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Review

Sphingosine kinases, sphingosine 1-phosphate, apoptosis and diseases

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Abstract

Sphingolipids are ubiquitous components of cell membranes and their metabolites ceramide (Cer), sphingosine (Sph), and sphingosine-1-phosphate (S1P) have important physiological functions, including regulation of cell growth and survival. Cer and Sph are associated with growth arrest and apoptosis. Many stress stimuli increase levels of Cer and Sph, whereas suppression of apoptosis is associated with increased intracellular levels of S1P. In addition, extracellular/secreted S1P regulates cellular processes by binding to five specific G protein coupled-receptors (GPCRs). S1P is generated by phosphorylation of Sph catalyzed by two isoforms of sphingosine kinases (SphK), type 1 and type 2, which are critical regulators of the "sphingolipid rheostat", producing pro-survival S1P and decreasing levels of pro-apoptotic Sph. Since sphingolipid metabolism is often dysregulated in many diseases, targeting SphKs is potentially clinically relevant. Here we review the growing recent literature on the regulation and the roles of SphKs and S1P in apoptosis and diseases.
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Keywords: Sphingosine kinase; Sphingosine-1-phosphate; Apoptosis; Cancer; Allergy; Asthma; Development

Contents

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1. Synthesis and metabolism of sphingolipids

De novo synthesis of sphingolipids is initiated at the cytoplasmic face of the endoplasmic reticulum (ER) by the condensation of serine and palmitate to produce 3-keto-sphinganine catalyzed by serine palmitoyl transferase (Fig. 1) [1]. In two rapid enzymatic reactions, 3-keto-sphinganine is reduced to sphinganine (dihydro-sphingosine, DHS) by 3-keto-sphinganine reductase in a NADH-dependent reaction. DHS is then N-acylated by dihydroceramide synthase using various fatty acyl CoAs to form dihydroceramide (DHCer) which is converted to Cer by a desaturase. The insertion of the trans 4,5-double bond into Cer by the desaturase is an important reaction because Cer, but not DHCer, mediates apoptosis [2]. Cer (and DHCer) is translocated from the ER to the Golgi apparatus in a non-vesicular transport manner by CERT [3], a cytoplasmic protein with a phosphatidylinositol-4-phosphate-binding domain and a putative catalytic domain for lipid transfer. Once in the Golgi, Cer and DHCer can be used to form sphingomyelin (SM) and dihydrosphingomyelin (DH-SM), respectively, by sphingomyelin synthase on the lumenal side of the Golgi, or to glucosylceramide (GlcCer) and dihydroglucosylceramide (DH-GlcCer) on the cytosolic surface of the Golgi [4]. After translocation into the Golgi lumen, GlcCers are further converted to lactosylceramides and more complex glycosphingolipids [5]. The sphingoid base sphingosine (Sph) is not produced de novo and is only formed from Cer by ceramidase-catalyzed hydrolysis. Sph can also arise during degradation of plasma membrane glycosphingolipids and SM in the endocytic recycling pathway.

Sph and DHS can be phosphorylated by SphKs to form S1P and dihydro-S1P, which are both substrates for specific S1P phosphatases that reside in the ER. Yeast SphKs, the products of two genes, LCB4 and LCB5, are required for the efficient utilization of exogenously added sphingoid bases [6]. LCB4 is principally required for this process and is found on the cytoplasmic face of internal membranes, including the ER, Golgi and probably endosomes [7]. There are also two mammalian isoforms, SphK1 and SphK2, that differ in sequence, catalytic properties, localization, and in their functions [8]. SphK1 has pro-survival functions and is mainly a cytosolic protein, whereas SphK2 is a putative BH3-only protein, inhibits cell growth and enhances apoptosis [9]. Recent results from our lab demonstrated that SphK1 decreases and SphK2 increases ceramide levels in HEK 293 cells.

From a mechanistic point of view, our results support the notion that SphK2, similar to yeast LCB4 [10], might play a role in the sphingosine salvage pathway of mammalian cells, acting in concert with S1P phosphatase (SPP-1) to convert S1P back to sphingosine and then to ceramide in the ER. Ceramide generated in the ER has been linked to increased Ca^{2+} release, leading to apoptosis. Moreover, cytosolic S1P formed by SphK1 inhibits *de novo* ceramide biosynthesis as a cellular sensing mechanism to minimize unneeded biosynthesis of ceramide [8]. S1P can also be further degraded by S1P lyase (SPL), an integral ER membrane protein facing the cytoplasm [11], which yields the cleavage products, hexadecenal and ethanolamine phosphate, the major exit route of metabolism of sphingolipids (Fig. 1).

