Thrombin selectively engages LIM kinase 1 and slingshot-1L phosphatase to regulate NF-κB activation and endothelial cell inflammation

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INFLAMMATION IS A HOST DEFENSE response against infection or injury and it tends to resolve once the invading pathogen has been cleared from the system (6, 38). When inflammation goes haywire it results in many inflammatory disease states such as acute lung injury (ALI), atherosclerosis, arthritis, and chronic obstructive pulmonary diseases (COPD). A hallmark of inflammation is the recruitment of polymorphonuclear leukocytes (PMN) to the site of inflammation (11, 50, 58). The movement of PMN from the blood to the inflammatory site is a highly ordered process. A key step in this process is the stable adhesion of PMN to the inflamed endothelium, which requires the expression of intercellular cell adhesion molecule-1 (ICAM-1) on endothelial cell (EC) surface and activation of its counterreceptor β2 integrins (CD11/CD18) on the surface of PMN. The interaction between ICAM-1 and β2 integrins enables PMN and other leukocytes to migrate across the endothelial to underlying tissues (15). Vascular cell adhesion molecule-1 (VCAM-1) is another inducible adhesive protein that interacts with its counterreceptor, very late antigen-4 (VLA-4; CD49d/CD29), present on the circulating monocytes and lymphocytes to promote their adhesion and migration across endothelial barrier (13, 58). The expression of ICAM-1 and VCAM-1 is under the tight control of the nuclear factor-κB (NF-κB) (3, 48).

NF-κB is a ubiquitously expressed family of transcription factors involved in various biological effects, including immune, inflammatory, and stress-induced responses, and cell fate decisions such as proliferation, differentiation, apoptosis, and tumorigenesis (9, 24). Importantly, recent studies have revealed surprising and previously unrecognized roles of NF-κB in the resolution phase of inflammation, tissue repair, and homeostasis and in mitochondrial respiration (12, 26, 32, 37). Indeed, the improper functioning of this transcription factor has been implicated in a wide range of human diseases including cancer, ALI, atherosclerosis, arthritis, and COPD (1, 14, 52, 59, 61). The mammalian NF-κB family consists of five members: RelA (p65), RelB, c-Rel, p50, and p52 (9, 24). The mammalian NF-κB family consists of five members: RelA (p65), RelB, c-Rel, p50, and p52 (9, 24). NF-κB exists as a dimer (typically a heterodimer of p50 and RelA or homodimer of RelA) and is retained in the cytoplasm through interaction with its inhibitor IκB (9, 24). Activation of NF-κB involves phosphorylation of its inhibitor IκBα on two specific serine residues (Ser32 and Ser36) by a cytoplasmic IkB kinase (IKK) complex (21, 65). Phosphorylation triggers the ubiquitination of IκBα, which in turn marks it for degradation by the 26S proteasome (27). The unleashed NF-κB migrates to the nucleus, where it activates the transcription of target genes including ICAM-1 and VCAM-1 (33, 39, 48, 49). Although the regulatory events responsible for signal-induced release of NF-κB are well studied, much less is known about the mechanisms controlling the nuclear translocation of the released NF-κB.

The actin cytoskeleton is dynamic and can modulate various signaling pathways including the NF-κB pathway (5, 29, 47). In response to a number of external and internal stimuli, the state of actin dynamics is regulated by a number of actin binding proteins (60). One such protein is cofilin, which functions as an actin depolymerizing protein to regulate actin dynamics (4, 7). Cofilin activity is reversibly regulated by its phosphorylation and dephosphorylation at Ser3 via engagement of LIM kinase and slingshot (SSH) phosphatase, respectively (7). LIM kinases (LIM is an acronym of the three gene products Lin-11, Isl-1, and Mec-3), composed of LIMK1 and LIMK2, are expressed in most tissues but with different subcellular localization. LIMK1 is known to...
phosphorylate and negatively regulate cofilin activity (31, 35, 45) and thereby stabilize the actin cytoskeleton. The Ser3-phosphorylated inactive cofilin is dephosphorylated and reactivated by Slingshot family of cofilin phosphatases to destabilize the actin cytoskeleton. The SSH family includes SSH-1L, -2L, and -3L (43, 46) or chronophin, a member of haloacid dehalogenases (23). All members considerably differ in their subcellular distribution, F-actin-binding activity, and phosphatase activity, implying that they have related but separate functions in various cellular and developmental events (46). We recently showed that cofilin-1 is a critical determinant of thrombin-induced NF-κB activation and EC inflammation (17). However, it remains unclear whether LIMK1 and SSH-1L contribute to EC inflammation. In this study, we provide novel evidence that thrombin selectively engages LIMK1 and SSH1L to promote EC inflammation by facilitating RelA/p65 nuclear translocation via dy-

