Cdc42 Regulates the Restoration of Endothelial Barrier Function

Panos Kouklis, Maria Konstantoulaki, Stephen Vogel, Michael Broman, Asrar B. Malik

Abstract—The mechanisms involved in the restoration of endothelial cell junctions subsequent to barrier disruption remain unclear. It is known that formation of adherens junctions (AJs) affects cytoskeletal actin arrangement and that Rho GTPases regulate the state of actin polymerization. In the present study, we examined the role of the Rho GTPases, Rho, Rac, and Cdc42 in the reannealing of AJs. We studied the response to thrombin, which increases endothelial permeability through disassembly of AJs, followed by recovery of barrier function through junctional reannealing within 2 hours. Cdc42 was activated late, at ≈1 hour after thrombin exposure, concurrent with its translocation from the cytoplasm to the membrane. Activation and translocation of Cdc42 preceded the reformation of AJs. Expression of the dnCdc42 mutant (N17Cdc42) significantly delayed the reformation of the VE-cadherin–containing AJs and restoration of endothelial barrier function. We also studied the lung microcirculation to address the in vivo relevance of Cdc42 signaling in barrier restoration. N17Cdc42 expression in the mouse lung endothelium markedly attenuated the endothelial barrier recovery after the permeability increase induced by activation of the thrombin receptor protease-activated receptor-1. These findings demonstrate the critical function of Cdc42 in restoring AJ-dependent, endothelial cell homotypic adhesion and barrier function. The delayed activation of Cdc42 represents a negative-feedback mechanism that signals AJ reassembly after the increase in endothelial permeability induced by inflammatory mediators such as thrombin. (Circ Res. 2004;94:159-166.)

Key Words: barrier reannealing ■ VE-cadherin ■ endothelium ■ cell shape ■ Rho GTPases

Endothelial adherens junctions (AJs) regulate the transendothelial flux of liquid and plasma proteins.1–3 The endothelial cell–specific VE-cadherin is a component of AJs involved in mediating cell-cell interactions.4,5 Endothelial cell AJs disassemble in response to proinflammatory mediators such as thrombin, resulting in increased transendothelial permeability.6–9 Thrombin, a protease involved in blood clotting, activates signaling cascades in endothelial cells through protease-activated receptor-1 (PAR-1), a member of the 7-transmembrane G-protein–coupled receptor family.10,11 PAR-1 activation increases intracellular Ca2+ and stimulates protein kinase C and myosin light chain kinase, thereby activating the endothelial actin-myosin contractile machinery.12 Thrombin also has stereotypic effects on AJs. The endothelial junctional barrier is disrupted within 5 to 10 minutes, and VE-cadherin complex is redistributed in the membrane5 in association with increased endothelial permeability.13 AJs disappear and then reform within 2 hours14 to restore endothelial junctional integrity and normal vasopermeability.15 Tyrosine and serine/threonine kinases and phosphatases acting on catenins, the proteins linking VE-cadherin to the actin cytoskeleton, seem to play an important role in the disassembly of AJs.14–16

Activation of two members of the Rho family of GTPases, Rho and Rac, was linked to the loss of endothelial barrier function. The increased permeability resulted from actin polymerization, stress fiber formation, and cell rounding induced by these GTPases.17–19 Inhibition of Rho or Rac by expression of dominant-negative (dn) mutants had no effect on VE-cadherin localization or stability of AJs.20 Inhibition of all Rho GTPases (by glycosylation induced with toxin B) disrupted VE-cadherin and increased endothelial permeability in cell monolayers as well as in isolated vascular tissue.21

The Rho family of GTPases temporally and spatially orchestrates a variety of cellular functions in response to stimuli. These alterations occur through modifications of the cytoskeleton, vesicular transport, and transcription.22 Studies in endothelial cells have addressed the role of the Rho GTPases either during an induced increase in endothelial permeability or in unchallenged cells.17–19 In the present study, we investigated a novel function of Rho GTPases in the reformation of endothelial AJs subsequent to the loss of the barrier induced by thrombin. We showed that Cdc42 was activated and translocated from the cytosol to the membrane during the recovery phase of AJs. Expression of dnCdc42 interfered with VE-cadherin junction reformation, as demonstrated by impaired VE-cadherin localization to cell junctions, delayed restoration of transendothelial electrical resistance (TER), a measure of junctional permeability, and...
delayed recovery to normal permeability of intact microvessels. These results from endothelial cell monolayers and perfused mouse lungs demonstrate the critical role of Cdc42 in the restoration of AJs and endothelial barrier function.

