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CLIC4 regulates endothelial barrier control by mediating PAR1 signaling via RhoA

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Abstract

Background: Endothelial chloride intracellular channel proteins CLIC1 and CLIC4 are required for the G protein-coupled receptors (GPCRs) sphingosine-1-phosphate (S1P) R1 and S1PR3 to activate the small guanine triphosphatases (GTPases) Rac1 and RhoA. To determine whether CLIC1 and CLIC4 function in additional endothelial GPCR pathways, we evaluated CLIC function in thrombin signaling via the thrombin-regulated protease activated receptor 1 (PAR1) and downstream effector RhoA.

Methods: We assessed the ability of CLIC1 and CLIC4 to re-localize to cell membranes in response to thrombin in human umbilical vein endothelial cells (HUVEC). We examined CLIC1 and CLIC4 function in HUVEC by knocking down (KD) expression of each CLIC protein and compared thrombin-mediated RhoA or Rac1 activation, ezrin/radixin/moesin (ERM) phosphorylation, and endothelial barrier modulation in control and CLIC KD HUVEC. We generated a conditional murine allele of *Clic4* and examined PAR1-mediated lung microvascular permeability and retinal angiogenesis in mice with endothelial-specific loss of *Clic4*.

Results: Thrombin promoted re-localization of CLIC4, but not CLIC1, to HUVEC membranes. Knock-down of CLIC4 in HUVEC reduced thrombin-mediated RhoA activation, ERM phosphorylation, and endothelial barrier disruption. Knock-down of CLIC1 did not reduce thrombin-mediated RhoA activity but prolonged the RhoA and endothelial barrier response to thrombin. Endothelial specific deletion of *Clic4* in mice reduced lung edema and microvascular permeability induced by PAR1 activating peptide.

Conclusions: CLIC4 is a critical effector of endothelial PAR1 signaling and is required to regulate RhoA-mediated endothelial barrier disruption in cultured endothelial cells and murine

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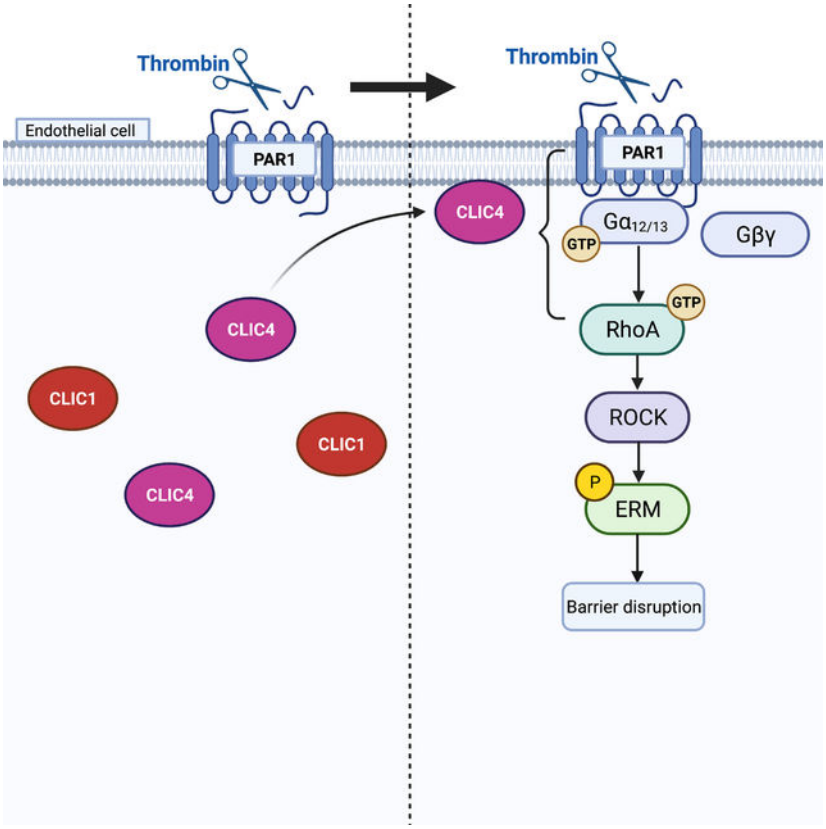
lung endothelium. CLIC1 was not critical for thrombin-mediated barrier disruption but contributed to the barrier recovery phase after thrombin treatment.

Graphical Abstract



Introduction

Chloride intracellular channels (CLICs) are a family of metamorphic proteins with multiple proposed biochemical activities [1]. CLICs were first described as ion channels, consistent with their primary sequence [2, 3], and several CLIC family members have been shown to form non-selective ion channels in artificial bilayers. However, the ability of CLICs to form functional chloride ion channels has not been established in physiological settings [4–6]. CLICs display structural similarity to the omega class of glutathione-S-transferases (GSTs) [7, 8]. Purified CLIC1, CLIC2, and CLIC4 have been shown to exhibit glutaredoxin-like activity that is dependent on a catalytic cysteine residue conserved between other putative glutaredoxin proteins [8]. The physiological relevance of these diverse biochemical activities remains unclear.

We have recently established a novel biochemical function for CLIC proteins in G protein-coupled receptor (GPCR) signaling, specifically in the sphingosine-1-phosphate (S1P) receptor pathway activated by the bioactive lipid S1P. Of the six mammalian CLIC proteins, CLIC1 and CLIC4 are expressed in endothelial cells [9–11] and both play a role in S1P receptor signaling. S1P promoted re-localization of CLIC1 and CLIC4 from the cytoplasm

to the plasma membrane in human umbilical vein endothelial cells (HUVECs) [9]. S1P preferentially binds to and activates the endothelial S1P receptor 1 (S1PR1) which couples with the $G\alpha_i$ subunit [12], activating the small GTPase Rac1, and leading to downstream endothelial responses including cell migration and barrier enhancement [13]. We determined that both CLIC1 and CLIC4 are necessary for S1P-induced Rac1 activation, cell migration, and endothelial barrier enhancement downstream of S1PR1/$G\alpha_i$. S1P also activates S1PR2 and S1PR3, which couple to $G\alpha_{12/13}$ [14], promoting the activation of the small GTPase RhoA, and resulting in the formation of actin stress fibers and increased cell contractility [15, 16]. We observed that CLIC1, but not CLIC4, is necessary for S1P-induced RhoA activation and stress fiber formation downstream of S1PR2 and S1PR3 [9].

Dynamic regulation of endothelial barrier permeability is required to maintain homeostasis and can be positively or negatively regulated by a variety of GPCR signaling pathways, in addition to S1P receptor signaling. Thrombin treatment is well known to cause endothelial barrier disruption [17–19]. Thrombin cleaves and activates several of the protease activated receptor (PAR) family of GPCRs, which are critical for the regulation of endothelial permeability [18–20]. Thrombin is the most well characterized activator of PARs and is known to cleave and activate PAR1, PAR3, and PAR4 [21–23]. PAR1 is thought to serve as the main receptor for thrombin in the vasculature [24, 25]. PAR1 has been found to activate several groups of heterotrimeric G proteins including $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12/13}$ [26], but thrombin signaling is primarily thought to be mediated by signaling events downstream of $G\alpha_{12/13}$ [18, 25]. Activation of $G\alpha_{12/13}$ by PAR1 promotes the activation of RhoA and leads to actin cytoskeletal reorganization, cell contraction, and overall barrier disruption [17].

Previous studies using neuroblastoma cells demonstrated that thrombin receptor-activating peptide (TRP), a synthetic peptide agonist of PAR1 [25, 27], can induce the translocation of CLIC4 to the plasma membrane [28], potentially linking CLIC function with thrombin signaling. We thus hypothesized that endothelial CLICs may be required for thrombin-mediated cell functions. In this study, we found that in endothelial cells CLIC4, but not CLIC1, relocates to the plasma membrane in response to thrombin treatment and that CLIC4, but not CLIC1, is required for thrombin-induced RhoA activation and endothelial barrier disruption. However, CLIC1 and CLIC4 are both necessary for the full range of thrombin downstream signaling and in the recovery of signaling to baseline levels. Using a newly generated *Clic4* floxed mouse strain, we discovered that murine *Clic4* is required in the endothelium for PAR1-mediated microvascular permeability in the lung. These findings establish that CLICs function as essential effectors in several endothelial GPCR pathways that regulate small GTPases and endothelial barrier integrity.

Methods

Data and materials availability

Data and materials that support the findings of this study are available from the corresponding author (Dr. Jan Kitajewski) upon reasonable request.

Primary cells and cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords following standard protocols [29]. Cells were grown on tissue culture plates coated with rat tail type I collagen (Corning) in Endothelial Cell Growth Medium-2 (EGM-2) (Lonza). HUVECs used in experiments were passage 5 or lower. Human embryonic kidney-293T cells (HEK-293T) were acquired from the American Type Culture Collection (ATCC) and maintained in high glucose Dulbecco's modified Eagle medium (Gibco) supplemented with 10% heat inactivated fetal bovine serum (FBS). Unless otherwise noted all cells were grown under standard culture conditions in a humidified incubator set at 37°C and 5% CO_2.

