Selective coupling of the S1P3 receptor subtype to S1P-mediated RhoA activation and cardioprotection.

Permalink
https://escholarship.org/uc/item/8g92584d

Journal
Journal of molecular and cellular cardiology, 103

ISSN
0022-2828

Authors
Yung, Bryan S
Brand, Cameron S
Xiang, Sunny Y
et al.

Publication Date
2017-02-01

DOI
10.1016/j.jmcc.2016.12.008

Peer reviewed
Original article

Selective coupling of the S1P₃ receptor subtype to S1P-mediated RhoA activation and cardioprotection

Bryan S. Yung, Cameron S. Brand, Sunny Y. Xiang, Charles B.B. Gray, Christopher K. Means, Hugh Rosen, Jerold Chun, Nicole H. Purcell, Joan Heller Brown, Shigeki Miyamoto

Department of Molecular and Cellular Neuroscience, Dorris Neuroscience Center, Scripps Research Institute, La Jolla, CA 92037, United States

Abstract

Sphingosine-1-phosphate (S1P), a bioactive lysophospholipid, is generated and released at sites of tissue injury in the heart and can act on S1P₁, S1P₂, and S1P₃ receptor subtypes to affect cardiovascular responses. We established that S1P causes little phosphoinositide hydrolysis and does not induce hypertrophy indicating that it does not cause receptor coupling to Gq. We previously demonstrated that S1P confers cardioprotection against ischemia/reperfusion by activating RhoA and its downstream effector PKD. The S1P receptor subtypes and G proteins that regulate RhoA activation and downstream responses in the heart have not been determined. Using siRNA or pertussis toxin to inhibit different G proteins in NRVMs we established that S1P regulates RhoA activation through Gα₁₃ but not Gα₁₂, Gα₁₆, or Gα₁₉. Knockdown of the three major S1P receptors using siRNA demonstrated a requirement for S1P₁ in RhoA activation and subsequent phosphorylation of PKD, and this was confirmed in studies using isolated hearts from S1P₃ knockout (KO) mice. S1P treatment reduced infarct size induced by ischemia/reperfusion in Langendorff perfused wild-type (WT) hearts and this protection was abolished in the S1P₃ KO mouse heart. CYM-51736, an S1P₃-specific agonist, also decreased infarct size after ischemia/reperfusion to a degree similar to that achieved by S1P. The finding that S1P₃ receptor- and Gα₁₃-mediated RhoA activation is responsible for protection against ischemia/reperfusion suggests that selective targeting of S1P₃ receptors could provide therapeutic benefits in ischemic heart disease.

© 2016 Elsevier Ltd. All rights reserved.

1. Introduction

Restoration of blood flow after an ischemic episode (e.g. myocardial infarct) is necessary to prevent catastrophic heart failure but reperfusion can itself increase cardiomyocyte death, a process referred to as reperfusion injury [1]. Previous studies have shown that the circulating S1P is itself increased cardiomyocyte death, a process referred to as reperfusion injury (I/R). Previous studies have shown that the circulating S1P is itself increased cardiomyocyte death, a process referred to as reperfusion injury (I/R). Previous studies have shown that the circulating S1P is itself increased cardiomyocyte death, a process referred to as reperfusion injury (I/R).
decreases tolerance to ischemic damage [26]. We also showed that S1P could confer cardioprotection through RhoA and its downstream effectors [6]. In this study, we examined whether S1P regulates PLC activation and hypertrophy through S1P2 or S1P3 receptors and/or whether these receptor subtypes regulate activation of RhoA and in turn S1P-mediated cardioprotection. The data presented here demonstrate that S1P primarily signals through coupling to Gαq, and activation of RhoA, that this pathway does not strongly activate PLCβ or contribute to development of cardiac hypertrophy, and that it is the S1P3 receptor that regulates RhoA activation to mediate cardioprotection.

2. Materials and methods

2.1. Animals

All animal procedures were performed in accordance with NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of California – San Diego. Generation of global homozygous C57BL/6 S1P2 KO and S1P3 KO mice has been previously described [27]. All experiments were performed on age-matched male WT and KO littermates.

