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Regulation of Vascular Permeability by Sphingosine 1-Phosphate

Lichun Wang and Steven M. Dudek

Section of Pulmonary & Critical Care Medicine, University of Chicago Pritzker School of Medicine, Chicago, Illinois

Abstract

A significant and sustained increase in vascular permeability is a hallmark of acute inflammatory diseases such as acute lung injury (ALI) and sepsis and is an essential component of tumor metastasis, angiogenesis, and atherosclerosis. Sphingosine 1-phosphate (S1P), an endogenous bioactive lipid produced in many cell types, regulates endothelial barrier function by activation of its G-protein coupled receptor SIP₁. S1P enhances vascular barrier function through a series of profound events initiated by SIP₁ ligation with subsequent downstream activation of the Rho family of small GTPases, cytoskeletal reorganization, adherens junction and tight junction assembly, and focal adhesion formation. Furthermore, recent studies have identified transactivation of SIP₁ signaling by other barrier enhancing agents as a common mechanism for promoting endothelial barrier function. This review summarizes the state of our current knowledge about the mechanisms through which the S1P/SIP₁ axis reduces vascular permeability, which remains an area of active investigation that will hopefully produce novel therapeutic agents in the near future.

Keywords

Sphingosine 1-phosphate (S1P); Sphingosine 1-phosphate receptor 1 (SIP₁); vascular permeability; G-protein coupled receptor (GPCR); Rac GTPase; actin cytoskeleton; adherens junction; tight junction; focal adhesion

Introduction

Endothelial cells lining the vasculature regulate a variety of functions such as vascular tone, blood coagulation, inflammation, angiogenesis, and tissue fluid homeostasis (Garcia et al., 2001; Mehta and Malik, 2006). In the lung, endothelial cells provide a semi-permeable barrier between vascular contents and the pulmonary interstitium and airspaces that is particularly important for maintenance of normal fluid homeostasis and adequate gas exchange. However, a significant and sustained increase in vascular permeability is a hallmark of acute inflammatory diseases such as ALI and sepsis and is an also essential component of tumor metastasis, angiogenesis, and atherosclerosis (Dudek and Garcia, 2001; Jacobson and Garcia, 2007). The size-selective characteristic of the barrier to plasma proteins and other solutes is a key factor in maintaining fluid balance of tissues. In general, the transport of fluids and solutes is determined by two separate pathways, the transcellular and paracellular routes. The

Corresponding address: Steven M. Dudek, MD, Section of Pulmonary & Critical Care Medicine, Department of Medicine, University of Chicago, 5841 South Maryland Ave., Chicago, IL 60637, Phone: 773-834-2390, Fax: 773-702-6500, Email: E-mail: sdudek@medicine.bsd.uchicago.edu.

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transcellular pathway actively transports macromolecules with molecular radii above 3 nm, such as albumin (Milici et al., 1987; Predescu et al., 2007). In contrast, solutes with molecular radii less than 3 nm move passively across the barrier via the paracellular route that is considered the primary determinant of endothelial barrier permeability (Michel and Curry, 1999). Paracellular permeability is regulated by a complex balance of intracellular contractile forces generated by actinomyosin and cellular adhesive cell-cell and cell-matrix forces. Inflammatory mediators such as thrombin, bradykinin, histamine, tumor necrosis factor alpha (TNF- α), and the angiogenic factor vascular endothelial growth factor (VEGF) increase vascular permeability by opening paracellular gaps and disrupting endothelial junctions and focal adhesion complexes (Andriopoulou et al., 1999; Aschner et al., 1997; Hippenstiel et al., 1998; Rotundo et al., 2002). Sphingosine 1-phosphate (S1P), a serum bioactive lipid, has been identified as a robust barrier-enhancing agent with great potential to serve as a novel and specific therapy for endothelial cell barrier dysfunction during inflammatory illnesses such as ALI and sepsis (Garcia et al., 2001; Jacobson and Garcia, 2007). Although S1P regulates multiple cell functions such as cell proliferation, differentiation, survival, migration, and morphogenesis, this review will focus on the mechanisms by which S1P promotes barrier function.

S1P and S1P receptors

S1P is stored primarily in platelets at a high concentration and is released upon their activation (Yatomi et al., 1995), although other cells, such as erythrocytes, neutrophils, mononuclear cells, and mast cells may also contribute to the release of S1P (Yang et al., 1999). In general, the serum concentration of S1P is approximately 0.4–1.1 μM (Venkataraman et al., 2006). The degradation of sphingomyelin (SM), a membrane structural component, is a major pathway involved in producing S1P (Figure 1). Ceramide can be synthesized from sphingomyelin via the action of sphingomyelinases. Subsequently, ceramide is deacylated by ceramidase to generate sphingosine, which is rapidly phosphorylated to S1P by sphingosine kinases since high levels of sphingosine are toxic to cells. Because of its phosphate group, S1P is more polar than sphingosine. Its level is regulated by dephosphorylation via S1P phosphatase, or it can be irreversibly degraded by S1P lyase into phosphoethanolamine and hexadecanal (Hait et al., 2006; Tani et al., 2007).