![Fig. 1](https://example.com/figure1.jpg)  
Fig. 1. Thrombin regulates cofilin phosphorylation via LIM kinase 1 (LIMK1) and slingshot-1Long (SSH-1L). A: time course of thrombin-induced LIMK1 phosphorylation and cofilin phosphorylation in endothelial cell (EC). Human pulmonary artery endothelial cells (HPAEC) were challenged with thrombin (5 U/ml) for the indicated time periods. Total lysates were separated by SDS-PAGE and immunoblotted with an anti-phospho-LIMK1 (Thr508), anti-phospho-cofilin (Ser3), and anti-LIMK antibody. For total cofilin, the same lysates were reelectrophoresed and immunoblotted with an anti-cofilin antibody. Results are representative of 2–3 separate experiments. B: bar graph represents the ratio of phospho-cofilin (p-cofilin) to cofilin upon thrombin treatment for the indicated time points. Data are means ± SE (n = 3). *Difference from untreated control (P < 0.05). C: effect of RNA interference (RNAi) knockdown of SSH-1L on thrombin-induced cofilin phosphorylation. HPAEC were transfected with control siRNA or SSH-1L siRNA for 24–36 h followed by thrombin (5 U/ml) challenge for the indicated time periods. Total cell lysates were separated by SDS-PAGE and immunoblotted with anti-phospho-cofilin and anti-cofilin antibody. For monitoring the knockdown of SSH-1L, the lysates were also immunoblotted with an anti-SSH-1L. Anti-RelA/p65 antibody was used to monitor loading. Bar graph represents the effect of SSH-1L depletion on thrombin-induced cofilin phosphorylation. Data are means ± SE (n = 3 for each condition). *Difference from control siRNA (P < 0.05). D: time course of thrombin-induced F-actin formation in EC. Confluent HPAEC monolayers grown on coverslips were left untreated or treated with thrombin for the indicated time periods. Cells were then fixed, permeabilized, and stained with Alexa Fluor 488-labeled phalloidin to visualize actin filaments. The cells were also stained and anti-RelA/p65 antibody and a secondary antibody were conjugated to Texas red. DNA was stained with Hoechst dye. Coverslips were mounted on the slide and analyzed by fluorescence microscopy. Results are representative of 3 experiments.
namic regulation of cofilin-1-dependent changes in actin cytoskeleton. We further show that LIMK1 and SSH-1L also mediate phosphorylation of RelA/p65 to increase its transcriptional activity. Additionally, LIMK1 regulates IKKβ-dependent release of RelA/p65 from IκBα for its nuclear transport.

**MATERIALS AND METHODS**

**Reagents.** Human thrombin was purchased from Enzyme Research Laboratories (South Bend, IN). Polyclonal antibodies to RelA/p65, IκBα, and β-actin and a monoclonal antibody to ICAM-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A rabbit polyclonal antibody to cofilin-1 was obtained from Cytoskeleton (Denver, CO) and a rabbit polyclonal antibody that detects cofilin-1 when phosphorylated at Ser3 was from Santa Cruz Biotechnology (Santa Cruz, CA). SSH-1L antibody was from Abcam and total LIMK1, p-LIMK1, and serine536 phospho-NF-κB antibody were from Cell Signaling. Plasmid Maxi Kit was from Qiagen (Valencia, CA), and the protein assay kit and nitrocellulose membrane were from Bio-Rad. Alexa Fluor 488-phalloidin and Texas red phalloidin were purchased from Molecular Probes (Eugene, OR). Goat serum (MP Biomedicals, Solon, OH) goat F(ab′)2 anti-rabbit IgG (H+L)-FITC (Southern Biotech, Birmingham, AL) DRAQ5 (Cell Signaling Technology, Danvers, MA). CellTracker Green CMFDA and CellTracker red CMTTPX were from Invitrogen. All other materials were from VWR Scientific Products (Gaithersburg, MD).

**Endothelial cell culture.** Human pulmonary artery endothelial cells (HPAEC) were purchased from Lonza (Walkersville, MD). Cells were cultured in 0.1% gelatin-coated flasks by using endothelial basal medium 2 (EBM2) with bullet kit additives (BioWhittaker, Walkersville, MD) as described (63). For treatment, cells were washed twice with serum-free medium and incubated in serum-free medium for 0.5–1 h prior to thrombin challenge. Cells used in the experiments were between 3 and 7 passages.

**Fig. 2.** RNAi-mediated depletion of SSH-1L augments whereas depletion of LIMK1 prevents actin filament formation. HPAEC were transfected with control siRNA (A and B), SSH-1L siRNA (C and D), or LIMK1 siRNA (E and F) for 24–36 h. Cells were treated with thrombin (5 U/ml) (B, D, and F) and then fixed, permeabilized, and stained with Texas red-labeled phalloidin and Alexa Fluor 488-labeled DNa51 to visualize the actin filaments and G-actin, respectively. Images were analyzed by fluorescence microscopy. Results are representative of 3 experiments.

