UNIVERSITY OF CALIFORNIA, SAN DIEGO

$S1P_3$ Receptor Coupling to RhoA Activation and Cardioprotection

A Thesis submitted in partial satisfaction of the

requirements for the degree in Master of Science

in

Biology

by

Bryan Shing Hei Yung

Committee in Charge:

Joan Heller Brown, Chair
Nicholas C. Spitzer, Co-Chair
Aaron B. Coleman

2015

The Thesis of Bryan Shing Hei Yung is approved and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego

2015

DEDICATION

This Thesis is dedicated to my family for their unconditional love and support; words cannot express the appreciation I have for you all. This is also dedicated to all my friends without whom this thesis would have been finished much earlier.

Thank you all so much.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AC	Adenylate cyclase
Akt	Protein kinase B
AMVM	Adult mouse ventricular myocytes
ANF	Atrial natriuretic factor
ANOVA	Analysis of variance
ARB	Angiotensin II receptor blockers
BNP	Brain natriuretic peptide
BW	Body weight
cAMP	Cyclic adenosine monophosphate
DAG	Diacylglycerol
DMEM	Dulbecco-modified Eagle's medium
DTT	Dithiothreitol
GAPDH	Glyceraldehyde-3-phoshpate dehydrogenase
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GPCR	G protein-coupled receptor
GST	Glutathione *S*-transferase
GTP	Guanosine triphosphate
HW	Heart weight
I/R	Ischemia/reperfusion
InsPs	Inositol phosphates
IP_3	Inositol triphosphate
IPOST	Ischemic postconditioning
KO	Knock out
MAPK	Mitogen-activated protein kinase
MEF	Mouse embryonic fibroblasts
NRVM	Neonatal rat ventricular myocytes
LARG	Leukemia-associate RhoGEF
LPA	Lysophosphatidic acid
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phenylephrine
PI	Phosphatidylinositol
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKD	Protein kinase D
PLC	Phospholipase C
PTX	Pertussis toxin
qPCR	Quantitative polymerase chain reaction
RH	Regulator of G protein signaling homology
RhoA	Ras homolog gene member A
ROCK	Rho Kinase
S1P	Sphingosine-1-phosphate
SDS	Sodium dodecylsulfate
Ser744/748	Serine-744/748

SRF	Serum response factor
TAC	Transverse aortic constriction
TTC	Triphenyl tetrazolium chloride
WT	Wild-type

LIST OF SCHEMES

LIST OF FIGURES

ACKNOWLEDGEMENTS

The last two years would not have been possible without such a great group of people who have been so instrumental in advancing my scientific education. I would first like to thank Dr. Joan Heller Brown for allowing me to be a part of the Brown lab family and for her support as my committee chair and principal investigator. Working in your lab has been a wonderful and invaluable experience. Furthermore, I want to thank my other committee members, Dr. Nicholas Spitzer and Dr. Aaron Coleman, for their time and support. They have been extremely accommodating despite their busy schedules. Thank you for volunteering to be a part of my thesis committee.

Next, I would also like to recognize my mentors, Dr. Shigeki Miyamoto and Dr. Sunny Xiang, for their guidance, patience, and insight. There are no words to express my gratitude for all that they have done for me and I could not imagine having better teachers.

I want to acknowledge Dr. Hugh Rosen and his laboratory for generously providing the $S1P_3$ selective agonist CYM-51736. I would like to thank Dr. Jerold Chun and his laboratory for providing the $S1P_2$ knock out and $S1P_3$ knock out mice.

Lastly, I would like to thank all the members of the Brown, Miyamoto, and Purcell labs for their support, friendship, and generosity. Thank you Dr. Nicole Purcell for your insight and help with the hypertrophy studies and Charlie Gray for helping me with the ischemia/reperfusion experiments. I would like to thank Jeffery Smith, Melissa Barlow, and Dr. Valerie Tan for technical support and animal care, and Dr. Stephanie Dusaban Gonzales for editing my writing. I am grateful for all the past and present members of the lab for making the overall experience so unforgettable: Amy Taylor, Eric Ding, Olivia Yu,

Andrew Willeford, Courtney Moc, Kinnary Doshi, and all the other members over the years. You have all been like a family to me and I will never forget all the memories we shared together.

ABSTRACT OF THE THESIS

$S1P_3$ Receptor Coupling to RhoA Activation and Cardioprotection

by

Bryan Shing Hei Yung

Master of Science in Biology

University of California, San Diego, 2015

Professor Joan Heller Brown, Chair
Professor Nicholas C. Spitzer, Co-Chair

Sphingosine-1-phoshpate (S1P) is a lysophospholipid that can signal through multiple G protein-coupled receptor subtypes. S1P 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 effect cardiovascular responses. We established that S1P causes little phosphatidylinositol hydrolysis and does not induce hypertrophy in neonatal rat ventricular myocytes (NRVMs), indicating that it does not cause receptor coupling to Gq. We previously demonstrated that S1P confers cardioprotection against

ischemia/reperfusion (I/R) and mediates this cardioprotection by activating RhoA and its downstream effector PKD. The S1P receptors and G proteins that regulate RhoA activation and downstream response in the heart have not been determined. By inhibiting different G proteins in NRVMs, we established that S1P regulates RhoA activation through $G\alpha_{13}$. Knockdown of the three S1P receptors using siRNA demonstrated a requirement for $S1P_3$ in RhoA activation and subsequent PKD phosphorylation, and was confirmed in studies using isolated hearts from $S1P_3$ knockout (KO) mice. To investigate the functional role of $S1P_3$ receptors, we subjected isolated wild-type (WT) or $S1P_3$ KO mouse hearts to *ex vivo* I/R. Addition of S1P prior to I/R significantly reduced infarct size in WT hearts; this protection was abolished in the $S1P_3$ KO. To confirm the role of $S1P_3$ in cardioprotection, WT mouse hearts were perfused with $S1P_3$-specific agonist CYM-51736; infarct development was attenuated to a degree similar to that achieved by S1P. These findings suggest that $S1P_3$- and $G\alpha_{13}$-mediated RhoA/PKD activation are responsible for protection against I/R.

INTRODUCTION

Ischemic heart disease

Cardiovascular disease is the leading cause of death globally, claiming approximately 17 million lives every year (1). Of the many types of heart disease, ischemic heart disease, also known as coronary artery disease, is the most common type of heart disease and is characterized by reduced blood supply to the heart, leading to myocardial infarction. Restoration of blood flow after an ischemic episode (e.g. myocardial infarction) is necessary to prevent catastrophic heart failure, but reperfusion can itself lead to an additional increase in cardiomyocyte death; a process referred to as reperfusion injury (2-4). The primary therapies available for limiting myocardial infarction and preventing the onset of heart failure are thrombolytic therapy or percutaneous coronary intervention (i.e. coronary angioplasty). An adjunct therapy to uninterrupted reperfusion is ischemic postconditioning (IPOST), the employment of intermittent ischemic episodes during reperfusion, which has been shown in some studies to further reduce myocardial infarction (5,6), although not all clinical studies have been positive (7,8). Despite improvements in therapies that allow timely reperfusion of occluded arteries, there is still no reliable therapy for preventing reperfusion injury, suggesting the ongoing need for new therapeutic treatments. Experiments have been conducted to identify potential mediators of reperfusion injury, including oxidative stress (9,10), intracellular calcium overload (11,12), and inflammation (13-15), and have resulted in the development of pharmacological treatments to limit cardiomyocyte death during ischemia/reperfusion injury (I/R). Previous work has shown that the G protein-coupled receptor (GPCR) agonist sphingosine-1-phosphate (S1P) is endogenously released in

response to cardiac injury (16,17) and that S1P protects the heart from the oxidative damage that leads to I/R (18-20). However, the receptor subtypes and intracellular signaling pathways by which S1P mediates cardiac protection are not fully understood.

G protein-coupled receptor signaling

Over 200 GPCRs have been identified in the heart and they play an important role in the regulation of diverse cardiac functions such as heart contractility, heart rate, hypertrophy, heart failure development, and cardioprotection (21). GPCRs are seven-transmembrane domain receptors found on the surface of eukaryotic cells and are currently the largest family of proteins targeted by drug discovery (21-23). Antagonists of these receptors are the most commonly used pharmacological treatment for heart failure and includes the use of β-blockers to inhibit β-adrenergic receptors that couple to $G\alpha_s$ and Angiotensin II receptor blockers (ARBs) to block the activity of the Angiotensin II receptor, which couples to $G\alpha_q$.

GPCRs couple to heterotrimeric G proteins, consisting of α, β, and γ subunits, each existing as multiple isoforms (21-23). Upon external activation of the receptor by a ligand, the ligand-receptor complex facilitates the activation of the α subunit of the heterotrimeric G protein complex by exchanging guanosine diphosphate (GDP) for guanosine triphosphate (GTP). This causes a conformational change and results in the disassociation of the Gα subunit from the β and γ subunits, allowing the α subunit and βγ complex to interact with various downstream effectors and ion channels (23).

