Signals from type 1 sphingosine 1-phosphate receptors enhance adult mouse cardiac myocyte survival during hypoxia

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Zhang J, Honbo N, Goetzl EJ, Chatterjee K, Karliner JS, Gray MO. Signals from type 1 sphingosine 1-phosphate receptors enhance adult mouse cardiac myocyte survival during hypoxia. Am J Physiol Heart Circ Physiol 293: H3150–H3158, 2007. First published August 31, 2007; doi:10.1152/ajpheart.00587.2006.—Sphingosine 1-phosphate (S1P) is a biologically active lysophospholipid that serves as a key regulator of cellular differentiation and survival. Immune stimuli increase S1P synthesis and secretion by mast cells and platelets, implicating this molecule in tissue responses to injury and inflammation. Binding of S1P to G protein-coupled receptors activates phosphatidylinositol 3-kinase and Akt in a variety of tissues. To elucidate the mechanisms by which S1P enhances adult cardiac myocyte survival during hypoxia, we used a mouse cell culture system in which S1P receptors were observed to transduce signals from exogenous S1P, an S1P1 receptor antibody with agonist properties, and the pharmacological agents FTY720 and SEW2871. S1P1 receptor mRNA and protein were abundantly expressed by adult mouse cardiac myocytes. S1P-S1P1 receptor axis enhancement of myocyte survival during hypoxia was abolished by phosphatidylinositol 3-kinase inhibition. S1P1 receptor function was closely associated with activation of Akt, inactivation of GSK-3β, and reduction of cytochrome c release from heart mitochondria. These observations highlight the importance of S1P1 receptors on ventricular myocytes as mediators of inducible resistance against cellular injury during severe hypoxic stress.

Heart; lysophospholipids; ischemia; cardioprotection; mitochondria

SPHINGOSINE 1-PHOSPHATE (S1P) is a biologically active lysophospholipid that serves as a regulator of cell differentiation and survival (8, 33, 38, 66). S1P is produced by mast cells, platelets, cardiac myocytes, and other cell types in de novo biosynthetic pathways and by metabolism of the plasma membrane phospholipid sphingomyelin (8). Secreted S1P is present in extracellular fluids at nanomolar to micromolar concentrations bound to albumin and plasma lipoproteins (1, 51). Immune stimuli increase S1P synthesis and secretion, implicating the molecule in injury responses and inflammation (5, 13, 15, 51, 53).

Although S1P exerts intracellular effects, functions triggered by extracellular binding to plasma membrane G protein-coupled receptors (GPCRs) designated as S1P1, S1P2, S1P3, S1P4, and S1P5 are better understood. For example, Windh et al. (68) used both insect and mammalian heterologous expression systems to confirm that S1P1 activated only G_{i} family members. In contrast, S1P2 and S1P3 were coupled with G_{i}, G_{q}, and G_{13} (68). Signaling pathways involving S1P1 and S1P5, G_{i} subunits, phosphatidylinositol 3-kinase (PI3K), and Akt mediate the protective actions of S1P in a range of tissues (49).

Lecour et al. (34) used an ex vivo rat heart protocol to demonstrate that extracellular S1P administered at physiological concentrations reduced infarction during subsequent myocardial ischemia-reperfusion. S1P1 treatment mimicked cardioprotection conferred by ischemic preconditioning (34). Those investigators did not identify cardiac cell types or signaling pathways required for S1P-mediated protective effects. S1P1, S1P2, and S1P3 receptors are expressed by cardiac myocytes and fibroblasts and coronary artery endothelial and smooth muscle cells (1, 7, 49). S1P modulation of ion homeostasis and contraction (6, 18, 23, 32, 37, 39, 45, 55, 61, 70) may influence myocyte resistance to injury.

Our laboratory previously examined effects of S1P pretreatment on ex vivo mouse hearts subjected to ischemia-reperfusion (26). Physiological concentrations (10 nmol/l) of extracellular S1P infused before ischemia reduced infarction by one-half and augmented myocardial contractile recovery during reperfusion by several-fold. Treatment of hearts with ganglioside GM-1 (10 nmol/l), which increases intracellular concentrations of S1P through its activation of sphingosine kinase (7), stimulated equivalent protection against ischemia-reperfusion injury. Interestingly, GM-1 treatment was not protective in protein kinase Cε (PKCε) null hearts, leading us to postulate that PKCε is a critical modulator of sphingosine kinase activity and intracellular S1P production in intact myocardium (26).