Antibodies and reagents

Human α-Thrombin was purchased from Enzyme Research Laboratories (HT1002a). Alexa Fluor 488 and Alexa Fluor 647 were from Invitrogen. Antibodies to human CLIC1 (1:150, Novus, H00001192-M01), human CLIC4 (1:250, Novus, NBP1-85574), tubulin (1:1000, Sigma-Aldrich, T6074), ERM (1:1000, Cell Signaling Technology, 3142), pERM (1:1000 Cell Signaling Technology, 3141), and PAR1 (1:500, Bioss, BS-0828R) were used for immunoblotting. Antibodies to HA (1:500, Cell Signaling Technology, 3724) and VE-cadherin (1:250, Cayman Chemical, 160840) were used for immunofluorescence.

RNA sequencing

RNA was previously isolated from HUVEC, human retinal endothelial cells (HRECs), and human dermal lymphatic endothelial cells (HDLECs) and sequences were processed and analyzed [9]. This data was used to compare the mRNA expression profiles of the PARs. The raw RNAseq data are available in the NCBI Gene Expression Omnibus repository (accession number GSE163568).

Gene silencing and overexpression

Human CLIC1 and CLIC4 shRNA lentiviral constructs were purchased from MilliporeSigma and evaluated for effective knockdown in HUVEC by immunoblotting. Results of these screenings were previously reported [10, 11]. The CLIC1 shRNA construct used for these experiments targets the gene coding region with targeting sequence 5′-CCTGTTGCCAAAGTTACACAT-3′ (MilliporeSigma). The CLIC4 shRNA construct targets the 5'-untranslated region (UTR) with targeting sequence 5′-GCCGTAATGTTGAACAGAATT-3′ (MilliporeSigma). A lentiviral vector containing a scrambled shRNA sequence 5′-CCTAAGGTTAAGTCGCCCTCG-3′ was used as a control (MilliporeSigma). Wild-type CLIC1 and CLIC4 expression constructs were generated from cDNA isolated from HUVEC and modified to express the HA-tag on the N-terminus. HA-CLIC1 and HA-CLIC4 DNA sequences were placed into the lentiviral pCCL vector for expression in cells.

Lentivirus-mediated stable expression of constructs in HUVECs

For lentiviral gene transfer, HEK-293T cells were transfected using the calcium phosphate method with 3μg of pVSVG, 5μg of pMDLg/pRRE, 2.5μg of pRSV-Rev, and 10μg of pCCL or pLKO vectors containing the genes of interest. After 48hrs the cell media containing

the lentivirus was collected, passed through a 0.45μm filter, and then added onto HUVECs. HUVECs were incubated for 48hrs to allow for vector expression before use in experiments. A pCCL vector containing red fluorescent protein (RFP) was used as a positive control to ensure that infection efficiency was high.

Trans-endothelial electrical resistance (TEER) assay

An electric cell-substrate impedance sensing (ECIS) 8-well array plate (Applied Biophysics) containing ten circular 250-μm-diameter active electrodes connected in parallel on a common gold pad was coated with rat tail type I collagen (Corning). HUVECs were seeded at 50,000 cells per well and allowed to grow overnight in EGM-2 (Lonza). The next day, the media was changed to endothelial basal medium-2 (EBM-2) (Lonza) and cells were serum-starved for 2hrs. The array plate was attached to the ECIS instrument (Applied Biophysics, model 1600R) and baseline resistance was recorded for 30min. Thrombin (4U/mL) or vehicle was then administered, and resistance was recorded at a frequency of 4000 Hz for 2–4hrs.

G-LISA small GTPase activation assays

The RhoA (cat# BK124) G-LISA Activation Assay Kit was purchased from Cytoskeleton. Confluent HUVECs were serum starved in EBM-2 (Lonza) for 3hrs. The cells were then stimulated with 4U/mL thrombin for 5min before cell lysates were collected and snap-frozen in liquid nitrogen. The assay was then performed according to the manufacturer's protocol.

Immunofluorescence assays

HUVECs were plated onto collagen-coated 8-well chamber slides (ibidi) at a density of 25,000 cells per well and allowed to grow to confluency in EGM-2 (Lonza). The next day, the media was changed to endothelial basal medium-2 (EBM-2) (Lonza) and cells were serum-starved for 3hrs. Cells were treated with 4U/mL thrombin for indicated time points. Cells were then fixed with 4% paraformaldehyde (PFA) for 10min and washed three times with phosphate-buffered saline (PBS). Cells were permeabilized for 10min (0.1% Triton X-100 in PBS) and then blocked for 30min with gentle shaking (3% BSA and 0.02% Triton X-100 in PBS). Primary antibodies for HA-tag (1:500; Cell Signaling Technology, 3724) and VE cadherin (1:250, Cayman Chemical 160840) were added and incubated overnight at 4°C. Secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 647 (1:1000; Life Technologies) were added and incubated for 2hrs at room temperature with gentle shaking. Cells were washed three times with PBS and mounted with Antifade Mounting Medium with DAPI (Vectashield, H-1200). Slides were imaged using a Zeiss laser scanning confocal microscope (Zeiss LSM880) through a 63x Plan-Apochromat/1.4NA oil immersion objective.

Quantifying HA-CLIC re-localization

We imaged ≥5 fields from three technical replicates of HUVEC that formed monolayers showing VE-cadherin staining. For each field we imaged 8 to 12 optical z-sections (0.65μm thick). Using Fiji/ImageJ we first created Z-projections of each image and split the HA, VE-Cadherin and DAPI channels. We used the "Subtract Background" function, and

manually removed background nuclear staining, on the VE-Cadherin channel and converted this channel to a binary image which we used to create a region-of-interest (ROI) mask demarcating all the membrane localized VE-Cadherin. This ROI mask was then used to measure VE-Cadherin and HA signal intensities in the original Z-projected image (Max intensity) converted to binary. HA to VE-Cadherin ratios were normalized to the average of the 0min control group from same technical replicate.

Live RhoA and Rac1 FRET measurements in HUVEC

HUVECs were lentivirally infected with both shRNA and RhoA or Rac1 FRET sensor constructs [30–32]. 24hrs after infection HUVECs were seeded onto collagen-coated 8-well chamber slides (ibidi) at a density of 5,000 cells per well and allowed to grow overnight in EGM-2 (Lonza). The remaining cells were seeded onto a collage-coated 10cm plate for lysate collection the next day for later confirmation of CLIC-KD by Western blot. The day after seeding (48hrs after infection) the media was changed to endothelial basal medium-2 (EBM-2) (Lonza) and cells were serum-starved for 3hrs. Cells were changed to imaging media (10mM HEPES in Hank's balanced salt solution (HBSS) containing Ca^{2+} and Mg^{2+}) 30min before the start of imaging. Cells were imaged at 40X using a Zeiss laser scanning confocal microscope (Zeiss LSM880) with 37°C incubation. Cyan fluorescent protein (CFP) was excited using a 458nm laser and CFP emission (455–510nm) and yellow fluorescent protein (YFP) emission (>510nm) were simultaneously collected. At time 1.5min thrombin was added for a final concentration of 4U/mL. Images were collected every 10sec for 10–20min. FRET imaging and ratio analysis was performed in ImageJ using a macro adapted from Timmerman *et al.* (2015) [32].

Immunoblotting

HUVECs were serum starved in EBM-2 media for 3hrs before treatment with 4U/mL thrombin. Cells were then washed with ice-cold PBS and lysates were collected using TENT lysis buffer (50mM tris (pH 8.0), 2mM EDTA, 150mM NaCl, and 1% Triton X-100) supplemented with a protease inhibitor cocktail (Thermo Scientific). Lysates were prepared for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using sample buffer containing SDS and β-mercaptoethanol. SDS-PAGE was performed followed by wet transfer onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in tris-buffered saline (TBS)-Tween solution. Primary antibodies were incubated in 2.5% milk in TBST overnight at 4°C. Secondary antibodies conjugated to horseradish peroxidase (HRP) were incubated in 2.5% milk in TBST at room temperature for 1hr. The membrane was then developed using enhanced chemiluminescence detection reagent (GE Healthcare) and imaged using a BioRad imager.

Generation of *Clic4*^{flox} transgenic mice

All animal studies and experiments were approved by the Institutional Animal Care and Use Committee of the University of Illinois. *Clic4*$^{tm1a(EUCOMM)HMGU}$ targeted embryonic stem (ES) cells were obtained from the European Conditional Mouse Mutagenesis Program (EUCOMM)[33] and were injected into blastocysts for chimera production at the Transgenic & Targeted Mutagenesis Laboratory at Northwestern University. Two chimeric males were generated and transferred to our animal facility at the University of Illinois at Chicago (UIC)

Biologic Resources Laboratory (BRL). These chimeras were bred with wild type C57BL/6J mice to produce offspring. Germline transmission was established through genotyping by confirming the presence of the downstream LoxP site. Once germline transmission was confirmed, the F2 generation of *Clic4*^{tm1a} offspring were bred to a mouse line expressing flippase recombinase (Flp) [34] to remove the neomycin selection cassette and generate the *Clic4*^{flox} allele. *Clic4*^{flox} mice negative for the *rd8* mutation (originating in the C57BL6/N ES cells) were crossed with endothelial-specific *Cdh5-CreER*^{T2} mice [35]. *Clic4*$^{flox/flox}$; *Cdh5-CreER*^{T2} (Clic4^{ECKO}) and *Clic4*^{WT}; *Cdh5-CreER*^{T2} (control) offspring were used for experiments. Mice were maintained on a C57BL6/J background.

