Novel regulators of endothelial barrier function

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Submitted 29 October 2014; accepted in final form 5 November 2014

Mehta D, Ravindran K, Kuebler WM. Novel regulators of endothelial barrier function. Am J Physiol Lung Cell Mol Physiol 307: L924–L935, 2014. First published November 7, 2014; doi:10.1152/ajplung.00318.2014.—Endothelial barrier function is an essential and tightly regulated process that ensures proper compartmentalization of the vascular and interstitial space, while allowing for the diffusive exchange of small molecules and the controlled trafficking of macromolecules and immune cells. Failure to control endothelial barrier integrity results in excessive leakage of fluid and proteins from the vasculature that can rapidly become fatal in scenarios such as sepsis or the acute respiratory distress syndrome. Here, we highlight recent advances in our understanding of the regulation of endothelial permeability, with a specific focus on the endothelial glycocalyx and endothelial scaffolds, regulatory intracellular signaling cascades, as well as triggers and mediators that either disrupt or enhance endothelial barrier integrity, and provide our perspective as to areas of seeming controversy and knowledge gaps, respectively.

endothelial cell; permeability; edema; acute lung injury/acute respiratory distress syndrome

THE PRIMORDIAL FUNCTION of the vascular endothelium is the maintenance of an effective barrier for fluids, proteins, and cells while concomitantly allowing for efficient gas transport and the regulated transport of solutes as well as trafficking of inflammatory cells (112). The endothelium allows for unrestricted transfer of low-molecular-weight substances (<3 nm in radii) such as dissolved gases or ions through passive diffusion. In contrast, transendothelial trafficking of plasma proteins such as albumin or immunoglobulins (ranging from 7 to 11.5 nm radii) is dynamically controlled. Failure to maintain an intact barrier results in leakage of solutes, proteins, and fluid into the interstitial space and, in the case of the lung, alveolar space with detrimental and frequently fatal sequelae for lung mechanics and alveolo-capillary gas exchange (100). Mechanisms regulating barrier integrity, failure, or reconstitution and therapeutic strategies aimed at the preservation or recovery of an intact endothelial barrier function have therefore been a topic of intense research over past years (64).

Barrier function of the endothelial monolayer is regulated by cell-cell and cell-extracellular matrix adhesion as well as a wide variety of biological, chemical, or physical stimuli, and endogenous mediators, respectively. The glycocalyx, an extracellular covering on the apical side of endothelium, along with the monolayer adhesive property provided by the intercellular endothelial junctions, integrin receptors, and their protein partners, maintains the albumin-impermeable nature of the vessel wall under basal conditions. Barrier disruption often stems from compromised interendothelial junctions, resulting in the formation of gaps between normally contiguous cells, while barrier reinforcement arises from stabilization of junctional complexes (86). Canonically, barrier disruptive substances have included thrombin, platelet activating factor, tumor necrosis factor-α (TNF-α), vascular endothelial growth factor (VEGF), histamine and bradykinin, while sphingosine-1-phosphate (SIP) and angiopoietin-1 are regarded as barrier stabilizing (87). Endothelial barrier function is critically regulated by small GTPases such as RhoA, Rac1, and Cdc42, that serve as key integrators of signaling events between the plasma membrane and the endothelial cytoskeleton. In broadest terms, signaling via Rac1 and Cdc42 enhances cortical actin formation leading to increased barrier stability, while RhoA activation reorganizes actin filaments into stress fibers, destabilizing VE-cadherin and increasing barrier permeability (51). In parallel, changes in the intracellular Ca2+ concentration ([Ca2+]i) regulate endothelial permeability via activation of myosin light chain kinase and subsequent actin-myosin-induced endothelial cell contraction (152). The relative contribution of both GT-Pase and [Ca2+]i, signaling varies considerably under different experimental conditions as well as in response to different stimuli, and this variability likely accounts for some of the existing heterogeneity in regulation of endothelial barrier function by these mechanisms (141).

Over the past years, a series of seminal studies focusing on the regulation of lung endothelial barrier function and edema formation have been published in the American Journal of Physiology - Lung Cellular and Molecular Physiology. Herein, we discuss these results and provide our perspective, which may shed new light into the basic mechanisms regulating barrier function and/or may have important therapeutic implications for the prevention or treatment of diseases associated with lung endothelial leak and edema formation.

