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Original article

Selective coupling of the $S1P_3$ receptor subtype to S1P-mediated RhoA activation and cardioprotection

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ABSTRACT

Sphingosine-1-phosphate (S1P), a bioactive lysophospholipid, is generated and released at sites of tissue injury in the heart and can act on $S1P_1$, $S1P_2$, and $S1P_3$ 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 G_q. 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 $G\alpha_{13}$ but not $G\alpha_{12}$, $G\alpha_q$, or $G\alpha_i$. Knockdown of the three major S1P receptors using siRNA demonstrated a requirement for $S1P_3$ in RhoA activation and subsequent phosphorylation of PKD, and this was confirmed in studies using isolated hearts from $S1P_3$ 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 $S1P_3$ KO mouse heart. CYM-51736, an $S1P_3$-specific agonist, also decreased infarct size after ischemia/reperfusion to a degree similar to that achieved by S1P. The finding that $S1P_3$ receptor- and $G\alpha_{13}$-mediated RhoA activation is responsible for protection against ischemia/reperfusion suggests that selective targeting of $S1P_3$ receptors could provide therapeutic benefits in ischemic heart disease.

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 bioactive lysophospholipid sphingosine-1-phosphate (S1P) is endogenously released in response to cardiac injury [2,3] and that S1P helps to protect the heart from the oxidative damage that leads to reperfusion injury [4–7]. S1P is a high affinity ligand for five G protein-coupled receptor (GPCR) subtypes denoted $S1P_{1-5}$ [8,9]. The $S1P_{1-3}$ receptor subtypes are expressed in cardiomyocytes. $S1P_1$ is the predominant subtype expressed in the heart as well as in cardiomyocytes and it exclusively couples to $G\alpha_i$ [10,11]. Coupling to $G\alpha_i$ leads to inhibition of cyclic AMP formation and accounts for the ability of S1P to decrease cardiac contractility [9,10,12,13]. The $S1P_2$ and $S1P_3$ receptors can couple to $G\alpha_i$ signaling in the heart [10] but these subtypes are, in addition, able to couple to $G\alpha_q$, $G\alpha_{12}$, and $G\alpha_{13}$ [9,14,15].

Activation of $G\alpha_q$ stimulates phospholipase C-beta (PLCβ) and has been demonstrated to play a major role in the development of cardiac hypertrophy [16–20]. While $S1P_2$ and $S1P_3$ have been shown to couple to $G\alpha_q$ in other systems [9,14], it has not been determined whether stimulation of these receptors in cardiomyocytes activates $G\alpha_q$ and PLCβ to elicit signals that lead to cardiomyocyte hypertrophy. The $G\alpha_{12/13}$ family of G proteins regulates the low molecular weight G protein Ras homolog gene member A (RhoA) through direct stimulation of guanine exchange factors [21–25]. Recently we demonstrated that cardiac expression of RhoA protects the heart against oxidative stress and ischemia/reperfusion (I/R) injury whereas gene deletion of RhoA

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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 $S1P_2$ or $S1P_3$ 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 $G\alpha_{13}$ and activation of RhoA, that this pathway does not strongly activate PLCβ or contribute to development of cardiac hypertrophy, and that it is the $S1P_3$ 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 $S1P_2$ KO and $S1P_3$ 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 $3.0 \times 10^5/cm^2$ 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 Rho inhibitor C3 exoenzyme was obtained from Cytoskeleton (CT04). Pertussis toxin (PTX) was purchased from Calbiochem. Phenylephrine (PE) was obtained from Sigma Life Science. S1P was obtained from Avanti Polar. CYM-51736, is an allosteric agonist of $S1PR_3$ of the structure *N*,*N*-dicyclohexyl-5-(furan-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 VECTASHIELD 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 C_T method (i.e. the $2^{-\Delta\Delta C}{}_T$ method) [31]. GAPDH levels were used as the internal control.