2. The sphingosine kinase family

SphKs are an evolutionary conserved lipid kinase family that contains five conserved domains. SphK1 was originally purified to homogeneity from rat kidney as a 49-kDa protein. Based on tryptic peptides, murine SphK1 was then cloned [12]. Two isoforms, termed SphK1α and SphK1β, with 42.2 and 43.2 kDa predicted molecular mass were identified which only differed in a few amino acids at their N-termini, suggesting that they were derived by alternative splicing. SphK1 has a broad tissue distribution, with higher levels in brain, heart, lung and spleen. It has no trans-membrane domains, rather it has three calcium/calmodulin-binding consensus sequences and several potential protein kinase phosphorylation sites [12]. Indeed, the lack of a hydrophobic domain or an identifiable signal peptide is consistent with its predominant cytosolic localization [12]. SphK1 displays specificity for the natural trans isomer of D-*erythro*-sphingosine. Several SphK inhibitors have been discovered including threo-dihydrosphingosine (DHS), *N*, *N*-dimethylsphingosine (DMS) [13], fungal-derived inhibitors [14], and 2-(*p*-hydroxyanilino)-4-(*p*-chlorophenyl) thiazole [15], which have been widely used to implicate SphK and S1P formation in many biological processes. Yet, most of these results must be interpreted with caution as all of the known inhibitors block both SphK1 and SphK2.

Based on homology to SphK1, a second isoform, SphK2, was cloned and characterized from mouse and human [16]. SphK2 shares five conserved domains with SphK1 (about 80% similarity and 50% identity) but has an additional 200 amino acids, making it a predicted 68 kDa protein. SphKs have a conserved ATP-binding motif ($SGDGX_{(17–21)}K(R)$) found in diacylglycerol kinases [16] that has some similarity to the highly conserved glycine-rich loop involved in binding ATP in the catalytic site of many protein kinases [17]. Unlike SphK1, SphK2 has a somewhat lower substrate specificity and also can phosphorylate FTY720, an immunosuppressive drug currently in Phase 3 clinical trials for kidney transplantation and multiple sclerosis treatment [18–20]. Although SphK2 has four

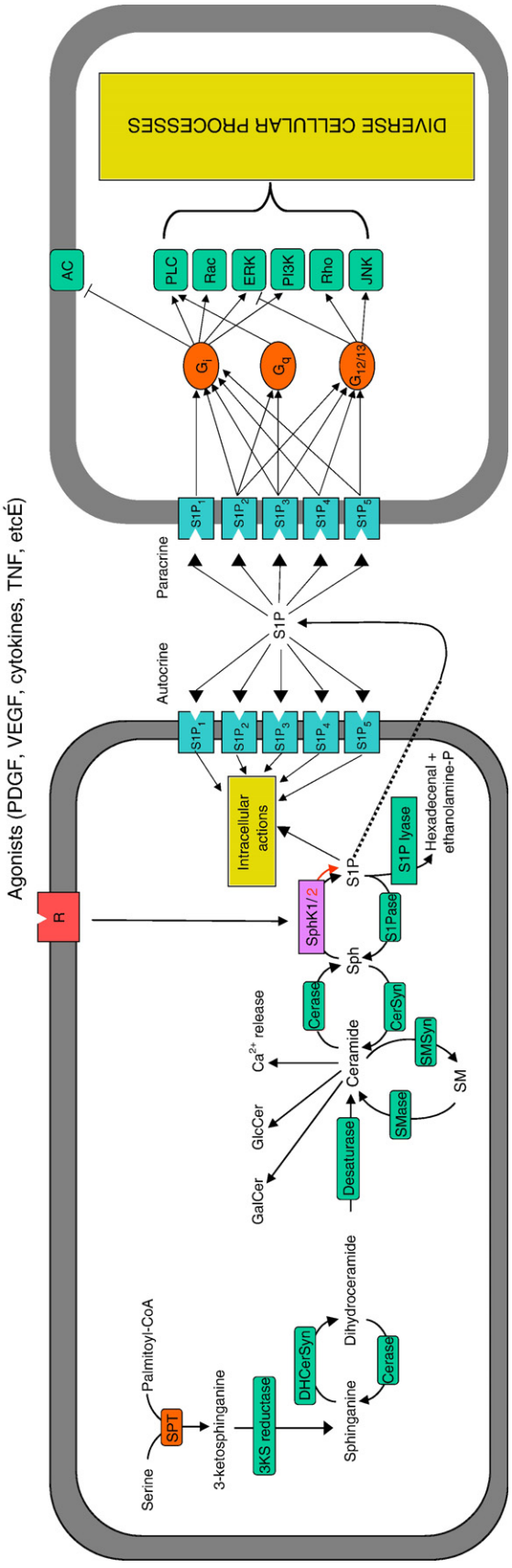

Fig. 1. Scheme showing ceramide biosynthesis and pathways leading to production of S1P. S1P in turn can act either in an autocrine or paracrine manner through five S1P receptors to regulate cellular functions. It may also have intracellular actions.

predicted trans-membrane domains, when ectopically expressed in HEK 293 cells, it is found mainly in the cytosol [16]. However, in MCF-7 breast cancer cells, endogenous SphK2 is almost exclusively nuclear localized [21], while it is mainly at the plasma membrane, at lower levels in internal membranes, and barely detectable in the cytosol of HEK 293 and MDA-MB-453 breast cancer cells [22].

