Role of Rho GTPases in thrombin-induced lung vascular endothelial cells barrier dysfunction

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Abstract

Thrombin-induced barrier dysfunction of pulmonary endothelial monolayer is associated with dramatic cytoskeletal reorganization, activation of actomyosin contraction, and gap formation. Phosphorylation of regulatory myosin light chains (MLC) is a key mechanism of endothelial cell (EC) contraction and barrier dysfunction, which is triggered by Ca2+/calmodulin-dependent MLC kinase (MLCK) and Rho-associated kinase (Rho-kinase). The role of MLCK in EC barrier regulation has been previously described; however, Rho-mediated pathway in thrombin-induced pulmonary EC dysfunction is not yet precisely characterized. Here, we demonstrate that thrombin-induced decreases in transendothelial electrical resistance (TER) indicating EC barrier dysfunction are universal for human and bovine pulmonary endothelium, and involve membrane translocation and direct activation of small GTPase Rho and its downstream target Rho-kinase. Transient Rho membrane translocation coincided with translocation of upstream Rho activator, guanosine nucleotide exchange factor p115-RhoGEF. Rho mediated activation of downstream target, Rho-kinase induced phosphorylation of the EC MLC phosphatase (MYPT1) at Thr686 and Thr850, resulting in MYPT1 inactivation, accumulation of diphospho-MLC, actin remodeling, and cell contraction. The specific Rho-kinase inhibitor, Y27632, abolished MYPT1 phosphorylation, significantly attenuated stress fiber formation and thrombin-induced TER decrease. Furthermore, expression of dominant-negative Rho and Rho-kinase abolished thrombin-induced stress fiber formation and MLC phosphorylation. Our data, which provide comprehensive analysis of Rho-mediated signal transduction in pulmonary EC, demonstrate involvement of guanosine nucleotide exchange factor, p115-RhoGEF, in thrombin-mediated Rho regulation, and suggest Rho, Rho-kinase, and MYPT1 as potential pharmacological and gene therapy targets critical for prevention of thrombin-induced EC barrier disruption and pulmonary edema associated with acute lung injury.

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Introduction

The vascular endothelium forms a selective permeable barrier between the blood and the interstitial space of all organs and participates in the regulation of macromolecule transport and blood cell trafficking through the vessel wall. Barrier maintenance is determined by the equilibrium of competing contractile and tethering forces generated by the cytoskeletal motor proteins such as actin and myosin and the adhesive molecules located at cell–cell and cell–matrix contacts (for review, see Bogatcheva et al., 2002; Dudek and Garcia, 2001; Lum and Malik, 1996). Similar to smooth muscle (SM), endothelial cell (EC) contraction is initiated by phosphorylation of regulatory myosin light chains (MLC20) (Garcia et al., 1995; Goeckeler and Wysolmerski, 1995; Kamm and Stull, 1986; Wysolmerski and Lagunoff, 1990). MLC kinases, which are able to phosphorylate MLC in vitro and in vivo include Ca2+/CaM-dependent MLC kinase (MLCK) and Rho-associated kinase (Rho-kinase), whose activity is dependent upon activation of the small G protein, Rho (Amano et al., 1996; Garcia et
Previous reports have described barrier-disruptive effect of thrombin on endothelial monolayer and demonstrated involvement of Rho-mediated pathway in EC response (Garcia et al., 1996, 1999; van Nieuw Amerongen et al., 2000; Wettchureck and Offermanns, 2002). However, different steps of Rho-mediated signaling triggered by thrombin have been studied in various cell types derived from different species (Carton et al., 2002; Essler et al., 1998; Greenberg et al., 2003; Wettchureck and Offermanns, 2002). Importantly, the majority of experiments utilizing endothelial culture have been performed on cells derived from human umbilical vein (HUVEC), the vessel, which is unique in many aspects and may not adequately represent the range of physiological responses observed in pulmonary circulation (Feoktistov et al., 2002; Garlanda and Dejana, 1997; Klages et al., 1999; Lokeshwar and Selzer, 2000; Wells et al., 2002; Yashima et al., 2001).

To more precisely characterize cellular events underlying lung permeability changes related to conditions of acute lung injury, in this study, we used endothelial cells derived from human pulmonary circulation and performed detailed analysis of Rho-mediated pathway involved in thrombin-induced EC barrier dysfunction.