2.5. Phosphatidylinositol (PI) hydrolysis

After overnight culturing, NRVMs were labeled with tritium-labeled ([^{3}H]) 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, $S1P_2$ KO, or $S1P_3$ 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 Rhotekin 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, $G\alpha_q$, $G\alpha_{12}$, and $G\alpha_{13}$ from Santa Cruz Biotechnology, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phospho-PKD ($Ser^{744/748}$), PKD, and α-actinin from Cell Signaling Technology. Peroxidase-conjugated secondary antibodies were used at a dilution of 1:2000 (Sigma) and the enhanced chemiluminescent 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, $S1P_2$ KO, or $S1P_3$ KO mice were removed quickly and perfused with modified Krebs-Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 0.5 mM EDTA, 1.2 mM $MgSO_4$, 11 mM glucose, 1.5 mM sodium pyruvate, and 2 mM $CaCl_2$) 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 $G\alpha_{12}$ or $G\alpha_{13}$. The following day, cells were transfected via Lipo2000 (Invitrogen) for 8 h with an SRE.L reporter (1 μg/well) as well as a *Renilla* plasmid (100 ng/well) to normalize the luminescence signal. After transfections, cells were serum starved for 24 h before stimulating with 1 μM S1P for 8 h. Reporter activity was then measured using the Dual-Luciferase Reporter Assay (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 μM S1P for 0, 1, 5, 10, 30, and 60 min. Responses were compared with those seen with 50 μM PE, an established hypertrophic agonist that activates α-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 PLCε 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 μg/mL). The response to S1P was blocked whereas the response to PE was not (Fig. 1B). In addition, knockdown of PLCε 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 $G\alpha_q$/PLCβ signaling pathway to elicit robust PI hydrolysis, the less robust PI response to S1P is mediated through RhoA signaling to PLCε.

