease in cell index induced by thrombin was significantly blocked (80%) by pretreatment with ASK1i (10 μM)

(Fig. 2Ai & Aii). Similar inhibition of permeability was seen (74%) in HMVECs (Fig. 2Bi & Bii) and (34%) in TIME cells (Fig. 2Ci & Cii). Inhibition of ASK1 increased the basal barrier function (Fig. 2D, Supplemental Fig. 1A). ASK1i inhibited thrombin-induced endothelial permeability in a dose-dependent manner (Supplemental Fig. 1 B). Addition of ASK1i after thrombin treatment also blocked the endothelial permeability as confirmed by CI measurement (Supplemental Fig. 1C).

To further confirm the inhibition of thrombin-induced permeability by ASK1i, we performed an FITC dextran transwell permeability assay in which HUVECs pretreated with ASK1i were stimulated with thrombin. We found a time-dependent increase in dextran permeability through the HUVEC monolayer upon stimulation with thrombin. This increase was blocked by ASK1i pretreatment (Fig. 2D).

To test endothelial permeability in murine ECs, we isolated MLECs from Ask1$^{-/-}$ and WT mice. Confluent cell monolayer from WT mice showed a sustained permeability, whereas Ask1$^{-/-}$ cells failed to show any endothelial permeability upon stimulation with thrombin (Fig. 2E). These results further support the essential role Ask1 plays in thrombin-induced EC permeability.

3.3. Thrombin-induced endothelial cell permeability is PAR1- and both $G_{\alpha}q$- and $G_{\alpha}12/13$-dependent

Thrombin regulates the function of target cells by cleaving and activating a family of G-protein-coupled receptors known as protease-activated receptors (PAR). ECs express PAR1, PAR2, PAR3, and PAR4 receptors [24]. PAR1, PAR3, and PAR4 are activated by thrombin, whereas PAR2 is activated by trypsin. To test if PAR1 (a major thrombin receptor on ECs) is responsible for thrombin-induced EC permeability, we used PAR1-activating peptide (SFLLRN, PAR1-AP), which specifically activates PAR1 and PAR2. PAR1-AP at 50 μM concentration increases the EC permeability to an extent similar to that of thrombin, which was completely blocked by pretreatment with ASK1i (Fig. 3Ai & Aii). Furthermore, thrombin-induced permeability was inhibited by vorapaxar (VPX, 100 nM) a PAR1-specific antagonist (Fig. 3Ci & Cii). These results suggest that thrombin induces EC permeability by activating PAR1, which was primarily dependent on ASK1.

It is known that in nucleated cells, ASK1 could be activated by reactive oxygen species (ROS) or by Ca^{2+}, depending on the cell type [25–28]. To determine through which of these mechanisms ASK1 is activated by thrombin, we pretreated HUVECs with BAPTA-AM to chelate Ca^{2+} or MnTMPyP to scavenge ROS, and then stimulated them with thrombin. We found that BAPTA-AM was able to significantly inhibit thrombin-induced permeability, while MnTMPyP did not (Fig. 3Bi & Bii). These results suggest that thrombin activates ASK1 in a Ca^{2+}-dependent mechanism to induce EC permeability. It is known that PAR1 is coupled to both $G_{\alpha q}$ (PLC/Ca^{2+} pathway) and $G_{\alpha 12/13}$ (RhoA/ROCK pathway) and both have been implicated in thrombin-induced endothelial permeability [29]. We used ROCK inhibitor (ROCKi) to confirm the role of RhoA/ROCK pathway in our experimental setting. We found a significant attenuation of EC permeability in the presence of ROCKi, which is comparable to VPX (Fig. 3Ci & Cii). These results suggest that thrombin-induced EC

permeability, which is dependent on ASK1 activity is regulated by both $G_{\alpha q}$ and $G_{\alpha 12/13}$ pathways.

3.4. Inhibition of ASK1 attenuates thrombin-induced disruption of junctions and para-cellular gap formation in EC monolayer

Thrombin-induced permeability is associated with cytoskeletal remodeling, which includes stress fiber formation (F-actin), dislocation of junctional proteins, and formation of para-cellular gaps [30]. To visualize such effects, HUVEC monolayers were immuno-stained for junctional proteins (JAM-A, VE-cadherin, and ZO-1) and F-actin (phalloidin). As expected, treatment of EC monolayer with thrombin caused a complete disappearance of JAM-A from the cell-cell junctions, whereas junctional distribution of VE-cadherin and ZO-1 was disturbed (Fig. 4 A & Supplemental Fig. 2). Pretreatment of ASK1i protected ECs from thrombin-induced dislocation of these junctional proteins (Fig. 4 A & Supplemental Fig. 2). We also observed significant paracellular gap formation as measured by reduction the in area covered by the cells upon thrombin treatment, which was restored in the presence of ASK1i (Fig. 4 B & C). These results suggest that inhibition of ASK1 protects thrombin-induced para-cellular gap formation.

During vascular permeability, VE-cadherin proteolysis led to shedding of VE-cadherin ecto-domain and generation of a C-terminal fragment (CTF) through calcium-mediated activation of ADAM10 [31]. Since inhibition of ASK1 significantly attenuated thrombin-induced endothelial permeability, we tested whether thrombin-induced VE-cadherin proteolysis is abolished using ASK1 inhibitor. We observed significant attenuation of VE-cadherin proteolysis as evidenced by CTF formation in immunoblotting using C-terminal antibody of VE-cadherin (Fig. 4D & E). These results suggest that ASK1 promotes VE-cadherin shedding to induce vascular permeability.

3.5. ASK1 increases thrombin-induced EC permeability independent of MLC2 activation and focal adhesion formation

Thrombin stimulation of EC leads to RhoA activation and phosphorylation of MLC leading to formation of stress fibers containing F-actin [30]. We therefore investigated if ASK1 regulates cytoskeletal rearrangement induced by thrombin. As expected, treatment with thrombin induced a robust stress-fiber formation which is associated with focal adhesion formation (Fig. 5A). Interestingly, pretreatment of ECs with ASK1i (10 μM) failed to significantly inhibit stress-fiber formation (Fig. 5A) or MLC2 activation (Fig. 5 B & C). These results suggest that ASK1 does not regulate thrombin-induced RhoA/ROCK-dependent cytoskeleton remodeling. Cortical actin filaments are essential for the maintenance of endothelial barrier function and S1P is known to support barrier function by restoring cortical F-actin. Similar effects were observed with use of ASK1i, where pretreatment of ECs with ASK1i results in localization of focal adhesions and P-MLC to the periphery along with the F-actin (Fig. 5A and Supplemental Fig. 3) further confirm the role of ASK1 in regulating EC barrier function.

