Mechanisms regulating endothelial permeability

Sukriti Sukriti, Mohammad Tauseef, Pascal Yazbeck, Dolly Mehta

Department of Pharmacology and Center for Lung and Vascular Biology, College of Medicine, University of Illinois, Chicago, Illinois, USA

Abstract: The endothelial monolayer partitioning underlying tissue from blood components in the vessel wall maintains tissue fluid balance and host defense through dynamically opening intercellular junctions. Edemagenic agonists disrupt endothelial barrier function by signaling the opening of the intercellular junctions leading to the formation of protein-rich edema in the interstitial tissue, a hallmark of tissue inflammation that, if left untreated, causes fatal diseases, such as acute respiratory distress syndrome. In this review, we discuss how intercellular junctions are maintained under normal conditions and after stimulation of endothelium with edemagenic agonists. We have focused on reviewing the new concepts dealing with the alteration of adherens junctions after inflammatory stimulus.

Keywords: vascular endothelium, adherens junctions, permeability, signaling mechanisms.

Pulm Circ 2014;4(4):535-551. DOI: 10.1086/677356.

The vascular endothelium forming the innermost lining of all blood vessels performs a vital task of providing nutrients to the underlying tissue as well as maintains tissue oncotic pressure.¹⁻³ Maintenance of the size-selective sieving property of endothelium is crucial for several physiological functions, including normal tissue-fluid homeostasis, angiogenesis, vessel tone, and host defense.²⁻⁹ Endothelium is permeable to molecules ranging from 0.1 nm (sodium ion) to 11.5 nm (immunoglobulin G [IgG]) in diameter.^{2,3,9,10} Interestingly, the sieving property of endothelium linearly correlated with molecular radii from 0.1 nm to 3.6 nm but became independent of the molecular radius above 3.6 nm.^{2,3} On the basis of these findings, two endothelial transport mechanisms were defined for solute/ion influx.^{2,3,7,11,12} The transport of macromolecules larger than 3 nm, such as albumin, IgG, and other macromolecules, occurs through the transcellular pathway, which is also called transcytosis or vesicular transport.^{3,12-14} Molecules smaller than 3 nm, such as glucose, water, and ions, can pass through interendothelial junctions (IEJs) through the paracellular pathway.^{2,3,14} Both transcellular and paracellular pathways work in concert to maintain tissue-oncotic pressure and thereby maintain the endothelial barrier.^{3,7,12}

Caveolae, a flask-shaped vesicle composed of several caveolins-1 along with other proteins, such as dynamin and intersectin, constitutes the transcellular pathway.^{2,12,15-18}

The paracellular pathway is regulated by a complex interaction of various junctional proteins and actomyosin motors.^{2,12,17} In an unperturbed endothelium, IEJs dynamically open to allow the passage of small molecules and inflammatory cells for tissue homeostasis and immune surveillance.^{2,8,9,12,14} Proinflammatory agonists such as thrombin, vascular endothelial growth factor (VEGF), and platelet-activating factor,¹⁹⁻²² by binding to their receptors, disorganize IEJs, leading to increase in endothelial permeability. Thus, to understand how tissue-fluid homeostasis is modulated under normal conditions and pathological processes, we must understand the signaling mechanisms that regulate IEJs.

ENDOTHELIAL BARRIER PERMEABILITY

Endothelial cells originate from embryonic precursor cells known as hemangioblasts.^{4,5,23,24} However, permeability of endothelial cells under basal conditions and in response to edemagenic agents varies remarkably in different vascular beds.² Vascular beds of coronary, pulmonary, splenchnic, and skeletal muscle are composed of continuous non-fenestrated endothelial cells, which form a restrictive barrier.^{5,25} Organs such as liver, kidney, and lymphatics are formed from discontinuous and highly permeable endothelial monolayers.² Also, segmental differences are observed between endothelial barrier permeability at the cell-culture

Address correspondence to Dr. Dolly Mehta, Department of Pharmacology, University of Illinois, College of Medicine, 835 South Wolcott Avenue, Chicago, IL 60612, USA. E-mail: dmehta@uic.edu.

Submitted December 19, 2013; Accepted March 3, 2014; Electronically published September 4, 2014.

© 2014 by the Pulmonary Vascular Research Institute. All rights reserved. 2045-8932/2014/0404-0002. \$15.00.

level and intact vessels of the same vascular bed.^{5,25-27} Measurement of the coefficient of vascular permeability (K_{fc}) across the endothelium in isolated perfused lung preparations under basal conditions in experimental animals showed a gradation of permeability across the microvascular bed, with the microvascular site constituting approximately 42%, the arterial region approximately 19%, and the venous area approximately 37%,²⁶ indicating that the arterial system was more restrictive than either venous or capillary linings. In pulmonary microvessels, endothelial cells formed a monolayer that was approximately 4 times less permeable to albumin compared with the confluent monolayer of the cells from venous or arterial regions.^{2,5} Furthermore, transendothelial electrical resistance (TER), which is a measure of interendothelial adhesion in real time, was lower in cells isolated from larger arteries than in microvascular endothelial cells.²

How can these differences in the endothelial permeability of various vascular beds be accounted for? Studies have shown that the constitution of IEJs, extracellular matrix (ECM), and cytoskeletal proteins may contribute toward segmental variability in the endothelium.^{4,5,25,28} The arterial segment has approximately 18 times more occludin, a component of tight junctions (TJs), than its venular counterpart, making the arterial endothelial barrier tighter than the venular barrier.² Also, occludin is found to be expressed in brain endothelial cells more than in any other organ.^{2,29-31} Microvascular endothelial cells express collagen 4 α 1, collagen 4 α 2, and laminin in their ECM, whereas collagen 5 α 1 and collagen 5 α 2 are expressed more in macrovascular endothelia.^{2,28} Moreover, there is an abundance of myosin light-chain kinase (MYLK; serine/threonine-specific protein kinase that phosphorylates the regulatory light chain of myosin II),^{1,2,32} LIM kinase (actin-binding kinases that phosphorylate members of the actin depolymerizing factor/cofilin family of actin-binding proteins),^{33,34} Rho-GTPases (Vav),^{2,32} and myosin motor cytoskeleton in microvascular cells. Microvascular endothelial cells also showed a higher density of caveolae. Besides the above-mentioned intrinsic mechanisms, extrinsic mechanisms, such as shear stress caused by linear versus pulsatile blood flow, were also shown to regulate permeability differences between microvascular and macrovascular endothelial cells. It has been shown that shear stress increases intracellular Ca²⁺ and inositol triphosphate (IP₃) generation and induces activities of small GTPases, such as Rac and RhoA, and reorganization of actin fibers.^{2,35,36} Thus, well-organized transcellular and paracellular pathways, by balancing extrinsic fluid dynamics, may impact endothelial barrier phenomena.

Starling and Kedem explained the transport of solute and water across the endothelial barrier.² Briefly, the rate

of the transport of fluid across the endothelial barrier is based on the Starling forces,

$$J_v = (L_p s)[(P_c - P_i) - \sigma(\Pi_c - \Pi_i)] \quad (1)$$

J_v denotes volume of flux of fluid, (mL/min); s is the capillary surface area (cm²); L_p is the hydraulic conductivity (cm · min⁻¹ · mmHg⁻¹); P_c and P_i are capillary and interstitial hydrostatic pressures (mmHg), respectively; Π_c and Π_i are capillary and interstitial colloidal osmotic pressures (mmHg), respectively; and σ is osmotic reflection coefficient of the capillary wall. If the $\sigma = 0$, vessel is completely permeable to fluid and solutes, whereas if the $\sigma = 1$, capillary is impermeable.^{2,3,37,38}

IEJ PROTEINS

IEJs are composed of adherens junctions (AJs), TJs, and connexins (Fig. 1).² IEJs are presumed to be covered by an extra layer of fibrous matrix composed of proteoglycans, glycosaminoglycans, and glycolipids known as glycocalyx.^{2,3,39-41}

TIGHT JUNCTIONS

TJs composed of claudin, occludin, and junctional adhesion molecules (JAMs) constitute about 20% of the total junctional proteins in endothelial cells.^{3,40-43} TJs are well-developed in arterial vessels compared with venular segments. Occludin was the first TJ protein identified in endothelial cells. Occludin interacts with zona occludens (ZO-1) in cytoplasm, which in turn associates with α -catenin and actin cytoskeleton and thus stabilizes endothelial barrier function.^{3,7,43} Occludin expression in endothelial cells was shown to regulate the tightness of the barrier.^{2,43} Depletion of occludin using small interference RNA (siRNA) increased endothelial barrier permeability as indicated by the decrease in TER.⁴⁴ However, occludin-lacking mice showed normal TJs morphology and no apparent change in intestinal epithelial barrier function, indicating compensation in TJs function by TJ proteins.⁴⁵

Several claudins have been identified to date, but endothelial cells predominantly express claudin 5.^{46,47} Claudin 5 also binds with ZO-1 protein through its cytoplasmic domain. ZO-1 thus allows TJ proteins to interact with each other and with actin.⁴⁸ Unlike occludin null mice which survive and grow well, claudin null mice die at 10 h after birth because of the increased permeability of blood brain barrier to small molecules.⁴⁷

JAM-A, JAM-B, and JAM-C, having 30%–40% sequence homology, belong to immunoglobulin superfamily and participate in formation of TJs in endothelial cells.⁴⁹⁻⁵¹ Al-

Figure 1. Structural organization of endothelial cell intercellular and matrix interactions. Interendothelial junctions (IEJs) comprised of tight junctions, adherens junctions, and gap junctions interact through actin cytoskeleton with integrin receptors enabling endothelial cell adhesion with contiguous cells and to the underlying matrix. Occludin, claudins, and junctional adhesion molecules (JAMs) are the backbones of tight junctions, whereas vascular endothelial cadherin (VE-cadherin) is required for the formation of adherens junctions. Connexins form gap junctions. Intracellular domains provide junctional stability through their linkages with the actin cytoskeleton via catenins (α , β , γ , and p120) or zona occluden 1 protein (ZO). Gap junctions allow the rapid exchange of information between cells. The cytosolic domains of integrins are linked with actin cytoskeleton through proteins talin and vinculin (Vin), involved in integrin-mediated signaling. Membrane metalloproteinases (MMPs) control remodeling of the extracellular matrix (ECM). FN: fibronectin; VN: vitronectin.

though JAM-B is specifically present in endothelial cells, JAM-A and JAM-C can form TJs in both endothelial cells and epithelial cells. JAM-C is also an important component of lymphatic venular endothelial cells.^{49,50} JAMs contain PDZ binding motif and may therefore form a platform for mediating the interaction between several proteins containing PDZ domain.⁵¹ JAM-A and JAM-C can also be cleaved by membrane-bound metalloproteinase ADAM 10 and ADAM 17 upon activation of endothelial cells by inflammatory agonist, leading to disruption of barrier function and inflammatory signaling.⁵² Consistently, destabilization of JAM-A protein using anti-JAM-A antibodies prevented the re-annealing of TJs in epithelial cell monolayer.^{53,54} JAM-A, JAM-B, and JAM-C also participate in transendothelial leukocyte transmigration. However, loss of JAM-A in mice reduced polymorphonuclear leukocyte diapedesis,^{51,55} indicating JAM-A may play a predominant role in regulating neutrophil transmigration. In line with this idea, it was

shown that JAM-A controls the internalization and recycling of $\beta 1$ integrin after chemotactic stimulation of neutrophils.

