**Caveolin-1–eNOS signaling promotes p190RhoGAP-A nitrination and endothelial permeability**

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Introduction

The endothelium lining all blood vessels plays an essential role in the maintenance of tissue fluid homeostasis (Mehta and Malik, 2006). It finely controls the permeability of the vessel wall barrier to plasma proteins and small solutes via transcellular, caveolae-mediated transcytosis and paracellular, inter-endothelial junctional (IEJ) routes. Whereas IEJs are permeant to molecules with radii of 3 nm or less, caveolae-mediated vesicle trafficking transports different-sized plasma proteins across the vascular barrier (Michel, 1996). The interrelationships between paracellular and transcellular pathways are not well understood. The loss of caveolin-1 (Cav-1) results in not only disappearance of caveolar structures but also destabilization of IEJs and formation of inter-endothelial gaps (Schubert et al., 2002; Miyawaki-Shimizu et al., 2006). We surmised from these previous studies that opening of the paracellular route for albumin transport in Cav-1−/− mice might represent a general mechanism by which Cav-1 influences IEJs and regulates junctional integrity.

In addition to the transport of plasma proteins and lipids, caveolae compartmentalize signaling molecules within lipid raft microdomains (García-Cardeña et al., 1997). Cav-1 interacts with a variety of signaling molecules such as EGF receptor (EGFR), c-Src, phosphoinositide 3-kinase (PI3K), G proteins, and endothelial nitric oxide synthase (eNOS; also known as NOS type 3), and binding of proteins to Cav-1 often leads to their inactivation (Minshall et al., 2002; Mehta and Malik, 2006). Interestingly, eNOS inhibitors were shown to restore the increase in paracellular permeability observed in Cav-1−/− mice (Miyawaki-Shimizu et al., 2006), which suggests that eNOS activity is required for the opening of IEJs. In the present study, we addressed mechanisms by which eNOS-dependent redox
signaling, which is normally held in abeyance by binding of eNOS to Cav-1 (Bucci et al., 2000), regulates IEJ integrity and thereby the permeability of the endothelial barrier.

**Results and discussion**

We first determined basal eNOS activity in murine lung vascular endothelial cells (MLVECs) isolated from wild-type (Wt) and Cav-1−/− mice (Fig. S1). We observed an approximately two-fold increase in eNOS phosphorylation at S1177 (Fig. 1a), the Akt-1 phosphorylation site known to regulate enzymatic activity of eNOS (Dimmeler et al., 1999). Phosphorylation of Akt-1 at S473 was also increased (Fig. 1a), which indicates marked Akt-1 activation in Cav-1−/− endothelium. Basal nitric oxide (NO) generation determined by L-arginine replenishment was fourfold greater in Cav-1−/− MLVECs (Fig. 1b). This finding
is consistent with the reported increased plasma NO concentration in Cav-1–deficient mice (Miyawaki-Shimizu et al., 2006). Nitro-L-arginine (L-NNA) treatment at an inhibitory concentration for nNOS, and eNOS prevented NO generation, whereas treatment with 1400W and N-propyl-L-arginine (NPA) in combination (at inhibitory concentrations for iNOS and nNOS) had a marginal effect (Fig. 1 b). We concluded that Cav-1 deficiency in the endothelium resulted in Akt-1 and eNOS activation and an augmented NO production, which is in agreement with the role of Cav-1 in inhibiting eNOS activity in endothelium (García-Cardeña et al., 1997).

Next we determined the effects of Cav-1 deficiency on adherens junction (AJ) integrity, the primary adhesive complex in the continuous endothelium responsible for regulating junctional permeability. We did not observe any significant change in the expression of AJ proteins in Cav-1–/– MLVECs compared with Wt cells (Fig. S1 b), which is in contrast to the difference reported for the brain endothelial cells (Song et al., 2007). This inconsistency might reflect tissue specificity (i.e., continuous vs. the highly restrictive brain endothelial barrier). Whereas AJs play a primary role in the mechanism of AJ integrity in the continuous endothelium, they are secondary in maintaining brain–blood barrier integrity might reflect tissue specificity (i.e., continuous vs. the highly restrictive brain endothelial barrier). Whereas AJs play a primary role in the mechanism of AJ integrity in the continuous endothelium, they are secondary in maintaining brain–blood barrier integrity.