3.6. ASK1 mediates thrombin-induced endothelial permeability by JNK-dependent, but p38-independent pathway

The phosphorylated Ser-967 residue in the C-terminal motif of ASK1 binds 14–3–3 proteins and inhibits ASK1 activation; thus, dephosphorylation of this residue serves as an indicator of ASK1 activation [32]. As expected, ASK1 is in an inactivated state in unstimulated ECs as observed by phosphorylated Ser-967 (Fig. 6Ai). Thrombin increases the activity of ASK1 (decreased Ser-967 phosphorylation) in a time-dependent manner and maximum activation was achieved at 10 min of treatment (Fig. 6Ai & Aii). This was further supported by a corresponding activation of p38 and JNK1/2, two known downstream effectors of ASK1. Interestingly, the activation of p38 appeared to occur much earlier (1 min) than ASK1 activation, whereas activation of JNK1/2 corresponded well with activation of ASK1 (Fig. 6Ai & Aii). Furthermore, inhibition of JNK1/2 showed attenuation of EC permeability induced by thrombin (Fig. 6Bi & Bii), whereas p38 inhibitor had no effect on thrombin-induced EC permeability. Additionally, the combination of JNK1/2/p38 inhibitors gave an extent of protection similar to that of JNK inhibitor alone (Fig. 6Bi & Bii). Treatment of these inhibitors (JNK and P38) alone on ECs do not affect the cell index values (Supplement Fig. 1D). These results indicate that ASK1 mediates thrombin-induced signaling through JNK1/2 and not p38 in ECs.

In summary, our data suggest a positive role of ASK1 in thrombin-induced endothelial permeability. Activation of ASK1 resulted in JNK activation that led to cleavage of VE-cadherin along with dissociation of ZO-1 and JAM-A from the junctions, which is independent of stress fiber formation (Fig. 6C).

4. Discussion

In this study, using *in vitro* and *in vivo* models, we demonstrated that ASK1 regulates thrombin-induced endothelial permeability. To best of our knowledge, this is the first direct demonstration that ASK1 is activated by thrombin in a confluent endothelial monolayer to induce vascular permeability. It is of interest to note that pharmacological inhibition of ASK1 by a serine/threonine kinase inhibitor (GS-4997) was able to significantly attenuate endothelial permeability both *in vitro* and *in vivo*. The endothelium acts as a semipermeable barrier and regulates vascular permeability and maintains tissue fluid homeostasis. Physiological increase in endothelial permeability is transient, transpired by post-tissue injury during the initial phases of healing, whereas pathological permeability is persistent, commonly witnessed in conditions such as atherosclerosis, chronic inflammation, tumor growth, diabetic retinopathy, sepsis, and many other conditions [2–8,33].

Thrombin is known to act on endothelial PAR receptors to cause increased endothelial permeability. Cleavage PARs results in a tethered ligand that triggers downstream signaling. Site of thrombin cleavage can therefore determine which signaling is triggered. Thus, cleavage at R41 and R46 of PAR1 results in very different signaling [34]. MAP kinases have been shown to be activated downstream of PARs. However, the mechanism of their activation is not well-understood. Using $Ask1^{-/-}$ mice and a highly specific ASK1 inhibitor, GS-4997, we have shown that thrombin induced endothelial permeability through PAR1 activation followed by ASK1/JNK signaling, leading to endothelial junctional breakdown

independent of stress fiber formation. A number of agents in addition to thrombin such as VEGF, bFGF, TNF-α, histamine, and bradykinin are known to induce endothelial permeability [35]. Whether ASK1 is common signaling molecule downstream of these agents are not known and is the topic of future research.

ECs express PAR1, PAR2 PAR3, and PAR4 receptors [36], and it is believed that PAR1 is activated by thrombin, whereas PAR2 is activated by trypsin [37]. However, Mihara et al. (2016) reported that thrombin, at concentrations that can be achieved at sites of acute injury, can also directly activate PAR2 [38]. The concentrations of thrombin (1–2 U/ml) used to induce EC permeability in this study was primarily through PAR1 activation. PAR1 is coupled to both $G_{\alpha q}$ and $G_{\alpha 12/13}$ trimeric G proteins. Activation of PAR1 can stimulate several pathways leading to endothelial permeability. These include calcium-dependent and independent activation of MLC2, activation of protein kinase C (PKC), disruption of association between junctional protein molecules [14,39], and activation of CaM kinase II and Erk or p38, leading to cytoskeletal rearrangement and contraction of cells [16,40]. Our finding that inhibition of ASK1 did not affect phosphorylation of MLC2 or stress fiber formation suggests that MLC-dependent cytoskeletal rearrangement is independent of ASK1. Although ASK1 is known to activate both JNK and p38 but not Erk MAP kinases through their respective MEKs [41], inhibition of thrombin-induced endothelial permeability by JNK inhibitor suggests that ASK1 mediates this function through JNK pathway. The role of p38 has been implicated in bovine pulmonary artery EC permeability [16]. However, we did not see any effects of p38 inhibition on thrombin induced HUVEC permeability. It is possible that p38 plays a role only in the later time of thrombin treatment or is specific to lung ECs [16]. Our results showed that ASK1 regulated the majority of thrombin-induced permeability effects through JNK.

In resting cells, ASK1 is bound to either thioredoxin or CIB1, two known endogenous inhibitors of ASK1 [26,27]. During oxidative stress, ROS oxidizes thioredoxin (Trx) to induce activation of ASK1, whereas increase in intracellular Ca^{2+} dissociates CIB1, thus allowing ASK1 activation. Although thrombin can induce generation of ROS, we found that in ECs the primary mechanism of ASK1 activation by thrombin was through intracellular Ca^{2+}, which dissociates CIB1 from ASK1, thus allowing TRAF2/6 to bind and initiate autophosphorylation of ASK1. Similar mechanisms have been reported to exist in neuronal cells and platelets [28,42].

Our observation of cleavage of VE-cadherin in response to thrombin supports the possibility of dislocation of junctional molecules and loss of barrier function. Cleavage of cytoplasmic domain of VE-cadherin results in its disassociation from β-catenin and thus disassembly of junctions and para-cellular gap formation between ECs [43]. Thrombin is known to mediate these effects through the activation of metalloproteinases such as ADAMs10 [31]. However, this is not limited to metalloproteinases since others have also reported the involvement of cysteine protease such as calpain in cleavage of VE-cadherin [43]. This suggests that thrombin-induced increase in intracellular Ca^{2+} increases the activity of ADAMs10/calpain through ASK1 and leads to disruption of barrier function by proteolysis of VE-cadherin. It is also possible that VE-cadherin is degraded *via* endocytosis/lysosomal degradation pathway, which is regulated by JNK, but not p38 [44]. Our finding that thrombin-induced

EC permeability was regulated by ASK1 and JNK resulted in disruption of VE-cadherin junctions in ECs is consistent with these reports.

Thrombin stimulation leads to RhoA activation and phosphorylation of MLC, leading to formation of F-actin stress fibers [30]. However, we did not see any significant changes in activation of MLC2 upon inhibition of ASK1/JNK. Interestingly, inhibition of ASK1 resulted in increased cortical actin formation and junctional localization of vinculin, similar to that seen upon treatment with angiopoietin-1 [45] and S1P [46]. At junctions, vinculin associates with VE-cadherin to protect contractile force induced para-cellular gap formation [47].

