Sources, metabolism and regulation of circulating sphingosine-1-phosphate

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Abstract

Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid that acts either as an intracellular messenger or as a ligand for its membrane receptors. S1P is a normal constituent of blood, where it is found both in plasma and blood cells. Compared to other cell types, sphingolipid metabolism in erythrocytes and platelets has unique features that allow them to accumulate S1P. In plasma, S1P is bound mainly to high-density lipoproteins and albumin. Of note, metabolism and biological activity of S1P is to a large extent affected by the type of its carrier. Plasma S1P is characterized by a short half-life indicating rapid clearance by degradative enzymes and the presence of high-capacity sources involved in maintaining its high concentration. These sources include blood cells, vascular endothelium and hepatocytes. However, the extent to which each of these contributes to the plasma pool of S1P is a matter of debate. Circulating S1P plays a significant physiological role. It was found to be the key regulator of lymphocyte trafficking, endothelial barrier function and vascular tone. The purpose of this review is to summarize the present state of knowledge on metabolism, transport and origin of plasma S1P, and to discuss the mechanisms regulating its homeostasis in blood.

Key words: dihydrosphingosine-1-phosphate, endothelial cells, HDL, red blood cells, thrombocytes
Introduction

Sphingosine-1-phosphate (S1P) is a bioactive intermediate of sphingolipid metabolism consisting of two main components: a polar head group – phosphate and a long-chain sphingoid base backbone – sphingosine (1). S1P acts not only intracellularly as a second messenger, but also extracellularly as a ligand for its membrane receptors (S1PRs). Moreover, intracellularly generated S1P can be exported and act in paracrine or autocrine fashion (2). S1P induces a wide spectrum of cellular effects including proliferation, differentiation, survival and migration (3). It is also implicated in several diseases, including cancer, myocardial infarction, autoimmunity, osteoporosis and atherosclerosis (4, 5).

Although S1P is constitutively present in all cells, its level in most tissues is low. This is in contrast to plasma where S1P is found in relatively high concentration (2). Circulating S1P serves an important physiological function and is a key regulator of lymphocyte trafficking, endothelial barrier function and vascular tone (6-8). It also plays an important role in bone homeostasis (9). This review summarizes the present state of knowledge on metabolism, transport, sources and regulation of S1P in the plasma and blood cells.

Overview of S1P metabolism

Sphingosine, the substrate for S1P synthesis, is produced by degradation of ceramide, not de novo biosynthesis. Subsequently sphingosine can be either reacylated back to ceramide or phosphorylated by sphingosine kinases (SPHK1 and SPHK2) to form S1P. Although both SPHK isoforms synthesize the same product, they display different catalytic properties, subcellular locations, tissue distribution, and possibly have unique and specific functions (2). SPHK1 is highly specific for sphingosine and dihydrosphingosine as substrates (10). It resides in the cytosol but translocates to the plasmalemma upon activation (11). Significant fraction of cellular SPHK1 (~8%) is constitutively released to the extracellular space. The secreted
enzyme is active which enables local production of S1P in vicinity to its cell surface receptors (2, 12). SPHK2 is mainly found in the nucleus, but it is also present in the cytosol, internal membranes and plasmalemma (13). SPHK2 is not secreted and is able to phosphorylate a broader range of substrates including sphingosine, dihydrosphingosine, phytosphingosine and FTY720 (14, 15).

S1P is degraded by three types of enzymes: S1P phosphatase (SPP), S1P lyase (S1PL) and lipid phosphate phosphohydrolase (LPP) (16). In most cells S1P is irreversibly degraded by S1PL to hexadecenal and ethanolamine-1-phosphate (17). S1P can be also dephosphorylated by two isoforms of SPP (SPP1 and SPP2), which are highly selective for sphingoid base-1-phosphates as substrates, to yield sphingosine (18).

SPPs and S1PL are localized in the endoplasmic reticulum, thus these enzymes cannot degrade extracellular S1P. The only enzymes that have the ability to do it belong to a family of three LPP isoforms. LPPs show broad substrate specificity and can degrade many phosphorylated lipids including phosphatidic acid, lysophosphatidic acid, S1P, FTY720, and ceramide-1-phosphate. LPP2 resides intracellularly, whereas LPP1 and LPP3 are mainly localized to the plasma membrane and function as ecto-enzymes, degrading lipid phosphate substrates in the extracellular space (19-24).

