Translational Review

Sphingosine-1–Phosphate, FTY720, and Sphingosine-1–Phosphate Receptors in the Pathobiology of Acute Lung Injury

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Acute lung injury (ALI) attributable to sepsis or mechanical ventilation and subacute lung injury because of ionizing radiation (RILI) share profound increases in vascular permeability as a key element and a common pathway driving increased morbidity and mortality. Unfortunately, despite advances in the understanding of lung pathophysiology, specific therapies do not yet exist for the treatment of ALI or RILI, or for the alleviation of unremitting pulmonary leakage, which serves as a defining feature of the illness. A critical need exists for new mechanistic insights that can lead to novel strategies, biomarkers, and therapies to reduce lung injury. Sphingosine 1–phosphate (S1P) is a naturally occurring bioactive sphingolipid that acts extracellularly via its G protein–coupled $S1P_{1-5}$ as well as intracellularly on various targets. S1P-mediated cellular responses are regulated by the synthesis of S1P, catalyzed by sphingosine kinases 1 and 2, and by the degradation of S1P mediated by lipid phosphate phosphatases, S1P phosphatases, and S1P lyase. We and others have demonstrated that S1P is a potent angiogenic factor that enhances lung endothelial cell integrity and an inhibitor of vascular permeability and alveolar flooding in preclinical animal models of ALI. In addition to S1P, S1P analogues such as 2-amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol (FTY720), FTY720 phosphate, and FTY720 phosphonates offer therapeutic potential in murine models of lung injury. This translational review summarizes the roles of S1P, S1P analogues, S1P-metabolizing enzymes, and S1P receptors in the pathophysiology of lung injury, with particular emphasis on the development of potential novel biomarkers and S1P-based therapies for ALI and RILI.

Keywords: sphingolipids; S1P receptors; sphingosine kinase; S1P lyase; sepsis

Acute and subacute inflammatory lung injuries are common and devastating disorders resulting from insults such as sepsis, ventilator-induced lung injury, ischemia/reperfusion, hyperoxia, and radiation therapy for thoracic malignancies. Unfortunately, despite recent advances in our understanding of the mechanisms

CLINICAL RELEVANCE

Acute lung injury (ALI) attributable to sepsis or mechanical ventilation and subacute lung injury attributable to ionizing radiation (RILI) share profound increases in vascular permeability as a key element and a common pathway driving increased morbidity and mortality. This translational review summarizes the roles of sphingosine 1–phosphate (S1P), S1P analogues, S1P-metabolizing enzymes, and S1P receptors in the pathophysiology of lung injury, with particular emphasis on the development of potential novel biomarkers and S1P-based therapies for ALI and RILI.

and pathophysiology of acute lung injury (ALI), mortality rates remain very high (30–50%) because of the dearth of specific therapies for the treatment of ALI (1, 2). The only therapy for radiation-induced pneumonitis is based on long-term treatment with a high dose of corticosteroids. However, it is encumbered by severe side effects and relatively low efficacy (3). Therefore, an urgent need exists for new mechanistic insights into the pathophysiology of ALI that are likely to reveal new potential therapeutic targets, discover novel biomarkers, and develop highly efficacious targeted therapies that will effectively reduce the morbidity and mortality associated with acute and subacute lung injury.

"Sphingosin," first described by J. L. W. Thudichum in 1884, derived its name from the Greek word "sphinx" meaning enigmatic, and it encompasses many compounds commonly referred to as "sphingoid bases" (4). Since their initial description, sphingoid bases have been found to be critical structural components of biological membranes and highly essential bioactive lipids that regulate diverse signaling pathways. The aberrant regulation of the sphingoid bases is known to contribute to a variety of pathologies that underlie cancer, inflammation, injury, edema, and infections (5–9). Of the several hundred sphingoid bases described to date, at least six, namely, sphingomyelin (SM), sphingosine (Sph), Sph-1–phosphate (S1P), ceramide, ceramide 1–phosphate (Cer1P), and sphingosylphosphorylcholine (lyso-SM), are considered key signaling and regulatory bioactive lipids (10). Furthermore, the importance of these lipids in human health and disease is underscored by the exponentially increasing number of publications that define key roles for these lipids in the areas of basic biology and translational and clinical research.

Among the various organs, the lung has been investigated to understand better the role of S1P, its receptors, and its metabolizing enzymes in cellular functions under physiological as well as pathological conditions such as acute and subacute lung injury, pulmonary barrier dysfunction/edema, emphysema, and airway inflammation (5, 6, 11). In view of the complexity of the sphingolipid metabolism, the interconversion of bioactive sphingolipids, and the varied expressions and differential functions of their multiple G protein–coupled receptors, we perceived the need for a comprehensive review of the literature that addresses the pathways that regulate the metabolism and mechanisms of action for S1P in ALI. This review addresses the regulatory mechanisms underlying S1P generation and signaling in the context of lung inflammation and injury, especially in conditions such as sepsis-induced and radiation-induced lung injury, with an emphasis on genomic, lipidomic, and metabolomic approaches. In addition to S1P, the roles of Sph and S1P analogues such as 2-amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol (FTY720), (S)-FTY720 phosphate (FTY720-P), and FTY720 phosphonates as novel therapeutic agents for acute lung injury will be discussed.
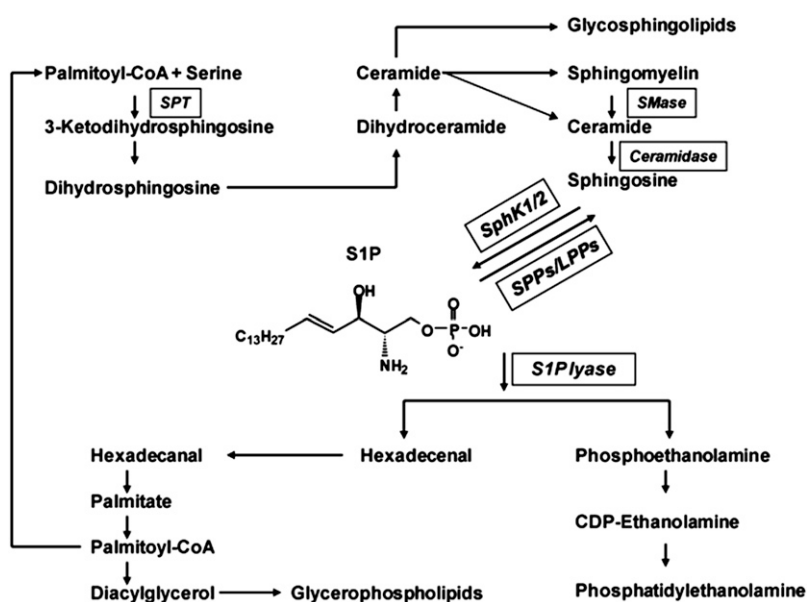
SPHINGOLIPID METABOLISM IN MAMMALIAN CELLS