ZOs are family members of membrane-associated guanylate kinases.⁵⁶ Three ZO subtypes have been identified in endothelial cells. ZO-1 interacts with claudins, JAMs, and occludin. Through PDZ, Src homology 3 and guanylate kinase domains, ZO-1 allow TJ proteins to interact with actin cytoskeleton. Besides TJ protein, ZO-1 is also known to interact with AJ proteins, such as α -catenin, gap junction (GJ) protein connexin-43, actin polymerizing proteins vasodilator-stimulated phosphoprotein (VASP), and spectrin.⁵⁷ ZO-1 may thereby regulate endothelial permeability by serving as a scaffold for mediating the interaction of TJs with AJs and connexin.⁵⁶ It has been speculated that ZO-1 stabilizes the endothelial barrier function through the stabilization of F-actin and F-actin itself stabilizes the localization of ZO-1.^{29,58}

GAP JUNCTIONS

GJs, formed by interaction of two connexins (Cx) from opposite cells, provide a means to facilitate direct cell-to-cell transfer of signaling molecules, ions, current, and transmembrane potential.⁵⁹ Each connexin is made of six connexin subunits (hexamers). Cx37, Cx40, and Cx43 are expressed in endothelial cells.⁶⁰ GJs are low-resistance pathways for the flow of current from one cell to another cell.⁶¹ Additionally, they allow exchange of information in the form of second messengers, such as Ca^{2+} , IP_3 , and so on. GJs form a pore size of about 2 nm. Phosphorylation of connexins is shown to regulate GJ function in endothelial cells.³⁴ Serine/threonine and tyrosine phosphorylation of connexins closes the channels, and this prevents intercellular communication. Moreover, phosphorylation of connexins also regulates the assembly and formation of channels. Whether phosphorylation of connexins is casually regulated to alteration in endothelial permeability by edemagenic agonist remains elusive. Recently, Bhattacharya group elegantly demonstrated that bone marrow-derived mesenchymal stromal cells (BMSCs) form GJ channels with the alveolar epithelium through connexin 43, enabling the transfer of mitochondria-containing microvesicles. They demonstrated that BMSC-induced transfer of mitochondria was required for alveolar bioenergetics, thereby preventing endotoxin-induced alveolar leukocytosis and protein leak as well as inhibition of surfactant secretion and mortality.⁶²

ADHERENT JUNCTIONS

AJs have a prominent role regulating endothelial barrier function.⁴¹ Homotypic adhesion between vascular endothelial (VE) cadherin from contiguous endothelial cells in a calcium-dependent manner forms AJs.^{2,8,12} VE-cadherin contains 5 extracellular cadherin-like repeats (Fig. 1).² These cadherin oligomerize to form cis-oligomers or trans-oligomers between adjacent cells. The cytoplasmic tail of the VE-cadherin contain two domains, namely juxtamembrane domain (JMD) and COOH-terminal domain (CTD).^{2,12,40} These cytoplasmic domains interact with three Armadillo family proteins known as β -catenin, plakoglobin (γ -catenin), and p-120 catenin. JMD binds with β -catenin, whereas CTD domain interacts with plakoglobin and p-120 catenin.^{40,63} β -catenin or plakoglobin then binds with α -catenin and links the whole complex with actin cytoskeleton.^{64,65}

VE-cadherin is required for forming vasculature, because loss of VE-cadherin embryonically induces lethality at E9.5 d.^{2,12,66} Neutralization of VE-cadherin with anti-VE-cadherin antibody or chelation of extracellular calcium using EDTA induce endothelial barrier leak. Truncation of the β -catenin binding site at the cytosolic domain of VE-cadherin or conditional inactivation of β -catenin in-

duces lethality in mice because of the disruption of endothelial junctions.⁶⁷ Expression of VE-cadherin mutant lacking the extracellular domain but with an intact β -catenin binding site was shown to disrupt endothelial barrier function. Overexpression of JMD of VE-cadherin, depleting endogenous p120-catenin, disrupted AJs, leading to the increase in endothelial permeability.^{40,68} Interestingly, depletion of p120-catenin in endothelial cells also modulated lung innate immune function by interfering with the association of Toll-like receptor 4 (TLR4) and its adaptor myeloid differentiation primary response gene (MyD88), preventing nuclear factor κ B cell (NF- κ B) activation and thereby the lung inflammatory response to endotoxin.⁶⁹ These findings suggest that β - and p120-catenin bound to VE-cadherin maintains endothelial permeability and innate response.

SIGNALING MECHANISMS REGULATING AJs

As discussed above, alteration of AJs adhesion primarily determines endothelial permeability to plasma proteins. In the following section, we describe the role of the signaling pathways that regulate the opening and resealing of AJs during the phases of increased permeability and of restoration of barrier function (Fig. 2).

Role of actin-myosin motor

Endothelial cells contract in response to permeability-increasing agonists, leading to opening of IEJs. Stress fibers composed of bundles of polymerized actin and myosin filaments form the contractile machinery of endothelial cells.² These fibers assemble in a characteristic manner in cultured endothelial cells in response to permeability-increasing mediators.^{12,70} Thrombin, an edemagenic agonist, increased isometric force in endothelial cell monolayers grown on a collagen matrix from a basal value of 70 to 132 dynes.^{71,72} However, endothelial cells developed comparatively less tension (1.3×10^5 dynes/cm²) than did contracting arterial smooth muscle cells (6.7×10^6 dynes/cm²),^{71,72} perhaps due to strong interendothelial adhesion between endothelial cells. Actinomyosin-generated contraction requires MYLK-dependent phosphorylation of the regulatory myosin light-chain (MLC) on Ser-19 (monophosphorylation) or Ser-19/Thr-18 (diphosphorylation). On thrombin stimulation of human umbilical vein endothelial cells, isometric contraction increased twofold to 2.5-fold within 5 minutes, whereas MLC phosphorylation increased from a basal value of 0.54 to 1.61 mol PO_4 /mol MLC within 60 seconds, primarily as the result of diphosphorylation of MLC.⁷³⁻⁷⁹ These studies showed diphosphorylation of MLC as an important determinant of endothelial contraction. MLC phosphorylation has also been shown to regulate

Figure 2. Signaling modulating adherens junctions (AJs). Agonists increase intracellular Ca^{2+} , which, by activating the myosin light-chain kinase (MLCK) and RhoA-Rho kinase pathway, induces actin stress fiber formation, leading to disruption of AJs. Additionally, protein kinase C (PKC), Src, and end-binding protein 3 (EB3) disrupt AJs adhesion by either phosphorylating them or by increasing microtubule dynamics. Several mechanisms are activated in parallel or synergistically to induce re-annealing of AJs. For example, focal adhesion kinase (FAK) phosphorylates neural Wiskott-Aldrich syndrome protein (N-WASP), enabling actin-related protein 2/3 (Arp2/3) to mediate cortical actin formation. Activated N-WASP also links p120-catenin to Arp2/3 and actin to stabilize AJs. FAK also induces Rac1 activity by inhibiting RhoA activity, which may further stabilize AJs. Activated Cdc42 may merge with FAK signaling to restore AJs formation. A color version of this figure is available online.

the permeability of intact isolated postcapillary venules and pulmonary microvessels in response to histamine and thrombin.⁸⁰⁻⁸² Mice lacking only the long isoform of MYLK present in endothelial cells support the *in vivo* role of MYLK-induced endothelial contraction in the mechanism of increased vascular permeability.⁸² *Mylk*^{-/-} mice were protected against increased lung vascular permeability and neutrophil adhesion induced by intraperitoneal injection of the bacterial product lipopolysaccharide (LPS),⁸² indicating a critical role of endothelial contraction induced by MYLK in mediating the increase in vascular permeability and inflammatory signaling.⁷⁸

Role of calcium

An increase in cytosolic Ca^{2+} precedes changes in endothelial cell shape and the opening of IEs.^{22,70,83,84} After cell stimulation with inflammatory mediators, an in-

crease in cytosolic Ca^{2+} concentration is apparent in two phases.^{22,70,84} There is an initial transient peak as the result of Ca^{2+} release from endoplasmic reticulum (ER) stores, which is followed by a more sustained response secondary to Ca^{2+} entry via plasmalemma channels. Ca^{2+} entry refills ER stores and sustains Ca^{2+} signaling.⁸⁴

Transient receptor potential (TRP) family has emerged as a predominant regulator of nonselective Ca^{2+} entry in endothelial cells.^{22,70,85,86} Approximately 28 TRP channels have been described so far in mammalian cells, which include TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin), and TRPA (ankyrin). TRPC, TRPM, and TRPV all have six transmembrane domains with a pore-forming unit located between transmembrane domains 5 and 6.^{2,87-89} TRPC and TRPM family members also contain protein-rich sequences in the C-terminal region of the TRP domain designated as TRP

box 2, which binds phosphatidyl inositol (PI) phosphates, such as PI (4, 5) P_2 .^{2,87,89} TRPC and TRPV family members also contain 3–4 ankyrin repeats at their N-terminus.^{2,87,90} The functional TRP channel is a tetramer and may be composed of homo- or heterotetramers. Evidence suggests that most of the TRP channels are modulated by Ca^{2+} itself, which may form positive or negative feedback signaling loops. In addition to their direct involvement in mediating Ca^{2+} entry, TRP channels are shown to be linked functionally with voltage-gated Ca^{2+} channels and to thereby amplify Ca^{2+} entry through them in a voltage-dependent manner.^{91,92}

TRPC channels are by far the most studied in endothelial cells.^{83,90,93,94} Expression of the various TRPCs differs among human endothelial cells, which may contribute to endothelial heterogeneity. The majority of human endothelial cells highly express TRPC1 and TRPC6, whereas TRPC3, TRPC4, and TRPC7 are expressed only weakly.^{70,90,95,96} TRPC1, TRPC4, and TRPC5 are activated by depletion of ER stores by IP₃ and are therefore called store-operated channels (SOCs), whereas TRPC3 and TRPC6 are activated by diacylglycerol (DAG) independently of store depletion and are referred to as receptor-operated channels (ROCs).^{70,83,95,96} SOCs and ROCs provide the primary structures by which Ca^{2+} enters endothelial cells. Multiple heterologous combinations of TRPCs (TRPC1 with TRPC4 or TRPC5 and TRPC3 with TRPC6) combine to produce tetrameric channels with unique properties.^{83,86,90,97}