Materials and Methods

Cell Culture
Human microvascular endothelial cells (HMECs; dermal) were provided by Dr E.W. Ades (National Center for Infectious Diseases, Center for Disease Control, Atlanta, Ga). Human pulmonary arterial endothelial (HPAE) cells were purchased from VEC Technologies, Rensselaer, NY.

Mice
We used pathogen-free male CD-1 mice (30 to 35 g; Harlan Co, Indianapolis, Ind). All animal experiments conformed to guidelines of the University of Illinois.

Thrombin Challenge
Human α-thrombin (Enzyme Research Laboratories) was added (4 U/mL) to confluent endothelial cells in serum-free MCDB-131 medium.

Antibodies
The following antibodies were used: anti–VE-cadherin monoclonal (Chemicon, Temecula, Calif), goat polyclonal (Research Diagnostics, Flanders, NJ), C-19 goat polyclonal (Santa Cruz, Santa Cruz, Calif); rabbit polyclonal anti-FLAG and anti-myec (Zymed, San Francisco, Calif); mouse monoclonal anti-FLAG (M2) (Sigma-Aldrich, St Louis, Mo); rabbit anti-Cdc42 polyclonal (Santa Cruz); HRP-conjugated secondary antibodies (Jackson Laboratories, West Grove, Pa); and FITC-conjugated or Texas Red–conjugated (Jackson Laboratories) or Alexa 488–conjugated and Alexa 568–conjugated (Molecular Probes, Eugene, Ore) secondary antibodies.

Transfection
Endothelial cells were transfected by electroporation.24 Endothelial cells (10⁶) were seeded in a 100-mm² dish, and after 20 to 24 hours, the cells were trypsinized and resuspended in 300 µL of medium. Plasmid DNA (10 µg) mixed with 14 µg of salmon sperm DNA was added, and cells were electroporated in 0.4-cm cuvettes (Gene Pulser, BioRad) at 180-mV and 950-millifarad capacitance. To obtain confluent monolayers, 50 µL of the transfected cells was applied to 1% gelatin-coated coverslips or electrodes (described below) and allowed to adhere for ≤24 hours. We typically observed ~40% transfection efficiency and reestablishment of AJs in vector-alone-transfected control cells during this period.24

Immunofluorescence Microscopy
Cells were grown on coverslips coated with 1% gelatin (Sigma-Aldrich), fixed at room temperature with 3.7% formaldehyde for 10 minutes, permeabilized with 0.4% Triton X-100 in PBS for 10 minutes, and incubated with blocking solution (20 mmol/L HEPES [pH 7.5], 1 mmol/L KCl, 1% BSA, 0.4% gelatin, 0.05% NaN₃) containing 0.1% Triton X-100 for 10 minutes. The cells were immunostained and visualized with a Nikon Diaphot 200 or Zeiss Pascal confocal microscope.

Protein Assay
Protein concentration of cell extracts was quantified using the BCA protein assay (Pierce).

Streptolysin-O Permeabilization
Cell monolayers, suspended in PBS with 50 to 75 U/mL streptolysin-O (Sigma-Aldrich), were incubated for 10 minutes (2°C) and 15 to 20 minutes (37°C) with gentle shaking. A cytosolic fraction and a membrane/cytoskeletal/nuclei pellet were recovered (12,000 rpm), and the latter was incubated with 1% Triton X-100 in PBS (4°C) containing protease inhibitors (Complete-Roche). After 20 minutes, the suspension was centrifuged (14,000 rpm) and the soluble membrane fraction was collected.

