Ezrin/radixin/moesin proteins differentially regulate endothelial hyperpermeability after thrombin

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Abstract

Endothelial cell (EC) barrier disruption induced by inflammatory agonists such as thrombin leads to potentially lethal physiological dysfunction such as alveolar flooding, hypoxemia and pulmonary edema. Thrombin stimulates paracellular gap and F-actin stress fiber formation, triggers actomyosin contraction and alters EC permeability through multiple mechanisms that include protein kinase C (PKC) activation. We previously have shown that the ezrin, radixin, and moesin (ERM) actin-binding proteins differentially participate in S1P-induced EC barrier enhancement. Phosphorylation of a conserved threonine residue in the C terminus of ERM proteins causes conformational changes in ERM to unmask binding sites and is considered a hallmark of ERM activation. In the present study we test the hypothesis that ERM proteins are phosphorylated on this critical threonine residue by thrombin-induced signaling events and explore the role of the ERM family in modulating thrombin-induced cytoskeletal rearrangement and EC barrier function. Thrombin promotes ERM phosphorylation at this threonine residue (Ezrin-567, Radixin-564, Moesin-558) in a PKC-dependent fashion and induces translocation of phosphorylated ERM to the EC periphery. Thrombin-induced ERM threonine phosphorylation is likely synergistically mediated by protease-activated receptors PAR\textsubscript{1} and PAR\textsubscript{2}. Using the siRNA approach, depletion of either moesin alone, or of all three ERM proteins, significantly attenuates thrombin-induced increase in EC barrier permeability (TER), cytoskeletal rearrangements, paracellular gap formation and accumulation of di-phospho-MLC. In contrast, radixin depletion exerts opposing effects on these indices. These data suggest that ERM proteins play important differential roles in the thrombin–induced modulation of EC permeability, with moesin promoting barrier dysfunction and radixin opposing it.
Keywords: thrombin; ERM; PKC; phosphorylation; endothelial cells; barrier dysfunction; cytoskeleton
Abbreviations: ERM, ezrin, radixin, and moesin proteins; PKC, protein kinase C; Thr, thrombin
Introduction

The pulmonary vascular endothelium serves as a semi-selective barrier between circulating blood and surrounding tissues. Endothelial cell (EC) barrier integrity is therefore critical to tissue and organ function. Disruption of the endothelial barrier by inflammatory mediators such as thrombin, histamine, and LPS leads to potentially lethal physiological dysfunction such as hypoxemia, atherosclerosis and pulmonary edema, a hallmark of acute lung injury and its more severe form, acute respiratory distress syndrome (12). Therefore, the preservation of vascular endothelial cell (EC) barrier integrity has the potential for profound clinical impact. Multiple studies have demonstrated that inflammation-induced EC barrier dysfunction involves cytoskeletal rearrangement, contraction of endothelial cells and intercellular gap formation, leading to increased paracellular permeability (18, 20, 55, 65). Thrombin, a multifunctional serine protease, proteolytically cleaves and activates PAR1, a member of a unique class of G protein–coupled receptors activated by proteolytic cleavage of their extracellular N-terminal domains and expressed at the surfaces of EC (43, 66). Thrombin can transactivate PAR2 through PAR1 in cultured human umbilical vein EC (HUVECs) (48). PAR1 and PAR2 activate heterotrimeric G-proteins Gq, G12/13, and Gi, all of which are involved in permeability regulation (42). Activation of Gq mobilizes Ca^{2+} and activates PKC, RhoA, and EC contraction, resulting in endothelial barrier disruption.

The widely distributed ERM family of membrane-associated proteins (ezrin, radixin, moesin) regulates the structure and function of specific domains of the cell cortex [reviewed in (2, 14, 46)]. The ERM proteins are actin-binding linkers that connect the actin cytoskeleton to the plasma membrane. This linker function makes ERM proteins essential for many fundamental cellular processes including cell adhesion, determination of cell shape, motility, cytokinesis and integration of membrane transport with signaling pathways (14, 47, 71). The three ERM proteins
share a high level of amino acid identity (70-85%) (14), and prior to activation exist in an auto-
inhibited conformation in which the actin-binding C-terminal tail binds and masks the N-
terminal FERM domain (band 4.1, ezrin, radixin, moesin homology domains) (50). The
activation state of ERM proteins is tightly regulated by phosphorylation events. Binding of the
protein to membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP$_2$) (15) and subsequent
phosphorylation of a conserved C-terminal threonine (T567 in ezrin, T564 in radixin, T558 in
moesin) (21, 41, 50) are believed to disrupt the intramolecular association, thus unmasking sites
for interactions with other proteins. In addition, phosphorylation of ezrin on other residues may
be required to direct specific targeted effects in cells (29, 36, 57). Several kinases have been
implicated in regulating ERM protein function through phosphorylation of the C-terminal
threonine residue (3, 10, 35, 40, 59, 67). However, the identity of kinases that directly
phosphorylate ERM in many cells remains to be clearly defined (14, 29).

ERM proteins also associate with cytoplasmic signaling molecules in cellular processes
that require membrane cytoskeletal reorganization. ERM proteins appear to act both downstream
and upstream of the Rho family of GTPases, which regulates remodeling of the actin
cytoskeleton (14, 29). However, information is limited concerning the possible role of ERM
proteins in the remodeling of endothelial cytoskeleton in response to different agonists. Koss and
coworkers (35) demonstrated that ERM proteins are phosphorylated on C-terminal threonine
residues by TNF-α-induced signaling events and likely play important roles in modulating the
cytoskeletal changes and permeability increases in human pulmonary microvascular EC. We
previously have shown that PKC isoforms are required for ERM phosphorylation in human
pulmonary EC induced by the potent barrier protective factor, platelet-derived phospholipid
sphingosine-1 phosphate (S1P) (1). Further, we previously demonstrated that ERM proteins,
despite their structural similarities and reported functional redundancy, differentially modulate S1P-induced changes in lung EC cytoskeleton and permeability (1). In the present study, we explored the potential involvement of ERM proteins in modulating thrombin-induced cytoskeletal rearrangement and EC barrier function.

Materials and methods

Reagents

Thrombin was obtained from Sigma Co. (St. Louis, MO). Antibodies (Ab) were obtained as follows: mouse monoclonal Ab against β-Tubulin (Covance, Berkeley, CA), rabbit polyclonal di-phospho-MLC and rabbit polyclonal phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558) Ab (Cell Signaling, Danvers, MA), ezrin specific mouse monoclonal Ab (Invitrogen Life Technologies, Carlsbad, CA), rabbit monoclonal anti-radixin Ab (Sigma, St. Louis, MO), mouse monoclonal anti-moesin Ab (BD Biosciences, San Jose, CA), mouse monoclonal anti-thrombin receptor WEDE15 blocking Ab (Beckman Coulter, Indianapolis, IN), mouse monoclonal thrombin R (ATAP2) blocking Ab (Santa Cruz Biotech., Santa Cruz, CA), Texas red phalloidin and Alexa 488-, Alexa 594-conjugated secondary Ab (Molecular Probes, Eugene, OR). ROCK inhibitors Y-27632 and H-1152, PKC inhibitors Ro-31-7549, Bisindolylmaleimide I, and Go 6976, p38 kinase inhibitor SB203580 were purchased from Calbiochem (San Diego, CA), Ca(2+) chelator BAPTA-AM was obtained from Sigma (St. Louis, MO), PI3 Kinase inhibitor LY294002 and MLCK inhibitor ML-7, PAR1 selective agonist TFLLR-NH₂, PAR2 selective agonist SLIGRL-NH₂ and reversed amino acid sequence control peptides RLLFT-NH₂
and LRGILS-NH₂ were obtained from TOCRIS (Bristol, UK). Unless specified, biochemical reagents were obtained from Sigma.

**Cell culture**

Human pulmonary artery endothelial cells (HPAEC) were obtained from Lonza Inc. (Walkersville, MD) and were utilized at passages 5–9.