**Materials and methods**

**Reagents**

Culture medium 199 was obtained from GIBCO BRL (Chagrin Falls, OH). Colustrum-free bovine serum was purchased from Irvine Scientific (Santa Ana, CA). EC growth supplement was from Collaborative Research (Bedford, MA). Antibiotic–antimycotic mixture and nonessential amino acids were purchased from Sigma (St. Louis, MO). Texas Red phalloidin-, Alexa 488-, and Alexa 594-conjugated secondary antibodies were purchased from Molecular Probes (Eugene, OR). MYPT1 polyclonal antibody was purchased from Covance Inc. (Beverly, CA). Site-specific phospho-MYPT1 (MYPT1) antibody (Upstate Biotechnology, Lake Placid, NY), HA rabbit polyclonal antibody were from Santa Cruz Biotechnology (Santa Cruz, CA), and diphospho-MLC antibody was obtained from Cell Signaling (Beverly, MA). p115-Rho-GEF mouse monoclonal antibody was purchased from Calbiochem (La Jolla, CA).

**Cell cultures**

Bovine pulmonary artery endothelial cells (BPAEC) were obtained frozen at 16 passages from American Type...
Tissue Culture Collection (Rockville, MD; culture line CCL 209) and were utilized at passages 19–24 as previously described. Cells were cultured in M-199 media (Gibco) supplemented with 20% (v/v) colostrum-free bovine serum (Irvine Scientific), 15 μg/ml EC growth supplement (Collaborative Research), 1% antibiotic and antimycotic (10,000 units/ml penicillin, 10 μg/ml streptomycin, and 25 μg/ml amphotericin B; K. C. Biologicals), and 0.1 mM nonessential amino acids (Gibco) and maintained at 37°C in humidified atmosphere of 5% CO₂–95% air. The EC grew to contact-inhibited monolayers with the typical cobblestone morphology. Human pulmonary artery endothelial cells (HPAEC) were obtained from Clonetics, BioWhittaker Inc. (Frederick, MD), propagated in culture medium EGM-2 and used at passages 6–10.

Immunofluorescent staining

Endothelial cells grown on glass coverslips were fixed after agonist treatment 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 antibodies (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 program, using a Pentium III PC.

Image analysis of gap formation and stress fiber formation

Texas Red-stained EC monolayers stimulated with either thrombin or vehicle were viewed under microscope using 20/0.4 objective, and images captured as described above. The 16-bit images were analyzed using MetaVue 4.6 (Universal Imaging, Downingtown, PA). Images were differentially segmented between gaps (black) and cells (highest gray value) based on image grayscale levels. The gap formation was expressed as a ratio of the gap area to the area of the whole image. Similarly, actin fibers were marked out, and the ratio to the cell area covered by stress fibers to the whole cell area was determined. The values were statistically processed using Sigma Plot 7.1 (SPSS Science, Chicago, IL) software.

Western immunoblotting

Protein extracts were separated by SDS-PAGE on 10% gels, transferred to nitrocellulose membrane (30 V for 18 h or 90 V for 2 h), and probed with specific antibodies as previously described (Verin et al., 2001). Immunoreactive proteins were detected using enhanced chemiluminescent detection system (ECL) according to the manufacturer’s protocol (Amersham, Little Chalfont, UK).

Endothelial cell protein fractionation

Cells grown on 100-mm petri dishes were stimulated with either vehicle (media), or thrombin (5 min, 100 nM),
washed with ice-cold PBS, lysed in 200 μl extraction buffer (20 mM Tris-HCl (pH 7.4), 125 mM sucrose, 50 mM NaCl, 2 mM EGTA, 1 mM PMSF), and collected in microtubes. After centrifugation (100,000 × g, 30 min, +4°C), supernatants containing cytosolic proteins and pellets containing particulate fraction were separated, solubi-

