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Co-Regulation of Transcellular and Paracellular Leak Across Microvascular Endothelium by Dynamin and Rac

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Increased permeability of the microvascular endothelium to fluids and proteins is the hallmark of inflammatory conditions such as sepsis. Leakage can occur between (paracellular) or through (transcytosis) endothelial cells, yet little is known about whether these pathways are linked. Understanding the regulation of microvascular permeability is essential for the identification of novel therapies to combat inflammation. We investigated whether transcytosis and paracellular leakage are co-regulated. Using molecular and pharmacologic approaches, we inhibited transcytosis of albumin in primary human microvascular endothelium and measured paracellular permeability. Blockade of transcytosis induced a rapid increase in paracellular leakage that was not explained by decreases in caveolin-1 or increases in activity of nitric oxide synthase. The effect required caveolin-1 but was not due to the general inhibition of endocytosis. Inhibiting transcytosis by dynamin blockade increased paracellular leakage concomitantly with the loss of cortical actin from the plasma membrane and the displacement of active Rac from the plasmalemma. Importantly, inhibition of paracellular leakage by sphingosine-1-phosphate, which activates Rac and induces cortical actin, caused a significant increase in transcytosis of albumin *in vitro* and in an *ex vivo* whole-lung model. In addition, dominant-negative Rac significantly diminished albumin uptake by endothe-

lia. Our findings indicate that transcytosis and paracellular permeability are co-regulated through a signaling pathway linking dynamin, Rac, and actin. (Am J Pathol 2012, 180: 1308–1323; DOI: 10.1016/j.ajpath.2011.12.002)

One of the critical functions of the endothelium is the maintenance of an intact barrier between the vascular lumen and the interstitium. Numerous inflammatory diseases, including acute lung injury, sepsis, and anaphylaxis, are characterized by prolonged and excessive microvascular leak.^{1–3} In such settings, the exodus of proteins and fluids has deleterious consequences,^{4,5} including the loss of serum albumin and oncotic pressure⁶ and culminating in the development of hypotension and tissue edema. There is great interest, therefore, in developing novel agents that decrease microvascular leak. However, for this to be successful, understanding the mechanisms and regulation of microvascular permeability is essential.³

Inflammatory mediators can induce vascular leak by opening gaps in endothelial cells, a process known as paracellular leak.^{7–9} Although the mechanisms of gap formation depend in part on the inflammatory stimulus, they can be broadly divided into three overlapping

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categories: i) injury and/or apoptosis of cells in the endothelial monolayer,^{10,11} ii) internalization or disassembly of intercellular junctions,^{12,13} and iii) remodeling of the actin cytoskeleton, leading to a change in cell shape. Cytoskeletal remodeling is regulated by members of the Rho GTPase family, which recruit downstream effectors when bound to GTP. For instance, the formation of the cortical actin fibers that normally buttress the plasmalemma is dependent on Rac,^{14,15} whereas the generation and contraction of cytoplasmic actinomyosin stress fibers require RhoA.^{16,17}

Although paracellular leak is more widely studied, leakage of plasma and proteins can also occur through the cytoplasm of endothelial cells by transcytosis.^{18,19} In fact, transcytosis is a physiologically important process that accounts for much of albumin transit across the resting endothelium.²⁰ This involves the traffic of caveolae, small intracellular vesicles that are often docked to the plasmalemma, and begins with binding of albumin by multiple receptors on the apical endothelial surface.^{19,21,22} This leads to the invagination of caveolae from the plasma membrane. The GTPase dynamin-2²³ forms a ring around the neck of the invaginating vesicles and mediates their scission from the plasmalemma.²⁴ The vesicles then move into the cell, fuse with the basolateral membrane, and release albumin to the interstitial space. Accordingly, caveolae contain SNARE proteins and the hexameric ATPase N-ethylmaleimide (NEM)-sensitive factor, both of which are required for the fusion of vesicles to the plasmalemma.¹⁹ In contrast to paracellular leak, nothing is known about the relationship between Rac and RhoA and transcytosis. In addition to albumin, transcytosis occurs for immunoglobulins, transferrin, aminopeptidase P,²⁵ and numerous other molecules.¹⁸

Although both paracellular and transcellular leak were described decades ago, remarkably little is known about the relationship between them. Endothelial cells from mice deficient in caveolin-1 lack caveolae and do not perform transcytosis.²⁶ The vasculature from these animals demonstrates an increase in permeability, although the mechanism of this change is controversial. Although some have described a compensatory induction of paracellular leak,²⁶ others attribute the effect to a change in capillary pressure or the endothelial glycocalyx.²⁷ Furthermore, caveolin-1 has numerous functions in cell signaling and growth,²⁸ making it impossible to know whether the increase in permeability is due to the loss of transcytosis per se or to the loss of caveolin-1. Thus, how paracellular and transcellular leak are related remains largely unexplored. We hypothesized that, independent of caveolin-1, inhibition of transcytosis would lead to a compensatory increase in paracellular leak. To address this issue, we first devised methods of measuring transcytosis of albumin by primary human microvascular endothelium in culture. We then inhibited transcytosis by multiple distinct molecular and pharmacologic approaches and determined the effect on paracellular leak. Reciprocally, we inhibited paracellular leak and monitored for changes in transcytosis both *in vitro* and using

an *ex vivo* lung model. Lastly, we determined the molecular mechanism for this relationship.

Materials and Methods

Cell Culture

Primary human microvascular endothelial cells of dermal origin were obtained from discarded human foreskin as previously described²⁹ and were used throughout the study. Primary human microvascular endothelial cells of pulmonary origin were obtained from Lonza (Basel, Switzerland) and were used to confirm the results of key transwell experiments, as indicated below. Cells were cultured in EBM-2 media with the recommended supplements (Lonza) and were used in passages 3 to 8.

Transwell Assays

Endothelial cells were seeded on gelatin-coated 0.4- μ m-pore polyester transwells (Costar, Corning, NY) and grown until confluency 1 to 3 days later. Only monolayers that appeared healthy by phase-contrast microscopy and had a transendothelial electrical resistance (TEER; measured using Endohm-12 from World Precision Instruments, Sarasota, FL) of $>19 \Omega\text{cm}^2$ were used for permeability experiments. Replacement of the TEER machine led to higher TEER values in some experiments, but permeability to dextran remained unchanged.

To measure transcytosis, endothelial monolayers were allowed to bind but not internalize fluorescein isothiocyanate (FITC)-conjugated albumin (Invitrogen, Eugene, OR; 0.4 mg/mL) for 10 minutes at 4°C. Unbound albumin was removed by washing the cells with cold media twice. Transcytosis was allowed to proceed by incubating the cells at 37°C for 2 hours. As controls, 80 $\mu\text{mol/L}$ Dynasore (Sigma-Aldrich, St Louis, MO) or 4 mmol/L histamine (Sigma-Aldrich) were added for the incubations at 37°C. Aliquots of the lower and upper chambers were then taken and measured for fluorescence in a Spectramax Gemini EM (Molecular Devices, Sunnyvale, CA) using the appropriate filter sets and after subtraction of appropriate blanks.

To measure transcytosis by a second method, serum-free media containing 0.3 mg/mL of biotin-tagged albumin (biotin-BSA; Thermo Scientific, Rockford, IL) and 50 $\mu\text{g/mL}$ of FITC-conjugated dextran (Invitrogen; molecular weight, 70 kDa) were added to the upper chamber of the transwell, whereas serum-free media alone was added to the lower chamber. After 4 hours of incubation, aliquots from the lower chamber were taken for measurement of biotin-BSA by enzyme-linked immunosorbent assay (ELISA) (see below). As a control for paracellular leak, aliquots were also taken for measurement of FITC-dextran fluorescence intensity in a Spectramax Gemini EM (Molecular Devices) using the appropriate filter sets and after subtraction of appropriate blanks.

