Sphingosine-1-Phosphate Receptor 1 Regulates Cardiac Function by Modulating Ca$^{2+}$ Sensitivity and Na$^+$/H$^+$ Exchange and Mediates Protection by Ischemic Preconditioning

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**Background—** Sphingosine-1-phosphate plays vital roles in cardiomyocyte physiology, myocardial ischemia–reperfusion injury, and ischemic preconditioning. The function of the cardiomyocyte sphingosine-1-phosphate receptor 1 (S1P1) in vivo is unknown.

**Methods and Results—** Cardiomyocyte-restricted deletion of S1P1 in mice (S1P1$^{αMHCCre}$) resulted in progressive cardiomyopathy, compromised response to dobutamine, and premature death. Isolated cardiomyocytes from S1P1$^{αMHCCre}$ mice revealed reduced diastolic and systolic Ca$^{2+}$ concentrations that were secondary to reduced intracellular Na$^+$ and caused by suppressed activity of the sarcolemmal Na$^+$/H$^+$ exchanger NHE-1 in the absence of S1P1. This scenario was successfully reproduced in wild-type cardiomyocytes by pharmacological inhibition of S1P1, or sphingosine kinases. Furthermore, Sarcomere shortening of S1P1$^{αMHCCre}$ cardiomyocytes was intact, but sarcomere relaxation was attenuated and Ca$^{2+}$ sensitivity increased, respectively. This went along with reduced phosphorylation of regulatory myofilament proteins such as myosin light chain 2, myosin-binding protein C, and troponin I. In addition, S1P1 mediated the inhibitory effect of exogenous sphingosine-1-phosphate on β-adrenergic-induced cardiomyocyte contractility by inhibiting the adenylyl cyclase. Furthermore, ischemic preconditioning was abolished in S1P1$^{αMHCCre}$ mice and was accompanied by defective Akt activation during preconditioning.

**Conclusions—** Tonic S1P1 signaling by endogenous sphingosine-1-phosphate contributes to intracellular Ca$^{2+}$ homeostasis by maintaining basal NHE-1 activity and controls simultaneously myofibril Ca$^{2+}$ sensitivity through its inhibitory effect on adenylyl cyclase. Cardioprotection by ischemic preconditioning depends on intact S1P1 signaling. These key findings on S1P1 functions in cardiac physiology may offer novel therapeutic approaches to cardiac diseases. (J Am Heart Assoc. 2016;5:e003393 doi: 10.1161/JAHA.116.003393)

**Key Words:** calcium sensitization • heart failure • ischemia reperfusion injury • Na$^+$/H$^+$ exchanger • preconditioning • signal transduction • sphingosine • sphingosine-1-phosphate

Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid that exerts major effects in cardiovascular physiology and disease. Plasma S1P levels have been associated with stable coronary artery disease, myocardial infarction, transient ischemia occurring during percutaneous coronary interventions, and coronary in-stent restenosis.1–5

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Accompanying Data S1, Table S1 and Figures S1, S2 are available at http://jaha.ahajournals.org/content/5/5/e003393/DC1/embed/inline-supplementary-material-1.pdf

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S1P1 Regulates NHE-1 and Ca2+

S1P is an integral constituent of high-density lipoproteins and has been demonstrated to causally contribute to several of their beneficial effects.6,7 Recently, we have shown that diminished S1P content in HDL from patients with coronary artery disease is a cause of HDL dysfunction and that raising HDL–S1P therapeutically restored HDL function.7

Mechanistically, S1P can act as an intracellular signaling molecule and as an extracellular ligand for 5 G-protein–coupled receptors. Three are expressed in the heart (S1P1, S1P2, and S1P3) and were shown to mediate the effects of S1P on different aspects of cardiomyocyte biology.8–10 In experimental myocardial ischemia–reperfusion models, S1P generated endogenously by cardiac sphingosine kinases or administered exogenously prior to ischemia protects against reperfusion injury, whereas endogenous S1P mediates the cardioprotective effect of ischemic pre- and postconditioning.8,10,11 Exogenous S1P has been shown to protect through nitric oxide produced following activation of the endothelial S1P3 receptor,12 whereas endogenous S1P required Akt activation by both S1P2 and S1P3 for efficient cardioprotection.13 Mice deficient for S1P2 or S1P3 have no obvious cardiac phenotype except for the resistance of S1P3–/– mice to the bradycardic effect of the S1P analog fingolimod (Gilenya; Novartis).14 The S1P receptor responsible for S1P-mediated preconditioning had not been identified prior to our study. In humans, S1P1 gene polymorphisms have been associated with coronary artery disease and stroke,15,16 but addressing its physiological role in the heart in vivo has been hampered by the embryonic lethality of global S1P1 knockout mice.

In this study, we have examined the role of S1P1 in normal and pathophysiological cardiac function by generating mice with a cardiomyocyte-specific deletion. We provided evidence that S1P1 is indispensable for normal cardiac function, ion homeostasis, activity of the Na+/H+ exchanger NHE-1, and myofibrillar Ca2+ sensitivity. Furthermore, we addressed the role of S1P1 in myocardial ischemia–reperfusion injury and ischemic preconditioning (IP).

Methods

Mice

Mice homozygous for a floxed S1P1 allele17 were crossed with C57Bl6J mice heterozygous for the Cre recombinase under the control of the α-myosin heavy chain (αMHCCre)18 to obtain S1P1αMHCCrefl/fl mice and littermate controls (S1P1αMHCCrefl/+) and S1P1floxf/+ mice. All procedures followed were in accordance with institutional guidelines.