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When NO is accompanied by \( O_2^- \) generation, it forms peroxynitrite. The diffusion-controlled reaction of NO with \( O_2^- \) is a likely source of peroxynitrite in cells and in vivo (Beckman et al., 1990; Jourd’heuil et al., 2001). The resulting peroxynitrite rapidly decomposes in the presence of ubiquitous \( CO_2 \) to produce nitrate (\( NO_3^- \)) as a stable end product as well as carbonate (\( CO_3^{2-} \)) and nitrogen dioxide (\( NO_2 \)) radicals (Bonini et al., 1999; Augusto et al., 2002), whose concerted action redirects peroxynitrite reactivity toward nitration of protein tyrosine residues (Santos et al., 2000). A direct measurement of peroxynitrite in cells is precluded by the extremely short lifetime of peroxynitrite at physiological pH and by the presence of metals, reductants, and \( CO_2 \). Because peroxynitrite formation depends on both \( O_2^- \) and NO and produces \( NO_3^- \) as the only stable product after oxidant decomposition, the use of superoxide scavengers followed by measurement of nitrite (\( NO_2^- \)) accumulation provides an assessment of peroxynitrite formation (Bonini et al., 2002). Agents such as polyethylene glycol-superoxide dismutase (PEG-SOD) and 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy (TEMPOL) in adequate concentrations outcompete NO for \( O_2^- \), thus favoring NO autoxidation and \( NO_3^- \) accumulation (Hoffman et al., 2003). Here we used TEMPOL/SOD-dependent \( NO_3^- \) accumulation to assess peroxynitrite formation. Both scavengers had marginal effects on accumulation of nitrate resulting from basal NO production in Wt cells (Fig. 2 b); in contrast, accumulation of nitrate was significantly higher in Cav-1–/– cells (Fig. 2 b). Consistent with this observation, Cav-1 deficiency was accompanied by a twofold increase in \( O_2^- \) generation (Fig. 2 c), as measured by formation of 2-hydroxyethidium (EOH), a product of dihydroethidium (DHE) oxidation. Furthermore, adding tetrahydrobiopterin (BH4) to these cells, but not L-arginine, restored \( O_2^- \) generation to the level seen in Wt cells (Fig. 2 c), which suggests that BH4 deprivation-mediated uncoupling eNOS from NO generation is the primary mechanism of \( O_2^- \) generation in the Cav-1–/– endothelial cells. We thus concluded that up-regulation of eNOS activity in Cav-1–/– endothelium results in an augmented generation of both NO and \( O_2^- \) and thereby the formation of peroxynitrite.

We next determined the nitration of AJ proteins by probing immunoprecipitated proteins with anti-nitrotyrosine antibody (3-N). We failed to detect any change in nitration of either \( \beta \)- or \( \gamma \)-catenins in Cav-1–/– endothelial monolayers (Fig. 2 d); however, nitration of p190A was significantly increased (Fig. 2 e).
We also found marked reduction in p190A nitration in Cav-1⁻/⁻ endothelial cells pretreated with TEMPOL and in MLVECs isolated from lung vessels of Cav-1/eNOS double knockout mice (Fig. 2 e). Nitration of p190A also appeared to be reversible because scavenging of O₂⁻• significantly reduced the nitrotrated protein levels, although the p190A expression level itself remained unchanged (Fig. 2 e). We speculate that nitration, which in some cases targets proteins for proteolytic degradation (Grune et al., 1998), does not regulate the metabolism and turnover of p190A. The dogma of irreversible nitration of proteins is under debate, and it is also now increasingly evident that the action of peroxynitrite might be specific (Aulak et al., 2004; Görg et al., 2007).

Nitration compromises protein function by altering tyrosine phosphorylation (Gow et al., 1996). The ability of p190A to hydrolyze RhoA is regulated by c-Src and FAK phosphorylation of p190A (Chang et al., 1995; Holinstat et al., 2006). Therefore, we tested whether nitration alters tyrosine phosphorylation of p190A, which could thereby inhibit its GAP activity. We found a marked decrease in p190A phosphorylation in Cav-1⁻/⁻ MLVECs (Fig. 3 a). We next addressed whether Y₁₁₀₅, the phosphorylation site critical for p190A activity (Roof et al., 1998), might be targeted for nitration. Mutation of Y₁₁₀₅, but not Y₁₀₈₇, to phenylalanine (Y→F) prevented the chemically induced nitration of p190A by 3-morpholinosydnonimine (SIN-1; Fig. 3 b). SIN-1 also induced nitration of endogenous and transiently expressed Wt p190A but not of p190RhoGAP-B (Fig. 3 b).