2.2. NRVM cell culture and reagents

Neonatal rat ventricular myocytes (NRVMs) were isolated from cardiac ventricles of 1- to 2-day-old Sprague-Dawley rat pups as described previously [28]. NRVMs were plated at a density of 3.0 × 10^5/cm^2 and maintained overnight in Dulbecco-modified Eagle’s medium (DMEM) containing 15% fetal bovine serum overnight. Cells were either serum-starved with DMEM for 24 h or transfected with siRNA for further analysis. Predesigned rat siRNA and scrambled control siRNA were purchased from Qiagen and used at 3 μg per 1 × 10^6 cells. Cardiomyocytes were transfected with siRNA using DharmaFECT-1 transfection reagent from Thermo Fisher Scientific based on the manufacturer’s instruction. The aortic inhibitor C3 exoenzyme was obtained from Cytoskeleton (CT04). Pertussis toxin (PTX) was purchased from Calbiochem. Phenylphrine (PE) was obtained from Sigma Life Science. S1P was obtained from Avanti Polar. CYM-S1736, is an allosteric agonist of S1PR4 of the structure N,N-dicyclohexyl-5-(furanyl-3-yl)isoxazole-3-carboxamide [29,30], was provided by Dr. Hugh Rosen (The Scripps Research Institute, La Jolla, CA).

2.3. Immunofluorescence

NRVMs were fixed in 3.5% paraformaldehyde solution, permeabilized in 0.2% NP-40 alternative, blocked in 2% bovine serum albumin (BSA) plus 10% goat serum, and then incubated in primary antibody against α-actinin (Sigma) or atrial natriuretic factor (Peninsula Laboratories) overnight at 4 °C. Secondary antibodies conjugated to Alexa Fluor 488 and Alexa Fluor 555 (Invitrogen) were applied for 2 h at room temperature. Cells were mounted with VECTASTAIN Hardset containing DAPI (Vector Labs). Images were acquired using confocal microscopy. Cell area was measured using a scale bar of 20 μm.

2.4. cDNA synthesis and qPCR analysis

RNA was isolated from NRVMs using Trizol. cDNA synthesis was carried out with the Verso cDNA synthesis kit (Thermo Scientific) and qRT–PCR was carried out using standard TaqMan primers and TaqMan Universal Mastermix II (Applied Biosystems) on a 7500 Fast Real-Time PCR system (Applied Biosystems). The data acquired was analyzed using the comparative Ct method (i.e., the 2^−ΔΔct method) [31]. GAPDH levels were used as the internal control.

2.5. Phosphatidylinositol (PI) hydrolysis

After overnight culturing, NRVMs were labeled with tritium-labeled ([3H]) inositol (2.5 μCi/mL) for 24 h. Cells were treated with agonists at various times in the presence of 25 mM lithium chloride, washed with cold PBS and incubated in cold 50 mM trichloroacetic acid (Sigma) for 40 min at 4 °C. Samples were centrifuged and trichloroacetic acid was extracted with water-saturated ether. [3H]InsPs were isolated by ion exchange chromatography, and radioactivity was then measured by liquid scintillation counting.

2.6. Transverse aortic constriction

Transverse aortic constriction (TAC) was used on 8- to 10-week-old WT, S1P2 KO, or S1P3 KO mice to induce pressure overload hypertrophy as previously described [32–35]. The transverse aortic arch was visualized by a median sternotomy and a 7-0 silk ligature was tied around the aorta (27-gauge constriction) between the right brachiocephalic and the left common carotid arteries for one week.

2.7. GTP-RhoA pull-down assay

RhoA activation was determined as described previously [36]. Briefly, cell lysate was incubated with Rho binding domain of Khoetkin and then subjected to series of washes and centrifugations. 4 × Laemmli buffer was added and boiled for 5 min prior to SDS-PAGE analysis. Activated GTP-bound RhoA was detected by Western blotting for RhoA and normalized to total RhoA in lysate. For GTP-RhoA pulldown on isolated perfused hearts, tissue was flash-frozen, homogenized in Rhoa lysis, debris pelleted via centrifugation, and the supernatant used for RhoA pull down assay as described above.

2.8. Western blotting

Western blot analysis was performed according to protocols previously described [28]. The antibodies used for immunoblotting were the following: RhoA, Gαq, Gα13, and Gα13 from Santa Cruz Biotechnology, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phospho-PKD (Ser744/748), PKD, and α-actinin from Cell Signaling Technology. Peroxidase-conjugated secondary antibodies were used at a dilution of 1:2000 (Sigma) and the enhanced chemiluminescence substrate was from Thermo Fisher Scientific.

2.9. Isolated perfused heart (Langendorff) ischemia/reperfusion

Hearts from age-matched 8- to 12-week-old male WT, S1P2 KO, or S1P3 KO mice were removed quickly and perfused with modified Krebs-Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 25 mM NaHCO3, 0.5 mM EDTA, 1.2 mM MgSO4, 11 mM glucose, 1.5 mM sodium pyruvate, and 2 mM CaCl2) in a Langendorff apparatus (Radnoti) at a constant pressure of 80 mm Hg. Hearts were stabilized for 10 min and then subjected to a period of global ischemia for 22 min followed by reperfusion for 60 min. To measure infarct size, triphenyl tetrazolium chloride (TTC) was used as described previously [28].