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**Fig. 2.** Effect of thrombin on endothelial monolayer integrity. Confluent ECs grown on glass coverslips were challenged either with vehicle (M199) or thrombin (100 nM) for the indicated periods of time. After stimulation, cells were fixed and stained with Texas Red phalloidin for F-actin (Panel A), or nonmuscle myosin A (Panel C). In control experiments, cells were stained with secondary antibody without prior anti-myosin staining (Panel C). Gap and stress fiber formation induced by thrombin was assessed by morphometric analysis of Texas Red phalloidin-stained HPAEC performed using MetaVue software as described in Materials and methods (Panel B). Gap formation was assessed by increases in the ratio of the integrated gap area to the area of the whole image. Actin fibers were marked out, and the ratio of the area covered by stress fibers to the whole cell area was calculated. Data are expressed as percentage of control corresponding to nonstimulated cells and represent results of three independent experiments. Thrombin-stimulated EC were stained for specific marker of endothelial adherence junctions, VE-cadherin (Panel D), and focal adhesion protein, vinculin (Panel E). Thrombin caused significant stress fiber formation, and development of intracellular gaps (shown by short arrows in Panel A) accompanied by disappearance of cortical ring (shown by long arrows in Panel A), disruption of cell–cell contacts, and remodeling of focal adhesion plaques. Shown are representative results of three independent experiments.
Fig. 3. Effect of thrombin on MLC phosphorylation. (Panel A) BPAEC were treated with either vehicle (M199) or thrombin (100 nM, 5 min). Shown is the Western blot of MLC separated by urea gel electrophoresis (un-P, unphosphorylated; mono-P, monophosphorylated; di-P, diphosphorylated MLC). (Panel B) HPAEC were treated with either vehicle (M199) or thrombin for the indicated time periods, and cell lysates were transferred to PVDF membrane and stained with anti-diphospho-MLC antibody. Control panel shows equal levels of total MLC in the samples. (Panel C) Cells were stained for F-actin (left column), unphospho-, monophospho-, or diphospho-MLC (right column) as described in Materials and methods. Thrombin increased levels of MLC phosphorylation in pulmonary endothelium. Phosphorylated MLC appears to be preferentially colocalized with actin stress fibers in control and stimulated cells. Shown are representative results of five independent experiments.
lized in 3× SDS sample buffer, and specific protein content in cytosolic and particulate fractions was analyzed by Western immunoblotting.

**Expression plasmids and transfection protocol**

Plasmids encoding dominant-negative Rho (N19Rho) and dominant-negative Rho-kinase (a Rb-PH(TT) mutant, which is the C-terminal fragment of Rho-kinase mutated at Rho-binding sites), have been previously described (Amano et al., 1998, 1999; Leung et al., 1996). EC grown in D35 petri dishes at 70% confluence were incubated with 2 ml of OPTI-MEM medium containing 2 μg DNA and 20 μl of Fugene 6 (Boehringer Mannheim-Roche, Indianapolis, IN) for 6 h in CO₂ incubator at 37°C. Following washing (DMEM + 10% FCS), cells were incubated for additional 24 h and used for experiments with thrombin stimulation. Control transfections were performed with empty vectors.

**Rho activation assay**

Rho activation in EC culture was analyzed using Rho assay kit available from Upstate Biotechnology. Briefly, pulmonary EC grown in 100-mm petri dishes were serum-starved for 2 h followed by stimulation with thrombin (100 nM) for the indicated periods of time. At the end of experiment, cells were rinsed with ice-cold PBS, and 500 μl of lysate buffer was added to each dish. Cells were scraped, and cell lysates were prepared according to manufacturer’s protocol. After centrifugation at 10,000 × g, 10 min, supernatants were incubated with rhotekin Rho-binding peptide immobilized on agarose, and activated GTP-Rho

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**Fig. 4. Thrombin-induced Rho activation in human pulmonary EC.** (Panel A) Cells were incubated with thrombin (100 nM) for the indicated periods of time, and Rho activation assay was performed as described in Materials and methods. Thrombin stimulation rapidly increased the levels of Rho-GTP indicating Rho activation. (Panel B) Immunofluorescent staining of Rho in EC after thrombin stimulation (100 nM, 5 min) demonstrates Rho peripheral translocation. (Panel C) Subcellular fractionation of EC after thrombin stimulation demonstrates Rho translocation in the particulate fraction. Shown are representative data of three independent experiments.
bound to rhotekin-agarose was detected by Western blot with anti-Rho antibody.