The signaling and downstream effects of these GPCRs depend on the G protein they couple (i.e. G_s, G_i, G_q, G_{12}, and G_{13}). While $G\alpha_i$ antagonizes the stimulation of adenylate cyclase (AC) and cardiac inotropic and chronotropic effects mediated through

$G\alpha_s$ (24), $G\alpha_q$ couples to phospholipase C-beta (PLCβ) and has been demonstrated to play a major role in the development of hypertrophy (21,25-28). The $G\alpha_{12/13}$ family of G proteins interact with various guanine exchange factors (GEFs) for the low molecular weight G protein Ras homolog gene member A (RhoA) (29-35). RhoA signals through downstream mediators, including Rho Kinase (ROCK) and serum response factor (SRF), to initiate a variety of cellular responses including actin cytoskeleton organization by inducing stress fiber and focal adhesion formation (36-38) as well as modulating cell migration, proliferation and gene expression (34,39,40). Our laboratory has previously shown that cardiac-specific expression of RhoA protects the heart against oxidative stress and I/R injury whereas gene deletion of RhoA decreases tolerance to ischemic damage (41).

Cardiac role of S1P

S1P is a circulating lysophospholipid that is bound to serum albumin and high-density lipoprotein (18,42). It has been shown to function in a variety of biological processes, including cell proliferation (43,44), development (45,46), and cell survival (47). S1P is a high affinity ligand for five GPCRs denoted $S1P_{1-5}$ (48-50). It has been demonstrated that cardiomyocytes primarily express $S1P_1$, $S1P_2$, and $S1P_3$. $S1P_1$ is the major subtype expressed in the heart as well as in cardiomyocytes and it exclusively couples to the heterotrimeric G protein G_i to inhibit cyclic adenosine monophosphate (cAMP) formation and cardiac contractility (24,51,52). Unlike $S1P_1$, the $S1P_2$ and $S1P_3$ receptors couple not only to G_i, but also to G_q, G_{12}, and G_{13} (51,53), as illustrated in Scheme 1.

The physiological and pathophysiological role of S1P-mediated RhoA activation in the heart has not been fully elucidated. It has been established that $G\alpha_q$ signaling is involved in development of cardiac hypertrophy *in vivo* (25,27,54), and while $S1P_2$ and $S1P_3$ have been shown to couple to $G\alpha_q$ in other systems (55,56) it has not been determined whether S1P regulates cardiac hypertrophy or plays a role in pressure overload induced hypertrophy *in vivo* through $S1P_2$ and $S1P_3$ receptor activation of $G\alpha_q$. Accumulating evidence has shown that S1P confers cardioprotection against stress including *in vitro* and *in vivo* I/R models (17-20,57). For example, S1P levels are increased in and released from cardiomyocytes in response to ischemic preconditioning and mediates cardioprotective effects of preconditioning (17,58). The Food and Drug Administration (FDA)-approved drug FTY720, which acutely stimulates $S1P_1$ and $S1P_3$, has been shown to provide cardioprotective effects *in vitro* (59,60). It has not been determined which receptor is responsible for the protective effect, although FTY720 eventually downregulates $S1P_1$ (61). In a separate study, antagonism of $S1P_1$ and $S1P_3$ receptors with VPC23019 prevented the cardioprotective effects of both pre- and post-conditioning on infarct size development after I/R (17). There have also been reports indicating that $S1P_1$ may be involved in protecting the heart against ischemic stress (60). However, it has been shown that the $S1P_1$ selective agonist SEW1781 perfusion provides very modest cardioprotection in rat hearts subjected to *ex vivo* I/R, compared to that provided by S1P (62).

We have previously demonstrated that S1P provides protection against oxidative stress or I/R in neonatal ventricular myocytes (NRVMs), in the isolated perfused heart, and in the *in vivo* heart (19,20). In our previous study, we showed that gene deletion of $S1P_2$ and $S1P_3$ in mice exacerbated infarct development, suggesting that these are the

major receptor subtypes mediating *in vivo* cardiac protection by S1P against I/R (19). In this study, we examined whether S1P regulates cardiomyocyte hypertrophy through $S1P_2$ or the $S1P_3$ receptors and which of these receptor subtypes are involved in S1P-mediated cardioprotection. We have previously shown that S1P conferred cardioprotection through RhoA and its downstream effectors phospholipase C-epsilon (PLCε) and protein kinase D (PKD) (20). Thus we also elucidated S1P receptor subtypes and G protein involvement in activation of RhoA/PKD signaling. The data presented here demonstrates that S1P does not contribute to development of cardiac hypertrophy, that $S1P_3$ is responsible for RhoA activation through coupling to $G\alpha_{13}$, and that this coupling plays a major role in S1P-mediated PKD activation and cardioprotection.

MATERIALS & METHODS

NRVM cell culture and reagents

NRVMs were isolated from cardiac ventricles of 1- to 2-day-old Sprague-Dawley rat pups and digested with collagenase, and cardiomyocytes were purified through a Percoll Gradient (63). NRVMs were then plated at a density of 3.0 x 10^4/cm^2 on 1% gelatin-coated dishes and maintained overnight in Dulbecco-modified Eagle's medium (DMEM) (4.5 g/L D-glucose) from Gibco containing 15% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL at 37°C with 10% CO_2 overnight. After overnight culture, cells were either serum-starved in serum-free DMEM for 24 hours or transfected with siRNA in Opti-MEM for 24 or 48 hours, depending on optimal knockdown levels, for further analysis. The Rho inhibitor C3 exoenzyme was obtained from Cytoskeleton (CT04) and used at 2 µg/mL for 6 hours before treatments. Pertussis toxin (PTX) from Calbiochem was used at 0.1 µg/mL overnight before treatments. Phenylephrine (PE) was obtained from Sigma Life Science and used at 50 µM. S1P was obtained from Avanti Polar Lipids (860492P) and used at 0.3 µM. $S1P_3$-specific agonist CYM-51736 was provided by Dr. Hugh Rosen (The Scripps Research Institute, La Jolla, CA) and used at 10 µM.

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 $S1P_2$ knock out (KO) and $S1P_3$ KO mice has been previously described (64).

All experiments were performed on age-matched (8- to 12-week-old) male wild-type (WT) and KO littermates.

Immunofluorescence

Following treatments, NRVMs were washed with cold phosphate buffered saline (PBS) and fixed in 3.5% paraformaldehyde solution for 15 minutes at room temperature. Cells were then rehydrated and permeabilized in 0.02% NP-40 alternative in PBS for 10 minutes, and blocked in 2% bovine serum albumin (BSA) and 10% goat serum in PBS for 1 hour at room temperature. Cells were then incubated in primary antibody against α-actinin or atrial natriuretic factor overnight at 4°C. The cells were then washed 3 times with 0.02% NP-40 alternative in PBS on a rocker for 5 minutes. Secondary antibodies conjugated to Alexa fluorophores were diluted in block solution described above and applied for 2 hours at room temperature in the absence of light. Cells were then washed 3 times in 0.02% NP-40 alternative in PBS and mounted with VECTASHIELD Hardset containing DAPI to visualize nuclei at room temperature in the absence of light. Images were visualized by confocal microscopy. Cell area was measured using a scale bar of 20 μm.

RNA isolation and measurements

NRVMs were harvested in Trizol and allowed to incubate at room temperature for 5 minutes. Chloroform was added to the samples and mixed by shaking followed by another room temperature incubation for 2 to 5 minutes. Samples were then centrifuged for 15 minutes at 4°C at 20,817 rcf. The clear supernatant phase was decanted into a clean microfuge tube containing isopropanol and incubated for 10 minutes at room

temperature. Samples were then centrifuged for 15 minutes at 4°C at 20,817 rcf and the supernatant was removed from the RNA pellet. The pellet was then rinsed with 70% ethanol made with nuclease free water. The samples were then centrifuged for 15 minutes at 4°C at 20,817 rcf. The ethanol was then removed and the pellet was allowed to air dry for 30 to 60 minutes. The pellet was resuspended in 25 µL of nuclease-free water and heated for 10 minutes at 55°C. RNA dilutions were made at 1:500 in 1 mL and the optical density at 260 nm was measured using a quartz cuvette and a spectrophotometer. To determine the concentration of RNA in µg/µL, the following equation was used: OD 260 x df (500) x 0.04.

cDNA synthesis and qPCR analysis

Total RNA was converted to cDNA using the Verso cDNA synthesis kit acquired from Thermo Scientific. From the isolated total RNA, 1 µg of RNA was added to polymerase chain reaction (PCR) tubes and brought up to a final volume of 11µL with nuclease free water. The samples were then heated to 70°C for 5 minutes and cooled to 4°C for 1 minute in a PCR thermocycler before the addition of kit components. Next, 4 µL of 5x synthesis buffer, 2 µL of dNTP mix, and 1 µL of random hexamers, RT Enhancer, and verso enzyme mix were added to each sample and heated to 50°C for 50 minutes followed by 95°C for 2 minutes before being cooled at 4°C. The cDNA mixture is then brought to a final volume of 40 µL with 20 µL of nuclease free water and then a subsequent 1:50 dilution in nuclease free water. Then, mRNA levels are assessed using the Applied Biosystem 7500 Fast Real-Time PCR system. A master mix containing 10 µL of 2x Taqman Universal Master Mix II with UNG from Applied Biosystems and 1 µL of the primer from Applied Biosystems was added to each well in a quantitative PCR

(qPCR) plate along with 9 µL of the 1:50 dilution of cDNA. Using the standard program, the data acquired was analyzed using the comparative C_T Method (i.e. the $2^{-\Delta\Delta C}{}_T$ method) (65). GAPDH levels were used as the internal control.