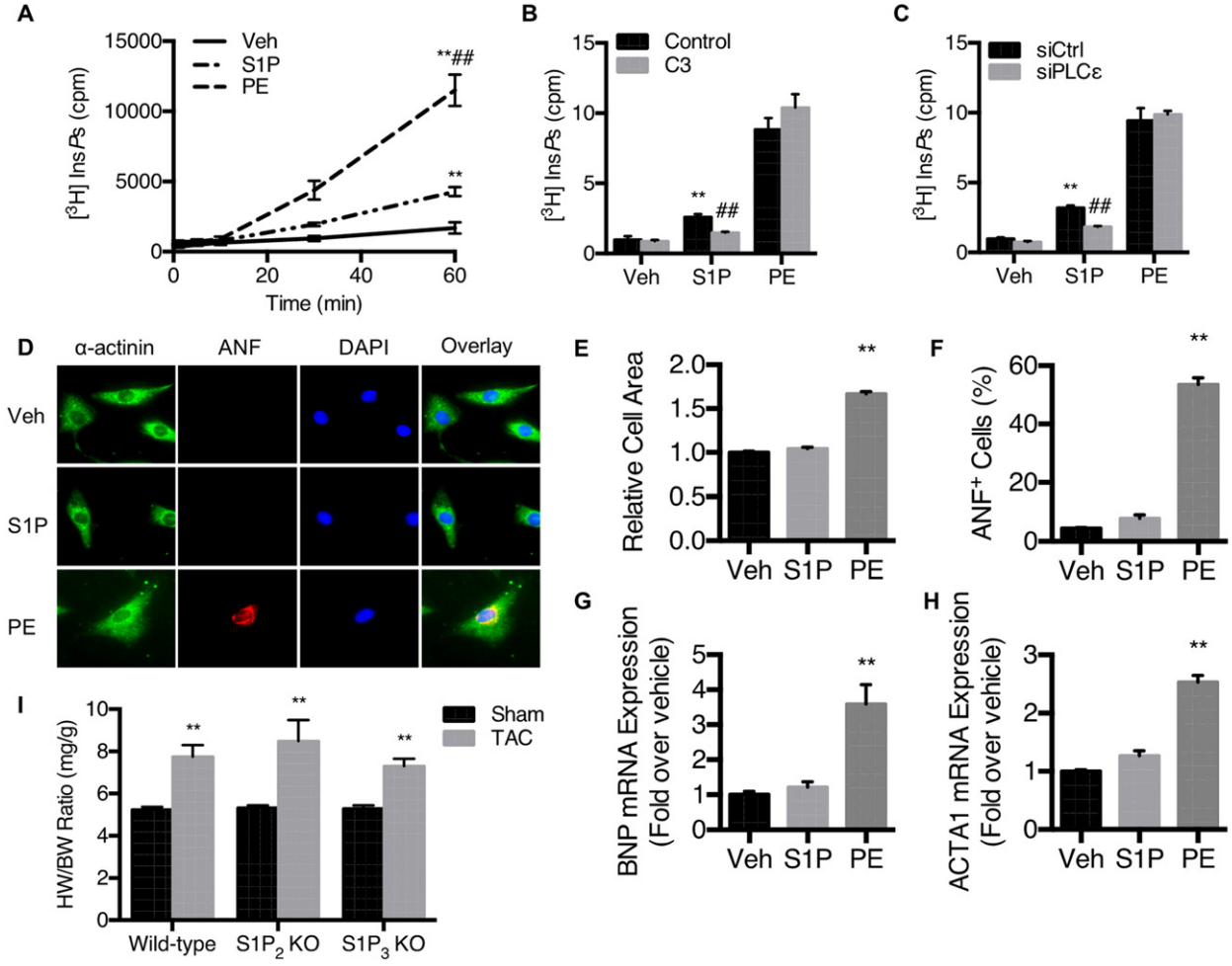

Fig. 1. S1P receptor signaling stimulates modest increases in PI hydrolysis and does not mediate in vitro or in vivo cardiac hypertrophy. (A) Time course of S1P- and PE-induced phosphatidylinositol hydrolysis. NRVMs were serum-starved overnight in the presence of [3H] inositol. Cells were then treated with agonists for 1, 5, 10, 30, and 60 min in the presence of LiCl before isolation of [3H] 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 μg/mL C3 exoenzyme (Rho inhibitor) for 6 h or (C) transfected with control siRNA (siCtrl) or siRNA against PLCε 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 α-actinin, atrial natriuretic factor (ANF), and nuclei with DAPI after treatment with either vehicle, 0.3 μM S1P, or 50 μM PE for 24 h. Scale bar: 20 μm. (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 α-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 ($n \geq 5$).

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 $G\alpha_q$/PLCβ elicits hypertrophy in NRVMs.

3.2. $S1P_2$ and $S1P_3$ receptors do not play a role in TAC induced hypertrophy in mouse hearts

Inhibition or genetic deletion of $G\alpha_q$ 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 $G\alpha_q$ ($S1P_2$ and $S1P_3$) mediate development of hypertrophy in vivo, we subjected WT, $S1P_2$ KO, and $S1P_3$ KO mice 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 $S1P_2$ and $S1P_3$ 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 $G\alpha_{13}$ 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 $G\alpha_i$ (for $S1P_1$) or to $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12/13}$ (for $S1P_2$ and $S1P_3$). The G protein subunits that are most dedicated in coupling GPCRs to Rho activation through RhoGEFs are $G\alpha_{12}$ and $G\alpha_{13}$. In some systems, however, RhoA can also be activated through $G\alpha_q$ and $G\alpha_i$. To test involvement of $G\alpha_i$ in Rho activation induced by S1P cells were pretreated with PTX overnight followed by 5-minute S1P stimulation. S1P elicited a significant increase in activated RhoA which was unaffected by pretreatment with PTX (Fig. 2B). To determine whether $G\alpha_q$, $G\alpha_{12}$ or $G\alpha_{13}$ were involved in S1P-mediated RhoA activation we