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Author(s)	Kawabori, Masahito; Kacimi, Rachid; Karliner, Joel S; Yenari, Midori A
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Sphingolipids in cardiovascular and cerebrovascular systems: Pathological implications and potential therapeutic targets

Masahito Kawabori, Rachid Kacimi, Joel S Karliner, Midori A Yenari

Masahito Kawabori, Rachid Kacimi, Midori A Yenari, Department of Neurology, San Francisco and San Francisco Veterans Affairs Medical Center, University of California, San Francisco, CA 94121, United States

Joel S Karliner, Department of Cardiology, VA Medical Center, University of California San Francisco, San Francisco, CA 94121, United States

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Correspondence to: Midori A Yenari, MD, Department of Neurology, San Francisco and San Francisco Veterans Affairs Medical Center, University of California, Neurology (127) VAMC 4150 Clement Street, San Francisco, CA 94121, United States. yenari@alum.mit.edu

Telephone: +1- 415-7502011 Fax: +1- 415-7502273

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Abstract

The sphingolipid metabolites ceramide, sphingosine, and sphingosine-1-phosphate (S1P) and its enzyme sphingosine kinase (SphK) play an important role in the regulation of cell proliferation, survival, inflammation, and cell death. Ceramide and sphingosine usually inhibit proliferation and promote apoptosis, while its metabolite S1P phosphorylated by SphK stimulates growth and suppresses apoptosis. Because these metabolites are interconvertible, it has been proposed that it is not the absolute amounts of these metabo-

lites but rather their relative levels that determine cell fate. The relevance of this "sphingolipid rheostat" and its role in regulating cell fate has been borne out by work in many labs using many different cell types and experimental manipulations. A central finding of these studies is that SphK is a critical regulator of the sphingolipid rheostat, as it not only produces the pro-growth, anti-apoptotic messenger S1P, but also decreases levels of pro-apoptotic ceramide and sphingosine. Activation of bioactive sphingolipid S1P signaling has emerged as a critical protective pathway in response to acute ischemic injury in both cardiac and cerebrovascular disease, and these observations have considerable relevance for future potential therapeutic targets.

Key words: Sphingolipids; Sphingosine-1-phosphate; Sphingosine kinase; Ceramide kinase

Core tip: The sphingolipid pathway has received considerable attention recently, because its active metabolites appear to have salutary effects on cytoprotection in experimental cardiac and cerebral ischemia. Both inhibitors and antagonists of the sphingolipid sphingosine-1-phosphate (S1P) pathway appear to limit ischemic injury through a variety of mechanisms. Because of the clinical availability of Fingolimod (FTY720), a S1P analog, for use in multiple sclerosis, preclinical and clinical studies should focus on the development of this and similar pharmaceuticals for a new indication.

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INTRODUCTION

Sphingolipids were first described in late 19th century and have long been viewed as merely ubiquitous components of the cell membrane. Recently, sphingolipids have been increasingly reevaluated because they are now recognized to not only regulate vital cell functions, but also form cell membrane microdomain “lipid rafts” for integrating cell signaling^[1,2]. Sphingolipids are formed *via* the metabolism of sphingomyelin, a ubiquitous constituent of the plasma membrane, or by *de novo* synthesis. Enzymatic pathways result in the formation of several different lipid mediators such as ceramide, sphingosine, and sphingosine-1-phosphate (S1P). Several studies now showed that these sphingolipid mediators and their enzymes, especially sphingosine kinase (SphK), are likely to have an integral role in different cell processes including proliferation, inflammation, apoptosis and migration. The mode of action of each sphingolipid is different. A significant body of research now indicates that sphingolipids are intimately involved in disease progression and that these lipids, together with associated enzymes and receptors, can provide effective drug targets for the treatment of pathological states. This review will highlight the current knowledge of research where sphingolipids are involved with focus on cardiovascular and cerebrovascular disease, and the mechanisms of action of each sphingolipid mediator. In addition, the therapeutic potential of drugs that alter sphingolipid actions with focus on SphK/S1P signaling pathway that appears to be a target of interest for therapeutic manipulation.

METABOLISM AND SIGNALING PATHWAYS OF SPHINGOLIPIDS

Sphingolipids are complex lipids comprised of a sphingoid base, and are one of the major lipid components of cell membrane as well as glycerophospholipid and cholesterol. A schematic diagram of sphingolipid metabolism is depicted in Figure 1. *De novo* biosynthesis of sphingolipids begins with the conversion of serine and palmitoyl-CoA into 3-ketosphinganine. 3-ketosphinganine is then converted to dihydrosphingosine. Dihydroceramide synthase acrylates dihydrosphingosine to form dihydroceramide, which is then reduced to ceramide by dihydroceramide desaturase. Sphingomyelin can also be converted to ceramide by sphingomyelin synthase, and a reverse reaction is catalyzed by sphingomyelinase. Ceramide can also be degraded by ceramidase to form sphingosine, which can, in turn, be phosphorylated to S1P by two enzymes SphK1 or 2. The bioactive lipid ceramide-1-phosphate (C1P) is formed by phosphorylation of ceramide by ceramide kinase; it can also be reverted to ceramide by ceramide phosphatase. The reverse reaction from S1P is catalyzed by sphingosine-1-phosphate phosphatases and ceramide synthase that yield sphingosine and ceramide respectively^[3]. S1P can be further metabolized by S1P lyase, yielding hexadecenal and ethanolamine phosphate^[4].