3. Sphingosine kinases and production of sphingosine-1-phosphate

SphK is stimulated by numerous external stimuli resulting in increased intracellular S1P concentration and increased release from certain cell types. These stimuli include agonists of growth factor receptors (including PDGF, VEGF, NGF, and EGF), ligands for GPCRs, transforming growth factor beta, the pro-inflammatory cytokine TNF-alpha and cross-linking of immunoglobulin receptors (reviewed in [5]). In most cases, the importance of SphK1 activation and concomitant production of S1P in the regulation of many biologically responses was only indirectly inferred by the ability of pan SphK inhibitors to block agonist-induced effects.

Intracellularly produced S1P can act in autocrine/paracrine manner to stimulate S1P receptors present at the cell surface (signaling "inside-out") and initiate downstream G protein-mediated signaling, including activation of Src, focal adhesion kinases and Rac, all important in cell migration (Fig. 1). Interestingly, binding of S1P to $S1P_1$ or $S1P_3$ stimulates chemotaxis whereas binding to $S1P_2$ generally has a negative effect [23,24]. Some evidence indicates that intracellularly produced S1P serves as a modulator of calcium responses by a mechanism primarily dependent on the phospholipase C (PLC)/inositol 1,4,5-triphosphate ($InsP_3$) pathway [25], while S1P-mediated intracellular calcium mobilization is independent of GPCRs [26] and $InsP_3$ formation [27]. SphK1/S1P also enhances endothelial cell survival through platelet endothelial cell adhesion molecule-1-(PECAM-1)-dependent activation of phosphatidylinositol 3-kinase/AKT and regulation of Bcl-2 family members [28]. TNF-α and other cytokines stimulate SphK1 leading to the activation of the transcription factor nuclear factor-κB (NF-κB) [29]. A recent study showed that endothelial nitric oxide synthase activation by TNF-α occurs through sequential activation of neutral sphingomyelinase 2 and SphK1/S1P and depends on both $S1P_1$ and $S1P_3$ receptors [30]. Activation of SphK1 by VEGF activates Ras, and, consequently, extracellular signal regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) signaling and cell growth [31]. In most of these reports, activation of SphK1 is transient, reaching a peak within a few minutes, and declining rapidly thereafter. We recently showed that EGF activates both endogenous SphK1 and SphK2 [22,32]. This is the first example of agonist-dependent regulation of SphK2 [22]. Treatment with EGF significantly increased endogenous S1P levels in naïve cells as well as in SphK2-expressing cells [22], which in turn, led to activation of S1PRs ("inside-out signaling"), to regulate cell movement towards EGF. Moreover, in MDA-MB-453 cells, SphK1 and SphK2 had overlapping and/or complementary functions in regulation of EGF-induced migration. In contrast, only SphK1, but not SphK2, was required for migration of mast cells toward antigen [24] and for C5a receptor-dependent chemotaxis of macrophages [33]. Similarly, although downregulation or overexpression of SphK2 had no effect on migration of HEK 293 cells toward EGF, in sharp contrast, down-regulation or overexpression of SphK1 reduced or enhanced migratory responses of these cells toward EGF [22].

4. Mechanisms of sphingosine kinase activation

Since SphK1 is mainly present in the cytosol and its substrate, Sph, is generated in membranes, it is not surprising that translocation of SphK1 to the plasma membrane appears to be an important and common feature of its activation [5]. The rapid onset of activation of SphK1 is most consistent with post-translational modification mechanisms. Of note, yeast LCB4 is regulated by several types of post-translational modifications, including phosphorylation, ubiquitination, and palmitoylation [34,35]. Indeed, the phorbol ester, phorbol 12-myristate 13-acetate (PMA) induces protein kinase C-mediated phosphorylation of SphK1 and its translocation to the plasma membrane, leading to enhanced release of S1P to the medium which, in turn, triggers autocrine/paracrine signaling [36]. Another report subsequently showed that PMA and TNF-α induce phosphorylation of SphK1 at Ser225 catalyzed by ERK2 and cyclin-dependent kinase 2 increasing its enzymatic activity, which was accompanied by its translocation to the plasma membrane [37]. Previous studies had shown that intracellularly generated S1P acts upstream of ERK1/2 in the signaling pathways initiated by TNF-α and VEGF [31,38,39]. Taken together, these results imply that some level of activation of SphK1 must occur prior to further activation by ERK kinases [37]. It is possible that the initial step in such an autocrine activation loop of SphK1 involves translocation to the cell membrane and/or interaction with other specific proteins or lipids that regulate its activity. In this regard, nearly a decade ago, we found that acidic phospholipids, particularly phosphatidylserine, dose-dependently increase SphK activity, whereas neutral phospholipids have no effect [40]. These interactions likely have biological significance since it was later shown that specific interaction of phosphatidic acid with SphK1 was required for its translocation to membrane compartments [41]. More recently, using surface plasmon resonance, Stahelin et al. showed that SphK1 selectively binds phosphatidylserine over other anionic phospholipids and that the highly conserved Thr54 and Asn89 residues in the putative membrane binding surface of SphK1 were essential for the lipid selectivity and membrane targeting both *in vivo* and in vitro and that S225 phosphorylation increases the selective membrane binding and lipid–protein interactions [42].