**Measurement of transendothelial electrical resistance**

Cellular barrier properties were measured using an electrical cell substrate impedance sensing system (ECIS) (Applied Biophysics, Troy, NY). HPAEC were seeded onto plates with small gold electrodes (10–4 cm²) and measurements of transendothelial electrical resistance (TER) across confluent HPAEC monolayers were performed as previously described (6, 19, 65).

**Real-time quantitative RT–PCR**

Endogenous transcript levels of ezrin (EZR), radixin (RDX), and moesin (MSN) in HPAEC were measured in a 384-well PCR plate with an ABI 7900 HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA). Total RNA (1 µg) were first reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen Life Technologies) and random hexamer primers (Applied Biosystems) to generate cDNA. Quantitative Real Time-PCR (qRT-PCR) was then performed using the Assay-on-Demand system (Hs00185574_m1 (EZR); Hs00267954_m1 (RDX); Hs00792607_mH (MSN) from Applied Biosystems according to the manufacturer's protocol. Purity and specificity of all products were confirmed by omitting the reverse transcriptase or template. Analysis of results is based on the average of triplicates. The standard
curve method was used for relative quantitation of target gene expression. Further information on
the method can be found on User Bulletin #2 on the ABI website.

**Depletion of specific EC proteins via siRNA**

To reduce the content of individual EC proteins, cultured EC were treated with specific
siRNA duplexes, which guide sequence-specific degradation of the homologous mRNA (13).
The following validated siRNAs were obtained from QIAGEN (Valencia, CA) in ready-to-use,
desalted, and duplexed form: duplex of sense 5'-CACCGUGGGAUGCUAAAGdTdT-3' and
antisense 5'-CUUUGAGCAUCCCACGGUGdTdT-3' siRNA was used for targeting sequences
that are part of the coding region for Homo sapiens ezrin: 5'-
AACACCGTGGATGCTCAAAG-3', duplex of sense 5'-
GAAAUAACCCAGAGACUCUdTdT-3' and antisense 5'-
AGAGUCUCUGGGUUAUUUCdTdT-3' was used for targeting sequences that are part of the
coding region for Homo sapiens radixin: 5'-AAGAAATAACCCAGAGACTCT-3', and duplex
of sense 5'-GGGAUGUCAACUGACCUAAdTdT-3' and antisense 5'-
UUAGGUCAGUUGACAUCCCdTdG-3' was used for targeting sequences that are part of the
coding region for Homo sapiens moesin: 5'-CAGGGATGTCAACTGACCTAA-3'. Duplex of
sense 5'-AGAGCUAAG-UAGAUGUGUAdTdT-3' and antisense 5'-
UACACAUCUACUUAGCUCUdTdG-3' siRNA was used for targeting sequences that are part of the
coding region for Homo sapiens PKCβI: 5'-CAAGAGCTAAGTAGATGTGT A-3', duplex
of sense 5'-GAAGCAUGACAGCAUUAAA dTdT-3' and antisense 5'-
UUUAUGCGUCAUGCUUCdCdG-3' was used for targeting sequences that are part of the
coding region for Homo sapiens PKCζ: 5'-CGGAAGCATGACAGCATTTAAA-3', duplex of
sense 5'-CUCUACCGUGCCACGUUU UdTdT-3' and antisense 5'-AAAACGUGGCACGGUAGAGdTdT-3' was used for targeting sequences that are part of the coding region for Homo sapiens PKCδ: 5'-AACTCTACCGTGCCACGTTT-3', duplex of sense 5'-CAAGAAGUGUAUUGAUAAAdTdT-3' and antisense 5'-UAUAUCAAUACACUUCUUGdTdT-3' was used for targeting sequences that are part of the coding region for Homo sapiens PKCδ: 5'-CACAAAGAATGTATGATGATATA-3', duplex of sense 5'-CGGAAACACCCGUACCUUAd TdT-3' and antisense 5'-UAAGGUACGGUGUUCCGdTdT-3' were used for targeting sequences that are part of the coding region for Homo sapiens PKCδ: 5'-CACAAAGAACCCGTACCTTA-3'. Silencer select pre-designed siRNA duplex (Life Technologies, Grand Island, NY) of sense 5'-CCCGUAACCUAAUUCCUAUdTdT-3' and antisense 5'-AUAGGAAUUAGGUUACCGGdCdC-3' was used for targeting sequences that are part of the coding region for Homo sapiens PKCδ: 5'-GGCCCGTAACCTAATTCCTAT-3'. Non-specific, non-targeting AllStars siRNA duplex (QIAGEN, Valencia, CA) was used as negative control treatment. HPAEC were grown to 70% confluence, and the transfection of siRNA (final concentration 50 nM) was performed using DharmaFECT1 transfection reagent (Dharmacon Research, Lafayette, CO) according to manufacturer's protocol. Forty eight hours post-transfection cells were harvested and used for experiments. Additional control experiments using EC transfections with fluorescently labeled nonspecific RNA showed that this protocol allowed us to achieve 90–100% transfection efficiency.

**Plasmid Constructs**
Moesin constructs (wild type and phosphorylation deficient mutant) were prepared as we have previously described (9).

Immunofluorescent staining

EC were plated on glass coverslips, grown to 70% confluence, and transfected with siRNA followed by stimulation with thrombin. Then cells were fixed in 3.7% formaldehyde solution in PBS for 10 min at 4°C, washed three times with PBS, permeabilized with 0.2% Triton X-100 in PBS-Tween (PBST) for 30 min at room temperature, and blocked with 2% BSA in PBST for 30 min. Incubation with antibody of interest was performed in blocking solution for 1 h at room temperature followed by staining with either Alexa 488-, or Alexa 594-conjugated secondary Ab (Molecular Probes). Actin filaments were stained with Texas Red-conjugated phalloidin (Molecular Probes) for 1 h at room temperature. After immunostaining, the glass slides were analyzed using a Nikon video-imaging system (Nikon Instech Co., Japan) consisting of a phase contrast inverted microscope Nikon Eclipse TE2000 connected to Hamamatsu (Hamamatsu Photonics K.K., Japan) digital camera and image processor. The images were recorded and processed using Adobe Photoshop 6.0.

Immunoblotting

Protein extracts were separated by 4-15% gradient SDS-PAGE, transferred to nitrocellulose or polyvinylidene difluoride membranes (30 V for 18 h or 100 V for 1.5 h), and reacted with Ab that recognizes ezrin, moesin, radixin, or other Ab of interest as indicated for individual experiments. The level of phosphorylated ERM was examined by using a single Ab that recognizes any of the three ERM proteins only when they are phosphorylated on the threonine
residue: ezrin (T567)/radixin (T564)/moesin (T558) (Cell Signaling). Immunoreactive proteins were detected with the enhanced chemiluminescent detection system (ECL) according to the manufacturer's directions (Amersham, Little Chalfont, UK). Intensities of immunoreactive protein bands were quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Statistical Analysis

Results are expressed as means ±SD of three to six independent experiments. We performed statistical comparison among treatment groups by unpaired Student’s t-test or by randomized-design two-way analysis of variance followed by the Newman-Keuls post hoc test for multiple-groups. Results with P <0.05 were considered statistically significant.

Results

Expression of ezrin, radixin and moesin in HPAEC

The mRNA expression profiles of individual ERMs were analyzed for confluent human pulmonary EC. RT-PCR analysis reveals differential expression with highest relative expression of moesin and lowest expression of radixin (Fig.1).

Thrombin induces threonine phosphorylation of ERM via a PKC-mediated pathway

To elucidate the effect of thrombin on phosphorylation of ERM at its critical C-terminal threonine, confluent human pulmonary EC were stimulated with thrombin (0.5 U/ml), and threonine phosphorylation then was evaluated by Western blot analysis utilizing phospho-specific ERM antibody (phospho-Ezrin Thr567/Radixin Thr564/Moesin Thr558). Thrombin
induced the sustained threonine phosphorylation of ERM, which reached maximum levels by 5 min and remained elevated for at least 120 min (Fig. 2).