Phosphatidylinositol (PI) hydrolysis

After overnight culturing, NRVMs were labeled with tritium-labeled ([^{3}H]) inositol (2.5 µCi/mL) in serum-free DMEM containing 0.1 % fatty acid free BSA for 24 hours. The media was then aspirated and cells were then treated with agonists at various times in the presence of 25mM lithium chloride, 0.1 % BSA, and 0.1 M HEPES in serum-free DMEM. Cells were then washed one time with cold PBS and incubated in cold 50 mM CH_2O_2 (Sigma) for 40 minutes at 4°C. Following incubation, 150 mM NH_4OH was added and the cells were stored in 4°C overnight. Samples were then run through BioRad Poly-Prep chromatography columns containing BioRad anion exchange AG1-X8 resin equilibrated in water. Columns were then rinsed two times with water followed by a rinse with 50mM $NH_3CH_2O_2$ (Sigma). Columns were then eluted with a solution of 1.2 M $NH_3CH_2O_2$ in 0.1 M CH_2O_2 (Fischer) into scintillation vials. Scinti-safe was then added to the vials and read in scintillation counter for [^{3}H] for 5 minutes.

Hypertrophy studies

Transverse aortic constriction (TAC) was used in 8- to 10-week-old WT, $S1P_2$ KO, or $S1P_3$ KO mice to induce pressure overload hypertrophy as previously described (66,67). 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. Mice were

anaesthetized under 2% isoflurane, intubated, and ventilated. Mice were euthanized by cervical dislocation and analyzed following one week TAC. Assessment following pressure overload hypertrophy was done by comparing the ratio of heart weight (HW) to body weight (BW).

GTP-RhoA pull-down assay

RhoA activation was determined by an affinity pull-down assay using a glutathione *S*-transferase (GST) fusion protein of the RhoA binding domain of the RhoA effector Rhotekin, as previously described (68). Cardiomyocytes were treated with Vehicle (DMEM), S1P, or PE for 5 minutes or with CYM-51736 for 20 minutes, rinsed with cold PBS, and then lysed in a buffer containing 50 mM Tris (pH 7.4), 0.1% Triton-X 100, 150 mM NaCl, 5 mM $MgCl_2$, and 10% glycerol, supplemented with protease and phosphatase inhibitors (Sigma). To determine RhoA activation in WT, $S1P_2$ KO, or $S1P_3$ KO hearts, hearts were perfused with either Vehicle (DMSO) or 0.3 µM S1P for 5 minutes, or 10 µM CYM-51736 for 20 minutes and homogenized in GTP-RhoA pull-down assay lysis buffer described above. The lysate was then clarified by centrifugation for 2 minutes at 4°C at 20,817 rcf and the supernatant was incubated with the Sepharose-bound GST-rhotekin-RhoA binding domain for 40 minutes at 4°C with mild nutation. The beads and precipitated proteins were washed with lysis buffer four times and the samples were prepared in 2x Laemmli sample buffer [33.3 mM $NaH_2PO_4 \bullet H_2O$, pH 7, 20% glycerol, 4% sodium dodecylsulfate (SDS), 1.2% Pyronin Y and 6% dithiothreitol (DTT)] and boiled for 5 minutes at 95°C to elute captured proteins.

Western blotting

Cardiomyocyte cell lysates or left ventricular lysates were prepared with either GTP-RhoA pull-down assay lysis buffer as described above or RIPA buffer (150 mM NaCl, 1% NP-40 alternative, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 50 mM NaF, 50 mM Tris, pH 7.4). Western blot analysis was performed according to protocols previously described (63). The antibodies used from immunoblotting were the following: RhoA from Santa Cruz Biotechnology Inc., glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phoshpo-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 Scientific.

Transfection of cardiomyocytes with siRNA

Predesigned PLCε, $G\alpha_q$, $G\alpha_{12}$, $G\alpha_{13}$, $S1P_1$, $S1P_2$, and $S1P_3$ siRNA for rat and scrambled control siRNA were purchased from Qiagen and used at 3 μg per 1 x 10^6 cells. Cardiomyocytes were transfected with siRNA using DharmaFECT-1 transfection reagent from Thermo Scientific based on the manufacturer's instruction. siRNA and DharmaFECT-1 (1:3 ratio) were individually incubated in tubes containing Opti-MEM medium from Gibco for 10 minutes at room temperature, mixed, and incubated for 20 minutes. Media in cardiomyocyte culture dishes were replaced with fresh media and siRNA/DharmaFECT-1 mixtures were added to the dishes. After overnight incubation, cells were washed and cultured for either 24 hours or 48 hours depending on optimal knockdown levels in serum-free DMEM supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL) before further treatment.

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 retrogradely 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 mmHg. To measure infarct size, hearts were stabilized for 10 minutes and then perfused with subjected to a period of global ischemia for 22 minutes followed by reperfusion for 60 minutes. Vehicle, S1P (0.3 μM) or CYM-51736 (10 μM) was perfused 10 minutes before the onset of ischemia and throughout reperfusion. At the end of reperfusion, ventricles were frozen and cut transversely into 5 slices of equal thickness and incubated in a solution of 1% triphenyl tetrazolium chloride (TTC) from Sigma in PBS and fixed in 10% formalin-PBS for 24 hours to measure infarct size. Fixed slices were scanned and ImageJ was used to measure and calculate the size of infarct area and the total slice area. To determine RhoA activation, hearts were stabilized in Krebs-Henseleit buffer for 10 minutes, perfused with either S1P for 5 minutes or CYM-51736 for 20 minutes, homogenized in GTP-RhoA pull-down assay lysis buffer and the protocol for the assay was followed as described above.

Statistical analysis

All experimental data was normalized to an internal control if available. The normalized values were all compared to non-treated control group and expressed as relative fold changes. All results are reported as means ± standard error (SE). Comparison of two groups with one characteristic was accomplished using an unpaired

Student's *t* test. Data with two groups with multiple characteristics 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 characteristic were compared by one-way ANOVA followed by Tukey's multiple comparison test. Probabilities values of < 0.05 were considered significant and are indicated by an asterisk or a pound in all figures.

RESULTS

S1P does not induce cardiac hypertrophy.

Stimulation of $G\alpha_q$ coupled-receptors with ligands such as PE or endothelin regulates PLC to increase PI hydrolysis and downstream signals implicated in development of cardiac hypertrophy (25,27). S1P is an endogenous regulator of cardiac function that can activate S1P receptor subtypes known to couple to $G\alpha_q$. The role of S1P in control of cardiac hypertrophy has not been thoroughly examined. Initially we assessed the ability of S1P receptor stimulation to activate PLC in NRVMs. NRVMs were serum-starved in the presence of ^{3}H-inositol and treated with 0.3 μM S1P for 0, 1, 5, 10, 30, and 60 minutes. Responses were compared with those seen with 50 μM PE, an established hypertrophic agonist that activates α-adrenergic receptors. We observed a robust increase the accumulation of inositol phosphates (InsPs) in response to PE whereas S1P elicited a significantly more modest increase in PI hydrolysis (Figure 1A). In previous studies we established that S1P receptor mediated PLC activation can occur through the novel PLC isoform PLCε and its activation by RhoA (69,70). Interestingly, the PI hydrolysis induced by S1P in NRVMs was fully inhibited by blockade of RhoA function with C3 exoenzyme (2 μg/ml) whereas the response to PE was not (Figure 1B). In addition, knockdown of PLCε with siRNA prevented the PI response to S1P but not that to PE (Figure 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.

To determine whether S1P treatment can elicit cardiomyocyte hypertrophy NRVMs were treated with S1P or PE for 24 hours. Cells were then subjected to

immunofluorescence analysis for α-actinin, a cardiomyocyte cytoskeletal protein, and atrial natriuretic factor (ANF), a hypertrophic marker (Figure 2A). Immunofluorescence analysis revealed a large increase in ANF positive cells following PE treatment, but not in S1P treated cardiomyocytes (Figure 2A, C). We also observed that PE, but not S1P, significantly increased the cell surface area (1.6 fold) assessed by α-actinin staining (Figure 2A, B). qPCR analysis was then 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 there was no significant increase in expression of either hypertrophic marker in response to S1P (Figure 2D, E). Thus S1P receptor stimulation appears unable to trigger the conventional hypertrophic signaling pathways by which activation of Gαq/PLCβ elicits hypertrophy in NRVMs.

$S1P_2$ 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 TAC (71). 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* WT, $S1P_2$ KO, and $S1P_3$ KO mice were subjected to TAC for one week to induce pressure overload hypertrophy. We observed a significant increase in HW relative to BW ratio, indicative of hypertrophy development, in WT mice and an equivalent increase in the $S1P_2$ KO and $S1P_3$ KO mice following TAC (Figure 3). This indicates that cardiac hypertrophy is still

induced in the absence of these receptors i.e. that S1P acting on these receptors is not a player in the development of cardiac hypertrophy induced by pressure overload.

S1P induced RhoA activation is $G\alpha_{13}$ dependent.