**Measurement of transendothelial electrical resistance (TER)**

The cellular barrier properties were measured using the highly sensitive biophysical assay with an electrical cell-substrate impedance sensing system (Applied Biophysics, Troy, NY) described previously (Garcia et al., 1997, 2000). Briefly, cells were cultured on small gold electrodes (10⁻⁴ cm²), and culture media were used as electrolyte. The total electrical resistance was measured dynamically across the monolayer and was determined by the combined resistance between the basal surface of the cell and the electrode, reflective of focal adhesion, and the resistance between the cells. As cells adhere and spread out on the microelectrode, TER increased (maximal at confluence), whereas cell retraction, rounding, or loss of adhesion was reflected by a decrease in TER. The small gold electrodes and the larger counter electrodes (1 cm²) were connected to a phase-sensitive lock-in amplifier with a built-in differential preamplifier (Applied Biophysics). A 1-V, 4000-Hz alternating current signal was supplied through a 1-MΩ resistor to approximate a constant-current source. Voltage and phase data were stored and computer-processed. Experiments were conducted only on wells that achieved >1000 Ω (10 microelectrodes per well) of steady-state resistance. Resistance was expressed by the in-phase voltage (proportional to the resistance), which was normalized to the initial voltage and expressed as a fraction of the normalized resistance value as previously described (Garcia et al., 1997).

**Fig. 5. Effect of Rho GTPase inhibition on thrombin-induced stress fiber formation and MLC phosphorylation.** (Panel A) HPAEC were transiently transfected with dominant-negative Rho A bearing HA-tag as described in Materials and methods, treated with either vehicle (M199) or thrombin (100 nM, 5 min), and stained with either Texas Red phalloidin to visualize F-Actin, or with anti-diphospho-MLC antibody. EC overexpressing dominant-negative Rho were detected by staining with anti-HA antibody and shown by arrows in Panel A. (Panel B) BPAEC monolayers were either first permeabilized by lipofectamine treatment in the presence or absence of specific Rho inhibitor, C3 exoenzyme (2.5 μg/ml) as previously described (Borbiev et al., 2000), or preincubated with toxin B (1 ng/ml, 1 h) followed by stimulation with thrombin (100 nM, 5 min). Shown are immunoblots of MLC species separated by urea gel electrophoresis and probed with anti-MLC antibody. Inhibition of Rho pathway completely abolished thrombin-induced stress fiber formation and MLC phosphorylation. Results are representative of three independent experiments.
Results

Effect of thrombin on pulmonary endothelium barrier properties

Previous studies have demonstrated that thrombin directly increases pulmonary EC permeability and plays a major role in the pathogenesis of acute lung injury (Dudek and Garcia, 2001; Ellis et al., 1999; Garcia et al., 1986, 1996, 1999; Johnson et al., 1983; Lo et al., 1985; Tiruppathi et al., 1992). However, the effect of thrombin on TER in human pulmonary endothelium has not been examined in detail. Measurements of TER across pulmonary EC monolayers after thrombin stimulation demonstrate that thrombin significantly decreases TER in both bovine and human pulmonary artery (BPAEC and HPAEC, respectively) endothelial cells reflecting dramatic EC barrier compromise (Fig. 1). The time course and dose dependence of EC responses to thrombin reveal similar patterns for HPAEC and BPAEC cultures with rapid onset and maximal TER decrease at 30 min of stimulation with 100 nM thrombin. Thrombin-induced decreases in TER were linked to actomyosin rearrangement. In unstimulated cells, F-actin primarily organized into cortical band and localized preferentially to cell periphery (long arrows). After thrombin stimulation, F-actin reorganized into shorter, thicker stress fibers in the center of the cells, whereas peripheral actin band disappeared. These changes were associated with appearance of paracellular gaps, indicating contraction (Fig. 2, Panel A). Quantitative analysis (Fig. 2, Panel B) confirmed increased gap formation and stress fiber formation indicative of cell contraction observed after thrombin stimulation. F-actin stress fiber formation was accompanied by activation of myosin filament assembly with appeared actomyosin colocalization (Fig. 2, Panel C), further suggesting enhancement of actomyosin interaction.