In conclusion, we have shown that ASK1 plays a positive role in thrombin-induced endothelial permeability by inducing cleavage of the cytoplasmic tail of VE cadherin. ASK1 achieves this independent of MLC2 activation through JNK pathway. Inhibition of ASK1 results in maintaining barrier function without affecting cytoskeletal rearrangement in response to thrombin. Thus, ASK1 inhibitor (GS-4997) has a potential therapeutic application in preventing vascular permeability and related complications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

This study was supported by grant # 5R01HL113188–08 to UPN from the National Heart, Lung, and Blood Institute (NHLBI) of the U.S. National Institutes of Health (NIH). The authors thank Meghna Naik for her help in manuscript editing and figures preparation.

References

[1]. Evans CE, Iruela-Arispe ML, Zhao YY, Mechanisms of endothelial regeneration and vascular repair and their application to regenerative medicine, Am. J. Pathol 191 (1) (2021) 52–65. [PubMed: 33069720]

[2]. Kim WY, Hong SB, Sepsis and acute respiratory distress syndrome: recent update, Tuberc. Respir. Dis 79 (2) (2016) 53–57.

[3]. Matthay MA, Zimmerman GA, Acute lung injury and the acute respiratory distress syndrome: four decades of inquiry into pathogenesis and rational management, Am. J. Respir. Cell Mol. Biol 33 (4) (2005) 319–327. [PubMed: 16172252]

[4]. Park-Windhol C, D'Amore PA, Disorders of vascular permeability, Annu. Rev. Pathol 11 (2016) 251–281. [PubMed: 26907525]

[5]. Lopez E, Srivastava AK, Pati S, Holcomb JB, Wade CE, Platelet-derived microvesicles: a potential therapy for trauma-induced coagulopathy, Shock 49 (3) (2018) 243–248. [PubMed: 28858139]

[6]. Johansson PI, Stensballe J, Ostrowski SR, Shock induced endotheliopathy (SHINE) in acute critical illness - a unifying pathophysiologic mechanism, Crit. Care 21 (1) (2017) 25.

[7]. Iba T, Connors JM, Levy JH, The coagulopathy, endotheliopathy, and vasculitis of COVID-19, Inflamm. Res 69 (12) (2020) 1181–1189. [PubMed: 32918567]

[8]. Oikonomou E, Souvaliotis N, Lampsas S, Siasos G, Poulakou G, Theofilis P, Papaioannou TG, Haidich AB, Tsaousi G, Ntousopoulos V, Sakka V, Charalambous G, Rapti V, Raftopoulou S, Syrigos K, Tsioufis C, Tousoulis D, Vavuranakis M, Endothelial dysfunction in acute and long standing COVID-19: a prospective cohort study, Vasc. Pharmacol 144 (2022), 106975.

[9]. Rodrigues SF, Granger DN, Blood cells and endothelial barrier function, Tissue Barriers 3 (1–2) (2015), e978720.

[10]. Ma L, Dorling A, The roles of thrombin and protease-activated receptors in inflammation, Semin. Immunopathol 34 (1) (2012) 63–72. [PubMed: 21809138]

[11]. Bogatcheva NV, Garcia JG, Verin AD, Molecular mechanisms of thrombin-induced endothelial cell permeability, Biochemistry 67 (1) (2002) 75–84. [PubMed: 11841342]

[12]. Chao CH, Chen HR, Chuang YC, Yeh TM, Macrophage migration inhibitory factor-induced autophagy contributes to thrombin-triggered endothelial hyperpermeability in sepsis, Shock 50 (1) (2018) 103–111. [PubMed: 28846570]

[13]. Mehta D, Malik AB, Signaling mechanisms regulating endothelial permeability, Physiol. Rev 86 (1) (2006) 279–367. [PubMed: 16371600]

[14]. Rabiet MJ, Plantier JL, Rival Y, Genoux Y, Lampugnani MG, Dejana E, Thrombin-induced increase in endothelial permeability is associated with changes in cell-to-cell junction organization, Arterioscler. Thromb. Vasc. Biol 16 (3) (1996) 488–496. [PubMed: 8630677]

[15]. Bogatcheva NV, Dudek SM, Garcia JG, Verin AD, Mitogen-activated protein kinases in endothelial pathophysiology, J. Investig. Med 51 (6) (2003) 341–352.

[16]. Borbiev T, Birukova A, Liu F, Nurmukhambetova S, Gerthoffer WT, Garcia JG, Verin AD, p38 MAP kinase-dependent regulation of endothelial cell permeability, Am. J. Phys. Lung Cell. Mol. Phys 287 (5) (2004) L911–L918.

[17]. Borbiev T, Verin AD, Birukova A, Liu F, Crow MT, Garcia JG, Role of CaM kinase II and ERK activation in thrombin-induced endothelial cell barrier dysfunction, Am. J. Phys. Lung Cell. Mol. Phys 285 (1) (2003) L43–L54.

[18]. Machino T, Hashimoto S, Maruoka S, Gon Y, Hayashi S, Mizumura K, Nishitoh H, Ichijo H, Horie T, Apoptosis signal-regulating kinase 1-mediated signaling pathway regulates hydrogen peroxide-induced apoptosis in human pulmonary vascular endothelial cells, Crit. Care Med 31 (12) (2003) 2776–2781. [PubMed: 14668614]

[19]. Yokoi T, Fukuo K, Yasuda O, Hotta M, Miyazaki J, Takemura Y, Kawamoto H, Ichijo H, Ogihara T, Apoptosis signal-regulating kinase 1 mediates cellular senescence induced by high glucose in endothelial cells, Diabetes 55 (6) (2006) 1660–1665. [PubMed: 16731828]

[20]. Tobiume K, Matsuzawa A, Takahashi T, Nishitoh H, Morita K, Takeda K, Minowa O, Miyazono K, Noda T, Ichijo H, ASK1 is required for sustained activations of JNK/p38 MAP kinases and apoptosis, EMBO Rep. 2 (3) (2001) 222–228. [PubMed: 11266364]

[21]. Hiraoka K, Inagaki A, Kato Y, Huang TT, Mitchell LA, Kamijima S, Takahashi M, Matsumoto H, Hacke K, Kruse CA, Ostertag D, Robbins JM, Gruber HE, Jolly DJ, Kasahara N, Retroviral replicating vector-mediated gene therapy achieves long-term control of tumor recurrence and leads to durable anticancer immunity, Neuro-Oncology 19 (7) (2017) 918–929. [PubMed: 28387831]

[22]. Loomba R, Lawitz E, Mantry PS, Jayakumar S, Caldwell SH, Arnold H, Diehl AM, Djedjos CS, Han L, Myers RP, Subramanian GM, McHutchison JG, Goodman ZD, Afdhal NH, Charlton MR, G.-U.−. Investigators, The ASK1 inhibitor selonsertib in patients with nonalcoholic steatohepatitis: a randomized, phase 2 trial, Hepatology 67 (2) (2017) 549–559. [PubMed: 28892558]

[23]. Bischoff I, Hornburger MC, Mayer BA, Beyerle A, Wegener J, Furst R, Pitfalls in assessing microvascular endothelial barrier function: impedance-based devices versus the classic macromolecular tracer assay, Sci. Rep 6 (2016) 23671. [PubMed: 27025965]