used small interfering RNA (siRNA)-mediated knockdown. Treatment with siRNA for 72 h reduced expression of each Gα subunit by >50% (Fig. 2C, D, E). Subsequent S1P treatment of cells transfected with control siRNA induced RhoA activation, which was not inhibited by knockdown of $G\alpha_q$ or $G\alpha_{12}$ (Fig. 2F). Knockdown of $G\alpha_{13}$, however, significantly attenuated RhoA activation by S1P. Using an SRE.L-luciferase readout for RhoA activation, we further demonstrated that S1P signaling in NRVMs was through $G\alpha_{13}$ but not $G\alpha_{12}$ (Fig. 2G). These combined results implicate $G\alpha_{13}$ in S1P induced activation of RhoA in cardiomyocytes.

3.4. S1P activates RhoA through the $S1P_3$ 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 the pulldown of GTP-bound active RhoA. Knockdown of $S1P_3$ receptors markedly reduced RhoA activation by S1P. In contrast, RhoA was activated to an equivalent extent in control siRNA-treated cells, and $S1P_1$ or $S1P_2$ knockdown cells (Fig. 3D). CYM-51736, an $S1P_3$ 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 $S1P_2$ receptor selective antagonist, JTE-013, which has been used at 1 μM to show $S1P_2$ selective signaling [41]. We observed that JTE-013 did not block RhoA activation by S1P (Fig. 3F), further indicating $S1P_3$ but not $S1P_2$ involvement.

To extend our finding of $S1P_3$ receptor-mediated RhoA activation to the intact adult heart, WT, $S1P_2$ KO, or $S1P_3$ 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 $S1P_2$ KO (Fig. 3G). In contrast, S1P treatment failed to activate RhoA in $S1P_3$ KO mouse hearts, indicating that the $S1P_3$ receptor plays a critical role in S1P induced RhoA activation in the adult mouse heart as it does in neonatal rat cardiomyocytes.

3.5. $S1P_3$ 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 $S1P_3$ receptor. To test this hypothesis, hearts from WT and $S1P_3$ 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 $S1P_3$ KO hearts (Fig. 4A). To further confirm the significant role of $S1P_3$ in cardioprotection we examined the effect of CYM-51736, a derivative of the previously reported $S1P_3$ 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 $S1P_3$ receptor in S1P-mediated cardioprotection against I/R injury.