Five closely related G protein-coupled receptors (GPCR)-S1P₁, S1P₂, S1P₃, S1P₄, and S1P₅, have been identified as high-affinity S1P receptors. These S1P receptors are expressed on various cell types including endothelial cells, neurons, leukocytes and smooth muscle cells (Peters and Alewijnse, 2007; Rosen and Goetzl, 2005). S1P₁ was first cloned from endothelial cells as an immediate early transcript induced by the tumor promoter phorbol 12-myristate 13-acetate (PMA) (Hla and Maciag, 1990). The discovery of the ligand for S1P₁ was a seminal event in blood lipid research (Lee et al., 1998). S1P₁ is a 382 amino acid protein that contains seven hydrophobic transmembrane spanning domains with significant structural similarities to other GPCRs. The third intracellular loop of S1P₁ specifically associates with G_i, which is pertussis toxin-sensitive, but not cholera toxin-sensitive (Lee et al., 1996). Transfection of human embryonic kidney 293 cells (HEK293), which express little if any endogenous S1P₁, induces a network of cell-cell aggregates. This morphology resembles the network formation of differentiated endothelial cells, and is completely prevented by the chelating agent EGTA and C3 exotoxin, a specific inhibitor of the small GTPase Rho. Furthermore, S1P greatly enhances the activity of ERK1/2 via a S1P₁/G_i dependent pathway (Lee et al., 1998). Vascular endothelial cells primarily express S1P₁, S1P₂ and S1P₃ (Hla et al., 2001), which have a high degree of sequence homology. However, they are coupled to different G proteins and therefore stimulate different downstream pathways. S1P₁ is specifically coupled to G_i whereas S1P₂ and S1P₃ couple to G_i, G_q and G_{12/13}. Stimulation of endothelial cells with S1P at physiological concentrations (0.5–1 μM) results in Rac1-dependent barrier protection by activation of S1P₁,

whereas S1P at a higher concentration ($\geq 5 \mu\text{M}$) mediates RhoA dependent barrier disruption through S1P3 ligation (Komarova et al., 2007; Shikata et al., 2003b).

The physiologic importance of the S1P/S1P₁ pathway to maintenance of vascular barrier integrity is demonstrated by the protective role of platelets, which have long been known to decrease endothelial permeability (Gimbrone et al., 1969; Roy and Djerassi, 1972). Recently, our group identified S1P as the major barrier-protective product responsible for this effect within platelets (Schaphorst et al., 2003). Platelet supernatants produce a similar barrier protective effect as S1P in a G_i dependent manner. More importantly, treatment of platelet supernatants with charcoal to deplete lipids, or interventions that decrease endothelial expression of S1P₁, significantly attenuates the barrier enhancing-effect of platelet supernatants. These results demonstrate that S1P is the critical barrier-protective mediator produced by human platelets (Schaphorst et al., 2003).

S1P and the Cytoskeleton

Endothelial barrier function is dependent upon a complex balance of tethering forces at cell-cell and cell-matrix junctional sites. Both of these tethering forces are linked to the underlying cytoskeleton and dynamically regulated by their interactions with it (McVerry and Garcia, 2004). Our group has extensively studied the effects of S1P on the endothelial cytoskeleton and was the first to demonstrate a dose-dependent enhancement of endothelial barrier function by S1P as measured in vitro by transmonolayer electrical resistance (TER) (Garcia et al., 2001). Maximal barrier enhancement is observed with 1 μM S1P that peaks after 10-20 minutes and is sustained for hours. Furthermore, S1P fully restores vascular barrier integrity when added subsequent to thrombin challenge. Thus, S1P not only increases baseline barrier integrity, but it also effectively protects the endothelium from the barrier disruptive effects of edemagenic agents such as thrombin (Garcia et al., 2001). Cytoskeleton rearrangement is the critical cellular event mediating endothelial barrier function. Morphologic studies demonstrate that S1P at 1 μM produces rapid and dramatic enhancement of polymerized F-actin and myosin light chain phosphorylation at the cell periphery (Garcia et al., 2001). The enhancement of TER by S1P is abolished by the actin depolymerizing agent, cytochalasin B, the actin polymerization inhibitor, latrunculin, but not by the microtubule disruptor, nocodazole (Garcia et al., 2001). These results demonstrate the essential role of the actin cytoskeleton in mediating endothelial barrier enhancement by S1P. More recently, we have further characterized the structural and mechanical changes in the cytoskeleton of cultured human pulmonary artery endothelial cells in response to S1P and thrombin by using atomic force microscopy techniques (AFM) (Teran Arce et al., 2008). The elastic modulus, an indicator of underlying structural force, is significantly elevated at the peripheral region of the cell by S1P treatment, whereas thrombin induces elevation within the central region of the cell. These force and elasticity maps correlate with F-actin rearrangements observed by immunofluorescence. Taken together, these studies support a critical functional role for dynamic actin assembly/disassembly and subsequent cortical redistribution in mediating S1P-induced barrier enhancement (Figure 2).

Further evidence suggests important roles for two actin-associated cytoskeletal proteins, cortactin and non-muscle myosin light chain kinase (nmMLCK), in mediating S1P-induced endothelial barrier enhancement (Dudek et al., 2004). Exposure of endothelial cells to S1P produces rapid and significant translocation of cortactin from the cytoplasm to a peripheral cortical distribution within 5 minutes. Moreover, cortactin depletion by antisense oligonucleotide techniques results in a 50% inhibition of peak S1P barrier enhancement, whereas endothelial cells overexpressing wild-type cortactin showed enhanced TER after S1P. This reduction in S1P barrier augmentation is similar in magnitude to that observed in other studies after downregulation of S1P₁ expression (Dudek et al., 2004). Furthermore, reduced expression of cortactin results in a shift in the S1P-mediated AFM elasticity pattern to more

closely resemble unstimulated endothelial cells (Teran Arce et al., 2008). Src mediated phosphorylation of cortactin at three critical tyrosine residues (Tyr421, Tyr466, and Tyr482) appears to be necessary for peak S1P induced barrier enhancement since a mutant cortactin deficient in these tyrosines attenuates the TER increase (Dudek et al., 2004). Similar to cortactin, nmMLCK is rapidly redistributed within 5 min of exposure to physiologic levels of S1P to areas of active membrane ruffling where it colocalizes with translocated cortactin (via SH3 domain of cortactin) (Dudek et al., 2004). The interaction of cortactin and nmMLCK appears to be necessary for optimal S1P induced barrier enhancement since a cell-permeable cortactin SH3 blocking peptide which abolishes cortactin interaction with nmMLCK significantly reduces both peak barrier enhancement and myosin light chain (MLC) phosphorylation without altering either cortactin or nmMLCK translocation (Dudek et al., 2004). These results strongly support a critical function for nmMLCK interaction with cortactin in enhancing vascular barrier integrity in response to S1P and highlight a novel role for nmMLCK in vascular barrier enhancement.