**S1P metabolism in blood cells**

**Platelets**

Platelets are characterized by high SPHK activity and lack of S1PL which allows them to accumulate large amounts of S1P (Fig. 1) (25). Thrombocytes express both SPHK1 and SPHK2, however, the former isoform is responsible for 75% of total SPHK activity (26). Other, yet unidentified, enzyme possessing sphingosine kinase activity with high specificity towards phytosphingosine may also be present in platelets (27). Activity of serine
palmitoyltransferase, the rate-limiting enzyme of de novo sphingolipid biosynthesis pathway, in thrombocytes is very low. Therefore, in order to synthesize S1P they have to incorporate sphingosine from the plasma. Alternatively, sphingosine can be generated on the outer leaflet of the plasmalemma (28). This process is initiated by degradation of the membrane sphingomyelin to ceramide (by acid sphingomyelinase released from activated platelets, or by secretory sphingomyelinase present in the plasma) which is then deacylated to sphingosine by neutral ceramidase localized to the plasma membrane of thrombocytes (28-30).

Concentration of S1P in human platelets decreases upon storage which is associated with elevation in ceramide level (31). It was shown that thrombocytes are able to dephosphorylate S1P (most likely with the help of LPP) and incorporate released sphingosine into ceramide and then sphingomyelin via the action of ceramide synthase and sphingomyelin synthase (25, 28). Human platelets are likely able to degrade also extracellular S1P since they express LPP1 on the outer surface of their plasma membrane (32).

**Erythrocytes**

Compared to platelets, red blood cells (RBCs) are characterized by 25-fold lower SPHK activity per mg of protein (3-fold lower per cell) (33). One study reported that erythrocytes express only SPHK1, whereas in another one both SPHK isoforms were found to be present (34, 35). Similarly to thrombocytes, RBCs obtain sphingosine either by incorporation from the plasma, or by generation on the outer leaflet of the cell membrane (Fig. 1). Human erythrocytes express alkaline ceramidase activity and it was suggested that this enzyme plays an important role in generation of sphingosine used for S1P synthesis (36). Neutral and acid sphingomyelinase, as well as sphingomyelin synthase were also found to be present in human erythrocytes (35, 37).
Early studies reported that RBCs lack S1PL and SPP and are, therefore, unable to degrade S1P (33, 38). However, recently low activity of these enzymes was detected in erythrocyte membranes which explains a marked decrease in S1P content in RBCs observed after prolonged storage. This phenomenon likely contributes to adverse clinical events such as pulmonary edema and impaired immune cell function occurring after transfusion (39).

We recently provided the first evidence that S1P synthesis in erythrocytes may be regulated. We found that prolonged reduction in plasma S1P concentration in patients with myocardial infarction is associated with increased erythrocyte SPHK activity and enhanced SPHK1 protein expression (40). This observation suggests that RBCs may be able to increase the rate of S1P production in response to reduction in its plasma concentration. In addition, increased activity of acid and neutral sphingomyelinase and accumulation of sphingosine and S1P was reported for erythrocytes of patients with sickle cell disease (35). Degradation of membrane sphingolipids may represent another regulatory point of S1P metabolism in RBCs.

**Leukocytes**

White blood cells (WBC) are also able to incorporate extracellular sphingosine and to convert it into S1P (41). As a nuclear cells, leukocytes possess full set of enzymes involved in sphingolipid metabolism. They are able to efficiently convert sphingosine into ceramide and then sphingomyelin. In addition, ceramidase and serine palmitoyltransferase were also found to be present in WBCs (41, 42). Both SPHK activity and S1P content in leukocytes is much lower than in platelets, and comparable to that of erythrocytes (33, 43, 44). In addition, considerable activity of S1P-degrading enzymes was found to be present in WBCs (38).

**S1P concentration and distribution in different blood compartments**
Plasma is characterized by relatively high S1P level compared to solid tissues, and it was proposed that significant S1P concentration gradient exists between plasma and interstitial fluid (45). In our studies, mean plasma S1P concentration in healthy subjects ranged from 100±22 to 372±142 nM depending on the population (40, 46-51). This is in line with the results of most other groups (44, 52-54). However, some authors reported much higher S1P concentrations of approximately 1 μM (55, 56). These differences are likely related to the protocol used to isolate plasma. In order to prevent S1P release from erythrocytes, blood should be put on ice immediately after sampling, and then centrifuged at 4°C (38). In addition, plasma separated by routine one-step centrifugation usually contains some platelets which can lead to overestimation of S1P concentration as well (M. Baranowski, unpublished observation).