Ceramide

Ceramides are a family of lipids that consist of sphingosine covalently linked to a fatty acid and are densely located at the cell membrane. Ceramides are the key component lipids that constitute sphingomyelin, the major source of the human sphingolipids, and one of the major components which form the phospholipid bilayer^[5]. Discovery over the last few decades reveal that all stress stimuli, such as inflammatory mediators, heat, ultraviolet radiation, hypoxia, chemotherapeutics, and oxidative stress increase ceramide production as part of an evolutionarily conserved cellular response^[6-13] and toll like receptor 4 seems to be involved in ceramide synthesis^[14]. Consecutively ceramides not only promote cell cycle arrest and promote apoptosis, a form of programmed cell death, but also play an important role in the regulation of autophagy, cell differentiation, and inflammatory responses^[9,15-18]. Ceramide is also involved in dephosphorylation and inactivation of one major mediator of cell survival; protein kinase Akt, (Akt/PKB)^[19-21]. On the other hand, recent data shows that phosphorylated ceramide, C1P seems to have the opposite effects from ceramide; by inducing prosurvival functions, such as cell growth and survival, control of inflammation and mediation of macrophage migration^[22-24].

Sphingosine

Sphingosine is also a bioactive sphingolipid formed from ceramide as a result of ceramidase activity. It was first described as the physiological inhibitor of the survival signal protein kinase C (PKC), and was also found to up-regulate caspase 3 in the cascade of apoptosis^[25-28]. There are many reports showing that PKC is inhibited by exogenous sphingosine, and it has been demonstrated that endogenously generated sphingosine is a potent PKC inhibitor^[29]. In turn, sphingosine can control the activity of other key enzymes involved in the regulation of metabolic or cell signaling pathways such as the Mg²⁺ dependent form of phosphatidate phosphohydrolase^[30,31], phospholipase D (PLD)^[32], or diacylglycerol kinase^[33,34]. Although there is abundant evidence that sphingosine is toxic to cells^[25,28], diverse function by concentration dependence of sphingosine has been reported. Vessey *et al.*^[35] recently reported that at lower dose (submicromolar), a more physiologic concentrations, sphingosine has been shown to be cardioprotective in isolated Langendorff-perfused rat hearts subjected to ischemia/reperfusion injury. Unlike S1P, sphingosine-induced cardioprotection seems to be mediated by cyclic nucleotide-dependent protein kinase A and G (PKA and PKG) pathways^[35]. While, at the higher concentrations usually employed, sphingosine is toxic to cells^[27].

S1P

S1P is a bioactive lipid signaling molecule formed when either one of two isoforms of the enzyme SphK1 or 2 catalyzes the addition of a phosphate group to sphingosine. S1P exerts a wide variety of biological activities in