ENDOTHELIUM CELL SURFACE AND SCAFFOLDS REGULATING PULMONARY BARRIER FUNCTION

Glycocalyx

The negatively charged glycocalyx is a surface layer of proteoglycans, glycosaminoglycans, and adsorbed plasma proteins on the apical side of the endothelial monolayer (84, 113). The thickness of the glycocalyx varies considerably depending on vascular beds as well as tissue types e.g., lungs, skeletal muscle, or heart (59, 67, 146). Additionally, studies indicate that negative charge distribution on the glycocalyx may be uneven, forming heterogeneous microdomains on the luminal cell surface (147, 148). Based on studies from vascular beds of systemic vessels, the glycocalyx has been shown to act as a
sieve allowing transendothelial transport of low-molecular-weight solutes, inhibiting red blood cell and neutrophil adhesion and extravasation, and as a mechanotransducer (67, 113). However, characterization of the glycocalyx in the pulmonary microcirculation remained controversial as most of the studies relied on using enzymatic degradation of glycocalyx constituents (24, 31, 35, 37, 45, 71, 102, 130). Recently, Schmidt and coworkers (125) used a model of intravital microscopy in the intact, ventilated mouse (136) to demonstrate that the pulmonary endothelial glycocalyx is \( \sim 1.7 \mu m \) thick, i.e., around two to three times the value of the glycocalyx in the systemic circulation. Schmidt and colleagues showed that the glycocalyx is rapidly lost following endotoxemia due to posttranslational activation of heparanase, resulting in exposure of adhesive molecules such as intercellular adhesion molecule-1 (ICAM-1) to circulating neutrophils and their extravasation into the interstitium, leading thereby to inflammatory injury. However, previous studies showed that formyl-methionyl-leucyl-phenylalanine-activated neutrophils trigger shedding of the glycocalyx around the postcapillary venules (97), raising the possibility that shedding of the glycocalyx may be a general phenomenon for regulating transendothelial migration of neutrophils following sepsis.

Neutrophil transmigration across the endothelium and the associated release of cytokines and chemokines are known to disrupt endothelial barrier function (138). Countertuitively, Schmidt and colleagues (125) showed that loss of glycocalyx following endotoxemia had no effect on protein permeability of the pulmonary vessel wall. In contrast, in 2012 Cioffi and colleagues (24) showed that sialic acids, negative charged carbohydrate moieties in complex sugars such as oligosaccharides and polysaccharides, within the glycocalyx, maintain endothelial barrier function by regulating cell-matrix and cell-cell interaction. They demonstrated that hydrolysis of sialic acid moieties using neuraminidase disrupted cell-cell and cell-matrix adhesions, suggesting that terminal sialic acids promote endothelial barrier integrity. Also, neuraminidase increased microvessel permeability in mice lungs. Similarly, Dull and colleagues (35–37) showed that degradation of heparan sulfate by heparanase in the perfused lungs increased microvessel permeability in response to pressure changes, secondary to induction of endothelial nitric oxide synthase (NOS) activity and actin cytoskeletal rearrangement. How can the above findings by Schmidt on the one side and Cioffi and Dull on the other then be reconciled? Is it because LPS induces heparanase activity in a tightly restricted spatiotemporal manner, limiting detection of endothelial barrier dysfunction?

Additionally, the above studies raise several novel questions that need to be addressed in favor of the idea that glycocalyx is an anti-inflammatory and barrier protective mechanism. 1) Are LPS-induced alterations in heparanase activity mediated through Toll-like receptors (TLR), and if so, which one(s)? 2) Does glycocalyx shedding alter endothelial cell-cell and cell-matrix attachment? 3) Are changes in nitric oxide (NO) levels, as seen by Dull and colleagues, linked to the posttranslational activation of heparanase? 4) Does shedding of the glycocalyx specifically expose endothelial cell surface receptors that maintain barrier homeostasis under basal conditions? Intravital two-photon microscopy and relevant transgenic mice models may prove useful if the glycocalyx interacts with endothelial cell junctions through NO signaling or via the actin cytoskeleton, and whether these linkages play a role in regulating transendothelial leukocyte transmigration in real-time under physiological and pathological conditions.

**Endothelial Scaffolds**

The interaction between contiguous endothelial cells and their underlying matrix provides the endothelial monolayer with adhesive strength that resists separation of cells from the substratum, a hallmark of barrier disruption. Interendothelial junctions (IEJ) are composed of adherens junctions (AJs), tight junctions (TJ), and gap junctions (GJs), whereas integrin receptors link the cells with the matrix through focal adhesion proteins (87). In contrast to the key role of TJ in the epithelium, endothelial barrier function is predominantly regulated by AJs (48, 104). Recent studies published in the *American Journal of Physiology - Lung Cellular and Molecular Physiology* showed that focal adhesion kinase (FAK), IQRas GTPase-activating protein-1 (IQGAP-1), and AMP-activated protein kinase (AMPK) have the potential to interfere with barrier disruptive mechanisms, limiting lung injury. On the other hand, prehaptoglobin 2, protein kinase C, ezrin, radixin, and moesin (ERM) proteins, or a disintegrin and metalloproteinase (ADAM) family member induces barrier disruption. Below, we compile these findings and pose fundamental questions that can be touted to analyze these signaling in a global manner.