Because several kinases, including members of PKC, ROCK, GRK2, p38, Mst4 and LOK, have been reported to phosphorylate the regulatory C-terminal threonine residue of ERM proteins in various systems (3, 10, 35, 40, 59, 67), experiments were performed to determine the signaling mechanisms leading to ERM phosphorylation in pulmonary EC upon thrombin treatment. The role of different kinases was examined by using specific pharmacological inhibitors: PKC-specific inhibitor Ro-31-7549, p38 MAPK inhibitor SB203580, Rho-associated protein kinase (ROCK) inhibitor Y-27632, phosphoinositide 3-kinases (PI3Ks) inhibitor LY294002, and chelator of intracellular Ca\(^{2+}\) BAPTA (Fig. 3). In our experiments, pretreatment with Ro-31-7549 effectively prevented the increase in ERM phosphorylation induced by thrombin (Fig. 3A), suggesting that this increase is PKC-dependent. Pretreatment with BAPTA, a chelator of intracellular Ca\(^{2+}\), partially inhibited ERM phosphorylation (Fig. 3A). We next explored whether additional signaling pathways previously reported to participate in ERM regulation are involved in thrombin-induced ERM phosphorylation. Pretreatment with Y-27632 did not exert a significant effect on ERM threonine phosphorylation. At the same time, phosphorylation of myosin light chain (MLC), regulated by the ROCK/myosin phosphatase-dependent signaling pathway, was significantly attenuated in ECs preincubated with Y-27632 (Fig. 3A). Because of the critical role of Rho activation, through its downstream effector ROCK, in thrombin-induced EC barrier dysfunction, we next studied how the inhibition of ROCK affects ERM phosphorylation using the more potent and selective cell-permeable pharmacological ROCK inhibitor H-1152. Pretreatment with either H-1152 or Y-27632 did not exert significant effects on ERM threonine phosphorylation after thrombin (Fig. 3B). In addition, preincubation of
HPAEC with the pharmacologic inhibitor of p38 MAPK SB203580 did not significantly affect ERM phosphorylation (Fig. 3A, B).

The role of PKC isoforms was examined by using two alternative approaches: pretreatment of EC with PKC-specific pharmacological inhibitors and using isoform-specific siRNAs. We utilized three PKC-specific pharmacological inhibitors that have different IC$_{50}$ values for different PKC isoforms, bisindolylmaleimide I (BIM), Go 6976, and Ro-31-7549. Bis I, Ro-31-7549 and Go 6970 are all competitive inhibitors for the ATP-binding site of PKC (39, 63, 69). BIM inhibits the conventional PKC isoforms $\alpha$, $\beta$I, $\beta$Ii and $\gamma$ (activated by phosphatidylserine, diacylglycerol and Ca$^{2+}$) with similar potency (IC$_{50} = 10$ nM) (63), and the unconventional isoforms $\delta$ and $\varepsilon$ (require phosphatidylserine and diacylglycerol but are Ca$^{2+}$-independent) and the atypical isoform $\zeta$ (require only phosphatidylserine), to a lesser extent (39). In contrast to BIM, Ro-31-7549 has slight selectivity for the $\alpha$ isoform (IC$_{50} = 53$ nM), but also affects $\beta$I, $\beta$Ii, $\varepsilon$ and $\gamma$ (69). Go 6970 inhibits Ca$^{2+}$-dependent PKC isoforms $\alpha$ and $\beta$I (39). In our experiments, pretreatment with Ro-31-7549, BIM and Go 6970 effectively suppressed ERM phosphorylation induced by thrombin (Fig. 3C). These pharmacological PKC inhibitor data combined with the BAPTA data suggest that multiple PKC isoforms may be required. Furthermore, our experiments demonstrate that incubation with Go 6976 significantly inhibited phosphorylation of MLC at Thr18 and Ser19 induced by thrombin (Fig. 3C).

To better characterize the PKC isoforms involved in ERM phosphorylation after thrombin, activation of individual PKC kinases was explored using isoform-specific phospho-antibodies. Thrombin stimulation (0.5 U/ml) of HPAEC significantly increased phosphorylation of PKC$\beta$ (Thr500) (Fig. 4A), PKC$\gamma$ (Thr514) (Fig. 4B), PKC$\varepsilon$ (Ser729) (Fig. 4C), PKC$\zeta$ (Thr410) (Fig. 4D), PKC$\theta$ (Thr538) (Fig. 4E), and PKC$\delta$ (Tyr311) (Fig. 4F). Previous
studies have indicated that these PKC isoforms play important roles in thrombin- and other inflammatory agonists- (TNF-α, IL-1β, VEGF, hypoxia) induced modulation of endothelial permeability (16, 38, 44, 51, 53, 56, 61, 62, 70) and therefore were selected for further experimentation using isoform-specific siRNA. We first validated that these siRNAs efficiently inhibit their respective targets: 65±7% depletion of PKCβ, 97±2.2% depletion of PKCδ, 75.5±2.4% depletion of PKCθ, 92±2.5% depletion of PKCε, and 65±2.5% depletion of PKCγ [Fig. 5, see also (1)]. It is important to note the limitations of this siRNA approach as it does not provide 100% protein suppression and therefore some, albeit reduced protein function likely remains. EC were transfected with siRNA for PKCβI, PKCγ, PKCε, PKCζ, PKCθ or PKCδ and then stimulated with thrombin to determine the effects on ERM threonine phosphorylation. Depletion of individual PKCγ, PKCε, PKCζ, PKCθ or PKCδ isoforms significantly reduced ERM phosphorylation after thrombin (Fig. 6 A, B). To further clarify the involvement of PKC activity in thrombin-induced ERM and MLC phosphorylation, we pursued simultaneous depletion of several PKC isoforms (pan-PKC) via siRNA. Combined depletion of several PKC isoforms markedly reduced ERM phosphorylation after thrombin (Fig. 6C, D), suggesting possible cooperative regulation in this phosphorylation response. Moreover, downregulation of pan-PKC significantly attenuates thrombin-induced MLC di-phosphorylation (Fig. 6C, E), suggesting that multiple PKC isoforms may be involved. Taken together, these data indicate that multiple PKC isoforms (conventional, unconventional and atypical) likely participate in thrombin-induced ERM and MLC phosphorylation. One limitation of these data is that combined silencing of multiple PKC isoforms may result in some nonspecific effects, e.g. nonspecific activation or/and inhibition of other signaling pathways. Therefore, further studies utilizing
alternative approaches (e.g. isoform specific PKC peptide inhibitors) will be needed to verify
these findings.

Effects of ERM depletion on thrombin-induced ERM threonine and MLC phosphorylation
Numerous studies have reported a critical role for activation of the contractile apparatus in
specific models of agonist-induced EC barrier dysfunction [reviewed in (12)]. A key contractile
event in thrombin-induced barrier dysfunction is the phosphorylation of regulatory MLC,
catalyzed by Ca$^{2+}$/CaM-dependent MLCK (60, 72) and regulated by small GTPase Rho. There are
no antibodies currently available that specifically differentiate among the C-terminal phospho-
threonines of individual ERM proteins. Therefore, to assess the contribution of individual
proteins in total ERM threonine phosphorylation and their roles in MLC phosphorylation after
thrombin, we used siRNAs targeting ezrin, radixin, and moesin. We first validated that these
siRNAs efficiently and specifically inhibited their respective targets (1). Pulmonary EC were
then transfected with nonspecific siRNA, or siRNA for moesin, radixin, or siRNA for the
combination of ezrin, radixin and moesin (pan-ERM), and then stimulated with thrombin. Pan-
ERM and MLC phosphorylation was evaluated by Western blot analysis utilizing phospho-
specific ERM antibody and phospho-myosin light chain 2 (Thr18/Ser19) antibody (di-phospho-
MLC). In contrast to cells transfected with nonspecific RNA, depletion of moesin alone or
downregulation of pan-ERM significantly reduced time-dependent ERM and MLC
phosphorylation after thrombin (Fig. 7A, D). Thrombin-induced ERM and MLC phosphorylation
was partially attenuated by depletion of ezrin (Fig. 7C). In contrast, depletion of radixin did not
have any significant effect on MLC phosphorylation after thrombin (Fig. 7B). Radixin siRNA
partially, but significantly, reduced ERM threonine phosphorylation (Fig. 7B). Taken together,
these data indicate that in pulmonary EC thrombin induces primarily threonine phosphorylation of moesin, followed by ezrin, and then radixin. These data also suggest that activated moesin and to a lesser degree ezrin, but not radixin, may play a role in MLC phosphorylation induced by thrombin via the ROCK/myosin phosphatase signaling pathway.