The S1P analogue, FTY720, is a synthesized derivative of the fungal compound, myriocin, and is currently in clinical trials as immunosuppression for transplant rejection and multiple sclerosis treatment (Budde et al., 2006; Kappos et al., 2006). Similar to S1P, FTY720 induces sustained and dose-dependent barrier enhancement in cultured pulmonary endothelial cells but with significantly delayed onset and intensity compared to the S1P response (Dudek et al., 2007). Interestingly, FTY720 does not induce either significant MLC phosphorylation or dramatic actin cytoskeletal rearrangement. Moreover, reduced expression of the cytoskeletal effectors, Rac1 and cortactin (via siRNA), does not inhibit FTY720-induced TER elevations as similar interventions do to the S1P barrier enhancing response (Dudek et al., 2007). Although both S1P and FTY720 rapidly induce SIP₁ accumulation in membrane lipid rafts, only S1P stimulates SIP₁ phosphorylation on a critical threonine residue. Reduction of SIP₁ expression and inhibition of PI3 kinase activity attenuates S1P- but not FTY720- induced TER elevations. However, the TER response to FTY720 is significantly attenuated by pertussis toxin and the lipid raft disrupting agent, methyl- β -cyclodextrin (M β CD) in a manner similar to the S1P response (Dudek et al., 2007). These results suggest a novel barrier-enhancing pathway for modulating vascular permeability without involvement of dramatic cytoskeletal rearrangement which still remains to be elucidated.

S1P and Rho Family GTPases

The Rho family of GTPases (Rho, Rac and Cdc42) is a group of regulatory molecules that link surface receptors to downstream effectors regulating actin cytoskeletal structure. In general, Rac activity results in the formation of focal contacts and lamellipodia, Rho induces the formation of stress fibers and focal adhesions, and Cdc42 regulates filopodia formation (Tzima, 2006). In smooth muscle cells (SMC), Rho regulates contraction and has been implicated in phenotypic modulation (Gosens et al., 2004). Rho GTPases play a crucial role in regulation of endothelial barrier function, endothelial migration and wound repair (Waschke et al., 2006). Activation of Rho increases the phosphorylation of MLC via MLCK and leads to the formation of actin stress fibers and actomyosin contraction (Garcia et al., 1999; Vouret-Craviari et al., 2002). In contrast, other studies have implicated Rac GTPase in lamellipodia formation and cortical cytoskeletal reorganization (Garcia et al., 2001). Inhibition of Rac leads to increased endothelial cell permeability and enhances thrombin-mediated barrier disruption (Waschke et al., 2006; Wojciak-Stothard et al., 2006). S1P, in physiologic concentrations, preferentially activates the small GTPase Rac via SIP₁ in a pertussis toxin-sensitive fashion and enhances barrier integrity, whereas thrombin preferentially stimulates Rho and causes cell contraction and barrier disruption (van Nieuw Amerongen et al., 2004; Vouret-Craviari et al., 2002). Rac activity is required for S1P-induced adherens junction assembly and cytoskeleton rearrangement (Lee et al., 1999). Microinjection of dominative negative Rac into endothelial

cells dramatically diminishes S1P-induced VE-cadherin and β -catenin enrichment at cell-cell junctions, while overexpression of active Rac (V12) reproduces changes in the cortical actin similar to those evoked by S1P. S1P-induced cortical rearrangement involves a cytoskeletal signaling sequence that includes binding of the p21-associated Ser/Thr kinase (PAK) with Rac, phosphorylation and activation of LIM kinase, and the subsequent inactivation of the LIM kinase target, cofilin, an actin-severing protein (Figure 2) (Garcia et al., 2001). Transient transfection of human endothelium with a PAK-1 dominant negative construct dramatically reduces S1P induced increases in the cortical distribution of polymerized actin, whereas adenoviral-mediated cofilin overexpression significantly inhibits the barrier-enhancing effects of S1P (Garcia et al., 2001). Rac activation is also associated with the translocation of cortactin, an 80/85 kD actin-binding protein involved in barrier regulation (Dudek et al., 2004). Dominative negative Rac prevents the translocation of cortactin and subsequent cortical actin polymerization. Moreover, reduction of Rac expression by siRNA significantly attenuates the S1P TER response (Dudek et al., 2007). These studies strongly support a pivotal role of Rac in barrier enhancement induced by S1P.

To further characterize upstream effectors of Rac that contribute to the regulation of barrier function, Singleton and colleagues examined S1P-induced signaling in human lung endothelial caveolin-rich microdomains (CEMs) (Singleton et al., 2005). S1P actively recruits SIP₁ and SIP₃, PI3 kinase catalytic subunits p110 α and β , Tiam1 and α -actinin1/4 to CEM fractions in a PI3 kinase-dependent manner. Methyl- β -cyclodextrin (MBCD), a cholesterol depletion agent that disrupts CEM, abolishes S1P-induced recruitment of S1P receptors and PI3 kinase p110 α and β to CEM fractions as well as subsequent barrier enhancement. Reduction in expression of either SIP₁ or Tiam1 (via siRNA techniques) or inhibition of PI3 kinase by LY294002 inhibits S1P-induced Rac1 activation, cortical actin rearrangement and endothelial barrier enhancement. These data illustrate the critical importance of the PI3 kinase-Tiam1-Rac1 pathway in mediating S1P signaling from SIP₁ to the EC cytoskeleton to produce rearrangements necessary for barrier enhancement. In a subsequent study utilizing quantitative proteomics analysis (iTRAQ), two additional proteins were identified as recruited to CEM by S1P, myristoylated alanine-rich protein kinase C substrate (MARCKS) and MARCKS-related protein (MRP) (Guo et al., 2007). Moreover, siRNA-mediated silencing of MARCKS or MRP (or both) attenuates S1P-mediated endothelial cell barrier enhancement. These data suggest novel molecular targets in S1P induced barrier enhancement. Additional studies have demonstrated that S1P induces an increase in intracellular Ca²⁺ concentration in endothelial cells via a G_i-dependent pathway (Mehta et al., 2005). Inhibition of G_i, phospholipase C (PLC), or inositol trisphosphate receptor prevents the S1P-activated increase in intracellular Ca²⁺, Rac activation, adherens junction assembly, and subsequent endothelial barrier enhancement. These results demonstrate the important role of endoplasmic reticulum Ca²⁺ release in S1P induced Rac activation.