FAK, a nonreceptor protein tyrosine kinase, was initially described as a regulator of cell-matrix attachment in endothelial cells (87). FAK is autophosphorylated on Tyr-397, but it can be further activated by tyrosine phosphorylation at several residues including Y576/577 residues in response to integrin activation induced by cell adhesion, antibody cross-linking, and permeability regulating mediators (124). FAK has a long list of interacting partners. Some of these proteins include the cytoplasmic domain of the β-subunit of integrins, band 4.1 containing proteins – ezrin, radixin, moesin-homology domain (FERM) domain, N-WASP, pp60Src, and pp59fyn, growth factor receptor-bound protein 2 (Grb2), Arf (ADP ribosylation factor), GAP containing SH3, Ankyrin repeats and PH domain (ASAP1), and p130 Crk-associated substrate (p130Cas), vinculin, talin, paxillin, 190RhoGEF, and GTPase regulator associated with FAK (GRAF) (50, 87, 108, 139). Several studies showed that FAK through N-WASP and p120-catenin interacts with AJs and reanneals them by dampening endothelial contraction (65, 75, 88, 118). FAK also promotes endothelial cell survival during cytotoxic stress (10, 60). RhoA is the major regulator of actin-myosin-induced contraction in endothelial cells and thereby is a key determinant of increased endothelial permeability (20, 25, 56, 60, 66, 83, 111, 119, 153, 163). However, studies also showed that dominant negative FAK or a kinase dead FAK mutant prevented AJ disruption in response to VEGF or oxidants indicating that FAK in fact disrupts barrier function (19, 116, 144). Thus, Schmidt et al. (125) used mice where FAK deletion can be conditionally induced in an endothelial cell-specific manner to assess the role of FAK in regulating acute lung injury (ALI). These authors showed that onset of ALI by ip administration of lipopolysaccharide (LPS) or cecal ligation and puncture markedly decreased FAK expression in mice lungs. Conditional deletion of FAK increased transvascular albumin influx, edema, and neutrophil accumulation in the lung. Hyperactivation of RhoA due to increased...
p115RhoGEF binding with RhoA was shown to be responsible for these effects because activated RhoA suppressed Rac1 activity, which maintains AJs and inhibition of Rho kinase, a downstream effector of RhoA, reinstated normal endothelial barrier function in EC-FAK null mice.

IQGAP-1 can interact with several proteins including polymerized actin, activated small GTPases such as Rac and Cdc42, β-catenin, calmodulin, and microtubule-associated cytoplasmic linker integral protein-170 (87). Using IQGAP1 null mice, Bhattacharya and colleagues (12) showed that IQGAP1 is required for maintaining barrier function in endothelial cells. They demonstrated that endotoxin as well as live bacteria induced fulminant edema formation in IQGAP1 null mice. IQGAP1 functioned by using αβ3-integrin as a new partner, which induces cortical actin formation thus strengthening AJs. Additionally, they showed that extracellular S1P, which ligates S1P receptor 1, recruits IQGAP1 at the AJs for inducing barrier enhancement by S1P. Previous studies showed that intracellular generation of S1P can also strengthen AJs by recruiting IQGAP1 and Rac1 to AJs (143). Similarly, Ang1 was shown to require IQGAP1 for inducing Rac1 activity and for promoting barrier defense (30). While these studies favor the idea that IQGAP1 induces AJs by promoting signaling through barrier protective agents such as Ang1 and S1P, depletion of IQGAP1 did not augment the barrier disruptive effect of thrombin in endothelial cells (30).

AMPK, a serine threonine kinase, regulates metabolic as well as anti-inflammatory functions (106, 126). AMPK was shown to suppress TLR4-dependent activation of nuclear factor NF-κB and the subsequent generation of cytokines (121, 137) and facilitated phagocytosis and clearance of apoptotic cells (107, 162), indicating that AMPK contributes to the resolution of inflammation. Creighton’s laboratory showed that activation of AMPK with N1-(α-D-ribofurano-syl)-5-aminoimidizole-4-carboxamide (AICAR) or metformin, a clinically approved antidiabetic drug, resealed wounds in LPS-exposed rat pulmonary microvascular endothelial cells. AICAR interacts with the γ-subunit of AMPK, resulting in phosphorylation of the catalytic α-subunit, thereby inducing AMPK activity. In line with this notion, AICAR or metformin failed to induce barrier repair in AMPK-α1 depleted endothelial cells, indicating these effects required AMPK-α1. Also, AICAR and metformin both blocked LPS-induced increases in microvessel permeability and sepsis induced mortality (70).