For the ELISA, 100 μL of media from the lower chamber and 50 μL of 0.5 $\mu\text{g/mL}$ of horseradish peroxidase-

conjugated sheep anti-BSA (Immunology Consultants Laboratory, Inc., Newberg, OR) were incubated in 96-well plates coated with streptavidin (Thermo Scientific) at room temperature for 2 hours with shaking. After washing with buffer (25 mmol/L Tris, pH 7.2, 150 mmol/L NaCl, 0.05% Tween-20, 0.1% gelatin), 50 μ L of 3,3',5,5'-tetramethylbenzidine (Thermo Scientific) was added to the well and incubated at room temperature for 15 minutes before adding 50 μ L of 2 mol/L sulfuric acid. Quantification of BSA was determined by measuring absorbance at 450 nm with a ThermoMax Microplate Reader (Molecular Devices) after verification with a standard curve using known amounts of biotin-BSA.

We measured paracellular permeability using two methods and in both dermal and pulmonary microvascular endothelium. After measurement of the baseline TEER, cells were exposed to 0.1 mmol/L NEM (Sigma) for 2 minutes followed by repeat measurement. Permeability of NEM-treated monolayers was also measured by the addition of FITC-dextran (50 μ g/mL; molecular weight, 70 kDa) to the upper chamber of the transwell with removal of aliquots of media from both the upper and lower chambers 40 minutes later. Using appropriate filter sets, we measured the fluorescence in aliquots of the lower and upper chambers after subtraction of the appropriate blanks. Permeability was calculated as $P = [(\Delta C_b / \Delta t) \times V_b] / (C_a \times A)$, where $\Delta C_b / \Delta t$ is the change in the basolateral concentration during the duration of the assay, V_b is the volume of the basolateral chamber, C_a is the apical concentration, and A is the area of the transwell.³⁰ Changes in V_b and C_a were negligible during the experiment, and C_b was much lower than C_a .

In other experiments, monolayers were incubated with 80 μ mol/L Dynasore [or dimethyl sulfoxide (DMSO) as a solvent control] for 40 minutes followed by repeat measurement of the TEER. Other cells were incubated with Dynasore or DMSO, and 50 μ g/mL of FITC-dextran was added simultaneously to the upper chamber of the transwell. Forty minutes later, flux of dextran into the lower chamber was calculated as explained above. To determine the effect of cooling to $<15^\circ\text{C}$, cells were incubated at 14°C or 37°C for 40 minutes followed by repeat measurement of TEER. Alternatively, FITC-dextran was added to the upper chamber immediately before incubation at 14°C or 37°C and flux was calculated 40 minutes later, as described above. In some experiments, cells were pretreated with the nitric oxide synthase inhibitor nitroglucine-nitro-L-arginine methyl ester (L-NAME) (Sigma; 100 μ mol/L) for 30 minutes before inhibition of transcytosis.

Permeability to dextran was also tested under 10 cm H_2O of pressure. A column was added to the upper chamber of the transwells and was filled with 10 cm of media. Transwells were acclimatized to the new pressure during 5 hours,³⁰ then the media in the column was replaced with media (also 10 cm in height) containing dextran and Dynasore or DMSO, and dextran flux was measured as described above with 80 μ mol/L Dynasore or DMSO control.

Electron Microscopy

Transcytosis experiments were performed as described above, using gold-tagged albumin (6 nm, Electron Microscopy Sciences, Hatfield, PA). Fifteen minutes after incubation at 37°C , cells on their transwells were fixed in 2% glutaraldehyde, after which the cells were washed four times with 0.1 mol/L sodium cacodylate buffer. After the wash, the membranes were cut out of the transwell inserts. The cells were then fixed for 45 minutes in 1% osmium tetroxide and 1% potassium ferrocyanide in cacodylate buffer. The samples were washed three times in buffer followed by twice in distilled water. The cells were stained for 30 minutes in 4% aqueous uranyl acetate then washed twice in distilled water. They were dehydrated through a graded series of ethanol and infiltrated and embedded in Epon (JEMBED 812). Sections were cut at 80 nm, picked up on copper grids, and stained with uranyl acetate and lead citrate. Sections were examined using an FEI Tecnai 20 transmission electron microscope. Images were recorded with an AMT 16000 digital camera.

Western Blot Analysis

Lysates were analyzed by SDS-PAGE using 6% to 12% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes, blocked for 1 hour, and probed overnight with the primary antibody at 4°C . After washing in PBS with Tween 20, blots were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour, washed, and then visualized by enhanced chemiluminescence (Amersham). For quantitation, blots were scanned using Image J (National Institutes of Health), and the integrated intensity was normalized to the amount of protein loaded after correction for background.

Microscopy and Immunofluorescence

Analysis of the distribution and density of caveolin-1, F-actin, vascular endothelial cadherin (VE-cadherin), claudin-5, heparan sulfate, and internalized albumin was performed using spinning disk confocal microscopy (Zeiss Axiovert 200M microscope with $25\times$ or $63\times$ objectives; Zeiss, Jena, Germany). The unit is equipped with diode-pumped, solid-state laser lines (440, 491, 561, 638, and 655 nm; Spectral Applied Research, Richmond Hill, ON), motorized XY stage (ASI), and a Zeiss focus drive. Images were acquired using back-thinned, electron-multiplied or conventional cooled, charge-couple device cameras (Hamamatsu, Shizuoka, Japan), driven by Volocity 4.1.1 software (Improvision, Coventry, England). In all cases, microscope settings were kept constant between conditions. All images were randomly chosen and were acquired as Z-stack projections (Z-interval, 0.2 to 0.5 μm).

Total internal reflectance fluorescence (TIRF) images were acquired on an Olympus cell[^]TIRF Motorized Multicolor TIRF module mounted on an Olympus IX81 microscope (Olympus, Hamburg, Germany). Samples were imaged using a $63\times/1.49$ objective with 491-nm excita-

tion lasers and MetaMorph acquisition software. Retention of the yellow fluorescent protein-tagged p21-binding domain (PBD-YFP) was determined by the ratio of lamellipodia or filopodia that still exhibited the PBD-YFP at 30 minutes compared with time 0.

To immunostain for F-actin, VE-cadherin, and heparan sulfate, cells were rinsed in PBS, fixed for 30 to 60 minutes in 4% paraformaldehyde in PBS, incubated in 0.15% glycine for 10 minutes, permeabilized in 0.1% Triton X-100 for 20 minutes, and blocked for 1 hour. Cells were stained with Alexa Fluor 488 phalloidin (Molecular Probes), anti-VE-cadherin (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-heparan sulfate (US Biological, Marblehead, MA) for 1 hour. For caveolin-1 and claudin-5, cells were fixed in methanol then put in blocking buffer for 1 hour at room temperature. The primary antibodies for caveolin-1 (Santa Cruz Biotechnology) and claudin-5 (Abcam, Cambridge, MA) were added for 1 hour at room temperature. Heparinase III treatment (0.2 U/mL for 1 hour) was used as a negative control for heparan sulfate.

In some experiments, the internalization of membrane-bound albumin was compared under different conditions. Briefly, fluorophore-tagged albumin was added to cells in cold media and spun down at $16 \times g$ for 10 seconds; then the cells were incubated at 4°C for 10 minutes. Afterward, cells were rinsed with cold media and incubated at 37°C for 15 minutes. Cells were then fixed and processed as above.

As a control for endocytosis, a lipophilic styryl dye, *N*-(3-triethylammoniumpropyl)-4-(*p*-diethylaminophenyl)-hexatrienyl pyridinium dibromide (FM 4-64, Invitrogen),³¹ was added to endothelial cells at a concentration of 5 μ g/mL for 1 hour. Cells were then rinsed, fixed, and processed as above.