In studies of cardioprotective signaling stimulated by ischemic preconditioning (IPC), we determined that IPC increased sphingosine kinase localization to tissue membrane fractions, elevated intracellular S1P concentrations, and reduced infarction size during ischemia-reperfusion (24). IPC also triggered sphingosine kinase translocation to tissue membrane fractions in PKCε null hearts but did not alter enzymatic activity or decrease infarction size, again suggesting functional interactions between PKCε and sphingosine kinase. We employed a pharmacological inhibitor, dimethylsphingosine (DMS), to show that sphingosine kinase function was necessary for the cardioprotective effects of IPC (24). However, inhibitory effects of DMS on PKCε (52) confounded data interpretation.

Findings from these previous investigations suggested autocrine and paracrine roles for S1P in constitutive and inducible cardioprotective mechanisms. However, available data did not distinguish cardiac cell types, membrane receptors, and signal-
EXPERIMENTAL PROCEDURES

Materials. S1P, wortmannin, LY294002, and pertussis toxin were purchased from Biomol International (Plymouth Meeting, PA). VPC23019 was purchased from Avanti Polar Lipids (Alabaster, AL). FTY720 and SEW2871 were purchased from Calbiochem (San Diego, CA). Primary antibodies directed against cytochrome c and the phospholysed and total endogenous forms of Akt and glycogen synthase kinase-3β (GSK-3β) were purchased from Cell Signaling Technology (Danvers, MA). S1P<sub>1</sub> polyclonal antibody for Western blotting was purchased from Abcam (Cambridge, MA). S1P<sub>1</sub> monoclonal antibody used for all in vitro experimental protocols was derived in the Goetzl laboratory at University of California, San Francisco, as published previously (14).

Animals. Male C57Bl/6 mice (20–25 g) were purchased from Charles River Laboratories (Hollister, CA). Animals were fed standard rodent chow and water ad libitum. All studies were approved by the Institutional Animal Care and Use Committee of the San Francisco Veterans Affairs Medical Center. All protocols conformed to the National Research Council’s Guide for the Care and Use of Laboratory Animals.

Adult mouse cardiac myocyte culture protocol. Ventricular myocytes were cultured with a modification of the method described by Zhou et al. (72). Mice were anaesthetized with heparin (50 units ip) and euthanized with pentobarbital sodium (200 mg/kg ip). Hearts were excised and cannulated via the aorta for retrograde perfusion for 2 min with Ca<sup>2+</sup>-free isolation buffer containing (in mmol/l) NaCl 120, KCl 5.4, MgSO<sub>4</sub> 1.2, NaH<sub>2</sub>PO<sub>4</sub> 1.2, glucose 5.6, NaHCO<sub>3</sub> 4.6, HEPES 10, taurine 5, 2,3-butanedione monoxime (BDM) 10. Hearts were subsequently perfused for 9 min with the same isolation buffer containing 50 μmol/l CaCl<sub>2</sub> and 1.5 mg/ml collagenase II (Worthington, Lakewood, NJ). Digested hearts were quickly removed from the perfusion apparatus. Atrial tissue and major vascular structures were trimmed away with scissors and discarded. Ventricles were teased apart with forceps, pipetted in collagenase II buffer, filtered through a cell dissociation sieve, and centrifuged at 4 g for 1 min. Cardiac myocyte pellets were serially resuspended in isolation buffers supplemented with 100, 250, 500, and 1,200 μmol/l CaCl<sub>2</sub>. The final Ca<sup>2+</sup>-resistant myocyte pellets were resuspended in minimal essential medium (MEM) containing HBSS, 10 μg/ml penicillin, 1.5 μmol/l vitamin B<sub>12</sub>, 2.5% bovine calf serum, and 10 mmol/l BDM.