2.10. Dual-luciferase reporter assay

NRVMs were plated onto 6-well plates. The following day, cells were transfected via Dharmafect1 (Dharmacon) for 8 h with control siRNA or siRNA for Gα13 or Gα13. The following day, cells were transfected via Lipofectamine 2000 (Invitrogen) for 8 h with an SRE.L reporter (Promega) based on the manufacturer’s protocol.
2.11. Statistical analysis

All results are reported as means ± standard error of the mean (SEM). Comparison of two groups with one variable was accomplished using an unpaired Student’s t-test, or if the control group was normalized to 1 with no variance, a paired t-test. Data with two groups with multiple variables were compared with two-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test. Data from experiments with more than two groups with one variable were compared by one-way ANOVA followed by Tukey’s multiple comparison test, or repeated measures ANOVA followed by Tukey’s multiple comparison test if the control group was normalized to 1 with no variance. Probabilities ≤0.05 were considered significant.

3. Results

3.1. S1P does not induce cardiac hypertrophy

Stimulation of G\(_{\alpha}\)q-coupled receptors with ligands such as phenylephrine (PE) or endothelin regulates PLC to increase PI hydrolysis and downstream signals implicated in development of cardiac hypertrophy [37]. S1P can activate S1P receptor subtypes known to couple to G\(_{\alpha}\)q, but the role of S1P in control of cardiac hypertrophy appears to be minimal [38]. To further demonstrate and explore the basis for the limited efficacy of S1P as a hypertrophic agonist we first assessed the ability of S1P receptor stimulation to activate PLC in neonatal rat ventricular myocytes (NRVMs). NRVMs were serum-starved overnight, labeled with \(^{3}\)H-inositol, and treated with 0.3 \(\mu\)M S1P for 0, 1, 5, 10, 30, and 60 min. Responses were compared with those seen with 50 \(\mu\)M PE, an established hypertrophic agonist that activates \(\alpha\)-adrenergic receptors. We observed a robust increase in the accumulation of inositol phosphates (InsPs) in response to PE whereas S1P elicited a much smaller response (Fig. 1A). S1P receptor-mediated PLC activation could occur through the novel PLC isoform PLC\(_{\varepsilon}\) and its activation by RhoA [39,40]. To determine if this was the mechanism by which S1P stimulated PI hydrolysis in NRVMs we inhibited RhoA function with C3 exoenzyme (2 \(\mu\)g/mL). The response to S1P was blocked whereas the response to PE was not (Fig. 1B). In addition, knockdown of PLC\(_{\varepsilon}\) with siRNA prevented the PI response to S1P but not that to PE (Fig. 1C). These data suggest that whereas PE works through the canonical G\(_{\alpha}\)q/PLC\(_{\beta}\) signaling pathway to elicit robust PI hydrolysis, the less robust PI response to S1P is mediated through RhoA signaling to PLC\(_{\varepsilon}\).

![Fig. 1. S1P receptor signaling stimulates modest increases in PI hydrolysis and does not mediate in vitro or in vivo cardiac hypertrophy. (A) Time course of S1P- and PE-induced phosphatidylinositol hydrolysis. NRVMs were serum-starved overnight in the presence of \(^{3}\)H inositol. Cells were then treated with agonists for 1, 5, 10, 30, and 60 min in the presence of LiCl before isolation of \(^{3}\)H inositol phosphates (InsPs). The data displayed are the mean ± SEM. **P < 0.01 vs. vehicle (Veh), ##P < 0.01 vs. S1P (n = 5). (B) NRVMs were pretreated with 2.0 \(\mu\)g/mL C3 exoenzyme (Rho inhibitor) for 6 h or (C) transfected with control siRNA (siCtrl) or siRNA against PLC\(_{\varepsilon}\) for 48 h before challenge with agonists for 60 min and assessed for InsPs production. **P < 0.01 vs. vehicle + control or siCtrl, ##P < 0.01 vs. S1P + control or siCtrl (n = 5). (D) Representative immunofluorescent images depicting NRVMs stained for \(\alpha\)-actinin, atrial natriuretic factor (ANF), and nuclei with DAPI after treatment with either vehicle, 0.3 \(\mu\)M S1P, or 50 \(\mu\)M PE for 24 h. Scale bar: 20 \(\mu\)m. (E) Quantified relative cell area and (F) quantified ANF positive cells (n = 400 cells). (G) mRNA expression of brain natriuretic peptide (BNP) and (H) skeletal muscle \(\alpha\)-actin (ACTA1). **P < 0.01 vs. vehicle (n = 4). (I) Heart weight (HW) to body weight (BW) ratio of WT, S1P\(_{2}\) KO, and S1P\(_{3}\) KO mice following transverse aortic constriction (TAC) to induce pressure overload hypertrophy for one week. **P < 0.01 vs. Sham (n ≥ 5).]
To determine whether S1P treatment can elicit cardiomyocyte hypertrophy, NRVMs were treated with S1P or PE for 24 h. Cells were subjected to immunofluorescence analysis for α-actinin, a cardiomyocyte cytoskeletal protein, and atrial natriuretic factor (ANF), a hypertrophic marker (Fig. 1D). Immunofluorescence analysis revealed a large increase in ANF positive cardiomyocytes following PE treatment, but not following S1P treatment (Fig. 1D, F). We also observed that PE, but not S1P, significantly increased cell surface area assessed by α-actinin staining (Fig. 1D, E). qPCR analysis was used to measure the expression of brain natriuretic peptide (BNP) and skeletal muscle α-actin (ACTA1), which are both up-regulated in NRVMs treated with hypertrophic stimuli. PE treatment resulted in a 3.6-fold increase in BNP and 2.5-fold increase in ACTA1 mRNA expression, but S1P did not significantly increase expression of either hypertrophic marker (Fig. 1G, H). Thus, S1P receptor stimulation is unable to trigger the conventional hypertrophic signaling pathways by which activation of receptors that couple to Gαq/PLCβ elicits hypertrophy in NRVMs.