S1P concentration in murine plasma is higher compared to the human one, the reported values range from 471±59 nM to 1,35±0,19 μM (36, 53, 56-58). It should be noted that serum S1P level is 2- to 3-fold higher than in plasma which is a consequence of S1P release from platelets activated during blood clotting (59, 60). There is no difference in plasma S1P between fasting and nonfasting subjects (52). However, S1P concentration is increased after 12h of fasting in mice (61).

One study showed that plasma S1P level is higher in males than in females (62), whereas the other found the opposite in both mice and humans (63). However, other authors did not observe any gender difference (52, 56). This discrepancy is likely related to differences in age of populations studied, as plasma S1P concentration in women decreases markedly after menopause (63). It was also reported that S1P level is elevated in obese humans and mice, and that it is negatively correlated with age (61, 63).

Ito et al. (33) found that one human platelet contains approximately 9-fold more S1P than one RBC. However, since erythrocytes constitute about 95% of total blood cell number,
the overall contribution of RBCs to S1P pool in whole blood is considerably higher than that of platelets. According to the results of the above study 14% of the total blood S1P is found in plasma, 32% in platelets and 54% in erythrocytes.

**S1P carriers in the plasma**

Most of plasma S1P is transported bound to high-density lipoproteins (HDL) (50-60%). The remaining part binds to albumin (30-40%), low density lipoproteins (LDL) (~8%), and very low density lipoproteins (VLDL) (2-3%) (56, 64). However, in some subjects more than 60% of S1P is transported by albumin (55). Although HDL is the major S1P carrier, there is no straightforward relationship between plasma HDL and total S1P level. We observed comparable S1P concentration in healthy subjects and patients with ischemic heart disease despite a 2-fold difference in HDL-cholesterol level (49). Similarly, there is no difference in total serum S1P level between groups of subjects with high and low HDL-cholesterol concentration (65). However, HDL-bound S1P is strongly positively correlated with plasma HDL-cholesterol, apo-AI and apo-AII content (66).

Interaction with plasma proteins seems to reduce bioavailability of S1P. Exposition of S1PRs to S1P concentration that is normally found in the plasma should result in their full activation since their $K_d$ values are in the range of 2-30 nM (64). However, the estimated level of bioactive S1P in plasma is approximately 10 nM (67).

S1P is not evenly distributed between HDL subclasses. Small dense HDL$_3$ particles carry ~78% of the lipoprotein-associated S1P whereas HDL$_2$ binds only 16% (52). Christoffersen et al. (68) found that apolipoprotein M (apoM) is the carrier of S1P in HDL. They showed that apoM-deficient fraction of human HDL contains no S1P, and that apoM-/- mice are characterized by a lack of HDL-bound S1P, whereas transgenic mice overexpressing human apoM show increased S1P content in HDL. However, according to a recent study,
apoM may not be the only HDL protein able to bind S1P (69). It was estimated that in average only 1-10% of HDL particles in human plasma transport S1P, and in mice the molar ratio between HDL-bound S1P and plasma apoM is ~1:3 (56, 68). The above data are in line with the fact that human apoM binds S1P with an IC$_{50}$ of 0.9 μM (70), a concentration that is 2- to 3-fold higher compared to plasma S1P level reported in most studies.

It is very likely that biological activity and metabolism of plasma S1P is highly dependent on its carrier. The half-life of HDL-bound S1P upon incubation with human umbilical vein endothelial cells (HUVEC) is 4-fold longer compared to albumin-bound S1P (71). This finding indicates that HDL prevents S1P degradation by ecto-phosphatase. In addition, protection against myocardial ischemia/reperfusion injury is induced by HDL- and albumin-bound S1P, whereas LDL-associated S1P is ineffective (72). Moreover, although both albumin- and HDL-bound S1P improves endothelial barrier function, duration of the barrier promotion elicited by HDL-bound S1P is much longer due to specific effects on S1PR$_1$ trafficking that prolong receptor signaling (73).