Assessment of murine neonatal retinal angiogenesis and vascular permeability

To assess murine CLIC4 function in retinal angiogenesis, weight-matched litters of control and Clic4^{ECKO} pups were administered tamoxifen (50μl 2mg/ml in corn oil) intra-gastrically to excise *Clic4* at postnatal days (P) 1–3. All weight-matched pups from a litter were used regardless of sex. Retinas were isolated at P5 and fixed in 4% paraformaldehyde (Thermo Fisher Scientific) for 1h at 4 °C with agitation. Retinas were dissected and stained in 1% BSA (Fisher Bioreagents) and 0.5% Triton X-100 (Fisher Bioreagents) using Biotinylated IB4 (1:250; Vector Laboratories, B-1205) and rat anti-mouse VE-Cadherin (1:200; BD Bioscience, #555289). For the albumin leakage analysis, pups were injected intraperitoneally with 40μL of 2mg/mL labeled Albumin-Alexa Fluor 488, which was allowed to enter the hepatic vessels and circulate for 2 hours prior to sacrifice.

Whole-mount stained retina images were acquired using Leica Dmi8 Platform. All images were analyzed using ImageJ (NIH). Vascular outgrowth was quantified by measuring the distance from the optic nerve to the angiogenic front. Vascular density was quantified by measuring the area of vessels in a 2000×600 pixel selection of the angiogenic plexus, and branchpoint density was quantified using the same selection but measuring the number of junctions in the given field with the ImageJ skeletonization tool. Tip cells were counted manually, and density was determined by dividing the number of tip cells by the total length of the angiogenic front.

To determine albumin leakage and VE-cadherin vessel coverage, a 1300×800 pixel region was selected for analysis from each retinal lobe. Total area coverage of albumin or VE-cadherin were determined within Isolectin B4-labeled vessels. For albumin, total area coverage was measured to calculate a ratio between vascular and extra-vascular signal. For each retinal assay, four measurements were taken per retina, averaged, and then averaged between both retina of the same animal to produce a single data point.

Assessment of lung edema and vascular permeability

To assess the role of CLIC4 in regulating endothelial barrier function mediated by PAR1 signaling, 4 cohorts of mice including 21 control mice (10 without PAR1-peptide and 11 with PAR1-peptide) and 24 Clic4^{ECKO} mice (12 without PAR1-peptide and 12 with PAR1-peptide) were used. Male and female mice were used in approximately equal numbers in all categories. Starting at P28 tamoxifen was administered (2mg in corn oil) to induce endothelial gene deletion. At P42 mice were randomized and blinded. To assess

PAR1-activating peptide (TFLLR) induced lung edema and vascular permeability, 100μL of Evans blue-tagged albumin (10mg/ml of Evans blue in 3% charcoal-stripped albumin) was injected retro-orbitally and after 15min the PAR1 peptide (1mg/kg) was injected intravenously (IV). Mice were sacrificed 30min after injection of the PAR1 peptide. Blood was collected in a heparinized tube and centrifuged for 5min at 5000rpm to collect plasma. Lungs were perfused with cold PBS. The left lung lobe was isolated and immediately weighed to determine the wet weight, then placed in an oven set at 55°C overnight. The right lung lobe was extracted and homogenized in 1mL of PBS then mixed with 2mL of formamide and incubated at 55°C overnight. After 24hrs the lung dry weight was taken and lung wet/dry weight ratio was calculated as described previously [36, 37]. Vascular permeability was quantified by centrifuging the plasma and lung homogenate to isolate the supernatant. The optical density was measured spectrophotometrically at 595nm (Evans blue) and 750nm (hemoglobin correction). Evans blue albumin extravasation was calculated by transendothelial albumin influx in lungs versus plasma [36, 37].

Statistical analysis

Tests used for each experiment are indicated in the figure legends. All data was tested for gaussian distribution using the Shapiro-Wilk or D'Agostino-Pearson tests and for equal variance using a Brown-Forsythe test, F test, or Bartlett's test. Only minor exceptions to normal distribution were detected which did not alter significance or non-significance of results. Non-parametric tests (i.e. Welch's t-test or Welch's ANOVA with Games-Howell corrections) were used in cases where data did not pass equal variance testing. One-way analysis of variance (ANOVA) with post hoc pairwise analyses with Bonferroni corrections were used for experiments with multiple conditions from one factor. Two-way ANOVA with post hoc pairwise analyses with Bonferroni corrections were used for experiments with multiple conditions from two factors. Area under the curve (AUC) averages and standard error were computed using the Gagnon method [38]. Gagnon analysis produces a single value with standard error for each condition, so individual AUC values are not determined. Unpaired two-tailed t-tests were used for comparisons of two conditions. Statistical tests were analyzed using GraphPad Prism (version 9.1.1). *P* values less than 0.05 were considered statistically significant.

Results

Endothelial CLIC4, but not CLIC1, localizes to the cell membrane in response to thrombin

We previously established that HA-tagged endothelial CLICs (CLIC1 and CLIC4) retain function and are primarily located in the cytoplasm of HUVECs and that in response to S1P treatment CLIC1 and CLIC4 transiently accumulate at the cell membrane within minutes of agonist addition [9]. To assess CLIC function in PAR signaling we first determined whether endothelial CLIC localization responds to thrombin/PAR signaling. We treated HA-tagged CLIC1 or CLIC4-expressing HUVEC with thrombin for up to 10 minutes, which is sufficient time to elicit activation of downstream effectors (see below), before fixing and immunostaining. Prior to thrombin treatment, HA-CLIC1 and HA-CLIC4 were observed throughout the cytoplasm (Fig. 1A, B), as previously described [9]. By 5min after thrombin addition we found significant accumulation of HA-CLIC4 at the plasma

membrane, overlapping with VE-cadherin (Fig. 1B, D), indicating that CLIC4 responds to thrombin signaling, and thus may play a role in this pathway. In contrast, HA-CLIC1 did not re-localize from the cytoplasm to the plasma membrane within 10 minutes (Fig. 1A, C), indicating that CLIC1 localization is not regulated by thrombin signaling and it may not play a role in this pathway.

Endothelial CLIC1 and CLIC4 regulate thrombin-induced changes in RhoA and Rac1 activity

Thrombin activates the GPCR PAR1, which then signals via the heterotrimeric G protein subunit $G\alpha_{12/13}$ to regulate RhoA activity [17, 25]. Endothelial RhoA acts as a critical effector in the PAR1 signaling pathway and is necessary for the barrier disrupting effects of thrombin [17]. Based upon our discovery that endothelial CLICs are required for activation of Rac1 and RhoA downstream of S1P receptors [9] we hypothesized that CLIC1 and CLIC4 participate in PAR1-mediated activation of RhoA, and we used loss-of-function studies to address this hypothesis. Analysis of RNA sequence data of multiple endothelial cell types revealed that PAR1 is the most highly expressed of the four PAR family members (Fig. S1C–E) suggesting that PAR1 is the primary receptor involved in endothelial thrombin signaling. Lentivirus vectors were used to introduce previously validated short hairpin RNA (shRNA) expression targeting either CLIC1 or CLIC4 in HUVEC [9–11]. Knockdown efficiency was evaluated by Western blot using CLIC1 and CLIC4 specific antibodies (Fig. S1A–B). We confirmed expression of PAR1 protein in HUVEC by Western blot and verified that expression levels of PAR1 do not change in CLIC1-KD and CLIC4-KD HUVEC compared to scramble shRNA control HUVEC (Fig. 2A).

To assess RhoA activity we used a G-LISA assay to quantify the amount of activated GTP-bound RhoA present in endothelial cell lysates after five minutes of thrombin treatment. At the five-minute time point, thrombin elicits activation of RhoA in control and CLIC1-KD HUVEC (Fig. 2B). In CLIC4-KD cells we observed a small increase in thrombin-induced GTP-bound RhoA, but this increase was significantly less than the thrombin response in control or CLIC1-KD cells and not statistically significant from untreated cells (Fig. 2B). This demonstrated that CLIC4, but not CLIC1, is required for thrombin-induced RhoA activation in cultured endothelial cells.