Role of ERM in thrombin-induced lung EC hyperpermeability

To evaluate the functional involvement of individual ERM proteins in thrombin-induced EC barrier dysfunction, we measured changes in TER, a highly sensitive in vitro assay of permeability, in lung EC treated with nonspecific siRNA or those treated with siRNA for ezrin, radixin, or moesin (either singly or in combination). Comparing the data expressed as normalized resistance (Fig. 8) to the time course of ERM threonine phosphorylation (Fig. 2) demonstrates that the increase in ERM phosphorylation is highly correlated with the onset of thrombin-induced hyperpermeability. Depletion of individual ERM proteins does not affect basal permeability. However, the thrombin-induced decrease in TER is markedly attenuated in EC transfected with moesin or pan-ERM-specific siRNA (Fig. 8A, D and E) compared with cells transfected with nonspecific RNA duplexes. The initial thrombin-induced decrease in TER was attenuated by siRNA depletion of ezrin, but this intervention markedly enhanced the recovery above baseline in the later stage (Fig. 8C, E). In contrast, depletion of radixin slightly augments the decrease in TER during the early phase and attenuates the later recovery phase after thrombin (Fig. 8B, E) compared with agonist-stimulated cells transfected with nonspecific RNA. These data clearly indicate differential roles for individual ERM proteins in mediating thrombin-induced lung EC hyperpermeability.
Role of PARs in thrombin-induced ERM phosphorylation

In human pulmonary EC, activation of PAR₁ and PAR₂ promotes PKC and RhoA activation and triggers the endothelial barrier-disruptive response (7, 22, 37, 43). We next utilized PAR₁-specific blocking antibodies ATAP2 and WEDE15, and PAR₁ (TFLLR-NH₂) and PAR₂ (SLIGRL-NH₂) selective agonist peptides, to determine the roles of these receptors in thrombin-induced threonine phosphorylation of ERM. HPAEC preincubation with both ATAP2 and WEDE15, either singly or in combination, significantly decreased ERM phosphorylation after thrombin relative to controls (Fig. 9). Stimulation of EC with both TFLLR-NH₂ and SLIGRL-NH₂ augmented the phospho-ERM signal (Fig. 10) compared with cells treated with control peptides. Interestingly, incubation of EC with a combination of TFLLR-NH₂ and SLIGRL-NH₂ markedly enhanced ERM phosphorylation, compared with cells treated with these two agonists alone (Fig. 10), suggesting that both PAR₁ and PAR₂ may have additive or synergistic effects in this phosphorylation response.

Involvement of ERM in thrombin-induced EC cytoskeletal remodeling

Compromised barrier function induced by thrombin is tightly associated with actin cytoskeletal rearrangements, F-actin stress fiber formation, increased MLCK-catalyzed MLC phosphorylation spatially co-localized with stress fibers, actomyosin contraction, opening of paracellular gaps, and hyperpermeability (12, 43). Within this context, the distribution of phosphorylated ERM in EC before and after thrombin treatment was examined via immunofluorescence. Before thrombin treatment, minimal phosphorylated ERM was observed in the cytoplasm of pulmonary EC. In some areas of quiescent monolayers, phospho-ERM localized in the spike-like structures overlapping the cell-cell contact areas (Fig. 11, image a,
Stimulation with thrombin induced an increase in the amount of phosphorylated ERM, consistent with immunoblotting studies (Fig. 2). During the early response (5 minutes after initial stimulation), phosphorylated ERM was localized primarily at the cell periphery and spike-like areas (Fig. 11, image b, arrows 1 and 2) with a smaller amount of phospho-ERM also observed in the cytoplasm (Fig. 11, image b). In contracting EC (15 minutes after initial challenge) the phospho-ERM signal markedly increased in the peripheral cytoplasmic areas (Fig. 11, image c). During partial restoration phases (60 and 120 minutes after the initial challenge), phosphorylated ERM primarily localized in cytoplasmic areas and spike-like structures that were originally seen in quiescent cells (Fig. 11, images d, e), but the overall level of phosphorylated ERM appeared slightly elevated compared to baseline. These dynamic changes in phosphorylated ERM localization during the phases of active cell contraction and partial barrier restoration indicate that ERM proteins may play a role in both processes. The observation that phosphorylated ERM proteins were primary concentrated along the EC periphery upon thrombin treatment led us to examine the role of these proteins in modulating endothelial cytoskeletal rearrangements that occur during thrombin-induced EC hyperpermeability.

In the next series of experiments we analyzed the effect of ERM depletion on the human endothelial actin cytoskeleton. EC were transfected with nonspecific RNA duplex oligonucleotides (Fig. 12) or ERM-specific siRNA (Fig. 12) followed by thrombin challenge (15 min, 0.5 U/ml). Double immunofluorescent staining using Texas red phalloidin to visualize F-actin and di-phospho-MLC antibody to detect phosphorylated MLC was performed. Unstimulated EC transfected with ezrin-, radixin-, or moesin-specific siRNAs, either individually or in combination, demonstrated no significant differences in the organization of actin cytoskeleton and levels of MLC phosphorylation compared with control EC exposed to
nonspecific RNA (Fig. 12, panel A, images a-n). However, thrombin stimulation of EC treated with nonspecific RNA induced robust F-actin stress fiber and gap formation and accumulation of di-phospho-MLC (Fig. 12, panel B, images c and d), which were nearly abolished by the combination of siRNAs for radixin/ezrin/radixin (pan-ERM depletion), or by siRNA for moesin alone. In contrast, radixin depletion alone slightly enhanced stress fiber formation and MLC phosphorylation (Fig.12, panel B, images k and l). We next explored the effects of overexpression of a phosphorylation-deficient mutant of moesin (Thr558Ala) on EC cytoskeletal organization. Scanning densitometry (Fig. 13A) demonstrated relatively equivalent expression of the wild type and mutant moesin in EC. Monolayers overexpressing mutant moesin exhibited decreased F-actin stress fibers after thrombin and prominent cortical actin compared to EC overexpressing wild type moesin (Fig.13, panel B, images c, g). These data together demonstrate that ERM proteins are downstream targets of thrombin-induced signaling mechanisms and play an essential and differential role in the regulation of the endothelial actomyosin cytoskeleton.

Discussion

Prior work has revealed that the procoagulant serine protease thrombin induces endothelial barrier compromise through G protein-coupled Ca\(^{2+}\) mobilization, MLCK, PKC and RhoA activation, which produces cytoskeletal rearrangement, dissociation of endothelial cell-cell junctions as well as cytoskeleton contraction resulting in paracellular hyperpermeability (12, 27, 33, 34, 43, 45, 66). Although ERM proteins act as signal transducers for agonists that induce cytoskeletal remodeling (14), the role of ERM in barrier regulation by thrombin is unknown. Moesin is the most expressed ERM protein in several types of endothelial cells (4, 24, 30, 35). Here we demonstrate that moesin is the most abundant ERM expressed in HPAEC as measured by mRNA content, with radixin being the least expressed. We previously have shown that the
angiogenic sphingolipid S1P induces ERM phosphorylation on a conserved threonine residue critical for ERM activation via a pathway that requires PKC, Rac1, Rho A, and p38 MAPK (1).