SM, the major sphingolipid of biological membranes, is synthesized de novo from serine and palmitoyl coenzyme A (CoA), which undergo condensation catalyzed by serine palmitoyltransferase (SPT) to form 3–keto-dihydroSph, which is reduced to dihydroSph, followed by ceramide synthase–mediated N-acylation to dihydroceramide and subsequent desaturation to ceramide (12). Ceramide is then channeled to complex sphingolipids such as SM or hydrolyzed to Sph through the action of ceramidases, or glycosylated to form glycosphingolipids (Figure 1). Ceramide is also phosphorylated by ceramide kinase to Cer1P (13). Mammalian cells do not convert dihydroSph to Sph. However, Sph is generated from ceramides by ceramidases (14). Sph is phosphorylated by two Sph kinases, SphK1 and SphK2, to S1P (15), and S1P is dephosphorylated to Sph through the action of specific S1P phosphatases 1 and 2 (S1PPases) (16) or through nonspecific lipid phosphate phosphatases (LPPs) (17, 18). Alternatively, S1P is irreversibly cleaved by S1P lyase (S1PL), a pyridoxal phosphate–dependent enzyme, to ethanolamine phosphate and trans–2-hexadecenal. Subsequently,

trans–2-hexadecenal is oxidized by fatty aldehyde dehydrogenase to trans–2-hexadecenoic acid, which is recycled into glycerolipid or sphingolipid metabolic pathways, whereas ethanolamine phosphate is used for the biosynthesis of ethanolamine phospholipids (Figure 1) (19–21). Moreover, in response to TNF-α and other agonists, SM is hydrolyzed to ceramides of variable N-acyl chain lengths by one of the three (acid, neutral, or alkaline) sphingomyelinases (SMases) (Figure 2) (22). Ceramide acts intracellularly and functions as a second messenger by modulating ceramide-activated protein phosphatases and kinases (23, 24). Thus, the complexity in the sphingolipid metabolism enables cells to orchestrate cellular responses by regulating the interconversions via the anabolic and catabolic enzymes that regulate their intracellular concentrations and spatiotemporal distributions.

SPHINGOSINE 1–PHOSPHATE IN VASCULAR PERMEABILITY

S1P is present in plasma and tissues, and the concentrations of S1P are 3–4 times higher in serum than in plasma (25). The source of plasma S1P is controversial. The initial notion that platelets are a major source of circulating S1P may be erroneous, because erythrocytes (26), hematopoietic cells (27), and vascular endothelial cells (ECs) (28) are known to contribute to plasma S1P. S1P, initially identified as a mitogen for fibroblasts (29), is a potent angiogenic factor and plays an essential role in vessel maturation, vascular permeability, the trafficking of T-lymphocytes, B-lymphocytes, and dendritic cells, reproduction, and central nervous system development (30, 31). The ability of platelets to decrease endothelial barrier permeability (32) may be mediated by S1P stored within the platelets (33). Pioneering studies by Dr. J. G. N. Garcia and others identified S1P as a major barrier-protective agent responsible for the maintenance of vascular barrier integrity in vitro and in vivo (33–36). The exogenous addition of S1P to human and bovine lung ECs increased transendothelial monolayer resistance. The barrier-enhancing effect of S1P was rapid, dose-dependent, and mediated mostly through S1P$_1$ (34). Agonists of S1P receptors such as 5-[4-phenyl-5-(trifluoromethyl)-2-thienyl]-3-[3-(trifluoromethyl)phenyl]-1,2,4-oxadiazole (SEW2871), FTY720-P, and FTY720 phosphonates were also effective in enhancing endothelial barrier function (37). Interestingly, the intracellular release of S1P by the photolysis of a caged S1P analogue also

Figure 1. Metabolism of sphingolipids in mammalian cells. Key enzymatic steps in the biosynthesis and degradation of sphingoid bases and recycling of trans–2-hexadecenal and ethanolamine phosphate from sphingolipids into glycerophospholipids are summarized. SPT, serine palmitoyltransferase; SMase, sphingomyelinase; S1P, sphingosine 1–phosphate; SphK, sphingosine kinase; SPP, S1P phosphatases; LPP, lipid phosphate phosphatase; CoA, coenzyme A; CDP, cytidine 5′-diphosphate.
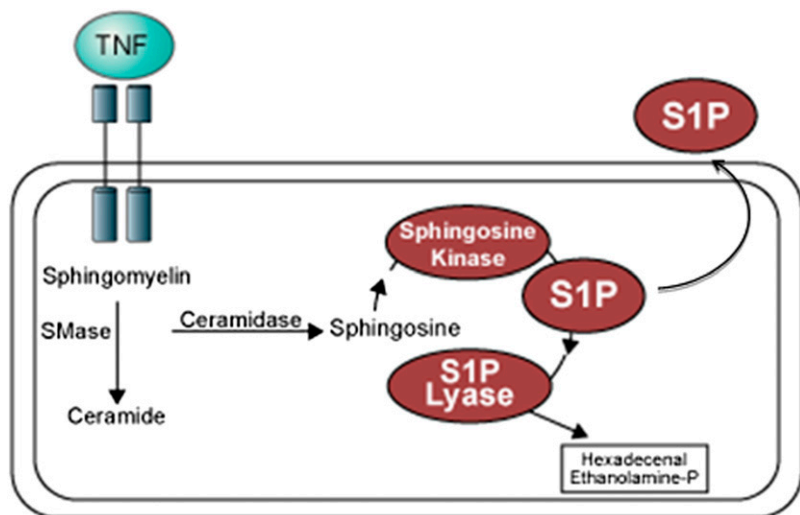
Figure 2. Intracellular generation and catabolism of S1P. The ceramide generated by the agonist-dependent hydrolysis of sphingomyelin (SM) by SMase is converted to sphingosine by ceramidases. Sphingosine is phosphorylated by SphK1 and/or SphK2 to S1P, which is transported outside the cell or catabolized to *trans*–2-hexadecenal and ethanolamine phosphate by S1P lyase. SMase, sphingomyelinase; S1P, sphingosine 1–phosphate.

rapidly and significantly enhanced endothelial barrier function (38). However, this effect of intracellular S1P was independent of $S1P_1$, and instead required Ras-related C3 botulinum toxin substrate 1 (Rac1) (38). That study delineated an important function for intracellular S1P and enzymes involved in the accumulation of S1P in cells in regulating barrier integrity. In fact, the extracellular action of S1P on endothelial barrier enhancement was dependent on intracellular S1P generation, because the blocking or down-regulation of SphK–1 activity or its forced expression attenuated S1P-induced barrier enhancement (Viswanathan Natarajan, unpublished data). In addition to *in vitro* barrier-protective effects, a barrier-regulatory role for S1P in murine and canine models of ALI was demonstrated. In an isolated perfused lung model, S1P infusion (1 μM) resulted in a significant decrease in the rate of edema formation, without a change in pulmonary artery pressure (39). Similarly, in mice suffering from LPS-induced lung injury or renal injury, pulmonary edema was significantly attenuated by an intravenous administration of S1P (39). The ability of S1P to confer protection against sepsis-induced barrier dysfunction was also confirmed in a canine model, wherein the infusion of S1P reduced bronchoalveolar lavage (BAL) fluid protein accumulation and alveolar edema, as measured by computed tomography (35).

MECHANISMS OF S1P-MEDIATED BARRIER PROTECTION

The mechanisms of the S1P-mediated regulation of vascular permeability are yet to be fully defined. S1P binding to $S1P_1$ or other S1P receptors activates Rac, cortactin translocation, peripheral myosin light chain phosphorylation, focal adhesion, adherens junction rearrangement, and recruits these signaling molecules and cytoskeletal effectors to lipid rafts. S1P also induces tight-junction assembly that further strengthens the endothelial barrier (Figure 3) (31, 34, 40). Because caged S1P–mediated barrier enhancement is Rac1-dependent (38), S1P may directly interact or bind with Rac1, and induce the dissociation of the Rho guanosine diphosphate (GDP) dissociation inhibitor (RhoGDI) from Rac1 for activation and redistribution to the cell periphery (41). This suggests that the action of S1P could be similar to that of another acidic phospholipid, phosphatidic acid (PA), generated by the phospholipase D signaling pathway, wherein PA acts as a membrane anchor of Rac1 by interacting with the polybasic motif in the carboxyl-terminal of Rac1, as shown in ovarian carcinoma-3 (OVCAR-3) cells (42). Further,

intracellularly generated S1P may bind to S1P receptors located in organelles such as the endoplasmic reticulum and nuclear membranes, and initiate signal transduction or signal "inside-out" via S1P receptors on the cell surface (20). Thus, localized, intracellularly generated S1P can play an important role in modulating signaling pathways and cell functions, and this role requires further investigation.

