Review

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2010;26:87-96

Accepted: February 04, 2010

Endothelial Functions of Sphingosine-1-phosphate

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Key Words
Sphingosine-1-phosphate (S1P) • Arterial tone • Sphingosine kinases • Inflammation • Permeability • Leak • FTY720

Abstract
The biologically active sphingolipid sphingosine-1-phosphate (S1P) plays key functions in the immune, inflammatory, and cardiovascular systems. In the vasculature, S1P and its receptors are involved in vessel morphogenesis and angiogenesis during embryonic development and in the adult organism both under normal and pathological conditions. Via its actions on endothelial and smooth muscle cells, S1P regulates arterial tone, vascular permeability, and tissue perfusion. Elevated local S1P levels during inflammation induce endothelial adhesion molecules, recruit inflammatory cells, and activate dendritic cells. At the same time, S1P activates a negative feedback loop that consecutively seals endothelial cell-cell contacts, decreases vascular leakage, and inhibits cytokine-induced leukocyte adhesion. Thus S1P determines not only the build-up, magnitude, and duration of an inflammatory reaction but also the pace of its resolution. This review focuses on the role S1P plays in endothelial function, its receptors and signalling pathways, and the role its major carrier high-density lipoproteins (HDL) play in its bioavailability and transport. We will also discuss the potential of interfering with S1P-S1P receptor interactions for the treatment of endothelial disorders and vascular pathologies.

S1P metabolism

In 1884, the German physician J. L. W. Tudichum first discovered a new biochemical substance by fractional crystallisation from human brain. Unable to assign it to any by then known class of molecules he named it "sphingolipids" after the enigmatic *Sphinx* from Greek mythology. For a long time since then, sphingolipids were merely regarded as structural components of biological membranes. However, the discovery of their signaling properties over the last 2 decades and the identification of a family of receptors they bind to has lead to their recognition as key players in the regulation of immunity, inflammation, and cardiovascular functions [1].

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S1P is synthesized intracellularly from sphingosine via phosphorylation of its primary hydroxyl group by two distinct sphingosine kinases: Sphk1 and Sphk2. While the major source of S1P in plasma are hematopoetic cells (erythrocytes, but also platelets, mast cells and leukocytes), vascular and lymphatic endothelial cells have also been shown to synthesise and release S1P [2, 3]. Inside the cell, S1P moves freely between different membranes but requires specific transport mechanisms for translocation to the outer leaflet of the cytoplasmic membrane [4, 5]. These transport mechanisms are still unknown in the majority of cell types but in some such as platelets and mast cells, ABC-type transporters have been implicated in S1P export [6, 7]. Clearly, other mechanisms must also exist as plasma S1P levels are unaltered in mice deficient for ABCA1, ABCA7, or ABCC1 [8]. Recently, a designated sphingolipid transporter named *Spns2* (*Two of Hearts*) has been found in zebra fish, the mutation of which results in cardia bifida [9, 10], resembling remarkably the phenotype of the *mil* (*Miles Apart*) mutant, the ortholog of the mammalian $S1P_2$ [11].

Plasma concentrations of S1P are between 200 and 1000 nM [12, 13]. Plasma S1P is bound mainly to high-density lipoproteins (HDL) (~50-70%) followed by albumin (~30%), LDL and VLDL (<10%) [14]. S1P binds with extremely high affinity to HDL, making these lipoproteins the primary acceptors and carriers of plasma S1P [15]. Accordingly, plasma S1P levels positively correlate with plasma levels of HDL-cholesterol (HDL-C) and those of apolipoproteins AI and AII, the two major apolipoproteins within HDL [13]. The degradation of S1P occurs either via dephosphorylation to sphingosine through the action of specific intracellular S1P phosphatases or through an irreversible cleavage of S1P by the S1P lyase to ethanolamine phosphate and hexadecenal [4, 16].