As thrombin-induced hyper-permeability has been suggested to be mediated by changes in cell–cell adhesion (Dejana, 1997; Sandoval et al., 2001; Winter et al., 1999) and focal adhesions (Schaphorst et al., 1997), we next examined thrombin effects on integrity of adherence junctions and remodeling of focal adhesions. Thrombin stimulation caused partial dissociation of key adherence junctions protein, VE-cadherin, from the areas of cell–cell interface, which indicates disruption of adherence junctions (Fig. 2, Panel D). Immunofluorescent analysis of vinculin redistribution in thrombin-stimulated pulmonary EC, which reflects focal adhesions remodeling, shows reduction in several smaller-size focal adhesions and formation of enlarged focal adhesions that serve as anchoring sites for actomyosin filaments (Fig. 2, Panel E). Quantitative analysis of focal adhesions was performed as described in Materials and methods and showed that thrombin stimulation decreased several focal adhesions per cell (129 ± 13 contacts per cell in thrombin stimulated EC versus 167 ± 18 contacts per cell in nonstimulated cells) but increased the average area of single focal contact (0.29 ± 0.17 µm² in thrombin stimulated cells versus 0.53 ± 0.24 µm² in nonstimulated EC). Taken together, these data demonstrate that thrombin induces dramatic barrier dysfunction in pulmonary endothelium, which involves contractile response, activation of focal adhesions, and partial disassembly of adherence junction complexes.

Effect of thrombin on EC MLC phosphorylation

Several studies have previously demonstrated that bovine EC barrier dysfunction is tightly linked to myosin-driven contraction initiated by MLC phosphorylation (Essler et al., 1998; Garcia et al., 1996; Wysolmerski and Lagunoff, 1990). In the next set of experiments, we characterized time dependence of MLC phosphorylation and examined intracellular localization of monophosphorylated MLC in human and bovine pulmonary EC. Like in bovine EC (Fig. 3, Panel A), thrombin stimulation of human pulmonary EC induced rapid increase
in mono- (data not shown) and di-MLC phosphorylation (Fig. 3, Panel B) with peak at 2 min followed by gradual decline which was evident at 60 min of stimulation. Nevertheless, even at 60 min, the level of di-MLC phosphorylation remained elevated compared to unstimulated cells. Immunofluorescent staining of EC monolayer suggested that mono- and diphosphorylated MLC preferentially colocalized with actin stress fibers, whereas unphosphorylated MLC showed cytoplasmic distribution (Fig. 3, Panel C). Thus, our results suggest that thrombin stimulation significantly increased intracellular levels of mono- and diphosphorylated MLC and their colocalization with stress fibers.

Effects of thrombin stimulation on Rho activity

Previous studies have demonstrated the involvement of both MLCK activation and Rho-mediated MYPT1 inhibition in thrombin-induced EC barrier dysfunction (Dudek and Garcia, 2001; Garcia et al., 1999; Velasco et al., 2002). Direct measurements of Rho activity in pulmonary EC indicated rapid activation of Rho observed after 2 min...
of thrombin treatment, which was sustained and remained elevated even after 60 min of stimulation (Fig. 4, Panel A). The time course of Rho activation correlated well with the time course of diphospho-MLC increase (Fig. 3, Panel B), suggesting direct link between thrombin-induced Rho activation and the level of MLC phosphorylation. Immunofluorescent staining of EC showed rapid Rho translocation to the cell membrane (Fig. 4, Panel B), which was further confirmed by subcellular fractionation and Western blot analysis of Rho distribution (Fig. 4, Panel C). These results suggest thrombin-induced increases in the interaction of Rho with membrane-bound regulatory proteins.

Effects of Rho inhibition on thrombin-induced cytoskeletal changes in pulmonary endothelium

To further evaluate the involvement of Rho in thrombin-mediated EC cytoskeletal remodeling, we overexpressed the dominant-negative RhoA in pulmonary EC. Cells were stimulated with either vehicle or thrombin, and subjected to double immunofluorescent staining for F-actin (red) and HA tag (green) (Fig. 5A, left columns), or phospho-MLC (green) and HA tag (red) (Fig. 5A, right columns). Staining for HA tag was performed to detect transfected cells (shown by arrows). Inhibition of RhoA significantly attenuated stress fiber formation in response to thrombin and abolished thrombin-induced MLC phosphorylation. Consistent with these results, inhibition of Rho activity using specific inhibitor C3 exoenzyme (Fig. 5, Panel B, left columns), or Toxin B, a broad inhibitor of Rho family small GTPases (Fig. 5, Panel B, right), completely abolished thrombin-induced MLC phosphorylation. Thus, these experiments further support a critical role for Rho in thrombin-induced pulmonary EC contractile responses.