S1P and Endothelial Junctions

Endothelial cells are connected to each other by three different junctional complexes including adherens junctions (AJ or zonula adherens), tight junctions (TJ or zonula occludens), and gap junctions (GJ). AJ and TJ primarily account for intercellular adhesion via formation of pericellular zipper-like structures along their transmembrane adhesion sites (Figure 2) (Bazzoni and Dejana, 2004). In general, AJ and TJ are composed of different junctional proteins with only a few exceptions.

Vascular endothelial cadherin (VE-cadherin) is the major structural protein of AJ in endothelial cells (Mehta and Malik, 2006; Vestweber, 2008). VE-cadherin possesses five extracellular cadherin domains and mediates homophilic interaction of adjacent cells in a Ca²⁺ dependent manner. The VE-cadherin cytoplasmic tail is highly homologous to that of other cadherins and

binds β -catenin or plakoglobin (γ -catenin). Then, β -catenin or plakoglobin binds α -catenin, an actin binding protein, which stabilizes the AJ anchorage to the actin cytoskeleton. α -catenin can also bind to α -actinin and vinculin, which further stabilizes AJ complexes. An additional VE-cadherin partner is p120 catenin, which binds to a membrane-proximal domain of VE-cadherin but does not associate with actin binding proteins such as α -catenin. VE-cadherin is required for endothelial barrier function and development of normal vasculature. VE-cadherin knockout mice are embryonically lethal (E9.5) due to immature vascular development (Carmeliet et al., 1999). In a mouse model, injection of anti-VE-cadherin antibodies induces a marked increase in pulmonary vascular permeability, but a similar effect is not observed in the brain vasculature (Corada et al., 1999). Overexpression of a VE-cadherin mutant lacking the extracellular domain, or chelation of extracellular Ca^{2+} by EDTA, also results in endothelial cell barrier disruption (Venkiteswaran et al., 2002). Furthermore, disruption of binding between β -catenin and VE-cadherin interferes with the association of AJ with the actin cytoskeleton and therefore inhibits proper AJ assembly, resulting in decreased cell adhesion strength and subsequent barrier disruption (Carmeliet et al., 1999).

In confluent human umbilical vein endothelial cells, S1P significantly increases the abundance of VE-cadherin and β -catenin at the cell-cell contact regions and enhances AJ assembly (Lee et al., 1999). Overexpression of S1P₁ in HEK293 cells markedly increases the expression level of P- and E-cadherin, but not α - and β -catenin, and induces cell-cell aggregation in a Ca^{2+} dependent manner (Lee et al., 1998). Furthermore, S1P₁ silencing leads to a reduction in expression of both VE-cadherin and platelet-endothelial cell adhesion molecule-1 (PECAM-1), with the degree of S1P₁ knockdown correlating with the expression levels of these cell surface proteins (Krump-Konvalinkova et al., 2005). Thus, the S1P/S1P₁ signaling pathway not only regulates the translocation of cadherin molecules and assembly of AJ but also modulates the expression of these important junctional molecules. However, the direct functional role of VE-cadherin in mediating S1P-induced endothelial barrier enhancement is complex and still poorly characterized as a recent study indicates that its expression is not necessary for the immediate TER increase (within minutes) after S1P but may participate in the sustained phase that lasts for several hours (Xu et al., 2007).

In addition to enhancing assembly of AJ, S1P also induces the formation of endothelial tight junctions (Lee et al., 2006). TJ are present on the outer leaflets of lateral membranes between adjacent endothelial cells. TJ restrict both the diffusion of solutes across intercellular spaces (barrier function) and the movement of membrane proteins between the apical and basolateral domains of the plasma membrane (fence function) (Bazzoni and Dejana, 2004). TJ are composed of claudins, occludin, and junctional adhesion molecules (JAM). The binding of claudins, occludin, and JAM with zona occludens proteins (ZO-1, ZO-2 or ZO-3) bridges TJ with the actin cytoskeleton and subsequently stabilizes TJ (Ebnet et al., 2000; Furuse et al., 1994; Furuse et al., 1999; Nitta et al., 2003). After S1P stimulation, ZO-1 is redistributed to the lamellipodia and cell-cell junctions via the S1P₁/G_i/Akt/Rac pathway, while the enhanced barrier function induced by S1P is attenuated by siRNA downregulation of ZO-1 expression (Lee et al., 2006). Thus, TJ proteins are functionally involved in mediating S1P-induced barrier enhancement.

S1P and Focal Adhesions

Focal adhesions (FA) are specific cellular sites that anchor cells to the underlying extracellular matrix (ECM) (Figure 2). Focal adhesion complexes are a complicated mix of integrin proteins, actin-binding structural proteins such as vinculin, talin, and α -actinin, adaptor proteins such as paxillin, and focal adhesion kinase (FAK) (Broussard et al., 2008). FAK is a non-receptor protein tyrosine kinase that functions as a major contributor to the regulation of focal adhesion formation and turnover and provides a crucial link between the endothelial cells and ECM.