S1P receptors

There are five cognate G-protein-coupled receptors, to which S1P specifically binds with a K_d of 8-20 nM designated $S1P_{1-5}$ [17]. The details on receptor binding and activation are complex as individual S1P receptors can couple to one or more G-proteins with considerable overlap: $S1P_1$ is coupled to $G_{i/o}$, preferentially $G_{i\alpha1}$ and $G_{i\alpha3}$; $S1P_2$ is associated to $G_{i/o}$,$G_{12/13}$ and G_q; $S1P_3$ activates either $G_{i/o}$, G_q or $G_{12/13}$ proteins, and $S1P_4$ and $S1P_5$ signal through $G_{i/o}$ or $G_{12/13}$ and $G_{i/o}$ or G_{12} subunits, respectively [4, 17]. The complexity of S1P receptor coupling to different G-proteins drives the multiplicity of downstream signalling pathways elicited by S1P: it activates phospholipase C and mobilizes Ca^{2+} via G_q, activates ERKs and PI3K while inhibiting adenylate cyclase via G_i, and activates Rho and actin cytoskeleton reassembly via $G_{12/13}$. Thus the relative expression levels of S1P receptors determine the net response of the cell to S1P [1]. In addition, S1P receptors have been shown to "trans-activate" tyrosine kinase receptors (VEGF-R, PDGF-R and EGF-R [18-21]) as well as G-protein coupled receptors (CXCR4 [22-24]) and even to "cross-activate" receptors (TGFβ-RII [25]), which enables yet another level of signalling cross-talk. S1P receptor signalling occurs at the full spectrum of the receptor occupancy curve: extremely low occupancies e.g. lead to transactivation of the PDGF receptor, those at the steep slope activate a toogle switch that turns off lymphocyte recirculation, and high concentrations (50-100-fold higher than the K_d) lead to receptor desensitization and degradation [26].

Much of the knowledge on S1P receptor signalling stems from studies that have focussed on the immune system: During lymphocyte recirculation, S1P is both necessary and required for T- and B-lymphocyte exit from the thymus and secondary lymphoid organs (spleen and lymph nodes) [27]. For lymph node exit, lymphocytes actively migrate along the S1P concentration gradient that exists between lymph node (less than pM S1P concentrations), lymph (S1P in the pM range) and plasma (μM concentrations of S1P). Ablation of this gradient by genetic or pharmacological manipulation leads to an exquisite lymphopenia in the peripheral blood by lymhocyte "trapping" in secondary lympoid organs [28]. In systemic inflammatory syndromes such as bacterial sepsis and viral hemorrhagic fever, S1P was identified to couple coagulation with inflammation downstream of PAR-1; there, S1P promotes the dissemination of inflammation by contributing to the coagulation-induced activation and trafficking of dendritic cells in the lymphatics [29]. Studies using genetic $S1P_1$ deficiency and employing $S1P_1$ agonists and antagonists have shown that the $S1P_1$ expressed on lymphocytes is responsible for mediating egress. Remarkably, only $S1P_1$ agonists but not antagonists induce lymphopenia, while the antagonists inhibit the lymphopenia induced by the agonists, thus indicating that activation rather than inhibition of $S1P_1$ impedes lymphocyte egress [30-32]. Therefore, the pharmacological development of synthetic S1P agonists over the last couple of years has been a novel approach to immunosuppressive therapy in transplantation medicine and multiple sclerosis that has lead to the filing of FTY720, a S1P analogue, for approval by the FDA [33].