Membrane Fraction Preparation

Subcellular fractionation was performed as previously described.³² Briefly, dermal endothelial cells were grown to confluency in 10 cm dishes. Cells were rinsed with PBS then scraped into PBS and centrifuged at $400 \times g$ for 5 minutes to pellet the cells. The pellet was resuspended in hypotonic lysis buffer containing 10 mmol/L Tris-HCl, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, protease inhibitor, and 1 mmol/L DTT. Cells were further lysed using a Dounce Homogenizer (Wheaton, Millville, NJ) with 20 strokes. The mixture was centrifuged at $400 \times g$ for 5 minutes to pellet cell debris, and the supernatant was collected and subjected to repeat centrifugation at $16,000 \times g$ for 30 minutes. The pellet was resuspended in radioimmunoprecipitation assay buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% NP-40, 0.5% deoxycholic acid, and 0.1% SDS) and incubated on ice for 30 minutes before analysis with Western blotting. Preparations were probed for the transferrin receptor as a positive control.

Lipid Raft Isolation and Quantitation

Dermal endothelial cells were grown to confluency in 6-cm dishes. After washing with PBS, cells were scraped

and precipitated by centrifugation. Cells were lysed in 140 μ L of lysis buffer containing 25 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 10% sucrose, 1% Triton X-100, 1 mmol/L dithiothreitol, and protease inhibitor cocktail (R) for 30 minutes on ice. The lysate was then added to 0.66 mL of 60% OptiPrep (Axis-Shield, Oslo, Norway). To ensure complete lysis, cells were homogenized in an ice-cold Dounce Homogenizer (Wheaton) with 10 strokes. Cell lysates were then placed in an ultracentrifuge and overlaid with 0.8 mL of 40%, 30%, and 20% OptiPrep. The gradients were centrifuged at $28,000 \times g$ at 4°C for 15 hours using an SW 55 Ti Rotor. Fractions were collected and analyzed by Western blot. Mouse anti-transferrin receptor antibody was from Invitrogen. Data were quantitated using ImageJ. After subtraction of background, the amount of caveolin-1 in the 20% fraction was divided by the total amount of caveolin-1.³³

Toxicity Assays

Endothelial cell death was detected using trypan blue by enumerating the number of stained cells in 10 random fields ($\times 20$ magnification). Apoptosis of endothelial cells was determined by staining with Annexin V-FITC (EBioscience, San Diego, CA) as per the manufacturer's instructions and analyzed by flow cytometry using a BD FACS Calibur cytometer (Becton Dickinson) and then imported into FlowJo 8.8.6 (TreeStar Inc., Ashland, OR).

Transfection and siRNA

For most experiments, primary human endothelial cells grown on coverslips or transwells were transfected with plasmids using Fugene HD (Roche) in accordance with the manufacturer's instructions and were used for experiments 24 hours later. For transfection of Rac constructs for cells on transwells, we used the Electro Square Porator according to the manufacturer's protocol (protocol 0394). The plasmids containing wild-type (WT) Rac, dominant-negative (DN) (T17N) Rac, dynamin-2 WT, K44A, and PBD-YFP were gifts from Sergio Grinstein and have been previously described.^{34,35} Dynamin-2 WT or K44A were co-transfected with green fluorescent protein (GFP) in a ratio of 9:1, and transfected cells were immunostained for dynamin to confirm that GFP-expressing cells also overexpressed dynamin-2. Clathrin heavy chain small interfering RNA (siRNA) (catalog no. SI00299880), caveolin-1 siRNA (FlexiTube Gene solution for catalog no. G5857), and the nontargeting siRNA control (catalog no. 1027280) were from Qiagen (Germantown, MD). Cells were transfected with siRNA using HiPerFect (Qiagen) in accordance with the manufacturer's instructions, subcultured to a transwell, and used in experiments 72 hours after transfection. Confirmation of knockdown by Western blotting was performed by making cell lysates directly from the transwells after the permeability experiments. Goat anti-clathrin heavy chain, rabbit anti-caveolin-1, mouse anti- β -actin, and horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology.

Fluorescence Imaging in Isolated Perfused Mouse Lungs

All experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, seventh edition, 1996) and were approved by the Animal Care and Use Committee of the local government authorities. Real-time *in situ* fluorescence imaging in isolated perfused lungs of male C57BL/6 mice (25 ± 1 g of body weight; Charles River Laboratories, Pointe-Claire, Quebec) was performed as previously described.³⁶ Briefly, isolated mouse lungs were constantly inflated with a gas mixture of 21% O₂, 5% CO₂, and balance N₂ at a positive airway pressure of 3 cm H₂O. Lungs were perfused with Krebs-Henseleit buffer containing 3% bovine serum albumin at 37°C and a constant perfusion rate of 1 mL/min. Membrane-permeant fura-2 AM (5 μ M; Invitrogen), FITC-conjugated albumin (1 mg/mL), and tetramethylrhodamine isothiocyanate (TRITC) dextran (molecular weight, approximately 70,000; 100 μ g/mL) (both from Sigma-Aldrich) were added to the perfusate and allowed to recirculate for 60 minutes in the presence or absence of

either sphingosine-1-phosphate (S1P; 1 μ M; Cayman, Ann Arbor, MI) or platelet-activating factor (50 nmol/L; Sigma-Aldrich). After 60 minutes, residual intravascular fluorescent dyes were washed out by lung perfusion with Krebs-Henseleit buffer.

Isolated perfused lungs were positioned on a custom-built, vibration-free, motorized XY stage under an upright intravital microscope (Axiotech^{Vario} 100 HD; Zeiss). The fluorescence of fura-2, FITC, and TRITC in the vascular wall and surrounding parenchyma of the lung venular capillaries was excited by monochromatic illumination (Polychrome IV; T.I.L.L. Photonics, Martinsried, Germany) at $\lambda = 360$ nm, 470 nm and 535 nm, respectively. Fluorescence was collected through an achromat objective (UAPO 40 $\times$ W2/340; Olympus) and appropriate dichroic and emission filters (FT 425 and BP 505-530 for fura-2, DCLP 500 and LP 515 for FITC, and DCLP 530 and BP 535-585 for TRITC; all Zeiss) by a CCD camera (Sensicam; PCO, Kehlheim, Germany) and subjected to digital image analysis (TILLvision 4.01; T.I.L.L. Photonics). In each lung, 10 to 15 single venular capillaries were viewed at a focal plane corresponding to their maximum diameter (16 to 32 μ m). Images of fura-2, FITC, and

Figure 1. Human microvascular endothelium performs transcytosis in culture. **A:** Endothelium was incubated with FITC-conjugated albumin with solvent control (DMSO) (left) or 80 μ M/L Dynasore (right). Nuclei were stained with DAPI. Internalization of albumin (arrow) is inhibited by Dynasore. Images are Z-stack projections. Scale bar = 16 μ m. Transcytosis assay was performed as described in *Materials and Methods*. Cells were treated with DMSO (control) or Dynasore ($n = 7$) (**B**) or histamine ($n = 6$) (**C**) ($*P < 0.05$). Mean $\pm$ SD (membrane-bound) albumin flux was $6.95 \pm 2.43 \times 10^{-6}$ cm/s for control and $1.72 \pm 1.13 \times 10^{-6}$ cm/s for Dynasore-treated monolayers. **D:** ELISA was performed using human pulmonary microvascular endothelium as described in *Materials and Methods*. Dextran is used as a measure of paracellular leak. The graph is representative of three independent experiments (x-axis is cell number; $*P < 0.05$ for all comparisons indicated). Mean $\pm$ SD dextran flux was $2.06 \pm 0.07 \times 10^{-6}$ cm/s, $0.89 \pm 0.19 \times 10^{-6}$ cm/s, and $0.65 \pm 0.20 \times 10^{-6}$ cm/s for transwells seeded with 50,000, 100,000, and 150,000 cells, respectively. Mean biotin-conjugated albumin flux was $1.36 \pm 0.79 \times 10^{-7}$ cm/s, $1.47 \pm 0.82 \times 10^{-7}$ cm/s, and $1.65 \pm 0.92 \times 10^{-7}$ cm/s for transwells seeded with 50,000, 100,000, and 150,000 cells, respectively. **E:** Endothelium grown on transwells was incubated with 6-nm gold-tagged albumin (Au-Alb) with or without Dynasore. **Left:** Arrow denotes caveola containing Au-Alb near the apical surface of a cell. **Center:** Caveola (arrow) containing Au-Alb that is unable to detach from the apical membrane of the cell due to Dynasore. **Right:** Caveola containing Au-Alb abutting the basolateral surface of control endothelial cells. Scale bar = 100 nm.