Depletion of FAK expression results in decreased tyrosine phosphorylation of paxillin in endothelial cells and an augmented and prolonged response to the barrier-disruptive agent, thrombin (Mehta et al., 2002). In addition, expression of a kinase-deficient FAK mutant blunts the barrier-strengthening effect of hyperosmolarity (Quadri et al., 2003). Recent evidence from our group suggests an important role for FA rearrangement in S1P-mediated barrier enhancement (Shikata et al., 2003b). S1P at physiologic concentrations (0.5 μ M) stimulates tyrosine phosphorylation of FAK at a specific site (Y576) and subsequently causes FA disruption and redistribution to the cell periphery. Furthermore, S1P induces a transient association of G protein-coupled receptor kinase-interacting protein 1 (GIT1) with paxillin and redistribution of the GIT2-paxillin complex to the cell cortical area. In contrast to S1P, thrombin induces distinct patterns of FAK phosphorylation with phosphorylation of three FAK sites (Y397, Y576, and Y925), causes the redistribution of FA proteins to the ends of the stress fibers, and disrupts the endothelial barrier (Shikata et al., 2003a). Pharmacological inhibition of Src with the Src-specific inhibitor PP2 abolished S1P-induced FAK (Y576) phosphorylation and translocation of FA proteins. However, PP2 exhibited only partial inhibition of FAK site-specific tyrosine phosphorylation induced by thrombin and failed to inhibit thrombin-induced stress fiber formation and redistribution of FAK (Shikata et al., 2003a). These observations indicate FAK may play differential roles in endothelial cell barrier function dependent upon different phosphorylation patterns induced by various signaling pathways.

Transactivation of SIP₁ signaling

Activated protein C (APC) has potent anti-inflammatory effects in animal models, and recombinant human APC is approved to treat patients with severe sepsis (Kapur et al., 2001). Upon ligation of the endothelial protein C receptor (EPCR), APC rapidly increases endothelial MLC phosphorylation and stimulates strong actin-phospho-MLC rearrangement at the cell periphery while reducing central stress fiber formation (Finigan et al., 2005). These APC-induced cytoskeletal changes are very reminiscent of those produced by S1P. Furthermore, APC induces rapid phosphorylation of SIP₁ on threonine residue 236 via an EPCR and PI3-kinase/AKT-dependent pathway and mediates Rac1-dependent cytoskeleton reorganization. Silencing of SIP₁ expression using siRNA significantly reduces APC-mediated barrier protection against thrombin (Finigan et al., 2005). These data suggest a critical role for transactivation of the SIP₁ receptor by EPCR in endothelial barrier protection induced by APC (Figure 2).

Another signaling molecule that utilizes this paradigm of SIP₁ transactivation is hyaluronan (HA), a major glycosaminoglycan (GAG) component of the extracellular matrix (Toole, 2004). Increased HA levels are observed in bronchoalveolar lavage fluid from patients with acute lung injury and chronic obstructive pulmonary disease (Bensadoun et al., 1996). HA consists of high molecule weight HA (>500 kDa) (HMW-HA) and low molecule weight HA (LMW-HA). Intratracheal administration of nebulized HMW-HA has been used to prevent injury in experimental emphysema (Cantor and Turino, 2004). HMW-HA induces the association of the CD44s isoform with SIP₁ within CEMs, AKT-mediated phosphorylation of SIP₁, and subsequent barrier enhancement (Singleton et al., 2006). However, LMW-HA induces sustained association of the CD44v10 isoform with the barrier-disrupting SIP₃ receptor, Src and ROCK 1/2-mediated phosphorylation of SIP₃, and subsequent barrier disruption. Silencing of SIP₁ blocks the barrier enhancing effects of HMW-HA whereas silencing SIP₃ blocks the barrier disruption induced by LMW-HA (Singleton et al., 2006). These data demonstrate that SIP₁ transactivation represents a novel paradigm for producing endothelial barrier protection in response to multiple agonists. Another promising approach for reducing vascular permeability is to inhibit the SIP₃ transactivation that occurs in response to various inflammatory agonists. For example, methylnaltrexone (MNTX), a μ opioid receptor (mOP-R) antagonist, has been reported to provide barrier protection against

edemagenic agonists such as thrombin and lipopolysaccharide (LPS) via inhibition of S1P₃ receptor activation (Singleton et al., 2007).

Effects of S1P in vivo

Acute lung injury (ALI) is a devastating inflammatory lung disease characterized by a marked increase in vascular permeability which is often exacerbated by the mechanical ventilation necessary to treat this lethal condition (Ware and Matthay, 2000). Intratracheal administration of LPS, a bacterial cell wall toxin component, is a well characterized experimental model of ALI. In an isolated perfused murine lung model, S1P infusion (1 μ M) results in a significant decrease in the rate of edema formation without a change in pulmonary artery pressures (Peng et al., 2004). Intratracheal instillation of LPS (2 mg/kg) produces significant murine inflammatory lung injury, alveolar wall thickening, infiltration of neutrophils into the lung interstitium and alveolar space. In sharp contrast, intravenous administration of S1P (1 μ M) delivered 1 hour after LPS exposure significantly reduces the inflammatory histologic changes produced by LPS and attenuates neutrophil infiltration in lung parenchyma (Figure 3) (Peng et al., 2004). Similar to S1P, the S1P analogue FTY720 when administered as a single intraperitoneal injection (0.1 mg/kg) 1 hour after LPS intratracheal instillation, significantly decreases LPS-induced pulmonary microvascular leakage. Furthermore, S1P also significantly reduces LPS-induced acute renal injury (Peng et al., 2004). Although rodent models provide an efficient means to establish the therapeutic potential of barrier-protective agents, large animal models are still essential since they allow for evaluation of regional differences in lung injury which is well recognized as characteristic of ALI in humans (Puybasset et al., 1998). As a result, the barrier-protective effects of S1P have been further evaluated in a canine model of ALI. Compared to injured controls after intrabronchial LPS administration, infusion of S1P in a canine model significantly reduces the formation of shunt, bronchoalveolar lavage (BAL) protein accumulation and the formation of alveolar edema as measured by computed tomography (McVerry et al., 2004). These exciting findings in murine and canine models of ALI indicate the great potential for S1P and related compounds in the treatment of vascular barrier dysfunction.

