Critical role of Cdc42 in mediating endothelial barrier protection in vivo

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Critical role of Cdc42 in mediating endothelial barrier protection in vivo. Am J Physiol Lung Cell Mol Physiol 295: L363–L369, 2008. First published May 30, 2008; doi:10.1152/ajplung.90241.2008.—Activation of the Rho GTPase Cdc42 has been shown in endothelial cell monolayers to prevent disassembly of interendothelial junctions and the increase in endothelial permeability. Here, we addressed the in vivo role of Cdc42 activity in mediating endothelial barrier protection in lungs by generating mice expressing the dominant active mutant V12Cdc42 protein in vascular endothelial cells targeted via the VE-cadherin promoter. These mice developed normally and exhibited constitutively active GTP-bound Cdc42. The increase in lung vascular permeability and gain in tissue water content in response to intraperitoneal lipopolysaccharide challenge (7 mg/kg) were markedly attenuated in the transgenic mice. To address the basis of the protective effect, we observed that expression of V12Cdc42 mutant in endothelial monolayers reduced the decrease in transendothelial electrical resistance, a measure of opening of interendothelial junctions, thus indicating that Cdc42 activity preserved junctional integrity. RhoA activity in V12Cdc42-expressing endothelial monolayers was reduced compared with untransfected cells, suggesting that activated Cdc42 functions by counteracting the canonical RhoA-mediated mechanism of endothelial hyperpermeability. Therefore, Cdc42 activity of microvessel endothelial cells is a critical determinant of junctional barrier restrictiveness and may represent a means of therapeutically modulating increased lung vascular permeability and edema formation.

Cdc42 mutant; lung vascular permeability; edema formation; Rho GTPases; RhoA; Rac1

The endothelial barrier lining blood vessels, which controls the exchange of fluid, small solutes, and plasma proteins (18), is compromised in inflammatory diseases. Disruption of endothelial barrier at the level of interendothelial junctions results in increased vascular permeability and leakage of plasma proteins and exudation of fluid, common in conditions such as sepsis, acute lung injury, and atherosclerosis (1, 18, 22). Angiogenic vessels in cancerous tissue also show leaky interendothelial junctions that may contribute to metastasis (28). The Rho family of monomeric GTPases, Rho, Rac, and Cdc42, have been shown to be crucial in the regulation of endothelial permeability through precise spatio-temporal signaling that alters actin stress fiber organization and adhesive junctions that form the endothelial monolayer (7, 29). Rho proteins are activated through GDP-GTP exchange induced by guanine nucleotide exchange factors and are inactivated by GTPase-activating proteins. Rho guanine nucleotide dissociation inhibitors mediate stabilization of the inactive GDP-bound form of Rho (12). Conformational changes activate the GTP-bound proteins, which translocate to the membrane and interact with specific effector molecules. This triggers the signaling pathways controlling actin cytoskeleton reorganization, thereby increasing vascular endothelial permeability and assembly of adherens junctions (AJs) of endothelial cells (18).

The AJ complex, which regulates homotypic adhesion of adjacent endothelial cells, is composed of VE-cadherin (VEC), β-catenin, and α-catenin proteins that link AJs to the actin cytoskeleton (6). AJs are implicated in a reversible modulation of endothelial permeability in response to inflammatory mediators such as LPS and thrombin (18, 23). AJs disassemble through a RhoA activation pathway resulting in activation of Rho-induced dependent endothelial contractility (19, 25). However, it has been shown in cultured endothelial monolayers that AJs also possess the intrinsic ability to assemble and thus restore the barrier function via Cdc42- and Rac1-dependent signaling (16, 29). We surmised that if such a GTPase-regulated mechanism of endothelial barrier protection is relevant to the intact microcirculation in instances such as sepsis, activation of Cdc42 in vivo may be useful in preventing vascular barrier dysfunction seen in inflammatory diseases such as sepsis-related acute lung injury. In the present study, we tested the hypothesis that activation of Cdc42 in vascular endothelial cells of mice induces lung vascular endothelial barrier protection and therefore is anti-edematogenic. We generated transgenic mice targeted to express a dominant-active mutant V12Cdc42 protein in vascular endothelial cells specified by the mouse VE-cadherin promoter. These mice exhibited a smaller rise in lung microvascular permeability (measured as microvessel filtration coefficient, Kt,e) and lung water content in response to LPS challenge. We showed that the barrier protection induced by the GTPase-defective V12Cdc42 mutant occurred at the level of interendothelial junctions.