TRITC fluorescence were recorded in random sequence, and the mean ratio of FITC to fura2 and TRITC to fura2 fluorescence within the field of observation was calculated.

Rac Activation Assay

Rac activation was measured using the NewEast Biosciences (Malvern, PA) Rac Activation Kit according to the manufacturer's instructions. Briefly, dermal endothelial cells were grown to confluency on 10-cm dishes. After appropriate experimental treatment, cells were washed with PBS, scraped into lysis buffer, kept on ice for 15 minutes, and then centrifuged. Supernatants were collected and analyzed by the Active Rac Pull-Down Assay. Samples containing GTP γ S and GDP were used as positive and negative controls, respectively.

Data and Statistical Analysis

Unless otherwise indicated, all experiments were performed three times and data are expressed as mean and SE. Student's *t*-tests and analyses of variance were used as appropriate. *P* < 0.05 was considered significant.

Results

Primary Human Microvascular Endothelial Cells Perform Transcytosis

We first devised assays to confirm that primary human microvascular endothelial cells grown in culture perform transcytosis.²² Endothelium grown on transwell filters was incubated with fluorescein-conjugated albumin in the upper chamber, and the transit of albumin to the

lower chamber was quantified using a spectrofluorometer. To exclude paracellular leak, we limited our analysis to the transit of *membrane-bound* albumin²¹ from the apical to the basolateral surface because this would eliminate passive diffusion of albumin across intercellular gaps. As a control, transcytosis was blocked by the cell-permeable small-molecule inhibitor of dynamin, Dynasore,³⁷ which prevents scission of caveolae from the apical plasma membrane. Dynasore is membrane permeable and has been shown to specifically inhibit dynamin with a reported half maximal inhibitory concentration of 15 μ mol/L. In initial experiments, we confirmed that Dynasore greatly attenuates the internalization of albumin by endothelial cells (Figure 1A).

As an additional control, we incubated cells with histamine after allowing them to bind albumin because histamine rapidly increases paracellular leak of endothelial monolayers by forming gaps between cells.⁸ We hypothesized that histamine should *not* increase the flux of albumin across the transwell if our assay was only measuring transcytosis. Indeed, our experiments demonstrate that cultured primary human microvascular endothelium performs transcytosis of albumin; as expected, inhibition of dynamin with Dynasore largely ablated transcytosis (Figure 1B), whereas incubation with histamine actually decreased it (Figure 1C).

To confirm our findings by a second method, we developed a streptavidin-based ELISA to measure transcytosis of biotin-conjugated albumin. We reasoned that the transcytosis of albumin would increase as a function of cell density until the monolayer was completely confluent. As a control, we measured the flux of dextran across the same transwell because dextran is not known to undergo transcytosis and passes via intercellular gaps.^{38,39} We observed that with increasing numbers of cells, the transcytosis of biotin-BSA across the transwell increased sig-

Figure 2. Inhibition of transcytosis by multiple methods induces paracellular leak. **A:** Endothelium grown on transwells was exposed to 0.1 mmol/L NEM or control then incubated with FITC-conjugated dextran (**P* < 0.05). Mean $\pm$ SD dextran flux was $0.24 \pm 0.17 \times 10^{-6}$ cm/s for control and $0.40 \pm 0.27 \times 10^{-6}$ cm/s for NEM. **B:** TEER was measured before and after exposure to NEM or control ($\pm$ SD baseline TEER: control, $25.0 \pm 1.6 \Omega\text{cm}^2$; NEM, $25.1 \pm 1.5 \Omega\text{cm}^2$; **P* < 0.05). **C:** Transfection with DN (K44A) dynamin-2 causes a marked decrease in TEER compared with transfection with WT dynamin-2. TEER was measured before and 24 hours after transfection. Transfection efficiency was approximately 70% and similar for both constructs (baseline TEER: WT, $21.6 \pm 0.7 \Omega\text{cm}^2$; K44A, $20.9 \pm 1.0 \Omega\text{cm}^2$; **P* < 0.05). **D:** Endothelium was incubated at 37°C (control) or 14°C. TEER was measured before and after the incubation (mean $\pm$ SD baseline TEER: control, $25.0 \pm 1.3 \Omega\text{cm}^2$; 14°C, $25.8 \pm 1.7 \Omega\text{cm}^2$; *n* = 5; **P* < 0.05). **E:** Endothelium was incubated with FITC-conjugated dextran for 40 minutes at 37°C (control) or 14°C (**P* < 0.05). **F:** Endothelium was incubated with FITC-conjugated albumin for 40 minutes at 37°C (left) or 14°C (right). Nuclei were stained with DAPI. Endosomes containing albumin (arrows, left) are absent in cells incubated at 14°C. Images are Z-stack projections.

nificantly, whereas the passage of dextran decreased (Figure 1D).

Finally, to confirm our findings visually, we performed transmission electron microscopy on endothelium that had been exposed to gold-tagged albumin (Figure 1E). As with the fluorescence assay, we used Dynasore as a control to block internalization (middle panel of Figure 1E). We observed internalization of gold-tagged albumin by caveolae and the apparent transcytosis of the albumin to the basolateral surface. Thus, taken together, our results convincingly demonstrate that primary human microvascular endothelial cells in culture both possess caveolae and perform transcytosis of albumin in a dynamin-dependent manner.

Inhibition of Transcytosis by Inhibiting SNARE Function Induces Paracellular Leak

Earlier studies have demonstrated that the absence of caveolin-1 greatly reduces transcytosis and is associated with increased vascular permeability^{26,40}; the latter has been attributed to increased paracellular leak or to changes in capillary pressure or the glycocalyx.²⁷ Because caveolin-1 has so many functions,²⁸ it is not clear whether the increased permeability is due to the loss of transcytosis per se or due to the loss of caveolin-1. To inhibit transcytosis without depleting caveolin-1 levels, we took advantage of the fact that, like other cellular processes that require membrane fusion, transcytosis requires the activity of SNARE proteins. Uncoupling of SNARE complexes is dependent on the hexameric ATPase NEM-sensitive factor, and inhibition of this enzyme using NEM is known to block vesicular traffic^{41–43} and transcytosis.⁴⁴ Incubation with NEM greatly inhibited internalization of albumin by both dermal and pulmonary endothelial cells (not shown). We found that exposure to NEM almost doubled the flux of dextran across the endothelial monolayer and significantly decreased the TEER (Figure 2, A and B). We considered the possibility that NEM might be toxic, even though higher concentrations and longer exposures have been used by others^{19,41,42,44} without untoward effects. Under the conditions used in our experiments, NEM caused no toxic effects as assessed by phase-contrast microscopy (not shown), trypan blue exclusion, and annexin V binding⁴⁵ to detect apoptosis (see Supplemental Figure S1, A and B, at <http://ajp.amjpathol.org>).

Inhibition of Transcytosis by a Molecular Approach Induces Paracellular Leak

Although NEM is a classic inhibitor of SNARE proteins, it lacks specificity. To inhibit transcytosis by a second, more specific method, we transfected endothelial cells with dynamin-2 K44A, a mutant form of dynamin-2 that is unable to bind GTP and acts in a DN manner.⁴⁶ As a control, we transfected other cells with WT dynamin-2. The transfection efficiencies for both constructs were equivalent and high (approximately 70% at 24 hours). DN dynamin-2 significantly reduced TEER compared

with WT, indicating an increase in paracellular permeability (Figure 2C). Thus, inhibition of transcytosis by a specific molecular method led to an increase in paracellular leak.