3.2. S1P2 and S1P3 receptors do not play a role in TAC induced hypertrophy in mouse hearts

Inhibition or genetic deletion of Gαq prevents pressure overload hypertrophy induced by transverse aortic constriction (TAC) [19, 20], suggesting that this pathway is stimulated through upstream GPCR agonists. To determine whether either of the S1P receptor subtypes known to couple to Gαq (S1P1 and S1P2) mediate development of hypertrophy in vivo, we subjected WT, S1P2 KO, and S1P3 KO mouse hearts to TAC for one week. We observed a significant increase in heart-weight relative to body-weight ratio, indicative of hypertrophy development, in WT mice and an equivalent increase in the S1P2 and S1P3 KO mice following TAC (Fig. 1). The finding that cardiac hypertrophy is still induced in the absence of these receptors suggests that S1P action on these receptors does not participate in the development of cardiac hypertrophy induced by pressure overload.

3.3. S1P induced RhoA activation is Gα13 dependent

We postulated that the differential PI hydrolysis pathways used by PE and S1P reflects differences in G protein and RhoA activation by these agonists. RhoA activation was assessed by immunoprecipitating the GTP-bound activated RhoA using the Rho binding domain of Rhotekin, a RhoA effector. S1P robustly activated RhoA whereas PE did not (Fig. 2A). S1P receptors have been shown to couple to Gαq (S1P1 and S1P2) mediated development of hypertrophy in vivo, we subjected WT, S1P2 KO, and S1P3 KO mouse hearts to TAC for one week. We observed a significant increase in heart-weight relative to body-weight ratio, indicative of hypertrophy development, in WT mice and an equivalent increase in the S1P2 and S1P3 KO mice following TAC (Fig. 1). The finding that cardiac hypertrophy is still induced in the absence of these receptors suggests that S1P action on these receptors does not participate in the development of cardiac hypertrophy induced by pressure overload.

3.4. S1P activates RhoA through the S1P3 receptor in NRVMs and in isolated perfused hearts

To determine which S1P receptor subtypes are required for the activation of RhoA in response to S1P, siRNA for each of the S1P receptor subtypes was transfected into NRVMs. Treatment with siRNA resulted in a >70% reduction in mRNA expression for each respective S1P receptor subtypes (Fig. 3A, B, C) and did not affect the expression of other subtypes (data not shown). The siRNA-treated cells were stimulated with S1P for 5 min and RhoA activity was assessed by pulldown of GTP-bound activated RhoA. Knockdown of S1P3 receptors markedly reduced RhoA activation by S1P. In contrast, RhoA was activated to an equivalent extent in control siRNA-treated cells, and S1P2 or S1P2 knockdown cells (Fig. 3D). CYM-51736, an S1P3 selective receptor agonist derived from CYM-5541 [29], also increased active RhoA (Fig. 3E), albeit to a lesser extent (approximately 1.5 fold) than S1P (approximately 2.5-fold). As further support, we measured active RhoA in the presence of an S1P3 receptor selective antagonist, JTE-013, which has been used at 1 μM to show S1P2 selective signaling [41]. We observed that JTE-013 did not block RhoA activation by S1P (Fig. 3F), further indicating S1P3 but not S1P2 involvement.

