S1P lyase (SPL): a novel therapeutic target for ischemia/reperfusion injury of the heart

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Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid that promotes cardiomyocyte survival and contributes to ischemic pre-conditioning. S1P lyase (SPL) is a stress-activated enzyme responsible for irreversible S1P catabolism. We hypothesized that SPL contributes to oxidative stress by depleting S1P pools available for cardioprotective signaling. Accordingly, we evaluated SPL inhibition as a strategy for reducing cardiac ischemia/reperfusion (I/R) injury. We measured SPL expression and enzyme activity in murine hearts. Basal SPL activity was low in wildtype cardiac tissue but was activated in response to 50 min of ischemia (n = 5, P < 0.01). Hearts of heterozygous SPL knockout mice exhibited reduced SPL activity, elevated S1P levels, smaller infarct size and increased functional recovery after I/R compared to littermate controls (n = 5, P < 0.01). The small molecule tetrahydroxybutylimidazole (THI) is an FDA-approved food additive that inhibits SPL. When given overnight at 25 mg/L in the drinking water, THI raised S1P levels and reduced SPL activity (n = 5, P < 0.01). THI reduced infarct size and enhanced hemodynamic recovery in response to 50 min of ischemia and to 40 min reperfusion in ex vivo hearts (n = 7, P < .01). These data correlated with an increase in Mnk1-, eIF4E- and S6-phosphorylation levels after I/R, suggesting that SPL inhibition enhances protein translation. Pretreatment with an S1P₁ and 3 receptor antagonist partially reversed the effects of THI. These results reveal for the first time that SPL is an ischemia-induced enzyme that can be targeted as a novel strategy for preventing cardiac I/R injury.
Keywords: sphingosine-1-phosphate, S1P lyase, ischemia/reperfusion, sphingolipid, Sgp11

Running head:
S1P lyase inhibition is cardioprotective
INTRODUCTION

Sphingosine-1-phosphate (S1P) is a sphingolipid metabolite that promotes cell survival and inhibits apoptosis through activation of its cognate G protein-coupled receptors, resulting in induction of multiple cell survival signaling pathways (4). S1P signaling contributes to cardiac development, angiogenesis, HDL-mediated effects, and regulation of vascular tone and permeability (7, 17, 21, 28). Recent studies have implicated sphingolipid signaling in protection from ischemia/reperfusion (I/R) injury in heart and other tissues, as well as activation by pre- and post-conditioning regimens (8-13, 15-18, 21, 29, 31, 32, 36). These studies collectively indicate that reduced S1P synthesis and signaling contribute to I/R injury, whereas increasing S1P signaling through specific S1P receptors may provide therapeutic benefit in the setting of I/R. Unfortunately, delivery of S1P is challenging due to binding by carrier proteins, ubiquitously expressed receptors and rapid metabolism. S1P receptor agonists such as FTY720 enhance functional recovery after I/R (6). However, FTY720’s clinical utility may be limited by bradycardia stemming from lack of receptor specificity. Thus, development of alternative methods for modulating S1P signaling for therapeutic benefit is warranted.

S1P lyase (SPL) catalyzes the irreversible degradation of S1P (14). By reducing S1P pools available for autocrine and paracrine signaling, SPL promotes apoptosis under stress conditions, whereas SPL downregulation promotes cell survival. We hypothesized that modulation of SPL would affect the
outcome of I/R injury in the heart. Accordingly, we measured SPL protein
eexpression and activity in cardiac tissue at baseline and during ischemia. We
further tested the contribution of SPL to cardiac injury after I/R using a genetic
SPL knockout mouse model and a chemical SPL inhibitor. Our results suggest
that SPL contributes to I/R injury and that SPL inhibition represents a promising
strategy to mitigate cardiac I/R injury.
METHODS

Animals and drug treatment

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The study was approved by San Francisco VAMC and CHORI Institutional Animal Care and Use Committees. Male wildtype C57Bl/6 mice weighing 19–25 gm were from Charles River (Hollister, CA). SPL gene-trap knockout mice were a kind gift from Philippe Soriano (Mount Sinai School of Medicine, NY) (22). These mice were maintained on a mixed C57BL6/129sv background by mating of heterozygotes, and wild type littermates were used as controls. Genotyping was performed as described previously (3). All mice received standard rodent chow. Wildtype C7Bl/6 mice received vehicle or 25 mg/L THI (roughly 200-fold more than would be ingested in a normal human diet per day) administered ad libitum in water containing 5.5 mmol/L glucose for 24h prior to euthanasia (23). Glucose was added to the water to improve palatability of the THI solution; water intake was not different between the vehicle- and THI-treated groups. SPL knockout mice received only vehicle.