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Imaging, Echocardiography, and In Vivo Hemodynamic Measurements

Magnetic resonance imaging was performed using a 7-T Bruker NMR spectrometer and 18F-fluorodeoxyglucose positron emission tomography on a high-resolution small-animal camera (quadHIDAC; Oxford Positron), respectively. High-resolution echocardiography with quantitative 3-dimensional assessment of cardiac function was performed on an ultrasound device with frame rates up to 280 Hz (Philips Medical Systems). Left ventricular (LV) catheterization was performed in closed-chest anesthetized mice, as described previously,19 with dobutamine administered via the cannulated left jugular vein accompanied by measurements of heart rate, maximal LV pressure, and the first derivative of LV pressure.

Cardiomyocyte Diameter, Fibrosis, Real-Time Polymerase Chain Reaction, and Western Blotting

The mean cardiomyocyte diameter was measured in 100 cardiomyocytes with longitudinally cut nuclei on periodic acid–Schiff–stained sections and interstitial fibrosis assessed on picrosirius red–stained sections with an image analysis program (KS 300; Zeiss). For gene expression, total RNA was isolated from the left ventricle, cDNA was synthesized using the Revert Aid First Strand cDNA Synthesis Kit (Qiagen), and real-time polymerase chain reaction was performed on a Bio-Rad CFX96 system using iQ SYBR Green. Relative gene expression was calculated by the 2−ΔΔCT method and normalized to GAPDH. For Western blotting, myocardial tissue was homogenized in RIPA buffer, run on SDS-PAGE, transferred to nitrocellulose membranes, and blotted with antibodies to phospholamban, which was phosphorylated at serine 16 and threonine 17, respectively, sarcoplasmic reticulum Ca2+ ATPase 2a, junctin, and calsequestrin (for normalization). The electrochemiluminescence signal was quantified by a Bio-Rad ChemiDoc MP Imaging System. The phosphorylation level of cardiac myosin light chain 2, troponin I, and myosin-binding protein C was determined using the Pro-Q Diamond/Sypro Ruby staining kit (Molecular Probes), as described.20 Briefly, proteins on gels were stained for 1 hour with Pro-Q Diamond to determine phosphoprotein expression and overnight with Sypro Ruby to measure total protein content. Signals were visualized using the LAS-4000 Image Reader (Fuji Science Imaging Systems) and analyzed using the manufacturer’s Multi Gauge version 3.2 software. Phosphorylation levels of myofilament proteins in knockout hearts were expressed relative to those of controls.
Functional Characterization of Single Cardiomyocytes

Detailed methods are provided in Data S1. LV myocytes were isolated by enzymatic dissociation. Cell size and membrane capacitance of the isolated cardiomyocytes were similar in all genotypes.

Action potential recordings

Action potentials (APs) were recorded using the perforated patch-clamp technique using an Axopatch 200B amplifier (Molecular Devices). APs were filtered and digitized at 10 and 40 kHz, respectively. APs were elicited at 1 to 10 Hz by 3-ms, \( \approx 1.2 \times \) threshold current pulses through the patch pipette. Resting membrane potential; maximal AP amplitude; maximal upstroke velocity; AP duration at 20%, 50%, and 90% repolarization; and plateau amplitude were analyzed and averaged from 10 consecutive APs.

Intracellular concentration measurements

Intracellular concentrations of Ca\(^{2+}\), Na\(^+\), and pH (Ca\(^{2+}\), Na\(^+\), and pH, respectively) were measured in indo-1, SBFI, and SNARF-1–loaded myocytes preincubated in HEPES-buffered solutions containing 10 \( \mu \)mol/L SBFI-AM or 5 \( \mu \)mol/L indo-1-AM or 10 \( \mu \)mol/L SNARF-1-AM and 0.01% pluronic. Incubation durations were 10, 30 and 120 minutes. Fluorescence of all indicators was measured in dual-wavelength emission mode at 405/505, 410/590, and 580/640 nm. Fluorescence signals were corrected for background recorded from probe-free myocytes and calibrated to obtain maximum and minimum ratio values of the emitted wavelengths and the constants \( \beta \) and \( k_s\). Na\(^+\) was averaged over the entire cardiac cycle. Cytosolic free Ca\(^{2+}\) was obtained by correction of measured overall cellular fluorescence signals for contributions of mitochondrially compartmentalized indo-1. pH was determined from calibration curves obtained with nigericin, and NHE–1–dependent proton flux was calculated from the rate of recovery of pH following acid loading. The sarcoplasmic reticulum Ca\(^{2+}\) content of cells was studied by 20 mmol/L caffeine application.

Sarcomere length shortening

Sarcomere length (SL) was measured using a fast Fourier transform algorithm of the video image (200 frames per second) of the contracting myocytes (IonOptix).

cAMP measurements

The LANCE cAMP kit (PerkinElmer) was used to determine cAMP concentrations using a Victor plate reader (Wallac; PerkinElmer). All experiments were performed in the presence of 0.05% bovine serum albumin and 500 \( \mu \)mol/L of isobutyl-methylxanthine. Force measurements at different Ca\(^{2+}\) concentrations were performed on single demembranated cardiomyocytes, as described previously.\(^{20}\) Briefly, isolated cells were bathed in relaxing solution (in mmol/L: free Mg, KCl 100, EGTA 2, Mg-ATP 4, and imidazole 10; pH 7.0) under an inverted microscope (Zeiss Axiovert 135) and attached with silicone adhesive between a force transducer and a piezoelectric motor as part of a permeabilized myocyte test system (1600A, with force transducer 403A; Aurora Scientific). The myocyte was adjusted to 2.2–μm SL and exposed to a series of solutions with different pCa values ranging from 9.0 (relaxing) to 4.5 (maximal activation) to obtain the force–pCa relation (pCa = −log of [Ca\(^{2+}\)]). Force values were calculated relative to the maximal force at pCa 4.5. Mean values on relative force–pCa diagrams were fit with the Hill equation. On the sigmoidal Hill curve, the pCa at 50% of maximum developed force is the Ca\(^{2+}\) sensitivity of the contractile apparatus.