PAK Binding Assay
GST-p21–binding domain (GST-PBD) of p21-activated kinase (PAK) (a gift of Dr G. Bokoch, Scripps), prepared from bacteria, was bound to glutathione-Sepharose beads (Amersham-Pharmacia). GST-PBD beads (20 µL) were incubated with endothelial extracts (200 to 300 µg total protein) in 400 µL membrane lysis buffer (25 mmol/L HEPES [pH 7.5], 150 mmol/L NaCl, 1% Igepal CA-630, 10 mmol/L MgCl₂, 1 mmol/L EDTA, 2% glycerol, protease inhibitors) for 16 hours (4°C). The eluates from washed beads were subjected to SDS-PAGE and Western blot analysis. The bands were quantified by densitometry using ImageJ version 1.30 (National Institutes of Health). The intensity of the protein band (Cdc42 or Rac) pulled down by GST-PBD was normalized to total Cdc42 or Rac at each time point.

Preparation of Cationic Liposomes for In Vivo Studies
Liposomes containing the cationic lipid dimethyldioctadecyl ammonium bromide (DDAB from Sigma) in a 1:1 molar ratio with cholesterol (Calbiochem) were prepared as described previously.25

Assessment of Protein Expression In Situ
Liposome-DNA suspension was injected into the intraglarial vein of mice. Lung endothelial cells were recovered by collagenase,26 cultured, and assayed by immunofluorescence for protein expression.

Transendothelial Electrical Resistance
Transfected cells were seeded on a gelatin-coated gold electrode (5.0×10⁶/cm²) and grown 40 to 48 hours to confluence and to allow AJs to form. The small electrode and the larger counter-electrode were connected serially to a 1-MΩ resistor between the small electrode and the larger counter-electrode. The voltage was monitored by a lock-in amplifier, stored, and processed by a personal computer. The same computer controlled the amplifier output and switched the measurement to different electrodes in the course of an experiment. Before each experiment, endothelial monolayers were washed with serum-free growth medium and used for measuring changes in TER. Data are presented as the change in resistive portions of the resistance normalized to its value at time 0.27

Measurement of Lung Capillary Filtration Coefficient (Kf,c)
Lungs of anesthetized mice were transferred en bloc to a perfusion apparatus, and Kf,c measurements were made as described previously.26

Reversible Increase in Lung Vascular Permeability Induced by PAR-1 Agonist Peptide
Lungs were perfused with the specific PAR-1 agonist peptide TFLLRNPPDK11,26 Kf,c was measured at 20-minute intervals, as described in the legend for Figure 6.

Results
Activation of Cdc42 During Reassembly of AJs
The presence of AJs in endothelial cells grown to confluence was evident from the anti–VE-cadherin immunostaining pattern (Figure 1, 0 minutes). Thrombin challenge induced cell rounding and disassembly of AJs within 15 minutes, as seen by the disrupted perimeter of VE-cadherin staining (Figure 1, 15 minutes). These responses were reversed as the cells
flattened, and AJs reformed after 1 hour of continuous thrombin exposure, thus restoring staining of the cell perimeter with the anti–VE-cadherin antibody (Figure 1, 1 and 2 hours).

We assessed the activation of Cdc42 (Cdc42-GTP) and Rac (Rac-GTP) by affinity purification at various times after thrombin addition. We used a pull-down assay with GST (PBD of PAK1), because PBD binds specifically to the GTP-bound Cdc42 and Rac but not Rho. Endothelial cells were cultured under the following conditions: confluent and confluent challenged with thrombin for 15 minutes, 1 hour, or 2 hours. Extracts were incubated with GST-PBD coupled to glutathione-Sepharose beads. The pull-downs were analyzed for the presence of activated Cdc42 or Rac by Western blotting. We observed Cdc42 binding to PBD in extracts from cells after challenge with thrombin for 1 or 2 hours (Figure 2A), which paralleled the reformation of AJs (Figure 1). In contrast, Rac was not activated during this period (Figure 2B). The basal level of activated Cdc42 remained constant in confluent untreated (0 minutes) and 15-minute thrombin-treated endothelial cells (Figure 2A).