MATERIALS AND METHODS

Mice. Mice developed in this study and experimental protocols were reviewed and approved by the Animal Care Committee at the University of Illinois at Chicago accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

Generation of VE-cadherin promoter driven VEC-V12Cdc42 expressing transgenic mice. The 2.5-kb mouse Cdh5 polyadenylation sequences transferred from pAmpho vector (BD Biosciences), and myc-tagged VE-cadherin promoter. These mice exhibited increased vascular permeability in response to inflammatory mediators such as LPS and thrombin (18, 23). AJs disassemble through a RhoA activation pathway resulting in activation of Rho-induced dependent endothelial contractility (19, 25). However, it has been shown in cultured endothelial monolayers that AJs also possess the intrinsic ability to assemble and thus restore the barrier function via Cdc42- and Rac1-dependent signaling (16, 29). We surmised that if such a GTPase-regulated mechanism of endothelial barrier protection is relevant to the intact microcirculation in instances such as sepsis, activation of Cdc42 in vivo may be useful in preventing vascular barrier dysfunction seen in inflammatory diseases such as sepsis-related acute lung injury. In the present study, we tested the hypothesis that activation of Cdc42 in vascular endothelial cells of mice induces lung vascular endothelial barrier protection and therefore is anti-edematogenic. We generated transgenic mice targeted to express a dominant-active mutant V12Cdc42 protein in vascular endothelial cells specified by the mouse VE-cadherin promoter. These mice exhibited a smaller rise in lung microvascular permeability (measured as microvessel filtration coefficient, Kt,e) and lung water content in response to LPS challenge. We showed that the barrier protection induced by the GTPase-defective V12Cdc42 mutant occurred at the level of interendothelial junctions.

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Generation of VE-cadherin promoter driven VEC-V12Cdc42 expressing transgenic mice. The 2.5-kb mouse Cdh5 promoter was isolated in our laboratory from a mouse BAC library and verified by sequencing (Invitrogen). The myc-tagged V12Cdc42 has been described (4). The transgenic vector comprises mouse VE-cadherin promoter, rabbit β-globin intronic and 3’ polyadenylation sequences transferred from pAmpho vector (BD Biosciences), and myc-tagged human V12Cdc42 mutant coding region (Fig. 1A). The construct was

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verified by sequencing for intactness and expression in endothelial cells (not shown). The vector was linearized by FauI digestion, and the 3.8-kb DNA fragment was microinjected into 1–2-cell stage mouse oocytes and implanted into pseudopregnant CD1 mice. The genomic DNA from litters was analyzed for the presence of the 3.8-kb DNA fragment by Southern analysis. EcoRI digested genomic DNA (10 μg) was used in a reverse transcription reaction and amplified using primers P5 and P6 stringently optimized for human Cdc42 in a PCR reaction vs. mouse endogenous product (Invitrogen). The transgenic vector served as a positive control for the PCR reaction; P5: 5'-CACAAACAGATGTATTTCTAGTC-3'; P6: 5'-CTTCTCTGTGTAAGTGCAGAACACTCC-3'.

Southern analysis. EcoRI digested genomic DNA (10 μg) from heterozygous VEC-V12Cdc42 (TG) or nontransgenic (NTG) mice tails were separated on a 0.9% gel and transferred to Protran nitrocellulose membrane and UV crosslinked. The template for the radiolabeled probe was obtained by using the primers P3 and P4 in a PCR reaction with human Cdc42 cDNA. P3: 5'-GTGCCTGAGATAACTCACCACTGT-3'; P4: 5'-CTTTCTGTGTAAGTGCAGAACACTCC-3'.