SOC activity. TRPC1 and/or TRPC4 have been clearly shown to regulate SOC activity and thereby IEJs.^{22,85,86,98,99} Alterations of TRPC1 function in endothelial cells using TRPC1 antisense or an anti-TRPC1-blocking antibody reduced Ca^{2+} entry by 50% and also decreased endothelial permeability in response to thrombin.²² Overexpression of TRPC1 in endothelial cells increased Ca^{2+} entry and, in turn, the formation of actin stress fibers, and it also increased endothelial permeability.¹⁰⁰ In addition, the in vivo relevance of TRPC-induced Ca^{2+} entry in regulating permeability of microvessels has been demonstrated using *TRPC4*^{-/-} mice.⁹⁰ The PAR-1 (thrombin receptor) activation-induced increase in lung microvessel permeability in mice was reduced by 50% using lanthanum (La^{3+}), a known Ca^{2+} channel blocker, indicating Ca^{2+} entry by this pathway contributed to a portion of the increased vascular permeability response. *Trpc4*^{-/-} mice also demonstrated a reduction in the thrombin-induced increase in lung microvessel permeability by ~50%, whereas this response was not affected by La^{3+} .⁸⁵

Evidence from human endothelial cells indicates that a coupling between the IP₃ receptor and TRPC1 could be mediated through small GTPase RhoA, because inhibition of RhoA prevented the thrombin-stimulated association of the IP₃ receptor with TRPC1 and influx of Ca^{2+} .^{2,22} RhoA may facilitate the interaction of the IP₃ receptor with TRPC1 by promoting actin polymerization.²² Studies also showed that TRPC1 activity was suppressed by depolymerization of actin filaments consistent with this model.^{22,101} In addition, SOC-induced Ca^{2+} entry (SOCE) was shown to require the interaction of spectrin, an actin cross-linking protein, with protein 4.1.^{102,103} Another signaling possibility is that phosphorylation by protein kinase C (PKC) α is required for TRPC1 activation.⁹⁸ MYLK may also be involved in the regulation of SOC-induced Ca^{2+} entry, because inhibition of MYLK with ML7 prevented the SOC-mediated Ca^{2+} entry in endothelial cells.¹⁰⁴ Together, these findings raise several possibilities concerning the mechanism of SOC activation and influx of Ca^{2+} in endothelial cells.

STIM1, a type 1A single-transmembrane protein originally identified as a tumor-suppressor protein, has been established as a Ca^{2+} sensor within the ER stores.^{105,106} STIM1 contains N-terminal EF hand domain, a Ca^{2+} binding domain, a sterile α motif or SAM domain, a single transmembrane domain (TM), an Ezrin-radixin-moesin (ERM) domain, a serine-proline-rich region (S/P-region), and a lysine-rich region (E-region).^{105,107,108} The Ca^{2+} -binding EF domain and SAM domain regulate aggregation of STIM1 as well as its translocation to the plasma membrane from ER. When bound with Ca^{2+} in ER lumen, STIM1 exists as an individual unit or a monomer. Upon store depletion, the EF hand domains and SAM domains from different STIM1 protein aggregate and form multimeric puncta, which leads to its interaction with store-operated Ca^{2+} channel and activation of SOCE.^{84,106,108} STIM1 has been shown to regulate TRPC channels, such as TRPC1, TRPC4, TRPC5, and ORAI channels, which are the important constituents of SOC and CRAC channels, respectively.^{109,110} EF hand mutant STIM1D76A does not bind with Ca^{2+} and is constitutively active, whereas Δ ERM-STIM1 mutant acts as dominant negative STIM1.¹¹¹ Tiruppathi's group showed that SOC activates p38 MAP kinase, which, by phosphorylating STIM1, suppresses the endothelial SOCE.⁸⁴ These findings suggest that a negative feedback loop may be important in terminating SOCE and thereby restoring AJs.

STIM2 shares a sequence homology between the N-terminal domain as well as transmembrane sequences with STIM1 and is also present in endothelial cells as well as other cells. STIM2 differs from STIM1 protein in the C-terminal region. STIM2 deletion seems to alter basal

cytosolic Ca^{2+} but not SOCE.^{108,112} However, overexpression of STIM2 inhibited SOCE in HEK and smooth muscle cells as well as abolished CRAC activity in Jurkat cell line.^{108,113,114} Thus, unlike STIM1, the expression of STIM2 is inversely linked with SOC and CRAC activities.^{108,114}

ROC activity. TRPC6 is highly expressed in lung and lung endothelial cells.^{70,83,115} Several studies showed that TRPC6 plays an important role in regulating endothelial permeability.^{20,70,83,93} Flufenamic acid, a TRPC6 activator, increased water conductivity in frog mesenteric vessels.^{20,93} Singh et al.⁷⁰ showed that siRNA-induced suppression of the TRPC6 channel in human pulmonary artery endothelial cells decreased endothelial cell permeability in response to thrombin. Recently, we showed that TRPC6 plays a key role in signaling both LPS-induced lung vascular permeability and inflammation.⁸³ Tauseef et al.⁸³ showed that LPS resulted in DAG production, which activated TRPC6. Activated TRPC6 induced MYLK activity that, by stimulating actomyosin cross-bridging, mediates endothelial cell contraction, leading to increased lung vascular permeability.⁸³ Additionally, activated MYLK promoted the interaction of MyD88 with interleukin receptor 1-associated kinase 4, which is involved in triggering NF- κ B signaling and pulmonary inflammation downstream of TLR4.⁸³ Thus, TRPC6 appears to be at the center of the signaling pathways mediating acute lung injury (ALI) owing to its dual role in increasing lung vascular permeability and mediating TLR4 signaling (Fig. 3).⁸³

Role of PKC

PKC forms the family of protein kinase enzymes that are involved in controlling the function of several other proteins through the phosphorylation of hydroxyl groups of serine and threonine amino acid residues on these proteins.⁶⁴ PKC are activated by signals such as increases in the concentration of DAG or cytosolic calcium ions (Ca^{2+}).^{2,70} The PKC family consists of conventional (or classical), novel, and atypical isoforms.¹¹⁶ Conventional PKCs (α , β , β _{II}, and γ) require Ca^{2+} , DAG, and a phospholipid, such as phosphatidylserine, for activation.¹¹⁶⁻¹¹⁸ Novel PKCs, which include δ , ϵ , η , and θ isoforms, are activated by DAG independent of Ca^{2+} , whereas atypical PKCs, such as PKC ζ , require neither Ca^{2+} nor DAG for activity.^{2,116,119} Thus, conventional and novel PKCs are activated through the same signal transduction pathway as phospholipase C.

PKC α has a crucial role in mediating IEJ disassembly,⁹⁸ although Ca^{2+} -independent isoforms, notably PKC ζ ,

may also be important in IEJ disruption.¹¹⁹ Upon activation by endothelial permeability-increasing agonists, PKC α translocated from the cytosol to the IEJ, where it phosphorylated p120 catenin at serine 879, which destabilized junctions and increased endothelial permeability. Vandembroucke St Amant et al.⁶⁴ showed that p120 catenin phosphorylation at serine-879 exposed the VE-cadherin binding site for endocytic protein AP1, leading to internalization of VE-cadherin. Expression of phosphorylation-deficient mutant of p-120 catenin mutant in mouse lung microvessel failed to induce lung vascular permeability in response to thrombin receptor activation, thereby identifying p-120 phosphorylation at serine 879 residue as a crucial regulator of VE-cadherin internalization and endothelial monolayer permeability.⁶⁴

PKC β and PKC ζ inhibition also decreased endothelial permeability through impairment of Ca^{2+} levels, MLC contraction, and RhoGTPases.¹²⁰⁻¹²³ Studies have shown that PKC δ is required for the permeability increase caused by stimulation with PKC activators or DAG, whereas others suggest that PKC δ has a barrier-protective function.¹²³ Overexpression of PKC δ decreases endothelial permeability by increasing focal adhesion contacts.¹²³ Additional approaches, such as using PKC β or PKC δ knockout mice models, will help to clarify the contribution of these isoforms in mediating increased endothelial permeability.

Role of Rho-GTPases

Evidence supports the general concept that each of the GTPases, RhoA, Rac, and Cdc42, contribute to the regulation of AJ function, but their relationship with AJs appears to be complex, because AJs themselves can modify RhoGTPase activity.^{7,8,124} RhoA was found to induce disassembly of endothelial AJs in response to thrombin, histamine, bradykinin, or PAF.¹²⁵ Inhibition of Rac with *Clostridium sordellii* toxin (a specific inhibitor of Rac) caused AJ disruption in cultured endothelial cells and increased L_p of rat venular microvessels,¹²⁶ implicating Rac in promoting barrier function by stabilizing AJs. Schmidt et al.¹²⁷ recently showed that a fine balance between the activities of RhoA and Rac1 GTPases is required for maintaining AJs in mice lungs. They showed, using a mouse model lacking endothelial focal adhesion kinase (FAK, described below), that, in the absence of FAK, activated RhoA antagonized Rac1 activity, destabilizing AJs. Inhibition of Rho kinase, a downstream effector of RhoA, reinstated normal endothelial barrier function in FAK^{-/-} endothelial cells and lung vascular integrity in EC-FAK^{-/-} mice. The mechanism by which RhoA antagonizes Rac1 remains to be identified. FilGAP, a GTPase, specifically inhibits Rac1 downstream

Figure 3. Ca^{2+} entry through canonical transient receptor potential (TRPC) mediates loss of endothelial barrier function and inflammatory signaling. Agonists such as thrombin and vascular endothelial growth factor (VEGF), by activating their receptors on the endothelial cell surface, lead to generation of inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 induces Ca^{2+} entry through store-operated channels (SOCs) such as TRPC1 and TRPC4. DAG directly activates the receptor-operated channel (TRPC6). Endotoxin lipopolysaccharide (LPS), by activating Toll-like receptor 4 (TLR4), induces DAG production, which activates TRPC6. Activated TRPC6 induces myosin light-chain kinase (MYLK) activity that, by stimulating actomyosin cross-bridging, mediates endothelial cell contraction, leading to increased lung vascular permeability. Additionally, activated MYLK promotes myeloid differentiation primary response gene (MyD88) interaction with interleukin-1 receptor-associated kinase 4 (IRAK4), triggering nuclear factor κ B cell (NF- κ B) signaling and pulmonary inflammation. CD14: cluster of differentiation 14; ER: endoplasmic reticulum; PAR-1: protease activating receptor 1.

of RhoA. Thus, it is possible that RhoA may induce FilGAP activity to suppress Rac1 and thereby disrupt AJs. Kouklis et al.¹²⁸ showed that the re-annealing of AJs following the increase in endothelial permeability by thrombin required the activation of Cdc42. Mice or ECs transducing activated form of Cdc42 showed reduced barrier leak, indicating that Cdc42 activity preserved junctional integrity. RhoA activity in V12Cdc42-expressing endothelial monolayers was reduced, compared with untransfected cells, suggesting that activated Cdc42 functions by counteracting the canonical RhoA-mediated mechanism of endothelial hyperpermeability.¹²⁹

Role of FAK

FAK, a 120-kD nonreceptor tyrosine kinase, regulates turnover of focal adhesion formation by binding to focal adhesion proteins, such as vinculin, talin, and paxillin.^{2,127} Global as well as endothelial cell-specific deletion of FAK induces lethality in embryos because of improper formation of blood vessels. Several studies have shown that FAK maintains basal endothelial barrier function and induces resealing of endothelial junctions following the increase in endothelial permeability by thrombin or H_2O_2 . For example, FAK was shown to bind with p120-catenin and

suppress the activity of the small GTPase RhoA to restrict endothelial contraction and thereby inducing AJs formation.¹⁰⁹ Quadri et al.¹³⁰ also showed that FAK prevents oxidant-induced barrier dysfunction by regulating VE-cadherin phosphorylation. Also, siRNA-induced depletion of FAK or expression of dominant FAK impaired AJ formation and enhancement of barrier function by sphingosine 1 phosphate and lysophosphatidic acid.¹³¹ However, expression of dominant negative FAK or kinase-dead FAK mutant in endothelial cells prevented AJ disruption in response to VEGF or oxidants, indicating FAK intact disrupts the barrier function.¹³² To resolve the role of FAK in regulating lung vascular barrier function, Schmidt et al.¹²⁷ generated tamoxifen-inducible endothelial-specific FAK knockout mice. These authors showed that loss of EC-FAK induced diffuse lung hemorrhage and increased transvascular albumin influx, edema, and neutrophil accumulation in the lung due to disruption of AJs. Induction of ALI by intraperitoneal administration of LPS or cecal ligation and puncture in wild-type mice markedly decreased FAK expression in lungs, indicating that decreased endothelial FAK expression is therefore a likely factor responsible for the pulmonary vascular hyperpermeability and edema formation seen during ALI.