Bioavailability and presentation

Although total S1P levels in plasma are 20- to 100-fold higher than the K_d value of its receptors [14, 34], its biologically active fraction is only 1-2% [14], suggesting that a large portion of the total plasma S1P is either buffered or withheld in a tightly "packaged" form, or both. All of these functions could be adopted by HDL as its major carrier. In fact, the capacity of HDL to take up exogenous S1P is enormous (up to 10-fold per milligram of protein; unpublished observations), so that these lipoproteins could easily remove excess S1P that is e.g. being produced during inflammation, buffer it and even carry it away from the inflammation site thus promoting resolution. Indeed, HDL are present in the interstitium in amounts that correspond to ~25% of their plasma concentration [35, 36], circulate with the lymph [37], and their concentration increases several-fold in inflammatory exsudates [38]. On the other hand, S1P associated with HDL is biologically active because several of the biological effects of HDL can be partially or entirely attributed to their S1P content: NO-dependent vasodilation, angiogenesis, and certain aspects of the anti-oxidative, anti-apoptotic and anti-inflammatory actions of HDL [39]. One plausible explanation may be that HDL-associated S1P is packaged in such a way that it is only partially accessible to S1P receptors. This would allow HDL to act both as a "sink" and a "presenter" of S1P. This presentation may be a highly selective and regulated process, as HDL require binding to their own receptor Srb1 to be able to engage S1P receptors via its S1P content [40-43]. This way, HDL would achieve a confined activation of S1P receptors dependent on: 1) distribution and expression levels of both HDL and S1P receptors, 2) the amount of S1P carried within the HDL particle, and 3) the plasma HDL level. Although hypothetical and expecting evidence from experimental studies, this hypothesis has been recently substantiated by the observation that exogenous loading of HDL with S1P increased their ability to inhibit oxidized LDL-induced apoptosis in endothelial cells [15].

Vascular morphogenesis

The S1P receptors most abundantly expressed in endothelial cells are $S1P_1$, $S1P_2$ and $S1P_3$ with $S1P_1 > S1P_2 \approx S1P_3$ [44]. Although not systematically analyzed, endothelial cells of different origin (arteries, capillaries, veins, lymphatics) and those from different arteries (aortic, cerebral, coronary, renal, and mesenteric) appear to express different levels of S1P receptors. In the endothelial cell, S1P exerts a variety of effects that affect vascular maturation and morphogenesis: it stimulates endothelial proliferation, migration, and angiogenesis, protects against apoptosis and controls vascular permeability [32, 45]. *In vitro*, S1P not only induces endothelial cell proliferation but also acts as a potent chemoattractant for endothelial cells [46]. Furthermore, it promotes directed migration, vascular differentiation, and formation of capillary networks on complex extracellular matrices [47, 48]. Small GTPases play an important role in mediating the effects of S1P: Rac1 activation by $S1P_1$ signalling induces focal contact assembly, membrane ruffling, and cortical actin formation via G_i, while RhoA activation by $S1P_3$ promotes stress fiber assembly via G_q. In contrast, $S1P_2$ signalling appears to have anti-migratory and anti-angiogenic effects: pharmacological $S1P_2$ blockade enhances the pro-migratory response of S1P elicited by $S1P_1$ in endothelial cells, while overexpression of $S1P_2$ inhibits S1P-induced migration by suppressing Rac1 activity [49].

These effects find their correlate *in vivo*, where S1P plays an important role in vascular morphogenesis during development. The $S1P_1$ receptor is particularly involved as both global and endothelial-specific $S1P_1$ knockout mice die in utero due to vessel rupture and hemorrhage caused by severe vascular maturation defects [48]. This phenomenon isn't due to defects in endothelial proliferation, migration or tube formation but to the inability of pericytes and smooth muscle cells to form the vascular sheath and thus stabilize the nascent vessel. Remarkably, this process is controlled by the endothelial and not smooth muscle $S1P_1$ because both the global and endothelial-specific $S1P_1$ knockout exhibit the same lethal phenotype caused by vascular hemorrhage [50]. Identical defects are also displayed by mice lacking S1P through genetic deletion of both S1P-synthesizing enzymes Sphk1 and Sphk2 [51]. $S1P_2$ and $S1P_3$ also play a role in vascular development as evidenced by additional defects in vasculogenesis becoming apparent in the double and triple (together with $S1P_1$) knockouts [52]. While loss of $S1P_1$ signalling impairs vascular development, the loss of $S1P_2$ renders mice resistant to the exuberant pathological angiogenesis triggered by hypoxia implying $S1P_2$ (itself a hypoxia-regulated gene) as a causal factor in ischemia-driven retinopathy [53]. S1P has also been shown to promote tumor-associated angiogenesis *in vivo* as revealed by reduced tumour progression after treatment with a monoclonal antibody that neutralizes