Ischemia/Reperfusion Injury

Ex vivo I/R studies were performed essentially as described (8). WT and SPL heterozygous null mice (males, weighing 22–25 g) were heparinized (500 U/kg, i.p.) and anaesthetized with sodium pentobarbital (60 mg/kg, i.p.). Hearts
were rapidly excised, washed in ice-cold arresting solution (NaCl 120 mmol/L, KCl 30 mmol/L), and cannulated via the aorta on a 20-gauge stainless steel blunt needle. Hearts were perfused at 70 mmHg on a modified Langendorff apparatus using Krebs–Henseleit solution containing (mmol/L) NaCl 118, KCl 4.7, CaCl2 2.5, MgSO4 1.2, KH2PO4 1.2, NaHCO3 24, glucose 5.5, and sodium pyruvate 5.0 bubbled with 95% O2/5% CO2 at 37°C.5 Platinum electrodes connected to a stimulus generator (Grass Instruments, West Warwick, RI) were used to pace hearts at 360 b.p.m. An isovolumic balloon filled with degassed distilled water was inserted into the left ventricle (LV) to record hemodynamics as previously described (9-11). The protocol consisted of a 20 min equilibration period, followed by 50 min of global ischemia and 40 min of reperfusion. In some studies, the index ischemia time was reduced to 40 min. For ischemic preconditioning (IPC), hearts were equilibrated for 16 min and then subjected to two short cycles of IR, each consisting of 2 min of global ischemia and 2 min of reperfusion, followed immediately by prolonged I/R as described above. Some hearts received the S1P receptor 1 and 3 antagonist VPC 23019 (Avanti Polar Lipids, Inc., Alabaster, AL) administered at 1 µmol/L for 20 min during the 20 min equilibration period.

For measurement of infarct size after I/R±IPC, hearts were infused with 15 ml 1% triphenyltetrazolium chloride (Sigma) in phosphate-buffed saline at a rate of 1.5 ml/min as previously described (9-11). Hearts were then removed from the cannula, weighed, and fixed overnight in 10% formalin. Hearts were removed from formalin and stored at −20 °C until sectioning for analysis of LV
The infarct size of each section was expressed as a fraction of the area at risk defined as the total area of the left ventricle in this global ischemia model.

**Antibodies and reagents**

Antibodies were raised against the murine SPL C-terminal peptide VTQGNQMNGSPKPR. Anti-S6-phosphoprotein, anti-phospho-eukaryotic translation initiation factor 4E (p-eIF4E), anti-p-Mnk1/2, and horseradish peroxidase-linked anti-rabbit IgG were from Cell Signaling (Danvers, MA). Anti-actin and triphenyltetrazolium chloride (TTC) were from Sigma-Aldrich. Rabbit polyclonal anti-β-galactosidase was from Abcam. Tetrahydroxybutyrimidazole (THI) was from Albany Molecular Research Inc. Omega(7-nitro-2,1,3-benzoxadiazol-4-yl)-D-erythro-S1P (NBD-S1P) and VPC 23019 were from Avanti Polar Lipids (Alabaster, AL)

**Western blotting and Immunohistochemistry (IHC)**

Measurement of SPL, p-eIF4E, p-S6 and p-Mnk1/2 were performed using standard SDS-PAGE Western blotting as previously described in our laboratory (20). Immunoreactive bands were detected by enhanced chemiluminescence (ECL) (Amersham Bioscience, Piscataway, NJ) and quantified by densitometric analysis of digitized autoradiograms with NIH Image 1.61 software.