Having observed that PI hydrolysis in response to S1P was Rho dependent in NRVM, we next compared RhoA activation by S1P and PE. S1P robustly activated RhoA (4.2-fold compared to vehicle) whereas PE did not significantly activate RhoA (Figure 4A). S1P receptors have been shown to couple to G_i (for $S1P_1$) or to G_i G_q, and $G_{12/13}$ (for $S1P_2$ and $S1P_3$). The G proteins subunits that are most dedicated in coupling GPCRs to Rho activation through RhoGEFs are $G\alpha_{12}$ and $G\alpha_{13}$ although RhoA can also be activated through $G\alpha_q$ and $G\alpha_i$. To test involvement of $G\alpha_i$ in RhoA activation induced by S1P, cells were pretreated with PTX for 24 hours followed by 5 minute S1P stimulation. RhoA activation was then assessed by immunoprecipitating the GTP-bound activated RhoA using the Rho binding domain of Rhotekin, a RhoA effector. S1P elicited a 3-fold increase in activated RhoA and pretreatment with PTX did not affect the activation of Rho by subsequent S1P stimulation (Figure 4B). To determine whether $G\alpha_q$, $G\alpha_{12}$, or $G\alpha_{13}$ were involved in S1P-mediated RhoA activation, we used siRNA-mediated knockdown of the various G protein α subunits. Treatment with siRNA for 72 hours reduced expression of each Gα subunit by greater than 50% (Figure 5 A, B, C). Subsequent S1P treatment of cells transfected with control siRNA elicited a 2-fold increase in RhoA activation, which was not inhibited by knockdown of $G\alpha_q$ or $G\alpha_{12}$ (Figure 6). Knockdown of $G\alpha_{13}$, however, significantly attenuated RhoA activation induced by S1P. These results implicate $G\alpha_{13}$ in S1P induced activation of RhoA in cardiomyocytes.

S1P activates RhoA through the $S1P_3$ receptor in NRVMs and in isolated perfused hearts.

As indicated above, the S1P receptor subtypes $S1P_{1-3}$ are expressed in cardiomyocytes. To determine which are important for the activation of RhoA in response to S1P, siRNA for each of the S1P receptor subtypes was transfected in NRVMs. siRNA knockdown of the S1P receptor subtypes assessed 48 hours later showed greater than 70% reduction in mRNA expression for each respective S1P receptor subtypes (Figure 7 A, B, C) and did not significantly affect the expression of other subtypes (data not shown). After 48 hours, cells were stimulated with S1P for 5 minutes and RhoA activity was assessed by GTP-RhoA pull-down assay as described above. Knockdown of $S1P_3$ receptors markedly reduced RhoA activation by S1P; in contrast a response equivalent to that seen with control siRNA was observed in $S1P_1$ or $S1P_2$ receptor knockdown cells (Figure 8A).

To extend the implications 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 minutes 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 heart isolated from $S1P_2$ knockout (Figure 8B). Remarkably, S1P treatment failed to activate RhoA in $S1P_3$ knockout mouse hearts, indicating that the $S1P_3$ receptor plays a critical role in S1P induced RhoA activation in the adult murine heart as it does in neonatal rat cardiomyocytes.

$S1P_3$ receptor mediates cardioprotection in Langendorff perfused mouse hearts against ischemia/reperfusion injury.

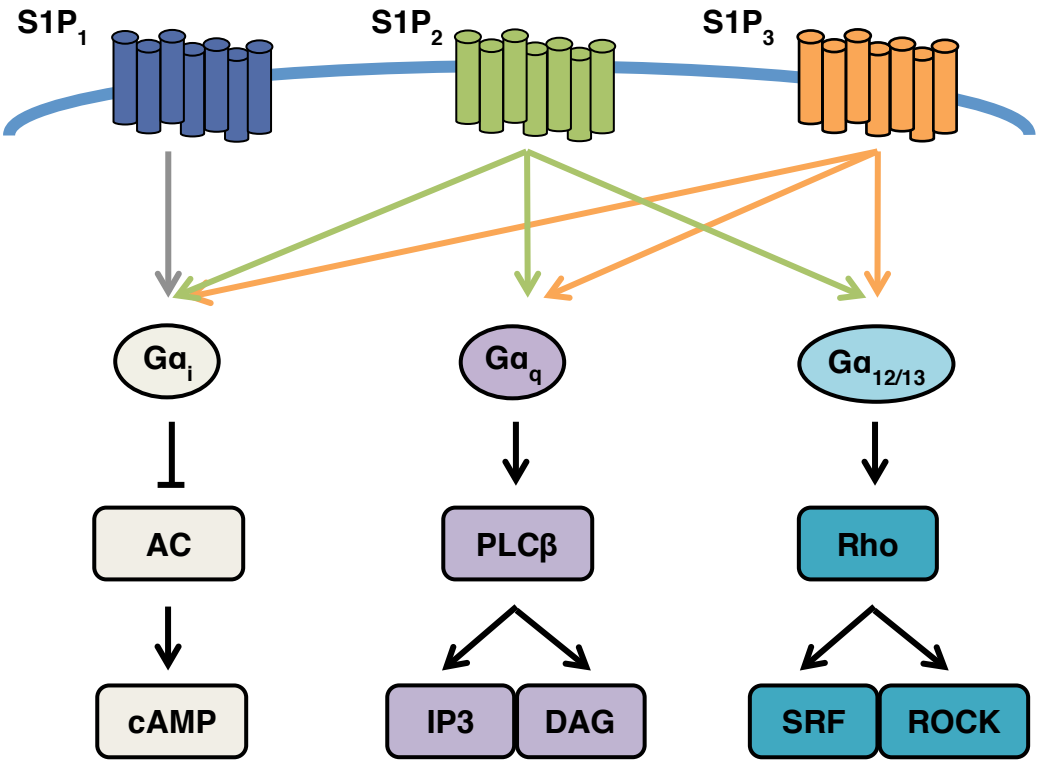
Our lab has previously reported that S1P confers strong cardioprotection in isolated perfused mouse hearts through signaling to RhoA and PKD. The findings above suggested that the cardioprotection induced by S1P would be mediated through activation of the $S1P_3$ receptor. To test this hypothesis, hearts from WT and $S1P_3$ KO mice were perfused with 0.3 μM S1P for 10 minutes, subjected to global ischemia for 22 minutes followed by reperfusion for 60 minutes, and cardiac damage assessed by staining with 1% TTC. S1P treatment significantly reduced myocardial infarction in the WT mouse heart, supporting previous observations (20). The protective effect of S1P was completely abolished in the $S1P_3$ KO hearts (Figure 9A). To further confirm the significant role of $S1P_3$ in cardioprotection, we examined the effect of CYM-51736, a $S1P_3$ receptor-specific agonist. Hearts isolated from WT mice were perfused with 10 μM CYM-51736 for 10 minutes followed by global I/R. CYM-51736 pretreatment resulted in a significant reduction in infarct size (Figure 9B), comparable to that observed in hearts perfused with S1P (Figure 9A) supporting the critical role of the $S1P_3$ receptor in S1P mediated cardioprotection against I/R injury.

$S1P_3$ also mediates the activation of PKD in NRVMs.

We previously demonstrated that PKD is downstream of RhoA activation and contributes to S1P/RhoA mediated cardioprotection (20). Knockdown of $S1P_3$ in NRVMs significantly attenuated PKD phosphorylation in response to S1P whereas knockdown of $S1P_1$ or $S1P_2$ did not (Figure 10A). In addition, the $S1P_3$ selective agonist CYM-51736 robustly increased PKD phosphorylation, and failed to do so following $S1P_3$ receptor

knockdown (Figure 10B). 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.

SCHEMES AND FIGURES



Scheme 1: S1P receptor coupling and downstream effectors. $S1P_1$, $S1P_2$, and $S1P_3$ are the three primary S1P receptors expressed in cardiomyocytes. Previous studies have shown that $S1P_1$ exclusively couples to $G\alpha_i$, whereas $S1P_2$ and $S1P_3$ couple to $G\alpha_i$, $G\alpha_q$, $G\alpha_{12}$, and $G\alpha_{13}$ in non-cardiac cell types. The preferential coupling of the S1P receptor subtypes to their G proteins and their downstream biological effects have not been well established in cardiomyocytes.

