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Sphingosine Kinase and Sphingosine 1-Phosphate in Cardioprotection

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Abstract

Activation of sphingosine kinase/sphingosine 1-phosphate– mediated signaling has emerged as a critical cardioprotective pathway in response to acute ischemia/reperfusion injury. Application of exogenous sphingosine 1-phosphate (S1P) in cultured cardiac myocytes subjected to hypoxia or treatment of isolated hearts either before ischemia or at the onset of reperfusion (pharmacologic preconditioning or postconditioning) exerts prosurvival effects. Synthetic congeners of S1P mimic these responses. Gene-targeted mice null for the sphingosine kinase 1 isoform whose hearts are subjected to ischemia/reperfusion injury exhibit increased infarct size and respond poorly either to ischemic preconditioning or to ischemic postconditioning. Measurements of cardiac sphingosine kinase activity and S1P parallel these observations. High-density lipoprotein is a major carrier of S1P, and studies of hearts in which selected S1P receptors have been deleted implicate the S1P cargo of high-density lipoprotein in cardioprotection. These observations have considerable relevance for future therapeutic approaches to acute and chronic myocardial injury.

Keywords

cardioprotection; cell signaling; dimethylsphingosine; high-density lipoprotein; sphingosine kinase; sphingosine 1-phosphate; S1P receptors

INTRODUCTION

Sphingolipids were first extensively characterized by a German neurochemist, J. L. W. Thudichum, in the late 19th century, who named the chemical backbone of sphingolipids for their enigmatic “Sphinx-like” properties. However, interest remained confined to a small number of investigators until relatively recently when the central importance of these compounds in pathophysiology became apparent. Among the most prominent of these is sphingosine 1-phosphate (S1P), a sphingolipid signaling molecule formed when an isoform of the enzyme sphingosine kinase (SK) catalyzes the binding of a phosphate group to sphingosine, which is in turn derived from the ubiquitous membrane lipid sphingomyelin (Fig. 1). The sphingosine kinase/sphingosine 1-phosphate (SK/S1P) pathway acts as a checkpoint involved in many cellular signaling cascades. Among other functions, signals generated by this pathway are critical for cell motility, cytoskeletal organization, vasculogenesis, cell growth, lymphoid trafficking, and immune function. These properties have led to extensive studies in cancer and

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immune and inflammatory cells and to observations that activation or inhibition of this pathway can determine cell fate by altering the balance between ceramide, which is proapoptotic, and S1P, which activates prosurvival signaling¹ (Fig. 1). In the cardiovascular system, considerable attention has focused on SK/S1P effects in endothelial cells and smooth muscle cells, both during development and in vitro.^{2–4}

Until recently, little was known about the role of the SK/S1P pathway in cardiac myocytes or in the heart. Moreover, there was no information regarding responses under conditions of oxidative stress such as acute or chronic ischemia or ischemia/reperfusion injury. Indeed, most recent reviews contain scant or no information on SK/S1P signalling in the heart under either normal or pathophysiologic circumstances,^{5–8} and none focus on cardioprotection. However, as a result of investigations published in the last few years, it has become abundantly clear that SK and S1P are crucial mediators of cardioprotection, indicating that these molecules or their synthetic analogues have potential as therapeutic modulators of cardiac responses to both acute and chronic myocardial injury.

Sphingosine Kinase(s)

There are 2 known isoforms of SK designated as SK1 and SK2, each of which contains several splice variants.⁹ Mouse and human SK1 exhibit substantial homology. In turn, SK2 is highly homologous to SK1 except for approximately 240 additional amino acids located at the N terminus and in the center of the enzyme, thereby accounting for its larger molecular mass.^{10,11} The genes encoding these isoforms are localized on different chromosomes.⁶ When both isoforms are genetically deleted, fetal death results from severe bleeding and inadequate vasculogenesis.¹² However, mice null for either SK1 or SK2 seem to exhibit normal development and are otherwise unremarkable in the basal state.^{13,14}