[24]. Macfarlane SR, Seatter MJ, Kanke T, Hunter GD, Plevin R, Proteinase-activated receptors, Pharmacol. Rev 53 (2) (2001) 245–282. [PubMed: 11356985]

[25]. Gotoh Y, Cooper JA, Reactive oxygen species- and dimerization-induced activation of apoptosis signal-regulating kinase 1 in tumor necrosis factor-alpha signal transduction, J. Biol. Chem 273 (28) (1998) 17477–17482. [PubMed: 9651337]

[26]. Liu H, Nishitoh H, Ichijo H, Kyriakis JM, Activation of apoptosis signal-regulating kinase 1 (ASK1) by tumor necrosis factor receptor-associated factor 2 requires prior dissociation of the ASK1 inhibitor thioredoxin, Mol. Cell. Biol 20 (6) (2000) 2198–2208. [PubMed: 10688666]

[27]. Yoon KW, Cho JH, Lee JK, Kang YH, Chae JS, Kim YM, Kim J, Kim EK, Kim SE, Baik JH, Naik UP, Cho SG, Choi EJ, CIB1 functions as a Ca(2+)-sensitive modulator of stress-induced signaling by targeting ASK1, Proc. Natl. Acad. Sci. U. S. A 106 (41) (2009) 17389–17394. [PubMed: 19805025]

[28]. Yoon KW, Yang HS, Kim YM, Kim Y, Kang S, Sun W, Naik UP, Parise LV, Choi EJ, CIB1 protects against MPTP-induced neurotoxicity through inhibiting ASK1, Sci. Rep 7 (1) (2017) 12178. [PubMed: 28939911]

[29]. van Nieuw Amerongen GP, van Delft S, Vermeer MA, Collard JG, van Hinsbergh VW, Activation of RhoA by thrombin in endothelial hyperpermeability: role of Rho kinase and protein tyrosine kinases, Circ. Res 87 (4) (2000) 335–340. [PubMed: 10948069]

[30]. Birukova AA, Smurova K, Birukov KG, Kaibuchi K, Garcia JG, Verin AD, Role of Rho GTPases in thrombin-induced lung vascular endothelial cells barrier dysfunction, Microvasc. Res 67 (1) (2004) 64–77. [PubMed: 14709404]

[31]. Schulz B, Pruessmeyer J, Maretzky T, Ludwig A, Blobel CP, Saftig P, Reiss K, ADAM10 regulates endothelial permeability and T-Cell transmigration by proteolysis of vascular endothelial cadherin, Circ. Res 102 (10) (2008) 1192–1201. [PubMed: 18420943]

[32]. Zhang L, Chen J, Fu H, Suppression of apoptosis signal-regulating kinase 1-induced cell death by 14–3-3 proteins, Proc. Natl. Acad. Sci. U. S. A 96 (15) (1999) 8511–8515. [PubMed: 10411906]

[33]. Adil MS, Somanath PR, Endothelial permeability assays in vitro, Methods Mol. Biol 2367 (2021) 177–191.

[34]. Heuberger DM, Schuepbach RA, Protease-activated receptors (PARs): mechanisms of action and potential therapeutic modulators in PAR-driven inflammatory diseases, Thromb. J 17 (2019) 4. [PubMed: 30976204]

[35]. Sukriti S, Tauseef M, Yazbeck P, Mehta D, Mechanisms regulating endothelial permeability, Pulm. Circ 4 (4) (2014) 535–551. [PubMed: 25610592]

[36]. O'Brien PJ, Prevost N, Molino M, Hollinger MK, Woolkalis MJ, Woulfe DS, Brass LF, Thrombin responses in human endothelial cells. Contributions from receptors other than PAR1 include the transactivation of PAR2 by thrombin-cleaved PAR1, J. Biol. Chem 275 (18) (2000) 13502–13509. [PubMed: 10788464]

[37]. Nystedt S, Emilsson K, Wahlestedt C, Sundelin J, Molecular cloning of a potential proteinase activated receptor, Proc. Natl. Acad. Sci. U. S. A 91 (20) (1994) 9208–9212. [PubMed: 7937743]

[38]. Mihara K, Ramachandran R, Saifeddine M, Hansen KK, Renaux B, Polley D, Gibson S, Vanderboor C, Hollenberg MD, Thrombin-mediated direct activation of proteinase-activated receptor-2: another target for thrombin signaling, Mol. Pharmacol 89 (5) (2016) 606–614.

[39]. Li X, Hahn CN, Parsons M, Drew J, Vadas MA, Gamble JR, Role of protein kinase Czeta in thrombin-induced endothelial permeability changes: inhibition by angiopoietin-1, Blood 104 (6) (2004) 1716–1724. [PubMed: 15172966]

[40]. Birukova AA, Birukov KG, Gorshkov B, Liu F, Garcia JG, Verin AD, MAP kinases in lung endothelial permeability induced by microtubule disassembly, Am. J. Phys. Lung Cell. Mol. Phys 289 (1) (2005) L75–L84.

[41]. Ichijo H, Nishida E, Irie K, ten Dijke P, Saitoh M, Moriguchi T, Takagi M, Matsumoto K, Miyazono K, Gotoh Y, Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways, Science 275 (5296) (1997) 90–94. [PubMed: 8974401]

[42]. Patel P, Naik MU, Golla K, Shaik NF, Naik UP, Calcium-induced dissociation of CIB1 from ASK1 regulates agonist-induced activation of the p38 MAPK pathway in platelets, Biochem. J 476 (19) (2019) 2835–2850. [PubMed: 31530712]

[43]. Su W, Kowalczyk AP, The VE-cadherin cytoplasmic domain undergoes proteolytic processing during endocytosis, Mol. Biol. Cell 28 (1) (2017) 76–84. [PubMed: 27798242]

[44]. Yin M, Zhou HJ, Zhang J, Lin C, Li H, Li X, Li Y, Zhang H, Breckenridge DG, Ji W, Min W, ASK1-dependent endothelial cell activation is critical in ovarian cancer growth and metastasis, JCI Insight 2 (18) (2017).

[45]. Mammoto T, Parikh SM, Mammoto A, Gallagher D, Chan B, Mostoslavsky G, Ingber DE, Sukhatme VP, Angiopoietin-1 requires p190 RhoGAP to protect against vascular leakage in vivo, J. Biol. Chem 282 (33) (2007) 23910–23918. [PubMed: 17562701]

[46]. Gonzalez E, Kou R, Michel T, Rac1 modulates sphingosine 1-phosphate-mediated activation of phosphoinositide 3-kinase/Akt signaling pathways in vascular endothelial cells, J. Biol. Chem 281 (6) (2006) 3210–3216. [PubMed: 16339142]

[47]. Huveneers S, Oldenburg J, Spanjaard E, van der Krogt G, Grigoriev I, Akhmanova A, Rehmann H, de Rooij J, Vinculin associates with endothelial VE-cadherin junctions to control force-dependent remodeling, J. Cell Biol 196 (5) (2012) 641–652. [PubMed: 22391038]