Summary

Significant and sustained increased vascular permeability is an essential pathophysiological feature of acute inflammatory diseases such as acute lung injury (ALI) and sepsis which have high morbidity and mortality. Unfortunately, efficient therapies to prevent or reverse established vascular leak are lacking. Our group is the first to demonstrate dose-dependent endothelial barrier enhancement by S1P in vitro and in vivo. By activating the G_i-protein coupled S1P₁ receptor and the downstream molecules of the small GTPase Rac, S1P induces cortactin translocation, peripheral MLC phosphorylation, strong cytoskeleton reorganization into the cortical actin ring, and assembly of adherens junctions, tight junctions, and focal adhesion complexes on the cell surface. Furthermore, in vivo studies have demonstrated that S1P significantly attenuates LPS-induced murine and canine models of ALI. Improved understanding of the mechanisms by which the S1P/S1P₁ signaling pathway promotes endothelial barrier integrity will hopefully benefit our future therapeutic efforts to treat acute inflammatory diseases.

References

- Andriopoulou P, et al. Histamine induces tyrosine phosphorylation of endothelial cell-to-cell adherens junctions. *Arterioscler Thromb Vasc Biol* 1999;19:2286–97. [PubMed: 10521356]
- Aschner JL, et al. Bradykinin- and thrombin-induced increases in endothelial permeability occur independently of phospholipase C but require protein kinase C activation. *J Cell Physiol* 1997;173:387–96. [PubMed: 9369952]

- Bazzoni G, Dejana E. Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis. *Physiol Rev* 2004;84:869–901. [PubMed: 15269339]
- Bensadoun ES, et al. Proteoglycan deposition in pulmonary fibrosis. *Am J Respir Crit Care Med* 1996;154:1819–28. [PubMed: 8970376]
- Broussard JA, et al. Asymmetric focal adhesion disassembly in motile cells. *Curr Opin Cell Biol* 2008;20:85–90. [PubMed: 18083360]
- Budde K, et al. FTY720 (fingolimod) in renal transplantation. *Clin Transplant* 2006;20(Suppl 17):17–24. [PubMed: 17100697]
- Cantor JO, Turino GM. Can exogenously administered hyaluronan improve respiratory function in patients with pulmonary emphysema? *Chest* 2004;125:288–92. [PubMed: 14718453]
- Carmeliet P, et al. Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. *Cell* 1999;98:147–57. [PubMed: 10428027]
- Corada M, et al. Vascular endothelial-cadherin is an important determinant of microvascular integrity in vivo. *Proc Natl Acad Sci U S A* 1999;96:9815–20. [PubMed: 10449777]
- Dudek SM, et al. Pulmonary endothelial cell barrier enhancement by FTY720 does not require the SIP1 receptor. *Cell Signal* 2007;19:1754–64. [PubMed: 17475445]
- Dudek SM, Garcia JG. Cytoskeletal regulation of pulmonary vascular permeability. *J Appl Physiol* 2001;91:1487–500. [PubMed: 11568129]
- Dudek SM, et al. Pulmonary endothelial cell barrier enhancement by sphingosine 1-phosphate: roles for cortactin and myosin light chain kinase. *J Biol Chem* 2004;279:24692–700. [PubMed: 15056655]
- Ebnet K, et al. Junctional adhesion molecule interacts with the PDZ domain-containing proteins AF-6 and ZO-1. *J Biol Chem* 2000;275:27979–88. [PubMed: 10856295]
- Finigan JH, et al. Activated protein C mediates novel lung endothelial barrier enhancement: role of sphingosine 1-phosphate receptor transactivation. *J Biol Chem* 2005;280:17286–93. [PubMed: 15710622]
- Furuse M, et al. Direct association of occludin with ZO-1 and its possible involvement in the localization of occludin at tight junctions. *J Cell Biol* 1994;127:1617–26. [PubMed: 7798316]
- Furuse M, et al. Manner of interaction of heterogeneous claudin species within and between tight junction strands. *J Cell Biol* 1999;147:891–903. [PubMed: 10562289]
- Garcia JG, et al. Sphingosine 1-phosphate promotes endothelial cell barrier integrity by Edg-dependent cytoskeletal rearrangement. *J Clin Invest* 2001;108:689–701. [PubMed: 11544274]
- Garcia JG, et al. Regulation of endothelial cell myosin light chain kinase by Rho, cortactin, and p60(src). *Am J Physiol* 1999;276:L989–98. [PubMed: 10362724]
- Gimbrone MA Jr, et al. Preservation of vascular integrity in organs perfused in vitro with a platelet-rich medium. *Nature* 1969;222:33–6. [PubMed: 5775827]
- Gosens R, et al. Role of Rho-kinase in maintaining airway smooth muscle contractile phenotype. *Eur J Pharmacol* 2004;483:71–8. [PubMed: 14709328]
- Guo Y, et al. Quantitative proteomics analysis of human endothelial cell membrane rafts: evidence of MARCKS and MRP regulation in the sphingosine 1-phosphate-induced barrier enhancement. *Mol Cell Proteomics* 2007;6:689–96. [PubMed: 17210631]
- Hait NC, et al. Sphingosine kinases, sphingosine 1-phosphate, apoptosis and diseases. *Biochim Biophys Acta* 2006;1758:2016–26. [PubMed: 16996023]
- Hippenstiel S, et al. VEGF induces hyperpermeability by a direct action on endothelial cells. *Am J Physiol* 1998;274:L678–84. [PubMed: 9612282]
- Hla T, et al. Lysophospholipids--receptor revelations. *Science* 2001;294:1875–8. [PubMed: 11729304]
- Hla T, Maciag T. An abundant transcript induced in differentiating human endothelial cells encodes a polypeptide with structural similarities to G-protein-coupled receptors. *J Biol Chem* 1990;265:9308–13. [PubMed: 2160972]
- Jacobson JR, Garcia JG. Novel therapies for microvascular permeability in sepsis. *Curr Drug Targets* 2007;8:509–14. [PubMed: 17430121]
- Kappos L, et al. Oral fingolimod (FTY720) for relapsing multiple sclerosis. *N Engl J Med* 2006;355:1124–40. [PubMed: 16971719]