Ischemia–Reperfusion Injury and IP

Ischemia–reperfusion injury and IP were performed, as described.\(^{21}\) In brief, mice of either sex were anesthetized with pentobarbital (80 mg/kg IP) and subjected to 30 minutes of occlusion of the left anterior descending coronary artery and 120 minutes of reperfusion without and with prior IP by 1 cycle of 5 minutes of occlusion of the left anterior descending coronary artery and 10 minutes of reperefusion. At the end of the experiment, the coronary artery was reoccluded, and Evans blue was injected intravenously to delineate the area at risk. After euthanizing the mice by KCl infusion, hearts were excised and cut into 4 to 5 transverse slices, and the infarct size was quantified by triphenyl tetrazolium chloride staining.

Statistical Analysis

Overall survival curves were estimated using the Kaplan–Meier method, and differences between genotypes were compared by the log-rank test. The Wilcoxon test was used in all experiments with small to moderate sample sizes (<10). ANOVA followed by a Bonferroni post hoc test was used to test for significant differences among groups. For the invasive hemodynamic measurements (Figure 2C), 2-way repeated-measures ANOVA was used, and the indicated significant differences are from the omnibus test. Average data are presented as mean±SEM. Values of \( P<0.05 \) were considered statistically significant.

Results

Impaired Cardiac Function in Mice Carrying a Deletion of S1P\(_1\) in Cardiomyocytes

In the murine heart, S1P\(_1\) is the most abundantly expressed S1P receptor (Figure 1A). To address its function in vivo, we
generated mice deficient for S1P1 specifically in cardiomyocytes (S1P1<sup>αMHC<sup>Cre</sup></sup> mice). S1P1<sup>αMHC<sup>Cre</sup></sup> mice were born with the expected mendelian frequency and appeared indistinguishable from their controls (S1P1<sup>fl<sup>x<sup/>f<sup>x</sup></sup> mice) at birth; however, S1P1<sup>αMHC<sup>Cre</sup></sup> mice began to die prematurely from 34 weeks on, with a median survival time of 45 weeks (Figure 1A). On autopsy, spontaneously deceased mice presented with enlargement of all cardiac chambers (Figure 1B). Cardiac echocardiography (Figure 1C) at 34 and 48 weeks and magnetic resonance imaging (Figure 1D) in S1P1<sup>αMHC<sup>Cre</sup></sup> mice aged 48 weeks revealed clearly impaired LV function compared with controls, as demonstrated by increased end-
diastolic and end-systolic volumes, increased LV mass, and reduced ejection fraction and stroke volume. Serial 18F-fluorodeoxyglucose positron emission tomography imaging excluded myocardial viability defects as a cause of impaired function (Figure 1E). Histomorphometry of the left ventricle revealed cardiac fibrosis in S1P1αMHCCre mice that increased with age but no alterations in cardiomyocyte size (Figure 2A). LV gene expression was altered in S1P1αMHCCre mice: Expression of atrial natriuretic peptide and β-myosin heavy chain was increased ≈6-fold, expression of brain natriuretic peptide was increased 2-fold, and expression of modulatory calcineurin inhibitory protein 1 was increased ≈5-fold compared with controls (Figure 2B).

Invasive cardiac catheterization was then performed to assess LV function in response to adrenergic stimulation in S1P1αMHCCre and control mice aged 38 to 40 weeks (Figure 2C). Maximal rates of contraction and relaxation were reduced with increasing doses of dobutamine in S1P1αMHCCre mice, whereas heart rates and maximal LV pressure were similar to controls (Figure 2C). Similar observations were made in 19-26 weeks old mice (Figure S1).

Reduced Ca2+ Concentration, Increased Ca2+ Sensitivity, and Attenuated Sarcomere Relaxation in S1P1αMHCCre Cardiomyocytes

To explore the role of S1P1 at the single-cell level, APs, Ca2+ transients, and sarcomere shortening were examined in single cardiomyocytes from S1P1αMHCCre mice and littermate controls. APs elicited at 1 to 8 Hz were significantly prolonged in S1P1αMHCCre cardiomyocytes (to 140% for AP duration at 90% repolarization), whereas all other AP parameters were unchanged (Figure 3A). Furthermore, a substantial decrease in diastolic and systolic Ca2+ was observed in S1P1αMHCCre cardiomyocytes compared with controls, whereas Ca2+ transient amplitude remained unchanged (Figure 3B). Time to peak (between 4 and 8 Hz) and time to 80% Ca2+ transient decay (at 4–6 Hz) were delayed (data not shown). Ca2+ concentration released following caffeine application was clearly reduced in S1P1αMHCCre cardiomyocytes, indicating reduced sarcoplasmic reticulum content (Figure 3C). Basal cAMP levels were increased in S1P1αMHCCre cardiomyocytes (Figure 3D), in agreement with the coupling of S1P1 to Gs and its inhibitory effect on adenylate cyclase. Despite the lower absolute Ca2+ values, no changes in diastolic or systolic sarcomere length (SL) or SL amplitude were observed in S1P1αMHCCre cardiomyocytes (Figure 3E); however, the rate of SL shortening was reduced, and the shortening durations increased (time to 90% relaxation at 2 Hz in controls 103±4 versus 120±5 ms in S1P1αMHCCre).

To test whether the preserved contractility of S1P1αMHCCre cardiomyocytes, despite their lower Ca2+, was caused by increased Ca2+ responsiveness of the contractile apparatus, we examined Ca2+ sensitivity in single skinned cardiomyocytes. Indeed, the Ca2+ sensitivity of force development was clearly increased in skinned S1P1αMHCCre cardiomyocytes compared with controls (Figure 4A). Furthermore, the phosphorylation status of
regulatory myofilament proteins such as myosin light chain 2 and myosin binding protein C was clearly reduced and that of troponin I showed a similar trend (Figure 4B).