Figure 3. Dynasore specifically inhibits dynamin and induces paracellular leak. **A:** Dynasore increases dextran flux in cells transfected with WT dynamin-2 ($*P < 0.05$). Mean $\pm$ SD dextran flux was $0.63 \pm 0.38 \times 10^{-6}$ cm/s for control and $1.01 \pm 0.58 \times 10^{-6}$ cm/s for Dynasore. **B:** Dynasore does not increase dextran flux in cells transfected with dynamin-2 K44A. Mean $\pm$ SD dextran flux was $0.99 \pm 0.28 \times 10^{-6}$ cm/s for control and $1.01 \pm 0.32 \times 10^{-6}$ cm/s for Dynasore. **C:** Endothelium was exposed to Dynasore or DMSO and FITC-conjugated dextran for 40 minutes. Permeability to dextran was calculated relative to control ($*P < 0.05$). Mean $\pm$ SD dextran flux was $0.55 \pm 0.21 \times 10^{-6}$ cm/s for control and $0.88 \pm 0.28 \times 10^{-6}$ cm/s for Dynasore. **D:** The TEER of endothelial cells was measured before and after incubation with Dynasore or DMSO for 40 minutes (mean $\pm$ SD baseline TEER: control, $25.0 \pm 0.4 \Omega\text{cm}^2$; Dynasore, $25.4 \pm 0.4 \Omega\text{cm}^2$) ($*P < 0.05$). **E:** Endothelium was exposed to 10 cm H₂O pressure for 5 hours then, while still under pressure, treated with Dynasore or DMSO and FITC-conjugated dextran for 40 minutes. Permeability to dextran was calculated relative to control ($*P < 0.05$). Mean $\pm$ SD dextran flux was $0.87 \pm 0.24 \times 10^{-6}$ cm/s for control and $1.22 \pm 0.30 \times 10^{-6}$ cm/s for Dynasore.

Inhibition of Transcytosis by Cooling also Induces Paracellular Leak

Many endocytic processes, such as transcytosis, are inhibited at temperatures $<15^{\circ}\text{C}$.^{24,47,48} Indeed, incubation at 14°C completely blocked the internalization of albumin by primary human microvascular endothelium (Figure 2F). Cooling also significantly increased paracellular flux of dextran and decreased the TEER of the monolayer (Figure 2, D and E). This effect was seen in both dermal and pulmonary microvascular endothelium (latter not shown). Importantly, incubation at 14°C was not toxic to the cells as assessed by trypan blue exclusion and annexin V staining (see Supplemental Figure S1, C and D, at <http://ajp.amjpathol.org>).

Inhibition of Transcytosis by Dynasore Induces Paracellular Leak

One disadvantage of a molecular approach is the inability to block transcytosis acutely, making it difficult to distinguish immediate from delayed compensatory effects. In addition, although incubation at 14°C acutely inhibits endocytic traffic, its lack of specificity makes the determination of the molecular mechanism(s) difficult. One solution is the dynamin inhibitor Dynasore, which blocks dynamin activity within minutes and has been shown to be highly specific.³⁷ We first confirmed its specificity in our own hands by transfecting cells with DN dynamin-2, reasoning that such cells should have no additional phenotype (ie, increased paracellular leak) when exposed to a dynamin inhibitor. Thus, although Dynasore induced paracellular leak in dynamin-2 WT transfected cells, it had no effect on the endothelial cells transfected with the K44A mutant (Figure 3, A and B).

Having confirmed its specificity, we then observed that Dynasore increased the flux of 70-kDa dextran across endothelial monolayers and decreased the TEER (Figure 3, C and D). This effect was also seen in primary pulmo-

nary endothelium (not shown). It is important to note that this experiment is different from that described in Figure 1 because it involves the flux of dextran (which is not transported by transcytosis³⁹) instead of membrane-bound albumin. In addition, Dynasore increased the flux of albumin across the transwell if binding to the endothelial membrane was *not* performed first (not shown).

Paracellular permeability across the endothelium *in vivo* is affected not just by diffusion gradients but also by transmural pressure and the resultant convective transport.⁴⁹ To determine whether our observations held under conditions that included the convective transport associated with vessels under pressure, we repeated the experiment at a transmural pressure of 10 cm H_2O . Under these conditions, Dynasore continued to induce a marked increase in paracellular leak (Figure 3E). Importantly, Dynasore did not induce cell death or apoptosis under the conditions tested, and cells treated with Dynasore appeared healthy; this finding suggests that the increase in paracellular leak was not due to toxicity (see Supplemental Figure S1, E and F, at <http://ajp.amjpathol.org>). Finally, although dynamin resides at both the plasma membrane and the Golgi, the effect of Dynasore was not due to impairment of Golgi function because dissolution of the Golgi by brefeldin A⁵⁰ did not induce paracellular leak (not shown).

Induction of Paracellular Leak Is Not Due to Interference With Clathrin-Mediated Endocytosis

Because SNAREs and dynamin are required for clathrin-mediated endocytosis, it was conceivable that the inhibitors used in this study were exerting their effects through blockade of clathrin-mediated endocytosis rather than inhibition of caveolar uptake. To resolve this issue, we knocked down clathrin heavy chain by siRNA in primary human microvascular endothelium before blocking transcytosis. We achieved a reduction in clathrin levels of

Figure 4. Inhibition of transcytosis induces paracellular leak in cells depleted of clathrin. **A:** Endothelium was transfected with siRNA to clathrin heavy chain or nontargeting control siRNA. Seventy-two hours after transfection, whole-cell lysates were probed for clathrin by Western blot. The lower panel shows β -actin as a loading control, whereas the histogram shows degree of knockdown ($n = 4$; $*P < 0.05$). **B–D:** Cooling (**B**; mean $\pm$ SD baseline TEER: negative control, $22.4 \pm 1.7 \Omega\text{cm}^2$; siRNA, $23.9 \pm 2.0 \Omega\text{cm}^2$), NEM (**C**; mean $\pm$ SD baseline TEER: negative control, $20.9 \pm 1.9 \Omega\text{cm}^2$; siRNA, $20.9 \pm 1.9 \Omega\text{cm}^2$), and Dynasore (**D**; mean $\pm$ SD baseline TEER: negative control, $24.3 \pm 1.0 \Omega\text{cm}^2$; siRNA, $23.1 \pm 0.4 \Omega\text{cm}^2$) induce leak to the same degree in cells depleted of clathrin compared with cells with normal levels of clathrin. Endothelium depleted of clathrin (siRNA) or not (control) was cooled to 14°C , incubated with 0.1 mmol/L NEM, or incubated with 80 $\mu\text{mol/L}$ Dynasore, and the effect on TEER was assessed. **E:** The effect of Dynasore is significantly attenuated in cells depleted of caveolin-1 compared with cells with normal levels. **F:** Endothelium was depleted of caveolin-1 (siRNA) or not (control) and the TEER was measured before and after treatment with Dynasore (mean $\pm$ SD baseline TEER: negative control, $25.4 \pm 2.9 \Omega\text{cm}^2$; siRNA, $22.7 \pm 2.2 \Omega\text{cm}^2$) ($*P < 0.05$).

almost 90% at 72 hours (Figure 4A). However, inhibition of transcytosis by Dynasore (and NEM and cooling to 14°C) still induced paracellular leak to the same degree as in cells with normal levels of clathrin (Figure 4, B–D). Thus, the effect we observed is not simply a nonspecific reaction to blockade of endocytosis. To prove that our findings reflected inhibition of transcytosis per se, we knocked down caveolin-1 by siRNA (Figure 4E). Under these conditions, endothelial cells are known to be depleted of caveolae and do not perform transcytosis.^{20,40} Despite seeding equal numbers of cells, the baseline TEER was slightly lower in cells knocked down for caveolin-1 compared with control. This finding is not unexpected given the reported increase in paracellular permeability under such conditions.⁴⁰ Under conditions in which caveolin-1 was depleted by approximately 90%, the effect of Dynasore was markedly reduced (Figure 4F). This finding suggests that transcytosis is required for the effect that we observed.