SK activity responds to stimulation by G-protein–coupled receptor agonists (muscarinic agonists, S1P itself, histamine, lysophosphatidic acid, angiotensin II), agonists at receptor tyrosine kinases (platelet derived growth factor, vascular endothelial growth factor, transforming growth factor- α , and transforming growth factor- β), immunoglobulin receptor cross-linking, interleukins, estrogen (for a review see Alemany et al⁶), and activators of PKC ϵ .¹⁵ Both tumor necrosis factor (TNF)- α and phorbol ester (which stimulates protein kinase C (PKC)) activate phosphorylation of SK1 at serine225 mediated by extracellular related protein kinase 1/2.¹⁶ The TNF- α effect requires binding by TNF receptor–associated factor-2.¹⁷ Other interacting proteins that stimulate SK include delta-catenin/neural plakophilin–related armadillo repeat protein,¹⁸ aminocyclase 1,¹⁹ and eukaryotic elongation factor 1A.²⁰ Reported inhibitory interacting proteins are sphingosine kinase 1–interacting protein,²¹ platelet endothelial adhesion molecule-1,²² and four and a half LIM domains 2/skeletal LIM3 (FHL2/SLIM3), a Lim only factor.²³ An interacting factor that is a putative adaptormolecule is ribosomal S6 kinase-like protein with two phytosulfokine domains (RPK118).²⁴ Additional regulators include phospholipase D and possibly calcium.^{25,26} In vitro evidence also has identified cellular export of SK, which may account for substantial enzyme activity in both mouse and human blood.^{27,28}

More recently and highly pertinent to acute ischemia/reperfusion injury is the observation that reactive oxygen species generated by the action of monoamine oxidase A on serotonin contained in platelets is responsible for the degradation of SK1.²⁹ Conversely, in glioma cells, hypoxia increases SK1 messenger RNA, protein levels, and activity that is dependent on hypoxia-inducing factor 2 α .³⁰ Thus, the balance of hypoxia and reactive oxygen species levels (and other factors as yet unidentified) could serve to regulate SK1 activity during oxidative stress in the heart (see below).

Numerous in vitro studies have demonstrated that SK1 promotes cell survival. These include an early report in cultured rat cardiac fibroblasts in which the monoganglioside GM-1, which activates SK via PKC, protected against apoptosis induced by the PKC inhibitor, staurosporine, and by C2-ceramide.¹⁵ These investigators also showed that GM-1 induced the synthesis of S1P, an effect that was partially blocked by the SK inhibitor, *N,N*-dimethylsphingosine (DMS).¹⁵ The latter has recently been shown to be predominantly an inhibitor of SK1.³¹ In addition to SK inhibitors, subsequent work has utilized dominant negative SK1 and small interfering RNA. Thus, knockdown of SK1 by the latter caused cell cycle arrest in MCF-7 cancer cells and induced apoptosis.³² Endogenous SK1 is an important regulator of intracellular ceramide levels (Fig. 1).³²

However, the evidence that either S1P or SK resides in mitochondria is sparse. In human leukemia cells, Cuvillier and Levade³³ concluded that S1P is an upstream of mitochondria. In contrast, Dindo et al³⁴ recently reported that a novel C₁₆-ceramide derivative induced selective upregulation of S1P in the mitochondria of SW403 human colon carcinoma cells. The authors suggested that this implied the presence of mitochondrial SK activity, which is selectively activated by treatment with exogenous long-chain ceramides. SK activity in heart mitochondria also has been characterized in response to ischemia/reperfusion injury and preconditioning (see below).

Other possible intracellular actions of SK and S1P have recently been investigated with respect to autophagy.^{35–37} The latter is a cellular process responsible for the degradation of long-lived proteins and organelles.³⁵ Normally, it occurs at low levels but is activated by cellular stress. In the heart, as in other cells and organs, autophagy may be either beneficial or detrimental, depending on its extent and timing. Autophagy associated with survival is regulated in part by SK1,^{36,37} whereas cell death-associated autophagy is promoted by ceramide.³⁷ As originally proposed by Cuvillier et al,¹ this represents an example of the sphingolipid “rheostat” at work. Lavieu et al³⁷ have suggested that autophagy may be a key mechanism by which sphingolipids control cell fate, but evidence for this possibility in the heart is not yet available.

Downregulation of SK1 results in enhanced ceramide synthesis via the de novo pathway and its accumulation in mitochondria, which may be key in initiating mitochondrial events, leading to cell death.