**Membrane Partitioning of Cdc42 During Reassembly of AJs**

Confluent endothelial cells incubated with thrombin from 0 to 60 minutes were permeabilized with streptolysin-O to preserve membranes and associated proteins. The cytosol was separated from the membrane/cytoskeletal pellet, which was extracted with Triton X-100. The cytosol and Triton X-100–soluble membrane fraction were assayed by Western blotting for the presence of Rac, Cdc42, and VE-cadherin. After thrombin challenge, Rac was distributed almost equally between the cytosolic and membrane fractions (Figure 3A). VE-cadherin was found exclusively in the membrane fraction at all times (Figure 3A). Before thrombin, Cdc42 was primarily in the membrane fraction, whereas thrombin exposure for 15 and 30 minutes resulted in decreased membrane-associated Cdc42 (Figure 3A). However, membrane-associated Cdc42 increased at 1 hour after thrombin during the AJ recovery phase (Figure 3A). We observed no significant change in the distribution of Rac1 or VE-cadherin at 1 hour (Figure 3A). We transfected HMECs with myc-tagged Cdc42 to follow Cdc42 and VE-cadherin movement at 0, 15, and 60 minutes after thrombin. Partial recovery of cell-cell contacts was detected only after 60 minutes (VEC, Figure 3B). At 60 minutes, Cdc42-myc accumulated at the plasma membrane (Figure 3B), consistent with the Cdc42 membrane partitioning data above. In contrast, the myc-tagged N17Cdc42 failed to localize to the plasma membrane, and AJs failed to reform in these cells (Figure 4A; N17Cdc42 localization shown 2 hours after thrombin; data were similar at 1 hour).

**Inhibition of Cdc42 Interferes With AJ Reassembly**

To address the role of the activated Cdc42 (GTP-Cdc42) in reformation of AJs, N17Cdc42-myc (Figure 4A) or FLAG-N19Rho (Figure 4B) was expressed in confluent HPAE cells. Cells were double-stained with anti-myc or anti-FLAG and anti–VE-cadherin Ab before and at 2 hours after thrombin challenge to monitor AJ reformation. In untreated cells, VE-cadherin was localized to AJs in >90% of cells, including the N17Cdc42-expressing cells (Figure 4A, 0 minutes). The cells were challenged with thrombin and fixed at 15 minutes and 1, 2, and 4 hours. As shown in Figure 1, AJs were disrupted 15 minutes after thrombin, and VE-cadherin was redistributed. At 1 hour, AJs began to reform, but the response was incomplete and no conclusions could be drawn (data not shown). However, at 2 hours, VE-cadherin relocalized to the AJs of nontransfected and N19Rho-transfected...
cells (Figure 4B, 2 hours), indicating the reestablishment of the junctional barrier. Conservatively, 50% of the N17Cdc42-transfected cells examined showed reduction of VE-cadherin AJs (higher magnification example shown in Figure 4A).

We measured TER of cells transfected with dn mutants of Rac1, Cdc42, or RhoA to quantify AJ reformation. TER measurements, which provide a real-time measurement of junctional integrity, were made in confluent monolayers of N17Cdc42, N17Rac1, N19RhoA, and nontransfected cells. Basal TER of each monolayer was in the 12 to 16 Ω cm² range. TER was monitored continuously for 5 hours after thrombin addition. TER decreased immediately after thrombin to the same extent in all transfected cells. N19RhoA-transfected cells recovered in 2 hours, as did the nontransfected cells (Figure 5, large arrowheads). However, recovery was significantly delayed in N17Cdc42-transfected cells (occurring after ~4 hours) (Figures 5A and 5B, large arrowheads). Recovery was also delayed, although less, in N17Rac-transfected cells (completed by ~3 hours). TER recovery was unaffected by N19RhoA expression (Figures 5A and 5B).