Inhibition of Transcytosis Does Not Decrease or Redistribute Caveolin-1 Levels

To determine the mechanism of the increased paracellular leak, we focused on the dynamin inhibitor Dynasore because it functions acutely and its mechanism of action is specific and known. Given that caveolin-1-deficient mice demonstrate increased vascular leak,²⁶ we considered the possibility that inhibition of transcytosis by Dynasore might have an effect on caveolin-1 levels or its cellular distribution. We assessed caveolin-1 by immunofluorescence and by Western blotting of whole cell lysates (not shown) and lipid raft preparations. Dynasore did not alter caveolin-1 density or distribution by immunofluorescence (Figure 5A) and did not deplete caveolin-1 levels in lipid rafts (Figure 5B).

The increase in paracellular leak observed in caveolin-1-deficient mice has been attributed to increased production of nitric oxide because endothelial nitric oxide synthase is constitutively inhibited by caveolin-1.^{26,51} In those experiments, treatment of caveolin-1-deficient mice with the nitric oxide synthase inhibitor L-NAME restored microvascular integrity.²⁶ Thus, we considered the possibility that inhibition of transcytosis might induce endothelial nitric oxide synthase activity, even though we were not able to detect any change in caveolin-1 and even though caveolar internalization has been reported to be required for endothelial nitric oxide synthase activation.⁵² Production of nitric oxide by resting endothelium is quite low (not shown), making it difficult to exclude small changes. However, we pretreated endothelial monolayers with L-NAME and observed no effect on the induction of paracellular leak that was induced by Dynasore (Figure 5C). Thus, although we cannot definitively exclude it, increased nitric oxide production is unlikely to account for the increase in paracellular leak.

Figure 5. Inhibition of transcytosis does not decrease or redistribute caveolin-1. **A:** Immunofluorescence staining for caveolin-1 (cav-1) in endothelial cells incubated with (right) or without (left) Dynasore. Images are Z-stack projections and are representative of three independent experiments. **B:** Levels of cav-1 in lipid rafts are not diminished with Dynasore treatment. Western blot for cav-1 in different density fractions of endothelial cells with or without incubation for 40 minutes with Dynasore. The transferrin receptor (TfR) was used as a control for nonlipid raft fractions. The fraction of protein in the lipid raft (20% fraction) was calculated relative to total protein ($n = 4$). **C:** Inhibition of nitric oxide synthase with L-NAME does not prevent induction of paracellular leak by Dynasore. Endothelium was preincubated with L-NAME for 30 minutes followed by Dynasore. TEER was measured before and after incubation. Data represent mean $\pm$ SD ($n = 2$; $*P < 0.05$).

Inhibition of Transcytosis Does Not Affect Junctional Proteins or the Glycocalyx

One of the canonical pathways regulating paracellular permeability is the internalization of VE-cadherin from the plasma membrane.¹³ *A priori*, this seemed unlikely to account for the increased paracellular leak because inhibition of transcytosis would not be expected to lead to increased uptake of VE-cadherin. As expected, Dynasore had no effect on the total amount of VE-cadherin, its fraction in the membrane, or its distribution as assessed by immunofluorescence (Figure 6, A and B). Similarly, Dynasore had no effect on the distribution of the tight junction protein claudin-5 (Figure 6C) or on the distribution or density of the glycocalyx component heparan sulfate (Figure 6D). Thus, at least initially, inhibition of transcytosis using Dynasore does not exert its primary effect on intercellular junctions or on the glycocalyx.

Figure 6. Treatment with Dynasore (Dyn) does not alter levels or distribution of VE-cadherin. **A:** Western blot for VE-cadherin in whole cell lysate (WCL) and membrane preparations in cells treated with Dynasore or DMSO [control (Ctrl)]. Membrane was stripped and reprobed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control or probed for the transferrin receptor (TfR) to confirm successful membrane isolation. Quantitation was performed using ImageJ. **B–D:** Treatment with Dynasore does not alter the distribution of VE-cadherin (**B**), claudin-5 (**C**), or heparan sulfate (**D**). Immunofluorescence staining for the junctional proteins (**B** and **C**) in endothelial cells incubated with (right) or without (left) Dynasore. **D:** Treatment with Dynasore does not alter the density or distribution of heparan sulfate. Cells were incubated with (middle) or without (left) Dynasore. Heparinase III treatment was used as a negative control (right). Images are Z-stack projections and are representative of three independent experiments.

Inhibition of Transcytosis Is Associated With Remodeling of the Actin Cytoskeleton

Instead, we reasoned that the rapid changes in paracellular permeability after inhibition of transcytosis were likely associated with remodeling of the actin cytoskeleton, similar to the leak induced by certain inflammatory mediators.^{53,54} We observed that inhibition of transcytosis by Dynasore was associated with a significant thinning of cortical actin (Figure 7, A and B). This loss of cortical actin was not due to generalized disassembly of actin because the total level of F-actin remained unchanged (Figure 7C).

We hypothesized that the loss of cortical actin likely stemmed from a change in Rac-GTP levels or its intracellular distribution. Treatment with Dynasore had no effect on total cellular Rac-GTP levels (Figure 7D). However, previous literature on phagocytosis had suggested that dynamin could regulate the intracellular traffic of Rac.⁵⁵ We therefore transfected primary endothelial cells with PBD-YFP; the latter construct consists of the p21 Rac-GTP-binding domain of PAK (a known effector of Rac) fused to YFP and is a sensitive indicator of Rac activity in live cells.³⁵ To distinguish plasmalemmal or juxta-plasmalemmal Rac-GTP from the cytosolic protein, we analyzed the distribution of PBD-YFP at the basal membrane of confluent endothelial cells by total internal reflectance fluorescence microscopy (TIRFM). This sensitive technique permits cellular imaging to be restricted to only the basal 100 to 150 nm of the cell,⁵⁶ decreasing confounding by cytosolic fluorescence. TIRFM revealed enrichment of PBD-YFP at the lamellipodia and filopodia of resting endothelium that was further enhanced by treatment with S1P, a known Rac agonist.⁵⁷ Remarkably, relative to control cells in which active Rac remained constant or increased over time, Dynasore-treated cells demonstrated a rapid and marked translocation of the Rac-GTP probe from cellular

edges, consistent with the effects of Dynasore on cortical actin and paracellular leak (Figure 7, E and F).

Decreasing Paracellular Leak Causes Induction of Transcytosis

We considered the possibility that induction of paracellular leak could be an indirect consequence rather than a direct result of the inhibition of transcytosis. We believe that this is unlikely given the consistent results obtained using multiple distinct methods of inhibition. However, a powerful way of establishing the connection between transcytosis and paracellular permeability would be to demonstrate the reverse relationship. In particular, we hypothesized that inhibition of paracellular leak would induce transcytosis. Furthermore, the changes we had observed in cortical actin and Rac were reminiscent of reciprocal changes induced by S1P. S1P is one of the few molecules that are well known to stabilize endothelial barrier integrity,¹ an action mediated in large part through Rac-dependent increased generation of cortical actin.⁵⁷ As expected, S1P caused a decrease in paracellular permeability (Figure 8A), whereas permeability to dextran (70 kDa), already low at baseline, was unaffected (Figure 8D). Treatment with S1P greatly attenuated the loss of cortical actin induced by Dynasore and the induction of paracellular leak (Figure 8, B, C, and E). Remarkably, the reduction in paracellular leak by S1P was associated with a significant increase in the transcytosis of albumin (Figure 8D).