Zonula occludens (ZO) proteins are family members of membrane-associated guanylate kinases (53), which form TJ and also allow TJs proteins to interact with AJ proteins such as α-catenin, GJ proteins such as connexin (Cx)-43, and actin polymerizing proteins such as vasodilator-stimulated phosphoprotein and spectrin (27, 40, 69, 98, 140). ZO-1 may thereby regulate endothelial permeability by serving as a scaffold for mediating the interaction of TJs with AJs and connexin. Prehaptoglobin2 has been identified as zonulin and is related to serine proteases (MASPs, C1qrs) that activate the complement system (120). A zonulin antagonist (AT-1001) in a dose-dependent manner, or zonulin neutralizing antibody, attenuated the intensity of ALI (as quantitated by albumin leak, neutrophil accumulation, and proinflammatory cytokines). Human immunodeficiency virus (HIV)-1 transgene expression also increased paracellular permeability of alveolar epithelial monolayers by decreasing the expression of ZO-1 expression. Interestingly, upregulating nuclear factor (erythroid-derived 2)-like 2 (Nrf2 or NFE2L2) (Nrf2) activity restored ZO1 expression normalizing barrier function. Nrf2 is a transcription factor constitutively expressed in all tissues and promotes cytoprotection by activating many proteins regulating metabolism of drugs and toxins, the protection against oxidative stresses and inflammation, the stability of proteins, and the removal of damaged proteins (149). However, whether AT-1001 or zonulin neutralizing antibody prevented ALI by augmenting Nrf2 activity remains unclear.

The protein kinase C (PKC) family consists of isoforms with different Ca2+ and phorbol ester requirements for activation: conventional (α, β, δ), novel (ε, γ), and atypical (ζ, η) (85, 128). PKCo has a crucial role in mediating IEJ disassembly either by phosphorylating IEJ components or through inducing the RhoA pathway (76, 78, 96). Ca2+-independent isoforms, notably PKCd, may also be important in IEJ disruption, which predominantly seems to require RhoA-induced signaling (91). PKCδ was shown to regulate focal adhesions and RhoA activity (57) as well as to induce NO generation via stimulating Akt activity (133). While some studies showed that PKCδ maintains basal barrier function, another showed that inhibition of PKCδ prevented phorbol ester-mediated barrier dysfunction (57). However, mice lacking PKCδ did not show any vascular leak in the lung under basal conditions and, intriguingly, resisted LPS-induced lung injury, indicating PKCδ also disrupt endothelial barrier function (22). Whether other PKC isoforms compensated for inducing barrier disruption in PKCδ null mice remains to be parsed out.

Adyshev and colleagues (3) showed that upon activation by thrombin receptors PKC also induces the phosphorylation of ERM proteins at canonical threonine residues (ezrin Thr567, radixin Thr564, moesin Thr558). Threonine phosphorylation within the COOH terminus unfolds the ERMs, leading to their activation and translocation to the periphery of endothelial cells (26). Activated ERMs cross-link actin and thereby play an important role in actin cytoskeletal remodeling. ERMs also act as a scaffold and interact with Rho-GDI-1, Gα13 protein, and FAK, which may modulate RhoA signaling and AJ function (26). Members of the stress-inducible small heat shock protein family (HSP) also regulate actin polymerization in vitro (164). Interestingly, depletion of moesin but not radixin markedly attenuated thrombin-induced increases in endothelial barrier permeability, cytoskeletal rearrangements, paracellular gap formation, and accumulation of phospho-myosin light chain. Inhibitors of Hsp90 also prevented LPS-induced lung endothelial barrier dysfunction by suppressing Src-mediated RhoA activity and signaling (18). In a recent study, Barabutis and coworkers (9) showed that LPS causes phosphorylation of HSP90 on the tyrosine Y309 residue in a Src-dependent manner. Expression of a nonphosphorylatable Hsp90 mutant reduced LPS-induced barrier dysfunction indicating HSP90 inhibition.

ADAM, single pass transmembrane glycoproteins, regulate several cellular functions in the lung vasculature and also regulate lung saccular formation by cleaving the ectodomains of cell surface protein and downstream signaling events (33, 154, 155). Several ADAMs such as ADAM 8, 9, 12, 15, and 19 have been shown to alter the expression of several vascular receptors and most of the time can cleave the same substrate (33). For example, ADAM 8, 10, 17, and 19 can cleave TNF-α.
endothelial barrier function?

MEDIATORS REGULATING ENDOTHELIAL BARRIER FUNCTION

While traumatic, infectious, or inflammatory insults have long been recognized as primary triggers of lung vascular barrier failure, the rise of new chemical and biological challenges such as the current Ebola epidemic has broadened the spectrum of potential causes. Below, we therefore discuss triggers and mediators that can cause barrier failure, as well as novel mediators and drugs that can enhance barrier maintenance and/or restoration.