The G-LISA assay measures overall RhoA activity at a given timepoint but lacks spatial and temporal information. We utilized a RhoA FRET sensor to collect more detailed information on the requirement of CLICs for rapid RhoA activation. The FRET sensor we employed is a unimolecular construct consisting of full length RhoA conjugated to cyan fluorescent protein (CFP) connected via a linker to yellow fluorescent protein (YFP) conjugated to the Rho binding domain of PKN1 [31]. When RhoA is GDP-bound and inactive it does not bind to PKN1 causing low FRET between CFP and YFP. In this state excitation of CFP mainly generates CFP emission. When RhoA is GTP-bound and active it binds to PKN1 causing high FRET between CFP and YFP, thus excitation of CFP now generates increased YFP emission. The ratio of YFP to CFP can be used as a readout for RhoA activity within a single cell and has previously been characterized in HUVEC [30]. An increase in the YFP/CFP ratio indicates an increase in RhoA activity. HUVEC expressing the RhoA

sensor were treated with thrombin at 1.5min after the start of imaging and monitored for a total of 20 minutes. In control cells and CLIC1-KD cells a comparable increase in RhoA activity can be seen localized mainly to the cell periphery (Fig. 2C). In CLIC4-KD cells no peri-membrane increase in RhoA activity was detected and overall thrombin-mediated induction of RhoA activity was markedly reduced (Fig. 2C). The overall YFP/CFP ratio shows that in control cells RhoA activity rapidly increased, reaching a maximum of about 1.24-fold above baseline one minute after thrombin treatment before gradually returning towards baseline (Fig. 2D). In the CLIC1-KD cells we see a slightly greater and prolonged peak in RhoA activity of about 1.25-fold with a slower rate of return towards baseline levels (Fig. 2D). In addition, CLIC1-KD results in a larger area under the curve suggesting an overall greater RhoA response to thrombin treatment compared to control cells (Fig. 2E). In contrast, the CLIC4-KD cells show significantly lower RhoA activity, with a peak of about 1.14-fold of baseline (Fig. 2D). The CLIC4-KD area under the curve is also reduced, signifying less overall RhoA response (Fig. 2E). Taken together we conclude that endothelial CLIC4 functions at the plasma membrane to promote rapid thrombin-mediated activation of RhoA. In contrast, CLIC1 appears to participate in the recovery phase and is important for the later return of RhoA activity to baseline levels, although this function does not appear to require plasma membrane localization of CLIC1.

While PAR1 primarily signals through RhoA [17, 39] cross-talk among Rho and Rac GTPases has been well documented in endothelial cells and RhoA is known to inhibit Rac1 activity in HUVEC [17, 30, 40]. To ask if thrombin/PAR1 dependent Rac1 activity is altered by loss of CLIC1 or CLIC4 we used a Rac1 FRET reporter [30, 32]. We found that thrombin elicited modest Rac1 activation (maximum 1.11-fold at one minute after thrombin addition) (Fig. S2A). CLIC4, but not CLIC1, knockdown caused a small, but consistent and significant, increase in Rac1 activation (Fig. S2A, B). Increased Rac1 activation in CLIC4-KD is consistent with the decrease in RhoA activity reported above, suggesting that plasma membrane localized CLIC4 may help modulate cross-talk between these GTPases in response to thrombin.

Endothelial CLIC1 and CLIC4 are necessary for thrombin-mediated phosphorylation of ERM proteins

Ezrin/radixin/moesin (ERM) are a family of proteins that, when phosphorylated, participate in membrane-cytoskeletal reorganization important in facilitating endothelial barrier disruption [41, 42]. Thrombin/PAR1 signaling promotes the phosphorylation of ERMs [41]. We evaluated how loss of CLIC1 or CLIC4 affects thrombin-mediated phosphorylation of ERMs using a phospho-specific ERM antibody to determine the amount of phosphorylated ERM (pERM) present five minutes after thrombin treatment. Both CLIC1-KD and CLIC4-KD cells had significantly less pERM after thrombin stimulation as compared to control cells, with CLIC4-KD cells showing the lowest induction of pERM (Fig. 3A and B). This indicates that both endothelial CLICs, and particularly CLIC4, function to enhance thrombin-mediated phosphorylation of ERM proteins.

Endothelial CLIC1 and CLIC4 are required to regulate thrombin-mediated endothelial barrier disruption

As a component of its physiological function, thrombin targets endothelial cells to disrupt the endothelial cell-cell barrier. To establish whether CLICs function in thrombin signaling to control endothelial barrier integrity we used trans-endothelial electrical resistance (TEER) to monitor changes in barrier integrity in response to thrombin. In control cells, barrier integrity decreased upon the addition of thrombin before recovering to baseline (Fig. 4A, S3A, D–E). In CLIC1-KD cells a more modest decrease in barrier integrity was observed as compared to control, with a more gradual rate of recovery of barrier integrity towards baseline (Fig. 4A, S3B, D–E). CLIC4-KD cells had a significantly smaller decrease in barrier integrity as compared to the control cells and with an earlier return to baseline (Fig. 4A, S3C–E). These differences in barrier response were evaluated using quantification of the area under the curve after thrombin stimulation. CLIC1-KD resulted in a larger total area under the curve compared to control, while CLIC4-KD caused a smaller area under the curve (Fig. 4B), indicating that the overall barrier disruption event caused by thrombin is greater in CLIC1-KD cells and is markedly reduced in CLIC4-KD cells.

The barrier response to thrombin can further be broken down into the initial thrombin-mediated barrier opening phase and the recovery phase [17, 43]. In the barrier opening phase, as defined by the period between thrombin addition and the point at which the lowest resistance was measured (approximately 25min), there is no significant difference between CLIC1-KD and control (Fig. 4C). In the barrier recovery phase, from the time of lowest resistance to the time of return to baseline resistance the area under the curve is greater, and the rate of barrier recovery is lower, in CLIC1-KD cells when compared to control (0.0104Ω-cm^2/min and 0.0358Ω-cm^2/min, respectively) (Fig. 4D–E). In CLIC4-KD cells, the area under the curve is significantly reduced in both the barrier opening phase and barrier recovery phase as compared to control (Fig. 4C–E). Taken together, these data demonstrate that CLIC4, but not CLIC1, is necessary for thrombin-mediated barrier disruption, while CLIC1 is critical in the recovery of barrier integrity following disruption by thrombin.

Endothelial *Clic4* is necessary for PAR1-mediated lung microvascular permeability in mice

We sought to determine whether thrombin/PAR1 mediated endothelial barrier disruption required CLIC function in the lung endothelium of mice, where thrombin/PAR signaling is known to regulate barrier integrity [44]. We generated a new conditional *Clic4* knockout allele (*Clic4^{flox}*)[33] to study the impact of loss of *Clic4* function in murine endothelium (Fig. S4A). To verify that we could induce deletion of *Clic4* using the new allele, we crossed *Clic4^{flox}* mice with a ubiquitous *R26R-Cre-ER^{T2}* expressing line (*R26R-Cre*) and tamoxifen (50μl at 2mg/ml in corn oil) was administered at post-natal days 1, 2, and 3 (P1-P3) to induce gene recombination in all cell types. Kidneys, which have abundant CLIC4 expression [45], were harvested at P10, homogenized, and the resulting protein lysates used for Western blots. Control mice showed no change in the expression of CLIC4 or CLIC1 (Fig. S4B). *Clic4$^{fl/fl}$;R26R-Cre+* mice showed near complete loss of CLIC4 protein with no change in expression of CLIC1 protein (Fig. S4B), indicating that the *Clic4^{flox}* allele reduces CLIC4 protein production in the presence of Cre activity.

Using this validated *Clic4*^{flox} allele, we characterized vascular phenotypes produced by loss of *Clic4* from the endothelium. We crossed the *Clic4*^{flox} allele with the endothelial cell specific *Cdh5-Cre-ER*^{T2} driver to generate *Clic4*$^{flox/flox}$*; Cdh5-CreER*^{T2} (Clic4^{ECKO}) to specifically delete *Clic4* from endothelium [35, 46]. *Clic4*^{WT}*; Cdh5-CreER*^{T2} were used as controls in all experiments. The mouse post-natal retina was used as a model for vascular development and endothelial barrier integrity [47, 48]. To excise *Clic4* specifically from endothelial cells, pups were administered tamoxifen as described above and retinas were examined at P5. We determined that during normal retinal angiogenesis, Clic4^{ECKO} mutants show a modest and significant reduction in tip cell formation, but no alterations in outgrowth, vascular coverage, or branching (Fig. 5A–E). These findings are consistent with the relatively modest phenotype observed in retinal angiogenesis in Clic4 null mutants [49]. To examine the endothelial barrier during retinal vascular development, we quantified VE-cadherin vessel coverage and retention of intravascular labeled albumin. Both measures appeared to be normal in Clic4^{ECKO} mutants, suggesting that endothelial barrier function does not require *Clic4* during retinal vascular development (Fig. 5F–G).