To extend our finding of S1P3 receptor-mediated RhoA activation to the intact adult heart, WT, S1P2 KO, or S1P3 KO mouse hearts were isolated and perfused in the Langendorff mode. S1P was perfused for 5 min and RhoA activation was assessed using the GTP-RhoA pull-down assay. S1P significantly increased the amount of active RhoA in WT hearts and a similar increase was observed in hearts isolated from the S1P3 KO (Fig. 3G). In contrast, S1P treatment failed to activate RhoA in S1P3 KO mouse hearts, indicating that the S1P3 receptor plays a critical role in S1P induced RhoA activation in the adult mouse heart as it does in neonatal rat cardiomyocytes.

3.5. S1P3 receptor mediates cardioprotection in Langendorff perfused mouse hearts against ischemia/reperfusion injury

Our lab has previously reported that S1P confers strong cardioprotection in isolated perfused mouse hearts through signaling to RhoA and PKD [6]. The findings above suggested that the cardioprotection induced by S1P would be mediated through activation of the S1P3 receptor. To test this hypothesis, hearts from WT and S1P3 KO mice were perfused with 0.3 μM S1P for 10 min, subjected to global ischemia for 22 min followed by reperfusion for 60 min, and cardiac damage assessed by staining with 1% TTC. S1P treatment significantly reduced infarct size in the WT mouse heart, supporting previous observations [6]. The protective effect of S1P was completely abolished in the S1P3 KO hearts (Fig. 4A). To further confirm the significant role of S1P3 in cardioprotection we examined the effect of CYM-51736, a derivative of the previously reported S1P3 receptor-specific agonist CYM-5541 [29]. Hearts isolated from WT mice were perfused with 10 μM CYM-51736 for 10 min followed by global I/R. CYM-51736 pretreatment resulted in a significant reduction in infarct size (Fig. 4B), comparable to that observed in hearts perfused with S1P (Fig. 4A) supporting the critical role of the S1P3 receptor in S1P-mediated cardioprotection against I/R injury.

3.6. S1P3 mediates the activation of PKD in NRVMs

We previously demonstrated that PKD is downstream of RhoA activation and contributes to S1P/RhoA-mediated cardioprotection [6]. Knockdown of S1P3 in NRVMs significantly attenuated PKD phosphorylation in response to S1P whereas knockdown of S1P2 or S1P2 did not (Fig. 5A). In addition, the S1P3 selective agonist CYM-51736 increased PKD phosphorylation, and failed to do so following S1P3 receptor knockdown (Fig. 5B). PKD activation by CYM-51736 was attenuated by siRNA knockdown of Gα13, or by inhibiting RhoA by treatment with C3 exoenzyme (Fig. 5C). The S1P3 antagonist JTE-013, shown above to have
no effect on S1P-stimulated RhoA activation, also failed to block S1P-mediated phosphorylation of PKD (Fig. 5D). Together, these results indicate that the cardiac S1P3 receptor plays a critical role in initiating the RhoA/PKD signaling cascade that contributes to cardioprotection [6].

4. Discussion

S1P is a pleiotropic bioactive lysophospholipid that couples to a variety of GPCR subtypes to regulate biological functions, including cell proliferation, inflammation, and cardiac function [24,42–44]. In the heart, S1P has been shown to confer cardioprotection against ischemic stress [3,45], and we recently reported that RhoA plays a crucial role in S1P-mediated cardioprotection against I/R injury [6,10,26,46]. Identifying the S1P receptor subtype through which cardioprotection is mediated would inform further consideration of potential therapeutic targets for ischemic heart diseases. We demonstrate here that S1P3 is the receptor subtype responsible for S1P-mediated RhoA activation, PKD activation and protection against I/R injury in cardiomyocytes and in the isolated

**Fig. 2.** S1P activates RhoA through Gα13 in NRVMs. (A) NRVMs were treated with 0.3 μM S1P or 50 μM PE for 5 min and RhoA activation was measured by a GTP-RhoA pull-down assay. The data displayed are the mean ± SEM (n = 5). **P < 0.01 vs. vehicle (Veh). (B) NRVMs were pretreated with 0.1 μg/mL pertussis toxin (PTX) overnight and then stimulated with 0.3 μM S1P for 5 min. RhoA activation was assessed by GTP-RhoA pull-down assay. NS indicates not significant (n = 4). (C) NRVMs were transfected with either control siRNA (siCtrl) or siRNA against Gαq. (D) Gα12 or (E) Gα13. G protein expression levels were assessed by Western blotting after 72 hour-knockdown. **P < 0.01 (n = 3). Cytoskeletal protein α-actinin was blotted as a loading control. (F) NRVMs were transfected with either siCtrl or siRNA against Gαq, Gα12, or Gα13 for 72 h and then stimulated with 0.3 μM S1P for 5 min. RhoA activation was assessed by GTP-RhoA pull-down assay. **P < 0.01 vs. siCtrl vehicle (Veh), ##P < 0.01 vs. siCtrl + S1P (n = 7). (G) Dual-Luciferase Reporter Assay was performed as described in Materials and Methods to assess RhoA activation. **P < 0.01 vs. siCtrl, ##P < 0.01 vs. siCtrl + S1P (n = 6).
Our results also suggest that $G_{\alpha_{13,b}}$ and not $G_{\alpha_{12}}$ couples S1P receptor stimulation to RhoA activation in the heart. We also provide extensive evidence that S1P signaling through this pathway causes limited PLC activation and is not sufficient to induce hypertrophic responses.