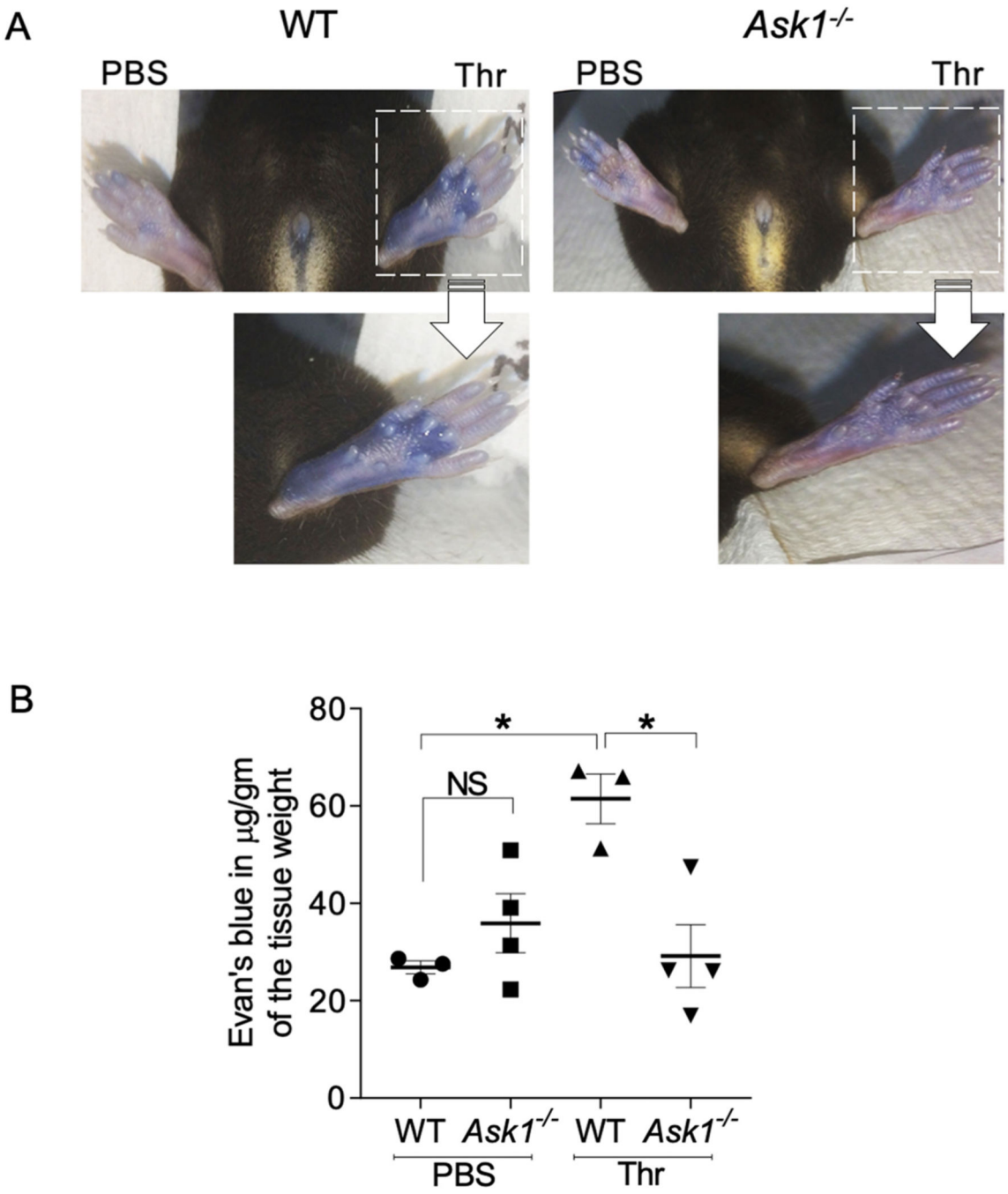

Fig. 1.

Lack of ASK1 in mice reduces thrombin-induced vascular permeability: (A) Representative images of hind-limb showing vascular leakage of Evan's blue in WT (left panel) and Ask1$^{-/-}$ mice (right panel) treated locally with thrombin (1 U/ml). (B) Quantification of Evans blue dye extravasation from the hind-limb in WT ($n = 3$) and Ask1$^{-/-}$ mice ($n = 4$). Data are represented as mean ± SEM. *$p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

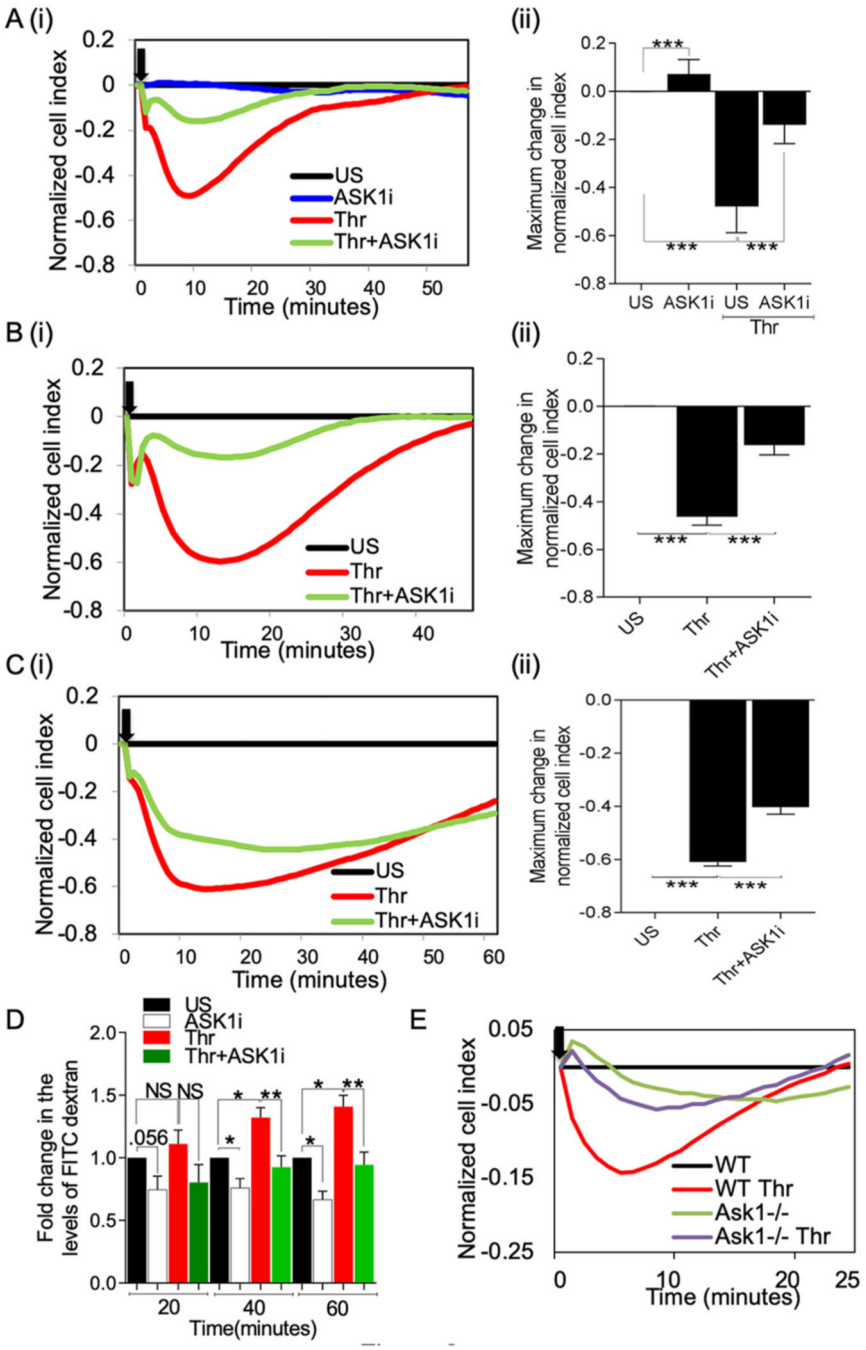

Fig. 2.