RT-PCR. Total RNA was isolated from genotyped 2-mo-old heterozygote or wild-type CD1 mice using TRIzol reagent. No DNase I treated and purified using RNeasy column (Qiagen). The RNA was used in the reverse transcription reaction and amplified using primers P5 and P6 stringently optimized for human Cdc42 in a PCR reaction vs. mouse endogenous product (Invitrogen). The transgenic vector served as a positive control for the PCR reaction; P5: 5'-CACAAACAGATGTATTTCTAGTC-3'; P6: 5'-CTTCTCTGTGTAAGTGCAGAACACTCC-3'.

Transgenic protein expression. Lungs were isolated from TG and NTG mice after PBS perfusion. Extracts were prepared from perfused mice in M-PER extraction reagent and affinity purified on anti-c-Myc antibody-coupled agarose as described in MATERIALS AND METHODS. The eluted proteins were analyzed by Western blotting with c-Myc-tag and Cdc42 antibodies. The myc-tagged Cdc42 protein was detected in TG mouse. The position of molecular markers is shown. All data are representative of at least 5 experiments.
10 mM MgCl₂, 2% IGEPA containing protease inhibitors). Antibodies used were polyclonal rabbit Cdc42, monoclonal RhoA, rabbit polyclonal actin (Santa Cruz Biotechnology), and c-Myc (rabbit polyclonal, Sigma, MO).

Transendothelial electrical resistance measurement. Cells plated on gold microelectrodes were transfected with CMV-mycV12Cdc42 plasmid using Superfect reagent (Qiagen) in a 400-µl volume for 5 h and grown for 36 h in EBM-2 medium. Transendothelial electrical resistance (TER) was monitored in MCDB-131 medium containing 0.1% serum after 50 nM thrombin (Enzyme Research Labs) stimulation using the ECIS system (Applied Biophysics).

Rho GTPase activation assays. PAK1-PBD or Rhotekin-PBD pull-down assays were performed for Cdc42 and RhoA activation studies, respectively. For analysis of Cdc42 activation in mice, lungs from TG and NTG were isolated, homogenized in assay buffer, pooled, and bound to GST-tagged PAK1-PBD or Rhotekin-RBD protein agarose beads (Cytoskeleton) in 1 ml of PBS, intraperitoneally. After 6 h, mice were anesthetized, and the lungs were transferred to the perfusion system for isogravimetric measurement. Basal TER of TG lungs was elevated by 6 cm of water. At the end of the measurement, lung tissue was dissected free of nonpulmonary tissue, and lung dry weight was determined.

Statistics. Differences between groups were examined for statistical significance using the Student’s t-test. Values of P < 0.05 were considered significant.

RESULTS

Characterization of transgenic mice. We identified founder mice incorporating the VEC-V12Cdc42 transgenic DNA fragment after screening genomic DNA in a genotyping PCR with primers P1 and P2 (Fig. 1A). The 422-bp PCR product was detected in the founder mice (TG), but absent in nontransgenic (NTG) CD1 mice, indicating successful mouse genomic integration (Fig. 1B). Southern blot analysis with EcoRI-fractionated genomic DNA (see MATERIALS AND METHODS, Fig. 1C) further confirmed this finding. The probe (Fig. 1A) hybridized to the 2.9-kb and 775-bp fragments, which represent mouse endogenous (chromosome 4) and transgenic Cdc42, respectively (Fig. 1C). The band intensities in blots using P32-radiolabeled Cdc42 probe was indicative of two copies of transgene integration in heterozygous mice.

Transgenic transcript (274-bp product) was detected in a reverse transcription PCR reaction using RNA isolated from vascularized tissues, heart, lung, liver, and kidney (Fig. 1D). Using an aggressive breeding protocol, transmission of transgene to the progeny was observed in a Mendelian ratio. We did not obtain viable homozygous VEC-V12Cdc42+/+ mice from heterozygote breeding. However, VEC-V12Cdc42+/− mice appeared normal and lived as long as NTG littermates and had no gross histological abnormalities of heart, lung, liver, kidney, spleen, and brain, and no obvious defects in vascular development.