Regulation of Endothelial Barrier by Mechanical Stretch

Mechanical forces imposed by circulating blood and respiratory cycles activate numerous signals including stretch-activated ion channels and calcium influx, secretory group V phospholipase A₂ (gVPLA2), small GTPases, Rho kinase, selectin expression, mitogen-activated kinases, and FAK (2, 4, 14, 43, 89, 94, 159), which may impact lung vascular barrier function. Critically ill patients with ALI are supported by mechanical ventilation with tidal volumes that typically range from 6 to 12 ml/kg body wt. However, it has become apparent that high-tidal-volume ventilator support leads to ventilator-induced lung injury, multiorgan dysfunction, and early mortality (158). Birukokva and coworkers (15) showed that mechanical stretch in the phase of on-going endothelial barrier injury induces RhoA signaling via interleukin-6 receptors that otherwise induce Jak and p38 MAP kinase and NF-κB signaling. NO, induced during pressure-induced shedding of glycocalyx by heparanase, is known to cause nitration of proteins including RhoA, which activates RhoA signaling (72, 117). Thus a number of questions remain unanswered. Does activation of heparanase induce RhoA GEFs to induce RhoA signaling? How does RhoA and p38 kinase signaling overlap? How do alterations in heparan sulfates influence the activity of endothelial mechanical sensors such as transient receptor potential vanilloid 4 (TRPV4) and platelet endothelial cell adhesion molecule? How does heparan sulfate communicate with sialic acid linkages during static vs. mechanical perturbations to maintain cell-cell and cell-matrix interaction and thereby endothelial permeability? Is restoration of heparan sulfates and sialic acid a viable means to limit ALI?

Elevation in arterial carbon dioxide tension (acidosis) during protective lung ventilation reduces ischemic lung injury and preserves lung compliance, but it may induce immunosuppression and worsen infection in sepsis (115, 158). For this and other reasons, buffering acidosis with tris-hydroxymethyl aminomethane but not sodium bicarbonate is recommended in patients with hemodynamic instability. Sayner and colleagues (101, 123) showed that bicarbonate in a dose-dependent manner decreased resistance across the pulmonary microvascular endothelial cell monolayer. Furthermore, perfusion of mice lungs ex vivo with bicarbonate increased vascular permeability while increasing osmolarity had no effect. These authors...
showed that bicarbonate can stimulate mammalian soluble adenyl cyclase (AC) isomorf 10 to generate cytosolic cAMP. While transmembrane AC activity is pulmonary endothelial barrier protective, cytosolic AC activity of bacterial toxins is endothelial barrier disruptive, leading to pulmonary edema (123). Whether bicarbonate may concomitantly compromise barrier integrity and promote lung edema formation by altering glycocalyx components or RhoA signaling remains to be addressed.

Mediators Disrupting the Endothelial Barrier

Chlorine gas (Cl₂) is a reactive gas that is considered a chemical threat agent, but exposure may equally result from chemical disasters, such as railway spills, or passive exposure, such as inhalation of disinfectants. Upon inhalation, Cl₂ gas is considered a chemical threat agent, but exposure may equally result from chemical disasters, such as railway spills, or passive exposure, such as inhalation of disinfectants. Upon inhalation, Cl₂ gas exposure in World War I was also frequently followed by bacterial superinfections, suggesting a potentially increased susceptibility to opportunistic infections postexposure (142). Indeed, as recently demonstrated by Gessner and colleagues (49), mice exposed to Cl₂ demonstrate a five-fold higher fungal burden following experimental inoculation with Aspergillus fumigatus. This increased susceptibility to opportunistic pathogens is associated with an increased permeability to plasma proteins, as evidenced by increased albumin and IgG levels in the bronchoalveolar lavage fluid, thus establishing a potentially vicious circle of endothelial barrier failure, leukocyte emigration, and microbial infection. It is important to note that opportunistic infections in this study were associated with enhanced recruitment of inflammatory cells into the lung, but the cellular infiltration failed to generate microbicidal superoxide. This finding highlights the dual role of oxidative stress in lung infection and vascular barrier function, in that reactive oxygen species (ROS) are essential to prevent alveolar pathogen invasion yet concomitantly themselves promote lung vascular inflammation and barrier failure. A recent study by Menden and colleagues (90) highlights this idea: These authors show that bacterial LPS induced an increase in endothelial ICAM-1 expression that was mediated by superoxide formation from NADPH oxidase (NOX) 2, subsequent phosphorylation of transforming growth factor-β-associated kinase 1 and inhibitor of κ-B kinase-β, and ultimately, activation of NF-κB. Increased susceptibility to oxidative stress also underlies alveolo-capillary barrier failure in HIV-1 infection, a finding that is of particular relevance in view of the fact that pulmonary complications constitute the leading cause of death in HIV-1-infected individuals. In HIV-1 transducing transgenic rats, Fan and colleagues (39) show that HIV-1 related viral proteins downregulate Nrf2, the key transcription factor that regulates a number of genes that comprise the antioxidant response element, thus effectively impairing lung antioxidant defenses and barrier function. It is noteworthy that the detrimental effects of oxidative stress on endothelial barrier function are further amplified by its concomitant effects on alveolar fluid clearance, an active epithelial mechanism that maintains alveolar fluid homeostasis via an ion transport-driven absorption of fluid from the distal air spaces. In a variety of experimental or clinical conditions associated with impaired lung barrier function and increased oxidative stress such as e.g., circulatory shock or bacteremia, alveolar fluid clearance is significantly impaired, thus contributing to impaired gas exchange and presumably, higher mortality (63, 160). It could therefore be speculated that ROS may play a critical role in inhibiting alveolar fluid clearance; however, it turns out that in vivo, ROS have the capacity to stimulate lung fluid clearance by increasing the activity of the epithelial sodium channel ENaC, the main driving force for alveolar fluid absorption (55). The opposing effects of ROS on invading pathogens, barrier integrity and alveolar fluid absorption, give rise to a characteristic U-shaped curve indicating that ROS level have to be uniquely titrated in dose, time, and space to prevent detrimental effects. Despite documented effectiveness of antioxidants such as N-acetylcysteine (NAC) to confer protection against ALI and lung edema in e.g., preclinical models of sepsis (17), the Janus-faced role of ROS in lung injury and barrier function has likely been the main reason why NAC has failed to improve outcome of acute respiratory distress syndrome (ARDS) patients in clinical trials (32). A recent preclinical study has now proposed an alternative and potentially, more promising antioxidative strategy by vitamin C supplementation (44). Ascorbic acid had previously been shown to reduce macromolecular permeability in cultured human umbilical vein endothelial cells (HUVECs) (145). Fisher and colleagues (44) now show that parenteral infusion of ascorbic acid in mice with experimentally induced abdominal peritonitis increases survival and reduces histological signs of lung injury, the expression of proinflammatory cytokines, and the extravasation of fluid and macromolecules in the lung. Notably, these effects were accompanied by an increased expression of key molecules involved in alveolar fluid absorption such as ENaC, the cystic fibrosis transmembrane conductance regulator CFTR, and the Na⁺/K⁺-ATPase and consequently, by an augmentation in alveolar fluid clearance. The molecular mechanisms that determine why inhibition of ROS production from NOX2 (55) attenuates fluid clearance in naïve mice, while the antioxidant ascorbic acid promotes it in lung injury (44) remain to be determined but likely reflect again the U-shaped homeostatic regulation that is characteristic for ROS. On this background, efforts to utilize nonselective antioxidants or inhibitors of ROS production seem hopeless but likely to fail unless we succeed in realizing and controlling both temporal and spatial targeting of these drugs.