We also have previously demonstrated that phosphorylation of ERM in response to the microtubule disruptor 2-methoxyestradiol (2ME) occurs in a p38/PKC-dependent manner (9). We therefore explored whether the ERM family of proteins plays a role in modulating the thrombin-induced endothelial barrier response. Our data demonstrate that thrombin increases ERM phosphorylation at a critical regulatory threonine site in HPAEC monolayers and strongly suggest important roles for the ERM proteins in mediating endothelial barrier dysfunction by thrombin. This phosphorylation requires thrombin-induced signaling pathways that include activation of PKC isoforms, but it is not regulated in a RhoA/ROCK- or p38-dependent manner.

Our results differ from those in our (1, 9), and others previous studies (23, 35, 68), in which the phosphorylation of ERM was PKC-, RhoA/ROCK- and-p38-dependent in response to S1P, PKC- and RhoA/ROCK-dependent in response to 2ME and TNF-α, and RhoA/ROCK-dependent in response to advanced glycation end products (AGE). Together, these observations suggest that ERM may be phosphorylated at the critical C-terminal threonine site by different upstream pathways that can vary from endothelium to endothelium and from stimuli to stimuli.

We and others previously have demonstrated that several PKC isoforms - conventional, unconventional and atypical—are likely to phosphorylate the C-terminal threonine residue of ERM proteins (1, 35, 52, 67). Our data now demonstrate that PKC isoforms ζ, γ, ε, θ and δ, which previously have been demonstrated to play roles in inflammation-induced changes in endothelial permeability (16, 44, 53, 56, 61, 62, 70), participate in thrombin-induced ERM phosphorylation at the C-terminal threonine site in human pulmonary EC (Figure 6). Moreover, our data indicate that these isoforms may synergistically regulate both ERM threonine and MLC
phosphorylation (Fig. 6C, D). One of the key events in the signaling cascade triggered by thrombin binding to PAR receptors is Ca\(^{2+}\)-dependent activation of PKC and Ca\(^{2+}\)/calmodulin-dependent MLCK, which phosphorylates myosin light chain (MLC) (12, 37). RhoA and its effector ROCK indirectly regulate MLCK activation and MLC phosphorylation and therefore also mediate endothelial hyperpermeability in response to thrombin (8, 49, 64). The RhoA/ROCK pathway is one of the downstream signals activated by PKC (8). Data demonstrating that ERM are phosphorylated by PKC in response to thrombin prompted us to examine the time-dependent phosphorylation status of individual ERM and whether ERM play a role in MLC phosphorylation after thrombin. The effects of ERM siRNA treatment, either singly or in combination, on the phosphorylated ERM and MLC phosphorylation before or after thrombin treatment were examined. We discovered that all three proteins were phosphorylated; however, knockdown of moesin alone or all three ERM proteins together significantly reduced thrombin-induced ERM and MLC phosphorylation (0-90 minutes). In contrast, depletion of radixin did not have any significant effect on MLC phosphorylation while slightly reducing ERM threonine phosphorylation after thrombin. Depletion of ezrin partially attenuated thrombin-induced ERM and MLC phosphorylation. Importantly, these data demonstrate differential roles for the ERM proteins in response to thrombin, despite their structural similarities and reported functional redundancy. These data also clearly suggest that moesin and to a lesser degree ezrin, but not radixin, are critically involved in MLC phosphorylation after thrombin. The underlying mechanism through which moesin and ezrin regulate MLC phosphorylation remains to be determined. ERM proteins are known to activate the Rho signaling in cell adhesion regulation via association with Rho regulator Rho GDP-dissociation inhibitor (GDI) (58). Our data indicate that ERM may be upstream of MLC phosphorylation. Depletion of PKC isoforms
(Fig. 6A-D) indicated that PKC-dependent moesin and ezrin phosphorylation on regulatory C-terminal threonine is involved in thrombin-induced RhoA activation and subsequent increased MLC phosphorylation. In addition, phosphorylation of moesin or/and ezrin at sites other then C-terminal threonine may contribute to this activation. For example, several additional sites have been reported for ezrin (threonine 235, tyrosines 145 and 353) (36, 73), but the functional roles of phosphorylation at these sites are still unclear. Interestingly, as we recently have been reported (31), each ERM protein has a distinct binding ability toward the subunits (CSIβ and MYPT) of MLC phosphatase (MLCP), a type 1 protein phosphatase (PPase 1) that regulate reversible phosphorylation of the MLC in intercellular gap formation and barrier dysfunction of EC. Our data demonstrated that the catalytical subunit CSIβ preferably bound to moesin, while the band that corresponds to ezrin detected in CSIβ immunoprecipitates is faint. In contrast, radixin did not bind to CSIβ, but strongly interacted with MLCP targeting subunit MYPT I. It is possible, that in addition to RhoA activation after thrombin, moesin may inhibit MLCP function leading to increased MLC phosphorylation. Our prior results indicated that MLCP play an important role in barrier protection in EC (31). Radixin may regulate MLCP activation through binding to MYPT I in S1P-induced barrier enhancement. Future studies will be needed to clarify the link between PKC/ERM and Rho/ROCK/MLCP pathways in thrombin-induced formation of stress fibers and increased endothelial permeability.

We next examined the role of ERM in thrombin-induced cytoskeletal changes. Thrombin induces translocation of phosphorylated ERM from the cytoplasm to EC periphery during the early stages of cells contraction and loss of monolayer integrity (Figure 11), which is consistent with previous studies (1, 9, 35). Most importantly, our data again demonstrate differential roles for the ERM proteins in response to thrombin. Moesin exerted a particularly prominent and
essential role in the promotion of EC barrier dysfunction by thrombin, while radixin appears to
have opposing effects. This observation is consistent with recent reports describing moesin
involvement in increased permeability induced by hypoxia and truncation of monocyte
chemoattractant protein 1 in the blood-brain barrier (25, 74) and AGE in human microvascular
EC (23, 68). Prior data obtained using knockout mice lacking individual ERM proteins largely
support the functional redundancy of the three ERM proteins (11, 32, 54). However recent
studies demonstrate differential biological functioning of these proteins. For example, ezrin and
moesin have distinct and critical functions in the T cell cortex during immunological synapse
formation (28). Moreover, ezrin, but not moesin, is phosphorylated on tyrosine in EGF-
challenged human A431 cells despite tyrosine 145 conservation in both proteins (17). In
addition, moesin has a non-redundant function in lymphocyte homeostasis (26). Our recent
findings also support the distinct biological roles of these proteins in agonist-mediated EC barrier
responses (1).

The observation that phosphorylated ERM is mostly localized to the peripheral area in EC
undergoing contraction after thrombin stimulation led us to examine the role of these proteins in
modulating permeability increases. We evaluated the role of ERM in thrombin-induced
hyperpermeability by measuring the TER in ERM depleted EC. The depletion of moesin alone or
triple ERM siRNA knockdown significantly attenuated the increase in permeability after
thrombin (Figure 8A, D and E). Ezrin knockdown also attenuated the decrease in TER induced
by thrombin, but to a lesser degree then moesin (Fig. 8C, E). In contrast, radixin depletion leads
to a slight increase in permeability during the early phase and delayed recovery during the later
phase of thrombin–mediated decreases in TER (Fig. 8B, E). These results suggest that ERM are
differentially involved in the development of thrombin-induced permeability with moesin and
ezrin promoting barrier permeability during the phase of active contraction (5-60 minutes of

treatment with thrombin). In contrast, despite the lower level of expression compare to moesin

and ezrin, radixin exerted a particularly prominent and essential role in the promotion of EC

barrier function in the restoration phase (60-120 minutes of treatment).