Detection of Cdc42 protein in lungs of VEC-V12Cdc42+/− mice was determined by immunofluorescence purification of lung extracts on c-Myc antibody-agarose beads (see MATERIALS AND METHODS). Specific Myc antibody-reactive 23-kDa band was observed only in TG bead eluates, which was absent in the NTG extracts (Fig. 1E). This 23-kDa band was immunoreactive towards a Cdc42-specific antibody.

Cdc42 activity in V12Cdc42-expressing mice impairs the increase in lung microvascular permeability and edema formation induced by LPS. We performed Rho pull-down assays in VEC-V12Cdc42+/− mouse lungs to establish biochemical activity of the dominant active Cdc42 transgene (Fig. 2A). Homogenized lung extracts in assay buffer were bound to GST-PAK1-PBD agarose beads, and Myc-tagged Cdc42-GTP associated with it was analyzed. The capacity to bind GST-PAK1-PBD agarose beads in pull-down assays was used to determine Rho-GTP-bound membrane associated Cdc42, which transitions between GTP- and GDP-bound states. As shown in Fig. 2A, Myc-tag-specific 23-kDa band was observed only in the pooled TG lung extracts. However, Cdc42-GTP and total Cdc42 reactive proteins were not different between NTG and TG lungs (Fig. 2A). The same analysis showed that Rac1-GTP was not different between TG and NTG lungs (Fig. 2A). Thus, VEC-driven Myc-tagged V12Cdc42 transgenic protein was expressed in lungs of VEC-V12Cdc42+/− mice, and it retained its dominant-active function.

To investigate alterations in lung vascular permeability induced by expression of the active Cdc42 mutant in vivo, we used the isogravimetric lung preparation (26). Basal lung capillary filtration coefficient, Kcl, was not different between TG and NTG groups (Fig. 2B). However, lungs of TG mice after 6 h of LPS administration showed significantly smaller increments in lung microvascular permeability compared with their NTG littermates (Fig. 2B). Lungs from TG mice also had reduced wet weight gain slope compared with NTG (Fig. 2C). Thus, VEC-V12Cdc42+/− mice exhibited markedly reduced LPS-induced vascular permeability response indicating the in vivo endothelial barrier protective effect of active Cdc42.

V12Cdc42 expression protects the endothelial junctional barrier. To address the possible basis of the barrier modulation induced by the activated Cdc42, we expressed Myc-tagged V12Cdc42 driven by the cytomegalovirus promoter in endothelial cells (see MATERIALS AND METHODS). In extracts from transfected cells, tagged Cdc42 reactive protein was detected as a 23-kDa protein compared with 21-kDa endogenous Cdc42 (Fig. 3A). The 23-kDa band alone was detected as a Myc-tagged protein. Rho GTPase pull-down assays using GST-PAK-PBD agarose beads identified GTPase-defective V12Cdc42 mutant in its GTP-bound form (Fig. 3A). We measured TER in cells expressing the activated mutant to assess junction formation in response to a standard concentration of thrombin, an agent used to increase endothelial permeability (16). Basal TER of V12Cdc42-expressing HMEC-1 was similar to the empty vector-transfected cells for a period of 36 h after transfection. Thrombin produced a decrease in normalized TER in V12Cdc42-expressing HMEC-1 that was significantly lower than control HMEC-1 (Fig. 3B). However, there was an upward shift in the recovery curve following thrombin challenge in cells transducing V12Cdc42 indicating that expression of active Cdc42 counteracts junction disruption and leads to faster barrier reannealing (Fig. 3B).
Increased Cdc42 activity reduces RhoA activation. To address the possibility that activated Cdc42 interfered with RhoA signaling responsible for increasing endothelial permeability (19, 25), we determined RhoA activity in the V12Cdc42-expressing endothelial cells (Fig. 4A). Cells were lysed with Rho assay buffer (MATERIALS AND METHODS) and bound to GST-rhotekin-RBD agarose beads for 1 h. As shown in Fig. 4B, expression of V12Cdc42 mutant significantly reduced basal RhoA activity as well as thrombin-induced RhoA activation. Rac1 activity was unaffected in these cells (data not shown). Thus, active V12Cdc42 modulation of hyperpermeability may involve countering the permeability-increasing effect of RhoA.