In experimental lung injury and its clinical correlate, the ARDS, lung vascular barrier failure, the invasion of immune cells, and the activation of inflammatory signaling pathways typically go hand in hand. The relevance of immune cells as important regulators of lung vascular barrier function is highlighted by the recent observation that inhibition of leukocyte-endothelial interaction in lung capillaries and venules by immunoneutralization of the adhesion molecule CD162 not only reduces the accumulation of neutrophils but also attenuates lung edema in murine models of lung injury that was triggered by intravenous injection of streptococcal M1 protein (161). Not only inflammatory cells, but also microparticles released from neutrophils, platelets, or lymphocytes, can increase endothelial permeability and trigger lung vascular barrier failure. Such microparticles are extracellular vesicles of 50 nm up to 1 μm in size with a lipid bilayer that can act as intercellular carriers for multiple membrane and cytosolic proteins, organelles, lipids, and RNA from their respective parental cells to various
target cells (86). As such, microparticles can shuttle, e.g., short-lived barrier-disruptive lipid mediators such as thromboxane A2 or platelet-activating factor to endothelial cells and, thus, promote barrier failure (86). Conversely, endothelial activation and expression of adhesion molecules are essential prerequisites for neutrophil invasion and emigration. For example, endothelial knock-down or inhibition of calpains, a family of Ca2+-dependent, nonlysosomal cysteine proteases, reduces endothelial NO synthase (NOS3)-mediated NO production, subsequent phosphorylation of ICAM-1, and thereby, neutrophil recruitment as well as lung edema and protein extravasation (80). Similarly, deficiency in the polymodal cation channel TRPV4, which is highly expressed in pulmonary endothelial (95) and smooth muscle cells (29, 157) but seemingly absent in neutrophils (8), prevented both edema formation and neutrophil invasion in murine models of chlorine gas- and acid aspiration-induced lung injury (8). However, in conditions where stimuli and/or inhibitors act in parallel on both endothelial and immune cells, cause and effect in the interaction between these cell types are often hard to differentiate. For example, the prehaptoglobin zonulin acts both as a modulator of tight junctions and as an activator of the complement system. In a recent study, Rittirsch and colleagues (120) could show that a zonulin antagonist or a zonulin neutralizing antibody both effectively attenuate albumin leak and neutrophil accumulation in two different models of ALI; however, given the duality of zonulin’s action, it is virtually impossible to dissect the individual roles of immune and endothelial cells in such an in vivo scenario. An abundant literature has documented that triggers such as thrombin, H2O2, or LPS can increase endothelial permeability in vitro in the absence of immune cells. Recent data by Leonard and colleagues (80) demonstrate that the underlying dynamic regulation of the actin cytoskeleton not only mediates the endothelial permeability response to thrombin but also modulates in turn proinflammatory signaling pathways including NF-κB. Modulation of actin filament stability through phosphorylation or dephosphorylation of the actin binding protein cofilin regulates not only endothelial barrier properties but also NF-κB activity and expression of its target genes such as ICAM-1. While these data provide compelling evidence for a mechanism by which endothelial barrier leak may precede the recruitment of immune cells, the situation is somewhat more complex in the in vivo scenario, where for reasons that are so far poorly understood many of the above-mentioned triggers of endothelial permeability in vitro fail to cause vascular leak in the absence of immune cells (141). Our understanding of the intricate interplay between immune and endothelial cells in the regulation of both neutrophil extravasation and endothelial barrier regulation is rudimentary at best, and we currently lack any approach that would allow us to target one yet not the other in an in vivo setting. We are in dire need, however, of such strategies, as they would potentially enable us to protect the lung vascular barrier while simultaneously allowing for extravasation of immunocompetent cells into the alveolar and interstitial space to fight off invading pathogens.