4.1. S1P and cardiac hypertrophy

$G_{\alpha_q}$ has been established to be essential in development of hypertrophy induced by GPCR agonists and by pressure-overload [19,20]. Our results demonstrate that S1P, in contrast to many other GPCR agonists, does not induce a hypertrophic response in NRVMs (Fig. 1D, E, F, and G) nor is S1P receptor stimulation involved in pressure-overload induced hypertrophy in vivo (Fig. 1). Compared to the adrenergic agonist PE, which activates $G_{\alpha_q}$-coupled $\alpha_1$-adrenergic receptors and PLCβ, S1P-induced PI responses are extremely modest and appear to be RhoA/PLCε dependent (Fig. 1A, B, and C). These results suggest that although S1P2 and S1P3 have the ability to activate $G_{\alpha_q}$ signaling [9,14,22], presumably through PLCε and its downstream mediators [17,47].

Fig. 3. S1P activates RhoA through S1P3 in NRVMs and in isolated perfused hearts. NRVMs were transfected with either siCtrl or siRNA against (A) S1P1, (B) S1P2, (C) or S1P3. S1P receptor levels were assessed by qPCR analysis after 48-hour siRNA transfection. Data shown represent the mean ± SEM, **$P < 0.01$ vs. siCtrl ($n = 3$) (D) NRVMs were transfected with siRNA to knockdown S1P receptor subtypes for 48 h and then stimulated with 0.3 $\mu$M S1P for 5 min. RhoA activation was assessed by GTP-RhoA pull-down assay. *, **$P < 0.05, 0.01$ vs. siCtrl, ###$P < 0.01$ vs. siCtrl + S1P ($n = 4$). (E) NRVMs were transfected with siRNA to knock down S1P3 receptor and then stimulated with 0.3 $\mu$M S1P or 10 $\mu$M CYM-51736 for 5 min. RhoA activation was assessed by the GTP-RhoA pulldown assay. **$P < 0.01$ vs. Veh ($n = 4$). (F) NRVMs were pretreated for 30 min with 1 $\mu$M of JTE-013, an S1P2 receptor antagonist, and then stimulated with 0.3 $\mu$M S1P for 5 min. RhoA activation was assessed by the GTP-RhoA pulldown assay. *$P < 0.05$ vs. Veh ($n = 3$). (G) Isolated wild-type (WT), S1P2 KO, and S1P3 KO mouse hearts were perfused with either vehicle or 0.3 $\mu$M S1P in Kreb-Henseleit buffer for 5 min in Langendorff mode and RhoA activation was assessed by GTP-RhoA pull-down assay. **$P < 0.01$ vs. Veh ($n = 3$ to 5).
**Fig. 4.** S1P protects against ex vivo I/R through S1P3 receptor. Representative images of TTC-stained cross sections of isolated perfused mouse hearts after I/R injury (top) and quantification of infarct size (bottom). White areas are infarcted tissue and red areas are viable tissue. (A) Isolated WT and S1P3 KO hearts were perfused with either Veh or 0.3 μM S1P for 10 min and subjected to 22 min global ischemia followed by 60 min reperfusion. *P < 0.05 vs. WT + Veh (n ≥ 5). The data shown are the mean ± SEM of each group's animals. (B) WT hearts were perfused with either vehicle or 10 μM CYM-51736 (S1P3 specific agonist) for 10 min and subjected to I/R. Infarct size was assessed by TTC staining. **P < 0.01 vs Vehicle (n = 5).