Role of Src kinase

Src activation occurs downstream of integrin activation that follows integrin clustering. Among various members of Src family kinases, pp60Src kinase has been shown to contribute to increased endothelial permeability in response to production of superoxide anion ($O^{\cdot -}$),¹³³ thrombin,¹³⁴ and VEGF.¹³⁵ VEGF, histamine, and thrombin resulted in Src activation and tyrosine phosphorylation of VE-cadherin at Y658 and Y731, which correspond to the binding sites for p120 and β -catenin, respectively.¹³⁶ Phosphorylation of VE-cadherin resulted in internalization of the protein leading to disruption of barrier. In addition, three other tyrosine residues, namely Y645, Y685, and Y733, in VE-cadherin were identified as phosphorylated. More importantly, point mutations Y658F and Y685F prevented internalization of VE-cadherin and thus blocked vascular permeability.¹³⁷ The endogenous phosphatase for VE-cadherin, VE-PTP, is frequently associated with the protein and prevents VE-cadherin phosphorylation, promoting an important stabilizing role of endothelial contacts *in vivo*.¹³⁸ How VE-PTP and p60Src compete for binding VE-cadherin to regulate AJs needs to be parsed out.

Proline-rich tyrosine kinase 2 (PYK2), a Ca^{2+} -dependent noneceptor tyrosine kinase structurally related to FAK, also binds to integrins and is highly expressed in pulmonary endothelial cells.¹³⁹ PYK2 is rapidly phosphorylated following exposure of endothelial cells to agonists (e.g., angiotensin) and mechanical stress (e.g., cyclic stretch).¹⁴⁰ Evidence indicates that PYK2 can activate RhoA in fibroblasts,¹⁴¹ suggesting that PYK2 may be involved in regulating endothelial barrier function by this mechanism.

Role of end-binding proteins

Microtubules form a lattice of rigid hollow rods spanning the cell in a polarized fashion from nucleus to periphery. Microtubules are presumed to resist compression of cells by opposing actin-myosin contractility.¹⁴² Microtubules (typically having a diameter of ~25 nm) are heterodimers organized by self-assembly of α - and β -tubulins (molecular weight of 55 kDa each).¹⁴² Microtubules undergo “dynamic treadmilling” (i.e., alternating addition and removal of tubulin dimers from the microtubule).⁸ The faster-growing end, the plus end, is made up of β -tubulin that binds to and hydrolyzes GTP to GDP, whereas α -tubulin constitutes the minus end and binds to but does not hydrolyse GTP. The plus ends of microtubules are attached to the cell cortex and exhibit “dynamic instability,” because they shift between phases of lengthening and shortening.¹⁴³ The minus ends are typically joined at a common attachment site in the cell called the microtubule-organizing center.¹⁴⁴ Disruption of MT is shown to impair

AJs. Likewise, stabilization of MTs prevented AJs disruption by permeability-increasing agonists.¹⁴⁴

Endbinding protein (EB) family members are evolutionary conserved proteins that bind MT⁺ ends.¹⁴⁵ EB1 and EB3 are ubiquitously expressed, whereas expression levels of EB2 vary between cell lines.¹⁴⁶ They can form homo- or heterodimers.¹⁴⁷ Komarova et al.¹⁴⁸ recently identified a key role of EB3 in regulating AJs adhesion by stabilizing MTs. They showed that phosphorylation of EB3 at S162 induced MT growth in confluent endothelial monolayers, which disassembled AJs. Whether EB3 bound directly with VE-cadherin and thereby orchestrated VE-cadherin homophilic interaction remains unclear.

Role of neural Wiskott-Aldrich syndrome protein

The cortical actin, the actin ring near the plasma membrane, stabilizes AJs. Disruption of preformed actin filaments by C2 toxin or cytochalasin is known to increase endothelial permeability in intact microvessels and cultured cells. Edemagenic agonists, such as thrombin, rapidly induce actin reorganization from cortical actin into actin stress fibers, which induce AJ disruption. In this regard, proteins that maintain cortical actin confirmation play a key role in maintaining AJs.

The actin-related protein 2/3 (Arp2/3) complex generates actin filament-driven protrusive force, which may stabilize AJs. Arp2 was shown to interact with E-cadherin, indicating protein-regulating Arp2 function may contribute to AJs stability. The family of Wiskott-Aldrich syndrome proteins (WASPs) share similar domain structure and are involved in transduction of signals from receptors on the cell surface to the actin cytoskeleton. Neural WASP (N-WASP) induces actin polymerization by activating Arp2.¹⁴⁹ In resting cells, N-WASP is auto-inhibited due to binding of C-terminus verprolin central acidic (VCA) domain with its GTPase domain. Several mechanisms have been implicated in facilitating the unfolding of the VCA domain from the GBD domain, which includes WASP phosphorylation by tyrosine kinases, interaction with PIP₃, or Cdc42 GTPases.¹⁵⁰ Rajput et al.¹⁵¹ showed that the depletion of N-WASP impaired AJ adhesion and promoted actin organization and stress fiber formation, leading to leaky endothelial barrier. These authors showed that N-WASP formed a complex with Arp2/3 and p120 catenin. Knockdown of Arp2 did not inhibit N-WASP interaction with p120 catenin, suggesting that N-WASP binds directly to p120 catenin and induces cortical actin formation, thereby stabilizing AJs.¹⁵¹ However, N-WASP phosphorylation at Y256 residue by FAK was required for reformation of cortical actin and p120-catenin interaction, because expression of phosphodeficient N-WASP mutant

impaired its interaction with p120-catenin and Arp2, leading to disruption of AJs and loss of endothelial barrier function. Overexpression of phosphomimicking mutant of N-WASP, on the other hand, enhanced basal barrier function and facilitated re-annealing of AJs and barrier restoration. These findings suggest that FAK-mediated N-WASP phosphorylation is a critical event in maintaining AJs and in restoration of endothelial barrier.¹⁵¹

MEDIATORS OF INCREASED ENDOTHELIAL PERMEABILITY

Inflammatory mediators

Histamine, thrombin, bradykinin, VEGF, and Ang2 are shown to disrupt AJs, thereby leading to increase in endothelial permeability. Thrombin disrupts AJs by binding to its receptors, PAR-1, on endothelial cells.⁹⁵ Thrombin induces proteolysis of the PAR-1 extracellular extension (between Arg 41 and Ser 42), generating PAR1 ligand which, by binding to the second extracellular loop of heptahelical PAR-1, initiates downstream signaling events.¹⁵² The synthetic 14-amino acid peptide, SFLLRNPNDKYEPF (TRP-14), can substitute for this tethered ligand.¹⁵³ Three other isoforms of PARs, PAR2, PAR3, and PAR4 have been identified in endothelial cells, of which only PAR3 and PAR4 are activated by thrombin.¹⁵⁴ PAR-2 is activated by tryptase.¹⁵⁵ The role of the other PARs in mediating increases in endothelial barrier permeability has not been fully elucidated, but those PARs may work in concert with PAR1 to facilitate changes in barrier function. For example, the transactivation of PAR2 by thrombin ligation of PAR1 has been demonstrated in COS7 cells and in HUVECs, which in turn contributes to thrombin signaling.¹⁵⁶ Selective activation and desensitization of PAR2 reduces subsequent responses to thrombin, suggesting that the transactivation of PAR2 by cleaved PAR1 occurs normally. A study showed that PAR2 activity was required for reversing PAR1-induced vascular leak following sepsis.¹⁵⁷ Similarly, it has been shown that PAR2 agonism exerts anti-inflammatory effects following LPS-induced lung injury in rodents.¹⁵⁸ Therefore, although PAR1 mediates thrombin-induced increase in lung vascular permeability, the role of other PARs in amplifying or suppressing thrombin-induced increase in endothelial permeability has yet to be understood. The role of PAR-3 and PAR-4 in mediating an increase in endothelial permeability has not been established, but evidence indicates that they may cooperate with PAR1 to increase endothelial permeability. PAR-1 induces a transient increase in vascular permeability that is followed by an equally rapid restoration of barrier function. PAR1 couples to the heterotrimeric G proteins of the G_q, G_{12/13}, G_i,

and G_s families,^{159,160} and initial studies predicted all of the G proteins to mediate increases in endothelial barrier permeability. It has been generally accepted that thrombin increases endothelial permeability by activating MYLK and RhoA via the G_{αq} and G_{α12/13} subunits; however, G_{αs} enhances barrier function through cAMP generation.⁸⁰ Additionally, dissociated G_{βγ} subunits have also emerged as barrier protective by inducing FAK activity.¹⁶¹ Studies also suggest that sphingosine kinase 1 maintains AJs and reverses the increase in endothelial permeability by thrombin by paracrine mechanism involving S1P generation.¹⁶²

Histamine increases endothelial permeability through increasing intracellular calcium and MYLK activation but, in addition, mediates Src-dependent phosphorylation of AJ and TJ proteins. Conversely, bradykinin, acting through B1 and B2 receptors, results in an eNOS/iNOS-dependent increase in permeability, although it is unclear whether nitrosylation of junctional proteins leads to barrier destabilization.⁶