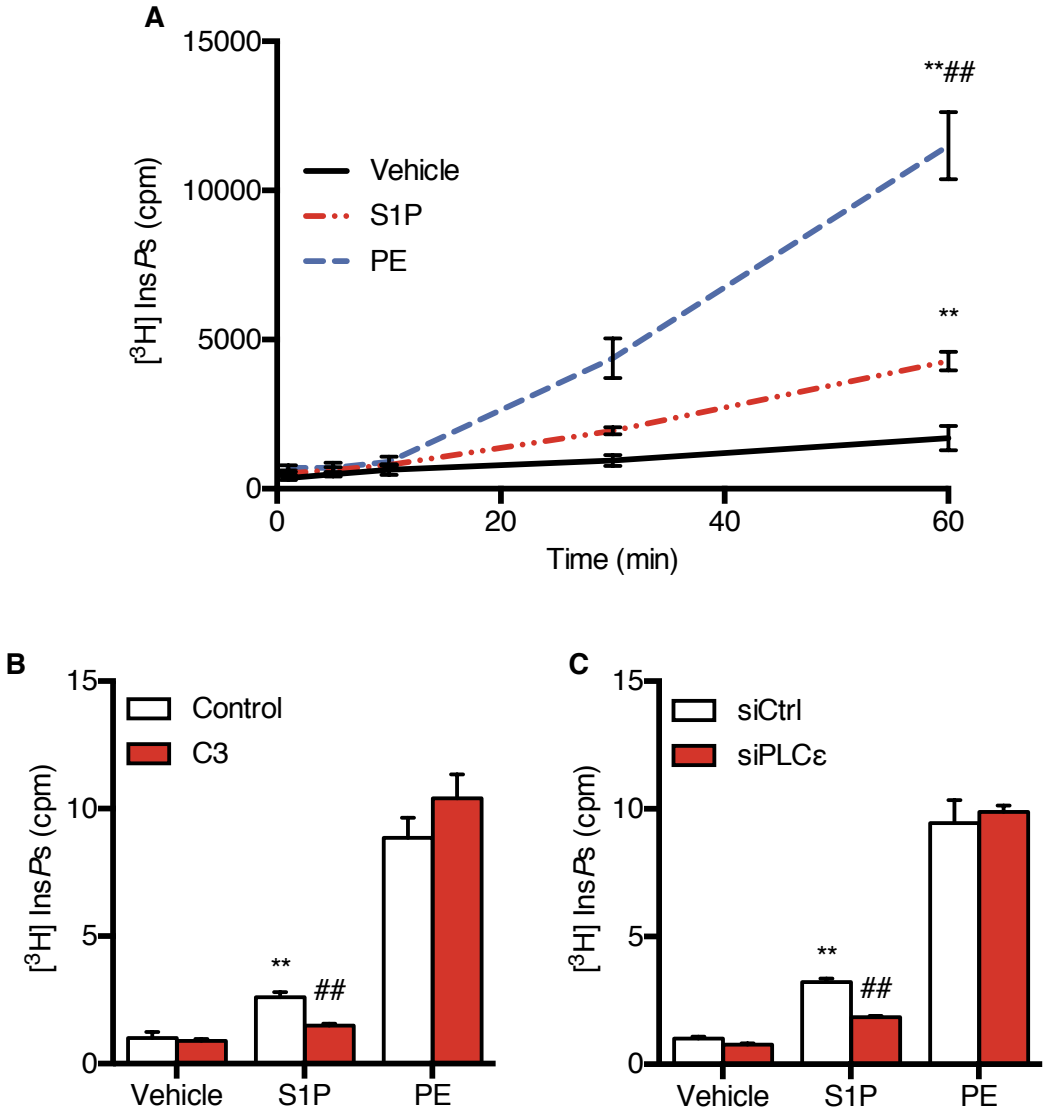


Figure 1: PLC activation measured by [H^3] inositol phosphate accumulation in NRVMs. **(A)** Time course of S1P- and PE-induced phosphatidylinositol hydrolysis. NRVMs were serum starved overnight in the presence of [^{3}H] inositol. Cells were then treated with agonists for 1, 5, 10, 30, and 60 minutes in the presence of LiCl before isolation of [^{3}H] inositol phosphates (InsPs). $^{**}P < 0.01$ vs. Vehicle, $\#\#P < 0.01$ vs. S1P ($n = 5$). **(B)** NRVMs were pretreated with 2.0 µg/mL C3 exoenzyme (Rho inhibitor) for 6 hours or **(C)** transfected with Control siRNA (siCtrl) or siRNA against PLCε for 48 hours before challenge with agonists for 60 minutes and assessed for InsPs production. $^{**}P < 0.01$ vs. Vehicle + Control or siCtrl, $\#\#P < 0.01$ vs. S1P + Control or siCtrl ($n = 5$).

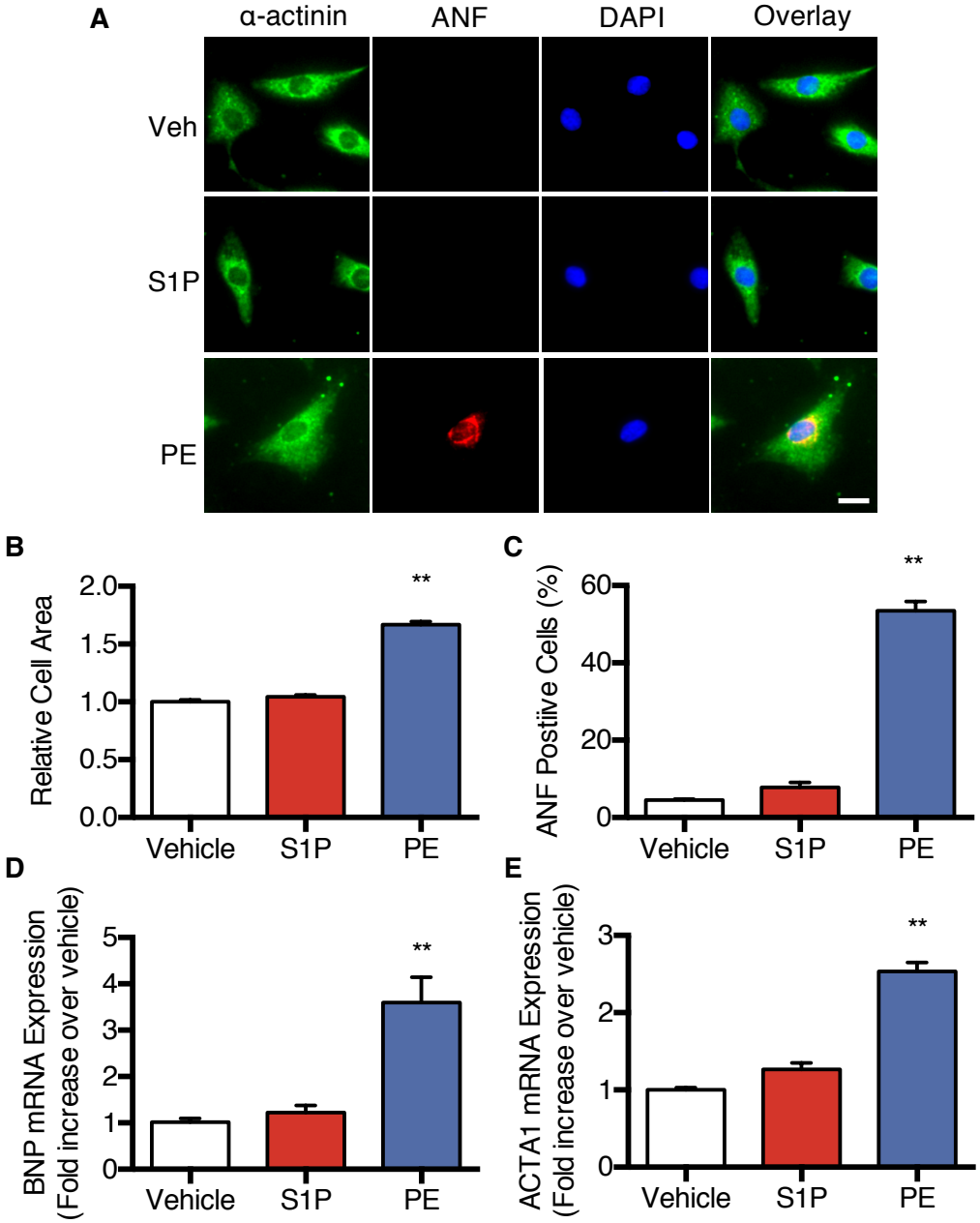


Figure 2: S1P does not induce cardiac hypertrophy in NRVMs. (A) NRVMs were treated with Vehicle (Veh), 0.3 μM S1P, or 50 μM PE for 24 hours. Representative immunofluorescent images depicting NRVMs stained for α-actinin, atrial natriuretic factor (ANF), and nuclei with DAPI. Scale bar: 20 μm. **(B)** Quantified relative cell area and **(C)** quantified ANF positive cells (n = 400 cells). **(D)** mRNA expression of brain natriuretic peptide (BNP) and **(E)** skeletal muscle α-actin (ACTA1). $^{**}P < 0.01$ vs. Vehicle ($n = 4$).

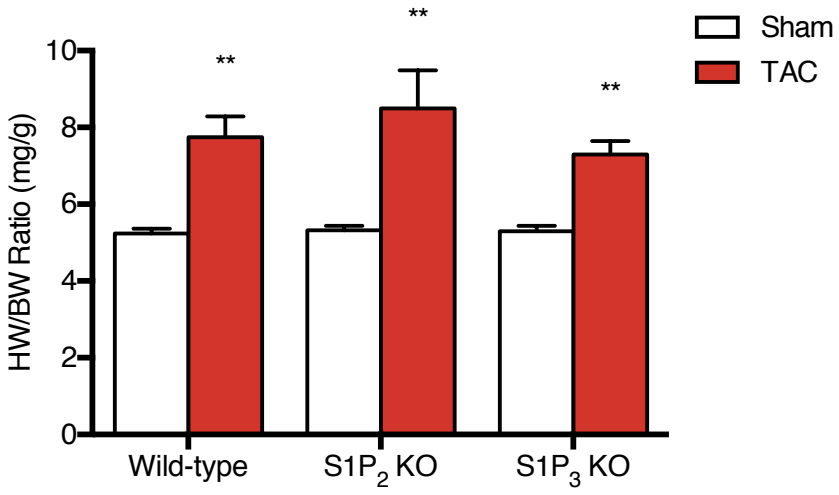


Figure 3: $S1P_2$ and $S1P_3$ receptors do not play a role in TAC-induced hypertrophy in mouse hearts. Representative graph of heart weight (HW) to body weight (BW) ratio of wild-type (WT), $S1P_2$ knockout (KO), and $S1P_3$ KO mice following transverse aortic constriction (TAC) for one week to induce pressure overload hypertrophy. $^{**}P < 0.01$ vs. Sham ($n \geq 5$).