Underlying the antiapoptotic effects of SK1 is its function as the terminal step in the intracellular synthesis of S1P. There is considerable evidence that intracellular S1P is then exported to activate prosurvival signaling pathways in an autocrine and/or paracrine manner, and this phenomenon of “inside-out signaling”⁶ has been described in cardiac myocytes.³⁸ A counterintuitive function of SK1 has recently been proposed in which apoptosis induces SK1 expression to release S1P that serves a “come-and-get-me” signal for scavenger cells to engulf them to prevent necrosis.³⁹ The data were obtained in Jurkat and U937 leukemia cells and required massive apoptosis to elicit this phenomenon.

In contrast, SK2 has opposing actions to SK1.⁴⁰ SK2 inhibits cell growth and enhances apoptosis, in part by regulating ceramide levels. Thus, downregulation of SK2 reduced conversion of sphingosine to ceramide, whereas downregulation of SK1 increased it.⁴⁰ Other effects of SK2 relating to apoptosis may be its nuclear localization resulting in inhibition of DNA synthesis^{41,42} or its BH3 domain.⁴³ In keeping with the designation of sphingolipids as Sphinx like or enigmatic,⁴⁴ it remains a mystery why SK2 is proapoptotic despite being responsible for S1P synthesis. Thus, SK1-null mice, which lack SK2, exhibit normal cardiac function until stressed (see below). In these mice, tissue S1P levels are normal but the serum level is reduced by half.¹³ One explanation of these observations has been the location of S1P

generated by SK isoforms. When the normally antiapoptotic SK1 form was targeted to the endoplasmic reticulum of NIH 3T3 fibroblasts, apoptosis ensued.⁴⁰ The authors suggested that the differences between prosurvival SK1 and proapoptotic SK2 are related in part to distinct subcellular localizations and spatially restricted production of S1P.⁴⁰ However, such localization has not been demonstrated in cardiac cells or in vivo in any organs.

Both the synthetic sphingosine analogue, FTY720, and the putative SK inhibitor, DMS, inhibit primarily the SK1 form but can activate the SK2 form at low substrate concentrations in rat heart.³¹ SK2 is also necessary to phosphorylate and thus activate FTY720, which is being used in clinical trials to prevent renal transplant rejection and reduce relapses in patients with multiple sclerosis.^{45,46}

Sphingosine 1-Phosphate

S1P is a bioactive lysophospholipid that regulates many important cellular processes including growth, survival, differentiation, cytoskeletal rearrangements, motility, angiogenesis, calcium mobilization, lymphocyte trafficking, and immune function.^{38,47–49} Most cells harbor the enzymatic machinery to synthesize S1P. In plasma and serum, S1P concentrations range between approximately 200 and 900 nM^{50–52} but these likely vary under pathologic conditions (see below). Sources of S1P in plasma include platelets,⁵³ red blood cells,⁴⁹ and endothelial cells.⁵⁴ Pertinent to cardioprotection is the observation that a major carrier of S1P in blood is high-density lipoprotein (HDL).^{50–51} S1P is subject to hydrolysis by lipid phosphatases and by a lyase enzyme.⁴⁸ Chronic inhibition of the latter can lead to persistently increased S1P levels and to lymphocyte sequestration and disruption of S1P gradients.⁵⁵

The existence of S1P receptors in the heart was first reported by Bünnemann et al⁵⁶ in 1995. It is now accepted that many S1P actions are mediated through subtypes of S1P G-protein–coupled receptors, which comprise S1P_{1–5}.^{57–59} In the heart, S1P binding to S1P₁, ₂, or ₃ receptors activates downstream signaling pathways that promote cell survival. Recent biochemical evidence supports this notion. S1P₁ interacts with G α_i , whereas S1P₂ and S1P₃ couple with G α_q and G α_{13} and G α_i , in a ligand-dependent manner.⁶⁰ Subsequent studies have shown that the S1P₁ receptor exhibits the most prominent expression pattern in cardiomyocytes.^{61,62} The S1P₁ receptor has been linked to proliferative/survival and migratory signaling in many different cell types.^{63–65} The S1P₂ and ₃ receptors are also important for regulation of vascular tone.^{66,67} Signaling responses to S1P during oxidative stress are described below.