Effects of Rho-kinase inhibition on thrombin-induced barrier dysfunction in pulmonary endothelium

We next examined a role of Rho effector, Rho-kinase, in thrombin-mediated signal transduction and cytoskeletal regulation. Rho-kinase inactivates myosin phosphatase via phosphorylation of its 130-kDa regulatory subunit (MYPT1) at Thr^{696} and Thr^{850} (Carbaljal et al., 2000; Kawano et al., 1999; Velasco et al., 2002). We next examined the effect of thrombin on MYPT1 phosphorylation in pulmonary EC. Fig. 6 demonstrates thrombin-induced MYPT1 phosphorylation at Thr^{696} and Thr^{850} in a time-dependent manner with maximum at 2 min. Pharmacological inhibition of Rho-kinase activity by Y27632 (5 μM) completely blocked thrombin-induced MYPT1 phosphorylation in HPAEC (Fig. 6, Panel B) and BPAEC (data not shown). Pretreatment of pulmonary EC with Y27632 completely abolished thrombin-induced MLC phosphorylation and significantly attenuated thrombin-induced decrease in TER (Fig. 7, Panels A and B). These effects were accompanied by decreases in the amounts of...
stress fibers, disappearance of diphospho-MLC staining, and reduced paracellular gaps (Fig. 7, Panel C). In the next series of experiments, Rho-kinase inhibition was performed using molecular approach. EC were transiently transfected with the RB-PH(TT) fragment of Rho-kinase, which functions as dominant-negative construct (Amano et al., 1998, 1999). Cells were stimulated with either vehicle or thrombin, and subjected to double immunofluorescent staining for F-actin (red) and c-myc tag (green) (Fig. 8A), or phospho-MLC (green) and c-myc tag (red) (Fig. 8B). Overexpression of dominant-negative Rho-kinase was monitored by anti-c-myc tag immunofluorescent staining (shown by arrows). Our data (Fig. 8) indicated that dominant-negative Rho-kinase reduced several stress fibers and decreased the level of diphospho-MLC in nonstimulated cells and completely abolished stress fiber formation and accumulation of diphospho-MLC in thrombin-treated cells. Collectively, these results clearly demonstrate that inhibition of Rho-kinase significantly attenuated thrombin-induced stress fiber formation and abolished thrombin-induced MLC phosphorylation.

Involvement of GEFs in thrombin-induced Rho activation

Upstream mechanisms of thrombin-induced RhoA activation by thrombin involve activation of thrombin receptor, PAR1, which triggers activation of PAR1-coupled heterotrimeric G proteins G12 and G13 (Barr et al., 1997; Bogatcheva et al., 2002; Garcia et al., 1993). In turn, G12 and G13 may stimulate Rho activity by interaction with specific guanosine nucleotide exchange factors (GEFs) (Zheng, 2001). However, precise molecular mechanisms of thrombin-induced Rho activation are not well described. In our studies, we examined expression and intracellular distribution of Rho-dependent GEF, p115-RhoGEF, in human pulmonary endothelium. Immunofluorescent staining revealed preferential cytosolic localization of p115-RhoGEF in nonstimulated pulmonary EC (Fig. 9, Panel A), whereas thrombin stimulation induced p115-RhoGEF translocation to cell membrane (Fig. 9, Panel B). Consistent with these results, subcellular fractionation showed that thrombin induced increased GEF accumulation in the membrane/cytoskeletal fraction (Fig. 9, Panel B). These data suggest involvement of p115RhoGEF in the mechanisms of thrombin-induced Rho-mediated pulmonary EC barrier dysfunction.

Taken together, our data provide comprehensive analysis of Rho-mediated signal transduction in pulmonary EC, and clearly demonstrate a critical role for Rho-dependent pathway in thrombin-mediated barrier dysfunction in pulmonary endothelium.

Discussion

Mechanisms of thrombin-induced EC hyperpermeability involving Ca²⁺, protein kinase C, phospholipase D, tyrosine phosphorylation, MYPT1 inhibition, and MLCK activation have been described in our previous works and by others (Bogatcheva et al., 2002; Garcia et al., 1992a,b; Greenberg et al., 2003; Klages et al., 1999; Lum and Malik, 1996; Mehta et al., 2001; Verin et al., 1995; Vouret-Craviari et al., 2002). Critical role for Rho-dependent mechanisms in cytoskeletal regulation has been shown in smooth muscle and nonmuscle cells (fibroblasts, platelets, endothelial cells). Effects of Rho or Rho-kinase inhibition on thrombin-induced barrier dysfunction in certain types of EC have been also demonstrated; however, the role of Rho-mediated signaling in human pulmonary EC barrier regulation has not been investigated, nor detailed analysis of Rho-mediated signaling cascade has been performed. This information is critical for understanding of cellular events underlying lung permeability changes related to conditions of acute lung injury.