There is accumulating evidence that the activity and subcellular localization of SphK1 are also influenced by its interaction with adaptor proteins. SphK1 interacting proteins comprise a heterogeneous array and include such diverse molecules as RPK118 [43], a protein related to protein kinase A

anchoring protein [44], aminoacylase 1 [45], PECAM-1 [46], and delta-catenin/NPRAP (neural plakophilin-related armadillo repeat protein) [47]. All of these interactors have been shown to co-immunoprecipitate with SphK1 but the physiological significance of their interaction with SphK1 remains unclear.

The important mast cell protein tyrosine kinases Lyn and Syk were recently identified as SphK1 interacting proteins by an antibody array method [48]. Although neither Lyn nor Syk phosphorylated SphK1, direct interaction of SphK1 with Lyn increased the activity of both kinases [48]. Moreover, this interaction results in the recruitment of SphK1 to FcεRI and shortly after stimulation, in the appearance of both Lyn and SphK1 in lipid rafts of the cell membrane. In mast cells isolated from Lyn knockout mice, there was no increase in SphK1 activity after FcεRI crosslinking, confirming that interaction with Lyn was required [48].

It has long been known that SphK1 plays an important role in cell survival and that its activation enhances cell survival in response to TNF-α [38,49]. SphK1 was subsequently shown to directly interact with the TNF receptor-associated factor 2 (TRAF2), resulting in activation of SphK1 that is required for TRAF2-mediated activation of NF-κB, but not for activation of c-Jun N-terminal kinase [29]. These findings suggest that SphK1 may be a component of the signal transduction pathway downstream of the TNF receptor and TRAF2.

In some cells, a more sustained increase of SphK activity has been observed following stimulation, lasting for hours or days, most likely resulting from increased transcription. Examples include NGF-induced increases in SphK activity in neuronal cells [50], PMA effects in HeLa cells [51], vitamin D3 treatment of HL60 cells [52], and stimulation of fibroblasts with TNF-α [53]. Thus, it is likely that regulation of SphK1 at the transcriptional level may also have important physiological functions.

Although much less is known of SphK2 interacting proteins, we found that the pro-apoptotic Bcl-x_L protein specifically interacts with SphK2 [9]. The IL-12 receptor was also identified as a SphK2 interactor by a yeast two-hybrid approach. This interaction was linked to IL-12 receptor signaling events [54], potentially associating SphK2 with Th1 effector functions.

5. Sphingosine kinases, S1P and apoptosis

SphKs and S1P phosphatases (SPPs) are essential cellular regulators of S1P levels [5]. Many studies in various cell types have confirmed our original observation that overexpression of SphK1 and increased S1P production promotes cell growth, enhances the G1/S transition and increases cells in S-phase [55]. For example, SphK1 overexpression in NIH 3T3 fibroblasts promotes growth of cells in soft agar and tumor formation in mice, probably owing to its role in Ras and ERK1/2 signaling, suggesting a potential oncogenic function [31,56,57]. Consistent with these observations, a dominant-negative form of SphK1 inhibited estrogen-mediated mitogenic signaling in MCF-7 cells and decreased their ability to form tumors in nude mice [58]. Furthermore, recently it was shown that phosphorylation of SphK1 and subsequent membrane translocation is required for its pro-oncogenic function [59]. Importantly, SphK1 is frequently overexpressed in a variety of solid tumors, suggesting an important role in human tumorigenesis [15,60]. SphK1 also mediates VEGF-induced Ras activation in bladder cancer cells by favoring inactivation of Ras-GAP, thereby promoting tumorigenic processes [61]. We have shown that decreasing SphK1 expression with siRNA markedly reduces EGF-dependent MCF-7 cell growth, while downregulation reduced the percentage of cells in S and G2/M phases, and increased cells in G0/G1 phase [32]. Together, these results suggest that endogenous SphK1 regulates the progression of MCF-7 cells through the cell cycle. Decreasing SphK1 expression by RNA interference or inhibiting its activity also significantly decreased the proliferation of U-1242 MG and U-87 MG glioblastoma cells [62]. Moreover, this prevented cells from exiting G1 phase of the cell cycle and marginally increased apoptosis. Surprisingly, knockdown of SphK2 expression inhibited glioblastoma cell proliferation more potently than did SphK1 knockdown. Thus, both SphK isoforms may be important regulators of glioblastoma cell proliferation.

We previously found that sensitivity of MCF-7 cells to the anti-cancer drug doxorubicin was potentiated by the pan SphK inhibitor, DMS [57]. Relevant to this, an interesting recent finding was that induction of apoptosis in MCF-7 cells by doxorubicin was accompanied by degradation of SphK1 [63]. MCF-7 cells are null for the executioner caspase-3 and depend mainly on caspase-7 for the execution of the cell death program [64]. In agreement, we found that activation of caspase-7 and cleavage of PARP induced by doxorubicin in MCF-7 cells were accelerated by downregulation of SphK1 [32]. As resistance to anti-cancer drugs is a common feature in breast cancer therapy, these observations suggest that the effectiveness of some chemotherapeutic agents might be related to the level of expression of SphK1.