**Fig. 3.** RNAi-mediated depletion of LIMK1 and SSH-1L inhibits thrombin-induced NF-κB activity. HPAEC were transfected with LIMK1-siRNA (A) or SSH-1L-siRNA (B) by use of DharmaFect1. Twenty-four hours later, cells were again transfected with NF-κB LUC construct by using DEAE-dextran as described in MATERIALS AND METHODS. Cells were then challenged with thrombin (5 U/ml) for 6 h, and the cell extracts were prepared and assayed for firefly and Renilla luciferase activities. The data were expressed as a ratio of firefly to Renilla luciferase activities. Data are means ± SE (n = 4–6 for each condition). #Difference from controls (P < 0.05); *difference from thrombin-stimulated controls (P < 0.05).
Cell lysis and immunoblotting. EC were lysed in radioimmune precipitation (RIPA) buffer containing 50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 0.25 mM EDTA, pH 8.0, 1% deoxycholic acid, 1% Triton X-100, 5 mM NaF, 1 mM sodium orthovanadate supplemented with complete protease inhibitors (Sigma), and the residual binding sites on the filters were blocked by incubating with 5% (wt/vol) nonfat dry milk in TBST or 5% BSA in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20) for 1 h at room temperature (RT). The membranes were subsequently incubated with the indicated antibodies and developed using an enhanced chemiluminescence (ECL) method as described (40). Representative blots shown in the result section come from the same membrane, which may have more samples in various groups.

Immunofluorescence. Cells grown on collagen I coated coverslips were fixed in 3.7% paraformaldehyde/PBS for 10 min at RT and then permeabilized with 0.1% Triton X-100 for 5 min at RT as described (19, 20). Permeabilized cells were then rinsed three times with 1× PBS and incubated in blocking solution (5% normal goat serum or 1% bovine serum albumin/PBS) for 1 h at RT to remove nonspecific binding of the antibody. All subsequent steps were carried out at RT and cells were rinsed three times with 1× PBS between each of the steps. To localize filamentous-actin (F-actin) and globular-actin (G-actin), cells were incubated with Texas red-labeled phalloidin and Alexa Fluor 488-labeled DNase1, respectively, for 20 min at RT in a humid chamber. The coverslips were rinsed in PBS and mounted on the slide with Vectashield mounting medium (Vector Laboratories, Lincolnshire, IL). Images were obtained via a Nikon Eclipse-TE2000-E fluorescent microscope.

RNAi knockdown. SMARTpool siRNA duplexes specific for SSH-1L, LIMK1, and a nonspecific siRNA control were obtained from Dharmacon (Lafayette, CO). Cells were transfected with siRNA by use of DharmaFect1 siRNA Transfection Reagent (Dharmacon) as described (17). Briefly, 50–100 nM siRNA was mixed with DharmaFect1 and added to cells that are 60–70% confluent. After 36 h, cells were treated with thrombin (5 U/ml), and cell lysates were prepared and immunoblotted as described above.

Reporter gene constructs, endothelial cell transfection, and luciferase assay. The construct pNF-κB-LUC containing 5 copies of consensus NF-κB sequences linked to a minimal E1B promoter-luciferase gene was purchased from Stratagene (La Jolla, CA). Transfections were performed by using the DEAE-dextran method essentially as described (43). Briefly, 5 μg of DNA was mixed with 50 μg/ml DEAE-dextran in serum-free endothelial basal medium 2 and the mixture was added onto cells that were 60–80% confluent. We used 0.125 μg of pTKRLUC plasmid (Promega, Madison, WI) as an internal control.

Fig. 4. RNAi-mediated depletion of LIMK1 differentially regulates thrombin and TNF-α-induced ICAM-1 and VCAM-1 expression. HPAEC were transfected with LIMK1-siRNA by use of DharmaFect1. After 24–36 h, the cells were challenged with thrombin (5 U/ml; A and B) or TNF-α (100 U/ml; C and D) for 6 h. Total cell lysates were immunoblotted with an anti-ICAM-1, anti VCAM-1, anti-LIMK1 antibody. Actin was used to monitor loading. Bar graph represents the effect of LIMK1 depletion on thrombin and TNF-α-induced ICAM-1 and VCAM-1 expression normalized to actin level. Data are means ± SE (n = 6 for each condition). #Difference from controls (P < 0.05); *diference from thrombin or TNF-α-stimulated controls (P < 0.05).
P-40 was added to a final concentration of 0.6%. Samples were comprised of DharmaFect1 and, 24 h later, cells were again transfected with control or SSH-1L siRNA. For experiments examining the effect of LIMK1 and SSH-1L knockdown on NF-κB activity, cells were first transfected with siRNA by use of DharmaFect1 and, 24 h later, cells were again transfected with SSH-1L siRNA by use of DEAE-dextran. After 16 h, cells were treated with DMSO in serum-free EBM2. The data were expressed as a ratio of firefly vs. Renilla luciferase activity normalized to Cu-Zn-SOD, RelA/p65, or Actin, whose expression is not affected by treatment, cell extracts were prepared and assayed for firefly luciferase activity. For experiments examining the effect of LIMK1 and SSH-1L knockdown on NF-κB activity, cells were first transfected with siRNA by use of DharmaFect1 and, 24 h later, cells were again transfected with SSH-1L siRNA by use of DEAE-dextran. After 16 h, cells were treated with thrombin and the luciferase activity was determined as described above.