Inhibition or deficiency of ASK1 attenuates thrombin-induced endothelial permeability: Representative baseline normalized tracings of CI of unstimulated (US), thrombin (Thr, 1 U/ml), ASK1 inhibitor (ASK1i, 10 μM), and ASK1i + thrombin in (Ai) HUVECs, (Bi) HMVECs, and (Ci) TIME cells. Each line represents the average of two technical replicates. Black arrow represents addition of thrombin (Aii, Bii, and Cii). Quantification of corresponding normalized cell index data ($n = 3$). (D) Fold change in the amount of FITC dextran passed through the HUVEC monolayer treated with or without thrombin in the

presence or absence of ASK1i (n = 4). (E) Representative baseline normalized tracings of cell index of MLECs isolated from WT or Ask1$^{-/-}$ mice, treated with or without thrombin. Black arrow represents addition of thrombin.
*$p < 0.5$, **$p < 0.001$, ***$p < 0.001$.

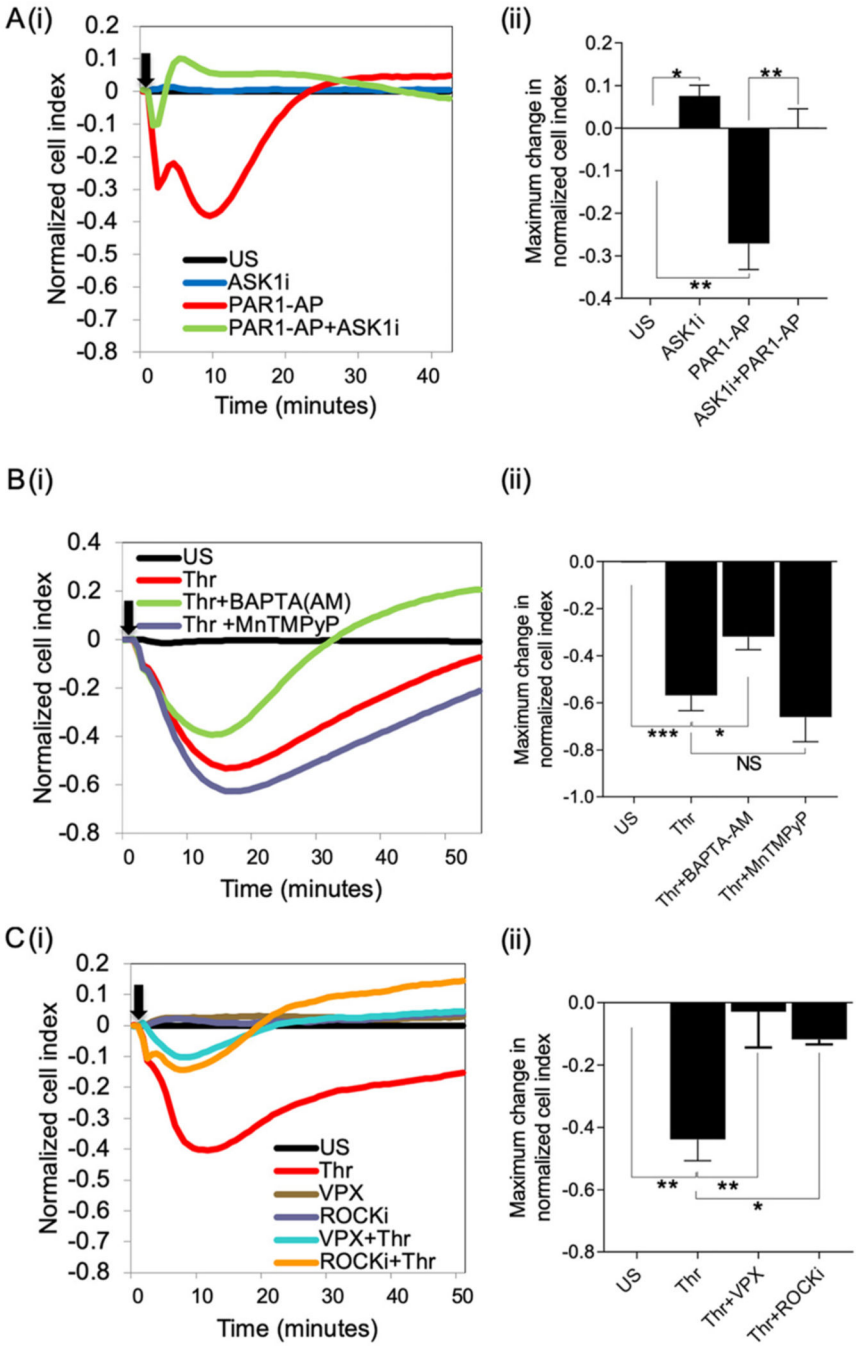

Fig. 3.

Thrombin-induced endothelial permeability is dependent on ASK1, Ca^{2+}, and ROCK, but not ROS: (Ai) Representative baseline normalized tracings of cell index of HUVECs unstimulated (US) or stimulated with PAR1-activating peptide, (PAR1-AP, 50 μM), in the presence or absence of ASK1i, 10 μM. (Aii) Quantification of CI data from (Ai) ($n = 3$). (Bi) Representative baseline normalized tracings of cell index of HUVECs unstimulated (US), pretreated with MnTMPyP (10 μM), BAPTA-AM (10 μM) and stimulated with thrombin (1 U/ ml). Black arrow represents addition of thrombin. (Bii) Quantification of cell index data

from (Bi) (n = 3). (Ci) Representative baseline normalized tracings of cell index of HUVECs unstimulated (US), pretreated with VPX (100 nM), ROCKi (10 μM), and stimulated with thrombin (1 U/ml). (Cii) Quantification of normalized cell index data from (Ci) (n = 3). Each line of tracing represents the average of two technical replicates. *$p < 0.5$, **$p < 0.001$, ***$p < 0.001$.