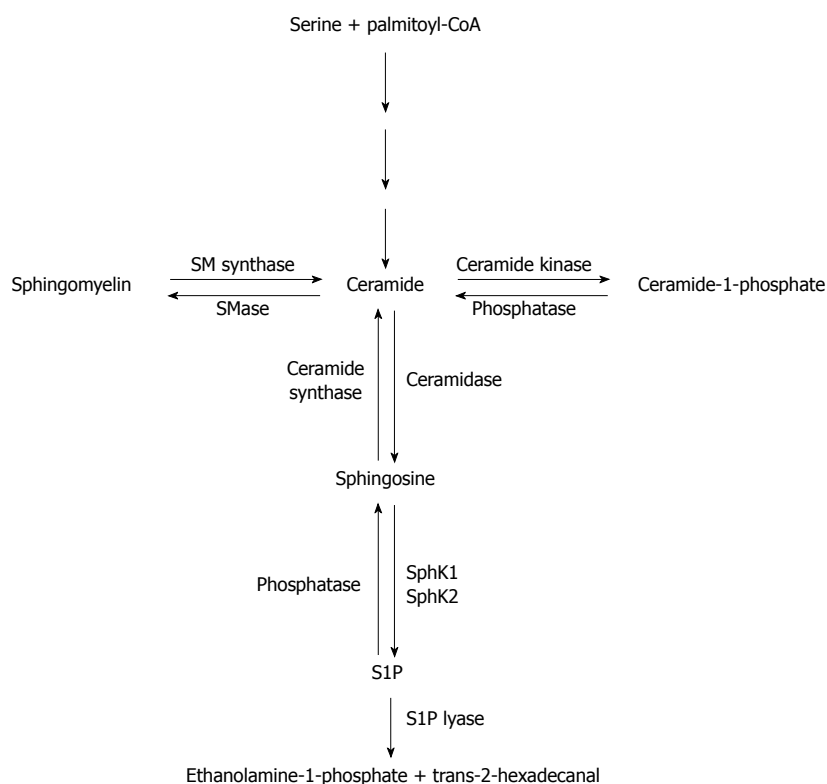


Figure 1 Schematic outline of sphingolipid metabolism. Names of the major intermediates and abbreviations of the enzymes involved are included. SM: Sphingomyelin; SphK: Sphingosine kinase; S1P: Sphingosine-1-phosphate.

many eukaryotic cell types^[36-38]. It was initially proposed to act as an intracellular second messenger, based on the ability of extracellular growth factors to activate SphK and increase intracellular S1P levels. The discovery and cloning of five G protein-coupled receptors (S1P₁, S1P₂, S1P₃, S1P₄, S1P₅) expressed on the cell membrane has stimulated the notion that S1P is an extracellular signaling ligand, regulating a host of cellular functions such as proliferation, survival, immunomodulation, apoptosis, migration, cytoskeletal organization, and differentiation/morphogenesis^[39]. Basal plasma and serum concentration levels of S1P are generally low ranging within 200-900 nmol/L, but can increase rapidly and transiently when cells are exposed to various agonists^[40,41]. The concentration of S1P is controlled by two enzyme, SphK and S1P lyase. While SphK activity can be upregulated by a variety of growth factors, S1P lyase activity in other hand is constantly at the high level, and this makes the intracellular S1P level very low in most tissues. However, erythrocytes and platelets have low S1P lyase activity resulting in high S1P concentration in blood plasma^[38,42]. This concentration gradient is presumed to provide the basis for the integral role for the bioactivity of S1P involved in lymphocyte trafficking^[43].

After the discovery of S1P receptors, there has been extensive work aimed at understanding the role of S1P as extracellular ligands. A schematic of the S1P receptors are shown in Figure 2. S1P mediates its effects through binding to G protein-coupled receptors (S1P₁₋₅) which

activates a variety of signaling *via* transduction of G proteins isoforms (G_s, G_i, G_q, and G_{12/13}). The prosurvival phosphatidylinositol-3-kinase (PI3K)/Akt have been shown to be downstream molecules regulated by the S1P₁ receptor signaling, Akt activation is a principal factor in the prevention of apoptosis^[44,45]. S1P also stimulates cell growth and proliferation *via* activation of mitogen-activated protein kinase extracellular signal-regulated kinases (ERK)^[46]. It is believed that elevated ERK phosphorylation plays a role in cell survival and proliferation in the penumbra, and ERK activity may block apoptosis by enhancing the level of the antiapoptotic protein Bcl-2 through cAMP responsive element binding protein activation^[44]. S1P is also assumed to prevent necrosis mediated by the PKC ϵ pathway^[47].

Sphingosine kinase

The synthesis of S1P is catalyzed by SphK which is responsible for linking a phosphate group to sphingosine. There are two isozymes of SphK designated as SphK1 and SphK2. SphK1 and SphK2 show different subcellular localizations and enzymatic properties as well as different expression in various tissues. Mouse and human SphK1 exhibit substantial homology and SphK2 is highly homologous to SphK1 except for 240 additional amino acids located at the N terminus and in the center of the enzyme. The genes encoding these isozymes are localized on different chromosomes^[48]. Genetic deletion of both isozymes results in fetal death from severe bleeding,