**Strategies to Enhance Lung Endothelial Barrier Function**

Regardless of the emerging challenge to counterbalance barrier protection versus antimicrobial defense in the alveolar compartment, a series of novel experimental strategies and therapeutic targets have recently emerged that present promising perspectives as potential stabilizers of the lung endothelial barrier. Aside from the classic mediators S1P and angiotensin-1, the neurotransmitter dopamine has recently emerged as a putative barrier stabilizer. Dopamine had previously been shown to inhibit barrier disruptive effects of VEGF in HUVECs by inhibiting the phosphorylation of VE-cadherin and ZO-1 (13). Recently, Vohra and coworkers (150) used this strategy now as a pretreatment in a murine model of LPS-induced lung injury and could demonstrate that dopamine increased survival and decreased lung edema, protein extravasation, and pulmonary infiltration of neutrophils. Similar to previous reports in HUVECs, these effects seem to be mediated via the VEGF-VEGF receptor axis, in that dopamine pretreatment reduced circulating VEGF levels in serum and attenuated phosphorylation of the VEGF receptor 2. As dopamine also increases transepithelial fluid flux in type II pneumocytes via translocation of the basolateral Na+K+ATPase in a MAPK/ERK-dependent manner (58), dopamine treatment may counteract lung edema formation at both the vascular endothelial and alveolar epithelial level. Like dopamine, the arachidonate metabolite prostaglandin E2 (PGE2) had previously been reported to prevent increases in endothelial permeability in vitro through its ability to activate Rac in a cAMP/PKA-dependent manner (16, 41). However, as PGE2 signals through any of four different receptors (EP1–EP4), which all belong to the family of G protein-coupled receptors, the biological effects of PGE2 become heavily dependent on relative expression of EP receptor subtypes. In isolated lungs, PGE2 can in fact contribute to barrier failure via activation of EP3, but not EP1, EP2, or EP4 receptors (51). Conversely, selective activation of EP4 in human pulmonary microvascular endothelial cells stimulates the production of the proinflammatory chemokine IL-8 in a p38-dependent manner (7), a mechanism that may contribute to neutrophil recruitment and secondary endothelial barrier failure in ARDS. In line with this notion, nimesulide, an inhibitor of prostaglandin H synthase-2, which is upstream of PGE2 synthesis, was recently shown to inhibit LPS-induced increases in both PGE2 and fetal lung IL-8 mRNA (151). Thus, while prostanoids such as PGE2 present interesting candidates for barrier modulation, these findings underline the need for agonists and/or antagonists that act more specifically on individual receptors. A similar case for higher therapeutic receptor specificity can be built for S1P, which signals through its receptors S1P1–S1P5, of which S1P1–S1P3 and potentially S1P4 are expressed in the pulmonary vasculature. S1P, by acting through its receptor S1P1, has long been recognized for its barrier-stabilizing effects that involve activation of Rac and result in a rearrangement of the endothelial cytoskeleton to form strong cortical actin rings in the cell periphery and enhanced cell-to-cell and cell-to-matrix tethering dynamics (1). However, activation of S1P3 increases endothelial leak in cultured HUVECs and in isolated perfused lungs stimulated with hydrogen peroxide (122), and single nucleotide polymorphisms (SNPs) in S1P3 were recently found to be associated with an increased risk for sepsis-associated ARDS (135). Conversely to these dichotomous effects of PGE2 and S1P and therefore, of particular therapeutic interest, the effects of adenosine in the lung seem rather uniformly beneficial, at least in the setting of ARDS. Adenosine acts through four receptors,
adenosine receptors A1, A2A, A2B, and A3, of which A3 is least expressed in endothelial cells (61). Ngamsri et al. (99) had previously shown A1AR to regulate neutrophil influx and microvascular permeability in lung injury, in that A1AR-deficient mice had increased, while mice treated with a specific A1AR agonist had decreased lung inflammation and edema following LPS challenge. More recently, Konrad et al. (77), from the same laboratory of Jörg Reutershan, showed similar effects for the A2b receptor, in that A2b-deficient mice showed more LPS-induced neutrophil infiltration and microvascular leakage, while a specific A2b receptor agonist decreased these effects. Generation of chimeric mice further revealed that A2b
receptor expression on hematopoietic cells was critical for polymorphonuclear leukocyte migration, while LPS-induced microvascular leakage was under the control of A2b on both hematopoietic and nonhematopoietic cells. Activation of the A2a receptor furthermore accelerates the resolution of alveolar edema, an effect that could be blocked by inhibition of epithelial ENaC, but not CFTR channels (46). Hence, adenosine as well as single, dual, or triple adenosine receptor agonists may indeed present a powerful new avenue for the treatment of ALI and lung endothelial barrier failure. However, in view of recent findings that sustained elevations of adenosine levels may contribute to cigarette smoke-induced lung endothelial apoptosis and emphysema formation (83), such interventions should probably be restricted to the acute exudative phase in lung edema.