- Kapur S, et al. Recombinant human activated protein C for severe sepsis. *N Engl J Med* 2001;345:219–20. [PubMed: 11463021]author reply 220-1
- Komarova YA, et al. Dual regulation of endothelial junctional permeability. *Sci STKE* 2007;2007:re8. [PubMed: 18000237]
- Krump-Konvalinkova V, et al. Stable knock-down of the sphingosine 1-phosphate receptor SIP1 influences multiple functions of human endothelial cells. *Arterioscler Thromb Vasc Biol* 2005;25:546–52. [PubMed: 15618544]
- Lee JF, et al. Dual roles of tight junction-associated protein, zonula occludens-1, in sphingosine 1-phosphate-mediated endothelial chemotaxis and barrier integrity. *J Biol Chem* 2006;281:29190–200. [PubMed: 16891661]
- Lee MJ, et al. The inducible G protein-coupled receptor edg-1 signals via the G(i)/mitogen-activated protein kinase pathway. *J Biol Chem* 1996;271:11272–9. [PubMed: 8626678]
- Lee MJ, et al. Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate. *Cell* 1999;99:301–12. [PubMed: 10555146]
- Lee MJ, et al. Sphingosine-1-phosphate as a ligand for the G protein-coupled receptor EDG-1. *Science* 1998;279:1552–5. [PubMed: 9488656]
- McVerry BJ, Garcia JG. Endothelial cell barrier regulation by sphingosine 1-phosphate. *J Cell Biochem* 2004;92:1075–85. [PubMed: 15258893]
- McVerry BJ, et al. Sphingosine 1-phosphate reduces vascular leak in murine and canine models of acute lung injury. *Am J Respir Crit Care Med* 2004;170:987–93. [PubMed: 15282202]
- Mehta D, et al. Sphingosine 1-phosphate-induced mobilization of intracellular Ca²⁺ mediates rac activation and adherens junction assembly in endothelial cells. *J Biol Chem* 2005;280:17320–8. [PubMed: 15728185]
- Mehta D, Malik AB. Signaling mechanisms regulating endothelial permeability. *Physiol Rev* 2006;86:279–367. [PubMed: 16371600]
- Mehta D, et al. Modulatory role of focal adhesion kinase in regulating human pulmonary arterial endothelial barrier function. *J Physiol* 2002;539:779–89. [PubMed: 11897849]
- Michel CC, Curry FE. Microvascular permeability. *Physiol Rev* 1999;79:703–61. [PubMed: 10390517]
- Milici AJ, et al. Transcytosis of albumin in capillary endothelium. *J Cell Biol* 1987;105:2603–12. [PubMed: 3320050]
- Nitta T, et al. Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. *J Cell Biol* 2003;161:653–60. [PubMed: 12743111]
- Peng X, et al. Protective effects of sphingosine 1-phosphate in murine endotoxin-induced inflammatory lung injury. *Am J Respir Crit Care Med* 2004;169:1245–51. [PubMed: 15020292]
- Peters SL, Alewijnse AE. Sphingosine-1-phosphate signaling in the cardiovascular system. *Curr Opin Pharmacol* 2007;7:186–92. [PubMed: 17280869]
- Predescu SA, et al. Molecular determinants of endothelial transcytosis and their role in endothelial permeability. *Am J Physiol Lung Cell Mol Physiol* 2007;293:L823–42. [PubMed: 17644753]
- Puybasset L, et al. A computed tomography scan assessment of regional lung volume in acute lung injury. The CT Scan ARDS Study Group. *Am J Respir Crit Care Med* 1998;158:1644–55. [PubMed: 9817720]
- Quadri SK, et al. Endothelial barrier strengthening by activation of focal adhesion kinase. *J Biol Chem* 2003;278:13342–9. [PubMed: 12556538]
- Rosen H, Goetzl EJ. Sphingosine 1-phosphate and its receptors: an autocrine and paracrine network. *Nat Rev Immunol* 2005;5:560–70. [PubMed: 15999095]
- Rotundo RF, et al. TNF-alpha disruption of lung endothelial integrity: reduced integrin mediated adhesion to fibronectin. *Am J Physiol Lung Cell Mol Physiol* 2002;282:L316–29. [PubMed: 11792637]
- Roy AJ, Djerassi I. Effects of platelet transfusions: plug formation and maintenance of vascular integrity. *Proc Soc Exp Biol Med* 1972;139:137–42. [PubMed: 5007449]
- Schaphorst KL, et al. Role of sphingosine-1 phosphate in the enhancement of endothelial barrier integrity by platelet-released products. *Am J Physiol Lung Cell Mol Physiol* 2003;285:L258–67. [PubMed: 12626332]