**Degradation of plasma S1P**

The half-life of albumin-bound C17-S1P (a 17-carbon analog of S1P) injected intravenously to mice is ~15 minutes (57). Even shorter half-life of ~1 minute was reported in another study (74). The above data indicate that circulating S1P is rapidly cleared by degradative enzymes and imply the presence of high-capacity sources of S1P involved in maintaining its high concentration in the plasma. Interestingly, S1P is stable in isolated plasma, but not in whole blood or in the presence of HUVECs (75). Zhao et al. (22) reported that HUVECs as well as endothelial cells (EC) from macro- and microvessels of human pulmonary circulation (but not epithelial cells, monocytes or macrophages) rapidly degrade extracellular S1P via the action of LPP1. Released sphingosine is then taken up and used for...
synthesis of intracellular S1P by SPHK1 (but not SPHK2). Although extracellular dihydrosphingosine-1-phosphate (dhS1P) undergoes the same pathway, S1P is the preferred substrate in human pulmonary artery endothelial cells (HPAEC) (22). As mentioned earlier, platelets express LPP1 (25, 32). However, plasma S1P concentration in NF-E2-deficient mice that lack thrombocytes is the same as in wild-type animals suggesting that platelets do not play a significant role in degradation of S1P in vivo (76). Moreover, in mice as much as 86% of intravenously administrated C17-S1P accumulates in the liver which strongly indicates the primary role of hepatic sinusoidal ECs in degradation of plasma S1P (74).

Pharmacological inhibition or knock-out of SPHK1 decreases S1P level in whole blood and plasma by approximately 50% suggesting that both SPHK isoforms are equally important in generation of the blood pool of this sphingolipid (77, 78). Unexpectedly, genetic or pharmacological inhibition of SPHK2 results in a striking increase in plasma and blood cell S1P concentration (77, 79). This observation indicates that regulation of plasma S1P level is more complex than initially presumed. Sensken et al. (79) proposed that accumulation of S1P in blood observed in mice lacking SPHK2 is a consequence of impaired intracellular rephosphorylation of sphingosine released from blood-borne S1P degraded by LPP1. Their results strongly suggest that SPHK2 plays a key role in regulation of S1P redistribution from erythrocytes into ECs.

**S1P release from blood cells**

*Erythrocytes*

S1P release seems to be cell specific, and the amount of exported S1P is much higher for blood cells than for other cell types (80). Although both platelets and erythrocytes store large amounts of sphingoid base-1-phosphates, only RBCs are considered to be an important source of plasma S1P since they release S1P spontaneously without any stimulation (38).
Transfusion of wild-type erythrocytes (but not leukocytes or platelets) to SPHK1/2-deficient mice is sufficient to restore normal plasma S1P level which proves the key role of RBCs in blood S1P homeostasis (76). Consistently, there is strong positive correlation between RBC-related parameters and S1P level in human plasma, and anemic patients show lower S1P concentration in plasma (but not in blood cells) than healthy subjects (39, 62, 81). Interestingly, anemia selectively depletes the HDL-bound pool of S1P (62). However, in mice neither severe hemolytic anemia nor blood loss affect plasma S1P level (82). These findings suggest that, compared to humans, mice possess more robust mechanisms to replenish circulating S1P when erythrocyte number declines.

When radiolabeled sphingosine was incubated with erythrocytes it was efficiently taken up and converted to S1P which was then released without the rate of export being affected by Ca\(^{2+}\), PKC activator or thrombin (83). S1P release is proportional to RBC number and is temperature-dependent (it does not efficiently occur at 4°C). It also requires the presence of S1P acceptors. Erythrocytes do not release S1P in plasma/serum free medium, and the rate of export decreases upon dilution of plasma (38). HDL and albumin are major endogenous triggers of S1P release from erythrocytes. However, HDL is much more effective in this regard compared to albumin which extracts only two S1P molecules per thousand molecules of albumin. VLDL and LDL are very weak inducers of S1P release (81). ApoM plays a significant role in HDL-induced S1P efflux from erythrocytes. Compared to normal lipoproteins, HDL isolated from mice overexpressing apoM induce markedly higher (and non-saturable) S1P release from RBCs. Surprisingly, HDL isolated from apoM-deficient mice do not exhibit impaired effectiveness in this regard. This finding strongly indicates the presence of an additional apoM-independent mechanism (most likely involving apoA-I) responsible for HDL-induced release of S1P from erythrocytes (69). It was also demonstrated...
that S1P transfer between RBCs and HDL is facilitated by phospholipid transfer protein (PLTP), and PLTP-deficient mice show a 60% decrease in plasma S1P concentration (84).