3.6. $S1P_3$ 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 $S1P_3$ in NRVMs significantly attenuated PKD phosphorylation in response to S1P whereas knockdown of $S1P_1$ or $S1P_2$ did not (Fig. 5A). In addition, the $S1P_3$ selective agonist CYM-51736 increased PKD phosphorylation, and failed to do so following $S1P_3$ receptor knockdown (Fig. 5B). PKD activation by CYM-51736 was attenuated by siRNA knockdown of $G\alpha_{13}$, or by inhibiting RhoA by treatment with C3 exoenzyme (Fig. 5C). The $S1P_2$ antagonist JTE-013, shown above to have

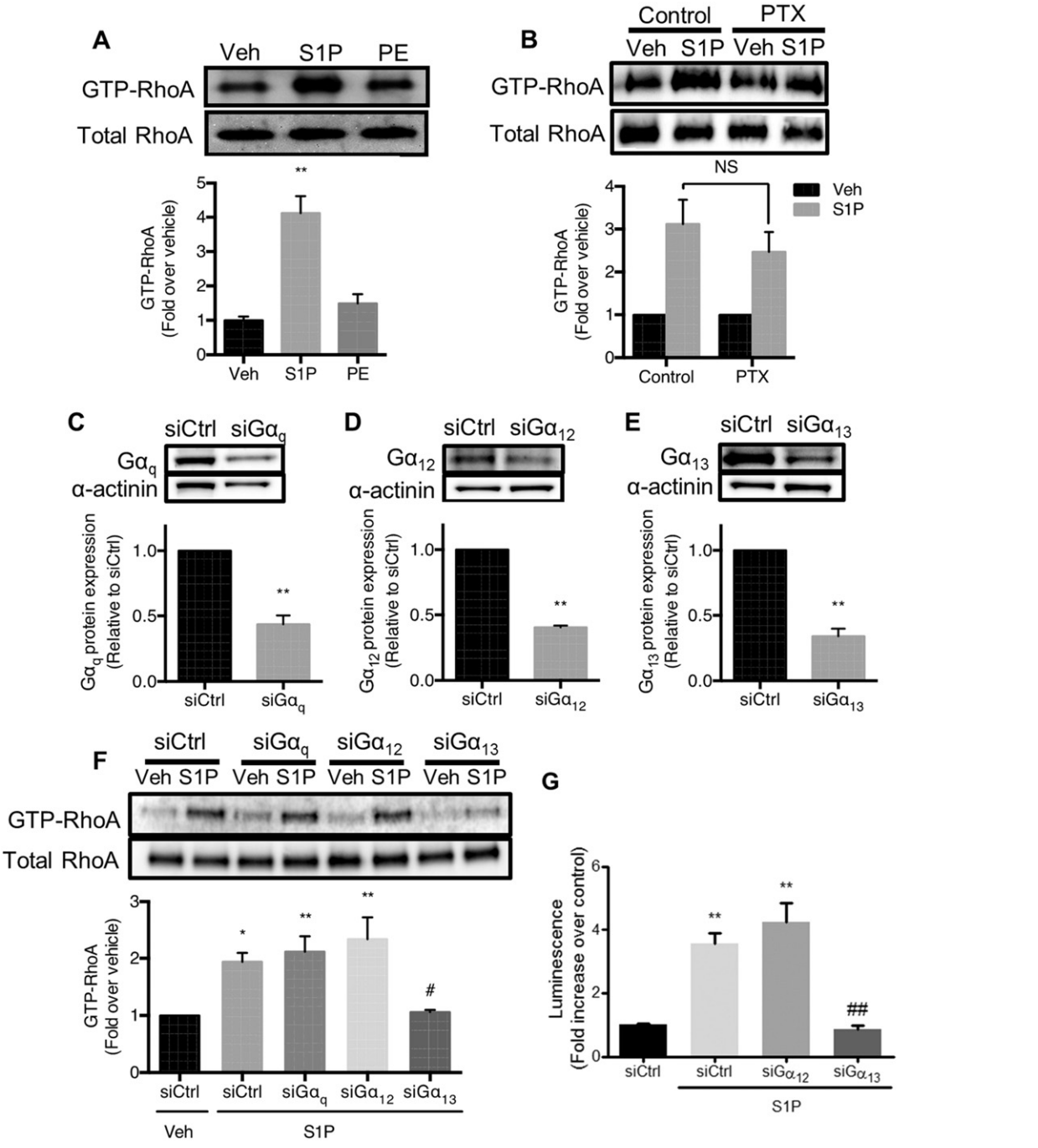

Fig. 2. S1P activates RhoA through $G\alpha_{13}$ in NRVMs. (A) NRVMs were treated with 0.3 μM S1P or 50 μM PE for 5 min and RhoA activation was measured by a GTP-RhoA pull-down assay. The data displayed are the mean ± SEM ($n = 5$), $^{**}P < 0.01$ vs. vehicle (Veh). (B) NRVMs were pretreated with 0.1 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 $G\alpha_q$, (D) $G\alpha_{12}$, or (E) $G\alpha_{13}$. 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 $G\alpha_q$, $G\alpha_{12}$, or $G\alpha_{13}$ for 72 h and then stimulated with 0.3 μM S1P for 5 min. RhoA activation was assessed by GTP-RhoA pull-down assay. *, $^{**}P < 0.05$, 0.01 vs. siCtrl vehicle (Veh), $\#P < 0.05$ 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 ($n = 6$).