Sphingosine 1 phosphate (S1P) circulates at high levels in the blood and signals through the G-coupled protein receptor S1P receptors (S1PR), including S1PR1, S1PR2, and S1PR3.¹⁶³ S1PR1 is predominantly studied in endothelial cells and is shown to strengthen endothelial barrier integrity (discussed below). Garcia's group recently showed a barrier-disruptive role of S1PR3.¹⁶⁴ They showed that human lung vascular ECs shed S1PR3 in microparticles following activation with LPS or low-molecular weight hyaluronan. Exposure of normal lung ECs to S1PR3-containing microparticles significantly reduced TER, consistent with increased permeability. These changes were attenuated by RNAi-mediated depletion of S1PR3 in ECs. Intriguingly, elevated total S1PR3 plasma concentrations were linked to sepsis and ALI mortality, indicating S1PR3 as a novel ALI biomarker linked to disease severity and outcome.¹⁶⁴

Long-term mediators of permeability, such as endotoxin, LPS, and tumor necrosis factor, result in NF-κB transcriptional expression of cytokines and leukocyte adhesion molecules.^{2,7} ICAM-1 cell surface activation results in RhoA-directed stress fiber formation as well as increased NO production, which further potentiates increased permeability. Many studies have shown that the ECs are crucial in mediating the lung's inflammatory response by LPS. LPS binds the endothelial TLR4 via CD14, a membrane-bound glycosylphosphatidyl inositol-anchored protein. TLR4 in turn activates signaling pathways responsible for the generation of proinflammatory cytokines via MyD 88, which in turn activates the NF-κB signaling and produces the proinflammatory cytokines. We have recently shown that TRPC6 mediates Ca²⁺ entry into ECs secondary to TLR4-induced

DAG generation, which participates in mediating both lung vascular barrier disruption and inflammation induced by endotoxin.⁸³

VEGF induces vascular permeability by several mechanisms, including AJ remodelling, induction of fenestrae, and VVOs formation.¹⁶⁵ VEGF concurrently activates multiple signaling pathways downstream of VEGFR2 that have been implicated in vascular permeability. These include PLC-dependent intracellular calcium release, Src kinase-mediated phosphorylation/internalization of junctional proteins, RhoGTPase activation, cytoskeletal rearrangement, and eNOS signalling.¹⁶⁶ More recently, *in vivo* data showed that VEGF mediates these effects by inducing VE-PTP/VE-cadherin dissociation¹⁶⁷ and FAK-dependent β -catenin phosphorylation.¹³² Furthermore, the T cell-specific adapter, TSAd, was found to be essential for Src activation and subsequent phosphorylation of junctional proteins downstream of VEGFR2.¹⁶⁸ The contribution of eNOS signaling upon VEGFR2 activation has remained elusive, but recent data suggest that nitrosylation of β -catenin by NO may be an additional mechanism of junctional destabilization by mediating the dissociation of β -catenin from VE-cadherin.¹⁶⁹

Ang-2 sensitizes the endothelium to both growth factors and inflammatory mediators, which increases vascular destabilization.¹⁷⁰ Although both Ang-1 and Ang-2 mediate Tie-2 clustering at cell-cell contacts, their differential signaling may explain their opposing effects on vascular stability. Several groups have also demonstrated that Ang-2 can act as a partial agonist of Tie-2 signaling through Tie-2 phosphorylation¹⁷¹ and can enhance barrier function following endothelial stress. Generation of mice with endothelial-specific deletion of Ang-2 will help to address its physiological role *in vivo* and enable a better understanding of the homeostatic functions of Ang-2 in the endothelium. To determine the role of Ang2 in sepsis, Ang2 antibody treatment attenuated LPS-induced hemodynamic alterations and reduced the mortality rate at 36 hours from 95% to 61%. These data clearly indicate that Ang2-mediated microvascular disintegration contributes to septic shock and suggest that inhibition of the Ang2/Tie2 interaction during sepsis is a potential therapeutic target.¹⁷²

ENDOTHELIAL BARRIER-STABILIZING MEDIATORS

Barrier restoration is critical for maintenance of basal permeability and recovery following exposure to acute inflammatory events, yet our understanding of how this process occurs at the molecular level has remained elusive. Here, we discuss some of the mediators of barrier stability and their known mechanisms of action.

Increase in cAMP levels reduces vascular leakage through activation of protein kinase A (PKA) and the guanine exchange factor, Epac. Epac-mediated Rap1 activation results in increased junctional adhesions and reorganization of actin filaments.¹⁷³ Emerging evidence on Rap1 suggests it has a cooperative association with VE-cadherin, because they can both modulate each other's responses. Interestingly, Rap1 can increase Krev interaction trapped gene 1 (KRIT-1) targeting to endothelial cell-cell junctions, suppressing stress fiber formation and stabilizing the barrier (KRIT-1 is known to bind to Rap1, a guanosine triphosphatase that maintains the integrity of endothelial junctions). Thus, defects in Rap1 signaling downstream of mutated KRIT-1 protein may explain the loss of vascular integrity seen in cerebral cavernous malformations.¹⁷⁴

More recently, fibroblast growth factor (FGF) has been found to play an important role in AJ integrity. Absence of FGF signaling was found to reduce expression of the phosphatase SHP2, resulting in increased phosphorylation of VE-cadherin, impairing its ability to bind p120 catenin.¹⁷⁵ VE-cadherin itself can affect barrier stability by inhibiting growth factor signalling pathways, including VEGF, TGF β , and PDGF, which promote permeability following angiogenic responses.⁴¹

Another emerging and potent barrier-stabilizing factor is sphingosine-1-phosphate (S1P). S1P mediates its effect on endothelial cells by ligating S1PR1 to induce cortical actin organization via a number of downstream targets, including Rac-1, cortactin, FAK, paxillin, and actinin 1 and 4.¹²⁹ Two recent studies have unequivocally demonstrated the effect of S1P in barrier stability *in vivo*. Pharmacological or genetic blockade of the S1P signaling axis results in AJs destabilization, permeability, and, in some cases, aberrant angiogenesis.

Tie receptors and their ligands (Ang1–4) are critical regulators of vascular maturation and quiescence.¹⁷⁶ One of the main questions in understanding Ang-1 signaling is how it can orchestrate both vascular remodelling and quiescence by signalling through the same receptor. Recent evidence suggests that Ang-1 stimulation leads to differential Tie-2 localization and signaling, depending on whether endothelial cells have engaged cell-cell contacts. Homotypic cell interactions between endothelial cells trigger recruitment of Tie2 to cell-cell contacts upon Ang-1 exposure, leading to enhanced vascular stability following Akt-mediated eNOS phosphorylation.¹⁷⁷

SUMMARY

In this review, we have emphasized the role of signaling mechanisms regulating AJs and thereby regulating endo-

thelial permeability. In general, increase in intracellular calcium by TRPC and consequent activation of RhoA and MLCK induce AJ disruption. In parallel, overlapping signals are initiated by kinases such as Src, PKC, and CAMK to induce AJ disruption either directly by mediating the phosphorylation of AJ components or through EB proteins. We have also discussed the roles of Rac1, Cdc42, FAK, SPHK1, N-WASP, and Rap1 in opposing AJ disruption in facilitating barrier formation. We believe underpinning of the commonalities of these complex signaling pathways will define targets for facilitating AJ formation and certainly will pave the way for many new therapeutic and prophylactic interventions.

Source of Support: This work was supported by National Institutes of Health grants HL71794, HL84153, and HL060678 as well as an American Heart Association postdoctoral fellowship to SS (12 Post 12080112).

Conflict of Interest: None declared.

REFERENCES

- Dudek SM, Garcia JG. Cytoskeletal regulation of pulmonary vascular permeability. *J Appl Physiol* (1985) 2001;91:1487–1500.
- Mehta D, Malik AB. Signaling mechanisms regulating endothelial permeability. *Physiol Rev* 2006;86:279–367.
- Vogel SM, Malik AB. Cytoskeletal dynamics and lung fluid balance. *Compr Physiol* 2012;2:449–478.
- Aird WC. Endothelial cell heterogeneity. *Cold Spring Harb Perspect Med* 2012;2:a006429.
- Aird WC. Phenotypic heterogeneity of the endothelium: I. Structure, function, and mechanisms. *Circ Res* 2007;100:158–173.
- Chavez A, Smith M, Mehta D. New insights into the regulation of vascular permeability. *Int Rev Cell Mol Biol* 2011;290:205–248.
- Vandenbroucke E, Mehta D, Minshall R, Malik AB. Regulation of endothelial junctional permeability. *Ann N Y Acad Sci* 2008;1123:134–145.
- Komarova YA, Mehta D, Malik AB. Dual regulation of endothelial junctional permeability. *Sci STKE* 2007;2007:re8.
- Pober JS, Sessa WC. Evolving functions of endothelial cells in inflammation. *Nat Rev Immunol* 2007;7:803–815.
- Wang Z, Malik AB. Nanoparticles squeezing across the blood-endothelial barrier via caveolae. *Ther Deliv* 2013;4:131–133.
- Dvorak AM, Kohn S, Morgan ES, Fox P, Nagy JA, Dvorak HF. The vesiculo-vacuolar organelle (VVO): a distinct endothelial cell structure that provides a transcellular pathway for macromolecular extravasation. *J Leukoc Biol* 1996;59:100–115.
- Komarova Y, Malik AB. Regulation of endothelial permeability via paracellular and transcellular transport pathways. *Annu Rev Physiol* 2010;72:463–493.
- Predescu D, Palade GE. Plasmalemmal vesicles represent the large pore system of continuous microvascular endothelium. *Am J Physiol* 1993;265:H725–H733.
- Predescu SA, Predescu DN, Palade GE. Endothelial transcytotic machinery involves supramolecular protein-lipid complexes. *Mol Biol Cell* 2001;12:1019–1033.
- Drab M, Verkade P, Elger M, et al. Loss of caveolae, vascular dysfunction, and pulmonary defects in caveolin-1 gene-disrupted mice. *Science* 2001;293:2449–2452.
- Miyawaki-Shimizu K, Predescu D, Shimizu J, Broman M, Predescu S, Malik AB. siRNA-induced caveolin-1 knock-down in mice increases lung vascular permeability via the junctional pathway. *Am J Physiol Lung Cell Mol Physiol* 2006;290:L405–L413.
- Machleidt T, Li WP, Liu P, Anderson RG. Multiple domains in caveolin-1 control its intracellular traffic. *J Cell Biol* 2000;148:17–28.
- Shajahan AN, Tiruppathi C, Smrcka AV, Malik AB, Minshall RD. G $\beta\gamma$ activation of *Src* induces caveolae-mediated endocytosis in endothelial cells. *J Biol Chem* 2004;279:48055–48062.
- Esser S, Lampugnani MG, Corada M, Dejana E, Risau W. Vascular endothelial growth factor induces VE-cadherin tyrosine phosphorylation in endothelial cells. *J Cell Sci* 1998;111(Pt 13):1853–1865.
- Bates DO, Harper SJ. Regulation of vascular permeability by vascular endothelial growth factors. *Vascul Pharmacol* 2002;39:225–237.
- Knezevic N, Roy A, Timblin B, et al. GDI-1 phosphorylation switch at serine 96 induces RhoA activation and increased endothelial permeability. *Mol Cell Biol* 2007;27:6323–6333.
- Mehta D, Ahmmed GU, Paria BC, et al. RhoA interaction with inositol 1,4,5-trisphosphate receptor and transient receptor potential channel-1 regulates Ca²⁺ entry. Role in signaling increased endothelial permeability. *J Biol Chem* 2003;278:33492–33500.
- Munoz-Chapuli R, Perez-Pomares JM, Macias D, Garcia-Garrido L, Carmona R, Gonzalez M. Differentiation of hemangioblasts from embryonic mesothelial cells? a model on the origin of the vertebrate cardiovascular system. *Differentiation* 1999;64:133–141.
- Park C, Kim TM, Malik AB. Transcriptional regulation of endothelial cell and vascular development. *Circulation Research* 2013;112:1380–1400.
- Stevens T, Phan S, Frid MG, Alvarez D, Herzog E, Stenmark KR. Lung vascular cell heterogeneity: endothelium, smooth muscle, and fibroblasts. *Proc Am Thorac Soc* 2008;5:783–791.
- Parker JC, Stevens T, Randall J, Weber DS, King JA. Hydraulic conductance of pulmonary microvascular and macrovascular endothelial cell monolayers. *Am J Physiol Lung Cell Mol Physiol* 2006;291:L30–L37.
- Lowe K, Alvarez D, King J, Stevens T. Phenotypic heterogeneity in lung capillary and extra-alveolar endothelial cells. Increased extra-alveolar endothelial permeability is sufficient to decrease compliance. *J Surg Res* 2007;143:70–77.
- Chi JT, Chang HY, Haraldsen G, et al. Endothelial cell diversity revealed by global expression profiling. *Proc Natl Acad Sci U S A* 2003;100:10623–10628.
- Bolton SJ, Anthony DC, Perry VH. Loss of the tight junction proteins occludin and zonula occludens-1 from cerebral vascular endothelium during neutrophil-induced blood-brain barrier breakdown in vivo. *Neuroscience* 1998;86:1245–1257.