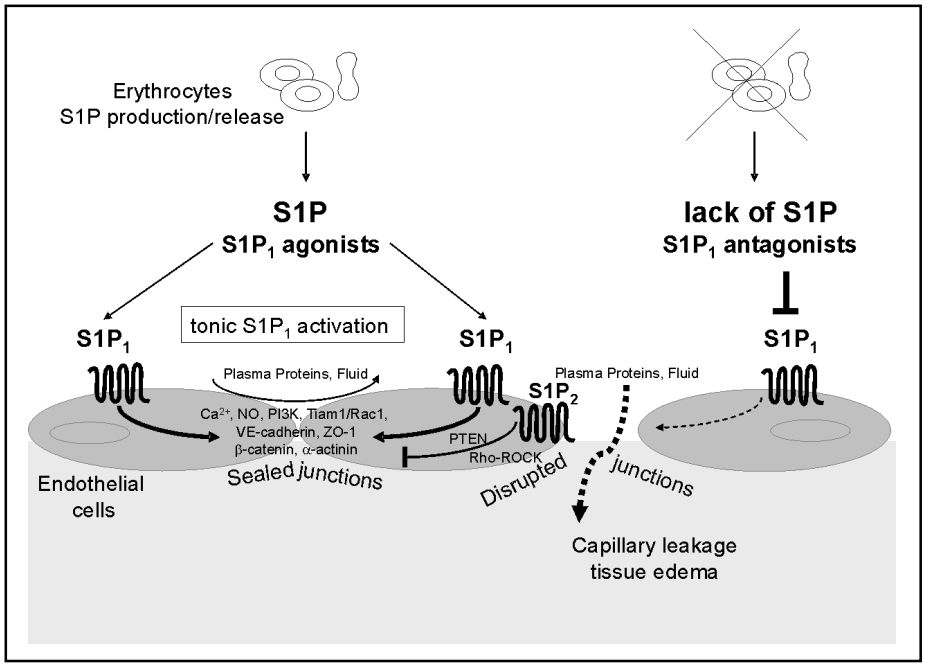

Fig. 1. Model of the effects of S1P and $S1P_1$ agonists and antagonists on endothelial permeability. Under physiological conditions, plasma S1P is produced and released by erythrocytes and acts via $S1P_1$ to strengthen endothelial junctions and prevent vascular leakage. This requires a tonic $S1P_1$ activation. Lack of plasma S1P due to decreased S1P provision by erythrocytes or administration of an $S1P_1$ antagonist weaken the tonic $S1P_1$ activation and lead to junction disruption, capillary leakage and edema. During pathological processes such as hypoxia, $S1P_2$ is also activated, disrupts junctions and enhances permeability.

bioactive S1P in several xenograft and allograft tumour models [54]. This has been attributed both to an inhibition of tumour angiogenesis and a direct impact on tumour cells [54]. *In vitro*, the same anti-S1P antibody inhibited endothelial cell migration and capillary formation as well as VEGF- and basic FGF-induced vessel formation. *In vivo*, the humanized version inhibited retinal and choroidal neovascularization in oxygen-induced ischemic retinopathy [55].