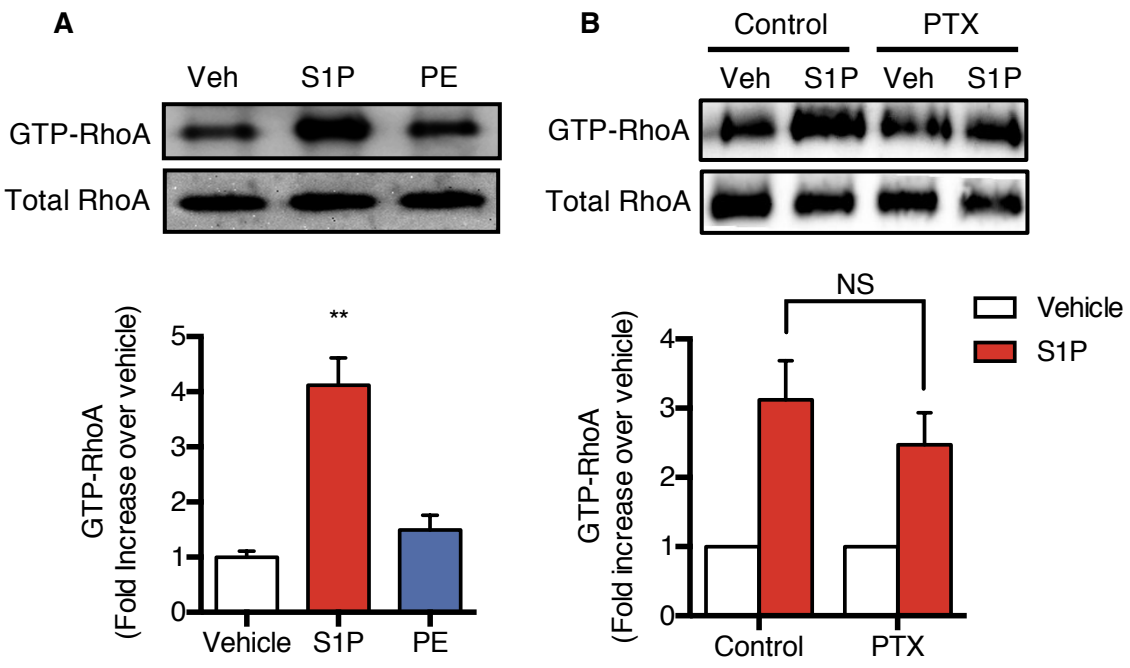


Figure 4: RhoA activation by S1P is robust and insensitive to pertussis toxin pretreatment. (A) NRVMs were treated with 0.3 μM S1P or 50 μM PE for 5 minutes and RhoA activation was measured by a GTP-RhoA pull-down assay. $^{**}P < 0.01$ vs. Vehicle ($n = 5$). **(B)** NRVMs were pretreated with 0.1 μg/mL pertussis toxin (PTX), a $G\alpha_i$ inhibitor, overnight and then stimulated with 0.3 μM S1P for 5 minutes. RhoA activation was assessed by GTP-RhoA pull-down assay. NS indicates not significant ($n = 4$).

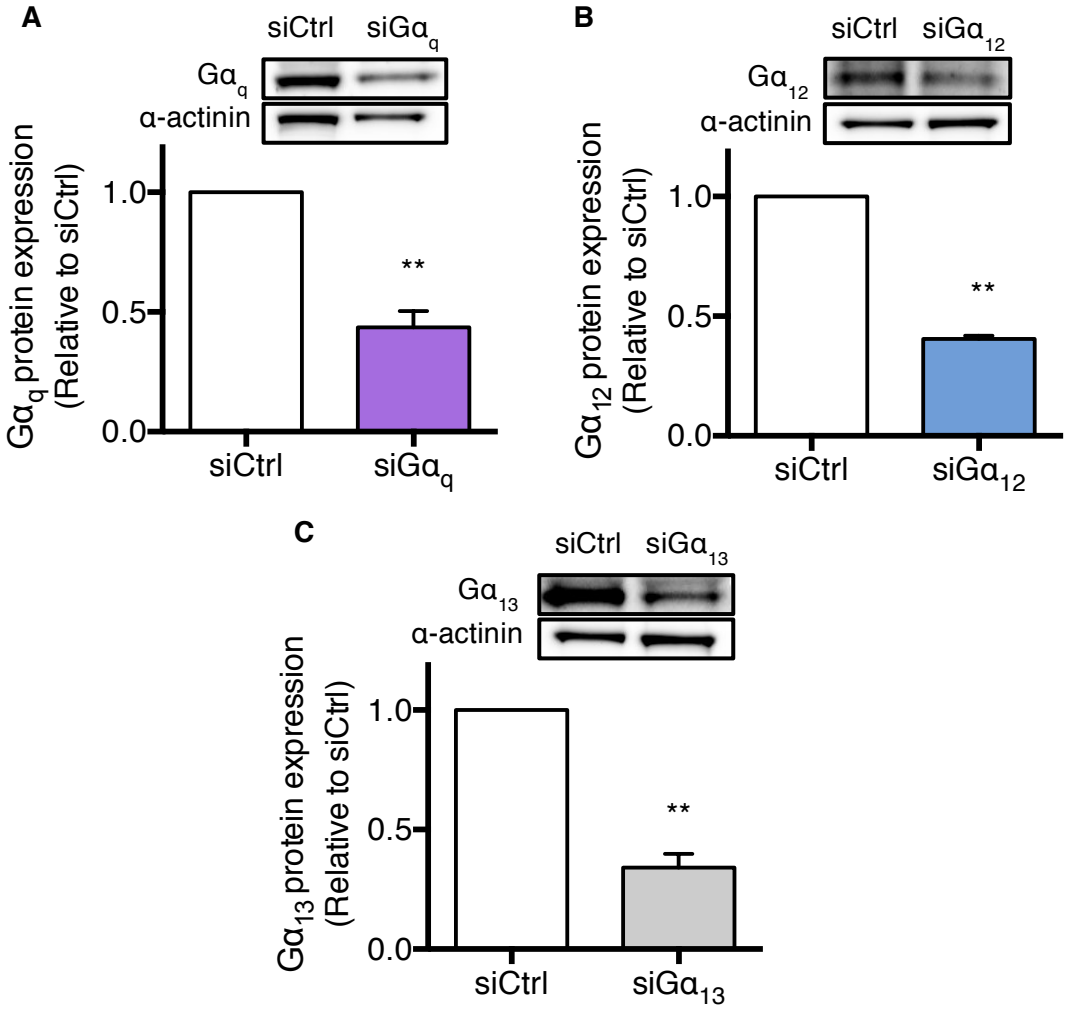


Figure 5: siRNA-mediated knockdown of G protein alpha subunits. NRVMs were transfected with either Control siRNA (siCtrl) or siRNA against **(A)** $G\alpha_q$, **(B)** $G\alpha_{12}$, or **(C)** $G\alpha_{13}$. G protein expression levels were assessed by Western blotting after 72 hour-knockdown, $^{**}P < 0.01$ ($n = 3$). Cytoskeletal protein α-actinin was blotted as a loading control.

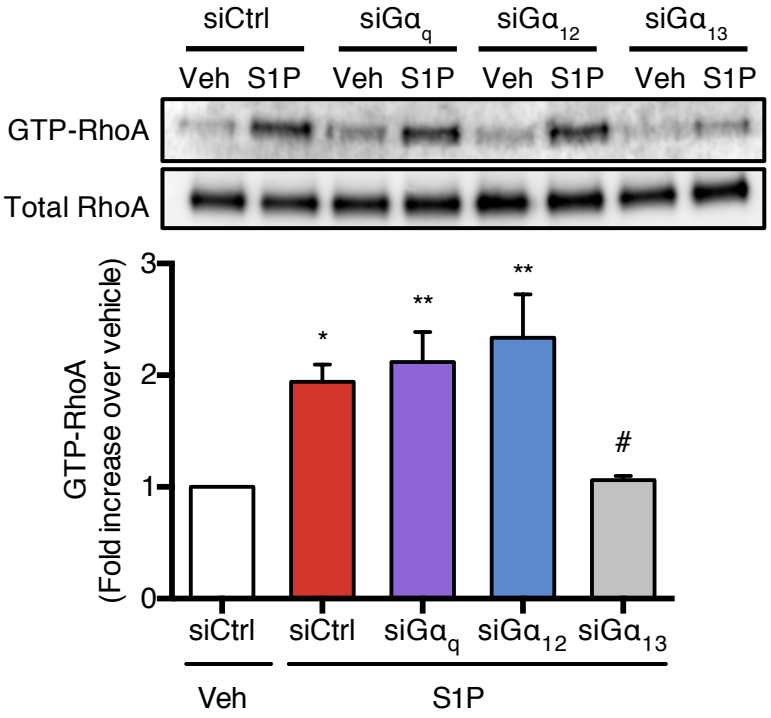


Figure 6: S1P-mediated RhoA activation is Gα$_{13}$ dependent. NRVMs were transfected with either Control siRNA (siCtrl) or siRNA against Gα$_q$, Gα$_{12}$, or Gα$_{13}$ for 72 hours and then stimulated with 0.3 μM S1P for 5 minutes. RhoA activation was assessed by GTP-RhoA pull-down assay. *, ***P* < 0.05, 0.01 vs. siCtrl, #*P* < 0.05 vs. siCtrl + S1P (*n* = 7).