Cytoplasmic and nuclear extract preparation. After treatments, cells were washed twice with ice-cold Tris-buffered saline and resuspended in 400 μl of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). After 15 min at 4°C, 10% Nonidet KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) and homogenized well with a motor-driven pestle. After 30 min at 4°C, lysates were centrifuged at 10,000 rpm for 5 min and the supernatants containing the cytosolic proteins. The pellets were washed two times with 1× PBS, and grown to confluence in complete medium (EBM-2, bullet kit additives, 10% FBS). We achieved transcription efficiency of 16 ± 3 (mean ± SD; n = 3) in these cells. After treatment, cell extracts were prepared and assayed for firefly luciferase activity by use of the Promega Biotech Dual Luciferase Reporter Assay System. The data were expressed as a ratio of firefly vs. Renilla luciferase activity. For experiments examining the effect of LIMK1 and SSH-1L knockdown on NF-κB activity, cells were first transfected with siRNA by use of DharmaFect1 and, 24 h later, cells were again transfected with pNF-κB-LUC by use of DEAE-dextran. After 16 h, cells were treated with thrombin and the luciferase activity was determined as described above.

Electrophoretic mobility shift assay. The EMSA was performed basically as described (33). Briefly, 10 μg of nuclear extract was mixed with 1 μg of poly(dI-dC) in a binding buffer [10 mM Tris·HCl (pH 7.5), 50 mM NaCl, 0.5 mM DTT, 10% glycerol (20 μl final volume)] for 15 min at RT. The double-stranded oligonucleotides were end labeled with γ-32P ATP. The reaction mixture was incubated with the end-labeled double-stranded oligonucleotides containing an NF-κB site (30,000 cpm each) for 15 min at RT. The DNA-protein complexes were resolved on a 5% native polyacrylamide gel in low ionic strength buffer (0.25 × Tris-borate-EDTA). The oligonucleotide used for the gel shift analysis was Ig-κB 5′-GTTGAGGGGA CTTTCCCA-

Fig. 5. RNAi-mediated depletion of SSH-1L differentially regulates thrombin and TNF-α-induced ICAM-1 and VCAM-1 expression. HPAEC were transfected with SSH-1L-siRNA by use of DharmaFect1. After 24–36 h, the cells were challenged with thrombin (5 U/ml; A and B) or TNF-α (100 U/ml; C and D) for 6 h. Total cell lysates were immunoblotted with an anti-ICAM-1, anti-VCAM-1, anti-SSH-1L antibody. In addition to actin, Cu-Zn-SOD, a housekeeping gene (18), and RelA/p65, whose expression is not affected by thrombin in EC, were used as loading control. Bar graph represents the effect of SSH-1L depletion on thrombin or TNF-α-induced ICAM-1 and VCAM-1 expression normalized to Cu-Zn-SOD, RelA/p65, or actin level. Data are means ± SE (n = 6 for each condition). #Difference from controls (Cont) (P < 0.05); *difference from thrombin or TNF-α-stimulated controls (P < 0.05).
polyclonal antibody for HD T then washed twice with PERM buffer PBS for 15 min at RT. Cells were incubated with the RelA/p65 rabbit 4% paraformaldehyde/PBS, and permeabilized with 0.1% Triton X-100 in PBS. Cells were then washed once with ice-cold PBS, fixed in nonradioactive kit from Cayman Chemical. Experiments were performed as per the manufacturer’s instructions.

RelA/p65 nuclear localization using ImageStreamX. After treatments, cells were washed twice with warm PBS, trypsinized, and transferred to 5 ml polystyrene tubes (Becton Dickinson Labware, Franklin Lakes, NJ). Cells were then washed once with ice-cold PBS, fixed in 4% paraformaldehyde/PBS, and permeabilized with 0.1% Triton X-100 in PBS. Cells were incubated with goat F(ab’)2 anti-rabbit IgG (permeabilization buffer) containing 10% goat serum and 0.1% Triton X-100 in PBS. Cells were incubated with goat F(ab’)2 anti-rabbit IgG (permeabilization buffer) containing 10% goat serum and 0.1% Triton X-100 in PBS. Cells were incubated with goat F(ab’)2 anti-rabbit IgG (permeabilization buffer) containing 10% goat serum and 0.1% Triton X-100 in PBS. Cells were incubated with goat F(ab’)2 anti-rabbit IgG (permeabilization buffer) containing 10% goat serum and 0.1% Triton X-100 in PBS. Cells were incubated with goat F(ab’)2 anti-rabbit IgG (permeabilization buffer) containing 10% goat serum and 0.1% Triton X-100 in PBS.