Binding capacity of an acceptor determines the amount of S1P released from RBCs. A highly specific anti-S1P antibody extracts S1P at a molar ratio of 1:1 (81). Interestingly, when erythrocytes are incubated in plasma/serum-free medium containing S1P, large amounts of the compound are taken up by RBCs (but not WBCs or platelets) (38). Moreover, addition of C17-S1P to erythrocytes incubated in the presence of albumin results in partial uptake of the compound (81). These findings suggest that there is a balance between the amount of S1P found in RBCs and bound to acceptors in the medium, and that incorporation or release of S1P occurs when this balance is disturbed (81). This hypothesis is supported by the data obtained from mice transfused with erythrocytes with either high or low S1P content. After 24h the amount of S1P in these cells equalized with that of endogenous RBCs (38).

Another mechanism of S1P export is its transcellular transportation from erythrocytes to ECs that is not mediated by plasma proteins. Both fluorescent analog of S1P and C17-S1P are readily exchanged between RBCs as well as between erythrocytes and HUVECs when they are coincubated in plasma/serum free medium (81). This transcellular transportation is, however, prevented by the presence of albumin or HDL. The blocking effect of albumin can be circumvented by tight contact between erythrocytes and HUVECs. It is, therefore, very likely that RBC-associated S1P is transferred to vascular ECs during erythrocyte passage through the capillaries. This exchange is facilitated by the fact that RBCs store S1P predominantly in their plasma membrane (38).

Recent studies identified several members of the ATP-binding cassette transporter (ABC) family and spinster homolog 2 (SPNS2) as putative S1P transporters in various cell types (85). SPNS2 is not expressed in murine erythrocytes, and the rate of S1P release from RBCs is not affected by SPNS2-deficiency (58, 86, 87). The transporter involved in S1P
export from rat erythrocytes is ATP-dependent, hydrolysis of ATP is, however, not required for its activity. It is also sensitive to glyburide (nonspecific ABC transporter inhibitor) and to vanadate (a phosphate analog, inhibitor of ATPases), but not to ABCB1 inhibitor cyclosporin or ABCC1/ABCC4 inhibitor MK571. Nevertheless, ABCC1, which is expressed in RBCs, was suggested as a candidate for erythrocyte S1P transporter (83). However, plasma S1P level is not affected by deficiency of ABCC1 (88). Interestingly, transport of S1P in erythrocyte inside-out membrane vesicles is not inhibited by the presence of closely related compounds such as dhS1P and ceramide-1-phosphate. This finding indicates that the transporter involved in S1P export from RBCs is highly specific to this sphingoid base-1-phosphate (83). Identification of the precise nature of erythrocyte S1P transporter requires further investigation. Analysis of the rate of S1P release from RBCs isolated from mice deficient in different types of ABC transporters could shed some light on this matter.

**Platelets**

In contrast to erythrocytes, platelets require activation to release significant amounts of S1P, and the rate of release depends on the agonist potency. Strong platelet agonists, like thrombin, collagen or convulxin, induce greater release compared to weak agonists such as ADP, which is barely effective in this regard (89, 90). S1P export from stimulated platelets is enhanced by increasing concentration of albumin in the medium (89). Interestingly, HDL extracts S1P from activated human thrombocytes less efficiently than albumin (75) suggesting that platelets and erythrocytes differ in their preferences for extracellular S1P acceptors.

There is some controversy as to whether thrombocytes show constitutive release of S1P. Two studies found no evidence for spontaneous S1P release (38, 91), whereas in other reports some S1P was released constitutively (90, 92, 93). However, Jonnalagadda et al. (89) found that considerable amounts of S1P are released without stimulation from human
thrombocytes, especially at higher albumin concentration in the medium. They also showed that increasing albumin concentration results in elevation of S1P stores in human and murine platelets. The authors postulated that albumin, by extracting S1P from resting thrombocytes, stimulates a homeostatic mechanism that maintains platelet S1P levels. A similar mechanism is likely activated upon thrombocyte stimulation as evidenced by accumulation of sphingosine and depletion of ceramide in murine platelets under these conditions (94). Jonnalagadda et al. (89) concluded that thrombocytes contain two pools of S1P, a passively extractable one, similar to the pool of S1P in erythrocytes, and a pool that is acutely mobilized by platelet activation. It was also reported that S1P concentration in plasma from platelet concentrates increases markedly upon storage, and that the incidence of transfusion reactions is associated with higher S1P level in the concentrate (95).