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 $S1P_3$ 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 [3,4,45], 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 $S1P_3$ is the receptor subtype responsible for S1P-mediated RhoA activation, PKD activation and protection against I/R injury in cardiomyocytes and in the isolated

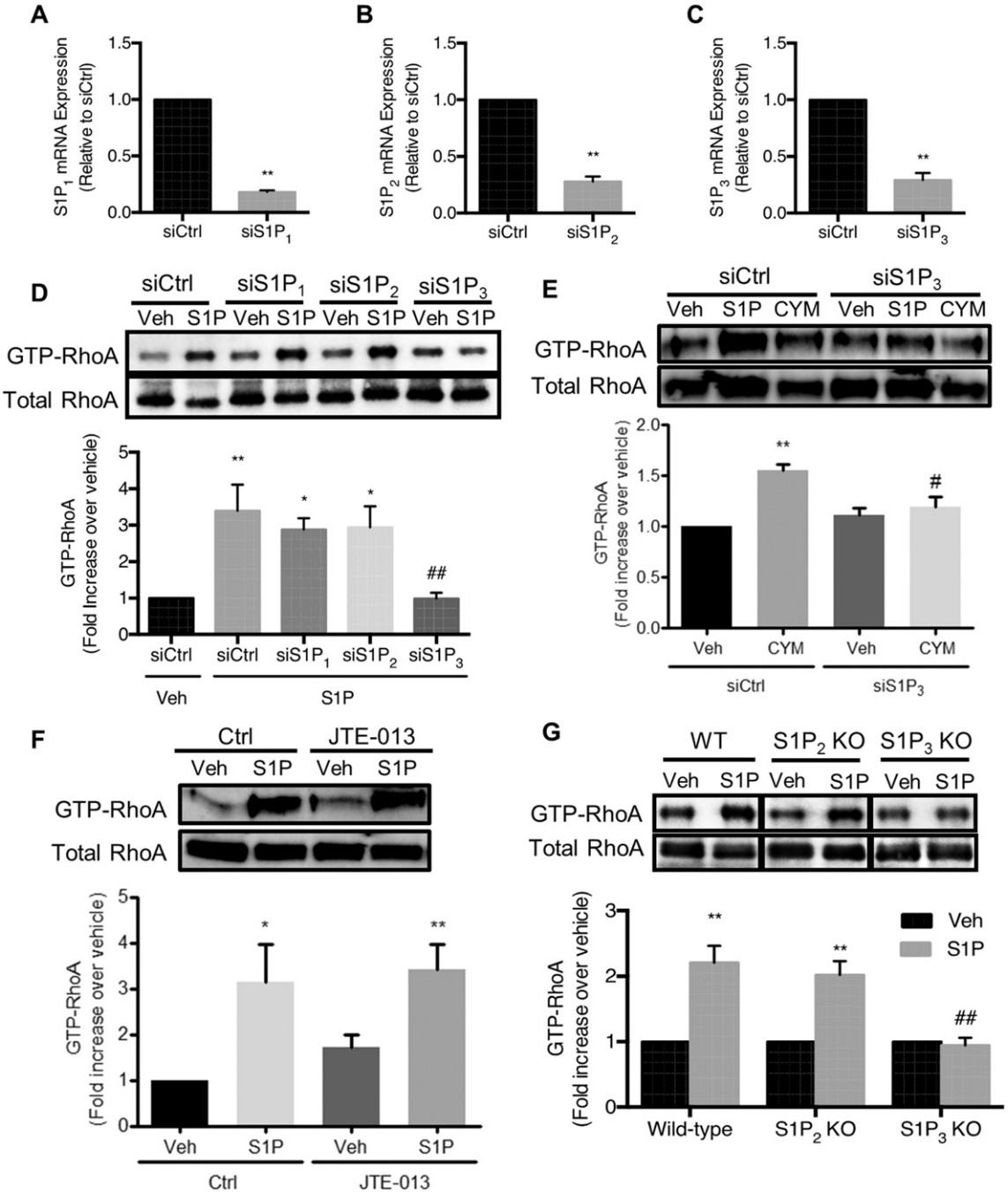

Fig. 3. S1P activates RhoA through $S1P_3$ in NRVMs and in isolated perfused hearts. NRVMs were transfected with either siCtrl or siRNA against (A) $S1P_1$, (B) $S1P_2$, (C) or $S1P_3$. S1P receptor levels were assessed by qPCR analysis after 48-hour siRNA transfection. Data shown represent the mean ± 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 μM 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 $S1P_3$ receptor and then stimulated with 0.3 μM S1P or 10 μM 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 μM of JTE-013, an $S1P_2$ receptor antagonist, and then stimulated with 0.3 μM 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), $S1P_2$ KO, and $S1P_3$ KO mouse hearts were perfused with either vehicle or 0.3 μM S1P in Krebs-Henseleit buffer for 5 min in Langendorff mode and RhoA activation was assessed by GTP-RhoA pull-down assay. $^{**}P < 0.01$ vs. vehicle, $\#\#P < 0.01$ vs. WT + S1P ($n = 3$ to 5).

perfused heart. Our results also suggest that $G\alpha_{13}$, but 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], presumably through PLCβ and its downstream mediators [17,47]. Our results demonstrate that S1P, in contrast to many other GPCR agonists, does not induce a hypertrophic response in NRVMs (Fig. 1D, E, F, G, and H) 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 α_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 $S1P_2$ and $S1P_3$ have the ability to activate $G\alpha_q$ signaling [9,14,

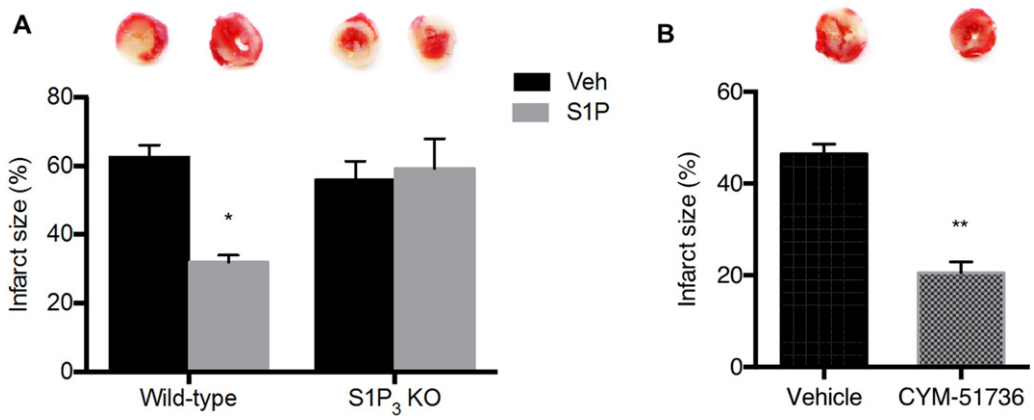

Fig. 4. S1P protects against ex vivo I/R through $S1P_3$ receptor. Representative images of TTC-stained cross sections of isolated perfused mouse hearts after I/R injury (top) and quantification of infarct size (bottom). White areas are infarcted tissue and red areas are viable tissue. (A) Isolated WT and $S1P_3$ 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 ($n \geq 5$). 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 ($S1P_3$ specific agonist) for 10 min and subjected to I/R. Infarct size was assessed by TTC staining. $^{**}P < 0.01$ vs Vehicle ($n = 5$).