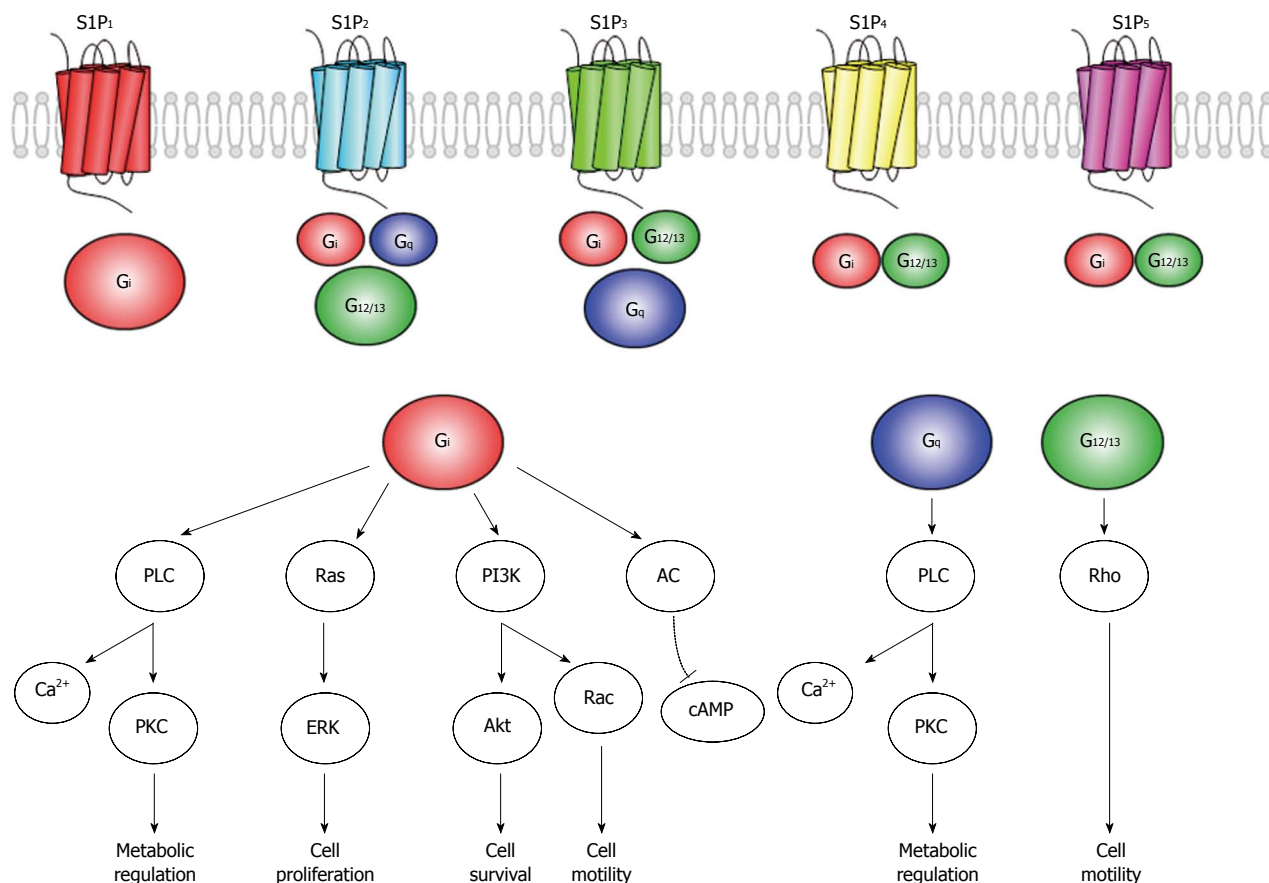


Figure 2 Schematic outline of sphingosine-1-phosphate signaling through receptors. S1P: Sphingosine-1-phosphate; ERK: Extracellular signal-regulated kinases; PKC: Protein kinase C; PLC: Phospholipase C.

inadequate vasculogenesis, and incomplete neural tube closure^[48,49]. In contrast, mice null for either the SphK1 or the SphK2 isozyme exhibit normal development and are otherwise unremarkable in the basal state^[49]. It is presumed that these isozymes have the complementary functions. The regulation of SphK activity is complex. It is stimulated by G-protein coupled receptor agonists (muscarinic receptor agonists^[50], formyl peptide^[51], nucleotides, bradykinin^[52], lysophosphatidic acid^[53], and S1P^[54]), agonists at receptor tyrosine kinases (platelet-derived growth factor^[55], endothelial growth factor^[56], nerve growth factor^[57], fibroblast growth factor^[57], vascular endothelial growth factor^[58]), immunoglobulin receptor crosslinking^[59], monoganglioside (GM1)^[60], estrogen^[61] and activators of PKCε^[62]. Both TNF-α and phorbol ester, which stimulates PKC, phosphorylate and thus activate SphK1 at serine225 mediated by ERK1/2^[63,64]. The tumor necrosis factor (TNF) α response requires binding by TNF receptor-associated factor-2 (TRAF2). Other interacting proteins that stimulate SphK include delta-catenin/NPRAP (neural plakophilin-related armadillo repeat protein), aminoacylase 1, and eukaryotic elongation factor 1A (EEFA1)^[65]. Reported inhibitory interacting proteins are SKIP (SK1-interacting protein), PECAM-1 (platelet endothelial adhesion molecule-1), and FHL-2 suppressed VEGF-induced PI-3 kinase/Akt activation *via*

interactions with SphK1^[66-69].