**Fig. 2.** Constitutive Cdc42 activity in transgenic VEC-V12Cdc42 mice markedly reduces the increase in lung vascular permeability induced by LPS. **A:** Cdc42 activity in transgenic VEC-V12Cdc42 mice. Activity of GTPase-defective myc-tagged Cdc42 expressed in mice lungs using GST-PAK-PBD bead pull-down assay. Lung extracts of the TG and NTG mice were bound to the beads for 1 h at 4°C, and the bead bound GTP-Cdc42 and GTP-Rac1 proteins were eluted and analyzed using Western blotting with myc-tag, Cdc42, and Rac1 antibodies. All data are representative of at least 5 experiments. **B** and **C:** Prevention of increased lung microvessel permeability and edema formation in VEC-Cdc42/V12Cdc42 mice. TG mice and NTG littermates were administered intraperitoneal LPS (7 mg/kg) in PBS or PBS alone. **B:** lungs were isolated after 6 h, and lung microvessel filtration coefficient, $K_{fv}$, was determined as described in MATERIALS AND METHODS. *Significantly reduced $K_{fv}$ value was obtained for LPS-challenged TG group ($n = 4$, $P < 0.05$, bars show means ± SE). **C:** representative trace of lung weight gains in TG and NTG groups challenged with LPS. These data are representative of 4 experiments.

**Fig. 3.** Transendothelial electrical resistance (TER) response in myc-V12Cdc42-expressing cells. **A:** expression of V12Cdc42 in endothelial cells. Myc-tagged mutant V12Cdc42 mutant driven by the cytomegalovirus promoter was expressed in HMEC-1. Cell extracts were prepared after 48 h using the assay buffer and bound to GST-PAK-PBD agarose beads. The bound proteins (Cdc42-GTP) and cell extracts (Cdc42, Myc-tag) were analyzed on SDS-PAGE and Western blotting for protein expression with Cdc42 and c-myc-tag antibodies. Arrowheads show position of molecular weight markers. The myc-tagged GTPase-defective mutant was detected as a 23-kDa band compared with the endogenous Cdc42 at 21 kDa. **B:** reduced decrease in TER in V12Cdc42-expressing cells. Endothelial cells plated on gold electrodes were transfected with myc-tagged V12Cdc42 mutant or the empty vector as described in MATERIALS AND METHODS. Endothelial TER was monitored after 50 nM thrombin stimulation using the ECIS system. Recorded values are plotted as the mean from triplicates as means ± SE.
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The hypothesis tested in the present studies stemmed from observations in which expression of V12Cdc42 in endothelial cells was shown to increase the interaction between α- and β-catenin leading to the enhancement of endothelial cell adhesiveness and barrier integrity (4). The present results in mice support earlier studies showing that the activation of Cdc42 occurring after disruption of interendothelial junctions is itself critical in signaling the reannealing of the endothelial barrier (16). Our results provide evidence of the obligatory role of activated Cdc42 in signaling vascular permeability restoration in mouse lung microvessels.

To validate the physiological results of the mouse model, we showed that Myc-tagged active V12Cdc42 GTP-bound protein was in fact expressed. Although these mice exhibited markedly reduced increases in lung vascular permeability and tissue water content after LPS challenge compared with NTG littermates, basal lung microvessel permeability was not different between the transgenic and NTG mice. This finding suggests active Cdc42 “strengthens” the endothelial barrier and thus mitigates the permeability increase induced by LPS rather than altering basal permeability per se. Junctional strengthening may involve activation of Cdc42-regulated signaling pathways that promote junctional barrier restoration (18). These observations with VEC-V12Cdc42 mice are consistent with studies in endothelial monolayers showing that the delayed activation of Cdc42 following stimulation of cells with thrombin was required for reversing the increase in endothelial permeability (16). Our study provides the first evidence in a mouse model of the potential value of targeting Cdc42 to repair the interendothelial junctional barrier in inflammatory diseases such as ARDS.