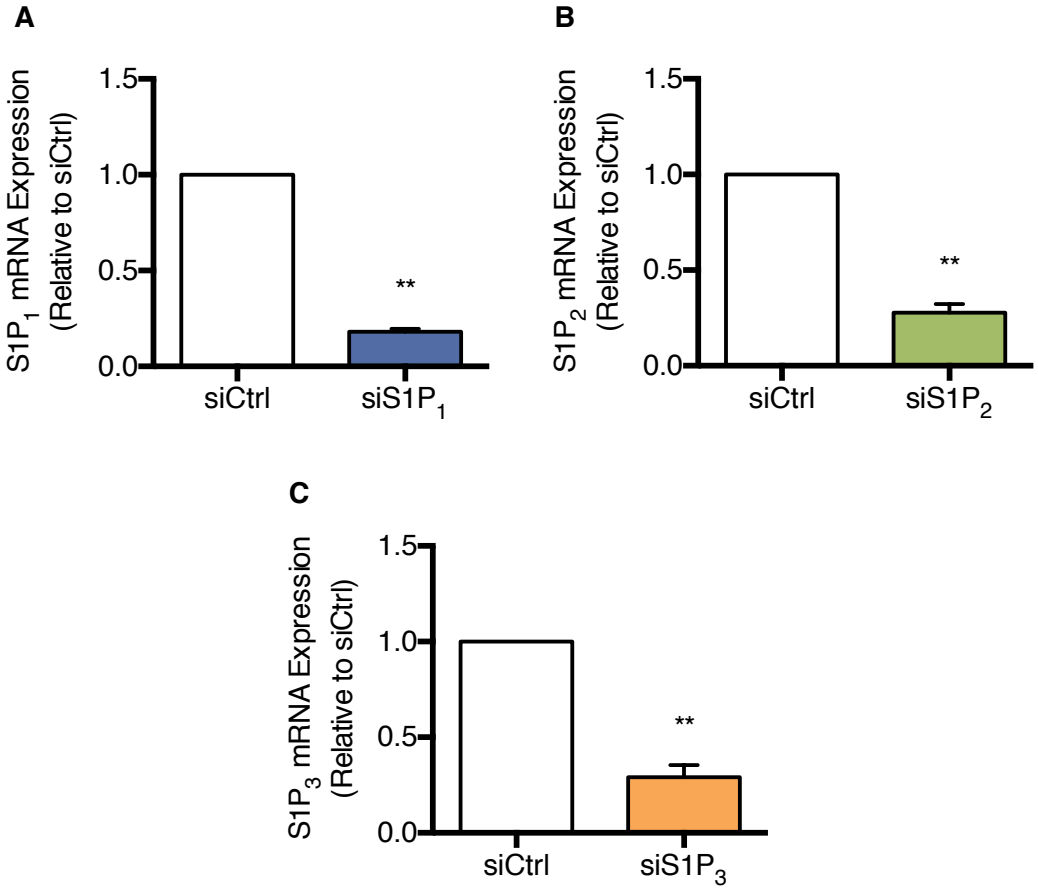


Figure 7: siRNA-mediated knockdown of the S1P receptor subtypes. NRVMs were transfected with either siCtrl or siRNA against **(A)** $S1P_1$, **(B)** $S1P_2$, **(C)** or $S1P_3$. S1P receptor levels were assessed by qPCR analysis after 48 hour siRNA transfection. $^{**}P < 0.01$ vs. siCtrl ($n = 6$).

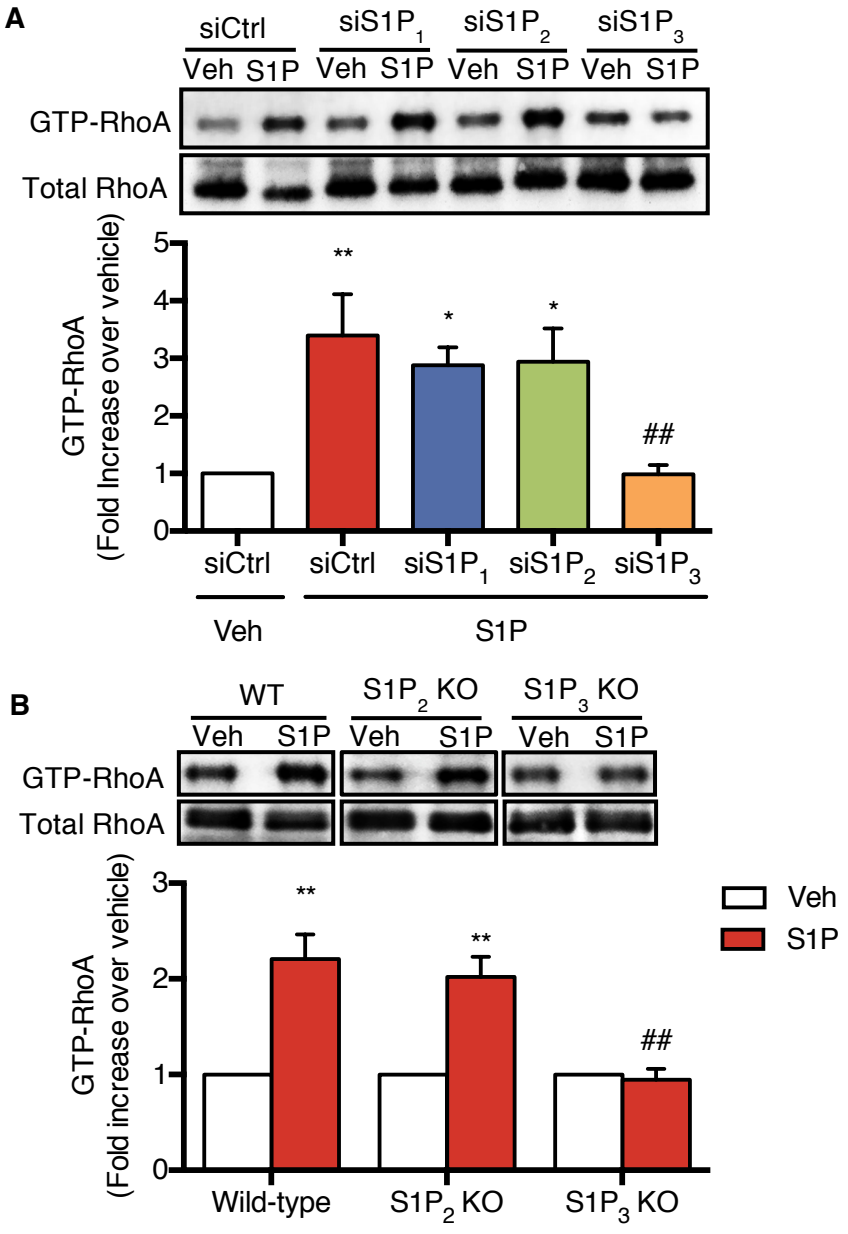


Figure 8: S1P activates RhoA through $S1P_3$ in NRVMs and in isolated perfused hearts. (A) NRVMs were transfected with siRNA to knockdown S1P receptor subtypes for 48 hours and then stimulated with 0.3 μM S1P for 5 minutes. RhoA activation was assessed by GTP-RhoA pull-down assay. *,**$P < 0.05$, 0.01 vs. siCtrl, ##$P < 0.01$ vs. siCtrl + S1P, ($n = 4$). **(B)** 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 minutes 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).