Protein levels of sarcoplasmic reticulum Ca\(^{2+}\) ATPase 2a, junctin, and total and phosphorylated phospholamban were not altered (Table S1).

**Figure 2.** Structural and biochemical changes. A, Cardiomyocyte diameters and cardiac fibrosis in S1P\(_{1}\)\(^{-}\)MHCCre and control hearts at different ages (1 day to 48 weeks). Inset shows representative picrosirius red staining of an S1P\(_{1}\)\(^{-}\)MHCCre mouse heart aged 11 months and its littermate control. B, Gene expression in total heart tissue from S1P\(_{1}\)\(^{-}\)MHCCre and controls as determined by real-time polymerase chain reaction and expressed relative to control in mice aged 45 to 48 weeks. *Statistical significance (P<0.05). C, Assessment of left ventricular function in S1P\(_{1}\)\(^{-}\)MHCCre mice aged 38 to 40 weeks (n=7) and their controls (n=7). Maximal rates of contraction and relaxation as well as heart rate and maximal left ventricular pressure were obtained from continuous recordings of left ventricular pressure and volume at basal state B and during continuous infusion of increasing doses of dobutamine. Values are mean±SEM. #Significant (P<0.05, 2-way repeated-measures ANOVA) difference in the obtained dobutamine response. ANP indicates atrial natriuretic peptide; βMHC, β-myosin heavy chain; BNP, brain natriuretic peptide; dP/dt\(_{\text{max}}\), maximal rate of contraction; dP/dt\(_{\text{min}}\), maximal rate of relaxation; mcip-1, modulatory calcineurin inhibitory protein 1; P\(_{\text{max}}\), maximal left ventricular pressure; S1P\(_{1}\), sphingosine-1-phosphate receptor 1.
Decreased Activity of the Na\(^+\)/H\(^+\) Exchanger NHE-1 in S1P\(_1\)\(^{\text{aMHCCre}}\) Cardiomyocytes

We next addressed the mechanism behind the low \(\text{Ca}^{2+}\)\(_i\) concentrations of S1P\(_1\)\(^{\text{aMHCCre}}\) cardiomyocytes. Considering the tight coupling of \(\text{Ca}^{2+}\)\(_i\) and Na\(^+\) through the Na\(^+/\text{Ca}^{2+}\) exchanger, we hypothesized that reduced \(\text{Ca}^{2+}\)\(_i\) was associated with reduced intracellular Na\(^+\) concentration (Na\(^+\))\(_i\). Indeed, S1P\(_1\)\(^{\text{aMHCCre}}\) cardiomyocytes exhibited 23% lower Na\(^+\)\(_i\) at all stimulation frequencies (1–8 Hz) (Figure 5A), leading us to test whether reduced \(\text{Ca}^{2+}\)\(_i\) might be secondary to lower Na\(^+\)\(_i\). The sarcolemmal NHE-1 and the Na\(^+\) channel are the 2 major routes for Na\(^+\) entry into cardiomyocytes, and because the upstroke velocity of the AP was unchanged, we did not expect alterations in Na\(^+\) channel activity. In contrast, the proton flux through NHE-1 as a measure of NHE-1 activity was dramatically decreased in S1P\(_1\)\(^{\text{aMHCCre}}\) cardiomyocytes (Figure 5B). NHE-1 expression was not altered (Figure 5B, inset). This suggested that Na\(^+\) entry through NHE-1 was compromised in the absence of S1P\(_1\), leading to reduced \(\text{Ca}^{2+}\)\(_i\).

Pharmacological S1P\(_1\) Inhibition and Blockade of S1P Production Both Diminish Na\(^+\)\(_i\) and Ca\(^2+\)\(_i\) by Inhibiting NHE-1

To test whether the low NHE-1 activity of S1P\(_1\)\(^{\text{aMHCCre}}\) cardiomyocytes could be simulated in control cardiomyocytes, we examined NHE-1 activity in the presence of the S1P\(_1\) antagonist W146.\(^{22}\) Furthermore, to test if endogenously produced S1P could be activating S1P\(_1\) signaling to control NHE-1 activity, we inhibited sphingosine kinase activity with dimethylsphingosine.\(^{23}\) Treatment with either W146 or dimethylsphingosine reduced systolic \(\text{Ca}^{2+}\)\(_i\), diastolic \(\text{Ca}^{2+}\)\(_i\), sarcoplasmic reticulum \(\text{Ca}^{2+}\) content, and Na\(^+\)\(_i\) down to values as low as those observed in S1P\(_1\)\(^{\text{aMHCCre}}\) cardiomyocytes (Figure 5C, 5D and 5E). Consecutive treatment with the NHE-1 inhibitor cariporide did not diminish Na\(^+\)\(_i\) any further (Figure 5F and 5G), suggesting that the effect was due to suppression of NHE-1 activity. Nevertheless, this experiment does not exclude the possibility of a “floor effect” below a certain Na\(^+\)\(_i\) level. Neither dimethylsphingosine nor W146 affected SL shortening (Figure 5H), completely resembling the phenotype of S1P\(_1\)\(^{\text{aMHCCre}}\) cardiomyocytes.