Despite the structural similarities and even though it catalyses the production of S1P, SphK2 has shown to have opposing actions to SphK1^[70]. Thus, SphK2 inhibits cell growth and enhances apoptosis, in part by regulating ceramide levels^[70]. Downregulation of SphK2 reduced conversion of sphingosine to ceramide, while downregulation of SphK1 increased it. The pathway of sphingosine into pro-apoptotic ceramide is dependent on SphK2, but not SphK1, acting in concert with S1P phosphohydrolase 1^[71]. And also interestingly, there are organ specific deviations of SphK. While SphK1 is the more abundant isozyme in lung, spleen, kidney, heart, renal proximal tubules and cardiomyocytes, SphK2 isozyme predominates in the brain^[72-74]. The intracellular locations of two enzymes are also different. SphK1 is localized predominantly in the cytoplasm while, SphK2 is mainly localized in the nucleus^[75]. The reason of this deviation difference is that SphK1 has two functional nuclear export signal (NES) sequences which positively direct SphK1 to an extranuclear location. On the other hand, a nuclear localization signal (NLS) sequence has been found in SphK2 which keeps SphK2 in the nucleus and NES shuttles SphK2 between the cytoplasm and the nucleus according to demand^[75]. The underlying reasons for this and its functions have yet to be elucidated.

ROLE OF SPHINGOLIPIDS IN CARDIOVASCULAR DISEASE

S1P in cardioprotection

The cardioprotective effect of S1P was first reported in 2001^[76]. In neonatal rat cardiac myocytes, exogenously applied S1P enhanced cardiac myocyte survival during hypoxia^[76]. Subsequent studies were undertaken using cultured adult mouse cardiac myocytes subjected to hypoxia *in vitro* that mimics ischemia *in vivo* during coronary artery occlusion. This system permitted measurements of S1P effects on myocyte viability during stress and activation of cell signaling from plasma membrane to mitochondria. There were three major findings that advanced understanding of S1P prosurvival effects during hypoxia^[77]. First, it was found that S1P₁ receptors are abundantly expressed by adult mouse cardiac myocytes^[78]. Second, exogenously applied S1P enhanced survival during prolonged *in vitro* hypoxia through mechanisms that required S1P₁ receptor function and G protein G_i-independent activation of the prosurvival kinase Akt/PKB. Finally, Akt-mediated phosphorylation of myocyte substrates that interact with mitochondria, such as GSK-3 and BAD, contributed to cardioprotection. In these studies the selective S1P₁ receptor agonist SEW2871 and the S1P analog FTY720 were as effective as S1P in preserving myocytes viability during hypoxia^[77]. In contrast, Means *et al.*^[79] were unable to demonstrate prosurvival signaling mediated by the S1P₁ receptor. The divergent observations surrounding the cardioprotective effects of S1P₁ agonism may result from methodologic differences. Even though, these data strongly suggest that the S1P₁ receptor, which is the most abundant S1P receptor subtype in cardiac myocytes, is at least partially responsible for S1P-mediated prosurvival signaling and for maintaining myocytes viability during hypoxia^[77], and during hypoxia/reoxygenation^[37]. In a study of the other receptors, it was shown that combined deletion of S1P₂ and S1P₃ receptors augmented infarct size in mice subjected to ischemia/reperfusion injury^[80]. In these hearts, activation of Akt was markedly attenuated compared to wildtype mice, but the absence of either receptor subtype alone affected neither infarct size nor Akt activation after ischemia/reperfusion injury. S1P augmented Akt activity in control murine myocytes, but was not effective in the double knockout cells^[80]. Thus, these observations suggest that the less abundant cardiac myocyte S1P receptors (S1P₂ and S1P₃) may also be necessary for cell survival during ischemia/reperfusion injury.

In SphK1 null ventricular myocytes subjected to *in vitro* hypoxia, cell death and cytochrome c release were greater than in wild-type controls^[36]. Exogenous S1P enhanced survival of both wild-type and SphK1 null cells. GM-1 treatment, which activates PKC domain and subsequently upregulates SphK to produce S1P, induced cytoprotection in wild-type cardiac myocytes but not in SphK1 null cells. These observations indicate that GM-1 activates SphK1, presumably *via* PKC ϵ -mediated phos-

phorylation. Interestingly, the beneficial effects of GM-1 on wild-type cardiac myocytes were abolished by pretreatment with either an S1P₁ receptor antagonist or pertussis toxin, which ADP-ribosylates and thereby inactivates G_i, suggesting that endogenous S1P was transported to the extracellular space for activation of its cognate G-protein coupled receptors^[36]. A potential mechanism for extrusion of S1P is *via* ABC transporters, which have been demonstrated in a variety of cell types^[81,82], as well as in murine and human hearts^[83,84]. Recently, a specific S1P transporter, SPN2, which also transports the phosphorylated form of FTY720, has also been described^[85]. Knapp *et al.*^[86] also mentioned the importance of the S1P/ceramide levels ratio which could be responsible for increased apoptosis in the myocardial infarction in the rat.