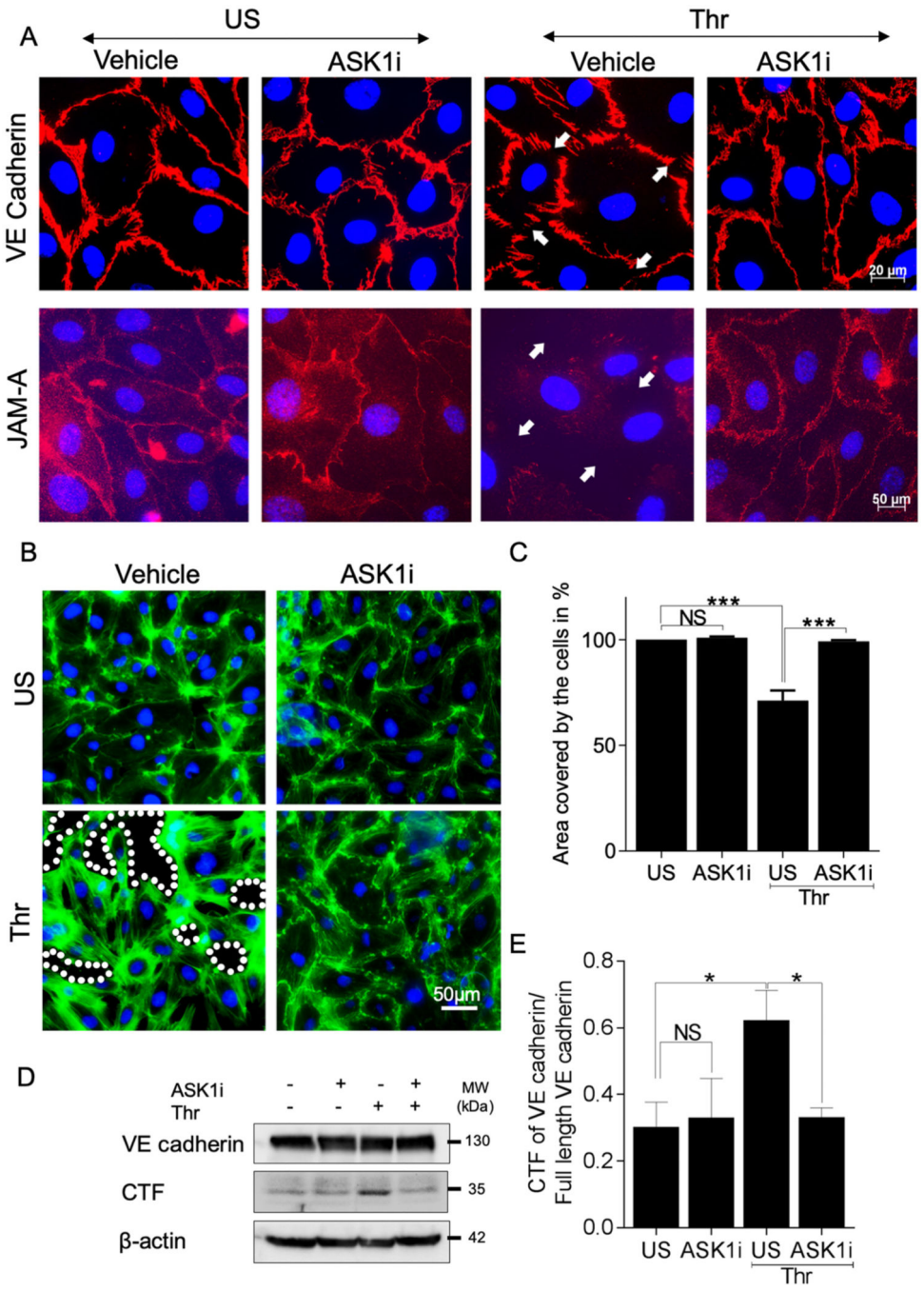

Fig. 4.

Inhibition of ASK1 attenuates thrombin-induced disorganization of junctional molecules, para-cellular gap formation in HUVEC monolayer: Immunofluorescence images of serum-starved confluent HUVEC monolayer pretreated with vehicle or ASK1i (10 μM) prior to stimulation with thrombin (2 U/ml) for 20 min. (A) HUVECs were stained (red) with VE-cadherin (upper row), JAM-A (lower row), and counter stained with DAPI (blue). White arrows show loss of junction molecules at the cell-cell contact upon treatment with thrombin (20× images with scale = 20 mm). (B) Following treatment, cells were fixed as usual and

stained for DAPI and F-actin (green) at different conditions; unstimulated (US), thrombin (1 U/ml), ASK1 inhibitor (10 μM), and ASK1i + thrombin (60× images with scale = 50 mm). Dotted lines represent boundary for gap area present in between the cells. (C) Quantification of percent area of cell coverage from ($n = 3$) individual experiments. (D) Representative western-blot image of VE-cadherin cleavage in the presence of thrombin, C-terminal fragment (CTF) of VE-cadherin, and β-actin as loading control. (E) Quantification of band density from (D) expressed as ratio of C-terminal fragment to full length VE-cadherin. ($n = 3$). *$p < 0.5$, ***$p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