In EC, thrombin-induced activation of PAR1 receptor initiate signaling through Gq- and

G12/13-coupled Ca2+ mobilization, PKC and RhoA activation, and MAPK signaling (5, 7, 27). In

addition, thrombin transactivates PAR2 through PAR1 (48). We next used PAR1 (TFLLR-NH2),

PAR2 (SLIGRL-NH2) selective agonists and the PAR1-specific blocking antibodies ATAP2 and

WEDE15 to determine the role of PARs in thrombin-induced threonine phosphorylation of

ERM. Our data indicate that thrombin primarily induces ERM threonine phosphorylation in

pulmonary EC by combined activation of both PAR1 and PAR2.

Conclusion

The present study demonstrates that thrombin induces PKC-dependent ERM

phosphorylation on a critical threonine residue (Ezrin-567, Radixin-564, Moesin-558) and

translocation of phosphorylated ERM to the EC periphery. ERM phosphorylation is mediated

by the combined actions of PAR1 and PAR2. Thrombin-induced barrier dysfunction in

pulmonary endothelium is associated with remodeling of the actin cytoskeleton that increases

permeability. ERM proteins are critically involved in the barrier-disruptive response induced in

the endothelium by thrombin and may modulate cytoskeletal changes and barrier

hyperpermeability via such intermediate signaling events as PKC mediated RhoA/ROCK-

dependent signaling. Our data demonstrate that depletion of either moesin alone, or of all three

ERM proteins, attenuates thrombin-induced F-actin cytoskeleton rearrangement, paracellular gap
formation, MLC phosphorylation and decrease in TER. In contrast, radixin depletion has the opposite effect on barrier function. Based on these results and our prior data (1), we propose the following model of ERM-dependent signaling in thrombin- and S1P-treated ECs (Fig. 14): thrombin treatment induces PAR₁ and PAR₂-mediated C-terminal threonine phosphorylation primarily of moesin and ezrin by PKC and PIP₂. Recently it has been reported that phosphorylation of the RhoA activator - guanine nucleotide exchange factor p115RhoGEF by PKC-α mediates TNF-α-induced RhoA activation and subsequent barrier dysfunction in mouse brain microvascular endothelial cells (51). We hypothesize that PKC isoforms may simultaneously phosphorylate both moesin and ezrin and p115RhoGEF. Activated moesin and ezrin may displace RhoGDI from RhoA, allowing p115RhoGEF mediated RhoA activation by GDP to GTP exchange. Increased intracellular concentration of Ca²⁺ activates MLCK. RhoA and MLCK phosphorylate MLC, allowing EC contraction and reorganization of the cytoskeleton, resulting in endothelial barrier dysfunction. In contrast, S1P induces S1PR₁-mediated radixin activation (primarily), resulting in Rac1 activation. Activated Rac1 via its downstream target PAK1 induces actomyosin remodeling, including formation of a prominent cortical actin rim, which stabilizes cell-cell junctions, peripheral accumulation of phosphorylated MLC, and disappearance of central stress fibers, resulting in endothelial barrier enhancement. Thus, despite their structural similarities and reported functional redundancy, the ERM proteins differentially modulate thrombin-induced changes in lung EC cytoskeleton and permeability. These results advance our mechanistic understanding of EC barrier regulation, identify the ERM family as potential targets for therapeutic manipulation in this clinically important physiologic process and extend previous knowledge about the involvement of PKC and ERM in endothelial barrier regulation.
Acknowledgments

The authors are grateful to Drs. D. Fulton and S. Black from Georgia Health Sciences University for help with moesin mutant preparation.

Grants

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31


70. Willis CL, Meske DS, and Davis TP. Protein kinase C activation modulates reversible increase in cortical blood-brain barrier permeability and tight junction protein expression during hypoxia and posthypoxic reoxygenation. *J Cereb Blood Flow Metab* 30: 1847-1859, 2010.


Figure Legends

Figure 1. Relative quantity of moesin (MSN), ezrin (EZR), and radixin (RDX) mRNA in HPAEC. Total RNA was isolated from human pulmonary artery endothelial cells (HPAEC) and quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed using target gene specific primers and probes and the relative amounts expressed using standard curve method as described in Methods. Each value represents the mean of triplicates.

Figure 2. Time-dependent effects of thrombin on threonine phosphorylation of ERM. (A) Confluent HPAEC were treated either with control vehicle or thrombin (0.5 U/ml) for the indicated times, and phosphorylated ERM (phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558) was detected via immunoblotting. (B) The bar graph represents relative densitometry. Data are presented as fold changes in phosphorylated ERM over vehicle-treated control and expressed as means ± S.E. from three independent experiments. *P < 0.05 vs. unstimulated control.

Figure 3. Thrombin-induced ERM phosphorylation requires activation of PKC. HPAEC were pretreated with either control vehicle or the following inhibitors: PKC inhibitors Ro-31-7549 (10 µM, A, C) for 30 min, bisindolylmaleimide (BIM, 1 µM, C) for 30 min, Go6976 (1 µM, C) for 1 h, Ca(2+) chelator BAPTA-AM (25 µM, A) for 1 h, p38 kinase inhibitor SB203580 (20 µM, A, B) for 30 min, Rho kinase inhibitors Y-27632 (10 µM, A, B) for 1 h and H-1152 (3 µM, B) for 1 h, PI3 Kinase inhibitor LY294002 (10 µM, A) for 1 hr. EC were then stimulated with EBM-2 medium alone or thrombin (0.5 U/ml) for the indicated time. Phosphorylation of ERM proteins and MLC were analyzed by immunoblotting of cell lysates.
with phospho-ERM (as in Fig. 2) or di-phospho-MLC (Thr18/Ser19) specific Abs. GAPDH or β-actin Abs were used as a normalization control. Rearranged lanes from the same blot are outlined by vertical dotted line. Results of scanning densitometry of Western blots are shown as fold changes of ERM or MLC phosphorylation relative to vehicle treated EC stimulated by thrombin. Results are representative of 3-6 independent experiments. Values are means ± S.E. *, significantly different from cells treated with vehicle (p < 0.05); **, significantly different from cells stimulated with thrombin (p < 0.05).

**Figure 4. Effects of thrombin on phosphorylation of PKC isoforms in HPAEC.** Confluent HPAEC were treated either with control vehicle or thrombin (0.5U/ml) for the indicated times, and phosphorylated PKCβ (A), PKCγ (B), PKCe (C), PKCζ (D), PKCθ (E), and PKCd (F) were detected via immunoblotting. Bar graphs represent relative densitometry of fold changes in phosphorylated PKC isoforms after thrombin relative to vehicle-treated control and expressed as means ± S.E. from three independent experiments. *, significantly different from cells treated with EBM-2 (p < 0.05); #, significantly different from cells treated with EBM-2 (p < 0.01).

**Figure 5. Depletion of PKC isoforms by siRNA.** PKCe (A) and PKCγ (B) depletion were induced by specific siRNA duplexes and assessed for silencing effects by immunoblotting with appropriate Ab, as compared with treatment with nonspecific (ns) siRNA. Immunoblotting with β-actin Ab was used as a normalization control. Rearranged lanes from the same blot are outlined by vertical dotted line. Quantitative analysis of protein expression was performed by scanning densitometry and expressed in relative density units (RDU). Results are means ± S.E. for three
independent experiments. #, significant difference \((p < 0.01)\) when compared with cells treated with ns siRNA.

**Figure 6. Depletion of PKC isoforms inhibits thrombin-induced ERM and MLC phosphorylation.** Confluent HPAEC were incubated with non-specific, PKCβI-, PKCζ-, PKCθ-, PKCδ-, PKCγ- and PKCε- specific siRNA (A) or with non-specific, combinations of PKCδ- and PKCε-, combinations of PKCγ-, PKCδ- and PKCε- and combinations of PKCβI-, PKCθ- and PKCζ- specific siRNAs (C) as described in Methods, and then stimulated by thrombin (0.5 U/ml, 5 min) or vehicle. Total lysates were analyzed by immunoblotting for phospho-ERM or di-phospho-MLC (Thr18/Ser19). Immunoblotting with β-tubulin Ab was used as a normalization control. Rearranged lanes from the same blot are outlined by vertical dotted line. (B, D, E) The bar graphs represent relative densitometry of fold changes in phosphorylated ERM and MLC after thrombin relative to vehicle-treated control. Results are means ± S.E. of four independent experiments. *, significantly different from cells treated with ns siRNA without thrombin \((p < 0.01)\); #, significantly different from cells treated with ns siRNA without thrombin \((p < 0.05)\). **, significantly different from cells treated with ns siRNA and thrombin \((p < 0.05)\).