**Fig. 5.** The S1P3 receptor mediates the activation of PKD by S1P in NRVMs. Representative Western blots of NRVMs that were transfected with siRNA against S1P receptor subtypes for 48 h followed by treatment with either (A) 0.3 μM S1P for 5 min or (B) 10 μM CYM-51736 for 20 min. The data displayed are the mean ± SEM. *P < 0.05 vs. siCtrl vehicle, #P < 0.05 vs. siCtrl + S1P or CYM-51736 (n = 4 to 5). (C) NRVMs were transfected with siRNA against Gα13 for 72 h or treated with 2 μg/mL C3 Rho inhibitor 12 h, followed by treatment with 10 μM CYM-51736 for 15 min. Data displayed are the experimental means ± SEM. **P < 0.01 vs. siCtrl + Veh, #P < 0.05 vs. siCtrl + CYM-51736, ##P < 0.01 vs. siCtrl + CYM-51736 (n = 5). (D) NRVMs were pretreated for 30 min with 1 μM of S1P2 receptor antagonist JTE-013, then stimulated with 0.3 μM S1P. Data displayed are the experimental means ± SEM. **P < 0.01 vs. vehicle, #P < 0.05 vs. S1P stimulation (n = 3).
15], recruitment of Goq signaling through these receptors in cardiomyocytes is limited and is not sufficient to drive hypertrophic responses in the heart. The observation that the hypertrophic response induced by pressure-overload was unaffected in S1P1 or S1P3 KO mice further indicates that activation of these S1P receptors does not contribute to hypertrophy in vivo (Fig. 1).

4.2. S1P receptor and Go subtype coupling in RhoA signaling

S1P1 exclusively couples to Goq, while S1P2 and S1P3 activate multiple G protein subtypes: Goq, Go12, and Go13 [12,14,15,46]. In the context of RhoA activation, Go12 and Go13, members of the Go12/13 subfamily of G proteins, are recognized to transduce G protein-coupled receptor stimulation to the activation of RhoA through direct regulation of RhoGEFs [21,24,48–50]. Interestingly, the data presented here reveal that Go13 but not Go12 knockdown significantly attenuates S1P-induced RhoA activation, suggesting that Go12 cannot compensate for the function of Go13 in cardiomyocytes. We cannot rule out the possibility that the Go12 knockdown achieved by siRNA is insufficient to observe the contribution of Go12 to S1P-mediated RhoA activation in NRVMs. It is clear, however, that Go12 and Go13 can signal through distinct and nonredundant mechanisms, as evidenced by the finding that Go13 KO mice die during embryonic development, while Go12 KO mice are viable, fertile, and without obvious phenotype [51–53].

There is considerable evidence for preferential activation of RhoA through Go13 in response to upstream GPCR activation in some systems. A previous study using G protein knockdown or knockout in cardiomyocytes demonstrated that Go13 was specifically required for endothelin-1 and AngII to activate RhoA [54]. Activation of RhoA by a TXA2 agonist has been observed in both wild-type and Go12 KO platelets, whereas Go13-ko platelets lacked RhoA activation [55]. Studies using Go12 and Go13 antibody microinjection in Swiss 3T3 cells demonstrated that lysophosphatic acid (LPA) stimulated stress fiber formation, a Go13-mediated response, through Go13 but not Go12 [56]. Using the SRE.L reporter assay, as demonstrated for S1P (Fig. 2G), we also observed preferential involvement of Go13 but not Go12 in RhoA activation by LPA (data not shown).

Selective utilization of Go13 versus Go12 may reflect the specific nature of the RhoA GTP exchange factors (GEFs) that predominate in the tissue under study. The regulator of G protein signaling homology (RH) family of Rho GEFs (p115RhoGEF, leukemia-associated RhoGEF a.k.a. LARG, and PDZ-RhoGEF) are among the best characterized downstream effectors of Go12 and Go13. Previous seminal in vitro reconstitution experiments revealed that Go13, but not Go12, stimulates the activity of p115 RhoGEF activity and PDZ-RhoGEF [21,23]. Leukemia-associated RhoGEF (LARG) is also selectively activated by Go13 and can only be activated by Go13 when this GEF is phosphorylated by tyrosine-kinase [57].

4.3. S1P receptor subtypes and Rho signaling

The expression of the five S1P receptor subtypes varies depending on cell type. Previous studies, including work from our laboratory and those of others showed that S1P1, S1P2, and S1P3 are the major subtypes expressed in the heart [8,27,46,58]. S1P1 is the most abundant subtype in mouse cardiomyocytes, followed by S1P2, while S1P1 is expressed at relatively lower levels [5,11,27,58]. The lack of involvement of the S1P1 receptor in RhoA activation is consistent with our finding that blocking Goq, the only G protein to which S1P1 couples, does not inhibit RhoA activation.