RESULTS

Thrombin engages LIMK1 and SSH-1L to tightly regulate cofilin 1 phosphorylation. We determined whether thrombin engages LIMK1 and SSH-1L to tightly regulate cofilin 1 activity. To address the temporal relationship between LIMK1 activation and cofilin 1 phosphorylation, we compared the time

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Fig. 6. RNAi-mediated depletion of LIMK1 and SSH-1L differentially regulates thrombin-induced IκBα phosphorylation/degradation and IKKβ phosphorylation. HPAEC were transfected with LIMK1 siRNA (A) or SSH-1L (B) siRNA by use of DharmaFect1. After 24–36 h, the cells were challenged for 1 h with thrombin (5 U/ml). Total cell lysates were prepared and immunoblotted with anti-phospho-IκBα (Ser18 and Ser19) and anti-IκBα to determine the phosphorylation and degradation of IκBα, respectively. The levels of RelA/p65 were used to monitor loading. Bar graph represents the effect of LIMK1 (A) or SSH-1L (B) depletion on thrombin-induced IκBα degradation normalized to RelA/p65 levels. C and D: total lysates were also immunoblotted with anti-phospho-IκBβ and anti-IκBβ. Bar graph represents the effect of LIMK1 (C) or SSH-1L depletion (D) on thrombin-induced IKKβ phosphorylation normalized to total IKKβ levels. Data are means ± SE (n = 3–6 for each condition). #Difference from controls (P < 0.05); *difference from thrombin-stimulated controls (P < 0.05).
To this end, cells were transfected with siRNA targeting SSH-1L (SSH-1L-siRNA) or control siRNA and analyzed for cofilin phosphorylation. Compared with control siRNA, cells transfected with SSH-1L-siRNA showed a marked augmentation in cofilin phosphorylation both in untreated as well as thrombin-treated samples (Fig. 1C). Furthermore, we assessed whether changes in cofilin phosphorylation correlate with dynamic alterations in actin cytoskeleton upon thrombin treatment. Cells were treated with thrombin for indicated time points and stained with Alexa Fluor-labeled phalloidin to visualize actin filaments (Fig. 1D). Results show a robust increase in F-actin formation at 5 and 15 min, which subsequently tapers down to baseline by 2 h. Together, these data indicate a temporal relationship between thrombin-induced changes in actin dynamics and tight regulation of cofilin activity via engagement of LIMK1 and SSH-1L. Interestingly, treatment of EC with another proinflammatory mediator TNF-α failed to induce changes in cofilin phosphorylation.
suggesting that regulation of cofilin activity by LIMK1 and SSH-1L is stimulus specific. RNA interference (RNAi) knockdown of SSH-1L augments whereas knockdown of LIMK1 prevents actin filament formation. Our findings that thrombin alters the actin cytoskeleton in EC and that cofilin plays a key role in mediating these alterations prompted us to determine the effect of SSH-1L and LIMK1 depletion on thrombin-induced changes in actin cytoskeleton. Cells were stained with Texas red-labeled phalloidin to visualize actin filaments and with Alexa Fluor 488-labeled DNase1 to visualize G-actin. Analysis by fluorescence microscopy of EC stained with Texas red-phalloidin showed that RNAi knockdown of SSH-1L augmented basal (Fig. 2; A vs. C), as well as thrombin-induced (Fig. 2; B vs. D) formation of actin filaments, consistent with its role as a cofilin phosphatase. By contrast, depletion of LIMK1 destabilized thrombin-induced (Fig. 2; B vs. F) actin filaments, which is in line with the role of LIMK1 as a kinase that phosphorylates cofilin to render it inactive (17, 31, 45).

RNA knockdown of LIMK1 and SSH-1L inhibits thrombin-induced NF-κB activity and EC inflammation. Since dynamic changes in actin cytoskeleton are required for thrombin-induced NF-κB activation and EC inflammation (17), we ascertained the role of LIMK1 and SSH-1L in these responses. Cells were transfected with pNF-κB-LUC in combination with LIMK1-siRNA, SSH-1L siRNA, or control siRNA. Results showed that thrombin challenge of cells transfected with control siRNA resulted in increased NF-κB reporter activity, and this response was inhibited in cells transfected with LIMK1 or SSH-1L-siRNA (Fig. 3, A and B). We also determined whether knockdown of LIMK1 and SSH-1L had a similar effect on thrombin-induced expression of NF-κB target genes, ICAM-1 and VCAM-1. We found that knockdown of LIMK1 (Fig. 4, A and B) or SSH-1L (Fig. 5, A and B) each attenuated thrombin-induced ICAM-1 and VCAM-1 expression consistent with the effect of depleting LIMK1 or SSH-1L on NF-κB activity. RNAi knockdown of LIMK1 and SSH-1L was also effective in inhibiting thrombin-induced expression of other NF-κB target genes.
RNK knockdown of LIMK1 and SSH-1L differentially regulates thrombin-induced IKKβ phosphorylation and IkBα degradation. To dissect the mechanism by which LIMK1 and SSH-1L specifically regulate thrombin-induced NF-κB signaling pathway, we analyzed the effect of LIMK1 and SSH-1L depletion on thrombin-induced IkBα degradation, a prerequisite for NF-κB activation. Because IkBα degradation is contingent on its phosphorylation on Ser32 and Ser36, we also evaluated the phosphorylation status of IkBα upon thrombin challenge of EC. Results showed that depletion of LIMK1 interfered with the ability of thrombin to induce IkBα phosphorylation and, consequently, its degradation (Fig. 6A). In contrast, depletion of SSH-1L had no effect on thrombin-induced IkBα phosphorylation and degradation (Fig. 6B). Since phosphorylation of IkBα is mediated by IKKβ, we also evaluated the role of LIMK1 and SSH-1L in IKKβ activation. Depletion of LIMK1 inhibited activation of IKKβ upon thrombin challenge, as determined by phosphorylation of IKKβ at Ser181 (Fig. 6C). In contrast, depletion of SSH-1L had no significant effect on thrombin-induced IKKβ phosphorylation (Fig. 6D), consistent with its effect on IkBα phosphorylation and degradation.