A recent report in renal mesangial cells showed that S1P-mediated signaling is rapidly desensitized upon S1P receptor activation.⁶⁸ In the latter study, there was complete loss of S1P receptors from the cell surface and receptor-mediated signaling responses after 10 minutes of S1P pretreatment that persisted for at least 8 hours.⁶⁸ In contrast, Oo et al⁶⁹ reported that the S1P₁ receptor recycled to the plasma membrane within 2 hours in S1P-treated human umbilical vein endothelial cells. The immune modulator and S1P analogue, FTY720, induced internalization and degradation of S1P₁ receptors.^{45,70} The timing and extent of S1P receptor internalization are highly dependent on FTY720 concentration.⁴⁵ Unlike FTY720, the S1P₁-selective synthetic agonist, SEW2871, induced S1P₁ internalization and recycling.⁷¹

Diverse mechanisms for S1P₁ receptor downregulation and trafficking have been described. These include translocation to perinuclear vesicles,⁷² plasmalemmal caveolae,⁷³ N-glycosylation,⁷⁴ and ubiquitination.⁷⁵ These studies have been performed in cell lines requiring transfection of the S1P₁ receptor. In adult mouse cardiomyocytes, we have recently found that S1P-mediated activation of ERK 1/2 is desensitized after 1 hour but responses are normal 24 hours later, suggesting receptor recycling (R. Tao, MD, PhD, H. E. Hoover, PhD, N. Honbo, MA, et al, 2008).

In experimental settings, acute effects of S1P include variable blood pressure and heart rate responses,⁷⁶ calcium dysregulation and myocyte hypertrophy,^{77,78} coronary artery vasoconstriction via the S1P₂ receptor,⁷⁹ and bradycardia via the S1P₃ receptor.⁸⁰ Other reported effects of the synthetic S1P₁ agonist, SEW2871, potentially pertinent to cardioprotection include a negative inotropic response in isolated adult mouse ventricular myocytes⁸¹ and exacerbation of reperfusion arrhythmias in an isolated rat heart preparation.⁸² Of note is that S1P also prevented allograft rejection in a rat heart transplantation model⁸³ and promoted in vivo angiogenesis in ischemic hind limbs of mice.⁸⁴ Serum S1P may also be a predictive marker for the presence of obstructive coronary artery disease in humans.⁵² Among other important functions of S1P in the cardiovascular system are regulation of stem cell development,⁸⁵ cytoskeleton dynamics,⁸⁶ and early heart development in conjunction with SK.³

The SK/S1P Pathway in Cardioprotection

Studies of S1P in Cardiomyocyte Cell Culture

In neonatal rat cardiac myocytes, initial observations suggested that exogenously applied S1P enhanced cardiac myocyte survival during hypoxia.⁸⁷ Subsequent work employed cultured adult mouse cardiac myocytes for hypoxia studies that serve as a model for in vivo conditions resulting from 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. Studies produced 3 major findings that advanced understanding of S1P prosurvival effects during hypoxia.⁸⁸ First, using a selective S1P₁ receptor antibody and VPC23019, a commercially available S1P analog that is predominantly an S1P₁ receptor competitive antagonist that also inhibits but has less affinity for S1P₃ receptors,⁸⁹ it was found that S1P₁ receptors are abundantly expressed by adult mouse cardiac myocytes. These findings were confirmed by quantitative real-time polymerase chain reaction assays. Second, exogenously applied S1P enhanced survival during prolonged in vitro hypoxia through mechanisms that required S1P₁ receptor function and G_i-dependent activation of the prosurvival kinase Akt (protein kinase B). Finally, Akt-mediated phosphorylation of myocyte substrates that interact with mitochondria, such as glycogen synthase kinase-3 β and Bcl-xL/Bcl-2-associated death promoter, contributed to cardioprotection. In these studies, the selective S1P₁ receptor agonist, SEW2871, was as effective as S1P in preserving myocyte viability during hypoxia.⁸⁸

In contrast, a recent study by Means et al⁹⁰ was unable to demonstrate prosurvival signaling mediated by the S1P₁ receptor. As noted above, the selective S1P₁ agonist, SEW2871, mediates myocyte survival during prolonged hypoxia⁸⁸ and also induces antiapoptotic signaling during hypoxia/reoxygenation (R. Tao, MD, PhD, H. E. Hoover, N. Honbo, et al, unpublished data). The divergent observations surrounding the cardioprotective effects of S1P₁ agonism remain unexplained. Nevertheless, previous and current experiments 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 myocyte viability during hypoxia⁸⁸ and during hypoxia/reoxygenation (R. Tao, MD, PhD, H. E. Hoover, PhD, N. Honbo, MA, et al, 2008).