For IHC, a β-galactosidase antibody was diluted to 1:500 and incubated for 1 hour with fixed cardiac tissue from SPL reporter mice or littermate control
mice lacking the reporter. Incubation with secondary antibody (anti-rabbit IgG
diluted 1:1000) followed for 1h. Four micrometer-thick paraffin sections were
stained with Mayer's H&E or immunostained. Detection of the secondary
antibody was performed with the VECTASTAIN ABC Elite Kit (Vector
Laboratories, CA). Images of slides were captured using 20x and 40x objectives
on a Carl Zeiss (Thornwood, NY) AxioScope microscope with AxioCam camera
and processed using Adobe Photoshop (Adobe Systems, Inc., San Jose, CA)
software.

SPL activity

SPL activity was determined using a fluorescent assay as described in our
laboratory (2). Briefly, 20 µM NBD-S1P in SPL reaction buffer (0.6 mM EDTA, 0.4
mM pyridoxal 5'-phosphate, 3 mM DTT, 70 mM sucrose, 36 mM potassium
phosphate buffer, and 36 mM NaF) was mixed with 0.08% of Triton X-100 and
sonicated briefly. Reaction was carried out by adding 25-50 µg protein at 37ºC
for 30 minutes. At the end of the reaction, lipids were extracted into organic
phase by two-phase separation. The lower organic phase was dried,
resuspended in methanol, and injected onto a 4.6 × 75 mm Luna C18 HPLC
column (Phenomenex, Torrance, CA). The mobile phase consisted of solvent A
(water) and solvent B (methanol/5 mM acetic acid in water/1 M tetra-n-
butylammonium dihydrogen phosphate (TBAP), 95:4:1 (v:v:v)). The peaks
corresponding to fluorescent, NBD-containing compounds were integrated for
quantification.
S1P quantification

For S1P measurements, mouse heart tissue, equivalent to 25% of a whole heart, was homogenized in 0.5 ml of methanol using a glass homogenizer and a tip sonicator. Following homogenization, 1.0 ml of chloroform/methanol 1:1 was added and the sample was incubated over night at 48°C. Sample was dried down and resuspended in 3 ml of chloroform/methanol 2:1, and the extract was made basic by adding 50 µl of 1 M KOH in methanol. A two-phase separation was obtained by adding 0.5 ml of water. The aqueous phase was recovered and made acidic by adding 0.1 ml of concentrated acetic acid. Another two-phase separation was obtained by adding 1 ml of chloroform/methanol 2:1 and the organic phase was recovered. S1P was extracted from 10 µl of mouse plasma by adding 0.4 ml of methanol followed by vortexing and incubation for 30 min at 30°C. The sample was spun at in tabletop centrifuge at 14,000 g and the supernatant was recovered. $^{17}$C-S1P was used as an internal standard. Lipids were separated on a C18 column (2.1 x 50 mm; Kinetex) (Phenomenex, Torrance, CA) at a flow rate of 0.25 ml/min. The gradient used was from 45% to 99% methanol containing 1% acetic acid and 5 mM ammonium acetate. The data were acquired in positive mode on a Micromass Quattro LCZ (Waters Corp.) mass spectrometer. Lipids were identified based on their specific precursor and product ion pair and quantitated using multiple reaction monitoring as described (27).

Statistical analysis
Statistical significance was determined by Student’s two-tailed t-test for comparison of the means of two samples. Significance is set at $p < 0.05$. 
RESULTS

SPL expression and activity in the mammalian heart.

The SPL gene Sgpl1 is transcriptionally active in murine heart tissues (37). Therefore, we compared immunohistochemical detection of a β-galactosidase reporter in hearts of SPL gene-trap knockout mice and wild-type littermate controls. Our results revealed specific signals in endothelium, endocardium lining the valve, and cardiomyocytes (Figure 1A). In contrast, fibroblasts comprising the valve stroma and scattered throughout the myocardium appeared negative. To confirm SPL expression in wildtype cardiomyocytes, immunoblotting of isolated adult murine cardiomyocytes was performed. As shown in the three control blots at the left of Figure 1B, SPL protein is readily expressed in isolated heart cells. Consistent with this finding, SPL activities of 2-5 pmol/mg/min were detected in adult cardiac tissues.