Our laboratory and others have demonstrated that in contrast to SphK1, overexpression of SphK2 suppresses growth and enhances apoptosis, preceded by cytochrome *c* release and activation of caspase-3 [9,21]. Moreover, SphK2-induced apoptosis was independent of activation of S1P receptors [9]. Sequence analysis revealed that SphK2 contains a 9 amino acid motif similar to that present in Bcl2-homology domain 3 (BH3)-only proteins, a pro-apoptotic subgroup of the Bcl-2 family. As with other BH3-only proteins, SphK2 interacted physically with the anti-apoptotic member Bcl-x_L [9]. However, mutation of the conserved leucine present in all BH3 domains, that is critical for their ability to induce apoptosis [65], only partially reduced SphK2-induced apoptosis [9], suggesting that the SphK2 protein possesses additional determinants important for this function. Recently, we found that the catalytic activity of SphK2 and its localization to the ER, where it functions to increase Cer levels, contribute to its apoptogenic effects [8]. Our results imply that the cellular location where S1P is produced determines how SphK1 and SphK2, two closely related enzymes that use the same substrate and generate the same product, have opposite effects on cell survival and opposing functions in regulation of sphingolipid metabolism and thus levels of ceramide [8].

Increases in cytosolic free calcium have been linked to both survival and apoptosis; while calcium is necessary for cell growth and survival, its inappropriate increase may also result in cell death

[66]. Both ER Cer [67] and BH3-only proteins have been connected to the ER-calcium apoptotic pathway [68]. The calcium concentration in the ER ($[Ca^{2+}]_{ER}$) is an important determinant of cellular responses to stress [68] and is regulated by the balance between anti- and pro-apoptotic Bcl-2/BAX family members [69]. Moreover, Cer itself causes the release of calcium from the ER and thus increases both cytosolic and mitochondrial calcium. The reduction of $[Ca^{2+}]_{ER}$ or buffering of cytoplasmic calcium changes can prevent mitochondrial damage and protect cells from Cer-induced apoptosis [67]. S1P may also contribute to regulation of calcium homeostasis in mammalian cells, particularly in response to stress, reminiscent of its function in yeast [70] and plants [71] that do not have S1P receptors.

Interestingly, a mutant *Dictyostelium discoideum* slime mold null for S1P lyase displayed higher viability in stationary growth phase than the wild-type and were resistant to the anti-cancer drug cisplatin [72], suggesting a role for S1P even in this primitive organism [73]. The SphK genes in *D. discoideum* – *sgkA* and *sgkB* – are homologous to those of other species and are biochemically equivalent to human SphK1 and SphK2, respectively. Disruption of *sgkA* and *sgkB* by homologous recombination (both single and double mutants) or overexpression of the SphK1 analogue resulted in altered growth rates while the null mutants showed increased sensitivity to cisplatin, and mutants overexpressing the SphK2 analogue had increased resistance. The increased sensitivity of the SphK2-null mutants was reversed by the addition of S1P, and the increased resistance of SphK1 overexpressors was reversed by DMS suggesting that SphKs might also be good targets for modulating the sensitivity to platinum-based anti-cancer drugs [74].

6. S1P regulates ceramide biosynthesis and apoptosis

Apoptotic Cer can be generated by the activation of acidic or neutral sphingomyelinases in response to numerous apoptotic stimuli [75]. In one example, it was recently shown that the acidic sphingomyelinase (ASMase) inhibitor imipramine or knock out of ASMase inhibited increases in pro-apoptotic C16-Cer and apoptosis induced by TNF-α [76]. Similarly, decreasing Cer levels by overexpression of neutral ceramidase inhibited TNF-α-induced apoptosis. Cer is also increased after induction of the mitochondrial apoptotic pathway as a result of increased *de novo* biosynthesis and leads to downstream activation of caspases and apoptosis [75]. Moreover, there is convincing evidence that Cer synthase and serine palmitoyltransferase are activated during apoptosis in response to certain stimuli [75].

As discussed above, the SPPs are also expressed in the ER and may indirectly regulate Cer and consequently, apoptosis [77–79]. An attractive hypothesis is that S1P formed by SphK1 may normally directly or indirectly act as a negative regulator of serine palmitoyltransferase or Cer synthase [8]. It is intriguing to speculate that this negative regulation of Cer biosynthesis by S1P might also be the mechanism for its intracellular pro-survival effects and its ability to suppress exogenous Cer-mediated apoptosis.

7. Sphingosine kinases, S1P and physiological functions

The role of S1P in various physiological processes is under intense investigation using pharmacological approaches, including treatments with S1P, S1PR agonists or antagonists, or SphK inhibitors. Some examples are discussed below. To date, in vitro studies have established: (1) the plasticity and adaptability of expression of S1P receptors (S1PRs) by cells as a function of activation, and (2) the role of opposing signals from different S1P receptors as a mechanism for fine tuning the effects of S1P. Additionally, the development of knock-out mice for specific S1P receptors has been a milestone in understanding the physiological functions of S1P.