Sphingosine kinase in cardioprotection

As noted above, GM-1 enhanced the survival of cardiac fibroblasts subjected either to PKC inhibition or to C2-ceramide (N-acetyl-sphingoid bases) treatment^[62]. GM-1 also increased S1P levels, an effect abrogated by the SphK inhibitor, DMS^[62]. Using isolated adult mouse hearts, exogenous S1P and GM-1 separately induced substantial resistance to ischemia-reperfusion injury in wild-type mice^[87]. Similar experiments were reported by Lecour *et al.*^[88] in isolated rat heart. The importance of the prosurvival kinase, PKC ϵ , was emphasized by experiments in which GM-1 proved to be ineffective in PKC ϵ -null hearts. In addition, GM-1, but not exogenous S1P, stimulated translocation of activated PKC ϵ to myocyte particulate fractions^[87]. Nevertheless, exogenously administered S1P was effective both in isolated PKC ϵ -null hearts subjected to ischemia/reperfusion injury^[87] and in isolated cardiac myocytes from these hearts subjected to hypoxia^[36]. Thus, S1P acting at cell surface receptors or activation of intracellular SphK confers cardioprotection during acute ischemia/reperfusion injury. Consistent with this hypothesis, it was shown that PKC ϵ activation is essential for cardioprotection induced by ischemic preconditioning (IPC)^[89]. PKC ϵ peptide agonists mimicked preconditioning effects on contractile recovery and tissue viability in wild-type hearts after prolonged ischemia-reperfusion injury^[90]. In contrast, inducible cardioprotection was blocked by PKC peptide antagonists and targeted deletion of the PKC ϵ gene^[91]. A subsequent series of experiments directly tested the hypothesis that SphK activation mediates IPC in isolated mouse hearts^[90]. It was determined that IPC sufficient to reduce infarction size in wild-type hearts increased SphK localization and activity in tissue membrane fractions. Interestingly, IPC triggered SphK translocation to tissue membrane fractions in PKC ϵ -null hearts but did not enhance enzymatic activity or decrease infarction size after ischemia-reperfusion injury^[90]. As noted above, DMS, the endogenous sphingolipid generated by N-methylation of sphingosine, inhibited tissue SphK activity, while 10 μ mol/L of DMS pretreatment abolished IPC-induced cardioprotection in wild-type hearts^[90]. Sub-

sequent experiments elucidated unpredicted effects of low DMS concentrations on SphK^[92]. In contrast to moderate dose DMS (10 $\mu\text{mol/L}$), low-dose DMS stimulated translocation of activated PKC ϵ to tissue particulate fractions and reduced cardiac ischemia-reperfusion injury. Importantly, low-dose DMS effects were abolished in PKC ϵ -null hearts, and SphK1 was found to co-immunoprecipitate with activated PKC phosphorylated at serine729. Low-dose DMS induced translocation of total Akt from Triton-insoluble fractions to cytosol and increased activated Akt phosphorylated at serine473^[92].

When tested with the classic SphK inhibitor, DMS, the activity of SphK2 was unaffected by concentrations as high as 20 $\mu\text{mol/L}$. Consistent with this observation, DMS was only a partial inhibitor of total cytosolic SphK activity^[93]. Also SphK2 was not inhibited by the sphingosine analogue, FTY720. As noted earlier, SphK1 was efficiently inhibited by both DMS and FTY720. Furthermore, when the cytosolic fraction from SphK1 knockout mouse hearts was tested, residual activity due to SphK2 was not inhibited by DMS or FTY720^[93]. These observations confirmed the specificity of SphK1 inhibition and indicated the lack of inhibition of SphK2 was not an artifact of purification. SphK2 from rat liver and spleen was also not inhibited by DMS. In contrast, l-sphingosine was an effective inhibitor of both forms^[93]. Taken together, along with data obtained in SphK1-null hearts, these observations indicated that DMS inhibits only the SK1 form in the heart. Thus, prior experiments in other cells and tissues in which DMS was used as inhibitor of SphK may require reinterpretation.

The time course of SphK activity in adult rat hearts subjected to ischemia/reperfusion injury and preconditioning has been reported^[94]. Cytosolic SphK activity declined by 61% during ischemia and did not recover upon reperfusion, paralleling the effects on left ventricular developed pressure (LVDP). IPC reduced the decrease in enzyme activity during ischemia by half and, upon reperfusion activity, returned to normal. LVDP recovered to 79% of control values, and infarct size was reduced. The low baseline-specific activity of SphK declined by 67% after 45 min of ischemia and remained at that level during reperfusion. IPC restored SphK activity almost to normal during reperfusion. Parallel effects were observed in mitochondria from the same hearts^[94]. In these experiments^[94], total S1P in cardiac tissue was quantified by liquid chromatography followed by tandem mass spectrometry^[38]. In non-preconditioned hearts, S1P content declined from base line after both ischemia and reperfusion. Preconditioned hearts had higher S1P levels after ischemia/reperfusion relative to control hearts. Treatment of non-preconditioned hearts at reperfusion (pharmacologic postconditioning) with 100 nmol/L of S1P improved recovery of LVDP. Thus, maintenance of SphK activity resulting from higher S1P levels is critical for recovery from ischemia/reperfusion injury. In this connection, the activity of S1P phosphatases and lyase has not been reported during experiments involving isch-

emia/reperfusion injury in the heart.