We then addressed whether stimulation with S1P affects contractile function in S1P\(_1\)\(^{\text{aMHCCre}}\) cardiomyocytes in the absence and presence of noradrenaline. In the absence of noradrenaline, S1P had no effect on SL in both control and S1P\(_1\)\(^{\text{aMHCCre}}\) cardiomyocytes (Figure 5I). In the presence of noradrenaline, S1P decreased SL amplitude and shortened time to 50% relaxation in control cardiomyocytes but failed to do so in S1P\(_1\)\(^{\text{aMHCCre}}\) cardiomyocytes (Figure 5J). Furthermore,
S1P inhibited noradrenaline-mediated cAMP generation in control but not in S1P<sub>1</sub><sup>aMHCCre</sup> cardiomyocytes (Figure 5K). Consequently, exogenous S1P exerted an indirect negative inotropic effect through S1P<sub>1</sub> in line with this hallmark feature of Gi-coupled receptors. These data are in agreement with previous studies showing attenuation of isoproterenol-induced cardiomyocyte contractility by S1P through adenylate cyclase inhibition and its reversal by pharmacological S1P<sub>1</sub> inhibition.24,25 In summary, S1P<sub>1</sub> activation by acute S1P stimulation reduced β-adrenergic contractility through adenylate cyclase inhibition, whereas continuous S1P<sub>1</sub> signaling by endogenous S1P was required for the maintenance of NHE-1 activity.

Loss of IP in S1P<sub>1</sub><sup>aMHCCre</sup> Mice

Several studies have shown that cardiac S1P produced endogenously by sphingosine kinases mediates the cardioprotective effect of IP<sup>8</sup>; however, the responsible S1P receptor has remained unknown. To test whether S1P<sub>1</sub> was involved, we compared the response to IP in S1P<sub>1</sub><sup>aMHCCre</sup> and control mice. As expected, IP conferred cardioprotection in controls, but the cardioprotective effect was completely absent in S1P<sub>1</sub><sup>aMHCCre</sup> mice (Figure 6A). Infarct size without IP was similar in both groups (Figure 6A). The survival kinase Akt is known to be instrumental for the effectiveness of IP in rodents (see review 26). Interestingly, sphingosine kinase 1–deficient mice (Spk1<sup>1<sup>−/−</sup></sup>) cannot be preconditioned and cannot activate Akt following IP.23 Consequently, we examined Akt signaling during IP in S1P<sub>1</sub><sup>aMHCCre</sup> and control mice and observed that Akt phosphorylation was clearly impaired in IP samples from S1P<sub>1</sub><sup>aMHCCre</sup> mice compared with controls (Figure 6B). In contrast, there was no difference in basal Akt phosphorylation in noninjured hearts (Figure S2). These data support...
Figure 5. Role of S1P₁ in the regulation of NHE-1. A, Na⁺ᵢ concentrations in control and S1P₁⁺MHCCre cardiomyocytes obtained at 1 to 8 Hz. B, NHE-1 activity shown as representative pHi traces of acid-load recoveries (left) and averaged pHi-NHE-1 profiles (right). Inset shows NHE-1 gene expression. C, Systolic Ca²⁺ᵢ; (D) sarcoplasmic reticulum Ca²⁺ content, and (E) Na⁺ᵢ in control cardiomyocytes with and without preincubation with W146 (1 μmol/L) and DMS (5 μmol/L), respectively, for 30 minutes. Values for S1P₁⁺MHCCre cardiomyocytes are shown for comparison. F and G, No further reduction of Na⁺ᵢ by cariporide (10 μmol/L) beyond that caused by DMS or W146. H, No effect of W146 and DMS on SL. I and J, SL shortening in the presence of exogenous S1P (100 nmol/L) under basal conditions and after stimulation with NA (100 nmol/L) with electrical pacing of 6 Hz. K, Cyclic AMP levels after NA stimulation without and with exogenous S1P (100 nmol/L) expressed as relative to basal in S1P₁⁺MHCCre cardiomyocytes and their controls. All mice were aged 45 to 48 weeks. *N* represents the number of animals used for isolation of cardiomyocytes and “n” indicates the number of total observations in single cardiomyocytes. *Statistical significance to vehicle or controls, respectively (P < 0.05). DMS, dimethylsphingosine; jHᵩₑₑ₁, NHE-1–dependent proton flux; NA, noradrenaline; Na⁺ᵢ, intracellular Na⁺ concentration; n.s., not significant; pHi, intracellular pH concentration; S1P₁, sphingosine-1-phosphate receptor 1; SL, sarcomere length.
the notion that S1P1 is responsible for IP and exerts its cardioprotective effect through Akt.

**Discussion**

In our study, we identified that continuous S1P1 signaling is necessary for the maintenance of NHE-1 activity and for dampening of the Ca\(^{2+}\) sensitivity of the contractile apparatus. The decrease in myofilament contractility caused by S1P1 signaling most likely takes place through inhibition of adenylate cyclase, consistent with the ability of S1P1 to inhibit cAMP formation through Gi. This is conceptually supported by the validity of the reverse because activation of the adenylate cyclase pathway is well known to cause the Ca\(^{2+}\)-sensitizing effect of many agents such as endothelin 1, noradrenaline, and levosimendan.\(^{27}\) The functional correlates of this finding were the shortened diastolic and systolic SLs, the reduced rate of SL shortening, and the increased shortening durations in S1P1\(^{\alpha MHC}\)Cre cardiomyocytes. Its biochemical manifestation was the reduced phosphorylation of myosin light chain 2, myosin binding protein C, and troponin I, which are all substrates for cAMP-activated protein kinase A. The source of S1P to continuously engage S1P1 was of cardiomyocyte origin and was generated by sphingosine kinases (with the cautionary note that sphingosine kinase inhibition data with dimethylsphingosine should be confirmed by genetic evidence). In this scenario, the lower Ca\(^{2+}\)\(^{2+}\) due to reduced NHE-1 activity in the absence of S1P1 may serve to balance elevated Ca\(^{2+}\) sensitivity. Conversely, any reduction in NHE-1 activity and thus of Ca\(^{2+}\) (eg, by decreased endogenous S1P production and thus lower S1P1 signaling) may be balanced by the simultaneous increase in Ca\(^{2+}\) sensitivity. In clear support, transgenic overexpression of NHE-1 has been shown to increase systolic and diastolic Ca\(^{2+}\) without changes in contractility, and lower Ca\(^{2+}\) sensitivity has been identified as the underlying cause.\(^{28}\) Although not shown directly in our study, the increase of cAMP in S1P1\(^{\alpha MHC}\)Cre cardiomyocytes may be suppressing NHE-1 activity because cAMP is a well-known inhibitor of NHE-1.\(^{29,30}\) In addition, S1P has been shown to stimulate Na\(^{+}/H^{+}\) exchange in thyroid cells.\(^{31}\) Nevertheless, complex compensatory changes in response to S1P1 deficiency during development should also be considered. A possible explanation (that needs experimental confirmation) of