It is generally thought that thrombocytes do not represent an important source of plasma S1P under normal conditions. This notion is supported by the fact that concentration of S1P in human plasma is not correlated with platelet count (62). This parameter is, however, the major determinant of S1P level in serum, which is reasonable considering that platelets are fully activated during blood clotting (96). Moreover, thrombocytopenic, as well as platelet-deficient mice, are characterized by normal plasma S1P concentration (57, 76). It should be noted, however, that murine thrombocytes, unlike human ones, store mostly dhS1P and contain only small (if any) amount of S1P (89, 94). Interestingly, the proportion between these two sphingoid base-1-phosphates in rat platelets resembles that of human thrombocytes (M. Baranowski, unpublished observation).

Although platelets do not seem to determine plasma sphingoid base-1-phosphate concentration in healthy subjects, it is possible that under pathological conditions, in which thrombocytes are activated, they play some role in this regard. For instance, in vivo platelet activation by anti-CD41 antibody resulted in elevation of plasma dhS1P in mice. S1P level
remained stable which is not surprising considering the fact that murine platelets lack this compound (94). In addition, rabbits with hypercholesterolemic atherosclerosis, a condition associated with platelet activation, are characterized by markedly increased S1P level in plasma and thrombocytes, as well as by enhanced S1P release from activated platelets (97). In humans, plasma S1P is correlated (albeit weakly) with concentrations of serotonin and β-thromboglobulin which are well known in vivo platelet activation markers (62).

There is only limited information on the signaling pathways mediating S1P release from platelets upon stimulation (Fig. 2). Thrombin-induced S1P release requires PKC activation as evidenced by the fact that it is mimicked by 12-O-tetradecanoylphorbol-13-acetate (PKC stimulator) and attenuated by staurosporin (PKC inhibitor) (93). There is conflicting evidence as to whether extracellular calcium affects thrombin-induced S1P release from platelets (89, 92).

Ulrych et al. (90) reported that S1P release from human platelets is mediated by thromboxane formation and subsequent activation of its receptor. They found that thrombin- and collagen-induced S1P release is suppressed by acetylsalicylic acid and thromboxane receptor antagonist, whereas thromboxane receptor activator stimulates S1P release from platelets in an acetylsalicylic acid-independent manner. Moreover, ADP, that caused only minor increase in thromboxane formation, did not induce S1P release. They also found evidence for the presence of additional thromboxane-independent mechanism of thrombin-induced S1P release. Constitutive S1P release was not affected by inhibition of thromboxane synthesis. In addition, thromboxane receptor-deficient mice showed normal plasma S1P concentration (90). The above data indicate that thromboxane mediates S1P release only under conditions of platelet activation. However, we (50) and others (98) found that aspirin and celecoxib reduce plasma S1P level in healthy subjects suggesting a role for cyclooxygenase in S1P homeostasis in blood.
Two studies concluded that S1P export from platelets is not mediated by exocytosis, and that S1P is stored in the plasma membrane and cytoplasm rather than in secretory granules (90, 93). However, recently Jonnalagadda et al. (89) provided strong evidence for the involvement of exocytosis in release of S1P from stimulated thrombocytes. They found that the kinetics of S1P release closely mirrored those of the α-granule marker, platelet factor 4, and subcellular fractionation confirmed the presence of S1P in α-granules, but not in dense granules of human thrombocytes. Moreover, Unc13d<sup>jinx</sup> mice, characterized by defective exocytosis, showed attenuated thrombin-induced S1P release from platelets. In the above study newly synthesized S1P required more than an hour to enter the stimulation-dependent, releasable pool. Of note, in the previous reports, where release of [³H]S1P instead of the natural compound was measured, platelets were incubated with [³H]sphingosine for not longer than ten minutes before stimulation (90, 93), which may explain why these authors did not find any evidence for granule-mediated S1P release.

Similarly to erythrocytes, the precise nature of the platelet S1P transporter remains unclear. S1P release from stimulated rat thrombocytes is inhibited by glyburide, suggesting involvement of an ABC transporter, however, cyclosporin or MK571 shows no effect (93). On the other hand, S1P release from stimulated human platelets is suppressed by MK571. Indomethacin and dipyridamole, which also inhibit ABCC4, show similar effect. It should be noted that basal S1P release is not affected by these compounds (90). ABCC4 is expressed in human thrombocytes at a high level, which further indicates that this isoform may mediate agonist-induced S1P release (99). It was also reported that SPNS2 transporter is not involved in S1P release from stimulated murine platelets (58).