After establishing that endothelial *Clic4* is not necessary for barrier integrity during retinal vascular development, we determined whether loss of *Clic4* from the lung endothelium would affect PAR1 signaling. To induce endothelial deletion, we administered tamoxifen (2 mg in corn oil) to Clic4^{ECKO} and control mice for five days starting at P28. Thrombin catalyzes multiple PAR1-indepenent pathways, such as blood coagulation, so we used a PAR1-specific activating peptide (TFLLRN) that specifically activates PAR1 to increase lung microvascular permeability and lung edema [44, 50] while avoiding effects on coagulation. At P42, mice were intravenously injected with PAR1-activating peptide (1mg/kg) or control, and lungs were harvested and assessed for lung vascular permeability after 30 minutes. All 4 treatment conditions (treated and untreated Clic4^{ECKO} and controls) were evaluated in 4 independent cohorts of mice, for a total of 24 Clic4^{ECKO} and 21 control animals. Both male and female mice were used.

The ratio between the weight of a freshly dissected lung and its dehydrated tissue defines the amount of fluid that has infiltrated the lung and is used to quantify lung edema [44, 50]. In control mice, the PAR1-activating peptide robustly increased the lung wet/dry weight ratio, confirming that PAR1 signaling increased lung microvascular permeability and resulted in lung edema (Fig. 5H). In Clic4^{ECKO} mice, no significant change in lung wet/dry weight ratio was observed, demonstrating that endothelial *Clic4* is necessary for PAR1-mediated changes in endothelial barrier integrity in the murine lung (Fig. 5H). Intravenously injected Evans blue-labeled albumin (EBA) enters the parenchyma through disrupted vascular barrier, and thus detection of EBA in tissue is an indicator of increased vascular permeability. In control mice, the PAR1-activating peptide significantly increased leakage of EBA into lung parenchyma, although this effect was modest and was not significant in all experimental cohorts, suggesting that this PAR1-activating peptide only weakly induces macromolecule leakage (Fig. 5I). In Clic4^{ECKO} mice, no significant change in EBA leakage was observed after PAR1-activating peptide administration, demonstrating a requirement of *Clic4* for PAR1-mediated endothelial barrier disruption (Fig. 5I).

Discussion

In this study, we identified endothelial CLIC4 as an effector in the thrombin/PAR1 signaling pathway. CLIC4 rapidly (within 5min) relocated to the plasma membrane in response to thrombin and was necessary for thrombin-mediated RhoA activation. In contrast, we did not detect significant CLIC1 membrane re-localization within the first 10 minutes after thrombin treatment, nor did CLIC1 knock-down significantly affect RhoA activation. CLIC4-KD caused impairment in thrombin-mediated ERM phosphorylation and endothelial barrier disruption. To study CLIC4 function in mice, we generated a *Clic4*^{flox} mouse line and crossed it to an inducible *Cdh5-Cre-ER*^{T2} to generate endothelial cell knock out mice (ECKO). Loss of endothelial *Clic4* significantly reduced numbers of angiogenic tip cells during retinal vascular development, but other features of retinal vessels were not noticeably affected by loss of *Clic4*. Using the Clic4^{ECKO} mice, we determined that retinal and lung vascular permeability were not altered during homeostasis, however, loss of endothelial *Clic4* caused a loss of the response of lung endothelial cells to a PAR1 agonist. Specifically, Clic4^{ECKO} mice challenged with PAR1-activating peptide failed to exhibit any increase in lung vascular permeability despite PAR1 activation, indicating that PAR1 signaling requires endothelial *Clic4*. Thus, our evidence defines CLIC4 as a central effector transmitting thrombin-PAR1 mediated signaling to RhoA in endothelium (Fig. 6).

ERM proteins are critical regulators of changes in vascular permeability in response to thrombin, and thrombin/PAR1 signaling induces ERM phosphorylation, in part, via RhoA activity [41, 51]. It was previously established that CLIC4 is required for ERM phosphorylation in the glomerular endothelium [51], but it remained unclear in this context how CLIC4 functioned to promote ERM phosphorylation. Here we show that CLIC4 is necessary for both thrombin-mediated RhoA activation and ERM phosphorylation. Thus, we propose that one way CLIC4 regulates barrier integrity is via RhoA-dependent ERM phosphorylation downstream of PAR1.

Although more modest than the effect seen with CLIC4-KD, we found a significant decrease in ERM phosphorylation in CLIC1-KD cells when compared to control. This is especially interesting in light of the fact that CLIC1 is not required for thrombin-mediated RhoA activation, and instead CLIC1-KD prolongs the duration of RhoA activity and barrier disruption in response to thrombin. Thus, although CLIC1 is not required for PAR1- RhoA-dependent barrier disruption, we propose that it does play a role in signaling pathway(s) involved in the re-establishment of barrier integrity after thrombin treatment. Notably, radixin, one of the ERM proteins, is also required for recovery of barrier integrity after thrombin treatment [41]. We speculate that the modest decrease in ERM phosphorylation caused by CLIC1-KD reflects a decrease in radixin phosphorylation, and that a CLIC1-radixin signaling axis is important for barrier recovery.

It has been suggested that barrier recovery after thrombin-mediated disruption is due to cross-talk between the PAR1 pathway and the S1P signaling pathway [17, 52]. HUVEC continuously produce and release S1P which helps to maintain endothelial barrier homeostasis [14]. After a barrier disrupting event, such as treatment with thrombin, endothelial cell derived S1P activates S1PR signaling, which increases Rac1 activity,

reduces RhoA activity, and thus facilitates recovery of barrier integrity [14, 17, 40]. Our previous studies established that CLIC1 is required for S1P-mediated Rac1 activation and barrier enhancement [9], leading us to speculate that barrier recovery and prolonged activation of RhoA after thrombin treatment of CLIC1-KD cells is likely due to loss of S1P pathway function.

Together, these results complement our previous findings of the role of CLICs in S1P signaling [9] and further support a model where the endothelial CLICs have distinct and specific functions in promoting Rho-family GTPase activity downstream of different GPCRs, and that their involvement is dependent on the signaling context. This is exemplified by the fact that in the S1P pathway only CLIC1 is required for RhoA activation and downstream functions [9], while our work here shows that in the PAR1 pathway it is CLIC4, and not CLIC1, that is required for RhoA activation. S1PR3 and PAR1 both signal through $G\alpha_{12/13}$ to promote the activation of RhoA [14–18, 25]. This is often mediated by Rho guanine exchange factors (RhoGEFs), which function to promote the exchange of GDP for GTP on Rho-family proteins in order to activate them [53]. There are several RhoGEFs that are known to mediate signaling between $G\alpha_{12/13}$ and RhoA, including p115-RhoGEF, PDZ-RhoGEF, and LARG [54, 55]. Different RhoGEFs can be involved in this function depending on the pathway being activated. It is not yet clear which particular RhoGEFs participate in S1PR3 and PAR1 signaling. Several studies suggest that p115-RhoGEF is responsible for PAR1-mediated RhoA activation [17, 56]. Some studies also suggest that PDZ-RhoGEF and LARG are also involved in linking PAR1 to RhoA [57]. Less is known about the RhoGEFs involved in S1PR2 and S1PR3 signaling. There is some evidence that LARG plays a major role in RhoA activation downstream of S1PR2 [58]. Future studies could focus on the hypothesis that the specificity for which CLIC mediates RhoA activation is related to the specific RhoGEFs involved in the given signaling pathway.

In conclusion, we demonstrate using human cells (HUVEC) and murine evaluation, that CLIC4 is required for PAR1-mediated endothelial barrier disruption. Taken together with our prior results [9] we report that CLICs function in several GPCR pathways and control the activity of members of the Ras superfamily (eg. Rac1 and RhoA). Endothelial CLIC4 has also been shown to play a protective role in pulmonary artery hypertension (PAH) by binding to and increasing the activity of the small GTPase Arf6, a member of the Ras superfamily [59, 60]. These results support a model where individual CLIC proteins regulate Ras proteins, possibly via multiple mechanisms, and are critical regulators of vascular health outcomes. Our future work will investigate how endothelial specific CLICs interact with GPCR signaling *in vivo* and how this regulates effective angiogenesis and vascular function in healthy states and in diseases such as PAH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations and Acronyms

CLIC	chloride intracellular channel
GST	glutathione-S-transferase
S1P	sphingosine-1-phosphate
GPCR	G protein-coupled receptor
HUVEC	human umbilical vein endothelial cell
TRP	thrombin receptor-activating peptide
PAR	protease activated receptor
shRNA	short hairpin RNA
YFP	yellow fluorescent protein
CFP	cyan fluorescent protein
ERM	ezrin/radixin/moesin
TEER	trans-endothelial electrical resistance
EBA	Evans blue-labeled albumin
ECKO	endothelial cell knock out
RhoGEF	Rho guanine exchange factor
HREC	human retinal endothelial cell
HDLEC	human dermal lymphatic endothelial cell
PAH	pulmonary artery hypertension
GTPase	guanine triphosphatase

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Highlights

- Chloride intracellular channel 4 (CLIC4), but not CLIC1, localizes to the endothelial cell membrane after thrombin treatment.
- CLIC4 is required for thrombin-mediated RhoA activation, ezrin/radixin/moesin (ERM) phosphorylation, and endothelial barrier disruption downstream of protease activated receptor 1 (PAR1).
- CLIC1 is necessary for thrombin-mediated ERM phosphorylation and knockdown of CLIC1 results in prolongation of thrombin-mediated RhoA activation and endothelial barrier disruption.
- Endothelial specific deletion of *Clic4* in mice results in reduced lung edema and microvascular permeability in response to a PAR1 activating peptide.