The 2.5-kb mouse VEC promoter was also used to target the Cdc42 protein expression in the adult vascular endothelium (10). VEC promoter has been used previously to force protein expression in the adult quiescent vasculature and for conditional targeting in mice (2, 17). Interestingly, it was reported that besides embryogenesis, this otherwise quiescent promoter is activated during ischemia-induced neovascularization (14).

We previously showed that destabilizing AJs by expressing a truncated mutant of VE-cadherin lacking the extracellular domains resulted in increased lung vascular permeability in mice (4). Junctional disruption in monolayers expressing this VEC mutant also exhibited increased Cdc42 activity without the involvement of RhoA or Rac1 (4). This resulted in increased lamellapodia formation in endothelial cells expressing the mutated VEC construct (4) suggesting that AJ disruption initiates the signaling responsible for Cdc42 activation. The formation of plasma membrane extensions induced by Cdc42 activation may represent the cellular basis of endothelial barrier reannealing (15). Our current study supports this model, and furthermore, it emphasizes the reparative capability of activated Cdc42 in restoring junctions in vivo.

Epithelial MDCK cells stably expressing the dominant active V12Cdc42 mutant induced E-cadherin adhesion and actin accumulation at the cell contacts independent of Rac1 and also inhibited HGF-induced disruption of the cadherin-catenin complex (13). We observed that HMEC-1 expressing the active V12Cdc42 mutant had significantly reduced thrombin-induced junctional barrier disruption as reflected by smaller decreases in TER (13). Thrombin was used in these endothelial monolayer permeability studies because it is known to disassemble
AJJs resulting in a sharp drop in TER (19). We speculate that the barrier protective effect of active Cdc42 mutant may be due to the observed downregulation of RhoA activity. Although the 2.4-fold RhoA activation was greater in V12Cdc42 expressing cells compared with the 1.6-fold increase in untransfected cells, the maximal thrombin-stimulated RhoA activity was comparable to the basal level observed in untransfected controls (Fig. 4B). Thus, counterregulation of RhoA-mediated hyperpermeability by the active Cdc42 may signal barrier integrity by modulating the lung vascular permeability response observed in VEC-V12Cdc42 mice.

The mechanism of LPS-induced increase in lung vascular endothelial permeability is complex. LPS can directly contribute to endothelial barrier dysfunction through caspase-mediated cleavage of AJ proteins (3). LPS may also activate neutrophils and other cells that mediate junction barrier disruption secondary to release of mediators such as oxidants and proteases (5). We did not observe the prevention of the LPS-induced increase in lung vascular permeability in VEC-V12Cdc42-expressing mice. One possible explanation for this may be a low level expression of active protein in the heterozygous viable mice. It is also likely that there are other endothelial barrier protective pathways besides Cdc42. Similar incomplete protection has been seen using pharmacological agents such as the ROCK inhibitor Y-27632, which blocked the RhoA signaling pathway in a murine model of LPS-induced lung injury (24); thus, there are redundant pathways regulating the increase in endothelial permeability in mice.

Given the lifelong expression of the cell type-specific active Cdc42 protein in transgenic mice, it is conceivable that the observed phenotype is the result of Cdc42-dependent transcriptionally regulated mechanisms (12, 21). Perez-Moreno et al. (20) showed that p120-catenin, an AJ protein, is a negative regulator of RhoA and a positive regulator of Cdc42 and Rac1. Until proven otherwise, it is possible that chronic Cdc42 activity may induce expression of p120-catenin, which would serve to negatively regulate RhoA (a permeability-increasing signal) and positively regulate Cdc42 (a permeability-decreasing signal). This could be another mechanism of the endothelial barrier restoration induced by the Cdc42 activity in our model.

In summary, we used genetically engineered mice expressing the active mutant of RhoGTPase Cdc42 in the vascular endothelium to show that these mice are resistant to LPS-induced increase in lung vascular permeability and edema formation. The barrier protective effect of active Cdc42 occurred at the level of interendothelial junctions. Thus, Cdc42 activation represents a potential therapeutic target in inflammatory diseases such as sepsis-induced acute lung injury.

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