The relative roles of S1P3 versus S1P2 in RhoA activation appear to be tissue specific since studies in non-cardiomyocytes implicate S1P2 in RhoA activation, or the combined effects of S1P2 and S1P3 on activation of RhoA [27,59,60]. Which subtype predominates could depend on subcellular localization of the receptor or its level of expression. The higher level of mRNA expression for S1P3 versus S1P2 in cardiomyocytes may explain the dominance of S1P3 signaling to RhoA in the heart [5,61]. There may also be as yet undefined differences in S1P1 and S1P3 coupling to Go12 versus Go13, for example differences in interaction with endogenous RhoGEFs in a given cell type as discussed above. Regardless of the mechanistic basis, our findings using siRNA-mediated S1P receptor knockdown and S1P3 KO hearts are the first to reveal that S1P3 is the receptor subtype that mediates RhoA activation by S1P in cardiomyocytes (Fig. 3D, G).

4.4. S1P receptor subtypes and cardioprotection

A wide range of molecules downstream of S1P receptor activation have the potential to mediate cardioprotection. Our laboratory previously demonstrated that S1P treatment of NRVMs leads, through RhoA and its effect on PLCc activation, to activation of PKD [6]. Furthermore, the protective effect of S1P was lost in hearts from PKD KO or PLCc KO mice [6]. In line with the aforementioned significant contribution of S1P3 to RhoA activation, we show here that PKD activation induced by S1P treatment also required the S1P3 receptor (Fig. 5A). These results place PKD downstream in the S1P3/RhoA signaling axis. We demonstrate that S1P-mediated cardioprotection against I/R injury is abolished in S1P3 KO hearts (Fig. 4A), and stimulated by the S1P3 agonist CYM-51736 (Fig. 4B). These results are consistent with previously published work demonstrating that S1P associated with high density lipoprotein protects the heart against I/R injury in vivo through effects on S1P3 [4]. We also demonstrate that selective activation of S1P3 by CYM-51736 leads to robust activation of PKD (Fig. 5C) and confers protection comparable to that observed with S1P (Fig. 4B), whereas inhibition of S1P2 by JTE-013 does not block PKD activation by S1P (Fig. 5D). Thus, this study defines early players in the S1P signaling pathway and suggests that coupling of the S1P3 receptor to Go13 and RhoA plays a major role in S1P mediated cardioprotection against U/R injury.

The involvement of S1P3 receptors in RhoA cardioprotective signaling is of additional interest since fingolimod (FTY720), a widely used drug for clinical treatment of multiple sclerosis, is an agonist for both S1P1 and S1P3 receptors. Fingolimod has been reported to induce cardioprotection in a porcine model of I/R injury [62], as well as to protect against reperfusion-induced cardiac arrhythmias [58,63,64]. Notably the S1P1 receptor which is predominant in the myocardium is downregulated by fingolimod treatment where S1P3 receptors may remain [63]. In light of the findings presented here, signaling through the S1P3 receptor could contribute to the protective effects of fingolimod.

All three S1P receptors expressed in the heart have been shown to couple to Goq. Our earlier studies using an in vivo I/R model concluded that both S1P2 and S1P3 activate Akt through Goq to confer cardioprotection [5]. Another study also demonstrated that S1P2 and S1P3 antagonists block S1P mediated cardioprotection [65]. The studies reported here show that signaling via the S1P3 receptor to RhoA in cardiomyocytes of the isolated, perfused heart is both sufficient and required for cardioprotection in ex vivo I/R. Akt activation by S1P2 and S1P3 could contribute, along with the S1P3-mediated RhoA activation, to the protective effect of S1P on cardiomyocytes observed in vivo, either by direct actions on cardiomyocytes or S1P-mediated effects on non-cardiac cells [41,63].

4.5. Conclusion

We show in this study that S1P receptor stimulation does not induce hypertrophic responses in cardiomyocytes and is not necessary for the development of hypertrophy in response to pressure-overload. Using siRNA-mediated knockdown and knockout mouse models, we demonstrate for the first time that the actions of the S1P on the S1P3 receptor and its coupling to Goq to activate RhoA and PKD. Using gene silencing and deletion, we also demonstrate that S1P3 is responsible for S1P-mediated cardioprotection against ex vivo I/R injury. These findings are supported by experiments using a recently developed S1P3 selective
agonist, CVM-51736. We suggest that specific drug targeting of S1P receptors could provide a therapeutic benefit in ischemic heart disease without the undesirable effects of global activation of other cardiac S1P receptors.

Conflicts of interest
The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions
B.S.Y. and C.S.B. performed, analyzed the experiments for the figures of this paper, and wrote the paper. S.Y.X. carried out experiments in Figs. 1A–H, 2, and 3A. J.H.B. and S.M. conceived and coordinated the study and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

Acknowledgments
This work was supported by National Institutes of Health Grants T32HL007444 to Cameron Brand, HL028143 and GM036927 to Joan Heller Brown, and R56 HL097037 to Shigeaki Miyamoto.

References