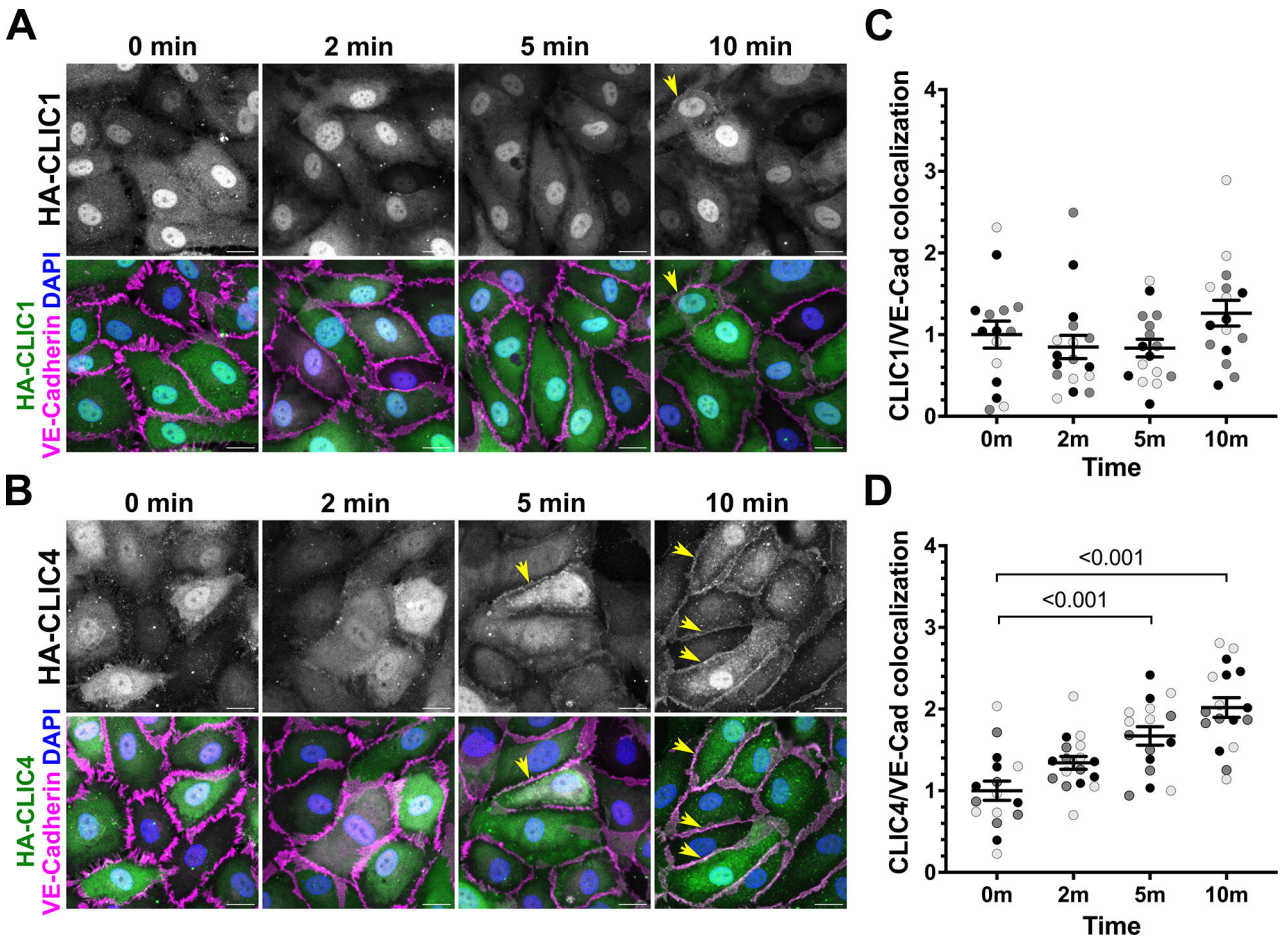


Figure 1. Thrombin promotes the relocation of CLIC4, but not CLIC1, to the cell membrane in endothelial cells.

Representative confocal micrographs of HUVECs transfected with HA-CLIC1 (**A**) or HA-CLIC4 (**B**) treated with thrombin for 2, 5, or 10 mins. Cells were immunostained with anti-HA (green) and anti-VE-cadherin (magenta) to mark the cell membrane and DAPI (blue) was used to mark the nucleus. Yellow arrowheads indicate HA-CLIC accumulation at the plasma membrane. Scale bar: 20μm. We quantified HA-CLIC1 (**C**) and HA-CLIC4 (**D**) membrane accumulation as a ratio of HA to VE-cadherin signal, and each data point was normalized to the average of the value at 0min. We found that HA-CLIC4, but not HA-CLIC1, significantly re-localized to the plasma membrane by 5 minutes after thrombin addition. This experiment was repeated three times with different fields evaluated for each timepoint. Data points obtained from each technical replicate (n≥5 fields per experiment) are denoted by distinct shades of grey. Horizontal bars represent the average, error bars represent the SEM, and p-values were determined by one-way ANOVA with Bonferroni correction for multiple comparisons.

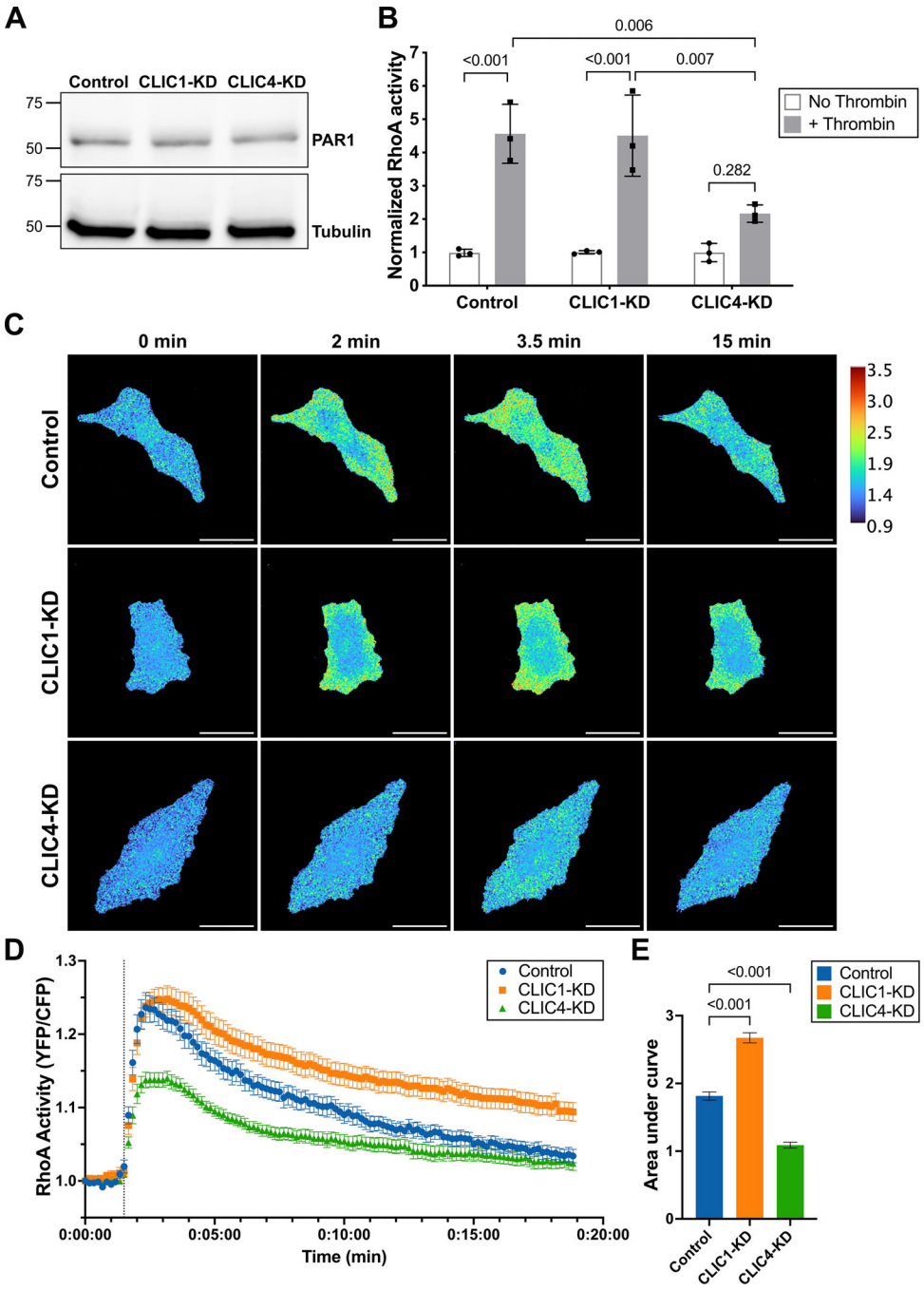


Figure 2. Endothelial CLICs are necessary to regulate RhoA activity after thrombin treatment.