Down-regulation of p190A activity results in RhoA activation (Vincent and Settleman, 1999), which may increase junctional permeability (Holinstat et al., 2006). We thus determined whether nitration of p190A results in RhoA activation in the Cav-1⁻/⁻ endothelium. For this, we assessed RhoA activity using the FRET-based RhoA biosensor (Pertz et al., 2006). Ratio images revealed a twofold increase in RhoA activity in Cav-1⁻/⁻ monolayers compared with Wt (Fig. 3, c and d). We detected uniform RhoA activity from the edge of the cell inwards with
To demonstrate the causal link between RhoA activity and destabilization of AJs, we attempted to restore integrity of AJs in Cav-1−/− MLVECs by inhibiting RhoA. Treatment of Cav-1−/− endothelium with the Rho inhibitor C3-transferase significantly reduced phosphorylation of myosin light chain (MLC), a very little and infrequent accumulation at cell edges and along extensions (Fig. 3 c), which is consistent with the distribution of active RhoA in other nonmigrating cells (Pertz et al., 2006). Notably, we observed a fourfold increase in RhoA activity by the pull-down of RhoA-GTP with Rhotekin beads (Fig. 3 e).

Figure 3. Nitration of p190A induces RhoA activation in endothelial cells. (a) p190A was co-immunoprecipitated and precipitates were probed for phosphotyrosine (PY20) and p190A; n = 3. Molecular mass standards are indicated next to the gel blots in kilodaltons. (b) Human microvascular endothelial cells (dermal) overexpressing HA-tagged p190A, p190B, or p190A mutants Y1105F and Y1087F were treated with SIN-1. Nitration of endogenous p190A and exogenously expressed proteins was determined with 3-N antibody. Exogenous proteins were co-immunoprecipitated with anti-HA antibody; n = 3. (c) Live imaging of a genetically encoded FRET-based RhoA biosensor. Representative YFP and FRET/CFP ratio confocal images. Pixel intensities of ratio images were scaled from 0 to 5 and color-coded as indicated on the left. Bar, 10 µm. (d) FRET/CFP emission ratio; mean and SEM are as in Fig. 1 e; n = 13; *, P < 0.01. (e) RhoA-GTP was pulled down with Rhotekin-RBD beads. Resultant precipitates and 5% of cell extracts were probed for RhoA; results of two independent experiments are shown; n = 4. Molecular mass standards are indicated next to the gel blots in kilodaltons.
To address the role of Cav-1–eNOS interaction in the mechanism of increased paracellular permeability in vivo, we recorded fluid shift in isolated-perfused murine lungs by the gravimetric method in response to a standard perturbation of the trans-endothelial oncotic pressure gradient \( \Delta \pi \). Elevating perfusate albumin from 0 to 10 g/100 ml caused significantly greater dehydration of lungs of Cav-1\(^{-/-}\) mice than in Wt control (Fig. 4 d), which indicates much faster equilibration of fluid across the endothelium devoid of Cav-1 expression. The lung weight gain (during the 40–50th min of the experiment) was fit to a double exponential function characterizing the fast and slow filtration processes (Table S1). The filtration rate calculated downstream effector of RhoA/ROCK, in Cav-1\(^{-/-}\) cells (Fig. S2).

It also rescued \( \beta \)-catenin accumulation at AJs and restored permeability of Cav-1\(^{-/-}\) monolayers to the Evans blue albumin (EBA) tracer (Fig. 4, a–c). Treatment of Cav-1\(^{-/-}\) endothelium with Cav-1 scaffold domain (CSD)–antennapedia (AP) fusion peptide, which binds to and suppresses eNOS catalytic activity (Bucci et al., 2000), superoxide scavenger TEMPOL, and l-NNA as well as Cav-1/eNOS deficiency (DKO) also restored the integrity of AJs and the permeability of Cav-1\(^{-/-}\) monolayers (Fig. 4, a–c). We concluded therefore that nitration of p190A provides a reversible switch mechanism regulating RhoA activity and thereby endothelial barrier integrity.