SPHINGOSINE KINASES AND S1P LYASE IN SEPSIS-INDUCED LUNG INJURY

The S1P-induced protection of endothelial barrier function in LPS-induced lung injury suggests a role for S1P-metabolizing enzymes in lung injury and repair. Circulating and cellular S1P concentrations are regulated by the synthesis and catabolism of S1P (15, 30, 43). The availability of Sph is a key event in the intracellular generation of S1P, and Sph is derived either from ceramides through ceramidases or from circulating plasma S1P through ecto-LPPs (9, 17, 18). Recent studies showed that human lung ECs have the ability to use exogenously added S1P to generate intracellular S1P by lipid phosphate phosphatases (18). In addition to these two pathways, S1P can also be generated in plasma by the lysophospholipase D/autotaxin–mediated hydrolysis of sphingosylphosphorylcholine (44). However, whether this pathway provides a major source of plasma S1P remains unclear. Thus, targeting SphKs, S1PPases, LPPs, and S1PL represent novel therapeutic approaches with the potential to minimize or ameliorate lung inflammation and injury.

ROLE OF Sph KINASES 1 AND 2 IN ACUTE AND SUBACUTE LUNG INJURY

The role of SphKs in lung inflammation and injury is somewhat controversial. The loss of SphK1 or SphK2 expression in mice exerted no significant effect on inflammatory responses, and normal neutrophil function was observed in *SphK1* and *SphK2* knockout mice. However, accelerated bacterial lung infection in SphK2, but not in SphK1, knockout mice compared with wild-type control mice was observed (45). The inhibition of SphK1 expression using an antisense or a SphK inhibitor such as *N,N*-dimethyl-Sph attenuated neutrophil activation, chemotaxis, and lung permeability (46), and disruption of the *SphK1* gene in mice exerted no effect on lymphocyte trafficking and lymphocyte distribution (47). The LPS challenge of C57BL/6 wild-type mice differentially up-regulated SphK1 and SphK2 expression levels.
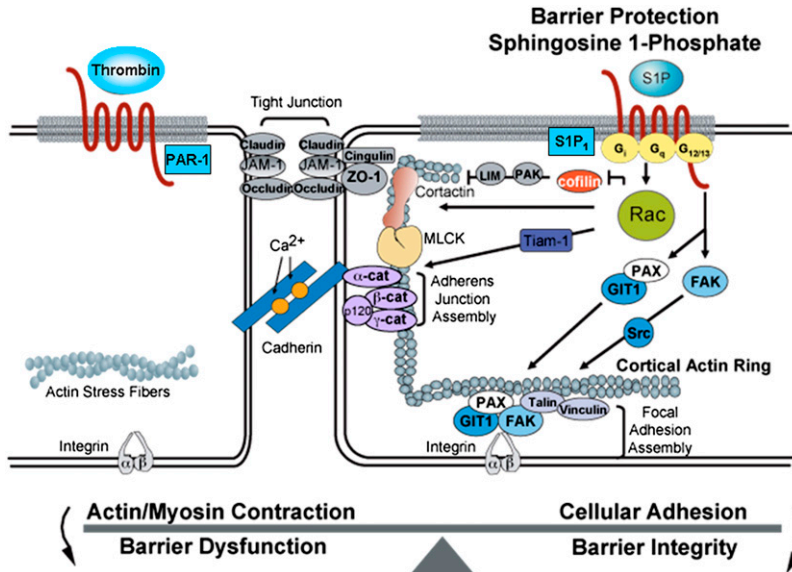
Figure 3. Regulation of endothelial barrier function by S1P. S1P binding to G protein–coupled S1P1 activates Rac1 and induces a series of signaling cascades, including cytoskeletal reorganization, the assembly of adherens junction and tight junction proteins, and the formation of focal adhesions that act together to enhance endothelial barrier function. However, cleavage of the protease-activated receptor (PAR-1) by thrombin induces actin stress fiber formation, and disrupts the assembly of adherens junctions and tight junction and focal adhesion proteins, resulting in barrier disruption. LIM, LIM kinase; PAK, p21-activated kinase–1; ZO, zona occludins; JAM, junctional adhesion molecule; PXN, paxillin; GIT, G protein–coupled receptor kinase–interacting protein; FAK, focal adhesion kinase; MLCK, myosin light chain kinase; cat, catenin; Src, Rous sarcoma oncogene cellular honolog; Rac1, Ras related C3 botulinum toxin 1.

However, $SphK1^{-/-}$ mice were more susceptible to LPS-induced lung injury compared with wild-type mice, whereas the overexpression of SphK1 (wild-type) delivered by an adenoviral vector to the lungs protected $SphK1^{-/-}$ mice from lung injury and blunted the severity of the LPS response (48). SphK1 was up-regulated in stimulated human phagocytes and in peritoneal phagocytes of patients with severe sepsis, and the inhibition of SphK1 with siRNA protected mice against sepsis-induced proinflammatory responses (49). In that study, LPS challenge or cecal ligation puncture increased the mortality rate in $SphK1^{-/-}$ mice, yet it was suggested that this phenomenon represents an "adaptive compensation during development" that contradicts several earlier studies demonstrating an anti-inflammatory role for Sphk1 in ALI (49). Because Sphk1 regulates S1P production, macrophages with an increased expression of Sphk1 should generate higher concentrations of S1P. However, the reported results in Sphk1 knockdown experiments are quite contradictory (49). Recently, a novel antimycobacterial role for Sphk1 was reported in macrophages wherein SphK1 knockdown increased sensitivity to *Mycobacterium smegmatis* infection (50). In phagocytes, SphK1 regulated C5L2 and CD88 expression, and dampened inflammatory responses to endotoxin (51). Therefore, additional ALI models are necessary to determine whether SphK1 has a protective or proinflammatory role in lung injury and inflammation. In contrast to the LPS-induced lung injury model, SphK1 deficiency protected mice from hyperoxia-induced lung inflammation and injury (Viswanathan Natarajan and colleagues, unpublished data). Thus, the role of SphK1 in lung inflammation and injury might depend on the type of insult and the degree of oxidative stress, because increased S1P modulates the generation of reactive oxygen species (ROS) via nicotinamide adenine dinucleotide phosphate–reduced oxidase activation.

In addition to ALI, SphK1 seems to play a role in sub-ALI, such as radiation-induced lung injury (RILI). The thoracic radiation of mice (25 Gy) enhanced the expression of SphK1 and SphK2 in mouse lungs after 6 weeks of treatment and increased the ratio of ceramide to S1P and dihydro-S1P in plasma, BAL fluid, and lung tissue (11). $SphK1^{-/-}$ mice exhibited a higher susceptibility to RILI after 6 weeks of treatment, indicating a protective role for SphK1 against RILI (11). However, pretreatment with myriocin, an inhibitor of SPT, decreased inflammation and fibrogenesis at 18 weeks after irradiation (52). The inhibition of SPT also decreased radiation-induced SphK

activity in the lung, which modulated the concentrations of S1P/dihydro-S1P in lung tissue and the circulation (52). Interestingly, simvastatin, a 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor, attenuated RILI in the mouse model by modulating the expression of SphK and S1PL proteins and the concentrations of ceramide to S1P/dihydroS1P in lung tissue (53). These results support the notion that S1P and S1P-metabolizing enzymes offer potential therapeutic targets against RILI.