![Figure 6](image_url)

**Figure 6.** Myocardial infarction and ischemic preconditioning in S1P1\(^{\alpha MHC}\)Cre mice. A, Infarct size/area at risk (INF/AAR) in S1P1\(^{\alpha MHC}\)Cre and control mice that underwent 30 minutes of coronary occlusion followed by 120 minutes of reperfusion without or with ischemic preconditioning (1 cycle of 5 minutes of occlusion and 10 minutes of reperfusion before occlusion). All mice were aged 18 to 20 weeks. *Statistical significance (P<0.05) between groups (2-way repeated measures ANOVA followed by Bonferroni). B, Akt phosphorylation (Ser473) in ischemic preconditioning samples of control and S1P1\(^{\alpha MHC}\)Cre mice at the time of sacrifice after ischemia–reperfusion. S1P1 indicates sphingosine-1-phosphate receptor 1.

![Figure 7](image_url)

**Figure 7.** Schematic of the role of S1P1 in the heart. β1-AR indicates β1-adrenoceptor; NCX, Na\(^{+}/Ca\(^{2+}\) exchanger; NHE-1, Na\(^{+}/H^{+}\) exchanger; S1P, sphingosine-1-phosphate; S1P1, sphingosine-1-phosphate receptor 1; Sphk1, sphingosine kinase 1; Sphk2, sphingosine kinase 2.
how this regulatory system may contribute to cardiac physiology is its potential ability to accommodate hemodynamic challenge by increasing Ca\(^{2+}\) sensitivity through a reduction of endogenous S1P production and thus S1P\(_1\) signaling; at the same time, lower S1P\(_1\) signaling would suppress NHE-1 activity, thereby reducing Na\(^+\) and Ca\(^{2+}\) and providing an elegant in-built safe-guarding mechanism against excessive Ca\(^{2+}\) sensitization (Figure 7).

IP relies on endogenous S1P synthesis by sphingosine kinases (see review 8), but the S1P receptor mediating IP was unknown. In this study, we identified S1P\(_1\) as the S1P receptor required for the cardioprotective effect of IP. In contrast, S1P\(_1\) played no role in the susceptibility of the heart to regular ischemia–reperfusion injury without IP. The exact mechanisms by which S1P\(_1\) mediates IP are unknown but should be sought in the signaling pathways common to S1P\(_1\) and IP. S1P produced by sphingosine kinases is increased during and indispensable for IP\(^8\) and acts by activating the reperfusion injury salvage kinase pathway and the survivor activating factor enhancement kinase pathway and the signaling pathways common to S1P\(_1\) and IP. S1P produced by sphingosine kinases is increased during and indispensable for IP\(^8\) and acts by activating the reperfusion injury salvage kinase pathway and the survivor activating factor enhancement pathway and by decreasing the cardiomyocyte susceptibility to mitochondrial permeability transition pore opening (see review 26). The inability of S1P\(_1\)\(^{MHCCre}\) mice to benefit from IP and their defective Akt activation during preconditioning are consistent with the lack of IP in Sphk\(^{1\text{−/−}}\) mice and their inability to activate the Akt pathway.\(^{23}\) Therefore, S1P\(_1\)/Akt signaling is essential for IP in mice (Figure 7).

A recent study showed that cardiac S1P\(_1\) was downregulated in isoproterenol-induced heart failure in rats and that its overexpression alleviated heart failure after myocardial infarction; the authors suggested downregulation of the \(\beta_1\)-adrenoreceptor by its cointernalization with S1P\(_1\) as possible cause.\(^{32}\) We did not see any enhanced contractile response to \(\beta\)-adrenergic stimulation in S1P\(_1\)^{MHCCre} as one might expect if there were a pool of \(\beta_1\)-adrenoreceptors constantly being cointernalized because of constitutive S1P\(_1\) activity—a pool that should be available in surplus for signaling in its absence. In fact, the response of S1P\(_1\)^{MHCCre} to \(\beta\)-adrenergic stimulation was even worse in vivo and unaffected in vitro. In support, 3 studies showed that long-term FTY70 treatment of mice (a regimen that is generally known to downregulate S1P\(_1\)) improved LV function and survival after myocardial infarction in mice and pigs.\(^{33–35}\) According to our observations, downregulation of cardiomyocyte S1P\(_1\) may have a beneficial role in heart failure because it would alleviate the compromised Ca\(^{2+}\) sensitivity of the failing heart and lessen its Ca\(^{2+}\) overload by subduing the pathologically elevated NHE-1 activity. Future studies in experimental models of heart failure are needed to address these questions. Clearly, the role of S1P and its receptors in cardiac function under physiological and pathophysiological conditions is an exciting new area of research that promises novel ramifications for cardiovascular medicine.

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Disclosures
None.