**dnCdc42 Expression in Mouse Lung Endothelium Delays Reversal of Thrombin-Induced Increase in Endothelial Permeability**

To determine the transfection efficiency of N17Cdc42-myc in intact mice, lungs were isolated 24 hours after injection of liposome/DNA complex and treated with collagenase to recover endothelial cells. The recovered endothelial cells were assessed for N17Cdc42-myc expression (Figure 6A). Cells positive for N17Cdc42-myc were also always positive for VE-cadherin. The transfection efficiency was determined to be 30% to 40%. We assessed the effects of N17Cdc42 on AJ reformation in vessels by measuring the lung capillary filtration coefficient, $K_{f,c}$. To stimulate the thrombin receptor PAR-1, the peptide agonist selective for PAR-1, TFLLRN-PNDK (60 μmol/L), was added to the perfusate over 2 minutes. $K_{f,c}$ was measured to evaluate the time and magnitude of the recovery to preagonist levels in mock-transfected and N17Cdc42-myc–transfected mice (Figure 6B). Control lungs showed a 3.5-fold increase in $K_{f,c}$ (Figure 6B) in the 20-minute period after peptide addition. Subsequent periodic measurements of $K_{f,c}$ showed that control lungs recovered fully within 1 hour of peptide addition, with a recovery half-time of 25 minutes. In contrast, in lungs from mice transfected with N17Cdc42, the recovery time was markedly increased (Figure 6B).

**Discussion**

AJs disassemble and reassemble in endothelial monolayers in response to proinflammatory mediators such as thrombin.29 AJ reassembly precedes the restoration of vascular endothelial barrier integrity.29–31 However, the signals responsible for recovery of endothelial barrier function are incompletely understood. Herein, we addressed the basis for the reformation of AJs and the subsequent recovery of endothelial barrier function. Reassembly of AJs in epithelial cells has been linked to activation of Rho GTPases.32 The Rho family of proteins, known to translocate to membranes on activation, is responsible for actin cytoskeleton rearrangements affecting chemotaxis, cell motility, vesicular trafficking, and cell-cell adhesion.32 We assessed the time course of activation of Rho GTPases after thrombin and found that Cdc42 was activated during the phase of AJ reformation. This finding is consistent with evidence that Cdc42 was not involved in signaling the increase in endothelial permeability induced by thrombin.19 Another study showed that thrombin did not activate Cdc42 within a 15-minute time period in endothelial cells,33 supporting our observation of delayed Cdc42 activation. In contrast, Rho was reported to be activated within 5 minutes after thrombin challenge and remained active for 30 minutes.17,34, ie, at a time when AJs were disassembling and endothelial permeability was in the increasing phase. In addition, Rho has been shown to participate in signaling the increased endothelial permeability.17–19 Our studies showed by subcellular
fractionation and immunofluorescence that Cdc42 shuttled from the cytoplasm to the plasma membrane during the period of AJ reformation. Reestablishment of endothelial junction integrity occurring between 1 and 2 hours after thrombin exposure corresponded with the time course of Cdc42 activation. Rac1 was reported to be present in the contact areas between epithelial cells during the initial stages of cell-cell junction formation and is involved in actin cytoskeleton remodeling and lamellipodia formation. In the present study, we observed that thrombin did not induce activation of Rac in endothelial cells under these conditions. AJ reformation in previous studies was addressed subsequent to the addition of Ca$^{2+}$ to the medium (ie, Ca$^{2+}$ switch) of epithelial cells or after random collisions of single cells. Recovery of endothelial AJs after thrombin in the present study was different from the de novo formation of AJs in epithelial cells in several aspects. The recovery occurred without external manipulations, and AJs did not disassemble over the entire cellular surface. The endothelial cells remained in partial contact throughout the entire period after thrombin challenge, and in this respect there was no de novo formation of AJs as in the epithelial cell studies.