7.1. Cancer-promoting roles of S1P

Overexpression of SphK1 in NIH 3T3 fibroblasts resulted in the acquisition of a transformed phenotype and tumor formation in nude mice, demonstrating the oncogenic potential of this enzyme [56]. Moreover, S1P promotes estrogen-dependent tumorigenesis of MCF-7 human breast cancer cells [57] and stimulates invasiveness of human glioblastoma cells [80]. We recently showed that endogenous SphK1 regulates motility, growth, and chemoresistance of MCF-7 cells [32], whereas both SphK1 and SphK2 are involved in EGF-mediated activation and migration of MDA-MB-453 breast cancer cells [22]. These results suggest that SphKs/S1P might be critical for growth, metastasis and chemoresistance of human breast cancers.

7.2. S1P in vasculogenesis and angiogenesis

S1P promotes endothelial cell growth, thereby promoting blood vessel formation through interaction with signaling by VEGF, a pivotal angiogenic growth factor [81]. VEGF stimulated SphK1 in T24 bladder tumor cells, which, in turn mediated VEGF-induced activation of Ras and MAPKs [61]. In an *ex vivo* model of angiogenesis, as well as in *in vivo* studies, a synergistic effect of S1P with other angiogenic factors such as bFGF and stem cell factor was observed on vascular sprouting, proliferation and tube formation during vasculogenesis and *de novo* vascularization [82,83]. Moreover, $S1P_1$ null mice die in utero between embryonic days 13.5 and 14.5 due to a defect in vascular stabilization [84]. Indeed, Chae et al. documented that $S1P_1$ receptor expression is also induced in angiogenic vessels *in vivo* [85]. Recent elegant studies by Proia and colleagues utilizing SphK1–SphK2 double knockout mice demonstrated an essential role for S1P in neural and vascular development [86]. These observations suggests that S1P should be included in a growing list of signaling molecules, such as VEGF, which regulate the functionally intertwined pathways of angiogenesis and neurogenesis [86].

7.3. S1P and atherogenesis

Plasma and serum concentrations of S1P have been estimated to be between 200 and 900 nM. S1P is mainly distributed in the lipoprotein fraction, with a rank order of high-density lipo-

protein (HDL) fraction, more than in the low-density lipoprotein (LDL), lower in very low-density lipoprotein (VLDL), and the lowest content associated with the albumin fraction [87]. Lipoproteins are therefore the major carriers of circulating S1P, perhaps protecting it from enzymatic degradation, cell uptake, and continuous ligation of S1P receptors on cells exposed to plasma or blood. S1P in serum is mainly released from stores in activated platelets [88], and when released can interact with endothelial cells thus playing a role in endothelial cell migration, proliferation and angiogenesis. It is noteworthy that other circulating cells, such as erythrocytes, neutrophils and mononuclear cells have relatively high SphK activity but only constitutively release small amounts of S1P [89]. HDL-associated S1P has cytoprotective effects, counteracting ox-LDL-triggered cytotoxicity, suggesting that it may have anti-atherogenic properties [90]. Increased permeability of the endothelium to plasma proteins such as LDL is one of the initiating steps of atherogenesis and S1P normally functions through $S1P_1$ and $S1P_3$ receptors to decrease the permeability of the endothelium, acting through both Rho and Rac [91,92]. In vascular smooth muscle cells, S1P stimulates DNA synthesis in association with ERK activation, and may play a central role in excessive fibroproliferative and inflammatory responses to vascular injury that are hallmarks of atherosclerosis progression [93,94]. In addition, platelet-derived S1P may contribute to chemotaxis of primary hematopoietic stem cells to tissue repair sites subsequent to myocardial infarction and other diseases [95]. Evidence for a central role of $S1P_1$ in VSMC migration was established by the phenotype of $S1P_1$-deficient mice in which VSMCs do not migrate properly to surround and reinforce nascent vessels [84]. Moreover, S1P signaling through endothelial $S1P_1$ protects the vasculature against TNF-α-mediated monocyte-EC interactions *in vivo*, suggesting that $S1P_1$ agonists may be efficacious in atherosclerosis [96].