Isolated cardiac myocytes were plated for 1 h on 35- and 60-mm tissue culture dishes coated with 10 μg/ml laminin. After an initial attachment period, myocytes were incubated overnight at 37°C in a humidified atmosphere of 1% CO<sub>2</sub> and air with serum-free plating medium supplemented with 1 mmol/l BDM. This concentration of BDM is lower than concentrations shown by other laboratories to inhibit L-type Ca<sup>2+</sup> channel function (2), activate ryanodine receptors (59), inhibit transient outward K<sup>+</sup> current (69), or disrupt gap junction communication (62) in cardiac myocytes. Nevertheless, all cells were incubated with BDM-free medium for 2 h before experimentation to reverse any residual effects of the agent. This cell culture protocol routinely yielded >90% cardiac myocytes at 50 cells/mm<sup>2</sup>; 80% of myocytes were rod shaped and viable at pH 7.2 for 48 h. All experiments were performed on the morning after cardiac myocyte isolation.

Cardiac fibroblasts were obtained from the 40 g supernatant of the ventricular cell suspension described above. Fibroblasts were pelleted at 500 g for 10 min, washed to remove collagenase, and resuspended in MEM supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and streptomycin). Following 30 min of plating on culture dishes, weakly attached cells (cardiac myocytes, endothelial cells, smooth muscle cells, leukocytes, and erythrocytes) were rinsed free and discarded. Adherent fibroblasts (>95% by immunophenotyping for vimentin) were incubated overnight under the same conditions described above for cardiac myocytes. Cardiac fibroblasts were harvested for quantitative PCR analysis without passaging on the day after isolation.

Quantitative real-time PCR. Total RNA was isolated from primary cultures of cardiac myocytes and fibroblasts using TRIzol (Life Technologies, Grand Island, NY), treated with DNase I, and amplified quantitatively in 50-ng replicates using TaqMan primers and probes for mouse S1P<sub>1</sub> receptors and a constitutive standard, GAPDH (Integrated DNA Technology, Coralville, IA). Analyses were conducted with the Prism 7700 Sequence Detection System and recommended optimal reagents and conditions, generating interanalysis coefficients of variation <4% (PE Applied Biosystems, Foster City, CA). Values for each unknown were determined by standard threshold cycle methods with GAPDH as reference sequence and calibration curves derived from known RNA amounts (16).

Hypoxia experimental protocol. On the morning after isolation, cardiac myocytes were incubated for 2 h in normoxic experimental medium consisting of serum-free, glucose-free MEM with HBSS that did not contain BDM. Cells were subsequently treated with pharmacological agonists and antagonists under normoxic conditions as indicated. For studies requiring inhibition of Gi-mediated signaling, cells were incubated with pertussis toxin at a final concentration of 100 ng/ml for 16 h before experiments (22). After treatment, myocytes were rinsed and incubated in fresh normoxic or hypoxic medium. Normoxic experimental medium was equilibrated overnight in water-jacketed incubators (Forma Scientific, Marietta, OH) under a humidified atmosphere of 1% CO<sub>2</sub> and air. Hypoxic medium was preequilibrated in Bactron I anaerobic chambers (Sheldon Manufacturing, Cornelius, OR) under a humidified atmosphere of 1% CO<sub>2</sub> and 99% N<sub>2</sub>. Hypoxic treatment groups were placed into the anaerobic chamber for 5 h at 37°C. Normoxic groups were placed into a water-jacketed incubator at 37°C during the same period.

Measurement of cell viability. Cardiac myocyte viability was measured by staining of cells in tissue culture dishes for 10 min at room temperature with Trypan blue solution (Sigma Chemical, St. Louis, MO) diluted to a final concentration of 0.04% (wt/vol). Myocytes were visualized at $\times100$ magnification by bright-field microscopy. The numbers of viable (unstained) and nonviable (blue stained) cardiac myocytes in 10 random microscopic fields were recorded, with at least 300 cells counted for each dish. Percent survival was...
defined as the number of unstained myocytes counted for each hypoxic treatment dish divided by the number of unstained myocytes counted for each corresponding normoxic control dish, a calculation that accounted for the detachment and loss of nonviable cells during the hypoxia experimental protocols. In preliminary studies, we demonstrated that measurements of cardiac myocyte survival derived with the Trypan blue exclusion assay correlated with other cell viability measurements published by our laboratory (17, 30, 31).