Leukocytes
In human leukocytes S1P is the major sphingoid base-1-phosphate, whereas in murine WBCs dhS1P predominates. However, unlike platelets, murine leukocytes contain also significant amounts of S1P (94). Hanel et al. (38) reported that human WBCs effectively degrade extracellular S1P but are not able to release it, whereas Yang et al. (41) found that human neutrophils and mononuclear cells show significant basal S1P release. This discrepancy likely results from the fact that Yang et al. determined the release of $[^3]$H$S1P, whereas Hanel et al. measured changes in S1P concentration in the incubation medium. The results of the above studies suggest that the rate of degradation of extracellular S1P by leukocytes exceeds the rate of its release by these cells. Plasma S1P concentration is not correlated with WBC count in healthy subjects (62), and is not affected by deficiency of both T and B cells in mice (76). These findings suggest that leukocytes do not play a significant role in S1P homeostasis in blood.

Stimulation of mast cells with IgE induces rapid activation of SPHKs, accumulation of intracellular S1P, and its release into the extracellular space (100). In murine and human mast cells, the export of S1P is markedly reduced by MK571 and by silencing of ABCC1 expression, suggesting an important role for this transporter in S1P release (101). However, it remains obscure whether similar mechanisms operate in circulating WBCs upon their activation. Murine lymphocytes lack SPNS2 suggesting that this transporter is not involved in S1P export from leukocytes (86).

**Other sources of circulating S1P**

**Vascular endothelium**

Similarly to erythrocytes, vascular ECs also spontaneously release S1P (Fig. 3). This was confirmed for HUVECs, HPAECs, endothelial cells from murine aorta, and mouse
embryonic ECs (57, 58, 63, 87, 88). However, in one study S1P export from HPAECs was not observed (22).

There is increasing body of evidence that S1P release from ECs is mediated by SPNS2. ECs isolated from the aorta of SPNS2-deficient mice are unable to release S1P, and silencing of SPNS2 expression markedly attenuates S1P export from HUVECs and HPAECs (58, 87). Consistently, whole-body or endothelium-specific SPNS2 deficiency reduces plasma S1P concentration in mice by approximately 40% (58, 86, 87). However, one study found no difference in plasma S1P level between SPNS2-null mice and wild-type animals (102).

Hisano et al. (58) reported that murine peripheral blood vessels express SPNS2 exclusively in the endothelium. In addition, they concluded that SPNS2 expression in the venous ECs is higher than in the arterial ones. In mice, the presence of this transporter in vascular ECs was confirmed for the thymus, heart, lungs and hypothalamus, but not for kidneys. SPNS2 is also expressed in human venous, aortic and microvascular ECs (87).

S1P release from HUVECs is attenuated by glyburide and MK571, which indicates involvement of an ABC transporter in addition to SPNS2. However, deficiency of either ABCA1, ABCA7 or ABCC1 does not affect plasma S1P concentration in mice (88). This observation, together with the finding that murine ECs are not able to export S1P in the absence of SPNS2, indicates that ABC transporters are not involved in S1P release by the vascular endothelium in mice.

There is only limited information on the mechanisms regulating S1P export from ECs. Estradiol increases intracellular content and release of S1P from HUVECs, which explains lower plasma S1P concentration in post-menopausal women. The effect of estradiol is related to enhanced expression of SPHK1, SPNS2, ABCC1 and ABCG2 (63). In addition, shear stress acutely increases S1P release from murine ECs, which is associated with downregulated expression of S1PL and SPP1 (57). Export of S1P from ECs is also enhanced by silencing of
S1PL expression (57). Interestingly, overexpression of SPHK1 increases release of dhS1P but not S1P (88).

The extent to which vascular endothelium contributes to the plasma pool of S1P is a matter of debate. S1P concentration in lethally irradiated wild-type mice transplanted with SPHK1/2-deficient bone marrow is reduced by 90%, and transplantation of SPHK1/2-deficient mice, that lack S1P in plasma, with wild-type bone marrow restores its concentration back to normal (76). These findings would indicate that endothelium is only a minor contributor to the plasma S1P pool in mice. However, it was also shown that adenoviral-mediated transduction of SPHK1 to SPHK1-null mice, characterized by 40% lower plasma S1P level, restores its normal concentration (57). The fact that recombinant SPHK1 is expressed in ECs (mainly in the liver) and not in blood cells suggests an important role for vascular endothelium in S1P homeostasis. This notion is also supported by the previously mentioned studies showing significant reduction in plasma S1P concentration in SPNS2-deficient mice. Recently Xiong et al. (103) provided strong evidence that vascular endothelium and RBCs are equally important sources of plasma S1P in mice. In their study erythrocyte-specific loss of three alleles of SPHK (SPHK1^{0/0} SPHK2^{+/−}) reduced S1P content in RBCs by 98%, whereas its plasma concentration decreased by only ~50%. Furthermore, endothelial-specific SPHK1/2 deletion caused a 30% reduction in plasma S1P.