**Figure 7. Effects of ERM depletion on thrombin-induced ERM and MLC phosphorylation.** Confluent HPAEC were incubated with non-specific, moesin- (panel A), radixin- (panel B), ezrin-specific (panel C) or combined siRNAs for ezrin, radixin, and moesin (panel D) as described in Methods then stimulated by thrombin (0.5 U/ml, 5 min) or vehicle. Total lysates were analyzed by immunoblotting with phospho-ERM or di-phospho-MLC (Thr18/Ser19) Abs. Immunoblotting with β-tubulin Ab was used as a normalization control. The bar graphs
represents relative densitometry of fold changes in phosphorylated ERM and MLC after thrombin relative to vehicle-treated control. Results are means ± S.E. of three independent experiments. *p<0.05, compared with corresponding pretreatment vehicle control.

**Figure 8. Effects of ERM depletion on thrombin-induced endothelial barrier hyperpermeability.** EC grown in chambers on gold microelectrodes were transfected with siRNA for moesin (panel A), radixin (panel B), ezrin (panel C), combined siRNAs for ezrin, radixin, and moesin (panel D), or treated with nonspecific (ns) siRNA, as described in Methods and used for transendothelial electrical resistance (TER) measurements. At time = 0, cells were stimulated with thrombin (0.5 U/ml) or vehicle control. Shown are pooled data of 5 independent experiments. The bar graph (E) depicts pooled TER data (n = 5) as maximal value of normalized TER elevation above base line achieved within 30 min ± S.E. *, significantly different from cells treated with ns siRNA reagent without thrombin (p < 0.05); **, significantly different from control cells stimulated with thrombin (p < 0.05).

**Figure 9. Effects of PAR1 blocking antibodies on thrombin-induced ERM phosphorylation.** (A) HPAEC were pretreated for 1 hour with either control vehicle or the PAR1 blocking Abs ATAP2 (25 µg/ml) or WEDE15 (25 µg/ml) or with combination of ATAP2 and WEDE15, then stimulated by thrombin (0.5 U/ml, 5 min) or vehicle. Total lysates were analyzed by immunoblotting for phospho-ERM. Immunoblotting with β-actin Ab was used as a normalization control. (B) The bar graph represents relative densitometry of fold changes in phosphorylated ERM after thrombin relative to vehicle-treated control. Results are means ± S.E. of four independent experiments. *, significantly different from cells treated with ns siRNA without S1P (p < 0.05); #, significantly different from cells treated with ns siRNA without
thrombin ($p < 0.01$). **, significantly different from cells treated with ns siRNA and thrombin ($p < 0.05$).

**Figure 10. Effects of PAR$_1$ and PAR$_2$ selective agonists on thrombin-induced ERM phosphorylation.** (A) EC were pretreated for 5 minutes with thrombin (0.5 U/ml), PAR$_1$ selective agonist TFLLR-NH$_2$ (50 μM), PAR$_2$ selective agonist SLIGRL-NH$_2$ (50 μM) or combination of TFLLR-NH$_2$ and SLIGRL-NH$_2$. Pretreatment with vehicle and reversed amino acid sequence peptides RLLFT-NH$_2$ and LRGILS-NH$_2$ used as controls. (B) The bar graph represents relative densitometry of fold changes in phosphorylated ERM after thrombin, TFLLR-NH$_2$ or SLIGRL-NH$_2$ relative to vehicle-treated control. Results are means ± S.E. of three independent experiments. *$p<0.05$, compared with corresponding pretreatment controls.

**Figure 11. Distribution of phospho-ERM in EC after thrombin.** EC grown on glass cover slips and treated with 0.5 U/ml thrombin for indicated time (images b-e) or non treated control cells (image a) were subjected to immunofluorescent staining with anti-phospho-ERM Ab. The phospho-ERM signal is very weak in quiescent monolayers and is evident only in spike-like structures in cell-cell border areas (image a, arrow 1). Threonine-phosphorylated ERM proteins predominantly localized to the periphery of ECs following thrombin stimulation (5-15 min, images b,c, arrow 2) and also are detectable in peripheral spike-like structures (image b, arrow 1). After 1-2 hrs phosphorylated ERM localized in spike-like structures characteristic of quiescent cells and in cytoplasm (images d,e). Images are representative of 3 independent experiments. Scale bar = 10 μm.
Figure 12. Effects of ERM depletion on thrombin-induced cytoskeletal remodeling. HPAEC grown on glass cover slips were incubated with siRNA to ezrin, radixin, moesin, or combination of siRNAs to all three proteins, or treated with non-specific siRNA duplex as described in Methods followed by thrombin treatment (0.5 U/ml, 5 min). ECs were subjected to double immunofluorescent staining with Texas Red phalloidin to visualize F-actin (panels A and B, upper images) and anti-pp-MLC Ab (Panels A and B, bottom images). Incubation with siRNA to moesin (g, h) and combined siRNAs to ezrin, radixin, and moesin (o, p) almost completely abolishes thrombin-induced F-actin stress fiber and gap formation and MLC phosphorylation compared with control (nsRNA) incubation (c, d, arrows). In contrast, pretreatment with siRNA to radixin slightly enhances the thickness of stress fibers and MLC phosphorylation (k, l, arrows) compared with incubation with nsRNA. Bar = 10 µM. Images are representative of three independent experiments.

Figure 13. Effects of overexpression of the phosphorylation-deficient mutant of moesin (Thr558Ala) on thrombin-induced cytoskeletal remodeling. (A) ECs were transfected with empty vector (control), V5 tagged wild-type or mutant moesin, which were then detected via immunoblotting with V5 Ab. Results of scanning densitometry of Western blots are shown as % of moesin relative to control. Immunoblotting with β-actin Ab was used as a normalization control. (B) After transfection with vectors expressing moesin (wild-type or mutant) tagged with V5, EC were grown on glass cover slips as described in Methods followed by thrombin treatment (0.5 U/ml, 5 min). ECs were subjected to double immunofluorescent staining with Texas Red phalloidin to visualize F-actin (panels B and C, upper images) and V5 Ab (Panels B and C, bottom images). Overexpression with mutant moesin abolishes thrombin-induced F-actin stress
fibers and induces cortical actin formation (panel C, image g) compared to EC overexpressed
with wild type moesin (panel C, image c). Arrow indicates cell transfected with mutant moesin
EC (panel C, image g). Images are representative of three independent experiments. Scale bar =
10 µm.