![Fig. 9. Effect of thrombin on p115-RhoGEF cellular localization. After stimulation with thrombin (100 nM, 5 min), immunofluorescent staining of HPAEC with anti-p115-RhoGEF antibody was performed (Panel A). Arrows depict translocation of p115-RhoGEF to the cell membrane upon thrombin stimulation. Panel B demonstrates results of subcellular fractionation and Western blot probing of cytosolic and particulate fractions with anti-p115-RhoGEF antibody. Results are representative of three independent experiments.](image-url)
We demonstrate in this study that thrombin induces rapid activation of Rho, which was accompanied by Rho translocation to the cell membrane and increased interaction with guanosine nucleotide exchange factor, p115-RhoGEF. Thrombin receptor PAR1 has been shown to couple with several heterotrimeric G-proteins: Gi, Go, G12/13, and Gq (Barr et al., 1997; Gilchrist et al., 2001; Manolopoulos et al., 1997). Activated G protein G13 regulates GEF activity of Dbl family protein, p115RhoGEF. Binding of p115RhoGEF through the N-terminal RGS domain to activated G13 \( \alpha \)-subunit could stimulate GTP-hydrolysis of \( \alpha_{13} \) and return it to the GDP-bound state, meanwhile causing an activation of GEF activity of p115RhoGEF and translocation to the plasma membrane, where it activates Rho-mediated pathways (Schmidt and Hall, 2002; Zheng, 2001). Our results are consistent with the proposed role for p115RhoGEF in receptor-mediated effects of thrombin on Rho activation. As demonstrated in this study, thrombin-activated Rho via its effector Rho-kinase triggers MLC phosphorylation, stress fiber formation, and actomyosin contraction. Rho inactivation by C3-exotoxin-induced ADP ribosylation or overexpression of dominant-negative Rho attenuated thrombin-induced MLC phosphorylation and stress fiber formation. MYPT1 has been previously shown to be a substrate for Rho-kinase-mediated phosphorylation at Ser\(^{686}\) and Ser\(^{850}\) (Amano et al., 1996; Kimura et al., 1996), which leads to MYPT1 inactivation (Kawano et al., 1999). Using site-specific antibodies to MYPT1 phospho-Ser\(^{686}\) and phospho-Ser\(^{850}\), we demonstrate that thrombin stimulation caused MYPT1 phosphorylation by Rho-kinase, which was linked to increased phospho-MLC accumulation in stimulated pulmonary EC. Direct involvement of Rho-Rho-kinase-MYPT1 pathway in thrombin-induced MLC phosphorylation and cytoskeletal remodeling was clearly demonstrated in our study, as pharmacological inhibition of Rho-kinase by Y27632 abolished MYPT1 phosphorylation at Ser\(^{686}\) and Ser\(^{860}\), decreased thrombin-induced MLC phosphorylation, and attenuated thrombin-mediated regulation of EC cytoskeletal rearrangement and barrier function induced by thrombin. See Discussion for details.

![Proposed schema of Rho-mediated regulation of EC cytoskeletal rearrangement and barrier function induced by thrombin](image-url)
induced decreases in TER. Consistent with these data, overexpression of dominant-negative Rho-kinase abolished thrombin-induced stress fiber formation and accumulation of diphospho-MLC in pulmonary EC. Fig. 10 summarizes results of this study and previous reports and depicts a potential mechanism of Rho-mediated pathway of thrombin-induced barrier dysfunction. Interaction of thrombin with PAR1 receptor activates G13 protein, which in turn binds and activates p115-RhoGEF exchange factor, that switches Rho from GDP- to GTP-bound state. GTP-Rho activates Rho-kinase, which phosphorylates and inactivates MYPT1, thus promoting MLC phosphorylation, actomyosin interaction, cell contraction, and gap formation, which results in EC barrier dysfunction.

Our results suggest that pharmacological inhibition or gene targeting of small GTPase Rho and its effector Rho-kinase in pulmonary vasculature as well as modulation of myosin phosphatase activity in pulmonary EC may represent important strategies for the prevention of thrombin-induced vascular barrier disruption, a key event in the physiological derangements associated with acute lung injury.

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