(A) Western blot of HUVECs confirm expression of PAR1 and reveals that knock down of CLIC1 or CLIC4 does not change level of PAR1 expression. (**B**) HUVECs knocked down for CLIC1 or CLIC4 and controls were serum starved for 3hrs then stimulated with 4U/mL thrombin or vehicle for 5min. Lysates were collected and the amount of active RhoA was determined by G-LISA assay. Amount of active RhoA is shown as normalized fold change in relation to the unstimulated sample for each cell line. Data are means ±SEM from three independent experiments. P-values determined by two-way ANOVA with Bonferroni

correction for multiple comparisons. **(C** and **D)** HUVECs transfected with a RhoA FRET biosensor and knocked down for CLIC1, CLIC4, or control plasmid were serum starved for 3hrs, then stimulated with 4U/mL thrombin. YFP and CFP intensity were captured by confocal microscope for 20min with thrombin addition occurring at time 1.5min (dashed line). **(C)** Ratiometric images of HUVECs. Warm colors represent high activation of RhoA. Scale bar: 50μm. **(D)** Normalized mean YFP/CFP ratio traces (±SEM) show total RhoA activity over time. Data are means of 16 individual cells for each condition from five independent experiments. **(E)** Mean area under the curve ±SEM was calculated for each condition from the time of thrombin addition until the last time point. P-values determined by Welch's ANOVA with Games-Howell correction for multiple comparisons.

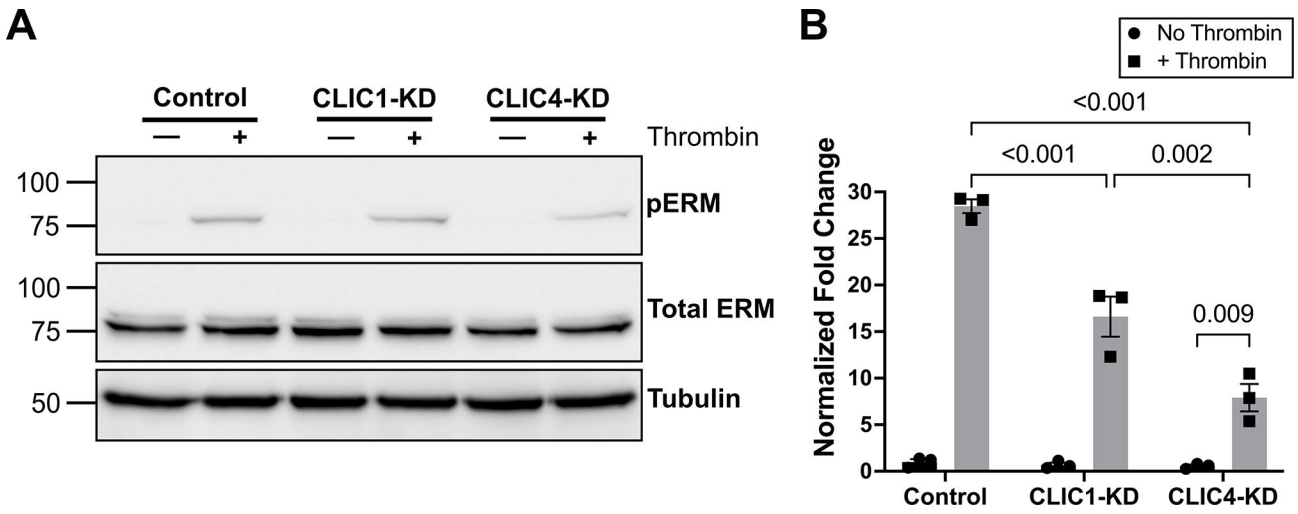


Figure 3. Endothelial CLICs are necessary for thrombin-mediated phosphorylation of ezrin/radixin/moesin.

HUVECs knocked down for CLIC1 or CLIC4 and controls were serum starved for 3hrs then stimulated with 4U/mL thrombin or vehicle for 5min. Lysates were collected and assessed for phosphorylation of ezrin/radixin/moesin (ERM) by Western blotting **(A)**. Densitometry of phosphorylated ERM was normalized to that of total ERM **(B)**. Data are means ±SEM from three independent experiments. P-values determined by two-way ANOVA with Bonferroni correction for multiple comparisons.

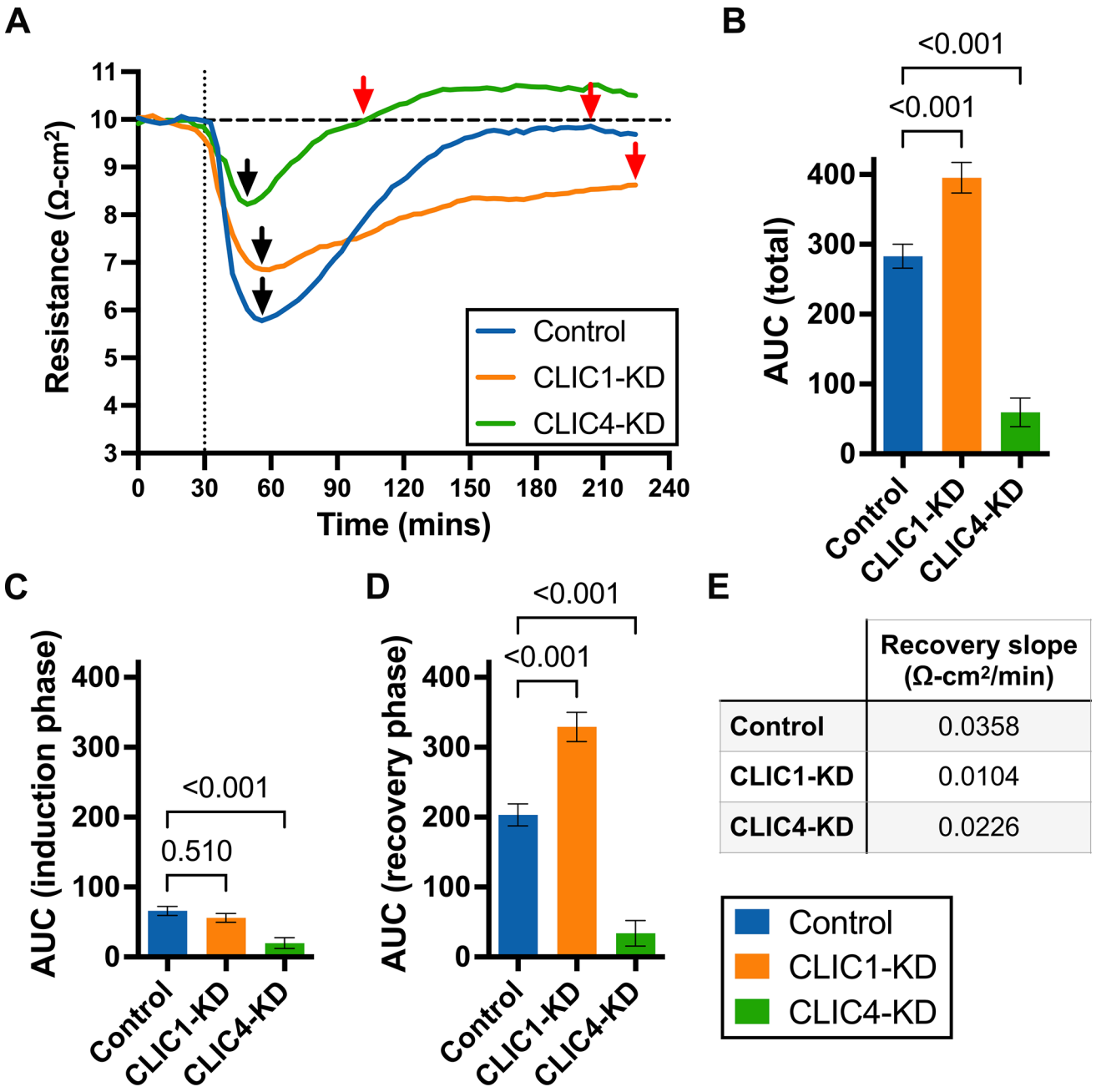


Figure 4. Endothelial CLICs regulate the change in endothelial barrier integrity in response to thrombin.