In another report, Means et al⁹¹ showed that combined deletion of S1P₂ and S1P₃ receptors augmented infarct size in mice subjected to 1 hour of ischemia and 2 hours of reperfusion. In these hearts, activation of Akt was markedly attenuated compared with wild-type mice but the absence of either receptor subtype alone affected neither infarct size nor Akt activation after ischemia/reperfusion injury. When myocyte preparations from control mice were studied, S1P augmented Akt activity but was ineffectual in the double knockout cells. Thus, these observations suggest that the less abundant cardiac myocyte S1P receptors (S1P₂ and 3) may

also be necessary for cell survival during ischemia/reperfusion injury. Because targeting of the S1P₁ receptor gene is lethal to the embryo,² studies in a conditional cardiac-specific S1P₁ receptor gene knockout^{92,93} will be necessary to further delineate the role of the various receptor subtypes in the heart.

During experiments in which ventricular myocytes from SK1-null hearts were subjected to in vitro hypoxia, it was found that cell death and cytochrome *c* release were greater in SK1-null myocytes than in wild-type controls.³⁸ Exogenous S1P enhanced survival of both wild-type and SK1-null cells. Monoganglioside GM-1 treatment, which activates PKC and subsequently SK to produce S1P, induced cytoprotection in wild-type cardiac myocytes but not in SK1-null cells. These observations indicate that GM-1 activates SK1, presumably via PKC ϵ -mediated phosphorylation (see below). 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 adenosine diphosphate ribosylates and thereby inactivates Gi, suggesting that endogenous S1P was transported to the extracellular space for activation of its cognate G-protein-coupled receptors.³⁸ A potential mechanism for extrusion of S1P is via binding cassette transporters, which has been demonstrated in a variety of cell types,^{28,94} but has not yet been reported in cardiac myocytes.

PKC ϵ is a critical modulator of SK activity and endogenous S1P production in cardiac tissue.

Studies on SK

As noted above, the monoganglioside, GM-1, enhanced the survival of cardiac fibroblasts subjected either to PKC inhibition or to C2-ceramide treatment.¹⁵ GM-1 also increased S1P levels, an effect abrogated by the SK inhibitor, DMS.¹⁵ Using isolated adult mouse hearts (Langendorff technique), exogenous S1P and GM-1 separately induced substantial resistance to ischemia-reperfusion injury in wild-type mouse hearts as determined by hemodynamic and infarct size measurements.⁹⁵ Similar experiments were reported by Lecour et al⁹⁶ 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. Nevertheless, exogenously administered S1P was effective both in isolated PKC ϵ -null hearts subjected to ischemia/reperfusion injury⁹⁵ and in isolated cardiac myocytes from these hearts subjected to hypoxia.³⁸ Thus, S1P acting at cell surface receptors or activation of intracellular SK confers cardioprotection during acute ischemia/reperfusion injury.

These experiments also provided evidence for the postulate that. Consistent with this hypothesis, it was shown that PKC ϵ activation is essential for cardioprotection induced by ischemic preconditioning (IPC). PKC ϵ peptide agonists mimicked preconditioning effects on contractile recovery and tissue viability in wild-type hearts after prolonged ischemia-reperfusion injury. In contrast, inducible cardioprotection was blocked by PKC ϵ peptide antagonists and targeted deletion of the PKC ϵ gene.⁹⁷

A subsequent series of experiments directly tested the hypothesis that SK activation mediates IPC in isolated mouse hearts.⁹⁸ It was determined that IPC sufficient to reduce infarction size in wild-type hearts increased SK localization and activity in tissue membrane fractions. Interestingly, IPC triggered SK translocation to tissue membrane fractions in PKC ϵ -null hearts but did not enhance enzymatic activity or decrease infarction size after ischemia-reperfusion injury.⁹⁸ As noted above, DMS, the endogenous sphingolipid generated by *N*-methylation of sphingosine, inhibited tissue SK activity. As predicted, 10 μ M of DMS pretreatment abolished IPC-induced cardioprotection in wild-type hearts.⁹⁸

Subsequent experiments elucidated unpredicted effects of low DMS concentrations on SK.⁹⁹ In contrast to moderate dose DMS (10 μ M), low-dose DMS (0.3–1.0 μ M) enhanced cytosolic SK activity. 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 SK1 was found to coimmunoprecipitate with activated PKC ϵ phosphorylated at serine729. In addition, low-dose DMS induced translocation of total Akt from Triton-insoluble fractions to cytosol and increased activated Akt phosphorylated at serine473.