Western blot analysis. Lysates prepared from cultured cardiac myocytes and HTC4 rat hepatoma cells retrovirolytransduced to express high levels of human S1P GPCRs were subjected to SDS-PAGE and transferred to nitrocellulose membranes as described (17, 26). Proteins of interest were detected with selective primary antibodies (see Materials), appropriate secondary antibodies conjugated with horseradish peroxidase, and enhanced chemiluminescence reagents (Amersham, Piscataway, NJ). Reactivity was quantitated by densitometry.

Statistical analysis. All results are reported as means ± SE. Comparisons between groups were made by one-way analysis of variance (ANOVA). Post hoc analysis was performed using the Student-Newman-Keuls test. P < 0.05 was considered significant.

RESULTS

*S1P* mRNA and protein are abundantly expressed by mouse ventricular myocytes. We performed quantitative real-time PCR assays on total RNA extracted from primary cultures of mouse ventricular myocytes and fibroblasts to measure relative expression of lysophospholipid receptors between these two cell types. As shown in Fig. 1A, *S1P*1 mRNA was the most abundant species, with a 10-fold greater expression in myocytes compared with fibroblasts isolated from the same heart. *S1P*3 mRNA was the next most abundant species, with myocyte expression approximately one-half that observed for *S1P*1 mRNA. Interestingly, *S1P*2 mRNA and type 1 lysophosphatidic acid receptor (LPA1) mRNA were more abundant in fibroblasts vs. myocytes. Other lysophospholipid GPCR mRNAs were minimally expressed.

In Western blot analyses of lysates prepared from myocytes and ventricular tissue, antibodies specific for *S1P*1 protein detected a single band at the predicted molecular mass of 43 kDa (Fig. 1B). Western blot analyses of lysates from HTC4 rat hepatoma cells retrovirolytransduced to express a high level of human *S1P*1 also generated an immunoreactive band at the same molecular mass. The commercial antibody did not react with lysates of HTC4 cells expressing human *S1P*2 or *S1P*3 receptors (Fig. 1B).

*SIP* and *S1P*1 monoclonal antibody enhance heart myocyte viability during hypoxia. In preliminary work, we adapted the adult mouse cardiac myocyte culture protocol that was originally described by Zhou et al. (72) for our investigation of severe hypoxia. As shown in Fig. 2A, this modified protocol consistently yielded >90% cardiac myocytes at 50 cells/mm². Approximately 80% of ventricular myocytes were rod shaped at pH 7.2 for 48 h. Cell viability quantitated with Trypan blue exclusion assays correlated closely with viability measurements previously published by our laboratory (17, 30, 31, 56).

As shown in Fig. 2B, incubation in hypoxic experimental medium for 5 h decreased myocyte viability by 40% compared with normoxic controls. In contrast, pretreatment with S1P (100 nmol/l) preserved cell viability after severe hypoxia. Pretreatment with S1P1 monoclonal antibody mimicked cytoprotection stimulated by S1P administration. Effects of simultaneous treatment with S1P and S1P1 antibody were no different from the effects of either agent given alone (data not shown). Wortmannin, an inhibitor of PI3K activity (3), did not alter cell viability during hypoxia (Fig. 2B). However, PI3K inhibition consistently blocked cytoprotection induced by S1P and S1P1 monoclonal antibody administration.

Additional experimental evidence further supported the importance of *S1P*1 receptor activation for enhancement of mouse ventricular myocyte viability during hypoxia. For the studies summarized in Fig. 2C, we used the S1P analog VPC23019 at 100 nmol/l as a competitive antagonist of *S1P*1 receptors. Davis et al. (11) demonstrated that this concentration of VPC23019 had no significant effect on *S1P*3 receptor activation with a broken cell assay that measured S1P-induced binding of γ-35S-GTP to membranes of HEK293T cells transfected with *S1P*3 receptor, Gβ1, Gβ2, and Gγ2 plasmid DNA. They also determined that this concentration of VPC23019 did not disrupt specific binding of radiolabeled S1P to *S1P*3 receptors in membranes from the same cell transfectants.