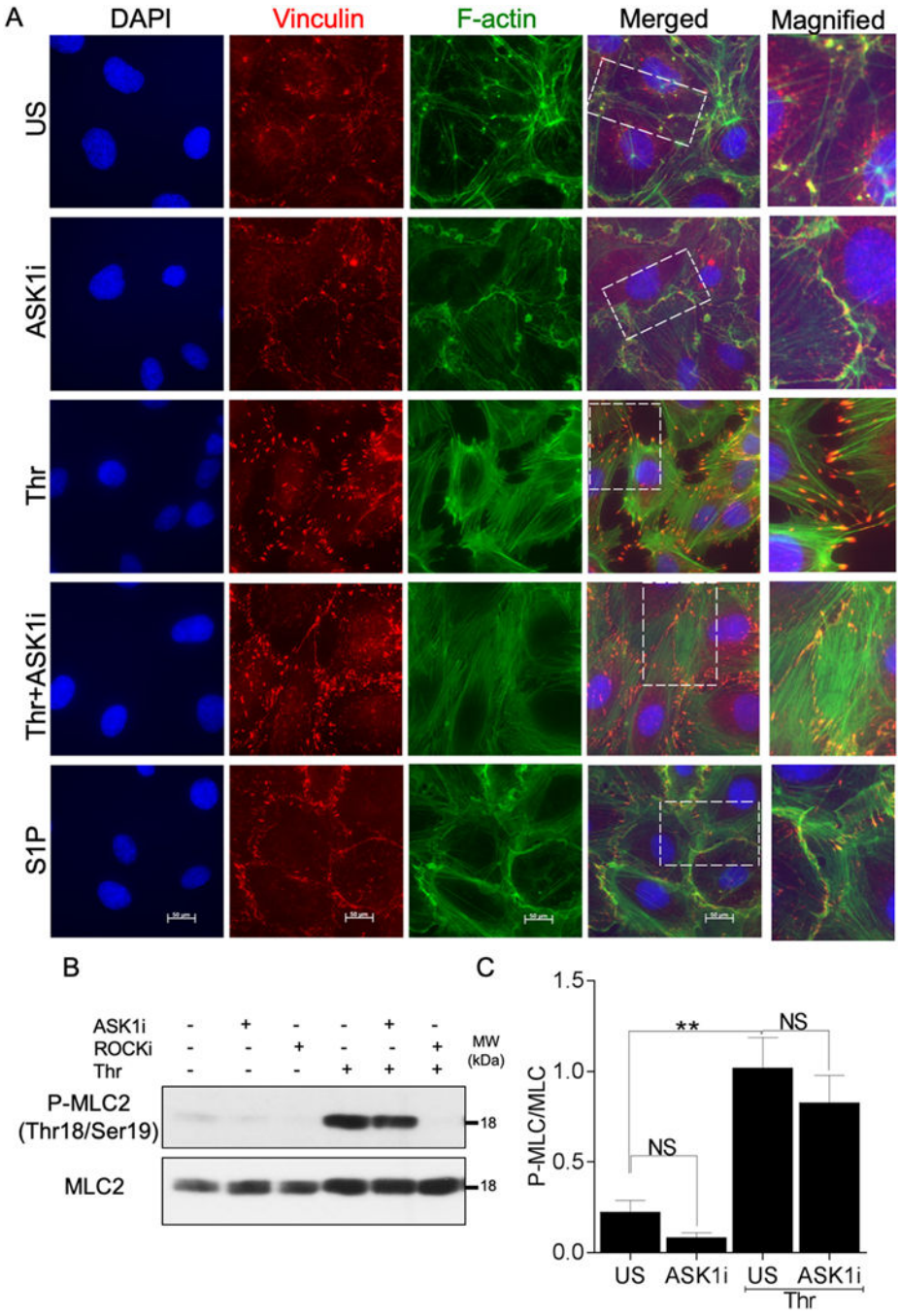

Fig. 5.

Inhibition of ASK1 does not affect thrombin-induced cytoskeletal remodeling or phosphorylation of MLC. (A) Immunofluorescence images of serum starved confluent HUVEC monolayer unstimulated (US) or pretreated with 10 μM ASK1i for 30 min prior to stimulation with thrombin (2 U/ml) for 20 min or S1P (5 μM) for 10 min. Cells were stained with vinculin (red), phalloidin (green), and DAPI (blue). Images represents n = 3 experiments. (63× images with scale = 50 μm). (B) Representative western blot showing effect of ASK1i or ROCKi on thrombin-induced MLC2 phosphorylation. (C) Quantification

of band density from (B) expressed as ratio of P-MLC2 to total MLC2. ($n = 3$). $^{**}P < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

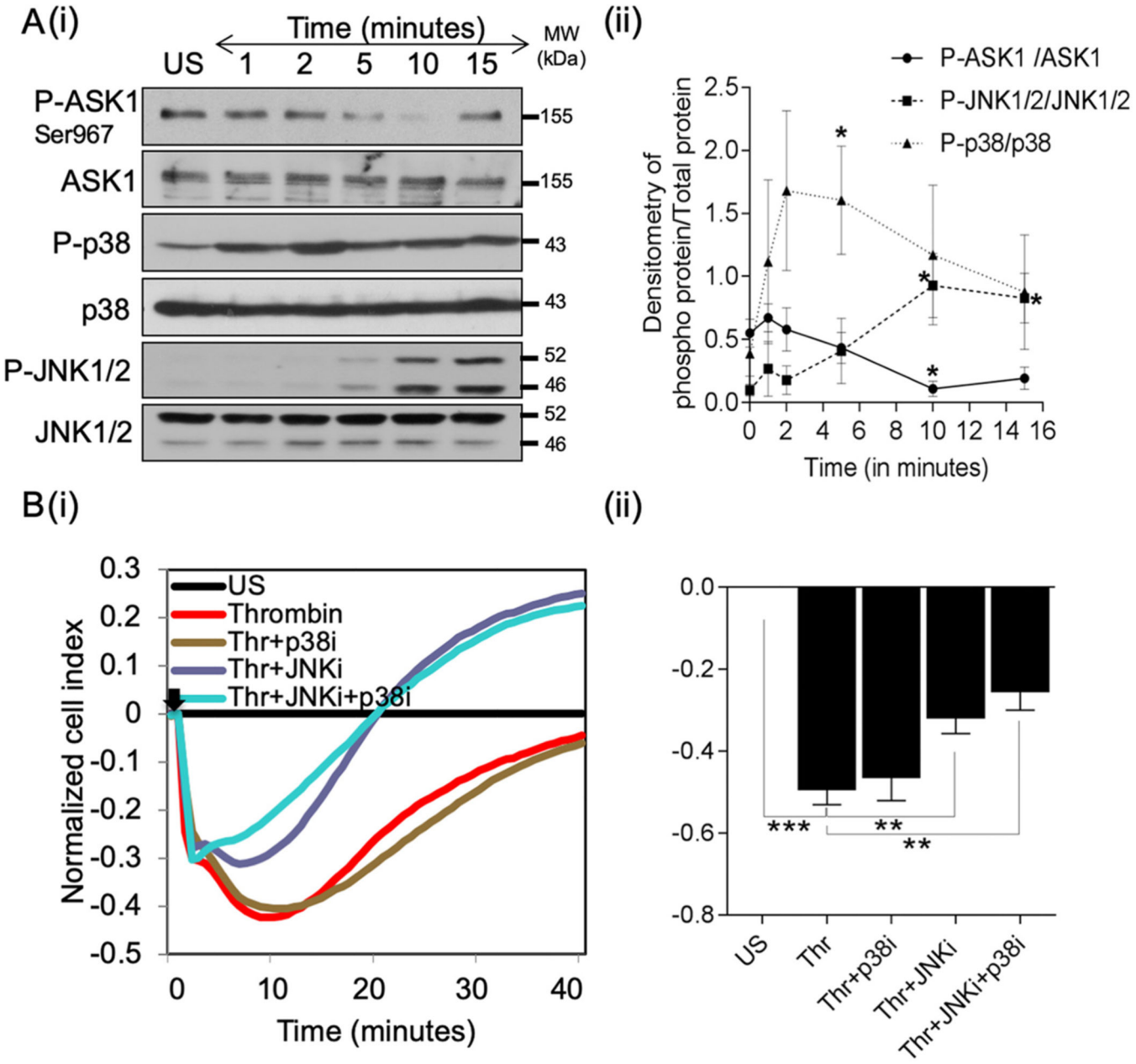

Fig. 6.

Thrombin induces permeability *via* ASK1/JNK, and not ASK1/p38-dependent pathway in HUVECs. (Ai) Representative western blot of HUVEC lysate treated with thrombin (1 U/ml) for various time points (1, 2, 5, 10, and 15 min) and probed with antibodies specific for phospho-ASK1 (Ser-967), P-p38 (Thr180/Tyr182), P-JNK1/2(Thr183/Tyr185). Blots were reprobed with anti-ASK1, anti-p38, and anti-JNK1/2 antibodies to ensure equal protein loading. (Aii) Quantification of band densities from (A) expressed as ratio of phospho-protein to total protein. ($n = 3$). (Bi) Representative baseline normalized tracings of cell index of HUVECs unstimulated (US), or pretreated with p38i, JNKi, or both prior to

stimulation with thrombin (1 U/ml). (Bii) Quantification of cell index data from (Bi) (n = 3). *$p < 0.5$, **$p < 0.001$, ***$p < 0.001$.