S1PL DEFICIENCY PROTECTS AGAINST LPS-INDUCED ALI

Current evidence suggests a role for S1PL in normal development, reproduction, cell survival, cancer, and immunity (19). S1PL function seems to be critical for mammalian survival, because S1P lyase knockout ($Sgpl1^{-/-}$) mice do not survive beyond a couple of weeks after birth, and exhibit vascular abnormalities (54). S1PL deficiency elevated S1P, Sph, ceramide, and SM concentrations in the serum and liver (55), and produced a proinflammatory response by impairing neutrophil trafficking (56). In $Sgpl1^{+/-}$ mice, LPS challenge increased lethality, serum concentrations of TNF-α, monocyte chemotactic protein 1 (MCP-1), and IL-6, and sphingoid bases compared with $Sgpl1^{+/+}$ mice (57), suggesting a detrimental effect of high-circulating S1P concentrations. The genetic and chemical inhibition of S1PL with the inhibitors 2-acetyl-4 (5)-[1R,2S,3R,4-tetrahydroxybutyl]-imidazole (THI) and LX2931 increased circulating and tissue S1P concentrations, reduced peripheral lymphocytes, and alleviated inflammatory responses in animal models of autoimmunity (58). A complete deficiency of S1PL in mice resulted in lesions in the lung, heart, urinary system, and bone, as well as T-cell depletion in the blood, thymus, spleen, and lymph nodes, which was attributed to very high circulating S1P concentrations (58). However, the partial restoration of S1PL activity in humanized knock-in mice harboring one ($Sgpl1^{H/-}$) or two ($Sgpl1^{H/H}$) alleles of human $Sgpl1$ offered protection from the lethal lymphoid lesions that developed in $Sgpl1^{-/-}$ mice (58).

A novel role for S1PL and intracellularly generated S1P in protecting against LPS-induced ALI was recently demonstrated *in vivo* and *in vitro* (59). An intratracheal instillation of LPS (5 mg/kg) to mice enhanced lung S1PL expression, decreased S1P

concentrations in lung tissue, and induced lung injury. *Sgpl1*$^{+/-}$ mice exhibited increased S1P concentrations in lung tissue and BAL fluid, with reduced lung injury and inflammation in response to LPS challenge. Furthermore, the reduction of S1PL activity by an oral administration of THI showed a direct correlation between elevated S1P concentrations in lung tissue and BAL fluid and reduced concentrations of neutrophils and IL-6 in mice receiving LPS intratracheally (59). The *in vitro* treatment of human lung microvascular ECs with LPS resulted in reduced concentrations of intracellular S1P and increased mRNA and protein expression of S1PL. The down-regulation of S1PL expression by small interfering RNA (siRNA) increased S1P concentrations in the cells and medium, attenuated the LPS-mediated phosphorylation of p38 mitogen-activated protein kinase (MAPK) and inhibitor of κ B (I-κB), and decreased IL-6 secretion, whereas the overexpression of S1PL enhanced the LPS-induced phosphorylation of p38 MAPK and I-κB, and enhanced IL-6 secretion. S1PL siRNA was more effective than exogenous S1P at attenuating LPS-induced IL-6 secretion. Furthermore, S1PL siRNA attenuated LPS-induced endothelial barrier disruption by inducing the activation and redistribution of Rac1 to the cell periphery. Some controversy persists regarding the role of S1PL-generated metabolites, namely, ethanolamine phosphate and *trans*–2-hexadecenal, in regulating physiological responses such as mitogenesis, inflammation, and apoptosis. In murine F9 embryonic carcinoma and Henrietta Lacks (HeLa) cells, the overexpression of SphK1 enhanced DNA synthesis. However, in *Sgpl1*$^{-/-}$ null cells or *Sgpl1*$^{-/-}$ null cells overexpressing SphK1, no effect on mitogenesis was evident, suggesting that the products of the S1PL pathway, and not S1P itself, stimulated mitogenesis (21, 60). The addition of *trans*–2-hexadecenal to human embryonic kidney293 (HEK293), National Institutes of Health 3-day transfer, inoculum 3 × 10,000 (NIH 3T3), and HeLa cells induced a cytoskeletal reorganization and apoptosis that was dependent on c-Jun N-terminal kinase (JNK) activation and c-Jun phosphorylation (61). In a recent study, *trans*–2-hexadecenal was shown to react readily with deoxyguanosine and DNA to form aldehyde-derived DNA adducts that may have potentially mutagenic consequences or trigger a previously unrecognized DNA damage response in living cells (62). Because both ethanolamine phosphate and *trans*–2-hexadecenal were added exogenously, it remains unclear whether intracellularly generated metabolites of S1P by S1PL exhibit similar physiological responses.

SM SYNTHASE 2 AND ALI

Sphingomyelin synthase (SMS) 1 and 2 catalyze the transfer of phosphorylcholine from phosphatidylcholine to ceramide and generate SM, a major component of all mammalian cell membranes. Recent studies demonstrated that SMS2 deficiency attenuates NF-κB signaling, thereby suggesting a role for ceramide in NF-κB signal transduction (63). However, ceramide also inhibits NF-κB activation (64), indicating a differential role
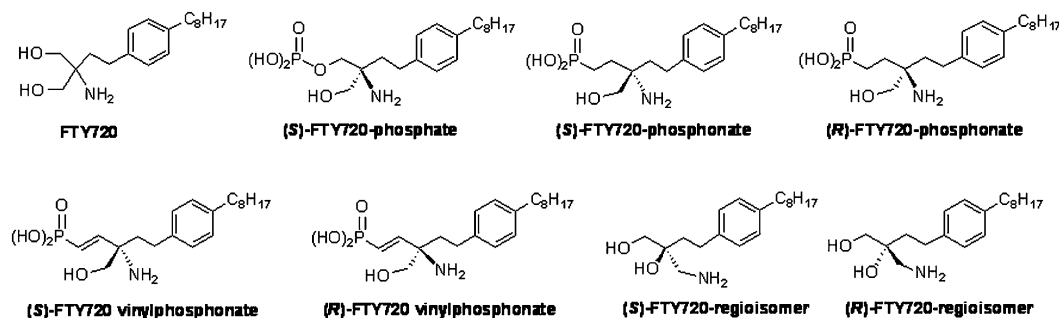
for ceramide in NF-κB activation in different cell types. *SMS2*$^{-/-}$ mice treated with LPS showed decreased inflammation, cytokine induction, and lung injury compared with *SMS2*$^{+/+}$ wild-type control mice (65). Furthermore, SMS2 depletion attenuated LPS-induced p38 MAPK and JNK activation and the transcriptional activity of NF-κB in human pulmonary artery endothelial cells, suggesting that blocking SM synthesis regulates endotoxin-induced inflammatory responses in a murine model of ALI. However, the effect of SMS2 knockdown on S1P, sphingosine, and ceramide concentrations in lung tissue and plasma was not determined (65).