Vascular tone

Several studies have shown that S1P stimulates nitric oxide (NO) production in endothelial cells *in vitro* through activation of the endothelial NO synthase (eNOS) [56]. In contrast, exogenously applied S1P promotes vasoconstriction in isolated mesenteric, cerebral, and coronary arteries in tension myograph studies, which has been attributed to actions of S1P on vascular smooth muscle cells (VSMC) [57, 58]. VSMC can endogenously produce S1P that acts in an autocrine/paracrine manner to regulate basal arterial tone as well as the myogenic response required for maintaining constant blood supply to tissues [59, 60]. However, if arterial tone is elevated e.g. by adrenergic stimulation, S1P can induce vasorelaxation by activating eNOS, thereby participating in the fine-tuning of vessel tone [61].

Endothelial permeability

S1P is well known to decrease endothelial permeability. *In vitro*, S1P increases the transmonolayer electrical resistance of microvascular endothelial cells as a direct measure of endothelial permeability. S1P exhibits its barrier-enhancing effect by several signalling events and cytoskeletal rearrangements that all promote adherens junction assembly such as Ca^{2+}, PI3K, Tiam1/Rac1, VE-cadherin, β-catenin, α-actinin and ZO-1 [36, 47, 62] (Fig. 1). The stabilization of endothelial barrier function by transient S1P-evoked intracellular Ca^{2+} increases has been linked to eNOS activation as both sealing of the endothelial barrier and prevention of microvascular leakage are inherent to the actions of NO [63-65]. While $S1P_1$ and $S1P_3$ strengthen the formation of endothelial cell junctions [35-37], $S1P_2$ weakens them [38, 66]. It promotes vascular permeability *in vitro* via disruption of adherens junctions mediated by its downstream effectors Rho-ROCK and PTEN [67], and contributes to the pathological retinal angiogenesis triggered by hypoxia *in vivo* [53] (Fig. 1). Virtually nothing is known on the effects of $S1P_3$ on endothelial permeability *in vivo* as the only study on this matter has been retracted [68].

The endogenous S1P required for maintenance of endothelial barrier function originates from the plasma compartment: "pS1Pless" mice that lack S1P in plasma display vascular leakage and die more frequently after

anaphylactic challenge, PAF and histamine administration [69] (Fig. 1). Transfusion of erythrocytes as S1P source to these "pS1Pless" mice restored plasma S1P levels and reversed the endothelial leak phenotype to a similar extent as the administration of $S1P_1$ receptor agonists [69]. Although these experiments show that erythrocyte-borne S1P is crucial for the maintenance of endothelial barrier function, they do not exclude that S1P produced by endothelial cells can also contribute. In fact, endothelial cells exposed to physiological laminar shear stress produce and secrete S1P *in vitro* [3], and the global knockout of Sphk1 exhibits a similar but less severe endothelial leak phenotype as the "pS1Pless" mice despite unaltered plasma S1P levels [70]. Sphk1 activity has been shown to be required for basal endothelial barrier function in "pS1Pless" mice [69] and to mediate the permeability-opposing effect of angiopoietin-1, albeit independently of S1P receptors [70]. Evidence for a role of $S1P_1$ in the homeostasis of endothelial permeability in the normal adult organism has been provided by studies with pharmacological $S1P_1$ antagonists *in vivo*, which were reported to induce capillary leakage in the lung, kidney, skin and intestine [26, 30, 71]. To the contrary, engagement of $S1P_1$ by S1P analogues was shown to inhibit VEGF-induced vascular leak in skin capillaries [30, 72]. This has suggested that continuous "tonic" $S1P_1$ receptor activation is required for maintenance of endothelial barrier function, and that the plasma levels of S1P regulate vascular permeability [73] (Fig. 1).