Figure 4. Restoration of normal paracellular permeability in Cav-1\(^{-/-}\) endothelial monolayers and vessels by inhibition of either RhoA or eNOS. (a) Immunofluorescent staining of MLVECs isolated from Cav-1\(^{-/-}\) and Cav-1/ eNOS double knockout (DKO) mice for \( \beta \)-catenin (green), F-actin (red), and nuclei (blue). Cav-1\(^{-/-}\) cells were treated with Rho inhibitor C3 transferase and AP-CSD peptide. Bar, 10 µm. (b) \( \beta \)-catenin accumulation at AJs as in Fig. 1 e; *, P < 0.01 as compared with Wt control; **, P < 0.01 as compared with Cav-1\(^{-/-}\); n = 10. (c) Endothelial permeability to EBA; mean and SEM are as in Fig. 1 e; *, P < 0.01 as compared with Wt control; **, P < 0.05 as compared with Cav-1\(^{-/-}\); n = 4. (d) Lung weight changes after a step increase in transvascular oncotic pressure gradient. Recordings were smoothed by averaging successive groups of five points. Lungs isolated from Wt and Cav-1\(^{-/-}\) mice were perfused with 0% BSA for 10 min, with 10% BSA for 30 min, and with 0% BSA for 10 min; an additional Cav-1\(^{-/-}\) group received AP-CSD peptide starting at 10 min of BSA perfusion. AP-CSD peptide reversed lung weight loss during high albumin perfusion in Cav-1\(^{-/-}\) lungs and largely restored the transvascular fluid filtration rate between 40 and 50 min. (e) The filtration rate was calculated from the initial slope of slow exponential component of lung weight gain; mean and SEM are as in Fig. 1 e; *, P < 0.05 as compared with Wt control; n = 5–9.
The NO redox endothelial permeability-increasing mechanism described in this paper may contribute to deregulation of tissue fluid homeostasis during inflammation, which is known to be accompanied by loss of p190A activity and activation of RhoA (Mammoto et al., 2007). Therefore, we determined whether stimulation of endothelial cell monolayers with a pro-inflammatory mediator such as the serine protease thrombin induces p190A nitration. Thrombin proteolytically cleaves and from the initial slope of the slow exponential component was significantly greater in $\text{Cav-1}^{-/-}$ (5.5 ± 0.6 µl/min; $n = 5$) than in Wt (4.1 ± 0.3 µl/min; $n = 9$) lungs (Fig. 4 e). Perfusion with AP-CSD peptide that reanneals open IEJs (Fig. 4 a) restored transvascular fluid filtration in $\text{Cav-1}^{-/-}$ lungs to the Wt values (Fig. 4, d and e). We infer therefore that Cav-1 regulates the permeability of the paracellular or AJ pathways mainly by functioning to restrain the eNOS activity.

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activates the protease-activated receptor PAR-1 on the endothelial surface, leading to increased intracellular calcium concentration and activation of eNOS (Motley et al., 2007). Challenge of human pulmonary artery endothelial cells (HPAECs) with α-thrombin resulted in translocation of eNOS to AJs and a transient interaction between eNOS and p190A and nitration of p190A (Fig. 5, a–d). Nitrated p190A also remained bound to p120-catenin (Fig. 5 d). Thus, we suggest that the signaling mechanism responsible for increased vascular permeability described in this study may be important in inflammatory diseases, and hence we propose it represents a novel anti-inflammatory therapeutic target.

In conclusion, our study demonstrates that paracellular permeability of the endothelium is finely regulated by constitutive Cav-1–dependent inhibition of eNOS activity (Fig. 5 e). Deficiency in Cav-1 or activation of eNOS in conjunction with O2•− production results in nitration of p190A and the concomitant activation of RhoA, which induces AJ destabilization and increased endothelial permeability. Because of the causal relationship of eNOS-specific signaling to the impairment of p190A activity, this mechanism induces RhoA activation in endothelial cells. In fibroblasts, however, Cav-1 depletion induces Src-dependent activation of p190A, resulting in loss of cell polarity and impaired wound healing (Grande-García et al., 2007). In contrast, in endothelial cells, Cav-1 knockdown increases directional migration (Gonzalez et al., 2004); thus, our findings help to inform the unique role of Cav-1 expression on p190A activity in endothelial cells and how Cav-1 regulates the permeability of the endothelial junctional barrier.