Another example of the concentration dependence of molecules usually considered to be inhibitory in the SK/S1P pathway is sphingosine, the immediate precursor of S1P. Although there is abundant evidence that sphingosine is toxic to cells, including cardiac myocytes,^{100–101} Vessey et al¹⁰² recently reported that at lower, more physiologic (submicromolar) concentrations, sphingosine was 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 kinases A and G) pathways.¹⁰² At the higher concentrations usually employed (eg, 5 μ M), sphingosine proved to be cardiotoxic.

In prior work, the study of the SK/S1P pathway had been hampered by the complexity of SK assays that required thin-layer chromatography and high-performance liquid chromatography. Accordingly, a new solvent extraction–based radioassay that exhibits equal or greater sensitivity but is much more rapid was developed.¹⁰³ Thus, it became feasible to perform numerous time point assays that are necessary when nonsaturating substrate concentrations and interfering enzymes are present in microsomal and mitochondrial fractions. In studies employing this assay, it was found that fractionation of cytosolic SK activity by gel filtration chromatography yielded 2 peaks of activity.¹⁰³ The early peak eluted as a 96-kDa protein and reacted with an SK2 antibody but not an SK1 antibody. This is larger than the molecular weight of SK2 based on sequence and suggests association with an accessory protein. The second peak seemed to be heterogeneous with a molecular weight centered on 46 kDa. This is consistent with the known existence and size of multiple forms of SK1.⁹ This fraction reacted with an SK1 antibody but not an SK2 antibody. Thus, these clearly separated enzymes were identified as SK2 and SK1, respectively.

When tested with the classic SK inhibitor, DMS, the activity of SK2 was unaffected by concentrations as high as 20 μ M. Consistent with this observation, DMS was only a partial inhibitor of total cytosolic SK activity.³¹ Also SK2 was not inhibited by the sphingosine analogue, FTY720. As noted earlier, SK1 was efficiently inhibited by both DMS and FTY720.³¹ Furthermore, when the cytosolic fraction from an SK1 knockout mouse was tested, residual activity due to SK2 was not inhibited by DMS or FTY720. These observations confirmed the specificity of SK1 inhibition and indicated that the lack of inhibition of SK2 was not an artifact of purification. SK2 from rat liver and spleen was also not inhibited by DMS. In contrast, l-sphingosine was an effective inhibitor of both forms.³¹ Taken together, along with data obtained in SK1-null hearts (see below), these observations indicate 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 SK may require reinterpretation.

In addition to signal transduction assays for studies of S1P receptor subtype function described above, the time course of SK activity in adult rat hearts subjected to ischemia/reperfusion injury and preconditioning has been reported.¹⁰⁴ Cytosolic SK activity declined by 61% during ischemia and did not recover upon reperfusion, paralleling 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 SK declined by 67% after 45 minutes of ischemia and remained at that level during reperfusion. IPC restored SK activity almost to normal during reperfusion. Parallel effects were observed in mitochondria from the same hearts.¹⁰⁴

In these experiments, total S1P in cardiac tissue was quantified by liquid chromatography followed by tandem mass spectrometry.⁴⁹ In non-preconditioned hearts, S1P content declined from baseline 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 nM of S1P improved recovery of LVDP. Thus, maintenance of SK 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 ischemia/reperfusion injury in the heart.

Despite strong corroborating evidence that DMS modulates resistance to injury by effects on SK, this agent alters PKC activity⁹⁹ and may confound interpretation of experimental data. Accordingly, SK1 knockout mice have been employed in a series of subsequent studies.^{105, 106} SK2 expression increased in hearts after SK1 gene disruption, resulting in total SK activity half that of wild type. Although SK1-null hearts exhibited normal hemodynamic performance under baseline conditions, contractile abnormalities and infarction were more severe after ischemia/reperfusion than in wild-type hearts. As predicted, targeted disruption of the SK1 gene abolished IPC-induced cardioprotection.¹⁰⁵ Importantly, when the index ischemia time was reduced from 50 to 40 minutes, 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 SK1-null hearts. Despite an increase in SK2 expression in the SK1-null hearts, infusion of DMS did not affect infarct size, confirming prior in vitro experiments and suggesting that the absence of SK1 rather than the increased presence of SK2 was critical to the loss of cardioprotection in myocardium null for SK1.¹⁰⁵

HDL is well recognized for its role in preventing atherogenesis, and one mechanism of this effect is the ability of S1P carried by HDL to preserve endothelium and inhibit proinflammatory responses in endothelial and vascular smooth muscle cells.