7.4. S1P in modulation of vascular barrier integrity

Acute lung injury (ALI), acute respiratory distress syndrome (ARDS), and multiple organ system failure are common consequences of pulmonary and systemic insults. The lung pathologies are characterized by early sequestration of neutrophils (polymorphonuclear cells or PMN) in the pulmonary microvasculature, followed by adhesion and migration [97]. Upon activation of PMN, S1P is rapidly synthesized by SphK, which, in turn, acts to mediate store-operated calcium entry [98,99]. Using a rat trauma/hemorrhagic shock model, it has been demonstrated that pre-shock inhibition of SphK with DMS decreased PMN activation both *in vitro* and *in vivo* [100]. To investigate the efficacy of S1P as a potential therapeutic avenue for vascular barrier dysfunction, experiments were conducted using isolated perfused murine lung models and bacterial endotoxin (LPS)-induced ALI in intact animals [101]. In these studies, intravenous S1P infusion resulted in rapid and significant reduction of lung weight gain and significantly reduced microvascular permeability and inflammation, consistent with systemic barrier enhancement. Interestingly, the S1P analog FTY720 also produced profound barrier enhancement and reduced lung inflammation [102]. Mechanistically, S1P induces cortical reorganization of the endothelial cytoskeleton and the distribution, assembly and stabilization of adherens junctions and focal adhesion complexes on the cell membrane *via* S1P receptor signaling and activation of Rac [103].

Activation of the complement system and production of anaphylatoxin C5a is associated with a variety of pathologies, including septic shock and ARDS, and in immune complex-dependent diseases, such as rheumatoid arthritis. C5a regulates inflammatory functions through its receptor C5aR (CD88) and increases intracellular calcium, degranulation, cytokine generation and chemotaxis. Importantly, C5a also stimulates SphK1 and its membrane translocation and generation of S1P and downregulation of SphK1 suppressed these effects of C5a [33]. Complementary *in vivo* data demonstrated that pretreating mice with DMS to inhibit SphK1 prior to C5a injection, prevented C5a-triggered neutropenia, increases in serum levels of TNF-α and IL-6, as well as neutrophil and monocyte infiltration [104].

7.5. S1P, inflammation, and immunity

Profound effects of S1P have been described on multiple aspects of differentiation, survival, migration, tissue homing, and effector functions of mononuclear phagocytes and T and B lymphocytes [105]. S1P and its $S1P_1$ receptor control emigration of thymocytes into the blood and the recirculation and tissue distribution of T and B cells exerting direct chemotactic effects. The same ligand/receptor system controls recirculation of naïve lymphocytes and S1P as well as $S1P_1$ agonists induce downregulation of $S1P_1$, altering the tissue distribution of effector lymphocytes [105]. Activation of T and B lymphocytes by various immunological stimuli also suppresses expression of $S1P_1$ and $S1P_4$ with a subsequent significant decrease of receptor-associated signaling [106,107]. The selective effect of S1P on T cell recruitment renders S1P a possible mediator to target, in combination with immunosuppressive agents aiming at T cell activation.

The specificity and the effectiveness of the control exerted by S1P on lymphocyte trafficking are determined by the local concentration of S1P. Typically, concentration gradients of S1P ranging from 3 to 30 nM induce chemotaxis of thymocytes, T and B lymphocytes, and presumably thymocyte emigration and lymphocyte movement from lymph nodes back into efferent lymph and then to the blood [108]. Developmental upregulation of $S1P_1$ expression in the thymus has been reported [105,109]. Expression of $S1P_1$ is first observed on $CD4^+CD8^+$ thymocytes, reaching the highest level of expression on single positive cells, which become ready to exit the thymus [109]. Indeed, lymphocyte-specific $S1P_1$ knockout mice display medullary hyperplasia of otherwise normal thymocytes [110]. Interactions between chemokine signaling and S1P receptor signaling are still undefined, but may be of importance for driving T cells into peripheral lymph nodes as splenic and lymph node T cells have distinct requirements for, and responses to both chemokines and S1P [111].

FTY720 is a novel immunosuppressive agent that is active in various models of graft versus host disease and autoimmunity and appears to be beneficial for treatment of multiple sclerosis [112]. FTY720 is a sphingosine analog, which after phosphorylation in vivo by SphK2 regulates lymphocyte trafficking through its interaction with $S1P_1$ [112,113]. FTY720-phosphate is a true agonist with nanomolar potency for all of the S1P receptors, except $S1P_2$ [102]. SphK1 null mice, but not SphK2 knockouts, become lymphopenic upon administration of FYT720 [110,114], substantiating previous results showing that FTY720 was a much better substrate for SphK2 than SphK1 [18]. The recent finding that FTY720 can also severely hamper migration of dendritic cells to the lymph nodes suggests additional mechanisms underlying the immunosuppressive effects of this sphingosine analog [115].

7.6. S1P and asthma

Mast cells play pivotal roles in immediate-type and inflammatory allergic reactions that can result in asthma a disease of chronic airway inflammation. Cross-linking of the high-affinity receptor for IgE (FcepsilonRI) on these cells leads to degranulation and the release of histamine and other preformed mediators, chemokines and cytokines, as well as eicosanoids (leukotrienes and prostaglandins), and S1P [116]. It has previously been suggested that the decisive balance between sphingosine and S1P determines the allergic responsiveness of mast cells [117]. Whereas sphingosine inhibits antigen-mediated leukotriene synthesis and cytokine production by preventing activation of ERK1/2, S1P activates it, stimulating degranulation and cytokine production [117]. S1P formed after antigenic stimulation is able to rapidly bind and activate its receptors $S1P_1$ and $S1P_2$ present on mast cells [24,118]. $S1P_1$ induces cytoskeletal rearrangements, leading to the movement of mast cells towards an antigen gradient, whereas transactivation of $S1P_2$ enhances the degranulation response. Thus, we have suggested that low antigen concentrations can attract mast cells to the site of action via $S1P_1$, and, as mast cells approach higher concentrations of antigen, a shift in the expression of S1P receptors (enhanced $S1P_2$ expression) resolves migration while promoting degranulation, a process known to require a stronger stimulus [24].