We next moved to an *ex vivo* model to validate our *in vitro* findings. To do so, we performed real-time *in situ* fluorescence imaging in isolated perfused mouse lungs.³⁶ Membrane-permeant fura-2 AM was used to label endothelial cells, and FITC-conjugated albumin and TRITC-conjugated dextran were added simultaneously to the lung perfusate (Figure 9A). As a positive control,

Figure 7. Inhibition of transcytosis by Dynasore (Dyn) results in a loss of cortical actin due to translocation of active Rac. **A:** Immunofluorescence staining for F-actin in endothelial cells incubated with (right) or without (left) Dynasore. Dashed white box shows a magnification of the portion of the image outlined with the solid white box. Arrows indicate cortical actin. **B:** Using ImageJ, three measurements of cortical actin thickness were taken for each cell (each at a different cell-to-cell junction) using Z-stack projections. Measurements were taken from >700 cells (* $P < 0.05$). **C:** Intensity of F-actin was measured for >50 randomly selected fields and divided by the number of cells. **D:** Endothelial cells were treated with Dynasore or DMSO (ctrl). Cell lysates were immunoprecipitated with an anti-Rac-GTP antibody ($n = 4$); lysates loaded with GDP or GTP γ S served as controls. Total Rac is shown in the lower panel. **E and F:** Cells transfected with PBD-YFP were imaged live using TIRFM to analyze the distribution of active Rac at the cell membrane (**F**; quantitation in **E**). Images were captured at 0 minutes and 30 minutes after treatment with DMSO or Dynasore. S1P (1 μ mol/L) was used as a positive control for Rac activation. Colored boxes denote areas enlarged for insets. Dynasore caused a significant translocation of active Rac from cellular edges (**E**). Monolayers were confluent, but only transfected cells are visible ($n = 5$; * $P < 0.05$).

treatment with the potent inflammatory mediator platelet-activating factor caused a marked extravasation of dextran from the vasculature (Figure 9C). In contrast, treatment with S1P caused a significant translocation of intravascular albumin with no extravasation of dextran (Figure 9B). Importantly, the fluorescent signals for albumin and fura-2 overlapped almost perfectly, indicating transcellular passage of albumin.

Inhibition of Rac Activity Reduces the Internalization of Membrane-Bound Albumin by Microvascular Endothelium

The data from Dynasore and S1P (a known Rac agonist) strongly implicated an unsuspected role for Rac in regulating albumin internalization. To validate this notion, we transfected primary human microvascular endothelium with GFP-tagged WT or DN (T17N) Rac.³⁵ We reasoned that if induction of transcytosis by S1P was mediated by

Rac activation, impairment of Rac should prevent the internalization of membrane-bound albumin. Endothelial cells that overexpressed WT Rac variably demonstrated normal or increased albumin internalization (Figure 10A). We observed that expression of DN Rac significantly attenuated the uptake of albumin compared with both untransfected and WT-Rac-transfected cells (Figure 10B), without affecting endocytosis (Figure 10C). Concordantly, despite incomplete transfection of the endothelial monolayer (approximately 75%), expression of DN Rac led to a significant increase in paracellular leak (Figure 10D). These data imply that active Rac, through its effects on actin and on albumin uptake, is a key player at the junction of paracellular leak and transcytosis.

Discussion

Increased vascular permeability is the hallmark of inflammation and is thought to facilitate the emigration of leu-

Figure 8. Reduction of paracellular leak by S1P causes induction of transcytosis. **A:** S1P decreases paracellular leak as measured by TEER. TEER was measured before and after incubation with methanol [solvent control (Ctrl)] or 1 $\mu\text{mol/L}$ S1P (mean $\pm$ SD baseline TEER: control, $35.5 \pm 1.3 \Omega\text{cm}^2$; S1P, $34.3 \pm 0.7 \Omega\text{cm}^2$). Results are normalized to control (* $P < 0.05$). **B:** Treatment with S1P attenuates Dynasore (Dyn)-induced loss of cortical actin compared with methanol-treated cells (control). Immunofluorescence staining for F-actin (**E**) in cells incubated for 40 minutes under control conditions (**left**), with Dynasore (**center**), or with Dynasore and S1P (**right**). **Arrows** indicate cortical actin. Images are Z-stack projections and are representative of three independent experiments (quantitation in **B**). Measurements were taken from >800 cells (* $P < 0.05$). **C:** The TEER of endothelial cells was measured before and after 40 minutes incubation with control, Dynasore, S1P, or Dynasore and S1P (mean $\pm$ SD baseline TEER: control, $35.6 \pm 1.7 \Omega\text{cm}^2$; Dynasore, $33.9 \pm 1.7 \Omega\text{cm}^2$; S1P, $33.6 \pm 1.8 \Omega\text{cm}^2$; Dynasore and S1P, $33.9 \pm 1.7 \Omega\text{cm}^2$) ($n = 4$; * $P < 0.05$). **D:** S1P increases transcytosis of albumin compared with methanol-treated cells (control) but not permeability to dextran. Black bars indicate relative permeability to albumin (transcytosis), whereas white bars indicate relative permeability to dextran (paracellular leak). Permeability is normalized to control. Transcytosis was measured by ELISA as described in *Materials and Methods*; * $P < 0.05$ for the effect of S1P on albumin permeability. Mean flux of dextran was $0.14 \pm 0.02 \times 10^{-6}$ cm/s for control and $0.15 \pm 0.02 \times 10^{-6}$ cm/s for S1P. Mean flux of albumin was $0.85 \pm 0.28 \times 10^{-7}$ cm/s for control and $1.10 \pm 0.33 \times 10^{-7}$ cm/s for S1P.

kocytes from the bloodstream to sites of infection. Nonetheless, it is now apparent that permeability of the endothelium to leukocytes and permeability to fluid and proteins are not necessarily synchronous.^{58,59} In fact, it is worth noting that the loss of fluid and proteins out of the vasculature during inflammation is far from benign.^{3,60,61} In acute lung injury, strategies that restrict fluid administration lead to improved lung function.⁶¹ In animal models of diseases characterized by disseminated microvascular permeability, such as sepsis and anaphylaxis, interventions that restore endothelial barrier integrity lead to improved blood pressure⁵ and increased survival.^{1,2} An understanding of how microvascular permeability is regulated is therefore essential for understanding inflammatory diseases and developing potential therapies.

Passage of fluid and proteins out of the vasculature can occur either between or through individual cells. Although both the paracellular and transcellular routes across the endothelium were described decades ago,^{62,63} remarkably little is known about the relationship between them. In particular, whether changes in one route can modulate the other is unknown. This paucity of literature about such a fundamental question in vascular biology stems in part from difficulties in measuring and quantifying transcytosis. Analyzing transcytosis and distinguishing it from paracellular leak has been challenging *in vivo*. Most studies have used electron microscopy of animal tissues, an arduous, expensive, and sometimes descriptive endeavor, although recent advances in intravital imaging appear promising.^{25,64} Dissecting out the molecular regulation of transcytosis and its link to paracellular leak would be more easily done *in vitro*; for instance, such a system would avoid the confounding effects of changes in blood pressure.²⁷ However, there has been little analysis of *in vitro* transcytosis, partly because the density of caveolae decreases in cell culture²²; furthermore, the frequency of transcellular events likely varies among different vascular beds and may be lowest in human umbilical vein endothelial cells,⁴³ which are commonly used in cell culture experiments. To overcome this barrier, our laboratory devised two independent assays to

Figure 9. Reduction of paracellular leak by S1P causes induction of transcytosis in an *ex vivo* lung model. **A:** Isolated mouse lungs were perfused with Krebs-Henseleit buffer containing membrane-permeant fura-2 AM, FITC-conjugated albumin (alb), and tetramethylrhodamine isothiocyanate (TRITC) dextran (dex) (molecular weight, approximately 70,000) in the presence or absence of either S1P (1 $\mu\text{mol/L}$) or platelet-activating factor (PAF; 50 nmol/L). After 60 minutes, residual dyes were washed out and images were captured at the appropriate wavelength by intravital microscopy. **B–C:** The fluorescence intensity of FITC (**B**) and TRITC (**C**) was quantified in the vascular wall and surrounding parenchyma of lung venular capillaries and expressed relative to the respective fura-2 fluorescence. Images have been pseudo-colored for clarity. In each experiment, 10 to 15 single venular capillaries were viewed ($n = 5$ experiments per group; * $P < 0.05$).