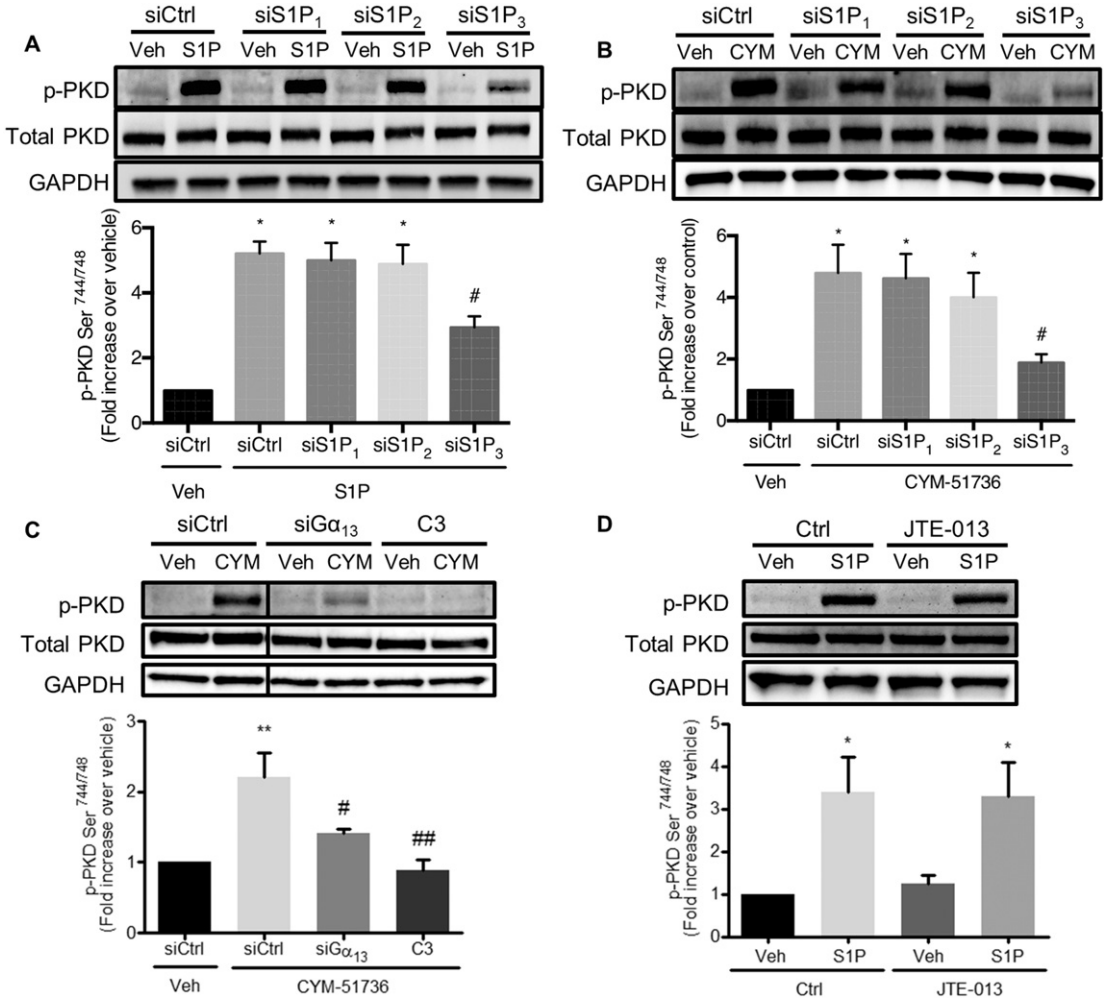

Fig. 5. The $S1P_3$ receptor mediates the activation of PKD by S1P in NRVMs. Representative Western blots of NRVMs that were transfected with siRNA against S1P receptor subtypes for 48 h followed by treatment with either (A) 0.3 μM S1P for 5 min or (B) 10 μM CYM-51736 for 20 min. The data displayed are 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 $G\alpha_{13}$ 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 $S1P_2$ 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 $G\alpha_q$ 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 $S1P_2$ or $S1P_3$ KO mice further indicates that activation of these S1P receptors does not contribute to hypertrophy in vivo (Fig. 1).

4.2. S1P receptor and Gα subtype coupling in RhoA signaling

$S1P_1$ exclusively couples to $G\alpha_i$, while $S1P_2$ and $S1P_3$ activate multiple G protein subtypes: $G\alpha_i$, $G\alpha_q$, $G\alpha_{12}$, and $G\alpha_{13}$ [12,14,15,46]. In the context of RhoA activation, $G\alpha_{12}$ and $G\alpha_{13}$, members of the $G\alpha_{12/13}$ 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 $G\alpha_{13}$ but not $G\alpha_{12}$ knockdown significantly attenuates S1P-induced RhoA activation, suggesting that $G\alpha_{12}$ cannot compensate for the function of $G\alpha_{13}$ in cardiomyocytes. We cannot rule out the possibility that the $G\alpha_{12}$ knockdown achieved by siRNA is insufficient to observe the contribution of $G\alpha_{12}$ to S1P-mediated RhoA activation in NRVMs. It is clear, however, that $G\alpha_{12}$ and $G\alpha_{13}$ can signal through distinct and nonredundant mechanisms, as evidenced by the finding that $G\alpha_{13}$ KO mice die during embryonic development, while $G\alpha_{12}$ KO mice are viable, fertile, and without obvious phenotype [51–53].