SPL activity is induced during ischemia.

To determine whether cardiac SPL activity is affected by ischemia, the Langendorff model was employed to induce ischemia and I/R in ex vivo wildtype murine hearts using the treatment scheme shown in Figure 2A. We have shown previously that IPC enhances the biosynthesis of S1P by increasing sphingosine kinase activity (11). Considering these previous findings, we also examined whether IPC influences the activity of the major enzyme responsible for S1P degradation, namely SPL. As shown in Figure 2B, SPL activity in ischemic
tissues increased three-fold over baseline. SPL activity returned to near baseline levels in I/R-treated tissues during reperfusion. SPL activity was lower in IPC plus ischemia-treated tissues than baseline, although results did not reach statistical significance. In contrast to the dynamic response of SPL activity, SPL protein expression remained constant under these conditions (Figure 1B). Based on these results, we conclude that SPL is activated by ischemia and may be inhibited by IPC.

**SPL loss-of-function mutant mice are resistant to I/R-induced cardiac injury.**

To ascertain whether SPL activation contributes to cardiac I/R injury we used a genetic model of SPL deficiency. Because mice homozygous for a targeted deletion of the murine SPL gene *Sgpl1* do not survive beyond approximately one month after birth, we compared mice heterozygous for the *Sgpl1* null allele to age- and gender-matched wild-type littermate controls. Further, to evaluate the importance of SPL in regulating cardiac S1P, we quantified S1P levels in wild-type and SPL-deficient mouse hearts at baseline and after I/R. As shown in Figure 3A, tissue SPL activity in heterozygous SPL null mice is approximately half that of littermate controls. Hearts were isolated from heterozygotes and wild-type littermate control mice, mounted on a Langendorff apparatus, equilibrated, and subjected to I/R. Hemodynamic function was followed by continuous monitoring of left ventricular developed pressure (LVDP) and infarct size was measured at the end of the experiment as described
under Methods. As shown in Figure 3B, SPL heterozygotes showed a two-fold
increase in cardiac S1P levels compared to wild-type littermates under baseline
conditions. After I/R, S1P levels decreased in both heterozygote and wild-type
hearts, consistent with SPL activation. Importantly, as shown in Figures 3 C and
D, mice constitutively deficient in SPL demonstrated significantly reduced infarct
size and increased hemodynamic recovery from I/R injury compared to controls.

Brief oral THI pretreatment affords cardioprotection from I/R-induced
injury.

In order to address whether the cardioprotective effects afforded by SPL
reduction observed in a genetic model has translational relevance, it became
important to assess the potential cardioprotective effects of pharmacological SPL
inhibition. Recently, THI, an imidazole compound found in FDA-approved
caramel food coloring #3, was shown to inhibit SPL when administered orally to
mice (23). The only other small molecule SPL inhibitor that could be used safely
in vivo is the vitamin B6 analog deoxypyridoxine, which has nonspecific activity
against all B6-dependent enzymes. Therefore, we employed THI to achieve
pharmacological SPL inhibition and test the effect of this strategy on ischemic
heart injury in wildtype hearts. Toward that end, THI was placed in the drinking
water for 24h prior to euthanasia. Initially we tested several different regimens of
THI administration including 24h treatment with 25 mg/L, 24h treatment with 50
mg/L, and 3 days of treatment at both 25mg/L and 50 mg/L concentrations.
Surprisingly, maximal cardioprotection was obtained using the lower dose of THI
for 24h, whereas increasing the concentration or the length of exposure resulted in diminished treatment efficacy (data not shown).

As shown in Figures 4A and B, THI at 25 mg/L given for 24 h reduced tissue SPL activity and raised cardiac and plasma S1P levels. THI treatment reduced the size of infarction from an average of 29.6 ± 2.4 % of the risk area in control hearts to 15.1 ± 4.0 % in hearts from the mice treated with THI (Figures 4C and 4E). Functional recovery was also improved by THI treatment. As shown in Figure 4D, LVDP at the end of the experiment averaged 27.8 ± 4.1 mmHg in control vs. 52.3 ± 7.5 mmHg in THI-treated hearts.