Figure 10. A: Inhibition of Rac inhibits albumin uptake. The internalization of membrane-bound rhodamine-conjugated albumin in endothelium transfected with WT- or DN-Rac-GFP was compared. Nuclei were stained with DAPI. Images are Z-stack projections. **Arrows** denote transfected cells. **B:** Fluorescence of endosomes was measured per cell and background fluorescence was subtracted. For both WT- and DN-Rac cells, measurements were normalized to untransfected cells. Measurements were taken of 95 transfected cells ($n = 3$; $*P < 0.05$). **C:** The endocytosis of FM 4–64 is not affected by transfection with WT- or DN-Rac. The internalization of FM 4–64 in endothelium transfected with WT- or DN-Rac-GFP was compared. Quantitation of FM internalization was performed using ImageJ; measurements were taken of >400 cells and >150 transfected cells. **D:** Cells transfected with DN-Rac have a higher permeability than those transfected with WT-Rac. Equal numbers of transfected cells were seeded on transwells, and TEER was measured 24 hours later (mean $\pm$ SD: WT, $26.3 \pm 1.5 \Omega\text{cm}^2$; DN, $24.0 \pm 0.9 \Omega\text{cm}^2$) ($*P < 0.05$).

measure transcytosis by primary human microvascular endothelium. These experiments, complemented by electron microscopy, convincingly demonstrate that transcytosis can in fact be studied *in vitro*.

Using this as a starting point, we sought to determine how transcytosis and paracellular leak were related. A connection seemed plausible because tissue beds that are the most sensitive to inflammatory mediators, such as postcapillary venules, also display a high density of caveolae.⁶⁵ Furthermore, signaling molecules that have been implicated in the generation of paracellular leak, such as Src kinase, are themselves essential for transcytosis.^{21,66} Lastly, given the critical nature of vascular integrity to homeostasis, there is a strong biological rationale for these two pathways to be co-regulated. Although ablation²⁶ or knockdown of caveolin-1⁴⁰ leads to inhibition of transcytosis, it has been controversial whether this results

in increased paracellular leak or simply causes an alteration in capillary pressure or the glycocalyx.²⁷ Furthermore, the pleiotropic effects of caveolin-1 make it difficult in such studies to attribute any change in permeability to the loss of transcytosis *per se*.

To answer this question, we inhibited transcytosis using different molecular and pharmacologic methods, each of which is well established to inhibit vesicular uptake. For instance, NEM is the prototypical inhibitor of SNARE function and has been used to inhibit vesicular traffic (including transcytosis)⁴⁴ since the 1980s.⁶⁷ Dynasore is a cell-permeable inhibitor of dynamin that has been extensively tested for specificity (and whose specificity was confirmed in our data).³⁷ The K44A mutant of dynamin-2 has also been widely studied and is commonly used to block dynamin-dependent events.⁶⁸ Finally, the inhibition of vesicular uptake at temperatures $<15^\circ\text{C}$ has been a feature of cell biological studies for decades.^{31,69} Remarkably, regardless of which of these methods was used to inhibit transcytosis, we observed an increase in paracellular leak. This was not simply due to the general inhibition of endocytosis because almost complete knockdown of clathrin by siRNA did not change the results. Furthermore, the increase in leak that we observed was not due to toxicity of the inhibitors or, in contrast to previous literature, due to depletion of caveolin-1²⁶ or increased nitric oxide synthase activity.

To determine the molecular mechanism of this relationship, we took advantage of the dynamin inhibitor Dynasore because its own mechanism of action is known and specific³⁷ (Figure 3). In addition, the acuity of its inhibition would allow us to distinguish the direct consequences of the loss of transcytosis from downstream compensatory events that might occur over time.

Our data demonstrate that the inhibition of transcytosis by blocking dynamin leads to a translocation of active Rac from the plasmalemma, culminating in the loss of cortical actin and the formation of intercellular gaps. Thus, the regulation of the localization of active Rac by dynamin forms the basis for a connection between transcytosis and paracellular leak. Such a relationship is plausible given the importance of Rac in the regulation of cortical actin,⁷⁰ the fact that caveolae and the actin cytoskeleton are closely associated,^{71,72} and existing data describing the influence of dynamin on actin during endocytosis in general.⁴⁸ The physiologic relevance of this observation is supported by the fact that the enhancement of paracellular integrity via the induction of Rac activity (by S1P) was sufficient to stimulate transcytosis. The fact that inhibition of Rac is sufficient to attenuate the internalization of membrane-bound albumin and to decrease endothelial resistance is also consistent with our model.

Our finding that Dynamin, Rac, and cortical actin constitute a critical link between paracellular leak and transcytosis is novel. Although cortical actin was previously thought to restrict the internalization of plasmalemmal vesicles,^{73,74} recent data suggest a more complex relationship.^{75,76} The role of Rac in regulating endocytosis is similarly nuanced; although activated Rac has been shown to inhibit clathrin-mediated endocytosis,⁷⁷ it has

also been reported to stimulate pinocytosis,⁷⁰ which, like transcytosis of albumin, is clathrin independent. The abundance of signals that regulate Rac activity indicates that the balance between paracellular and transcellular leak has the potential to be dynamic and exquisitely responsive to cellular cues.

To our knowledge, this is the first demonstration of a reciprocal relationship between paracellular and transcellular permeability in resting endothelium.⁷⁸ Previous literature has noted that some inflammatory stimuli can induce paracellular leak and up-regulate the number of caveolae or expression of caveolin-1, although most studies have not measured transcytosis itself. Furthermore, little mechanistic work has been performed to date. For instance, vascular endothelial growth factor and endotoxin are known to induce paracellular permeability^{13,16} while increasing caveolin-1 levels and/or the number of caveolae in endothelia.^{79,80} Similarly, leukotrienes have been reported to increase transcytosis⁶⁴ but have also been demonstrated to induce the leakage of dextran,⁸¹ likely by paracellular means. Thus, whether induction of paracellular leak necessarily decreases transcytosis or whether, under certain conditions, both pathways can be induced is likely to depend on the specific inflammatory stimulus or the specific tissue bed. However, we obtained similar results in resting microvascular endothelium of both dermal and pulmonary origin.

It is important to acknowledge that in addition to endothelial cells, there are other determinants of vascular permeability, including nonendothelial cells, such as pericytes.⁸² Compared with the importance of endothelial cells to barrier function, their contribution is less well defined. Similarly, although we observed a clear role for Rac, the GTPase RhoA is also known to contribute to vascular leak by inducing actin stress fibers.⁸³ Although we did not observe any change in stress fibers on inhibition of transcytosis, further studies will be needed to definitively address the role of RhoA in the co-regulation of transcytosis and paracellular leak.

The resting permeability of our endothelial monolayers to dextran was similar or lower to that described in the literature,^{30,84} reflecting the health and integrity of the cells. Permeability to albumin, as measured during the biotin-BSA assay (Figure 1D), was lower than the permeability to albumin reported by others.⁸⁴ This is likely because of the much higher molecular weight of biotin-BSA compared with BSA alone, which significantly decreases passage across transwells.⁸⁵

Although the occurrence of transcytosis is now widely accepted, its physiologic importance continues to be debated.¹⁹ *In vitro* models often neglect the contribution of convective transport that occurs when blood vessels are under pressure³⁰; however, we obtained similar results under conditions incorporating transmural pressure. Furthermore, our ability to replicate our findings in an intact (*ex vivo*) lung is important validation of our model. Other investigators have reported that transcytosis is more prominent *in vivo*²⁵ than in cell culture, which leads us to believe that the effects we observed will be proportionately more important *in vivo* as well.

In conclusion, we have demonstrated that transcytosis of albumin and paracellular leak are co-regulated in human endothelium. We hypothesize that such a relationship may allow for the fine regulation of total vascular permeability. For example, the ability to increase transcytosis at the expense of intercellular gap formation might be advantageous when blood vessels experience shear stress because it would theoretically decrease the risk of dissection of the vessel wall and, by shielding the underlying extracellular matrix, decrease the risk of intraluminal thrombus formation. We are currently investigating this notion. Finally, the identification of the specific molecular links between the two pathways of leak could have profound implications for the development of anti-inflammatory therapies. For instance, if attempts to inhibit one pathway cause the enhancement of the other, the therapeutic benefit may be limited.

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