30. Mavrakis AG, Havaki S, Marinos E, Chroni E, Konstantinou D. Occludin dislocation in brain capillary endothelium of rats with bile duct ligation induced cholestasis. *Neurosci Lett* 2012;528:180–184.
31. Van Itallie CM, Fanning AS, Holmes J, Anderson JM. Occludin is required for cytokine-induced regulation of tight junction barriers. *J Cell Sci* 2010;123:2844–2852.
32. Giorgi D, Ferraz C, Mattei MG, Demaille J, Rouquier S. The myosin light chain kinase gene is not duplicated in mouse: partial structure and chromosomal localization of Mylk. *Genomics* 2001;75:49–56.
33. Cheng AK, Robertson EJ. The murine LIM-kinase gene (*limk*) encodes a novel serine threonine kinase expressed predominantly in trophoblast giant cells and the developing nervous system. *Mech Dev* 1995;52:187–197.
34. Zhang J, Wang W, Sun J, et al. Gap junction channel modulates pulmonary vascular permeability through calcium in acute lung injury: an experimental study. *Respiration* 2010;80:236–245.
35. Malek AM, Jiang L, Lee I, Sessa WC, Izumo S, Alper SL. Induction of nitric oxide synthase mRNA by shear stress requires intracellular calcium and G-protein signals and is modulated by PI 3 kinase. *Biochem Biophys Res Commun* 1999;254:231–242.
36. Tseng H, Peterson TE, Berk BC. Fluid shear stress stimulates mitogen-activated protein kinase in endothelial cells. *Circ Res* 1995;77:869–878.
37. Kedem O, Katchalsky A. Thermodynamic analysis of the permeability of biological membranes to non-electrolytes. *Biochim Biophys Acta* 1958;27:229–246.
38. Kedem O, Katchalsky A. Thermodynamic analysis of the permeability of biological membranes to non-electrolytes. 1958. *Biochim Biophys Acta* 1989;1000:413–430.
39. Melchior B, Frangos JA. Shear-induced endothelial cell-cell junction inclination. *Am J Physiol Cell Physiol* 2010;299:C621–C629.
40. Giannotta M, Trani M, Dejana E. VE-cadherin and endothelial adherens junctions: active guardians of vascular integrity. *Dev Cell* 2013;26:441–454.
41. Dejana E, Giampietro C. Vascular endothelial-cadherin and vascular stability. *Curr Opin Hematol* 2012;19:218–223.
42. Taddei A, Giampietro C, Conti A, et al. Endothelial adherens junctions control tight junctions by VE-cadherin-mediated upregulation of claudin-5. *Nat Cell Biol* 2008;10:923–934.
43. Runkle EA, Mu D. Tight junction proteins: from barrier to tumorigenesis. *Cancer Lett* 2013;337:41–48.
44. Kevil CG, Okayama N, Trocha SD, et al. Expression of zonula occludens and adherens junctional proteins in human venous and arterial endothelial cells: role of occludin in endothelial solute barriers. *Microcirculation* 1998;5:197–210.
45. Saitou M, Furuse M, Sasaki H, et al. Complex phenotype of mice lacking occludin, a component of tight junction strands. *Mol Biol Cell* 2000;11:4131–4142.
46. Morita K, Sasaki H, Furuse M, Tsukita S. Endothelial claudin: claudin-5/TMVCF constitutes tight junction strands in endothelial cells. *J Cell Biol* 1999;147:185–194.
47. Nitta T, Hata M, Gotoh S, et al. Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. *J Cell Biol* 2003;161:653–660.
48. Tsukita S, Furuse M. The structure and function of claudins, cell adhesion molecules at tight junctions. *Ann N Y Acad Sci* 2000;915:129–135.
49. Aurrand-Lions M, Johnson-Leger C, Wong C, Du Pasquier L, Imhof BA. Heterogeneity of endothelial junctions is reflected by differential expression and specific subcellular localization of the three JAM family members. *Blood* 2001;98:3699–3707.
50. Aurrand-Lions MA, Duncan L, Du Pasquier L, Imhof BA. Cloning of JAM-2 and JAM-3: an emerging junctional adhesion molecular family? *Curr Top Microbiol Immunol* 2000;251:91–98.
51. Arcangeli ML, Frontera V, Aurrand-Lions M. Function of junctional adhesion molecules (JAMs) in leukocyte migration and homeostasis. *Arch Immunol Ther Exp (Warsz)* 2013;61:15–23.
52. Koenen RR, Pruessmeyer J, Soehnlein O, et al. Regulated release and functional modulation of junctional adhesion molecule A by disintegrin metalloproteinases. *Blood* 2009;113:4799–4809.
53. Liang TW, DeMarco RA, Mrsny RJ, et al. Characterization of huJAM: evidence for involvement in cell-cell contact and tight junction regulation. *Am J Physiol Cell Physiol* 2000;279:C1733–C1743.
54. Lippoldt A, Liebner S, Andbjer B, et al. Organization of chorioid plexus epithelial and endothelial cell tight junctions and regulation of claudin-1, -2 and -5 expression by protein kinase C. *Neuroreport* 2000;11:1427–1431.
55. Lakshmi SP, Reddy AT, Naik MU, Naik UP, Reddy RC. Effects of JAM-A deficiency or blocking antibodies on neutrophil migration and lung injury in a murine model of ALI. *Am J Physiol Lung Cell Mol Physiol* 2012;303:L758–L766.
56. Gonzalez-Mariscal L, Betanzos A, Avila-Flores A. MAGUK proteins: structure and role in the tight junction. *Semin Cell Dev Biol* 2000;11:315–324.
57. Fanning AS, Jameson BJ, Jesaitis LA, Anderson JM. The tight junction protein ZO-1 establishes a link between the transmembrane protein occludin and the actin cytoskeleton. *J Biol Chem* 1998;273:29745–29753.
58. Tsukita S, Katsuno T, Yamazaki Y, Umeda K, Tamura A. Roles of ZO-1 and ZO-2 in establishment of the belt-like adherens and tight junctions with paracellular permselective barrier function. *Ann N Y Acad Sci* 2009;1165:44–52.
59. Goodenough DA, Paul DL. Beyond the gap: functions of unpaired connexon channels. *Nat Rev Mol Cell Biol* 2003;4:285–294.
60. Van Rijen H, van Kempen MJ, Analbers LJ, et al. Gap junctions in human umbilical cord endothelial cells contain multiple connexins. *Am J Physiol* 1997;272:C117–C130.
61. Carter TD, Ogden D. Acetylcholine-stimulated changes of membrane potential and intracellular Ca²⁺ concentration recorded in endothelial cells in situ in the isolated rat aorta. *Pflügers Arch* 1994;428:476–484.
62. Islam MN, Das SR, Emin MT, et al. Mitochondrial transfer from bone-marrow-derived stromal cells to pulmonary alveoli protects against acute lung injury. *Nat Med* 2012;18:759–765.
63. Dejana E, Tournier-Lasserre E, Weinstein BM. The control of vascular integrity by endothelial cell junctions: molecular