Leukocyte adhesion

S1P appears to affect leukocyte-endothelial interactions in several ways in the context of adhesion. While exogenous S1P inhibited the induction of endothelial adhesion molecules following TNFα stimulation [74], the down-regulation of constitutive $S1P_1$ signalling by stable long-term siRNA knockdown decreased both basal expression of PECAM-1 and VE-cadherin and the induction of E-selectin after TNFα or LPS stimulation [75]. In agreement with an inhibitory function of S1P on adhesion molecule expression, S1P suppressed the adherence of inflammatory cells to TNFα-activated aortic endothelium *in vivo* [76]. However, this was not due to an inhibition of VCAM-1 or ICAM-1 but to inhibition of IL-8 and MCP-1 production [76]. One mechanism by which S1P inhibits MCP-1 production may be its suppression of the cytoplasmatic NADP(H) oxidase. MCP-1 release by thrombin is induced in a reactive oxygen species (ROS)-dependent manner, and S1P has been shown to abrogate it by inhibiting the NADP(H) oxidase [43]. In diabetic NOD mice, S1P and $S1P_1$ agonists were shown to abrogate monocytic cell adhesion to aortic endothelium in a partially NO-dependent manner and via VCAM-1 suppression due to their inhibitory effect on NF-κB [77].

However, there has also been evidence in favour of a pro-inflammatory effect of S1P in endothelial cells: S1P generated endogenously by Sphk1 after TNFα stimulation has been shown to mediate adhesion molecule induction, and S1P itself stimulated VCAM-1 and E-selectin via transcriptional activation of NF-κB [74, 78-80]. Furthermore, chronic overexpression of Sphk1 was shown to promote a pro-inflammatory phenotype in cultured endothelial cells as characterized by the higher constitutive expression of VCAM-1 and the augmented induction of VCAM-1 and E-selectin along with enhanced neutrophil adhesion in response to TNFα [81]. In addition, Sphk1 has been implicated in the induction of COX-2 and the subsequent production of inflammatory prostaglandins such as PGE_2 by TNFα [82]. In agreement, knockdown of the S1P degrading enzymes S1P phosphatase and S1P lyase augmented prostaglandin production and raised S1P levels [82], suggesting that S1P is mediating the COX-2-dependent pro-inflammatory effects of cytokines. Nevertheless, there have also been contradictory reports showing that TNFα-induced expression of adhesion molecules is S1P-independent [83].

Three explanations have been put forward to reconcile these views. The first proposes a pro-inflammatory role of intracellular S1P generated after TNFα-stimulation but without pinpointing the underlying mechanism [79]. The second suggests opposing effects of the different S1P receptors involved, with $S1P_1$ inhibiting and $S1P_3$ stimulating the induction of adhesion molecules, and $G_{12/13}$ being responsible because of its activation by $S1P_3$ and not $S1P_1$ [84]. There are arguments both in favour and against this: in favour are studies showing that S1P and $S1P_1$ agonists inhibit monocyte adhesion to $S1P_3$-deficient endothelium as effectively as to wild-type endothelium [77]; in apparent contradiction, S1P has been shown to lose its inhibitory effect on neutrophil recruitment in $S1P_3$-deficient mice during post-ischemic inflammation [85]. Finally, the third explanation may be simply due to the observation that supra-physiological levels of S1P often inhibit the very same processes that activated by lower concentrations [17]. Indeed, studies that have reported that S1P induces endothelial VCAM-1 and E-selectin expression [78, 79]

have used rather high concentrations of S1P (5 to 20 μmol/L), while studies describing VCAM-1 inhibition have used nanomolar concentrations of S1P [77]. When the same authors increased S1P concentrations to greater than 5 μmol/L, they noticed the opposite - an increase in monocyte adhesion [77]. Obviously, more work is required to dissect the different aspects of S1P signalling in adhesion.