We recently provided some in vivo evidence that S1P may be released by the vascular endothelium in humans (104). We found that plasma is enriched in S1P (but not in dhS1P) upon its passage through the vasculature of the leg. Interestingly, this effect was no longer present after 30 minutes of exercise, suggesting acute changes in the rate of S1P release and/or degradation by the endothelium.

*Liver*
Murine primary hepatocytes and HepG2 cells are able to export S1P, although at a lower rate than ECs (57, 105, 106). Moreover, plasma S1P concentration is reduced after partial hepatectomy in mice, upon the development of carbon tetrachloride-induced liver fibrosis in rats, and in chronic hepatitis C patients (105, 107). These findings suggest that liver is important for the maintenance of normal plasma S1P level. In addition to mere S1P export, the role of this organ in S1P homeostasis may be also related to the fact that liver is the source of plasma apoM, the major carrier of circulating S1P (108). Indeed, hepatic overexpression of apoM induces striking increase in plasma S1P concentration (105). This increase may result from several effects. First of all, isolated murine hepatocytes and HepG2 cells overexpressing apoM are characterized by enhanced export of S1P (105, 106). However, apoM overexpression not only stimulates S1P release from hepatocytes but also protects it from degradation by ecto-phosphatase (105). In addition, increased plasma apoM concentration may stimulate S1P export from extrahepatic sources.

It was recently found that insulin affects plasma S1P level via its action on hepatic apoM production. S1P and apoM concentration is elevated in mice with streptozotocin-induced diabetes, and administration of insulin decreases their levels in both healthy and diabetic mice. In addition, incubation of HepG2 cells with insulin reduces apoM content in the medium and cells (109).

**Secretory SPHK**

Apart from being released to the circulation by various cell types, S1P can be also produced directly in the plasma by secreted SPHK. Enzymatically active SPHK1 is secreted by HUVECs, and SPHK activity was detected in murine and human plasma (14). In mice contribution of plasma to total SPHK activity in blood is comparable to that of platelets. However, human plasma is characterized by 20-fold lower SPHK activity which argues
against significant role of secreted SPHK1 in generation of S1P. This notion is supported by the fact that phosphorylation of sphingosine was not detected in isolated human plasma (75).

**Concluding remarks and future perspectives**

Studies conducted over the last decade substantially expanded our knowledge on the origin, transport and metabolism of S1P in blood. Several new sources of circulating S1P were identified, and it is now evident that platelets do not play a major role in this regard. However, the relative contribution of erythrocytes, vascular endothelium and hepatocytes to plasma S1P pool remains unclear. Moreover, many fundamental questions about the mechanisms maintaining S1P homeostasis remain to be resolved. Which transporter is involved in S1P release from erythrocytes, and is it regulated? Are other sources able to “sense” abnormal S1P concentration and adjust the rate of its export? What exactly is regulated? Total plasma S1P concentration or the level of its specific pool (e.g. HDL-bound)? Answering these questions is essential for development of pharmacological tools to manipulate plasma S1P concentration for therapeutic purposes. Another important area for future research is better characterization of the biological activity of different pools of plasma S1P and factors that affect the relative distribution of S1P between these pools.

It is, however, important to note that the results of mice studies cannot be directly translated to humans due to significant differences in metabolism, and possibly also origin of circulating S1P. First of all, in murine blood cells, in contrast to the human ones, dhS1P is the dominant sphingoid base-1-phosphate. Secondly, plasma S1P concentration is correlated with RBC-related parameters only in humans. In addition, S1P release from human ECs and activated platelets seems to be mediated by distinct types of transporters than in mice. Yet another difference is high activity of secretory SPHK in the plasma of mice. Therefore, future studies in this area should be conducted on humans whenever possible.
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References


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Figure 1. Schematic representation of sphingolipid metabolism in erythrocytes and platelets.