LIMITATIONS OF S1P THERAPY IN ALI

Although S1P infusion has proven beneficial against LPS-induced lung injury in animal models (35, 39), it is an endogenous bioactive lipid that exerts pleiotropic effects, and some of these effects are likely to limit the usefulness of S1P in minimizing ALI. For example, an intravascular administration of S1P decreases the severity of ALI. However, intratracheal administrations can produce pulmonary edema through disruption of the epithelial/endothelial barrier via the ligation of S1P$_1$ or S1P$_3$. In human lung ECs, high concentrations of S1P (> 10 μM) can disrupt EC monolayer integrity *in vitro* through the ligation of S1P$_3$ and subsequent activation of Rho, suggesting a limited therapeutic window for S1P in barrier enhancement (31). S1P also exhibits well-described cardiac toxicity (bradycardia) through the activation of S1P$_3$ in the heart (66), directly stimulates the contraction of human airway and bronchial smooth muscle cells (67), and increases airway hyperresponsiveness in allergen-challenged mice (68), suggesting a potential for S1P to exacerbate airway obstruction in patients with asthma.

FTY720 AND FTY720-P AS BARRIER-PROTECTIVE AND ANTI-INFLAMMATORY AGENTS

Given the limitations of S1P as a therapeutic agent in ALI, considerable interest has arisen in the biologic effects of a structurally similar compound, FTY720 (Figure 4), a synthetic derivative of the fungal metabolite myriocin. FTY720 has attracted a great deal of clinical interest as an immunosuppressive agent, and in fact was approved by the United States Food and Drug Administration in 2010 for the treatment of multiple sclerosis (70). In addition to its immunomodulatory effects, FTY720 also decreased vascular permeability, both *in vivo* (39, 70) and *in vitro* (71). For example, a single intraperitoneal injection of FTY720 significantly attenuated murine pulmonary injury after LPS administration (35). Interestingly, although lower doses of FTY720 (0.01–1 μM) enhanced endothelial barrier function in human umbilical vein endothelial cells (HUVECs), higher concentrations of FTY720 (10–100 μM) induced irreversible barrier breakdown and apoptosis (72). Similarly, low concentrations of FTY720 (0.1 mg/kg) reduced lung permeability

FTY720

(S)-FTY720-phosphate

(S)-FTY720-phosphonate

(R)-FTY720-phosphonate

(S)-FTY720 vinylphosphonate

(R)-FTY720 vinylphosphonate

(S)-FTY720-regioisomer

(R)-FTY720-regioisomer

Figure 4. Structures of 2-amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol (FTY720) and FTY720 analogues. The chemical structures of FTY720, the (*R*) and (*S*) stereoisomers of FTY720 phosphate, FTY720 phosphonate, and FTY720 vinylphosphonate are illustrated.

in mechanically ventilated mice. However, higher concentrations (2 mg/kg) increased pulmonary leakage and apoptosis in ventilated mice, without affecting permeability in nonventilated mice (72). Because nonphosphorylated FTY720 demonstrates a low affinity for S1P receptors (73), current concepts related to mode of action for FTY720 invoke the phosphorylation of FTY720 *in situ* by SphK2 to produce the S1P analogue (S)-FTY720-P (74), thereby enhancing an affinity for the S1P family of receptors, and particularly $S1P_1$ and $S1P_3$. The phosphorylation of FTY720 to FTY720-P occurs rapidly both *in vitro* and *in vivo* (74). However, approximately 25% of FTY720 remains in a nonphosphorylated state in patients (75). Furthermore, FTY720-P reversed vascular endothelial growth factor–induced transcellular permeability in murine embryonic ECs (71). These investigations indicate that the FTY720-mediated protection against EC barrier dysfunction and lung inflammation is complex and dose-sensitive, and appears to involve both nonphosphorylated and phosphorylated forms of FTY720.

MECHANISMS OF BARRIER REGULATION BY FTY720 AND FTY720-P

The mechanisms of action by FTY720 and FTY720-P in preventing vascular leakage remain unclear. FTY720, unlike S1P, appears to internalize and down-regulate $S1P_1$ signaling, instead of activating this pathway. In contrast to S1P, FTY720 enhances EC barrier function without rapidly increasing intracellular calcium or cortical actin structure, or requiring the expression of proteins integral to the generation of the cortical actin ring (e.g., Rac and cortactin) (70). Furthermore, these studies suggest that FTY720 enhances EC barrier function via a novel $S1P_1$-independent mechanism, because abrogating $S1P_1$ expression using specific siRNA failed to block this effect, whereas embryonic ECs cultured from $S1P_1^{-/-}$ mice retained the ability to mount a FTY720-induced barrier-enhancing response (70). Because pertussis toxin completely abolished this effect (70), G_i protein–coupled receptors (GPCRs) appear to play a key role in this FTY720-conferred EC barrier enhancement. GPCRs comprise a large and diverse family of receptors with varying homologies to the S1P receptors that could potentially participate in transducing FTY720 responses (76). For example, FTY720 interacts with the cannabinoid family of GPCRs (77), but the primary cannabinoid receptors, CB_1 and CB_2, are not involved in the EC barrier–enhancing response (70). In addition, FTY720 (but not S1P) inhibits S1PL (78), cytosolic phospholipase A_2 (79), and ceramide synthases (80, 81), and activates protein phosphatase 2A (82). In human lung ECs, FTY720 increased c-Abelson tyrosine kinase (c-Abl) tyrosine kinase activity, and c-Abl siRNA attenuated FTY720-dependent barrier enhancement (Figure 5) (83). However, although FTY720 increased the expression of protein phosphatase 2A, it did not alter FTY720-induced barrier enhancement (83). FTY720 also up-regulated the expression of EC junctional proteins β-catenin and zonula occludens protein 1 (ZO-1) (71), and it promoted adherens junction assembly (84). However, ECs treated with specific siRNAs against claudin-5 or ZO-1/ZO-2 did not alter FTY720-induced barrier enhancement, suggesting that adherens junction or tight junction proteins are not involved in FTY720-induced barrier enhancement (83). A new paradigm has been developed in which FTY720 appears to function as an $S1P_1$ antagonist and exerts its observed inhibitory effects on lymphocyte circulation (85). In contrast to FTY720, FTY720-P increased $[Ca^{2+}]_i$ in ECs, and induced cortical actin distribution to the cell periphery and focal adhesion activity via Rac1 (Figure 5). Further evidence in support of the FTY720-induced down-regulation of $S1P_1$ derives from the ability of FTY720-P to induce the ubiquitination and proteosomal degradation of $S1P_1$ in cultured ECs to a greater extent than observed with S1P (86).
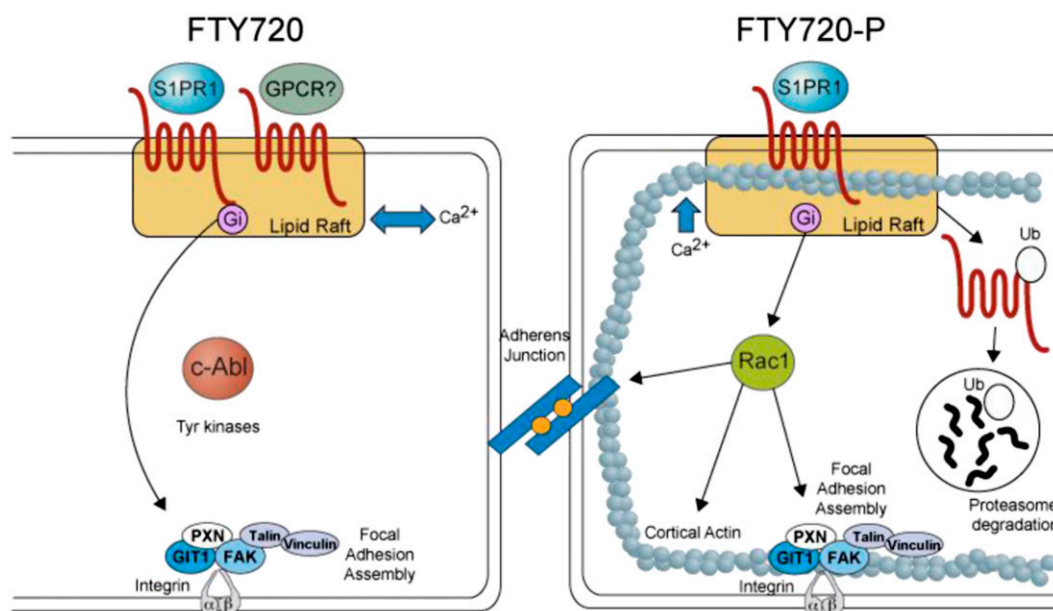
Figure 5. Comparative signaling pathways involved in endothelial cell barrier enhancement by FTY720 and FTY720-phosphate (P). Both FTY720 (*left panel*) and FTY720-P (*right panel*) potently increase endothelial cell (EC) barrier function *in vitro* when concentrations of less than 10 μM are used (higher concentrations or prolonged stimulation over hours to days may disrupt barrier function), but multiple aspects differ in the signaling pathways involved. FTY720-P rapidly induces a series of events similar to the actions of S1P to enhance barrier function, including $S1P_1$ ligation, Gi-coupled signaling, lipid raft membrane platforms, increased intracellular Ca^{2+}, Rac1 activation, and dynamic actin changes, producing increased cortical actin linked to adherens junction and focal adhesion complex formation and stabilization. However, FTY720-P also induces the ubiquitination and subsequent proteosomal degradation of barrier-promoting $S1P_1$, eventually leading to increased permeability after prolonged exposure. EC barrier enhancement by FTY720 is slower in onset and may involve an alternative, but not yet identified, G protein–coupled receptor (GPCR) in addition to $S1P_1$. Similar to FTY720-P, FTY720-induced barrier enhancement requires Gi and lipid raft–coupled signaling. However, no significant Ca^{2+} increase is observed in pulmonary ECs, nor does dramatic cytoskeletal rearrangement or cortical actin formation occur during the timeframe associated with maximal barrier effects. Tyrosine kinase activity is involved, and recent work indicates that c-Abl and FAK signaling are necessary for optimal barrier enhancement. Focal adhesion complexes also appear to participate in this process after FTY720. EC, endothelial cell; Ub, ubiquitination; PXN, paxillin; FAK, focal adhesion kinase; GIT, G protein–coupled receptor kinase interactor–1; Tyr, tyrosine; c-Abl, Abelson tyrosine kinase.