The upstream mechanisms of Cdc42 activation in endothelial cells are not clear. We showed recently that the expression of the cytoplasmic domain of VE-cadherin at the plasma membrane resulted in Cdc42 activation and plasma membrane protrusion specifically in endothelial cells. Therefore, it is possible that membrane accumulation of VE-cadherin induced by thrombin during AJ disassembly can stabilize activated Cdc42, specifically at the sites of AJ disruption. In endothelial monolayers, thrombin induced the transient aggregation of VE-cadherin in distinct foci at the plasma membrane. Thus, the aggregation of VE-cadherin after disassembly of AJs itself may participate in AJ reformation secondary to activation of Cdc42. Reintroduction of VE-cadherin in endothelial cell lines from VE-cadherin–null mice increased the level of activated Rac and its localization to the membrane. These cells had profound morphological alterations as the result of elevated Rac1 activity. Furthermore, there was an increase in protein level of the Rac-specific guanine nucleotide exchange factor Tiam-1, which was localized to intercellular junctions. Together with the hyperphosphorylated Rac effector protein PAK, Tiam-1 and Rac were found to be enriched in the membrane fraction. Therefore, VE-cadherin in the present study may similarly stabilize and activate Cdc42 and signal AJ reassembly. It remains to be seen whether Cdc42 activation is controlled by as-yet-unknown thrombin-activated signals. Studies in epithelial cells showed that Cdc42 was also activated during the reformation of AJs after an extracellular Ca$^{2+}$ concentration switch. However, it was not clear if Cdc42 activation occurred as the result of homotypic engagement of the extracellular domains of E-cadherin or secondary to the cadherin engagement and AJ formation.

We observed that Cdc42 was critical to the mechanism of AJ reassembly in endothelial cells. This was evident from the experiments in which AJs failed to reform normally in thrombin-challenged endothelial cells after the expression of dnCdc42. These findings cannot be ascribed to a direct disruptive effect on AJs by the Cdc42 mutant, because endothelial cells expressing N17Cdc42 formed normal VE-cadherin junctions that responded to thrombin by junction disassembly (Figure 4A). The Rho mutant, results nevertheless point to a crucial role of Cdc42 activity in the time-dependent reformation of endothelial AJs and barrier recovery.

To determine whether restoration of AJs was coupled to the return to the normal state of endothelial permeability, we...
measured changes in endothelial barrier function by TER.\textsuperscript{27} TER is a more sensitive and quantitative assay for the assessment of barrier recovery than are morphological studies based on localization of junction proteins by immunofluorescence. We showed that N17Cdc42 expression significantly delayed the TER recovery after thrombin challenge. N17Rac1 also delayed TER recovery, probably secondary to blocking guanine nucleotide exchange factors (GEFs) common to both Cdc42 and Rac1. Our functional data imply that Rac1 might also play a role in formation of endothelial cell AJs similar to that seen in epithelial cells,\textsuperscript{35,38} but its effect does not seem to be as important as that of Cdc42. In contrast to Cdc42 and Rac mutants, dnRho had no effect on TER recovery after thrombin.

Because endothelial permeability in monolayer studies may not always reflect vessel wall permeability in the intact microcirculation,\textsuperscript{21} we assessed the effects of N17Cdc42 on restoration of endothelial barrier function after activation of the thrombin receptor PAR-1. Previous studies showed that PAR-1 activation increased vascular permeability in the mouse lung.\textsuperscript{11} In the present study, we observed that expression of N17Cdc42 significantly delayed the recovery of vascular permeability consistent with the TER data. Thus, both cell culture and intact microvessel experiments showed an important signaling function of Cdc42 in restoring integrity of endothelial AJs and barrier function after the thrombin-induced increase in endothelial permeability.
A previous study examining the role of the Rho GTPases has implicated Rho and Rac in the mechanism of thrombin-induced increase in endothelial permeability.19 Another study showed that Rho and Rac regulated the early phase of AJ formation during vascular morphogenesis.45 The present findings are different in that we identified a role for Cdc42 specifically in signaling the reassembly phase of AJs after their disassembly induced by thrombin. Thus, Cdc42 was activated and localized to the membrane only during the period of AJ reassembly. This action of Cdc42 in regulating AJ reassembly is consistent with the versatility of Rho GTPase effectors in integrating multiple cell functions depending on the spatial and temporal activation.57

In summary, we have described a key role for Cdc42 activity in endothelial cells in signaling AJ reformation and restoration of endothelial barrier function. This activity was temporally and spatially restricted at the cell junctions during AJ reassembly. Thus, permeability-increasing mediators such as thrombin may activate Cdc42 and set in motion reannealing of AJs and return of endothelial permeability to basal levels. Cdc42 activation represents an important negative-feedback mechanism signaling the restoration of integrity of AJs and endothelial barrier function.

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