RNKi knockdown of LIMK1 and SSH-1L selectively impairs thrombin-induced nuclear translocation and subsequent DNA binding of RelA/p65. Since IkBα degradation is a requirement for NF-κB nuclear translocation, we next determined whether depletion of LIMK1 and SSH-1L leads to inhibition of this event. We took two different approaches to analyze thrombin-induced nuclear accumulation of RelA/p65. Our data using ImageStream X, which combines the high image content information of microscopy with high throughput and multiparameter analysis of flow cytometry (36), showed that depletion of LIMK1 and SSH-1L significantly inhibited thrombin-induced nuclear uptake of RelA/p65. In unstimu-

Fig. 8. RNKi-mediated depletion of LIMK1 and SSH-1L differentially regulates thrombin and TNF-α-induced DNA binding of RelA/p65. HPAEC were transfected with LIMK1-siRNA (A, C, and E) or SSH-1L-siRNA (B, D, and E) by use of DharmaFect1. After 24–36 h, the cells were challenged for 1 h with thrombin (A–D) or 0.5 h with TNF-α (E). Nuclear extracts were prepared and assayed for DNA binding of RelA/p65 by EMSA (A and B) or Cayman’s NF-κB (RelA/p65) Transcription Factor Assay kit (C–E) as described in MATERIALS AND METHODS. Data are means ± SE (n = 3 for each condition). #Difference from controls (P < 0.05); *difference from thrombin- or TNF-α-stimulated controls (P < 0.05).
lated cells, RelA/p65 was localized primarily in the cytoplasm, irrespective of whether they were transfected with control siRNA, LIMK1-siRNA, or SSH-1L-siRNA (Fig. 7, A and B, a vs. d; green staining). Exposure to thrombin caused a marked nuclear accumulation of RelA/p65 in cells transfected with control siRNA as indicated by the red vs. yellow nuclei (Fig. 7, A and B, a vs. b). In contrast, thrombin-induced RelA/p65 nuclear localization was prevented in cells transfected with LIMK1-siRNA or SSH-1L-siRNA as indicated by yellow vs. red nuclei (Fig. 7, A and B, b vs. c). Furthermore, immunoblotting of nuclear extracts also showed a substantial decrease in thrombin-induced nuclear uptake of RelA/p65 upon depletion of LIMK1 and SSH-1L (Fig. 7, C and D). Interestingly, immunoblotting of nuclear extracts showed that depletion of LIMK1 and SSH-1L did not inhibit TNF-α-induced RelA/p65 nuclear uptake (Fig. 7E). We next determined whether impaired nuclear translocation of RelA/p65 results in decreased DNA binding of NF-κB. We have previously shown that induced NF-κB complex is predominantly composed of RelA/p65 homodimer (8, 20, 48). EMSA showed that depletion of LIMK1 and SSH-1L each caused a marked reduction in thrombin-induced RelA/p65 binding to DNA (Fig. 8, A and B). Additionally, we used a nonradioactive, ELISA-based assay to monitor RelA/p65 binding to DNA in nuclear extracts. Results show that thrombin-induced RelA/p65 binding to DNA was inhibited upon depletion of LIMK1 and SSH-1L (Fig. 8, C and D), further substantiating our EMSA data. In contrast, TNF-α-induced binding of RelA/p65 to DNA was unchanged upon LIMK1 and SSH-1L knockdown (Fig. 8E).

RNAi knockdown of LIMK1 and SSH-1L regulates thrombin-induced RelA/p65 phosphorylation at Ser\textsuperscript{536}. Because phosphorylation of Ser\textsuperscript{536} in the transactivation domain of RelA/p65 regulates thrombin-induced phosphorylation of RelA/p65 and that inhibition of LIMK1 and SSH-1L prevents this response (Fig. 9, A and B). To test whether RelA/p65 phosphorylation at Ser\textsuperscript{536} is a direct target of LIMK1, we analyzed the immunoprecipitates of RelA/p65 from control and thrombin-challenged cells to determine the association of LIMK1 with RelA/p65. Western blot analysis using anti-LIMK1 antibody showed that LIMK1 does not associate with RelA/p65 (Fig. 9C). Control IgG immunoprecipitates were used to monitor the specificity of the interaction.