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Supplemental Material

Methods

Functional Characterization of Isolated Cardiomyocytes (Detailed Methods)

Left ventricular myocytes were isolated from adult mice (aged 30-38 weeks) by enzymatic dissociation. Cell size and membrane capacitance of the isolated cardiomyocytes were similar in all genotypes (data not shown). The following parameters were measured as described before\textsuperscript{1,2} and detailed here again. Action potential recordings: Action potentials (APs) were recorded using the perforated patch-clamp technique using an Axopatch 200B amplifier (Molecular Devices, Union City, CA). Tyrode’s solution (36±0.2°C) contained (in mM): 140 NaCl, 5.4 KCl, 1.8 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 5.5 glucose, 5 HEPES; pH 7.4 (NaOH). Patch pipettes (~2.0 M\textOmega) contained (in mM): 125 K-gluc, 20 KCl, 10 NaCl, 0.22 amphotericin-B, 10 HEPES; pH 7.2 (KOH). APs were filtered and digitized at 10 and 40 kHz, respectively. Custom-made software was used for data acquisition and analysis; potentials were corrected for the calculated liquid junction potential (15 mV). APs were elicited at 1 to 10-Hz by 3-ms, \approx 1.2\times \text{threshold current pulses through the patch pipette. Resting membrane potential (RMP), maximal AP amplitude (APA), maximal upstroke velocity (dV/dt\textsubscript{max}), AP duration at 20, 50, and 90% repolarization (APD\textsubscript{20}, APD\textsubscript{50}, and APD\textsubscript{90}, respectively), and plateau amplitude (Pla; defined as the potential difference between RMP and the potential 20-ms after the upstroke) were analyzed and averaged from 10 consecutive APs. Ca\textsuperscript{2+}, Na\textsuperscript{+}, and pH\textsubscript{i} measurements: Ca\textsuperscript{2+}, Na\textsuperscript{+}, and pH\textsubscript{i} were measured in indo-1, SBFI, and SNARF-1 loaded myocytes, preincubated in HEPES-buffered solutions containing 10 µM SBFI-AM or 5 µM indo\textsubscript{1}AM or 10 µM SNARF\textsubscript{1}AM and 0.01% pluronic. Incubation durations were 10:30 and 120 min. Myocytes were washed twice before use with fresh HEPES solution (without albumin) and kept for 15
min to ensure complete deesterification. Excitation wavelengths were 340, 340, and 515 nm, respectively. Fluorescence of all indicators was measured in dual wavelength emission mode at 405/505, 410/590 and 580/640 nm. Fluorescence signals were corrected for background recorded from probe-free myocytes and calibrated to obtain maximum and minimum ratio values of the emitted wavelengths and the constants $\beta$ and $k_d$ as described previously $^1$. Na$^+$ and Ca$^{2+}$ were calculated with the formulation of Grynkiewicz et al. Na$^+$ was averaged over the entire cardiac cycle because it does not vary on a beat to beat basis. Cytosolic free Ca$^{2+}$ was obtained by correction of measured overall cellular fluorescence signals for contributions of mitochondrially compartmentalized indo-1. pH$_i$ was determined from calibration curves obtained with nigericin, and NHE-1 dependent proton flux ($J_{\text{NHE-1}}$) was calculated from the rate of recovery of pH$_i$ following acid loading as described in detail in the supplement. The SR Ca$^{2+}$ content of cells was studied by 20 mM caffeine application. **Sarcomere length shortening:** Sarcomere length was measured using a fast Fourier transform algorithm of the video image (200 frames/s) of the contracting myocytes (IonOptix, Milton, MA). **cAMP measurements:** The LANCE cAMP kit (PerkinElmer, Zaventem, Belgium) was used to determine cAMP concentrations (according to the manufacturer's instruction) using a Victor plate reader (Wallac, PerkinElmer, Zaventem, Belgium). Cell suspension was added to a 96-well OptiPlate (PerkinElmer, Waltham, MA, USA; 5 µl per well) and incubated for 10 min at 37°C with vehicle (0.4% albumin), cariporide (10 µM), S1P (100 nM) or S1P (100 nM) + cariporide (10 µM) either in the absence or in the presence of noradrenaline (100 nM). All experiments were performed in the presence of 0.05% bovine serum albumin (BSA) and 500 µM of the phosphodiesterase inhibitor isobutyl-methyl-xanthine (IBMX) to allow accumulation of cAMP. The obtained cAMP values were normalized to the control cAMP value measured in the absence of noradrenaline.
pH$_i$, Fluorescence Measurements in Myocytes

All procedures have been performed as described previously. In brief, wells of a 96 well plate were initially filled with 200 µl Tyrode's solution containing either 0, 1.25, 12.5, 37.5, 125, 375 nmol/l S1P (dissolved in BSA), 0, 1.25, 12.5, 37.5, 125, 375 nmol/l SEW (dissolved in DMSO), or 0, 1.25, 12.5, 37.5, 125, 375 nmol/l FTY720-P (dissolved in BSA) and stored at 37°C until use. The pH$_i$-$J_{NHE-1}$ flux profile obtained at 0 nM S1P, FTY720-P and SEW2817 contained the same vehicle concentration, (BSA, BSA and DMSO) as used at 300 nM S1P, FTY720-P and SEW2817. Each plate contained also wells filled with 200 µl “high K⁺-nigericin” solutions set at different pH values (5.0, 6.0, 6.5, 7.0, 7.5, 8.0 and 9.0) for calibration. Rod-shaped cardiomyocytes were exposed for 30 minutes to 10 µmol/l BCECF-AM (dissolved in DMSO). Excess BCECF-AM was removed by centrifugation (300 G for 2 minutes) and supernatant was removed. To acid-load the cardiomyocytes, the pellet was first resuspended in 20 mM NH$_4$Cl containing Tyrode’s solutions (37°C) and after 15 minutes NH$_4$Cl was replaced (300 G for 2 minutes) by 5 ml Na⁺-free Tyrode’s solution(37°C). In the absence of Na⁺, cardiomyocytes are unable to recover from the acid-load. To all wells of the 96-well plate, except for the blanco wells, 50 µl cell suspension (Na⁺-free Tyrode’s solution) was rapidly added with a multichannel pipette. To the wells that serve as blanco controls 50 µl cell suspension of cardiomyocytes were added that were not loaded with BCECF. Addition of 50 µl Tyrode´s solution diluted the S1P, SEW and FTY720-P concentrations to 0, 1.0, 10, 30, 100, 300 nmol/l. The 96 well plate was quickly placed in a plate readed (Novostar) that measured pH fluorescence (excitation 440 and 480 nm; emission at 540 nm) of each well every 20 seconds. During the 30 minutes acid-load recovery temperature was kept at 37°C. $J_{NHE-1}$ was calculated as described in the materials and methods section.
**Reagents**