Materials and methods

Antibodies and reagents

We used anti-HA (clone 16B12; Covance), anti-phosphotyrosine (PY20), and PECAM-1 (BD); anti–VE-cadherin, anti–α-actin, anti-β, and anti–p120 catenin; anti-RhoA, anti-eNOS, and anti–Cav-1 (Santa Cruz Biotechnology, Inc.); anti-p190RhoGAP (Sigma-Aldrich); anti-nitrotyrosine, anti-CAT, and anti–MLC 2; and phospho-eNOS-Ser 1177, phospho-Akt-Ser 473, and phospho-Crk-Ser 365 (Cell Signaling Technology). Secondary Abs were HRP-conjugated donkey anti–mouse, anti–rabbit, and anti–goat (Jackson ImmunoResearch Laboratories, Inc.); TRITC-, FITC-, and Cy-5-conjugated donkey anti–mouse, anti–goat, and anti–rabbit (Jackson ImmunoResearch Laboratories, Inc.), Alexa Fluor 532 phalloidin, and DAPI (Sigma-Aldrich).

We used protein A/G beads (Santa Cruz Biotechnology, Inc.), DHE, and Dynabeads M-450 (Invitrogen). We also used a Rho activation assay kit and Rho inhibitor C3 transferase (Cytoskeleton), SIN-1 (EMD), NPA (Cayman Chemical), PEG-SOD, TEMPO, 2 µg/ml Rho inhibitor C3 transferase, and 6 µM AP-CEP peptide for 2 h. 70–80% confluent monolayers were transfected using FuGENE HD (Roche) and Lipofectamine 2000 (Invitrogen) transfection reagents in accordance with the manufacturer’s instructions.

Expression constructs, primers, and RT-PCR

HA-tagged rat p190A and p190B, Y1105F, and Y1087F mutants of HA-tagged rat p190A and p190B, Y1105F, and Y1087F mutants of Cav-1 forward, 5′-GCTTCCAACTGAACTGTGAGACCT-3′ and reverse, 5′-GCTTCTCATTCACCTCGTCT-3′. The relative mRNA expression was normalized to the β2 gene (Applied Biosystems).

Cell culture, treatments, and transfection

Cav-1−/− and matching control mice of C57Bl/6 background were obtained from The Jackson Laboratory (JAX mice and services). Cav-1/eNOS double knockout was produced as described previously (Zhao et al., 2009). MVECs were isolated as described previously (Garreau et al., 2006), with some modification. In brief, 3–4-wk-old mice were anesthetized by using isoflurane (2.5% in room air) according to the protocol approved by the University of Illinois Animal Care Committee. Depth of anesthesia was ascertained by loss of pain reflex to a vigorous paw pinch. After exsanguination, the lung was perfused with sterile HBSS supplemented with antibiotics. Lung was removed, minced, and digested with collagenase A (1.0 mg/ml in HBSS) for 60 min at 37°C on shaker. The released cells were centrifuged at 200 g for 10 min. The pellet was suspended in 10 ml of suspension buffer (Ca2+- and Mg2+-free PBS containing 0.5 g/100 ml bovine serum albumin, 2 mM EDTA, and 4.5 mg/ml B-EGF) and filtered through a 200-µm mesh sterile filter. The cell suspension was incubated with 10 µg of anti–mouse PECAM-1 antibody on ice for 1 h, centrifuged to remove unbound antibody, and washed once with suspension buffer. The cells were incubated with Dynabeads M-450 (Sheep anti-rat IgG) for 60 min at 4°C. The cells attached to Dynabeads were trapped in a magnetic column to separate a subpopulation of PECAM-1–positive cells, washed, and resuspended in endothelial growth medium EGM-2 MV medium (Lonza), supplemented with 10% FBS (Invitrogen), and seeded in Matrigel-coated culture dishes (BD).

MVECs and human lung microvascular endothelial cells (HMVECs; Lonza) were grown in EGM-2 MV medium (Lonza) supplemented with 15% FBS (Invitrogen). Endothelial cells were used at passages 2–6. Human microvascular endothelial cell (HMVECs; dermal; Lonza) were grown in MCDB 131 medium (Invitrogen) supplemented with 10% FBS, 0.003 µg/ml h-EGF, 0.001 mg/ml hydrocortisone, and 2-μg/ml sodium nitrite.