LIMITATIONS OF FTY720 AND FTY720-P AS THERAPEUTIC AGENTS IN ALI

Similar to S1P, FTY720 exhibits physiological responses that may limit its therapeutic utility in patients with life-threatening inflammatory diseases such as ALI. Its immunosuppressant effects may be detrimental to patients with ALI, many of whom exhibit sepsis as a triggering event (87). FTY720 induces bradycardia through its effects on $S1P_3$, similar to the effects of S1P in animal models, and this induction of bradycardia has been confirmed in patients with ALI (87). Further, in a recent multiple sclerosis clinical trial, FTY720 increased rates of dyspnea and decreased lung function (lowered forced expiratory volume in 1 second) (88), perhaps mediated through mechanisms similar to those seen in the S1P-induced contraction of bronchial smooth muscle in mice (67). Importantly, FTY720-P induced the ubiquitination and proteasomal degradation of $S1P_1$ in cultured ECs (84) and HEK293 cells (89). Prolonged exposure to FTY720 resulted in the down-regulation of the EC surface expression of $S1P_1$ and decreased responses to S1P (90). An administration of FTY720 (0.5–5.0 mg/kg) to wild-type C57BL/6 mice induced a dose-dependent $S1P_1$ degradation and an increase in vascular permeability (91). This *in vivo* barrier-disruptive effect of FTY720 is in contrast to its barrier-protective effect observed *in vitro* (37, 70, 83), suggesting differential responses in mouse and human endothelium. However, the *in vivo* effect in mice points to a direct link between $S1P_1$ degradation and vascular leakage, which may account for the recent report of increased lung injury and mortality in bleomycin-injured mice receiving prolonged FTY720 treatment (91). In summary, these studies suggest that FTY720 itself is unlikely to be an optimal therapeutic agent for ALI.

FTY720 PHOSPHONATES AND ENDOTHELIAL BARRIER FUNCTION

As a result of these limitations of FTY720 and FTY720-P, significant interest has arisen in FTY720-P analogues and related compounds that may exert fewer side effects. Several groups have synthesized multiple derivatives of FTY720-P, including phosphonates (73, 92), phosphothioates (93), and 4(5)-phenylimidazole–containing (94) and conformationally constrained (95) analogues, primarily for the purposes of characterizing their S1P-receptor affinity and their ability to induce lymphopenia (96). A number of novel analogues of FTY720-P, namely, the (*R*)-enantiomers and (*S*)-enantiomers of FTY720 phosphonates (Figure 4) (97), have been partly evaluated *in vitro* and *in vivo* for protection against endothelial barrier dysfunction and pulmonary leakage in three murine models of lung injury. The (*R*)-enantiomers and (*S*)-enantiomers of FTY720 phosphonate and of their unsaturated derivative, FTY720-vinylphosphonate, in contrast to the (*R*)-enantiomers and (*S*)-enantiomers of the FTY720 regioisomers (in which the positions of the amino group and one of the hydroxyl groups are reversed), enhanced endothelial barrier integrity in human lung ECs (37). Consistent with these *in vitro* responses, in a murine model of lung injury, (*S*)-FTY720 vinylphosphonate significantly reduced LPS-induced vascular leakage and the infiltration of leukocytes into the alveolar space (37). Similarly, a prolonged administration of (*S*)-FTY720 vinylphosphonate significantly improved the survival of bleomycin-injured in mice (98). In a preclinical model of RILI, (*S*)-FTY720 vinylphosphonate, but not FTY720, conferred protection against radiation-induced pulmonary leakage and inflammation (11). In human pulmonary artery smooth muscle, breast cancer, and androgen-independent prostate cancer cells, (*S*)-FTY720 vinylphosphonate regulated SphK1 activity via the induction of proteasomal degradation of SphK1 (99). Furthermore, (*S*)-FTY720 vinylphosphonate did not activate $S1P_{1–5}$, whereas the

(*R*)-enantiomer was an agonist of $S1P_1$ and a partial antagonist of $S1P_2$ and $S1P_5$ (100).

## MECHANISMS OF BARRIER PROTECTION BY FTY720 PHOSPHONATES

Compared with S1P and FTY720, very little is known about the potential mechanisms underlying the barrier protection promoted by FTY720 phosphonates. *In vitro*, (*S*)-FTY720 phosphonate maintained the basal expression of $S1P_1$, in contrast to the significant reduction (> 50%) induced by S1P or FTY720 treatment (89), suggesting an inhibition of the ubiquitination-mediated degradation of $S1P_1$ by (*S*)-FTY720-phosphonate (98). Furthermore, the recruitment of β-arrestin to $S1P_1$ by (*S*)-FTY720 phosphonate was significantly lower than that via S1P or FTY720 treatment (98), indicating prolonged signaling through $S1P_1$ by (*S*)-FTY720 phosphonate (98).