In another recent study, it was reported that previous adenoviral gene transfer of SK1 protected against hemodynamic deterioration and reduced creatine kinase release and arrhythmias during acute ischemia/reperfusion injury in isolated rat hearts.¹⁰⁷ When gene transfer was performed at the time of acute left anterior descending coronary artery ligation, studies 2 weeks later revealed improved left ventricular function in the treated mice, reduced infarct size, more neovascularization, and reduced collagen content.

It has been known for some time that the widely used anesthetic agent, isoflurane, protects against organ damage. In a recent report, Kim et al¹⁰⁸ noted that an important mechanism of protection induced by isoflurane is activation of the SK/S1P pathway. Using an ischemia/reperfusion model of renal injury, these investigators reported that isoflurane anesthesia reduced the degree of renal failure and necrosis. Mice deficient in SK1 were not protected, and in wild-type mice, protection was abrogated by DMS and the S1P₁ antagonist, VPC2309. The authors also demonstrated that isoflurane increased SK1 messenger RNA in HK-2 cells.

Like IPC, ischemic postconditioning is cardioprotective,¹⁰⁹ and this observation has recently been extended to patients undergoing percutaneous coronary interventions.¹¹⁰ To ascertain

whether the SK/S1P pathway is a determinant of successful postconditioning, isolated wild-type and SK1-null mouse hearts were subjected to ischemia/reperfusion injury.¹¹¹ At the onset of reperfusion, hearts selected for treatment underwent 3 brief cycles of postconditioning (5 seconds of ischemia followed by 5 seconds of reperfusion). Results were similar to the preconditioning studies cited above: Hemodynamics were improved and infarct size reduced compared with untreated hearts. Phospho-Akt and phospho-ERK were enhanced. None of these findings were present in SK1-null hearts. Thus, SK1 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 minutes of ischemia.¹¹²

Cardiac fibroblasts are critical for the maintenance of extracellular matrix deposition and turnover in the normal heart and are key mediators of inflammatory and fibrotic myocardial remodeling in the injured and failing heart. As noted above, SK activation is a well-recognized determinant of cell fate in cardiac myocytes and other cells, but SK responses have not previously been studied in cardiac fibroblasts except for the earlier study by Cavallini et al¹⁵ cited above. Initially, it was found that total SK activity is more than 10-fold higher in cardiac fibroblasts than in adult mouse cardiac myocytes.¹¹³ In cardiac fibroblasts isolated from SK1 knockout mice, SK activity was greatly reduced, indicating that SK1 is the major isoform expressed in these cells. To determine whether SK regulates cell proliferation and the proinflammatory protein-inducible nitric oxide synthase (iNOS), cultured cardiac fibroblasts were incubated with the cytokine, interleukin-1 β (IL-1 β), in the presence or absence of hypoxia. Hypoxia did not alter fibroblast SK activity, whereas IL-1 β enhanced enzyme activity. In wild-type cardiac fibroblasts, hypoxia induced proliferation, but in SK1-null fibroblasts, this response was blunted even in the presence of serum. In contrast, iNOS expression and NO production were enhanced in SK1-null fibroblasts during hypoxia. In wild-type fibroblasts, IL-1 β was only a weak inducer of iNOS and of NO accumulation and hypoxia alone had no significant effect on iNOS activation. However, IL-1 β in combination with hypoxia stimulated both iNOS and NO production, and this stimulation was enhanced in SK1-null fibroblasts. Thus, activation of endogenous SK1 serves a dual regulatory function: It is required for optimal cardiac fibroblast proliferation but is a negative modulator of proinflammatory responses during hypoxia.¹¹³