For measurement of basal NO generation, cells were incubated in l-arginine–free EBM media (Lonza) for 2 h. Cells were treated with 4 µM NPA, 4 µM 1400W, 4 µM l-NAME, and 100 µM SIN-1 for 30 min; and with 150 µM l-NAME, 100 µM TEMPO, 2 µg/ml Rho inhibitor C3 transferase, and 6 µM AP-CEP peptide for 2 h. 70–80% confluent monolayers were transfected using FuGENE HD (Roche) and Lipofectamine 2000 (Invitrogen) transfection reagents in accordance with the manufacturer’s instructions.

Co-immunoprecipitation and Western blotting

Cells were lysed in modified RIPa buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM Na3VO4, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 0.1 mM PMSF). For co-immunoprecipitation experiments, 350 µg of total protein was incubated with 15 µg of antibody at 4°C, and precipitates were collected with protein A/G agarose beads. Densitometry of blots was performed using ImageJ software (National Institutes of Health).

RhoA-GTP pull-down assay

RhoA activity was determined by pull-down assays using GST-Rhoetkin beads (Knezevic et al., 2007). Cells were lysed in 50 mM Tris–HCl, pH 7.5, 10 mM MgCl2, 0.5 M NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM Na3VO4, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 0.1 mM PMSF. Cell lysates were incubated with GST-RBD beads at 4°C and bound proteins were analyzed by Western blotting. 5% of cell lysates were used to detect total RhoA.

NO measurement

NO formation was measured using porphyrinic NO electrodes (Bromley et al., 1999). Electrodes were calibrated using a stock solution of NO formation was measured using porphyrinic NO electrodes (Brovkovych et al., 2007). The electrode potential. The system was coupled to a FAS1 femtostat and a personal computer with electrochemical software (Gamry Instruments). The electrode potential was ascertained by loss of pain reflex to a vigorous paw pinch. After exsanguination, the lung was perfused with sterile HBSS supplemented with antibiotics. Lung was removed, minced, and digested with collagenase A (1.0 mg/ml in HBSS) for 60 min at 37°C on shaker. The released cells were centrifuged at 200 g for 10 min. The pellet was suspended in 10 ml of suspension buffer (Ca2+- and Mg2+-free PBS containing 0.5 g/100 ml bovine serum albumin, 2 mM EDTA, and 4.5 mg/ml B-EGF) and filtered through a 200-µm mesh sterile filter. The cell suspension was incubated with 10 µg of anti–mouse PECAM-1 antibody on ice for 1 h, centrifuged to remove unbound antibody, and washed once with suspension buffer. The cells were incubated with Dynabeads M-450 (Sheep anti-rat IgG) for 60 min at 4°C. The cells attached to Dynabeads were trapped in a magnetic column to separate a subpopulation of PECAM-1–positive cells, washed, and resuspended in endothelial growth medium EGM-2 MV medium (Lonza), supplemented with 10% FBS (Invitrogen), and seeded in Matrigel-coated culture dishes (BD).

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Measurement of intracellular superoxide

Cells plated in a 96-well plate were left untreated or incubated with 50 µM BH4 or 500 µM L-arginine for 30 min, then treated with 50 µM DHE for 1 h. Emission of EOH, a product of DHE oxidation, at λ = 580 nm, was measured using a SpectraMax M5e Multi-Mode Microplate Reader ( Molecular Devices).

SNO measurement

SNO content of junctional proteins was detected by biotin-switch method using the S-Nitrosylated Detection Assay kit (Cayman Chemical) according to the manufacturer’s instructions. The biotin-labeled proteins were precipitated with iodoacetamide, and 350 µg of total protein was used for co-immunoprecipitation with specific Abs. SNO content was detected by SNO detection reagent I on Western blots. The same blot was reprobed for protein of interest.