**S1P cell surface receptor antagonism reduces the effect of THI.**

We next asked whether inhibition of cell surface S1P receptors would blunt the effects of THI. In isolated mouse hearts we infused the S1P1 and 3 receptor antagonist VPC 23019 at a concentration of 1 µmol/L for 20 min during 20 min of equilibration. Hearts (n = 4/group) were then subjected to 50 min of ischemia and 50 min of reperfusion. Hearts were from mice treated with THI ± VPC 23019 or controls treated with the S1P receptor antagonist alone. As shown in panels A and B of Figure 5, VPC 23019 alone had no effect on infarct size and hemodynamic recovery compared to hearts from mice not treated with this agent (compare with Figure 4, panels C and D). As expected, THI treatment by itself reduced infarct size dramatically and improved hemodynamic recovery markedly, also consistent with the data in Figure 4, panels C and D. VPC 23019 pretreatment of hearts from mice that received THI showed an intermediate
response: infarct size was significantly reduced but not to the level of THI alone.

The same was true for LVDP, which was augmented but not to the level of the hearts treated with THI alone. These data establish receptor involvement in THI-mediated cardioprotection but do not exclude an intracellular role for S1P.

SPL inhibition potentiates activation of key regulators of protein translation after I/R.

Cell survival and containment of tissue damage after I/R are facilitated by stress-induced signaling pathways that activate the protein translational machinery. To explore whether SPL inhibition influences this process, we measured the activation status of two key components of the translational machinery, ribosomal S6 protein and eIF4E, by monitoring their phosphorylation status in the hearts of wildtype THI-treated and vehicle-treated mice. As shown in Figure 6A, phosphorylation of eIF4E and S6 were both elevated after I/R in control and THI-treated hearts. However, phosphorylation of both these proteins was significantly higher in THI-treated hearts. Similarly, hearts of THI-treated mice subjected to I/R exhibited elevated levels of phosphorylated Mnk1/2, the protein kinase responsible for eIF4E phosphorylation, compared to vehicle-treated I/R control hearts. Quantitative analysis of this immunoblot is shown in Figure 6B. Quantitated differences in protein levels between THI-treated and vehicle-treated hearts reached statistical significance (p < 0.05) except for eIF4E (p = 0.05). Based on these results, we conclude that THI treatment activates a signaling pathway that involves Mnk/eIF4E and ribosomal S6 protein.
Importantly, we found that the levels of eIF4E, Mnk1/2 and S6 phosphorylation observed in heart tissues exposed to the combination of THI pretreatment administered *in vivo* and VPC 23019 given *ex vivo* prior to I/R were markedly lower than that observed in heart tissues exposed to THI alone. In the case of eIF4E, phosphorylation was almost completely blocked whereas Mnk1/2 and S6 phosphorylation levels were intermediate between those observed in hearts receiving either agent alone, as shown in Figures 6C and D. We observed some variability in our results, some of which may be accounted for by the use of different antibody lots between the two experiments. Despite these variations, our results show clearly that THI effects on the protein translational machinery are attenuated by VPC 23019. These results demonstrate that the activation status of the Mnk/eIF4E and S6 signaling pathway correlates with THI-mediated cardioprotection. Further, both THI-mediated cardioprotection and its effects on the protein translation machinery appear to be at least partially dependent upon S1P receptor signaling.
DISCUSSION

The principal and most remarkable finding of this study is that we show for the first time that short-term inhibition of SPL protects mouse hearts against I/R injury. Our study also reveals that SPL is expressed in murine heart. Although cardiac SPL protein levels remained constant under different ex vivo conditions, SPL activity was rapidly induced in hearts subjected to I/R, presumably through a post-translational mechanism. This possibility is consistent with the identification of SPL in a screen for nitrosylated proteins, and with the presence of potential sites for protein kinase phosphorylation within mammalian SPL protein sequences and the recent identification of SPL as a target of phosphorylation by ATM/ATR (19, 24, 35).