- basis and pathological implications. *Dev Cell* 2009;16:209–221.
64. Vandenbroucke St Amant E, Tauseef M, Vogel SM, et al. PKC α activation of p120-catenin serine 879 phospho-switch disassembles VE-cadherin junctions and disrupts vascular integrity. *Circ Res* 2012;111:739–749.
 65. Zebda N, Tian Y, Tian X, et al. Interaction of p190RhoGAP with C-terminal domain of p120-catenin modulates endothelial cytoskeleton and permeability. *J Biol Chem* 2013;288:18290–18299.
 66. Carmeliet P, Lampugnani MG, Moons L, et al. Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. *Cell* 1999;98:147–157.
 67. Tian Z, Dong B, Blackwell JW, Stewart PW, Egan TM. Effect of a vascular endothelial cadherin antagonist in a rat lung transplant model. *Ann Thorac Surg* 2013;95:1028–1033.
 68. Broman MT, Kouklis P, Gao X, et al. Cdc42 regulates adherens junction stability and endothelial permeability by inducing α -catenin interaction with the vascular endothelial cadherin complex. *Circ Res* 2006;98:73–80.
 69. Wang YL, Malik AB, Sun Y, et al. Innate immune function of the adherens junction protein p120-catenin in endothelial response to endotoxin. *J Immunol* 2011;186:3180–3187.
 70. Singh I, Knezevic N, Ahmmed GU, Kini V, Malik AB, Mehta D. Galphaq-TRPC6-mediated Ca^{2+} entry induces RhoA activation and resultant endothelial cell shape change in response to thrombin. *J Biol Chem* 2007;282:7833–7843.
 71. Kolodney MS, Wysolmerski RB. Isometric contraction by fibroblasts and endothelial cells in tissue culture: a quantitative study. *J Cell Biol* 1992;117:73–82.
 72. Morel NM, Petruzzio PP, Hechtman HB, Shepro D. Inflammatory agonists that increase microvascular permeability in vivo stimulate cultured pulmonary microvessel endothelial cell contraction. *Inflammation* 1990;14:571–583.
 73. Katoh K, Kano Y, Amano M, Kaibuchi K, Fujiwara K. Stress fiber organization regulated by MLCK and Rho-kinase in cultured human fibroblasts. *Am J Physiol Cell Physiol* 2001;280:C1669–C1679.
 74. Kaye AD, Nossaman BD, Ibrahim IN, Kadowitz PJ. Influence of PLC and MLCK inhibitors and the role of L-calcium channels in the cat pulmonary vascular bed. *Am J Physiol* 1995;269:L507–L513.
 75. Lazar V, Garcia JG. A single human myosin light chain kinase gene (MLCK; MYLK). *Genomics* 1999;57:256–267.
 76. Rao K, He JA, Halayko AJ, Pan N, Kepron W, Stephens NL. Increased ATPase activity and myosin light chain kinase (MLCK) content in airway smooth muscle from sensitized dogs. *Adv Exp Med Biol* 1991;304:369–376.
 77. Totsukawa G, Yamakita Y, Yamashiro S, Hartshorne DJ, Sasaki Y, Matsumura F. Distinct roles of ROCK (Rho-kinase) and MLCK in spatial regulation of MLC phosphorylation for assembly of stress fibers and focal adhesions in 3T3 fibroblasts. *J Cell Biol* 2000;150:797–806.
 78. Yanase M, Ikeda H, Ogata I, et al. Functional diversity between Rho-kinase- and MLCK-mediated cytoskeletal actions in a myofibroblast-like hepatic stellate cell line. *Biochem Biophys Res Commun* 2003;305:223–228.
 79. Goeckeler ZM, Wysolmerski RB. Myosin light chain kinase-regulated endothelial cell contraction: the relationship between isometric tension, actin polymerization, and myosin phosphorylation. *J Cell Biol* 1995;130:613–627.
 80. Vogel SM, Gao X, Mehta D, et al. Abrogation of thrombin-induced increase in pulmonary microvascular permeability in PAR-1 knockout mice. *Physiol Genomics* 2000;4:137–145.
 81. Yuan Y, Huang Q, Wu HM. Myosin light chain phosphorylation: modulation of basal and agonist-stimulated venular permeability. *Am J Physiol* 1997;272:H1437–H1443.
 82. Wainwright MS, Rossi J, Schavocky J, et al. Protein kinase involved in lung injury susceptibility: evidence from enzyme isoform genetic knockout and in vivo inhibitor treatment. *Proc Natl Acad Sci U S A* 2003;100:6233–6238.
 83. Tauseef M, Knezevic N, Chava KR, et al. TLR4 activation of TRPC6-dependent calcium signaling mediates endotoxin-induced lung vascular permeability and inflammation. *J Exp Med* 2012;209:1953–1968.
 84. Sundivakkam PC, Natarajan V, Malik AB, Tiruppathi C. Store-operated Ca^{2+} entry (SOCE) induced by protease-activated receptor-1 mediates STIM1 protein phosphorylation to inhibit SOCE in endothelial cells through AMP-activated protein kinase and p38 β mitogen-activated protein kinase. *J Biol Chem* 2013;288:17030–17041.
 85. Tiruppathi C, Freichel M, Vogel SM, et al. Impairment of store-operated Ca^{2+} entry in TRPC4(-/-) mice interferes with increase in lung microvascular permeability. *Circ Res* 2002;91:70–76.
 86. Sundivakkam PC, Freichel M, Singh V, et al. The Ca^{2+} sensor stromal interaction molecule 1 (STIM1) is necessary and sufficient for the store-operated Ca^{2+} entry function of transient receptor potential canonical (TRPC) 1 and 4 channels in endothelial cells. *Mol Pharmacol* 2012;81:510–526.
 87. Birnbaumer L. The TRPC class of ion channels: a critical review of their roles in slow, sustained increases in intracellular Ca^{2+} concentrations. *Annu Rev Pharmacol Toxicol* 2009;49:395–426.
 88. Montell C, Birnbaumer L, Flockerzi V. The TRP channels, a remarkably functional family. *Cell* 2002;108:595–598.
 89. Abramowitz J, Birnbaumer L. Physiology and pathophysiology of canonical transient receptor potential channels. *FASEB J* 2009;23:297–328.
 90. Tiruppathi C, Ahmmed GU, Vogel SM, Malik AB. Ca^{2+} signaling, TRP channels, and endothelial permeability. *Microcirculation* 2006;13:693–708.
 91. Curry FR, Glass CA. TRP channels and the regulation of vascular permeability: new insights from the lung microvasculature. *Circ Res* 2006;99:915–917.
 92. Nilius B, Droogmans G. Ion channels and their functional role in vascular endothelium. *Physiol Rev* 2001;81:1415–1459.
 93. Pocock TM, Foster RR, Bates DO. Evidence of a role for TRPC channels in VEGF-mediated increased vascular permeability in vivo. *Am J Physiol Heart Circ Physiol* 2004;286:H1015–H1026.
 94. Dietrich A, Kalwa H, Gudermann T. TRPC channels in vascular cell function. *Thromb Haemost* 2010;103:262–270.
 95. Tiruppathi C, Minshall RD, Paria BC, Vogel SM, Malik AB. Role of Ca^{2+} signaling in the regulation of endothelial permeability. *Vascul Pharmacol* 2002;39:173–185.

96. Ahmmed GU, Malik AB. Functional role of TRPC channels in the regulation of endothelial permeability. *Pflügers Arch* 2005;451:131–142.
97. Obukhov AG, Schultz G, Luckhoff A. Regulation of heterologously expressed transient receptor potential-like channels by calcium ions. *Neuroscience* 1998;85:487–495.
98. Ahmmed GU, Mehta D, Vogel S, et al. Protein kinase C α phosphorylates the TRPC1 channel and regulates store-operated Ca $^{2+}$ entry in endothelial cells. *J Biol Chem* 2004;279:20941–20949.
99. Jho D, Mehta D, Ahmmed G, et al. Angiopoietin-1 opposes VEGF-induced increase in endothelial permeability by inhibiting TRPC1-dependent Ca $^{2+}$ influx. *Circ Res* 2005;96:1282–1290.
100. Paria BC, Vogel SM, Ahmmed GU, et al. Tumor necrosis factor- α -induced TRPC1 expression amplifies store-operated Ca $^{2+}$ influx and endothelial permeability. *Am J Physiol Lung Cell Mol Physiol* 2004;287:L1303–L1313.
101. Holda JR, Blatter LA. Capacitative calcium entry is inhibited in vascular endothelial cells by disruption of cytoskeletal microfilaments. *FEBS Lett* 1997;403:191–196.
102. Wu S, Sangerman J, Li M, Brough GH, Goodman SR, Stevens T. Essential control of an endothelial cell ISOC by the spectrin membrane skeleton. *J Cell Biol* 2001;154:1225–1233.
103. Hartwig JH. Actin-binding proteins. 1: Spectrin super family. *Protein Profile* 1995;2:703–800.
104. Watanabe H, Tran QK, Takeuchi K, et al. Myosin light-chain kinase regulates endothelial calcium entry and endothelium-dependent vasodilation. *FASEB J* 2001;15:282–284.
105. Soboloff J, Rothberg BS, Madesh M, Gill DL. STIM proteins: dynamic calcium signal transducers. *Nat Rev Mol Cell Biol* 2012;13:549–565.
106. Putney JW. Pharmacology of store-operated calcium channels. *Mol Interv* 2010;10:209–218.
107. Bird GS, Hwang SY, Smyth JT, Fukushima M, Boyles RR, Putney JW Jr. STIM1 is a calcium sensor specialized for digital signaling. *Curr Biol* 2009;19:1724–1729.
108. Cahalan MD. STIMulating store-operated Ca(2+) entry. *Nat Cell Biol* 2009;11:669–677.
109. Yuan JP, Kim MS, Zeng W, et al. TRPC channels as STIM1-regulated SOCs. *Channels (Austin)* 2009;3:221–225.
110. Huang GN, Zeng W, Kim JY, et al. STIM1 carboxyl-terminus activates native SOC, I(crac) and TRPC1 channels. *Nat Cell Biol* 2006;8:1003–1010.
111. Yuan JP, Zeng W, Huang GN, Worley PF, Muallem S. STIM1 heteromultimerizes TRPC channels to determine their function as store-operated channels. *Nat Cell Biol* 2007;9:636–645.
112. Brandman O, Liou J, Park WS, Meyer T. STIM2 is a feedback regulator that stabilizes basal cytosolic and endoplasmic reticulum Ca $^{2+}$ levels. *Cell* 2007;131:1327–1339.
113. Smyth JT, Hwang SY, Tomita T, DeHaven WI, Mercer JC, Putney JW. Activation and regulation of store-operated calcium entry. *J Cell Mol Med* 2010;14:2337–2349.
114. Hogan PG, Lewis RS, Rao A. Molecular basis of calcium signaling in lymphocytes: STIM and ORAI. *Annu Rev Immunol* 2010;28:491–533.
115. Dietrich A, Mederos YSM, Gollasch M, et al. Increased vascular smooth muscle contractility in TRPC6 $^{-/-}$ mice. *Mol Cell Biol* 2005;25:6980–6989.
116. Asaoka Y, Nakamura S, Yoshida K, Nishizuka Y. Protein kinase C, calcium and phospholipid degradation. *Trends Biochem Sci* 1992;17:414–417.
117. Smart EJ, Ying YS, Anderson RG. Hormonal regulation of caveolae internalization. *J Cell Biol* 1995;131:929–938.
118. Tang ZL, Scherer PE, Lisanti MP. The primary sequence of murine caveolin reveals a conserved consensus site for phosphorylation by protein kinase C. *Gene* 1994;147:299–300.
119. Minshall RD, Vandenbroucke EE, Holinstat M, et al. Role of protein kinase C ζ in thrombin-induced RhoA activation and inter-endothelial gap formation of human dermal microvessel endothelial cell monolayers. *Microvasc Res* 2010;80:240–249.
120. Rigor RR, Shen Q, Pivetti CD, Wu MH, Yuan SY. Myosin light chain kinase signaling in endothelial barrier dysfunction. *Med Res Rev* 2013;33:911–933.
121. Grinnell KL, Harrington EO. Interplay between FAK, PKC δ , and p190RhoGAP in the regulation of endothelial barrier function. *Microvasc Res* 2012;83:12–21.
122. Huang F, Subbiah PV, Holian O, et al. Lysophosphatidylcholine increases endothelial permeability: role of PKC α and RhoA cross talk. *Am J Physiol Lung Cell Mol Physiol* 2005;289:L176–L185.
123. Harrington EO, Brunelle JL, Shannon CJ, Kim ES, Mennella K, Rounds S. Role of protein kinase C isoforms in rat epididymal microvascular endothelial barrier function. *Am J Respir Cell Mol Biol* 2003;28:626–636.
124. Chava KR, Tauseef M, Sharma T, Mehta D. Cyclic AMP response element-binding protein prevents endothelial permeability increase through transcriptional controlling p190RhoGAP expression. *Blood* 2012;119:308–319.
125. Wojciak-Stothard B, Potempa S, Eichholtz T, Ridley AJ. Rho and Rac but not Cdc42 regulate endothelial cell permeability. *J Cell Sci* 2001;114:1343–1355.
126. Schlegel N, Burger S, Golenhofen N, Walter U, Drenckhahn D, Waschke J. The role of VASP in regulation of cAMP- and Rac 1-mediated endothelial barrier stabilization. *Am J Physiol Cell Physiol* 2008;294:C178–C188.
127. Schmidt TT, Tauseef M, Yue L, et al. Conditional deletion of FAK in mice endothelium disrupts lung vascular barrier function due to destabilization of RhoA and Rac1 activities. *Am J Physiol Lung Cell Mol Physiol* 2013;305:L291–L300.
128. Kouklis P, Konstantoulaki M, Vogel S, Broman M, Malik AB. Cdc42 regulates the restoration of endothelial barrier function. *Circ Res* 2004;94:159–166.
129. Ramchandran R, Mehta D, Vogel SM, Mirza MK, Kouklis P, Malik AB. Critical role of Cdc42 in mediating endothelial barrier protection in vivo. *Am J Physiol Lung Cell Mol Physiol* 2008;295:L363–L369.
130. Quadri SK, Bhattacharya J. Resealing of endothelial junctions by focal adhesion kinase. *Am J Physiol Lung Cell Mol Physiol* 2007;292:L334–L342.
131. Sun X, Shikata Y, Wang L, et al. Enhanced interaction between focal adhesion and adherens junction proteins: involvement in sphingosine 1-phosphate-induced endothelial barrier enhancement. *Microvasc Res* 2009;77:304–313.