LIMITATIONS OF FTY720 PHOSPHONATES AS THERAPEUTIC AGENTS IN ALI

As already stated, FTY720 is approved by the United States Food and Drug Administration for the oral treatment of multiple sclerosis. In contrast to FTY720, studies with FTY720 phosphonates in preclinical animal models are limited, and its metabolism *in vivo* is unknown. Further studies on its cytotoxicity, pharmacokinetics, and efficacy in animal models are necessary to advance these phosphonate analogues to a Phase 1 trial in humans.

S1P RECEPTORS IN LUNG INJURY AND BARRIER REGULATION

S1P elicits its cellular effects through a family of G protein–coupled $S1P_{1–5}$ receptors, formerly known as endothelial differentiation gene receptors, which are expressed in various cell types, including endothelial cells (101). Although these receptors share significant homology and overlap in their biological functions, distinct receptor subtype spatial distributions, coupling to different G proteins, and differences in receptor-complex internalization and recycling may provide specificity for each of the S1P receptors (Figure 6). The deletion of $S1P_1$ in mice is embryonically lethal (on Embryonic Days 12.5 and 14.5) (102), whereas $S1P_2$ and $S1P_3$ deletions appear to exert no discernible effect on normal phenotypes (103). However, $S1P_1$/$S1P_2$ double-knockout and $S1P_1$/$S1P_2$/$S1P_3$ triple-null embryos showed a more severe vascular phenotype than did $S1P_1$ knockout embryos, suggesting that the three S1P receptors cooperate to promote vascular development during embryonic angiogenesis (104). The evidence is overwhelming for the role of S1P/$S1P_1$ in preventing the vascular leakage induced by many edemagenic agents, including LPS in the lung (35, 39, 72, 89, 91, 105, 106). Consistent with the barrier-protective role of $S1P_1$, the pretreatment of wild-type mice with an $S1P_1$ inverse agonist, Smith Kline and Beecham-649146 (SB-649146), or the use of $S1P_1^{+/−}$ mice, reduced S1P/SEW2871-induced barrier protection after LPS challenge (106). Similar to the protective role of $S1P_1$, $S1P_2$-null mice and mice with a reduced expression of $S1P_3$ (generated by treating with a specific siRNA against $S1P_3$) also offered significant protection against LPS-induced barrier disruption and leakage, compared with wild-type mice (106). However, $S1P_2$ seems to mediate enhanced vascular permeability in newborn mice exposed to a hypoxia-induced model of retinopathy (107) and a hydrogen peroxide–induced model of barrier dysfunction (103). In RILI, the roles of $S1P_2$ and $S1P_3$ in barrier regulation appear to be conflicting, as observed in a preclinical model of LPS-induced ALI. In a murine RILI model, the knockdown or reduced expression of $S1P_1$, $S1P_2$, and $S1P_3$
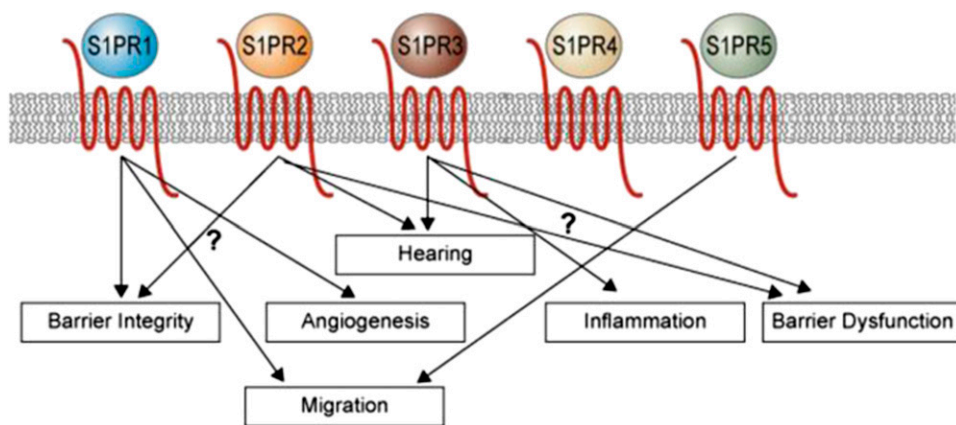
Figure 6. Potential role of S1P receptors in cellular and biological processes. Extracellular S1P signals via G protein–coupled S1P receptors, and regulates a number of cellular and biological processes such as barrier integrity, barrier disruption, inflammation, migration, and angiogenesis in mammalian cells. S1PR, S1P receptor.

increased susceptibility to lung injury (11). These results suggest a differential role for $S1P_{1–3}$ in these two models of lung injury because of the differential transduction of signals via multimeric G proteins (Figure 7). In addition to the genetic engineering of S1P receptors, S1P-receptor agonists and antagonists could be useful in studying the roles of $S1P_{1–5}$ in lung inflammation and injury. However, many agonists such as SEW2871 and 3-[[2-[4-phenyl-3-(trifluoromethyl)phenyl]-1-benzothiophen-5-yl]meth-ylamino]propanoic acid (AUY954) for $S1P_1$ exhibit poor water solubility, thereby limiting their use in animal models of lung inflammation and injury. A systematic study of S1P receptor–mediated signaling and the study of intracellular targets that regulate S1P concentrations in vascular cells may provide further insights into the mechanisms underlying the barrier function disruption seen in acute and subacute lung injury.

FUNCTIONAL POLYMORPHISM OF S1P RECEPTORS AND SphK1 AND SphK2 AND THEIR ASSOCIATION WITH ALI

Association studies on the functional consequences of single-nucleotide polymorphisms (SNPs) in S1P receptors, SphKs, S1PL, and sphingomyelinase that are linked to acute and sub-acute lung injury/inflammation are limited. The DNA sequencing of both African American and European American populations consisting of 378 control subjects and 218 cases of sepsis/ALI revealed that $S1P_3$ promoter SNPs rs7022797 (−1899 T/G) and rs11137480 (−1785 G/C) in European Americans conferred decreased susceptibility to both severe sepsis

and sepsis-induced ALI. Furthermore, $S1P_3$ promoter SNPs −1899G and −1785C, singly or together, significantly decreased the luciferase promoter activity triggered by the TNF-α–induced binding of transcriptional factors caudal related homeobox1 (CDX1) and early B cell factor 1 (EBF1) to the S1PR3 promoter (108). Thus, multiple SNPs in $S1P_3$ alter promoter activity and confer susceptibility to sepsis/ALI in multiethnic populations.