![Figure 9](http://ajplung.physiology.org/)

**A**

**B**

**C**

![Table](http://ajplung.physiology.org/)
The major finding of this study is that thrombin engages LIMK1 and SSH-1L to promote EC inflammation by ensuring tight regulation of cofilin-1, which in turn causes dynamic alterations in the actin cytoskeleton to mediate nuclear translocation of RelA/p65 (Fig. 10). Our data show that LIMK1, a cofilin kinase and downstream effector of Rho-associated kinase ROCK (31, 35), serves dual function in regulating NF-κB activation and EC inflammation by coordinating IKKβ-mediated IkBα degradation-dependent release of RelA/p65 and cofilin-dependent changes in actin cytoskeleton to facilitate the translocation of the released RelA/p65 to the nucleus. Unlike LIMK1, the action of cofilin phosphatase SSH-1L is restricted to nuclear translocation of the released RelA/p65 to cause EC inflammation (Fig. 10). However, both LIMK1 and SSH-1L confer transcriptional competency to NF-κB by mediating the phosphorylation of RelA/p65 at Ser326. Cofilin being a central regulator of actin dynamics is important to numerous vital cellular processes and thus is emerging as a mediator of cellular homeostasis. In addition, its role in mitochondrial-dependent apoptosis, phospholipid metabolism, cell migration, and gene expression are latest revelations (4, 7).

We recently identified a novel role of cofilin-1 in regulating EC inflammation (17). In the present study, we addressed the possibility that LIMK1 and SSH-1L are critical mediators of thrombin-induced NF-κB activation and EC inflammation by their ability to tightly regulate cofilin activity. We found that thrombin induced LIMK1 activation and cofilin phosphorylation with a similar time course, suggesting a role of LIMK1 in cofilin phosphorylation. Furthermore, the loss of cofilin phosphorylation in LIMK1-depleted cells confirmed the involvement of this kinase in cofilin phosphorylation/inactivation (17). Consistent with the loss of cofilin phosphorylation that renders it active, LIMK knockdown was associated with destabilization of basal as well as thrombin-induced actin filaments.

We also determined whether thrombin engages SSH-1L in regulating cofilin-dependent changes in the actin cytoskeleton. Depletion of SSH-1L augmented the basal as well as thrombin-induced phosphorylation of cofilin-1. In accordance with the augmented phosphorylation of cofilin-1 that renders it inactive, SSH-1L knockdown caused stabilization of the actin filaments under both basal and thrombin-treated conditions. Studies have shown that the activation of SSH-1L occur in conjunction with activation of LIMK in vascular smooth muscle cells (VSMC) and neuronal cells (16, 42, 54, 57). Recently, San Martin et al. (54) showed that PDGF induced LIMK1 phosphorylation at 5 min that returns to baseline at 30 min, whereas SSH-1L phosphatase activity reaches its peak at 30 min following PDGF treatment in VSMC. Our data indicate a similar kinetics for thrombin-induced LIMK1 and SSH-1L activation in EC.

Because dynamic changes in the actin cytoskeleton play an important role in NF-κB signaling (17, 20), we evaluated the effect of SSH-1L or LIMK1 depletion on NF-κB activity and EC inflammation. Indeed, manipulating the actin cytoskeleton by depleting either LIMK1 or SSH-1L inhibited thrombin-induced NF-κB reporter activity as well as expression of ICAM-1 and VCAM-1. Interestingly, in parallel studies knockdown of LIMK1 and SSH-1L failed to prevent TNF-α-induced ICAM-1 and VCAM-1 expression. Given the involvement of RhoA/ROCK pathway in thrombin-induced EC inflammation (2), these data indicate that LIMK1/cofilin/SSH-1L/actin pathway acts downstream of RhoA/ROCK to mediate NF-κB activation and EC inflammation, and this effect appears to be stimulus specific. Although TNF-α activates RhoA/ROCK in EC (64), it fails to induce actin filament formation comparable to thrombin (20). Moreover, the kinetics of actin filament formation by TNF-α appears to be slower than its ability to induce RelA/p65 nuclear accumulation and ICAM-1 expression (20, 41). It is likely that stimulation of RhoA by thrombin and TNF-α activates distinct downstream effectors or the same effectors but with different kinetics, leading to differential responses by these agonists. In contrast to findings, studies have shown that disruption of actin cytoskeleton in airway epithelial cells and myelomonocytic cells (HL-60) enhanced NF-κB-mediated transcription induced by both TNF-α and LPS but via different mechanisms (28). Thus the regulation of NF-κB by actin dynamics occurs not only in a stimulus-specific but also in a cell-specific manner.