Another promising strategy for endothelial barrier enhancement builds on the well-recognized barrier-stabilizing effects of subplasma membrane cAMP generated by transmembrane adenylate cyclase (131). In a murine model of ALI secondary to polymicrobial sepsis, Oishi and colleagues (103) tested the effectiveness of two different approaches to increase endothelial cAMP levels, either by enhancing cAMP generation by the water-soluble adenylate cyclase stimulator colforsin or by attenuating cAMP degradation by the specific phosphodiesterase III inhibitor, olprinone. Treatment with both agents prevented the onset of ALI through a mechanism that involved activation of Akt, but not the cyclic AMP response element binding protein CREB, as inhibition of the Akt upstream regulator phosphatidylinositol 3-kinase, yet not a CREB decoy binding protein CREB, as inhibition of the Akt upstream regulator phosphatidylinositol 3-kinase, yet not a CREB decoy oligodeoxynucleotide, blocked the effects of both compounds. Finally, gaseous transmitters and inhaled gases have received great interest as potential therapeutic avenues to alleviate lung injury and restore endothelial barrier function. While sobering results from a series of clinical trials failed to show significant improvements in clinical outcome in ARDS patients treated with inhaled NO, preclinical evidence is accumulating for potential protective roles of carbon monoxide, hydrogen sulfide, or hydrogen gas. In a recent study, Fernandez-Gonzalez and colleagues (42) could now show that constitutive lung-specific overexpression of the carbon monoxide-generating enzyme heme oxygenase 1 (HO-1) confers vasculoprotective effects in a murine model of hyperoxia-induced bronchopulmonary dysplasia, in that pulmonary inflammation, arterial remodeling, and pulmonary edema were markedly improved. In adult lungs, Kawamura and colleagues (73) found the detrimental effects of hyperoxia on lung injury, inflammation, and edema to be markedly alleviated in animals inhaling 2% hydrogen, and this effect was associated with an increased expression of HO-1. Notably, protection by hydrogen gas inhalation was alleviated in mice deficient in the main transcriptome factors for antioxidative genes, Nrf2, which regulates HO-1 expression, suggesting that hydrogen gas alleviates hyperoxic lung injury through induction of Nrf2-dependent genes, such as HO-1.

CONCLUSION

As summarized in Figs. 1 and 2, great strides have been made in our understanding of signaling mechanisms and mediators that regulate endothelial permeability. However, the emerging overall picture of endothelial barrier regulation has several fundamental gaps that need to be filled systematically to foster the development of new therapeutic targets and drugs for the treatment of ARDS. A combinatorial, parallel strategy at the cellular, tissue, and organ level using key barrier-disrupting and barrier-stabilizing mediators that induce acute or chronic changes in barrier properties may inform us better about “signaling nodes” that can be targeted than single hit studies in individual models (Figs. 1 and 2). These nodes can then be verified by integration of novel technologies such as intravital microscopy in conditional mice along with gene editing tools and epigenetics assessment. These types of integrative studies can be expected to significantly advance our understanding of the mechanisms regulating microvasculature and endothelial permeability in the normal state and during inflammation. For example, suppression of RhoA emerges as a central player in various scenarios of endothelial barrier disruption. The real challenge that emerges is whether we will be able to identify strategies to target, e.g., RhoA in the endothelium only to such a degree that it prevents disruption of AJs following injury, without interfering with basal barrier formation and functions including the emigration of immune cells to sites of injury and inflammation? Similarly, can we modulate ROS production in a way that we maintain cellular and physiological homeostasis at the alveolo-capillary membrane yet prevent the detrimental effects of excessively high (or low) ROS levels? And along these lines, how safe are barrier-protective strategies such as metformin, AICAR, sulfophosphamide, adenosine, colforsin, or olprinone in scenarios of, e.g., pathogen invasion? The regulation of endothelial permeability and lung vascular barrier homeostasis turns out to be much more complex than often anticipated, and, as a result, so is the development of novel and safe therapeutic strategies.

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