SphK1 translocates to the plasma membrane within minutes of FcεpsilonRI clustering [24,119]. SphK1 interacts with Lyn and Fyn, FcεpsilonRI proximal kinases that initiate the signaling events following cross-linking of this receptor [48]. Recently, a cooperative role between Fyn and Lyn kinases in the activation of both SphK1 and SphK2 has been demonstrated, which contributes to mast cell responses [120]. Intracellular S1P is also thought to mobilize calcium from intracellular stores [27,119], independently of phospholipase C (PLC) activation and inositol 1,4,5-trisphosphate ($InsP_3$) generation, and this is necessary for mast cell degranulation.

Because expression of MCP-1, MIP1-α, MIP1-β, MIP2 (belonging to the CC-chemokine family), and MIF, all important modulators of monocyte and eosinophil recruitment and inflammation, were significantly increased by S1P in mast cells [24], it is also possible that secretion of S1P by mast cells can also promote inflammation by activating and recruiting other immune cells involved in allergic and inflammatory responses. S1P also profoundly affects endothelial cell function and promotes adhesion molecule expression in endothelial cells [49,92], induces contraction and proliferation of smooth muscle cells and stimulates IL-6 production in airway smooth muscle cells [121], and shifts maturing dendritic cell-induced polarization of T cells into a Th2 phenotype [122]. Because its levels are elevated in the broncholavage fluid of asthmatic individuals after Ag challenge, secretion of S1P by mast cells is of great relevance in inflammatory responses and asthma [24,121].

7.7. S1P and the reproductive system

While not yet extensively studied, evidence has begun to accumulate that S1P also has important functions in male and female reproductive systems. $S1P_1$ receptors are constitutively expressed in the seminiferous tubules of the testes but not on primary spermatocytes or Sertoli cells [123]. S1P can provide protection against radiation-induced male germ cell loss, evoking a role for S1P in the survival of male germ cells [124]. Similarly, S1P administration prevents oocyte apoptosis and infertility subsequent to cytotoxic chemotherapy treatment in females [125]. Such studies open new avenues of clinical relevance for the control of ovarian germ cell dynamics and potential management of ovarian failure in women, as well as for preservation of ovarian function in female cancer patients.

7.8. S1P and the central nervous system

In vitro studies as well as gene knockouts have begun to elucidate important functions for S1P (and ceramide) in neurons and in glial cells. In neuronal cultures, S1P acts through two S1PRs, $S1P_2$ and $S1P_5$, to regulate neurite retraction and soma rounding [126]. In the CNS, S1P receptor expression is regulated throughout development, with a specific distribution profile within the brain [126]. $S1P_5$ expressed by oligodendrocytes might play a role in normal myelination and in demyelinating diseases, such as multiple sclerosis, as its expression is restricted to oligodendrocytes throughout development from immature stages to myelin-forming cells [127]. Studies from Sato-Bigbee's lab demonstrated that neurotrophin (NT)-3 is a potent activator of the transcription factor CREB [128,129] and more recently, that CREB phosphorylation is stimulated by S1P in oligodendrocyte progenitors, demonstrating crosstalk between NT-3 and S1P signaling [130]. It has been difficult to ascertain the importance of S1P production in the brain since neither SphK1 nor SphK2 knockout mice have any obvious CNS phenotypes, likely due to complementation since the S1P levels are not seriously compromised and knockout of both SphKs results in a severe brain developmental defect [86]. The SphK1/SphK2 double knockout is embryonically lethal and embryos have no S1P and exhibit severely disturbed neurogenesis, including neural tube closure, and angiogenesis. A dramatic increase in apoptosis and a decrease in mitosis were seen in the

developing nervous system. $S1P_1$ receptor-null mice also showed severe defects in neurogenesis [86]. The neural defects of increased cell death and decreased proliferation in the neuroepithelium of almost all brain regions, particularly in the telencephalon, that correlated with thinning of the neuroepithelial layer, was much more severe in the double SphK knockout embryos compared to $S1P_1$ knockout embryos. Moreover, in contrast to SphK1/SphK2 null embryos, neural tube defects were not observed in $S1P_1$ null embryos. These results suggest that S1P may also have intracellular function to regulate cell survival independent of $S1P_1$. Alternatively, other S1P receptors might be important as it is known that functional S1P receptors are expressed in the embryonic brain in areas where active neurogenesis takes place [131]. Of note, S1P can be secreted by astroglial cells [132] and by cells from the cerebellum [133] and thus might function in an autocrine and/or paracrine manner within the CNS.

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