In our study, a combination of genetic and pharmacological models of SPL inhibition was used to test the role of SPL in I/R, since no specific inhibitor of SPL is available to date. We observed cardioprotection in a genetically altered mouse model exhibiting reduced SPL expression and activity and increased S1P levels. This model was generated by gene trap mutagenesis resulting in a null allele (22). In the homozygous state, SPL mutants are runted and die early, whereas heterozygotes are reproductive and live a normal lifespan. In this mouse model, reduced SPL activity observed in the heterozygote does not confer a phenotype in the heart under resting conditions, but the response to acute ischemia is markedly enhanced.
Importantly, in wildtype C57/Bl6 mice inhibition of SPL by placing the FDA- approved food additive THI (caramel coloring #3) in the drinking water for 24 h increased S1P levels, reduced infarct size and improved functional recovery after I/R. The results we have obtained using both of these models strongly implicate SPL as the target of THI that is responsible for mediating cardioprotection. Some differences in the extent of responses between THI administration and SPL heterozygosity were noted. This may be accounted for by the fact that these two experiments were performed in mouse lines of different genetic backgrounds. However, the most likely explanation for this difference is that the SPL heterozygote null mouse represents a chronic state of inhibition of the sphingolipid degradative pathway. In homozygotes, this has been shown to result in accumulation of other sphingolipid intermediates upstream of S1P that may be damaging to the myocardium, such as ceramide or sphingosine (3). We suspect that targeting SPL using a small molecule inhibitor for a brief time period avoids the potential untoward complications of the genetic model, thereby unmasking the full effect of S1P as a cardioprotectant.

The Langendorff system we utilized for these experiments is a constant pressure system set at 70 mmHg (11). Since coronary perfusion pressure was constant, coronary flow reflects coronary vascular resistance. At baseline, coronary flow as measured by coronary sinus effluent over time did not differ between the control and THI groups (2.9+/−0.04 vs 2.92+/−0.03 ml/min). After recovery from ischemia/reperfusion injury, the coronary flow was higher in THI group (1.88 ± 0.07 vs 1.52 ± 0.06 ml/min, n = 6, P < 0.01). The following factors
may be involved: control hearts had a larger infarct size, which leads to a larger area of no flow and hence an overall reduction in coronary flow. Also, as shown in Figure 4, ischemia/reperfusion injury resulted in higher diastolic pressures and lower systolic pressures in the control vs. the THI-treated hearts. The marked reduction in developed pressure would also be expected to contribute to a reduction in coronary flow. Similar results were found in the SPL heterozygous hearts subjected to I/R injury compared to wildtype controls.

As noted above, there is substantial evidence that administration of S1P acutely is cardioprotective (8-12, 17, 21, 29, 31, 32, 36). Although the degree of S1P elevation in response to THI pre-treatment was modest, it exceeds endogenous levels relative to baseline achieved by ischemic preconditioning. These levels are sufficient to mediate cardioprotection in rat models of I/R, as we have reported previously (32, 33). We observed that pre-treatment of mice with a low concentration of THI for 24h afforded greater protection than did a higher dose and/or longer treatment periods such as those used by Schwab et al. to induce lymphocyte depletion (23). In this connection, it has recently been reported that acute \textit{in vitro} S1P degradation is primarily driven by lyase cleavage, a process that takes only 40-100 minutes (25). The translational relevance of these findings will require further testing using SPL inhibitory strategies in post-ischemic delivery regimens and/or through the use of inducible SPL knockout mouse models.

The shortened life-span and congenital anomalies observed in homozygous SPL null mice demonstrate that complete SPL inhibition is not well-
tolerated and that SPL is an essential protein (3, 22, 34). In contrast, mice exhibiting low levels of SPL expression and activity do not exhibit notable phenotypes, and pharmacological inhibition of SPL has proven useful for immunomodulation (1, 34). Thus, we interpret our data to indicate that brief and incomplete inhibition of SPL may serve to raise and maintain S1P during I/R injury at levels sufficient to capitalize on the protective effects of S1P signaling. Accordingly, the complications that may be associated with long-term total inhibition of sphingolipid degradation can be avoided.