Figure 14. Proposed model of ERM-dependent signaling in thrombin- and S1P-challenged
lung endothelium (see explanation in Conclusion).
Figure 1
Figure 2

A

Thrombin, 0.5 U/ml

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* Indicates significance.
Figure 3A

Thrombin, 5 min

Fold increase in Thr-pERM

Fold increase in di-pMLC (Thr18/Ser19)

GAPDH
Figure 3B

B

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Thr          -     +       -      +      -      +       -       +

Fold increase in pERM

- DMSO
- Y-27632
- H-1152
- SB203580

* indicates significant increase in pERM

Legend:
- no Thr
- Thr
Figure 3C

C

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Fold increase in pERM

Fold increase in di-pMLC

* p < 0.05
** p < 0.01
*** p < 0.001
Figure 4A-D

A

pPKCβ

β-actin

Fold increase in phosphorylation

0 0.5 1 1.5 2 2.5

0 min 2 min 5 min

B

pPKCγ

β-actin

Fold increase in phosphorylation

0 0.5 1 1.5 2 2.5

0 min 2 min 5 min

C

pPKCε

β-actin

Fold increase in phosphorylation

0 0.5 1 1.5 2

0 min 2 min 5 min

D

pPKCζ

β-actin

Fold increase in phosphorylation

0 1 2 3 4

0 min 2 min 5 min
Figure 4E, F

**E**

- **pPKC\(\theta\)**
- **\(\beta\)-actin**

**Fold increase in phosphorylation**

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**F**

- **pPKC\(\delta\)**
- **\(\beta\)-actin**

**Fold increase in phosphorylation**

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Figure 5

A

nsRNA  PKCε siRNA

PKCε

β-actin

RDU

0  50  100  150

nsRNA  PKCε siRNA

B

nsRNA  PKCγ siRNA

PKCγ

β-actin

RDU

0  50  100  150

nsRNA  PKCγ siRNA
Figure 6A, B

A

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Thr-pERM

β-actin

B

Fold increase in pERM

- nsRNA
- PKCβ1si
- PKCαsi
- PKCθsi
- PKCζsi
- PKCεsi
- PKCγsi

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<td>2.0</td>
</tr>
<tr>
<td>PKCεsi</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>PKCγsi</td>
<td>1.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* p < 0.05
** p < 0.01
Figure 6C, D, E

C

<table>
<thead>
<tr>
<th>nsRNA</th>
<th>PKCδ/εsi</th>
<th>PKCγ/δ/εsi</th>
<th>PKCβ/θ/ζsi</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Thr-pERM" /></td>
<td><img src="image2" alt="Thr-pERM" /></td>
<td><img src="image3" alt="Thr-pERM" /></td>
<td><img src="image4" alt="Thr-pERM" /></td>
</tr>
<tr>
<td><img src="image5" alt="ppMLC (Thr18/Ser19)" /></td>
<td><img src="image6" alt="ppMLC (Thr18/Ser19)" /></td>
<td><img src="image7" alt="ppMLC (Thr18/Ser19)" /></td>
<td><img src="image8" alt="ppMLC (Thr18/Ser19)" /></td>
</tr>
<tr>
<td><img src="image9" alt="β-actin" /></td>
<td><img src="image10" alt="β-actin" /></td>
<td><img src="image11" alt="β-actin" /></td>
<td><img src="image12" alt="β-actin" /></td>
</tr>
</tbody>
</table>

D

![Bar graph showing fold increase in pERM](image13)

E

![Bar graph showing fold increase in di-pMLC](image14)
Figure 7A, B

A

|                | nsRNA | MSNs|i |
|----------------|-------|------|
| Thr (min)      | 0     | 1    | 5    | 15   | 30   | 90   |
| Thr-pERM       | ![Image](image1.png) |
| ppMLC band intensity | ![Image](image2.png) |
| β-Tub | ![Image](image3.png) |

B

<table>
<thead>
<tr>
<th></th>
<th>nsRNA</th>
<th>RDXsi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thr (min)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Thr-pERM</td>
<td><img src="image4.png" alt="Image" /></td>
<td></td>
</tr>
<tr>
<td>ppMLC band intensity</td>
<td><img src="image5.png" alt="Image" /></td>
<td></td>
</tr>
<tr>
<td>β-Tub</td>
<td><img src="image6.png" alt="Image" /></td>
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</tbody>
</table>
Figure 7C, D

C

<table>
<thead>
<tr>
<th>Thr</th>
<th>0</th>
<th>1</th>
<th>5</th>
<th>15</th>
<th>30</th>
<th>90</th>
</tr>
</thead>
<tbody>
<tr>
<td>nsRNA</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
<tr>
<td>EZRsi</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
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</tbody>
</table>

D

<table>
<thead>
<tr>
<th>Thr</th>
<th>0</th>
<th>1</th>
<th>5</th>
<th>15</th>
<th>30</th>
<th>90</th>
</tr>
</thead>
<tbody>
<tr>
<td>nsRNA</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
<tr>
<td>pan-ERMsi</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
</tbody>
</table>
Figure 8A, B
Figure 8C, D
Figure 8E

[Bar graph showing normalized resistance levels for different conditions: Control, Msn siRNA, Rdx siRNA, Ezr siRNA, pan-ERM siRNA, with no Thr and Thr conditions, indicated by asterisks (*) and double asterisks (**) indicating statistical significance.]

Normalized Resistance

- Control
- Msn siRNA
- Rdx siRNA
- Ezr siRNA
- pan-ERM siRNA

- no Thr
- Thr

* and ** indicating statistical significance.
Figure 9

A

<table>
<thead>
<tr>
<th></th>
<th>Cntr</th>
<th>ATAP2</th>
<th>WEDE15</th>
<th>ATAP2 &amp; WEDE15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thr</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Thr-pERM

β-actin

B

Fold increase in ERM phosphorylation

- Cntr
- ATAP2
- WEDE15
- ATAP2 & WEDE15

no Thr
Thr
Figure 10

A

![Western blot image showing Thr-pERM and β-actin](image)

B

![Bar graph showing fold increase in ERM phosphorylation](image)

Legend:

- Cntr1
- Cntr1, Thr
- Cntr2
- TFLR
- Cntr3
- SLGRL
- Cntr4
- TFLR&SLGRL

* indicates statistically significant difference.
Figure 11

Thr-pERM

Control

Thr, 5 min

Thr, 15 min

Thr, 60 min

Thr, 120 min
Figure 12

A

<table>
<thead>
<tr>
<th>nsRNA</th>
<th>Moesin siRNA</th>
<th>Radixin siRNA</th>
<th>Pan-ERM siRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="a" alt="F-actin" /></td>
<td><img src="e" alt="F-actin" /></td>
<td><img src="i" alt="F-actin" /></td>
<td><img src="m" alt="F-actin" /></td>
</tr>
<tr>
<td><img src="b" alt="ppMLC" /></td>
<td><img src="f" alt="ppMLC" /></td>
<td><img src="j" alt="ppMLC" /></td>
<td><img src="n" alt="ppMLC" /></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>Thrombin</th>
<th>F-actin</th>
<th>Moesin siRNA</th>
<th>Radixin siRNA</th>
<th>Pan-ERM siRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="c" alt="F-actin" /></td>
<td><img src="g" alt="F-actin" /></td>
<td><img src="k" alt="F-actin" /></td>
<td><img src="o" alt="F-actin" /></td>
<td></td>
</tr>
<tr>
<td><img src="d" alt="ppMLC" /></td>
<td><img src="h" alt="ppMLC" /></td>
<td><img src="l" alt="ppMLC" /></td>
<td><img src="p" alt="ppMLC" /></td>
<td></td>
</tr>
</tbody>
</table>

Legend:
- F-actin
- ppMLC

Arrows indicate changes or differences in the images due to the treatments.
Figure 13

A

Vector control moesin (WT)-V5 moesin (Mut)-V5

V5

β-Actin

B

moesin (WT)-V5

F-actin

Vehicle

V5

moesin (Mut)-V5

F-actin

C

F-actin

Thrombin

V5
Figure 14

**S1P**

- S1PR1
  - Gi
  - PKC, PIP<sub>2</sub>
  - Dormant Radixin
  - Active Radixin
  - RhoGDI<sub>α</sub>
  - Rac
  - GTP
  - PAK
  - VE-cadherin (Cytoskeletal rearrangements)
  - EC Barrier Enhancement

**Thrombin**

- PAR1
  - Gi, Gq, G<sub>12/13</sub>
  - PKC, PIP<sub>2</sub>
  - Dormant Moesin & Ezrin
  - Active Moesin & Ezrin
  - RhoGDI<sub>α</sub>
  - RhoA
  - GDP
  - GTP
  - MLCK
  - MLC
  - MLC-P
  - EC contraction
  - Acute Lung Injury

Transactivation from PAR1 to PAR2.