Despite compelling evidence that DMS modulates resistance to injury by inhibiting SphK1, however, this drug also has been shown to alter other kinases such as PKC activity^[92]. Accordingly, SphK1 knockout mice have been employed in a series of subsequent studies^[95,96]. SphK2 expression increased in hearts after *SphK1* gene disruption, resulting in total SphK activity half that of wild type. Although SphK1-null hearts exhibited normal hemodynamic performance under baseline conditions, contractile abnormalities and infarction were more severe after ischemia/reperfusion than in wild-type hearts^[95]. As predicted, targeted disruption of the *SphK1* gene abolished IPC-induced cardioprotection^[95]. Importantly, when the index ischemia time was reduced from 50 to 40 min, infarct size in the *SK1* knockout hearts declined to the level seen in the wild type hearts subjected to ischemia/reperfusion injury. At this reduced level of injury, IPC was still ineffective in producing cardioprotection in the knockout hearts. However, exogenous S1P retained the ability to induce cardioprotection in these *SphK1*-null hearts. Despite an increase in SphK2 expression in the *SphK1*-null hearts, infusion of DMS did not affect infarct size, confirming prior *in vitro* experiments and suggesting that the absence of SphK1 rather than the increased presence of SphK2 was critical to the loss of cardioprotection in myocardium null for SphK1^[95]. However, Vessey *et al.*^[97] recently demonstrated that myocardial damage is enhanced after ischemia/reperfusion in mice null for SphK2 and that the cardioprotective intervention of preconditioning is abolished by deletion in the *SphK2* gene. These observations are contrary to prior suggestions derived from *in vitro* models that SphK1 and SphK2 drive opposing functions that regulate cell fate^[97].

In another recent study, it was reported that previous adenoviral gene transfer of *SphK1* protected against hemodynamic deterioration and reduced creatine kinase release and arrhythmias during acute ischemia/reperfusion injury in isolated rat hearts^[96]. When gene transfer was performed at the time of acute left anterior descending coronary artery ligation, studies 2 wk later revealed improved left ventricular function in the treated mice, reduced infarct size, more neovascularization, and reduced collagen content^[96].

Like IPC, ischemic postconditioning is cardioprotective^[98], and this observation has recently been extended to patients undergoing percutaneous coronary interventions^[99]. To ascertain whether the SphK/S1P pathway is a determinant of successful postconditioning, isolated wild type and *SphK1*-null mouse hearts were subjected to ischemia/reperfusion injury^[100]. At the onset of reperfusion, hearts selected for treatment underwent 3 brief cycles of postconditioning (5 s of ischemia followed by 5 s of reperfusion). Results were similar to the preconditioning studies cited above: hemodynamics were improved and infarction size reduced compared with untreated hearts^[100]. Phospho-Akt and phospho-ERK were enhanced. None of these findings were present in *SphK1*-

null hearts. Thus, SphK1 is also critical for successful ischemic postconditioning. In this connection, it has recently been found that a ramped ischemic postconditioning protocol combined with low-dose sphingosine + S1P given at the time of reperfusion can rescue isolated hearts from as much as 90 min of ischemia^[101].

ROLE OF SPHINGOLIPIDS IN Cerebrovascular Disease

Distribution and function of S1P and SphK in the brain

While S1P signaling has long been known to mediate protection in peripheral and cardiac ischemia, only recently has this bioactive lipid pathway drawn attention in cerebral ischemia.

S1P has shown many neuroprotective mechanisms in both *in vitro* and *in vivo*. S1P is presumed to protect central nervous system through many different ways^[102,103]. In addition to the above mentioned prosurvival effect of S1P, S1P may also protect the brain vasculature by reducing leukocyte adhesion secondary to altering endothelial adhesion molecule expression and preventing endothelial apoptosis through Bcl-2 activation. There is also evidence that S1P may act as a proximal trigger of cerebroprotection (both neuronal and vascular) through activation of signaling molecules such as endothelial nitric oxide synthase^[102].

In models of stroke, Kimura *et al.*^[104] found that S1P concentrations in the brain were significantly decreased 3 d after ischemia. However, S1P in the brain was increased thereafter and reached a maximum 14 d after the insult. Upregulation of S1P was observed at the infarct border zone and at the infarct core, and mostly colocalized to microglia and some astrocytes, indicating that microglia may be the main source of S1P production in ischemic brain^[104].