We hypothesize that cardioprotection associated with SPL inhibition or reduced SPL expression can be attributed primarily to accumulation of S1P and resulting intracellular and/or receptor-mediated effects. Because THI increases S1P levels both in tissue and in the plasma, we sought to determine whether cell surface S1P receptors are involved in its beneficial effects. We found that the S1P$_1$ and 3 receptor antagonist VPC 23019 significantly reduced the cardioprotective effects of THI feeding. However, there was still a residual reduction of infarct size and an increase in hemodynamic recovery. One possibility is that there is an additional effect of THI on intracellular or even on mitochondrial S1P. Recent evidence has identified a pool of S1P generated by sphingosine kinase-2 that regulates gene transcription (5). Of direct pertinence to the present study is that S1P produced by sphingosine kinase-2 in the mitochondria interacts with prohibitin 2 to regulate the assembly and function of a key member of the electron transport chain, complex IV or cytochrome oxidase.
(26). By raising intracellular S1P levels, THI might affect one or both of these processes. Whether this is the case awaits future investigation.

However, it is possible that other factors may be involved. For example, SPL catalyzes the formation of the reactive long-chain aldehyde hexadecenal. The latter could enhance oxidant stress produced by I/R, thereby potentiating tissue injury. Conversely, oxidant stress during ischemia may be equivalent or even additive to pharmacologic post-conditioning with S1P, which is cardioprotective (8, 32). Future studies will be required to clarify how modulation of SPL expression and activity influence the heart in the context of I/R and whether S1P and S1P receptors are necessary for mediating the cardioprotective effects of THI. SPL is expressed in cardiomyocytes, fibroblasts and endothelial cells, as well as in other cells and tissues extrinsic to the heart. Further studies employing tissue-specific SPL knockout models may be helpful in identifying the specific tissue source of SPL that is required for modulating the cardiac response to I/R.

We observed no obvious differences in apoptotic or autophagic programmed cell death between THI-treated and vehicle-treated ischemic heart tissues, based on levels of the cleaved form of the caspase-3 substrate poly ADP ribose polymerase (PARP) as a marker of apoptosis and the processed form of the autophagy marker LC3 by immunoblotting (data not shown). These were not unexpected findings, since it would be unlikely for either intrinsic or extrinsic apoptotic pathways to be activated within the short time frame in which our
studies were conducted. Based on our results, we attribute the protection from myocardial tissue injury afforded by THI to prevention of necrotic cell death. The effects of THI pre-treatment on I/R recovery and infarct size correlated with elevated levels of phosphorylated S6, Mnk1/2 and eIF4E. Furthermore, both THI-mediated cardioprotection and protein phosphorylation effects were partially reversed by inhibition of signaling through S1P₁ and 3. Thus, the effects of THI on S1P receptor signaling appear to contribute to its effects on the protein translational machinery. All three of these proteins are downstream targets of stress-activated pro-survival signaling pathways that are influenced by S1P, including PI3K, and MEK/ERK MAPK pathways (29, 30, 36). Our cumulative findings suggest that pretreatment with THI primes specific stress activated signaling pathways, thereby preparing tissues to respond to ischemic injury and contributing to its cardioprotective effects. Preservation of protein translational activity may contribute to the ability of THI to mediate cardioprotection, but it is likely that other factors affected by the immediate response to stress signaling are also involved.

In summary, our findings demonstrate that short-term SPL inhibition may be a novel strategy to protect against acute cardiac ischemia/reperfusion injury. Small molecule inhibitors of SPL are currently in clinical trials as immunomodulatory agents. Based on our findings, we hypothesize that these agents could potentially mitigate I/R injury and other forms of cardiac insult or ischemic tissue damage. In conclusion, this is the first demonstration that short-term SPL inhibition using an FDA-approved food additive is cardioprotective.
ACKNOWLEDGMENTS

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Figure 1: S1P lyase (SPL) expression and activity in murine heart. (A) Immunohistochemical detection of a β-galactosidase reporter in hearts of SPL gene-trap reporter mice and reporter-less littermate controls. β-galactosidase is detected in endothelium (arrows), endocardium lining the valve (v) and cardiomyocytes. (B) SPL protein expression in wildtype heart tissue. See text for details. MSPL = murine S1P lyase.