There is considerable evidence for preferential activation of RhoA through $G\alpha_{13}$ in response to upstream GPCR activation in some systems. A previous study using G protein knockdown or knockout in cardiomyocytes demonstrated that $G\alpha_{13}$ was specifically required for endothelin-1 and AngII to activate RhoA [54]. Activation of RhoA by a TXA_2 agonist has been observed in both wild-type and $G\alpha_{12}$ KO platelets, whereas $G\alpha_{13}$ KO platelets lacked RhoA activation [55]. Studies using $G\alpha_{12}$ and $G\alpha_{13}$ antibody microinjection in Swiss 3T3 cells demonstrated that lysophosphatidic acid (LPA) stimulated stress fiber formation, a RhoA-mediated response, through $G\alpha_{13}$ but not $G\alpha_{12}$ [56]. Using the SRE.L reporter assay, as demonstrated for S1P (Fig. 2G), we also observed preferential involvement of $G\alpha_{13}$ but not $G\alpha_{12}$ in RhoA activation by LPA (data not shown).

Selective utilization of $G\alpha_{13}$ versus $G\alpha_{12}$ 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 $G\alpha_{12}$ and $G\alpha_{13}$. Previous seminal in vitro reconstitution experiments revealed that $G\alpha_{13}$, but not $G\alpha_{12}$, stimulates the activity of p115 RhoGEF activity and PDZ-RhoGEF [21,23]. Leukemia-associated RhoGEF (LARG) is also selectively activated by $G\alpha_{13}$ and can only be activated by $G\alpha_{12}$ 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 $S1P_1$, $S1P_2$, and $S1P_3$ are the major subtypes expressed in the heart [8,27,46,58]. $S1P_1$ is the most abundant subtype in mouse cardiomyocytes, followed by $S1P_3$, while $S1P_2$ is expressed at relatively lower levels [5,11,27,58]. The lack of involvement of the $S1P_1$ receptor in RhoA activation is consistent with our finding that blocking $G\alpha_i$, the only G protein to which $S1P_1$ couples, does not inhibit RhoA activation.

The relative roles of $S1P_3$ versus $S1P_2$ in RhoA activation appear to be tissue specific since studies in non-cardiomyocytes implicate $S1P_2$ in RhoA activation, or the combined effects of $S1P_2$ and $S1P_3$ 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 $S1P_3$ versus $S1P_2$ in cardiomyocytes may explain the dominance of $S1P_3$ signaling to RhoA in the heart [5,61]. There may also be as yet undefined differences in $S1P_2$ and $S1P_3$ coupling to $G\alpha_{12}$ versus $G\alpha_{13}$, 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 $S1P_3$ KO hearts are the first to reveal that $S1P_3$ 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 PLCε activation, to activation of PKD [6]. Furthermore, the protective effect of S1P was lost in hearts from PKD KO or PLCε KO mice [6]. In line with the aforementioned significant contribution of $S1P_3$ to RhoA activation, we show here that PKD activation induced by S1P treatment also required the $S1P_3$ receptor (Fig. 5A). These results place PKD downstream in the $S1P_3$/RhoA signaling axis. We demonstrate that S1P-mediated cardioprotection against I/R injury is abolished in $S1P_3$ KO hearts (Fig. 4A), and stimulated by the $S1P_3$ 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 $S1P_3$ [4]. We also demonstrate that selective activation of $S1P_3$ 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 $S1P_2$ 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 $S1P_3$ receptor to $G\alpha_{13}$ and RhoA plays a major role in S1P mediated cardioprotection against I/R injury.

The involvement of $S1P_3$ 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 $S1P_1$ and $S1P_3$ 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 $S1P_1$ receptor which is predominant in the myocardium is downregulated by fingolimod treatment where $S1P_3$ receptors may remain [63]. In light of the findings presented here, signaling through the $S1P_3$ receptor could contribute to the protective effects of fingolimod.

All three S1P receptors expressed in the heart have been shown to couple to $G\alpha_i$. Our earlier studies using an in vivo I/R model concluded that both $S1P_2$ and $S1P_3$ activate Akt through $G\alpha_i$ to confer cardioprotection [5]. Another study also demonstrated that $S1P_2$ and $S1P_3$ antagonists block S1P mediated cardioprotection [65]. The studies reported here show that signaling via the $S1P_3$ 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 $S1P_2$ and $S1P_3$ could contribute, along with the $S1P_3$-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 S1P on the $S1P_3$ receptor and its coupling to $G\alpha_{13}$ activate RhoA and PKD. Using gene silencing and deletion, we also demonstrate that $S1P_3$ is responsible for S1P-mediated cardioprotection against ex vivo I/R injury. These findings are supported by experiments using a recently developed $S1P_3$ selective

agonist, CYM-51736. We suggest that specific drug targeting of $S1P_3$ 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 the paper, and wrote the paper. S.Y.X. carried out experiments in Fig. 1A–H. C.B.B.G. provided technical assistance for Figs. 3 and 4. J.C. provided S1P receptor KO mice and insightful comments (Figs. 1, 3 and 4). H.R. provided CYM-51736 and helpful discussion (Figs. 4 and 5). N.P. and C.M carried out the experiments in Fig. 1I. 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.

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