We used the immunomodulator FTY720 as an activator of S1P GPCRs that does not differentiate among four S1P receptor subtypes (5). FTY720 is phosphorylated by sphingosine kinase, and the resultant phosphate ester (FTY720-P) is an agonist of nanomolar potency for *S1P*1, *S1P*3, *S1P*4, and *S1P*5 receptors. We used SEW2871 as a selective agonist for *S1P*1.
S1P and S1P1 monoclonal antibody increase Akt activation in ventricular myocytes. Our findings of 1) abundant expression of S1P1 in cardiac myocytes and 2) inhibition of S1P effects on viability during hypoxia by wortmannin led us to test the hypothesis that S1P stimulates cardioprotective signaling involving S1P1, G\(_i\) complexes, PI3K, and Akt. As shown in Fig. 3, A and B, treatment of myocytes with S1P or S1P1 antibody for 20 min doubled phosphorylation of Akt at serine-473. In parallel studies, we observed that S1P and S1P1 antibody also doubled Akt phosphorylation at threonine-308 (data not shown).

As indicated in Fig. 3C, S1P1 receptor inhibition prevented phosphorylation of Akt at serine-473 in response to either S1P or S1P1 receptor monoclonal antibody stimulation. Inhibition of G\(_i\)-mediated signaling by an overnight incubation of myocytes with pertussis toxin abolished Akt activation caused by S1P or S1P1 receptor antibody (Fig. 3, A and B). Decreased PI3K function following administration of the competitive inhibitor LY294002 (20 \(\mu\)mol/l) or the irreversible inhibitor wortmannin (100 \(\mu\)mol/l) also blocked Akt activation.

We tested the hypothesis that cardioprotective signaling persists throughout hypoxia by harvesting myocytes for Western blot analysis after 60-min normoxic incubations with either S1P or S1P1 antibody followed by 5-h incubations under hypoxic conditions. As shown in Fig. 4, A and B, hypoxia alone caused a robust increase in Akt phosphorylation compared with normoxic controls. S1P or S1P1 antibody treatment stimulated additional Akt phosphorylation. Interestingly, wortmannin prevented Akt activation in response to hypoxia alone and to treatment with S1P or S1P1 monoclonal antibody (Fig. 4, A and B).

SIP inactivates GSK-3\(\beta\) and reduces cytochrome c release from heart mitochondria. Juhaszova et al. (29) determined that the beneficial effects of Akt activation on myocyte viability during hypoxia-reoxygenation were caused in large part by phosphorylation and inactivation of GSK-3\(\beta\). In the present study, we determined that S1P or S1P1 antibody treatment increased GSK-3\(\beta\) phosphorylation (Fig. 4C). Conversely, inhibition of PI3K prevented GSK-3\(\beta\) inactivation following S1P or S1P1 antibody administration (Fig. 4C). S1P1 receptor inhibition with VPC23019 also blocked GSK-3\(\beta\) inactivation (not shown).

GSK-3\(\beta\) inactivation has been shown to preserve mitochondrial function under stress conditions by disrupting translocation of BH3 domain-containing proteins, enhancing the outer membrane stabilizing effects of MCL-1, and inhibiting permeability transition pore opening (4, 10, 40, 43, 60, 65). In the
inhibitor.

The present study generated three principal findings that advance understanding of S1P actions on cardiac myocytes subjected to hypoxic stress. First, S1P1 receptors are abundantly expressed by adult mouse ventricular myocytes. Second, S1P is protective during hypoxia through mechanisms that require S1P1 receptor activation. Finally, PI3K is a key mediator of S1P-S1P1 receptor axis enhancement of myocyte viability, possibly via modulation of Akt activity, GSK-3β activity, and mitochondrial membrane integrity.

The existence of S1P1 receptors on cardiac myocytes has been controversial. Using subtype-selective antibodies against S1P1 and S1P3 receptors for immunofluorescence staining, Forrest et al. (13) determined that S1P1 protein was undetectable on myocytes in adult rat and mouse heart sections. S1P1 antibody stained vascular endothelial cells, identified by double labeling with a platelet-endothelial cell adhesion molecule antibody. In contrast, S1P3 receptor antibody stained cardiac myocytes without labeling vascular endothelial cells (13). Those investigators proposed that S1P1 receptor activity on endothelial cells determines S1P effects on blood pressure regulation, whereas S1P3 receptor activity on cardiac myocytes might be responsible for S1P effects on heart rate.