Figure 2: S1P lyase (SPL) activity and expression in response to ischemia/reperfusion injury in wildtype hearts. (A) Ischemia reperfusion (I/R) protocols. IPC = ischemic preconditioning. (B) SPL enzyme activity. n = 5 hearts per treatment. Con-Total = 110 min equilibrium normoxic control. C vs. I = control vs. ischemia; C vs. IR = control vs. ischemia followed by reperfusion (I/R); I vs. IPC = ischemia vs. ischemic preconditioning (IPC). * = P < 0.05.

Figure 3: Mouse models with reduced S1P lyase (SPL) expression are less susceptible to I/R injury. (A) Tissue (intestine) SPL activity and (B) cardiac S1P levels in mice heterozygous for a gene-trap targeted disruption of Sgpl1 and littermate controls. Infarct size (C) and left ventricular developed pressure (LVDP) (D) after I/R. Four WT and 5 heterozygous mouse hearts were used. * = P < 0.05.
Figure 4: THI pretreatment protects against ischemia/reperfusion injury of hearts from wildtype mice. (A) Tissue (thymus) SPL activity and (B) cardiac and plasma S1P levels in vehicle- and THI-treated mice. (C) Infarction size in control (CON) and THI-treated (THI) mice as percentage of the area at risk, which is the entire left ventricle in this global ischemia model. Seven control and 6 THI-treated mice were used. (D) Left ventricular developed pressure at the end of reperfusion (LVDP). (E) Typical infarcts in control and THI-treated hearts. Arrows point to representative infarct areas. (F) Representative examples of left ventricular developed pressure in control and THI-treated hearts. As can be seen the developed pressure is substantially greater in the THI-treated heart at the end of reperfusion. See panel D for group details. SPL = sphingosine 1-phosphate lyase; THI = tetrahydroxybutylimidazole. * indicates statistical significance. (P < 0.05).

Figure 5. The S1P cell surface receptor antagonist VPC 23019 reduces the effect of THI. Mouse hearts were subjected to ischemia/reperfusion injury as described under Methods (n = 4/group). (A). Compared with THI, the S1P antagonist VPC 23019 had no significant effect on left ventricular developed pressure (LVDP) at the end of reperfusion. Values expressed as a percent of baseline were similar to those obtained in the absence of VPC [compare with panel D of Figure 4]. In hearts harvested from THI-treated mice, the results were intermediate. (B). Infarct size expressed as a percent of the risk area, which is
the entire left ventricle in this global ischemia model, was reduced by treatment with THI and VPC alone had no effect on the extent of infarction [compare with panel C of Figure 4]. However, in the presence of VPC, the results in THI-treated mice were intermediate. These data establish S1P receptor involvement but also suggest a possible intracellular role for S1P. Statistics: One-way ANOVA with post-hoc Student-Newman-Keuls testing. * = P < 0.05 vs. all other conditions.

**Figure 6. THI-treatment potentiates activation of protein translation after I/R in wildtype mouse hearts.** (A) phosphorylated eIF4E (p-eIF4E), phosphorylated S6 (p-S6), phosphorylated Mnk1/2 (p-Mnk), and actin levels in heart tissues from THI-treated and control mice before and after I/R. (B) ImageJ quantification of post-IR signals normalized to actin. These blots are representative of four separate experiments. THI = tetrahydroxybutylimidazole. * indicates P < 0.05 vs. Con I/R. (C) phosphorylated eIF4E (p-eIF4E), phosphorylated S6 (p-S6), phosphorylated Mnk1/2 (p-Mnk), and actin levels in tissues from THI-treated, VPC-treated and THI plus VPC-treated hearts after I/R. (D) ImageJ quantification of results shown in (C). * indicates P < 0.05 vs. THI I/R.
Figure 2
Figure 4
Figure 5
Figure 6