We next delineated the mechanism by which LIMK1 and SSH-1L controls NF-κB activity. Intriguingly, our experiments showed that thrombin-induced IKKβ activation and subse-

**DISCUSSION**

**Fig. 10. Schematic showing the role of LIMK1 and SSH-1L in mediating thrombin-induced EC inflammation.** Thrombin activation of RhoA/ROCK results in parallel activation of IKKβ mediating the release of RelA/p65 via phosphorylation/degradation of IkBα and dynamic reorganization of the actin cytoskeleton via regulation of cofilin activity; together these events serve to facilitate nuclear translocation of NF-κB and subsequent ICAM-1 expression (17). The present study further identifies that thrombin specifically engages LIMK1 and SSH1L downstream of RhoA/ROCK to tightly regulate cofilin phosphorylation, causing dynamic reorganization of the actin cytoskeleton required for RelA/p65 nuclear translocation and proinflammatory gene expression. LIMK1 and SSH-1L also mediate RelA/p65 phosphorylation at Ser326, a critical event conferring transcriptional competency to the bound NF-κB. Furthermore, LIMK1 links RhoA/ROCK pathway to IKKβ-mediated IkBα degradation-dependent release of RelA/p65 for its actin cytoskeleton-dependent translocation to the nucleus and EC inflammation.

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quent IkBα phosphorylation and degradation were sensitive to depletion of LIMK1 but insensitive to depletion of SSH-1L. Because the effects of LIMK1 depletion are similar to RhoA/ROCK inhibition and inhibition of ROCK prevents LIMK1 activation (17), these findings indicate that Rho/ROCK mediates its effects on IKKβ and IkBα (2) via its downstream effector LIMK1. Thus LIMK1, in addition to regulating cofillin activity via Ser3 phosphorylation, serves to relay the RhoA/ROCK signaling to mediate IkBα degradation, a requirement for NF-κB activation. On the contrary, depletion of SSH-1L failed to inhibit thrombin-induced IKKβ phosphorylation and the resultant IkBα phosphorylation/degradation, suggesting that SSH-1L exerts its effect on NF-κB activation and EC inflammation largely through regulation of actin dynamics. Because IkBα degradation results in nuclear localization and DNA binding of NF-κB, we determined whether inhibition of LIMK1 and SSH-1L would lead to inhibition of these events. As expected, depletion of LIMK1 inhibited nuclear localization of RelA/p65 following thrombin challenge of EC. However, depletion of SSH-1L also showed impairment in nuclear localization of RelA/p65, despite having no effect on its release from IkBα in the cytosol. Consistent with nuclear translocation, a significant reduction in thrombin-induced RelA/p65 DNA binding was observed in LIMK1- or SSH-1L-depleted cells. Our data further indicate that LIMK1 and SSH-1L are involved in thrombin-induced phosphorylation of RelA/p65, an additional regulatory pathway activated in parallel with IkBα degradation, and that plays an essential role in conferring transcriptional competency to DNA-bound NF-κB (53, 66, 67). Our results also show that LIMK1 does not associate with RelA/p65, excluding the possibility that it directly phosphorylates RelA/p65. Similarly, SSH-1L, which is a phosphatase, is unlikely to directly mediate RelA/p65 phosphorylation. Thus the data suggest the involvement of intermediate kinases(s) in causing RelA/p65 phosphorylation.

Our finding that a functional and dynamic actin cytoskeleton as regulated by coordinate action of LIMK1 and SSH-1L is necessary for RelA/p65 nuclear translocation finds support from previous reports. For example, cofillin-dependent changes in actin dynamics are implicated in agonist-induced endocytosis of β-adrenergic (βAR) and the β isoform of thromboxane A2 (TPβ) receptors (30, 62). Similarly, activation of Rho/ROCK/LIMK/Cofilin pathway is required for shear stress-induced sterol regulatory element binding proteins (SREBP) in EC (34). Importantly, microtubule dynamics is also implicated in nuclear localization of the tumor suppressor protein p53, and, consistent with this, p53 nuclear uptake is inhibited upon stabilization or destabilization of the microtubule cytoskeleton (22).

In summary, this study defines the role of LIMK1 and SSH-1L in NF-κB signaling pathway and EC inflammation. The role of LIMK1 and SSH-1L is distinct to thrombin response because TNF-α-mediated EC inflammation occurs independently of this mechanism. Thus specific targeting of LIMK1 or SSH-1L may be a useful strategy for dampening EC inflammation associated with intravascular coagulation. Additionally, these findings may have implications in designing therapeutic strategies for prevention of other diseases whose etiology involves actin cytoskeletal rearrangement (10, 51).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

A.L., C.M., and F.F. performed experiments; A.L., C.M., and F.F. analyzed data; A.L., C.M., A.R., and F.F. approved final version of manuscript; A.R. and F.F. interpreted results of experiments; A.R. and F.F. edited and revised manuscript; F.F. conception and design of research; F.F. prepared figures; F.F. drafted manuscript.

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