In silico analyses provide limited information on polymorphic variants in the genomic sequences of SphK1 and SphK2, and on linking the variations to ALI among various ethnic groups. The genotyping of patients with severe sepsis (African Americans, $n = 75$; European Americans, $n = 143$) and healthy control subjects (African Americans, $n = 187$; European Americans, $n = 190$) revealed 30 SphK1 SNP variants, including 20 novel SNPs with seven SphK1 tagging SNPs whose minor allele frequencies were equal to or greater than 5%. African American patients with the SNP rs3744037 CC genotype (exon 6, Tyr407Tyr) demonstrated greater odds of developing severe sepsis-induced ALI than did carriers of the T allele (odds ratio, 3.93; 95% confidence interval, 1.05–14.77; $P = 0.05$). A stronger association was found in European American patients, in whom the rs2247856 AA genotype (exon 2, Ala30Thr) demonstrated significantly greater odds of developing severe sepsis than did carriers of the G allele (odds ratio, 1.94; 95% confidence interval, 1.15–3.25; $P = 0.013$). The resequencing of the SphK2 gene and flanking sequences revealed 54 polymorphisms, and association data analysis revealed five data base single nucleotide polymorphism (dbSNPs) and one novel SNP, rs12610339, located in the promoter region that was significantly associated
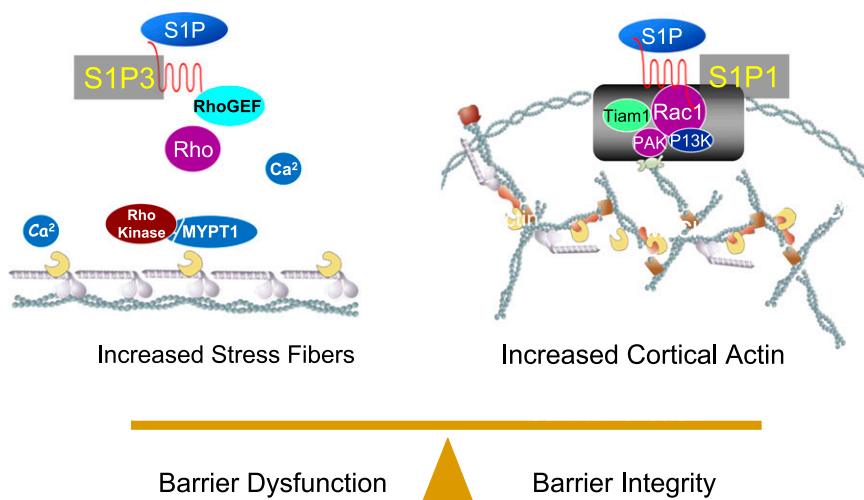
Figure 7. Comparative signaling pathways involved in endothelial barrier integrity and dysfunction by S1P via S1P1 and S1P3. S1P enhances endothelial barrier functions that include S1P1 ligation, Gi-coupled signaling, lipid raft membrane platforms, increased intracellular Ca^{2+}, Rac1 activation, Tiam 1, PAK1 and PI3K recruitment to lipid rafts, and dynamic actin changes, producing increased cortical actin, which is linked to adherens junction and focal adhesion complex formation and stabilization. However, the ligation of S1P to $S1P_3$ enhances RhoGEF recruitment to lipid rafts, and Rho activation leads to cytoskeletal reorganization, decreased cortical actin, and barrier dysfunction. S1P, sphingosine 1–phosphate; Tiam 1, T-cell lymphoma invasion and metastasis factor–1; PAK, p21–activated kinase; GEF, guanine nucleotide exchange factor; MYPT1, myosin phosphatase–targeting subunit; PI3K, phosphatidylinositol 3–kinase.

with sepsis and sepsis-induced ALI in the African American cohort for sepsis and ALI. The strength of the association with ALI suggests that the SphK2 gene may elicit multiple effects in patients with ALI (109). These initial polymorphism studies associated with the $S1P_3$, SphK1, and SphK2 genes suggest a potential association between the SNPs and susceptibility to sepsis and ALI in African Americans and European Americans that should be validated with larger groups of control and patient populations.

S1P HOMEOSTASIS IN ALI

S1P concentrations in circulation are higher compared with intracellular concentrations, and are tightly regulated by synthesis, secretion, and uptake by different cell types, including endothelial cells. Most likely, circulating S1P helps maintain endothelial barrier integrity under basal conditions. However, pathological conditions such as sepsis may alter S1P homeostasis and offset the critical balance from tight endothelial junctions to barrier dysregulation. In a murine model of ALI, an intratracheal instillation of LPS (5 mg/kg body weight for 24 hours) reduced S1P concentrations in lung tissue (S1P, fmol/nmol lipid phosphorous, vehicle, 296 ± 24; intratracheal LPS, 138 ± 16) and in plasma (S1P, fmol/nmol lipid phosphorous, vehicle, $1,126 \pm 36$; intratracheal LPS, 825 ± 29) that paralleled the increased expression of S1PL in lungs and lung inflammation and injury (59). Blocking S1PL activity by the administration of THI in mice increased S1P concentrations in lungs without altering plasma concentrations, and reduced LPS-induced lung inflammation (59). In a radiation model of RILI, the ceramide/S1P ratios in BAL, plasma, and lung tissue were significantly increased and remained elevated as RILI progressed throughout a 12-week period of RILI assessment (11). Intriguingly, plasma concentrations of S1P from patients with ALI and sepsis were significantly decreased compared with control subjects. However, no significant differences were evident between African American and Caucasian populations in terms of the association of ALI or sepsis with circulating plasma S1P concentrations (Joe G. N. Garcia and colleagues, unpublished data). An analysis of S1P concentrations from control, sepsis, and sepsis-induced ALI patients revealed a potential correlation between lung injury/edema and a decrease in circulating plasma S1P concentrations (110). Whether lower plasma S1P concentrations serve as a potential biomarker in sepsis and ALI pathologies remains to be confirmed.

CONCLUSIONS AND FUTURE DIRECTIONS

Acute and subacute lung injuries share profound increases in vascular permeability as a key element and a common pathway driving the increased morbidity and mortality in these disorders. Recent studies suggest that the bioactive sphingolipid S1P and its receptors, and enzymes of S1P metabolism, are important modulators of lung injury and inflammation. S1P is a potent angiogenic factor, enhances EC integrity *in vitro*, and is a robust *in vivo* modulator of the vascular permeability and alveolar flooding induced by endotoxemia. Concentrations of S1P are regulated by its synthesis, catalyzed by SphKs, and its degradation, mediated by S1PL, S1PPases, and LPPs. In an LPS-induced murine model of lung injury inflammation, the downregulation of SphK1 potentiated ALI, whereas the knockdown of S1PL offered partial protection, suggesting that S1PL may constitute a potential therapeutic target in ameliorating sepsis-induced pulmonary edema. S1P-mediated endothelial responses are driven via G protein–coupled S1P receptors, and current studies using knockout mice and S1P-receptor antagonists show that $S1P_1$ is the primary S1P receptor effecting EC barrier protection, whereas $S1P_3$ and to a lesser extent $S1P_2$ are barrier-disruptive. Although S1P exerts a potentially beneficial effect in restoring endothelial barrier integrity and suppressing the pulmonary leakage attributable to sepsis, there are limitations to the use of S1P as a therapeutic agent in a clinical setting. Among several S1P analogues evaluated for their efficacy in barrier protection against acute and subacute lung injury animal models, (*S*)-FTY720 phosphonate produced rapid and increased endothelial barrier function *in vitro* and decreased LPS-induced and radiation-induced lung permeability *in vivo*. Association studies in Caucasian and African American ALI cohorts revealed novel SNPs in S1P receptors and S1P-metabolizing enzymes. Future studies on functional polymorphisms in sphingolipid pathway genes should provide new approaches to the development of drugs against ALI.

Although the targeting of S1P receptors and metabolizing enzymes is promising in preclinical animal models of sepsis-induced lung injury, the outcome of S1P targeting to minimize alveolar flooding and pulmonary leakage in patients with ALI needs to be evaluated. The development of specific small-molecule agonists and antagonists of S1P receptors and S1P metabolizing enzymes is critical in modulating endothelial barrier dysfunction. This would require not only the development of selective and potent inhibitors of S1P receptors and metabolizing enzymes with minimal cytotoxicity, but the pinpoint targeting of these agents to specific cell types in the lung. Attempts to target S1P receptors and metabolizing enzymes simultaneously should provide a synergistic approach to conferring protection against ALI. Furthermore, the identification of novel S1P-signaling biomarkers and a systems biology approach will greatly facilitate the development of novel S1P-based therapies for patients with severe inflammatory lung injury.

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