Other investigations of S1P1 receptor expression in cardiovascular tissue generated conflicting data. For example, Robert et al. (50) used polyclonal antibodies against an S1P1 receptor domain displaying high homology across different mammalian species to establish that the receptor subtype was present in neonatal rat heart homogenates and membranes prepared from cultured neonatal cardiac myocytes. In subsequent studies, Mazurais et al. (41) used the same antibodies to demonstrate that S1P1 receptors were localized on human ventricular myocytes in addition to coronary artery endothelial cells. Concurrent experiments based on Northern blot, in situ hybridization, and Western blot analyses confirmed immunohistochemical localization of human S1P1 receptors (41).

Findings reported by Nakajima et al. (44) strongly support S1P1 receptor localization and function on neonatal and adult cardiac myocytes. Using neonatal cells, investigators identified an S1P1 receptor isoform by RT-PCR with 100% homology to rat brain sequences. Quantitative RT-PCR analysis demonstrated sevenfold higher expression of S1P1 receptor mRNA in myocytes vs. fibroblasts from the same neonatal rat heart (44). Antibodies against the extracellular domain of S1P1 receptors labeled the plasma membranes of neonatal cardiac myocytes and sarcolemma of adult rat cardiac papillary muscles. Importantly, transfection of neonatal myocytes with antisense vector reduced both S1P1 receptor expression and S1P modulation of cellular calcium transients (44).

Zhang et al. (71) cloned a mouse homolog of the S1P1 receptor gene and observed abundant expression of S1P1 receptor mRNA in adult C57BL/6J hearts by Northern blot analysis. Liu et al. (38) disrupted the S1P1 gene by inserting a LacZ-neo cassette into the open reading frame. Although S1P1 receptor null mice died in utero from bleeding, β-galactosidase activity was prominent in embryonic cardiac myocytes and endothelial cells (38). Thus results from the present study and a growing consensus in the published literature favor S1P1 receptor expression by cardiac myocytes throughout development.

S1P is widely recognized as a modulator of T cell and B cell trafficking between the systemic circulation and lymphoid organs (15, 16, 51, 53). Therefore, the effects of S1P treatment on immune responses can be difficult to distinguish from more direct actions on ventricular myocytes during in vivo cardiac

DISCUSSION

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ischemia-reperfusion. Similarly, effects of S1P on myocardial contractile function and viability in ex vivo perfusion protocols result from the combined activation of S1P receptor subtypes expressed by mixed populations of heart cells. Although we recognize the limitations of all in vitro experimental systems, our group established cardiac myocyte culture models as powerful and complementary tools for elucidation of cardioprotective signaling mechanisms that are operative during hypoxia (17, 30, 31, 56). Independent groups validated the utility of adult mouse cardiac myocyte cultures for studies of intracellular signaling and genetic physiology (19, 21, 72).

After interruptions in coronary blood flow, myocardial injury is caused in large part by the oxidative stress of reperfusion. However, severe ischemia alone has been shown to produce substantial tissue damage. For example, Borutaite et al. (4) demonstrated that global ischemia without reperfusion stimulated cytochrome c release from mitochondria, respiratory chain complex dysfunction, and ventricular myocyte apoptosis in isolated rat hearts. Similarly, Lesnfsky et al. (35) found that reactive oxygen species generated by respiratory chain complex III in ischemic rabbit hearts depleted mitochondrial cardiolipin, triggering cytochrome crelease and cytochrome oxidase dysfunction. Our investigation measured cardiac myocyte viability during hypoxia and highlights interactions between the endogenous cytoprotective molecule S1P and one major component of ischemia.

As depicted schematically in Fig. 5, our findings strongly support S1P-S1P1 receptor axis enhancement of adult mouse cardiac myocyte survival during hypoxia. We showed that the S1P1 receptor antagonist VPC23019 blocked protection generated by pretreatment with S1P itself or the synthetic compound FTY720 (Fig. 2C), whereas the S1P1 receptor-activating antibody 4B5.2 and the S1P1 receptor agonist SEW2871 con-
Inactivation of the redox-sensitive phosphatase PTEN and increased Akt phosphorylation at serine-473

Li et al. (36) observed that hypoxia triggered phosphorylation on nuclear translocation of mammalian target of rapamycin (mTOR) in rat aortic endothelial cells and in mouse embryonic fibroblasts followed by mTOR-dependent Akt phosphorylation at serine-473, validating work by other groups that identified mTOR as a cellular oxygen sensor (9, 54).