Inflammation

The increased S1P levels at inflammation sites [86, 87] have been attributed to the activation of Sphk1 by inflammatory cytokines such as TNFα and IL-1β, LPS, PAR-1 and thrombin [86, 88]. High local S1P concentrations may boost inflammation by augmenting PGE_2 release, inducing adhesion molecules, recruiting neutrophils [89, 90] and monocyte/macrophages [91], retaining T-cells at sites of inflammation [87] and promoting activation of dendritic cells in the lymphatics [29]. At the same time, S1P may limit the local inflammatory response in a negative feedback manner by decreasing endothelial permeability, enhancing barrier function, and inhibiting leukocyte adhesion. Thus locally produced S1P appears to be an important determinant of the build-up, magnitude, and duration of the inflammatory response, and may also play a role in its restraint and resolution. This sequence of events can be observed during inflammatory lung injury: there, the initial increase in vascular permeability induced by LPS or PAR-1 is gradually counteracted by an increase in S1P levels by concomitant Sphk1 activation that re-seals the endothelial cell barrier via $S1P_1$ [86, 92]. Vice versa, the barrier-enhancing functions of agents that protect against lung injury such as activated protein C (APC) can be partially attributed to their activation of Sphk1, production of S1P and subsequent engagement of $S1P_1$ [93]. The APC receptor EPCR has even been described to transactivate $S1P_1$ directly [94]. The endothelium-sealing effects of S1P in inflammation are not restricted to the local vascular beds of particular organs but are also present under conditions of generalized inflammation: Administration of $S1P_1$ agonists has been shown to protect mice devoid of plasma S1P from PAF-induced generalized vascular leakage and death [69].

S1P-based therapeutic approaches to endothelial pathologies

Most of the experimental and clinical experience with S1P analogues has come from studies with FTY720, an orally administered immunosuppressant that, after endogenous phosphorylation to FTY720-P, engages four out of five S1P receptors except $S1P_2$. The initial agonistic action of FTY720-P on $S1P_1$ is followed by an antagonistic one due to its induction of $S1P_1$ internalization and proteasomal degradation. Originally considered a novel immunosuppressive agent for prevention of renal allograft rejection, FTY720 has been assessed in Phase III clinical studies but did not prove superior to standard treatments. Much in contrast, FTY720 has yielded extremely promising results in proof-of-concept trials for the treatment of multiple sclerosis [95] despite the remaining uncertainty about the actual mechanism underlying its therapeutic effectiveness. FTY720 is also active in several settings of endothelial inflammation: it confers protection against ischemia/reperfusion injury [96, 97], decreases endothelial leakage [72], inhibits ROS and MCP-1 production [43], promotes NO-dependent vasodilation [98], and attenuates the development of atherosclerosis [99, 100]. However, its immunosuppressive effect remains a major drawback for a potential use in endothelium-targeted therapies.

A different approach to a potential therapeutic application of S1P may be the modulation of endogenous S1P bioavailability. Although agents that inhibit S1P degradation are immunosuppressive [101], their careful dosage together with individual evaluation of risk and benefit may make them additional therapeutic options. Vice versa, blocking S1P by monoclonal antibodies has proven successful in experimental cancer models and may also become interesting in disease syndromes such anaphylaxis. In this respect, the resistance to anaphylaxis in Sphk1-deficient mice has been attributed to their lower plasma S1P level, suggesting that circulating S1P may be a factor that elicits an overly extensive degranulation of mast cells and thus promotes the anaphylactic response [102]. Altering HDL levels and application of HDL-mimetics may also lead to shifts in S1P bioavailability, which could become another way of interfering with endogenous S1P effects. Finally, inhibitors specific for the two sphingosine kinases are currently under development by several pharmaceutical companies.

S1P has clear regulatory effects on vascular tone and organ perfusion including that of the heart, brain, mesenterium and kidney [61]. However, these effects differ in magnitude and amplitude in the different vascular beds. Although challenging, this opens perspectives for selective modulation of organ-specific perfusion and for interventions at the level of the microvasculature without affecting systemic blood pressure. For this, agonists and antagonists of the S1P receptors participating in the regulation of vascular tone - $S1P_1$, $S1P_2$, and $S1P_3$ - may need to be employed in variable combinations assembled to target only particular vascular beds while leaving others unaffected. In summary, the modulation of endothelial S1P-signalling at different levels may emerge as a new concept in the treatment of vascular pathologies.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft [LE940/4-1].

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