Immunological detection of phospholamban (PLB), SERCA2a, calsequestrin and junctin and the fraction of PLB phosphorylated at serine 16 and at threonine 17 were determined as described. We thank Dr. L.R. Jones, Indianapolis, for providing calsequestrin, SERCA2a and junctin antibodies. Other antibodies used for Western blotting were from Santa Cruz Biotechnology (p38, #sc-535) or from Cell Signaling (pERK1/2 (#9101), ERK1/2 (#4695) and pp38 (#9211)).

**Mice**

All mice used in the experiments were of similar age and either all male or age and sex-matched. In studies involving age-related phenomena (such as the progressive development of cardiomyopathy), the specific age of the examined mice has been specified in text and figure legend. All animal experiments were in compliance with ethical regulations as approved by the Animal Ethics Committee of the Academic Medical Center, Amsterdam, The Netherlands, and the Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV) Nordrhein-Westfalen, Recklinghausen, Germany, respectively, and are in accordance with EU guidelines (2010/63/EU) on the care and use of laboratory animals.

**Statistics**

Overall survival curves were estimated using the Kaplan-Meier method and differences between genotypes were compared by the log-rank test. Average data are presented as mean±SEM in all figures but Fig. 6a where the median (minimum to maximum) has been shown. For all cellular (*in vitro*) and animal (*in vivo*) work, two-sided ANOVA for multiple comparisons (repeated measurements) were used to test whether relationships were significantly different, a two-side paired Student’s t-test
was used to test for significant differences within groups (e.g. effect of a drug or echocardiographical measurements in the same animal before and after treatment) and a two-sided unpaired t-test to test for significant difference between two groups, depending on the existence of equal variances and normal distribution of data. Mann-Values of $P<0.05$ were considered statistically significant. In all echocardiographical measurements, the investigator was blinded to genotype and treatment. For in vitro experiments, sample size has been chosen based on own experience from in previous experiments $^4$. For all in vivo experiments, sample size was based on established (literature) method-specific sample sizes and our own work on I/R and ischemic preconditioning $^4$.

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Supplemental Tables

Table S1. Expression of Ca$^{2+}$-Regulatory SR Proteins in the Left Ventricle of S1P$_1^{\alpha}$MHCCre Mice and Controls.

Values are expressed in percent of the mean of the control group. CSQ, calsequestrin; SERCA2a, cardiac isoform of sarcoplasmic Ca$^{2+}$-ATPase; PLN, total phospholamban; S16-PLN, phospholamban phosphorylated at serine 16; T17-PLN, phospholamban phosphorylated at threonine 17; JCN, junctin. *$P < 0.05$ vs. control.

|                  | control ($n=10$) | S1P$_1^{\alpha}$MHCCre ($n=6$) |
|------------------|------------------|---------------------------------|
| CSQ              | 100              | 90 ± 13                         |
| SERCA2a          | 100              | 96 ± 4                          |
| PLN              | 100              | 114 ± 9                         |
| S16-PLN          | 100              | 98 ± 11                         |
| T17-PLN          | 100              | 110 ± 6                         |
| S16-PLN/PLN      | 100              | 90 ± 15                         |
| T17-PLN/PLN      | 100              | 97 ± 12                         |
| PLN/SERCA2a      | 100              | 118 ± 6 *                       |
Supplemental Figures

**Figure S1.** Western blotting for phosphorylated (active) kinases in cardiac tissue of control and S1P_1^{αMHCCre} mice (n=5 each).
Figure S2. Assessment of left-ventricular function in $S1P_1^{\alpha MHCCre}$ mice and controls by left ventricular catheterization in 19-26 weeks-old $S1P_1^{\alpha MHCCre}$ mice ($n=7$) and controls ($n=10$). Heart rate (HR), left-ventricular pressure ($LVP_{\text{max}}$), contraction velocity ($dLVP/dt_{\text{max}}$), relaxation velocity ($dLVP/dt_{\text{min}}$), stroke volume (SV), cardiac output (CO), and stroke work (SW) were obtained from continuous recordings of left-ventricular pressure and volume at basal state (B) and during continuous infusion of increasing doses of dobutamine. $S1P_1^{\alpha MHCCre}$ mice had normal baseline cardiac function but displayed reduced maximal rates of contraction ($dLVP/dt_{\text{max}}$), relaxation ($dLVP/dt_{\text{min}}$) and stroke work (SW) with increasing doses of dobutamine. Basal and dobutamine-stimulated HR did not differ from controls, while $LVP_{\text{max}}$ was lower in $S1P_1^{\alpha MHCCre}$ mice. Non-floxed mice with and without the Cre transgene were indistinguishable in all parameters excluding Cre effects. Values are mean ± SEM. ‘#’ denotes a significant ($P<0.05$) difference in the dobutamine response; ‘+’ denotes a significant ($P<0.05$) difference in values (2-way repeated measures ANOVA).