(**A**) HUVECs knocked down for CLIC1 or CLIC4 and controls were serum starved for 2hrs then stimulated with 4U/mL thrombin or vehicle. Trans-endothelial electrical resistance (TEER) was recorded for a total of 4hrs with thrombin or vehicle addition occurring at time 30min (dotted line). This time point was also considered the start of the induction phase of barrier disruption. Black arrowheads indicate the time of lowest resistance and denotes the end of the induction phase and beginning of the recovery phase. Red arrowheads indicate the time that resistance returns closest to baseline (dashed line) denoting the end of the recovery phase. Data are means from four independent experiments with SEM omitted for

clarity. **(B-D)** Mean area under the curve ±SEM was calculated for each condition. P-values determined by Welch's ANOVA with Games-Howell correction for multiple comparisons. **(B)** Total area under the curve from the time of thrombin addition to the time of return to baseline. **(C)** Area under the curve of the induction phase. **(D)** Area under the curve of the recovery phase. **(E)** Slope of the recovery phase.

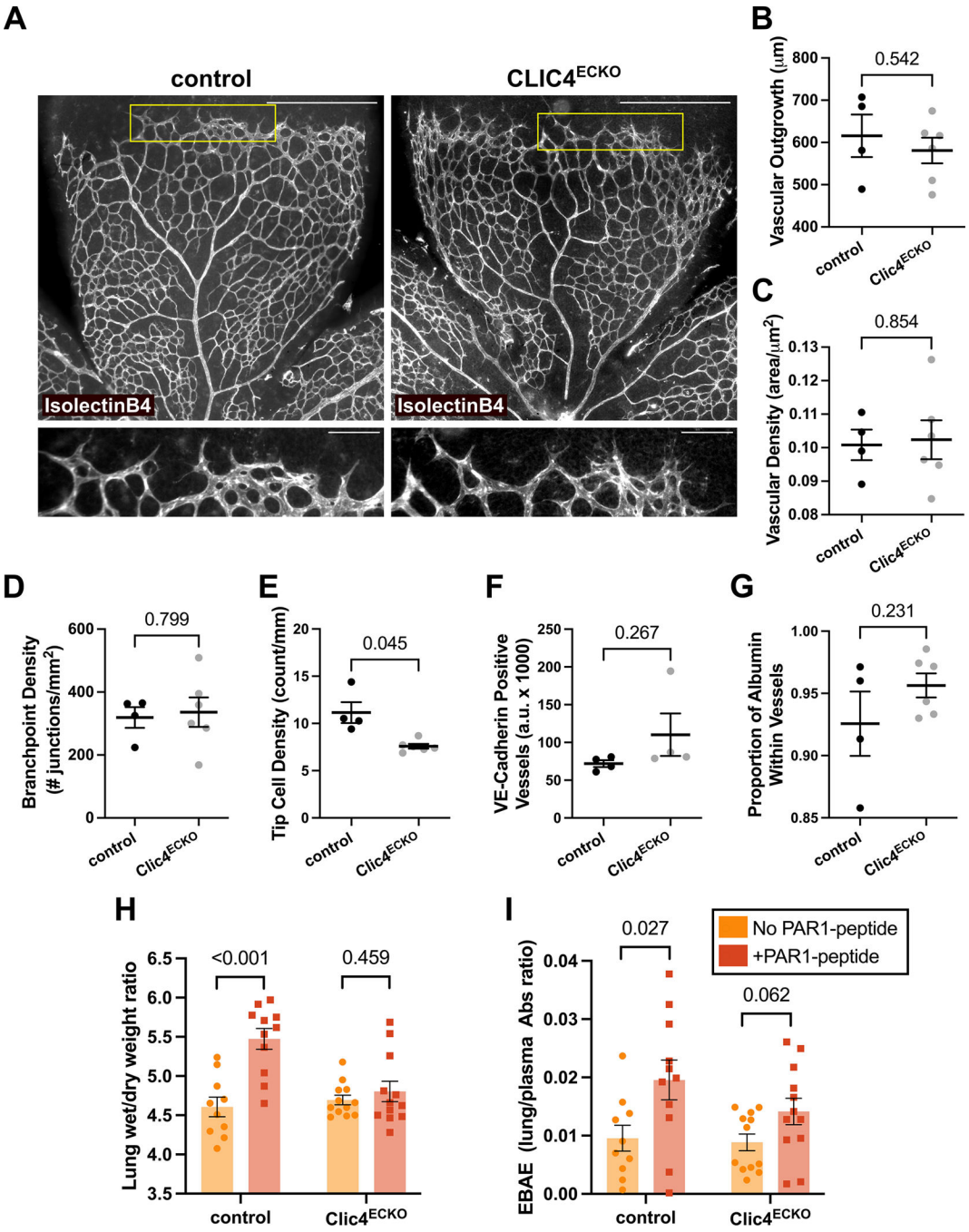
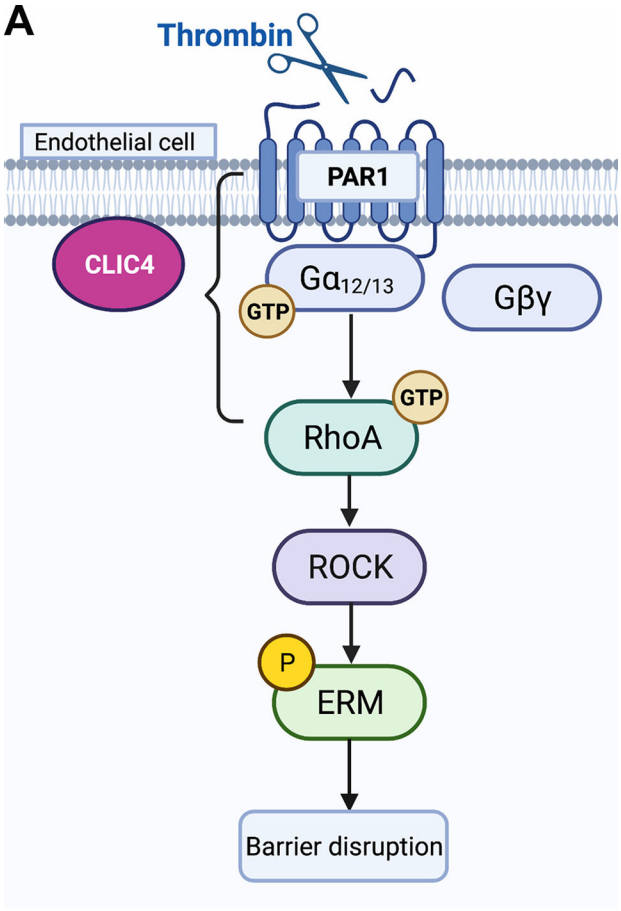


Figure 5. Endothelial *Clic4* is not required for retinal vascular development and endothelial barrier maintenance but is necessary for PAR1 induced lung microvascular permeability.

(A) Retina from P5 control (n=4) and Clic4^{ECKO} (n=6) pups stained with the vascular marker Isolectin B4 (white). Representative tip cells are shown in yellow box with a higher magnification view in the bottom panel. Scale bars: 500μm for top panels and 100μm for bottom panels. No significant phenotypic difference in retinal vascular development was observed between control and Clic4^{ECKO} mice with respect to vascular outgrowth **(B)**, vascular density **(C)**, or branchpoint density **(D)**. Tip cell density **(E)** was reduced in

Clic4^{ECKO} as compared to control mice. **(F)** Retina from P5 control (n=4) and Clic4^{ECKO} (n=4) pups were stained for Isolectin B4 and the endothelial specific adhesion molecule VE-cadherin. No significant difference in VE-cadherin vessel coverage was observed between control and Clic4^{ECKO} retina. **(G)** P5 control (n=4) and Clic4^{ECKO} (n=6) mice were injected intraperitoneally with AF488-labeled albumin 2 hours prior to harvest and retinal vasculature stained with Isolectin B4. No significant difference in albumin leakage was observed between control and Clic4^{ECKO} retina. **(H** and **I)** P42 control and Clic4^{ECKO} mice were injected with Evans blue-labeled albumin (EBA) and treated with PAR1-activating peptide or control for 30min. Lungs were collected, and lung vascular permeability was determined by measuring lung wet/dry weight ratio **(H)** and albumin accumulation in the lung parenchyma versus plasma **(I)**. Data represent 4 independent experiments with a total of n=10 control untreated, n=11 control PAR1-peptide treated, n=12 Clic4^{ECKO} untreated, and n=12 Clic4^{ECKO} PAR1-peptide treated. In all panels, data are presented as mean ±SEM with p-values determined by Welch's t-test for panels E and F, and p-values determined by two-tailed unpaired t-test for all other panels.

A



B

Thrombin-mediated event	CLIC1-KD	CLIC4-KD
CLIC localization to cell membrane	Not observed	Observed
RhoA activation	Prolonged	Reduced
ERM phosphorylation	Reduced	Reduced
Barrier disruption	Prolonged	Reduced

Figure 6. Endothelial CLICs mediate PAR1 signaling via regulation of RhoA activity to facilitate endothelial barrier control.

(A) Model of the PAR1 pathway in endothelial cells demonstrating location of CLIC4 function. CLIC4 is required for PAR1 signaling and functions upstream of RhoA. **(B)** Table summarizing the findings of *in-vitro* knock-down experiments.