In our experiments, hypoxia-induced Akt activation did not increase myocyte viability during stress. However, S1P<sub>1</sub> receptor activation increased Akt phosphorylation above values measured after hypoxia alone (Fig. 4, A and B), a response that correlated with increased cell survival (Fig. 2B). These results suggest that signaling through the S1P-S1P<sub>1</sub> receptor axis augmented Akt activity above the threshold necessary for protection against hypoxia-induced cell death. An alternative interpretation is that S1P<sub>1</sub> receptor activation of PI3K signaling stimulated cardioprotection through molecular targets other than Akt, such as protein kinase C and p70S6-kinase (17, 25, 26, 28, 46, 49, 58). Future studies can test the relative importance of downstream effector molecules using pharmacological and genetic approaches beyond the scope of the present investigation.

Ion channels constitute an important category of proteins regulated by S1P that may contribute to resistance against hypoxic injury. In studies focused on hypoxia and cell electrophysiology, Henry et al. (20) enveloped isolated adult rat ventricular myocytes in mineral oil droplets saturated with nitrogen gas. The investigators found that simulated ischemia produced both inhibition and activation of distinct inward rectifier K<sup>+</sup> currents. Diaz et al. (12) later determined that isolated adult rabbit ventricular myocytes could be preconditioned, thereby enhancing cell viability during simulated ischemia-reperfusion. The investigators showed that pharmacological inhibition of inward rectifier K<sup>+</sup> current (<i>I<sub>K1</sub></i>) and knockdown of <i>I<sub>K1</sub></i> channel subunits Kir2.1 and Kir2.2 were equally effective in blocking preconditioning-induced cardioprotection. Ochi et al. (45) demonstrated that S1P activated the G protein-gated inward rectifier K<sup>+</sup> current (<i>I<sub>K(S1P)(G)</sub></i>) in guinea pig atrial myocytes carried by the same channel proteins as those activated by acetylcholine.

Importantly, MacDonnell et al. (39) found that S1P had no significant effect on <i>I<sub>K1</sub></i> in adult rat ventricular myocytes. Their data suggest that <i>I<sub>K(S1P)</sub></i> activation is unlikely to be a major component of S1P-mediated cardioprotection in mouse ventricular myocytes. However, S1P treatment was shown to reduce inward sodium current (<i>I<sub>Na</sub></i>) and to increase stimulus currents required to elicit action potentials (39). Intracellular Na<sup>+</sup> accumulation during ischemia facilitates cardiac injury by causing cytosolic and mitochondrial Ca<sup>2+</sup> overload, whereas agents that block sarcolemmal Na<sup>+</sup> entry are cardioprotective (64, 67). We look forward to experiments designed to test the hypothesis that S1P enhances myocyte viability during hypoxia through <i>I<sub>Na</sub></i> inhibition.

In summary, we used a mouse cardiac myocyte culture model to study mechanisms responsible for protection against hypoxia-induced cell death. We determined that S1P<sub>1</sub> receptors were abundantly expressed by adult ventricular myocytes and necessary for S1P-mediated cardioprotection. S1P<sub>1</sub> receptor axis enhancement of cell viability during hypoxia required PI3K activation and was associated with Akt activation, GSK-3B

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Fig. 5. S1P<sub>1</sub> receptor activation and enhanced myocyte survival during hypoxia. Schematic summarizing experimental tools used to show that S1P preserves cardiac myocyte viability during severe hypoxia through signaling mechanisms involving S1P<sub>1</sub> receptors, G<sub>i</sub> complexes, and PI3K. Although Akt activation and GSK-3B inactivation are considered strongly cardioprotective, other molecular targets of PI3K signaling are likely to stimulate additional resistance against hypoxic injury in ventricular myocytes.
inactivation, and reduced cytochrome c release from mitochondria. Our data advance understanding of S1P1 receptors as mediators of resistance to hypoxic injury in cardiac myocytes and may have broader implications for the prevention of ischemic heart disease.

GRANTS

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Sphingosine 1-phosphate and cardiac myocyte viability