Immunofluorescence staining and image analysis

Cell were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and stained as described previously (Komarova et al., 2002). Samples were mounted with Prolong-Gold antifade reagent (Invitrogen). Z-stack images were obtained using a confocal microscope (LSM 510 Meta; Carl Zeiss) equipped with a 63×, 1.2 NA water immersion objective lens and Ar ion and dual HeNe lasers, and driven by LSM software. 12-bit images were analyzed using MetaMorph software (Molecular Devices) and prepared for illustration in Photoshop (Adobe). Projection images were generated by collecting the maximum pixel intensity from each image of the z stack and by projecting pixel intensity onto the single (projection) image. The relative accumulation of β-catenin (mean fluorescence intensity) at AJs and the area of gaps were measured on projected images. The 12-bit images were thresholded by subtracting intracellular background, and the mean fluorescence intensity at AJs was measured. For measurement of en-NOS accumulation at AJs, the corresponding VE-cadherin image was used to generate a binary mask within 0 (outside of AJs) and 1 (AJs area) values after subtracting intracellular background, and the eNOS image was multiplied by a binary mask image for VE-cadherin. The mean fluorescence intensity of en-NOS at AJs was then measured as described for β-catenin. Gap area was expressed as a percentage of the area outside of the cell.

Live cell imaging and image processing

Cells were imaged in phenol-free EBMT media (Lonza) supplemented with 5% FBS at 37°C maintained by the stage heater (Tempcontrol-37; Carl Zeiss). Z-stack images were obtained using a confocal microscope (LSM 510 Meta; Carl Zeiss) equipped with a 63×, 1.2 NA water immersion objective lens and Ar ion and dual HeNe lasers. For emission ratio imaging, we acquired 12-bit CFP (λ = 458 nm; BP500/20), FRET (λ = 458 nm; LP530), and YFP (λ = 514 nm; LP530) images. All images were processed and analyzed using MetaMorph Software. The FRET image was used to generate a binary mask within 0–1 values. To generate a ratio image reflecting RhoA activity, the FRET image was first multiplied by a binary mask image and then divided by the CFP image. The ratio images were rescaled to the lower value and a linear pseudocolor table was applied to generate the color-coded image map. The z stack FRET/CFP emission ratio images were used to generate the projection image. The relative RhoA activity was expressed as mean pixel intensity.

Endothelial barrier permeability measurement

The permeability of MVECs monolayer to EBA was determined as described previously (Patterson et al., 1992). Cells were grown to confluence on transwell inserts with a 0.4 µm pore size. After a 30-min equilibration period, the luminal chamber was loaded with 0.057% EBA. Samples from the abluminal chamber were collected every 5 min for 1 h. EBA concentration was calculated from the optical density measured at 620 nm with the aid of the SpectraMax Plus microplate spectrophotometer ( Molecular Devices). The rate of EBA clearance from luminal chamber was determined by linear regression between 5 and 60 min.

Perfusion of mouse lungs and measurement of transvascular fluid filtration

Murine lung preparations from WT and Cav-1+/- male mice (C57Bl/6 strain) were performed according to an approved protocol of the University of Illinois at Chicago Animal Care Committee (Vogel et al., 2006). In brief, isolated lung preparations were perfused with RPMI medium at constant flow (2 ml/min), temperature (37°C), and venous pressure (4 cm H2O).

The preparation was ventilated at a rate of 120 breaths per min, at constant peak inspiratory (~10 cm H2O) and end expiratory pressures (2 cm H2O). The lung weight change was recorded by a force-displacement transducer (Model FT03C; Grass Technologies). All lung preparations underwent a 10-min equilibration perfusion to establish isogravimetric conditions followed by a 30-min step change in perfusate albumin concentration (g/100 ml) of albumin from 0 to 10. In some cases, AP-CSD (final concentration of 10 µM) was added into the high-albumin perfusate between minute 20 and 40 from the beginning of perfusion. The fluid filtration rate was obtained by fitting a double exponential growth curve, \( y = a(1 - e^{-kt}) + b(1 - e^{-kt}) \), to the lung weight gain upon removal of albumin. The fast exponential component, which was of relatively low amplitude and coincident with the change of solution, most likely represented the effect of the shift in solution viscosity. The slow exponential component represented fluid filtration across the vessel wall induced by the change in oncotic pressure. The filtration rate was determined from the initial slope of this component extrapolated to zero time.

Statistical analysis

Data handling was performed using Sigma Plot software (SPSS). For statistical analysis, a paired two-tailed Student’s t-test was used to compare data between two groups.

Online supplemental material

Fig. S1 provides a characterization of primary MVECs. Fig. S2 shows the effect of a Rho inhibitor and phospho-Ab. Am. J. Physiol. 274:10802–10806. doi:10.1152/ajpheart.00743.2003

References