S1P, HDL, and Cardioprotection

As noted above, a major carrier of S1P in serum is HDL.^{50,51,114–116} The synthetic sphingosine analogue, FTY720, which when phosphorylated by SK2, acts as an agonist at S1P receptors, has similar effects, and has been shown to reduce atherosclerosis both in low-density lipoprotein receptor-deficient mice¹¹⁷ and in apolipoprotein E-deficient mice.¹¹⁸ These responses would be expected to confer chronic cardioprotection and might also constitute part of a therapeutic prevention strategy. In this connection, statins have been reported to induce S1P₁ receptors and enhance endothelial NO production in response to HDL.¹¹⁹

In acute studies, HDL is cardioprotective.¹²⁰ It has been proposed that at least part of this cardioprotection can be attributed to the S1P content of HDL.¹²⁰ A postulated mechanism is S1P-mediated suppression of inflammation that includes inhibition of adhesion molecule expression and impaired recruitment of polymorphonuclear cells to the infarcted area.¹²¹ Both genetic alterations in S1P receptors and responses to HDL have provided additional insight into the role of SK/S1P pathways in cardioprotection. In these studies, the focus has been on the role of the S1P₃ receptor. Nofer et al⁶⁶ showed that HDL induced NO release in human endothelial cells and caused NO-dependent vasorelaxation via the S1P₃ receptor. These effects could be suppressed in mice lacking this receptor. Based on these observations, S1P₃ seems

to be a major regulator of arterial vasodilation as compared with the S1P₂ receptor whose stimulation results in vasoconstriction.⁶⁷

Thus, HDL would be expected to improve myocardial perfusion and protect the heart against ischemia/reperfusion injury in vivo via the S1P₃ receptor. This indeed may be so, but the evidence is somewhat conflicting. Using a radionuclide technique, Levkau et al¹²² reported that human HDL stimulated murine myocardial perfusion in vivo but this effect was abolished in S1P₃ receptor-null mice. Moreover, in this study, it was reported that S1P inhibited myocardial perfusion through the S1P₃ receptor.¹²² In a subsequent report, however, Theilmeier et al¹²³ demonstrated that HDL and its constituent S1P acutely protected the murine heart against ischemia/reperfusion injury via an S1P₃-mediated and NO-dependent pathway. As noted above, Means et al⁹¹ noted that deletion of neither the S1P₂ receptor nor the S1P₃ receptor alone affected infarct size or Akt activation. However, in double-knockout mice, infarct size after ischemia/reperfusion was increased by 50%, and Akt activation was markedly attenuated. As described earlier, other work has emphasized the role of the S1P₁ receptor in preserving myocyte viability.⁸⁸ Although these studies are not in agreement as to how S1P receptor subtypes preserve myocardial viability, regulate the coronary circulation, and influence atherosclerosis, they all point to the importance of S1P in cardioprotection and to a critical role for HDL in transporting S1P, both acutely and chronically, to sites within the coronary vasculature.

In summary, during the past few years, a plethora of new information identifying the importance of sphingolipid signaling pathways in the cardiovascular system has accumulated. The potential for the development of new therapeutic agents based on this understanding is high, but the Sphinx still harbors many riddles that require solution.¹²⁴

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FIGURE 1.

Regulation and function of SK1. The diagram depicts some of the negative and positive regulators of SK1 activity and the synthesis and location of the SK1/S1P pathway either present in the heart or derived experimentally. A key feature of this pathway is the conversion of the ubiquitous membrane lipid sphingomyelin by several sphingomyelinases manufactured intracellularly that act both at the membrane level and extracellularly. This results in the generation of ceramide that is deacylated by neutral ceramidase, yielding sphingosine, which is then converted to S1P by the action of SK1. As noted in the text, activation of SK1 in the heart is inhibited by I/R injury and is stimulated by monoganglioside GM-1 that like IPC requires activation of PKCε. SK1 activity can also be influenced negatively or positively by the interacting proteins shown and by many other agonists described in the text. As shown in the figure, SK1 generates intracellular S1P that may act in as yet poorly defined ways on mitochondria, nuclei, or other targets. Intracellular S1P is known to be exported ("inside-out signaling") to act as an autocrine or paracrine messenger via G-protein-coupled cell surface receptors. A second isoform of SK (SK2) is also responsible for S1P generation (not shown). The location of S1P generation within the cell may determine its effect, but the outcomes of such location-based effects have not been determined. As shown in the figure, S1P is also generated extracellularly by a sequence of enzyme reactions similar to those described above. I/R, ischemia/reperfusion.