132. Chen XL, Nam JO, Jean C, et al. VEGF-induced vascular permeability is mediated by FAK. *Dev Cell* 2012;22:146–157.
133. Chen Z, Bakhshi FR, Shajahan AN, et al. Nitric oxide-dependent Src activation and resultant caveolin-1 phosphorylation promote eNOS/caveolin-1 binding and eNOS inhibition. *Mol Biol Cell* 2012;23:1388–1398.
134. Harper MT, Sage SO. Src family tyrosine kinases activate thrombin-induced non-capacitative cation entry in human platelets. *Platelets* 2010;21:445–450.
135. Wu T, Zhang B, Ye F, Xiao Z. A potential role for caveolin-1 in VEGF-induced fibronectin upregulation in mesangial cells: involvement of VEGFR2 and Src. *Am J Physiol Renal Physiol* 2013;304:F820–F830.
136. Potter MD, Barbero S, Cheresh DA. Tyrosine phosphorylation of VE-cadherin prevents binding of p120- and beta-catenin and maintains the cellular mesenchymal state. *J Biol Chem* 2005;280:31906–31912.
137. Orsenigo F, Giampietro C, Ferrari A, et al. Phosphorylation of VE-cadherin is modulated by haemodynamic forces and contributes to the regulation of vascular permeability in vivo. *Nat Commun* 2012;3:1208.
138. Vestweber D, Winderlich M, Cagna G, Nottebaum AF. Cell adhesion dynamics at endothelial junctions: VE-cadherin as a major player. *Trends Cell Biol* 2009;19:8–15.
139. Arnold KM, Goeckeler ZM, Wysolmerski RB. Loss of focal adhesion kinase enhances endothelial barrier function and increases focal adhesions. *Microcirculation* 2013;20:637–649.
140. Loot AE, Schreiber JG, Fisslthaler B, Fleming I. Angiotensin II impairs endothelial function via tyrosine phosphorylation of the endothelial nitric oxide synthase. *J Exp Med* 2009;206:2889–2896.
141. Ying Z, Giachini FR, Tostes RC, Webb RC. PYK2/PDZ-RhoGEF links Ca²⁺ signaling to RhoA. *Arterioscler Thromb Vasc Biol* 2009;29:1657–1663.
142. Field JJ, Diaz JF, Miller JH. The binding sites of microtubule-stabilizing agents. *Chem Biol* 2013;20:301–315.
143. Stec-Martyna E, Ponassi M, Miele M, Parodi S, Felli L, Rosano C. Structural comparison of the interaction of tubulin with various ligands affecting microtubule dynamics. *Curr Cancer Drug Targets* 2012;12:658–666.
144. Vignaud T, Blanchoin L, Thery M. Directed cytoskeleton self-organization. *Trends Cell Biol* 2012;22:671–682.
145. Akhmanova A, Hoogenraad CC. Microtubule plus-end-tracking proteins: mechanisms and functions. *Curr Opin Cell Biol* 2005;17:47–54.
146. Bajer S, Kasprzak AA. [Plus end tracking proteins and their role in mitotic spindle organization]. *Postepy Biochem* 2009;55:223–231.
147. Li W, Miki T, Watanabe T, et al. EB1 promotes microtubule dynamics by recruiting Sentin in *Drosophila* cells. *J Cell Biol* 2011;193:973–983.
148. Komarova YA, Huang F, Geyer M, et al. VE-cadherin signaling induces EB3 phosphorylation to suppress microtubule growth and assemble adherens junctions. *Mol Cell* 2012;48:914–925.
149. Padrick SB, Rosen MK. Physical mechanisms of signal integration by WASP family proteins. *Annu Rev Biochem* 2010;79:707–735.
150. Takenawa T, Suetsugu S. The WASP-WAVE protein network: connecting the membrane to the cytoskeleton. *Nat Rev Mol Cell Biol* 2007;8:37–48.
151. Rajput C, Kini V, Smith M, et al. Neural Wiskott-Aldrich syndrome protein (N-WASP)-mediated p120-catenin interaction with Arp2-Actin complex stabilizes endothelial adherens junctions. *J Biol Chem* 2013;288:4241–4250.
152. Coughlin SR. Thrombin signalling and protease-activated receptors. *Nature* 2000;407:258–264.
153. Vu TK, Hung DT, Wheaton VI, Coughlin SR. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* 1991;64:1057–1068.
154. Ishihara H, Connolly AJ, Zeng D, et al. Protease-activated receptor 3 is a second thrombin receptor in humans. *Nature* 1997;386:502–506.
155. Mirza H, Yatsula V, Bahou WF. The proteinase activated receptor-2 (PAR-2) mediates mitogenic responses in human vascular endothelial cells. *J Clin Invest* 1996;97:1705–1714.
156. Wells CD, Liu MY, Jackson M, et al. Mechanisms for reversible regulation between G13 and Rho exchange factors. *J Biol Chem* 2002;277:1174–1181.
157. Kaneider NC, Leger AJ, Agarwal A, et al. ‘Role reversal’ for the receptor PAR1 in sepsis-induced vascular damage. *Nat Immunol* 2007;8:1303–1312.
158. Klarenbach SW, Chipiuk A, Nelson RC, Hollenberg MD, Murray AG. Differential actions of PAR2 and PAR1 in stimulating human endothelial cell exocytosis and permeability: the role of Rho-GTPases. *Circ Res* 2003;92:272–278.
159. Barr AJ, Brass LF, Manning DR. Reconstitution of receptors and GTP-binding regulatory proteins (G proteins) in Sf9 cells. A direct evaluation of selectivity in receptor.G protein coupling. *J Biol Chem* 1997;272:2223–2229.
160. Hung DT, Wong YH, Vu TK, Coughlin SR. The cloned platelet thrombin receptor couples to at least two distinct effectors to stimulate phosphoinositide hydrolysis and inhibit adenylyl cyclase. *J Biol Chem* 1992;267:20831–20834.
161. Knezevic N, Tauseef M, Thennes T, Mehta D. The G protein betagamma subunit mediates reannealing of adherens junctions to reverse endothelial permeability increase by thrombin. *J Exp Med* 2009;206:2761–2777.
162. Tauseef M, Kini V, Knezevic N, et al. Activation of sphingosine kinase-1 reverses the increase in lung vascular permeability through sphingosine-1-phosphate receptor signaling in endothelial cells. *Circ Res* 2008;103:1164–1172.
163. Thennes T, Mehta D. Heterotrimeric G proteins, focal adhesion kinase, and endothelial barrier function. *Microvasc Res* 2012;83:31–44.
164. Sun X, Ma SF, Wade MS, et al. Functional promoter variants in sphingosine 1-phosphate receptor 3 associate with susceptibility to sepsis-associated acute respiratory distress syndrome. *Am J Physiol Lung Cell Mol Physiol* 2013;305:L467–L477.
165. Weis SM, Cheresh DA. Pathophysiological consequences of VEGF-induced vascular permeability. *Nature* 2005;437:497–504.
166. Bates DO. Vascular endothelial growth factors and vascular permeability. *Cardiovasc Res* 2010;87:262–271.
167. Nottebaum AF, Cagna G, Winderlich M, et al. VE-PTP maintains the endothelial barrier via plakoglobin and be-

- comes dissociated from VE-cadherin by leukocytes and by VEGF. *J Exp Med* 2008;205:2929–2945.
168. Sun Z, Li X, Massena S, et al. VEGFR2 induces c-Src signaling and vascular permeability in vivo via the adaptor protein TSAd. *J Exp Med* 2012;209:1363–1377.
 169. Thibeault S, Rautureau Y, Oubaha M, et al. S-nitrosylation of beta-catenin by eNOS-derived NO promotes VEGF-induced endothelial cell permeability. *Mol Cell* 2010;39:468–476.
 170. Scharpfenecker M, Fiedler U, Reiss Y, Augustin HG. The Tie-2 ligand angiopoietin-2 destabilizes quiescent endothelium through an internal autocrine loop mechanism. *J Cell Sci* 2005;118:771–780.
 171. Yuan HT, Khankin EV, Karumanchi SA, Parikh SM. Angiopoietin 2 is a partial agonist/antagonist of Tie2 signaling in the endothelium. *Mol Cell Biol* 2009;29:2011–2022.
 172. Ziegler T, Horstkotte J, Schwab C, et al. Angiopoietin 2 mediates microvascular and hemodynamic alterations in sepsis. *J Clin Invest* 2013;123(8):3436–3445.
 173. Cullere X, Shaw SK, Andersson L, Hirahashi J, Luscinskas FW, Mayadas TN. Regulation of vascular endothelial barrier function by Epac, a cAMP-activated exchange factor for Rap GTPase. *Blood* 2005;105:1950–1955.
 174. Glading A, Han J, Stockton RA, Ginsberg MH. KRIT-1/CCM1 is a Rap1 effector that regulates endothelial cell cell junctions. *J Cell Biol* 2007;179:247–254.
 175. Hatanaka K, Simons M, Murakami M. Phosphorylation of VE-cadherin controls endothelial phenotypes via p120-catenin coupling and Rac1 activation. *Am J Physiol Heart Circ Physiol* 2011;300:H162–H172.
 176. Augustin HG, Koh GY, Thurston G, Alitalo K. Control of vascular morphogenesis and homeostasis through the angiopoietin-Tie system. *Nat Rev Mol Cell Biol* 2009;10:165–177.
 177. Fukuhara S, Sako K, Minami T, et al. Differential function of Tie2 at cell-cell contacts and cell-substratum contacts regulated by angiopoietin-1. *Nat Cell Biol* 2008;10:513–526.