- Shikata Y, et al. Involvement of site-specific FAK phosphorylation in sphingosine-1 phosphate- and thrombin-induced focal adhesion remodeling: role of Src and GIT. *FASEB J* 2003a;17:2240–9. [PubMed: 14656986]
- Shikata Y, et al. S1P induces FA remodeling in human pulmonary endothelial cells: role of Rac, GIT1, FAK, and paxillin. *J Appl Physiol* 2003b;94:1193–203. [PubMed: 12482769]
- Singleton PA, et al. Regulation of sphingosine 1-phosphate-induced endothelial cytoskeletal rearrangement and barrier enhancement by SIP1 receptor, PI3 kinase, Tiam1/Rac1, and alpha-actinin. *FASEB J* 2005;19:1646–56. [PubMed: 16195373]
- Singleton PA, et al. Transactivation of sphingosine 1-phosphate receptors is essential for vascular barrier regulation. Novel role for hyaluronan and CD44 receptor family. *J Biol Chem* 2006;281:34381–93. [PubMed: 16963454]
- Singleton PA, et al. Attenuation of vascular permeability by methyl naltrexone: role of mOP-R and S1P3 transactivation. *Am J Respir Cell Mol Biol* 2007;37:222–31. [PubMed: 17395891]
- Tani M, et al. Ceramide/sphingosine/sphingosine 1-phosphate metabolism on the cell surface and in the extracellular space. *Cell Signal* 2007;19:229–37. [PubMed: 16963225]
- Teran Arce F, et al. Regulation of the Micromechanical Properties of Pulmonary Endothelium by S1P and Thrombin: Role of Cortactin. *Biophys J*. 2008
- Toole BP. Hyaluronan: from extracellular glue to pericellular cue. *Nat Rev Cancer* 2004;4:528–39. [PubMed: 15229478]
- Tzima E. Role of small GTPases in endothelial cytoskeletal dynamics and the shear stress response. *Circ Res* 2006;98:176–85. [PubMed: 16456110]
- van Nieuw Amerongen GP, et al. GIT1 mediates thrombin signaling in endothelial cells: role in turnover of RhoA-type focal adhesions. *Circ Res* 2004;94:1041–9. [PubMed: 15016733]
- Venkataraman K, et al. Extracellular export of sphingosine kinase-1a contributes to the vascular S1P gradient. *Biochem J* 2006;397:461–71. [PubMed: 16623665]
- Venkiteswaran K, et al. Regulation of endothelial barrier function and growth by VE-cadherin, plakoglobin, and beta-catenin. *Am J Physiol Cell Physiol* 2002;283:C811–21. [PubMed: 12176738]
- Vestweber D. VE-cadherin: the major endothelial adhesion molecule controlling cellular junctions and blood vessel formation. *Arterioscler Thromb Vasc Biol* 2008;28:223–32. [PubMed: 18162609]
- Vouret-Craviari V, et al. Distinct signals via Rho GTPases and Src drive shape changes by thrombin and sphingosine-1-phosphate in endothelial cells. *J Cell Sci* 2002;115:2475–84. [PubMed: 12045218]
- Ware LB, Matthay MA. The acute respiratory distress syndrome. *N Engl J Med* 2000;342:1334–49. [PubMed: 10793167]
- Waschke J, et al. Activation of Rac-1 and Cdc42 stabilizes the microvascular endothelial barrier. *Histochem Cell Biol* 2006;125:397–406. [PubMed: 16195887]
- Wojciak-Stothard B, et al. Rac1 and RhoA as regulators of endothelial phenotype and barrier function in hypoxia-induced neonatal pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol* 2006;290:L1173–82. [PubMed: 16428270]
- Xu M, et al. Sphingosine 1-phosphate rapidly increases endothelial barrier function independently of VE-cadherin but requires cell spreading and Rho kinase. *Am J Physiol Cell Physiol* 2007;293:C1309–18. [PubMed: 17670896]
- Yang L, et al. Metabolism and functional effects of sphingolipids in blood cells. *Br J Haematol* 1999;107:282–93. [PubMed: 10583213]
- Yatomi Y, et al. Sphingosine-1-phosphate: a platelet-activating sphingolipid released from agonist-stimulated human platelets. *Blood* 1995;86:193–202. [PubMed: 7795224]

Figure 1. Biosynthesis and metabolism of S1P

Depicted are the primary cellular components responsible for S1P generation and degradation. SM, Sphingomyelin; SMase, Sphingomyelinase; Cer, Ceramide; CDase, Ceramidase; Sph, Sphingosine; S1P, Sphingosine 1-phosphate; SphK, Sphingosine kinase; S1Pase, Sphingosine 1-phosphate phosphatase; S1PL, S1P lyase; P-Eth, Phosphoethanolamine; Hex, Hexadecanal.

Figure 2. Regulation of Vascular Permeability by S1P/ SIP₁ signaling

Binding of S1P to the SIP₁ receptor stimulates the G_i-dependent recruitment of PI3 kinase, Tiam1 and Rac1 to lipid rafts (CEM), which serves to activate Rac1 in a G_i-PI3K-Tiam1 dependent manner. In addition, S1P induces an increase in intracellular Ca²⁺ concentration via a G_i-PLC pathway with additional activation of Rac1. After the activation of Rac1, S1P induces a series of profound events including adherens junction and tight junction assembly, cytoskeletal reorganization, and formation of focal adhesions that combine to enhance vascular barrier function. Furthermore, the transactivation of SIP₁ signaling by other barrier enhancing agents is recently recognized as a common mechanism for promoting endothelial barrier function. TJ, tight junction; AJ, adherens junction; S1P, Sphingosine-1-phosphate; SIP₁, Sphingosine-1-phosphate receptor 1; PI3K, Phosphoinositide 3-kinase; Tiam1, T-lymphoma invasion and metastasis gene 1; Rac1, Rho family of GTPase Rac1; PAK1, p21-activated protein kinase 1; LIMK, LIM kinase; PLC, Phospholipase C; ZO-1, Zona occluden protein-1; nmMLCK, non-muscle myosin light chain kinase; VE-Cad, Vascular endothelial cadherin; α-Cat, α-Catenin; β-Cat, β-Catenin; Vin, Vinculin; Pax, Paxillin; FAK, focal adhesion kinase; GIT2, G protein-coupled receptor kinase interactor-1; ECM, Extracellular matrix; APC, Activated protein C; LMW-HA, high molecule weight hyaluronan.

Figure 3. S1P reduces lung permeability in vivo

24 hours after mice received intratracheal LPS (2 mg/kg) or control saline, lung tissue specimens were harvested, fixed in paraformaldehyde, and histologically evaluated by hematoxylin and eosin stain. The top panel demonstrates a pronounced inflammatory cell infiltrate in response to LPS (inset: control lung for comparison). However, in mice receiving intravenous S1P (1 μ M final concentration) one hour after LPS administration, the inflammatory cell infiltrate is markedly reduced (bottom panel). This effect demonstrates the potent ability of S1P to decrease vascular permeability to inflammatory cells in vivo (Peng et al., 2004).