Moreover, the S1P regulating enzymes SphKs show differential tissue expression patterns and different subcellular localization^[72]. Although SphK1 has greater expression and activity than SphK2 in many organs such as lung and spleen, SphK2 expression levels are greater than SphK1 in the brain, suggesting a more prominent physiological role for SphK2 in the brain and brain vasculature^[73,74]. Among brain resident cells, primary glial cells express more SphK2 mRNA than primary neurons, and the highest mRNA concentrations were found in cortex, while mRNA was least abundant in striatum^[74]. Increased SphK2 was observed in response to cerebral ischemia both *in vitro* and *in vivo*^[74]. As mentioned earlier, SphK2 could promote apoptosis, instead of cell survival as SphK1 shows at the non-central nervous system^[70], there is also accumulated evidence that SphK2 could play an important role as a prosurvival factor in the central nervous system^[73,105,106].

A widely used anesthetic agent isoflurane is now considered to be one of the promising therapeutic strategies for many neurological diseases including ischemic stroke^[107]. Zhou *et al.*^[107] reported that isoflurane given post injury attenuated brain damage after subarachnoid hemorrhage, and that the neuroprotective effect was

associated with decreased neuronal apoptosis partly through the antiapoptotic effect of sphingosine-related pathway activation including SphK1 and S1P_{1,3} receptors. Isoflurane-mediated neuroprotection has also been examined during neonatal hypoxia ischemia through the S1P/PI3K/Akt signaling. The PI3K/Akt signaling cascade has been shown to play a key role in preventing apoptosis under hypoxic or ischemic conditions. Hypoxic preconditioning (HPC) has also been investigated in the context of cerebral ischemia^[105,108]. With respect to elucidating the molecular basis of preconditioning-induced tolerance, Yung *et al.*^[106] showed that hypoxic preconditioning significantly reduced infarct volume and improved neurological outcome in wild-type and SphK1^{-/-}, but not in SphK2^{-/-} mice. Wacker *et al.*^[105,108] also documented HPC-induced ischemic tolerance and the concomitant protection of the blood-brain-barrier depended on SphK2 signaling. SphK2-generated S1P participates in both the normal maintenance of occlusion at cytoskeletally linked cell junctions, as well as the mediation of HPC-induced increases in the expression of claudin-5 and VE-cadherin at these junctions, which may be compulsory for induction of the vasculoprotective phenotype by HPC. The present data demonstrates that SK2 is a universal mediator of isoflurane- and hypoxia-induced preconditioning.

Role of FTY720 in cerebrovascular disease

FTY720 (Fingolimod) is a novel immunomodulatory agent, which in its phosphorylated form acts as a high affinity agonist of S1P receptors^[109,110]. It became the first oral drug to be FDA-approved for clinical use in the treatment of multiple sclerosis. FTY720 readily crosses the blood-brain barrier and exerts a number of direct effects in the central nervous system. FTY720 is phosphorylated by SphK, mainly by SphK2^[73,111], into the active compound phospho-FTY720, which then acts on 4 of the 5 known S1P receptor subtypes (S1P₁, S1P₃, S1P₄, S1P₅), and shows neuroprotective effect against many central nervous system disease including cerebral ischemia^[73,112-115]. Mechanisms include regulation of myelination and microglial activation following injury, proliferation and migration of neural precursor cells toward injury sites, and potentiation of growth-factor regulated neuronal differentiation, survival, and process extension, and also antiapoptotic and anti-inflammatory pathways^[104,113,115-119]. FTY720 also exerts immunomodulatory actions by affecting lymphocyte production, trafficking, and apoptosis through S1P receptors which induces a depletion of circulating lymphocytes by preventing the egress of lymphocytes from the lymph nodes. Mechanistically, this is due to a downregulation of the S1P type 1 receptor (S1P₁). Expression levels of endothelial adhesion molecules such as E-selectin, P-selectin, intracellular adhesion molecule-1 or vascular cell adhesion molecule-1 were shown to be induced by FTY720 treatment, and therefore might contribute to the prevention of early infiltration of neurotrophils and activation of microglia/macrophages. These findings suggest that anti-inflamma-

tory mechanisms, and possibly vasculoprotection, rather than direct effects on neurons, underlie the beneficial effects of fingolimod after stroke. Most of the past reports have shown beneficial effect of S1P in the field of ischemia, but by contrast, Liesz *et al.*^[120] showed opposite results. These authors found that S1P treatment did show a reduction of lymphocyte brain invasion but could not achieve a significant reduction of infarct volumes and behavior dysfunction^[120]. Liu *et al.*^[121] recently published a systematic meta-analysis of the efficacy of FTY720 in animal model of stroke. In this study, they concluded that FTY720 reduced infarct volume and improve functional outcome. However, the authors also indicated that more experimental studies should be performed to evaluate the safety of FTY720 in the future. Thus, taken this recent scientific highlights together, it is obvious that S1P receptor pathways and sphingolipids regulating enzymes are a highly promising target in stroke treatment.

CONCLUSION

During the past few years, a plethora of new information identifying the importance of sphingolipid signaling pathways in the cardiovascular and cerebrovascular diseases has accumulated. The potential for the development of new therapeutic agents based on this understanding is high, but this is clearly a new area of investigation that is still in its infancy.

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