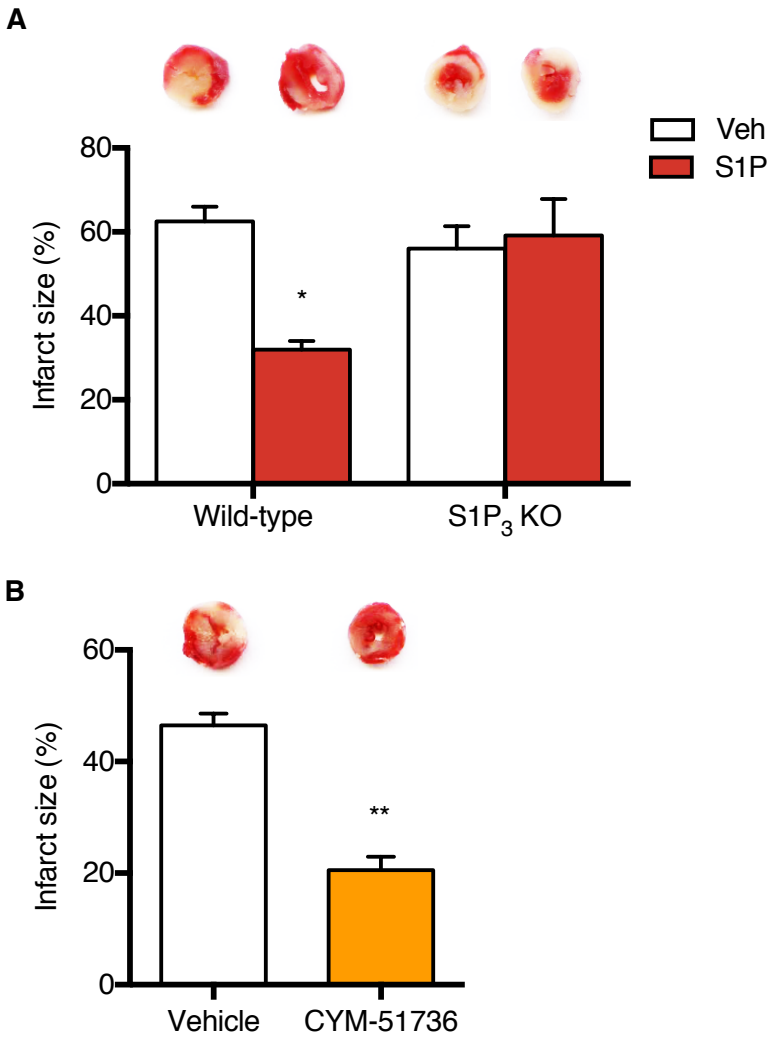


Figure 9: $S1P_3$ mediates the cardioprotective effects of S1P on I/R injury in the isolated perfused mouse heart. Representative images of TTC-stained cross sections of isolated perfused mouse hearts after I/R injury (top) and quantification of infarct size (bottom). White areas are infarcted tissue and red areas are viable tissue. **(A)** Isolated WT and $S1P_3$ KO hearts were perfused with either Vehicle or 0.3 μM S1P for 15 minutes and subjected to 22 minute global ischemia followed by 60 min reperfusion. $^*P < 0.05$ vs. WT + Vehicle ($n \geq 5$). **(B)** WT hearts were perfused with either Vehicle or 10 μM CYM-51736 ($S1P_3$ specific agonist) for 15 minutes and subjected to I/R. Infarct size was assessed by TTC staining. $^{**}P < 0.01$ vs Vehicle ($n = 5$).

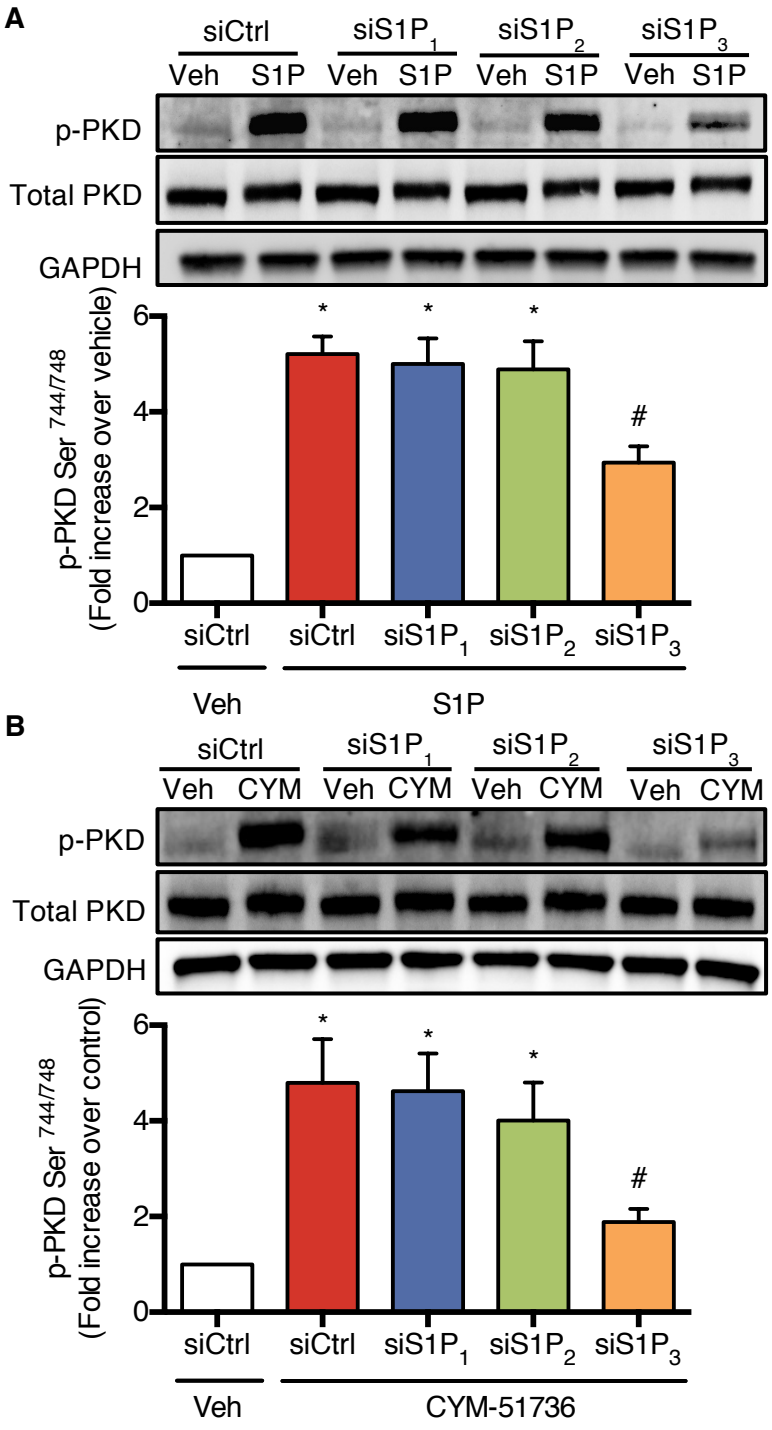
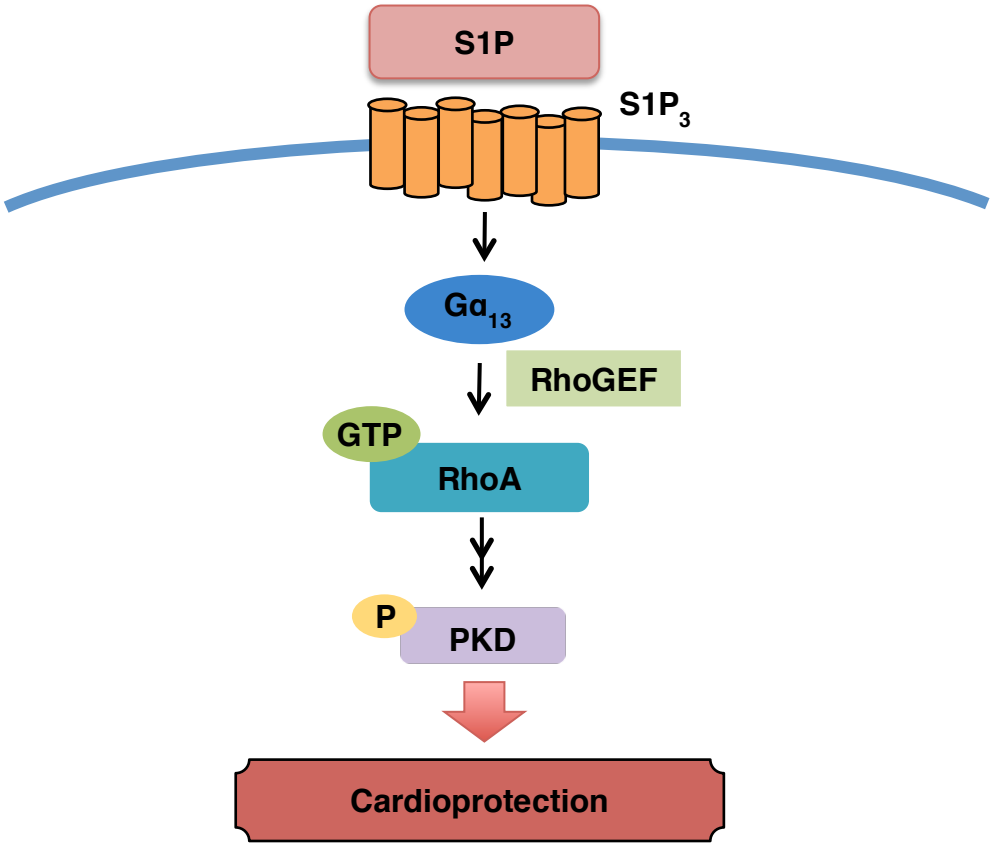


Figure 10: $S1P_3$ mediates the activation of PKD by S1P in NRVMs. NRVMs were transfected with siRNA against S1P receptor subtypes for 48 hours followed by treatment with either **(A)** 0.3 μM S1P for 5 minutes or **(B)** 10 μM CYM-51736 for 20 minutes. **P* < 0.05 vs. siCtrl, #*P* < 0.05 vs. siCtrl + S1P or CYM-51736 (*n* = 4 to 5).



Scheme 2: Proposed scheme of $S1P_3$-mediated RhoA activation and cardioprotection.

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 (21,22,32,49). In the heart, S1P has been shown to confer protection against ischemic stress, and we recently reported that RhoA plays a crucial role in S1P-mediated cardioprotection against I/R injury (17-20,57). 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 and protection against I/R injury in cardiomyocytes and in the isolated 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. Studies using NRVMs also demonstrated that the $S1P_3$ regulates RhoA as well as PKD but that S1P causes limited PLC activation and does not induce hypertrophic responses. $S1P_2$ and $S1P_3$ are also dispensable for *in vivo* pressure-overload induced hypertrophy.

G protein-coupled receptors and RhoA

As previously mentioned, $S1P_1$ exclusively couples to $G\alpha_i$, while $S1P_2$ and $S1P_3$ activate multiple G-protein subtypes: $G\alpha_q$, $G\alpha_i$, $G\alpha_{12}$, and $G\alpha_{13}$ (48,51-53). In the context of RhoA activation, G_{12} and G_{13}, members of the $G\alpha_{12/13}$ subfamily of G proteins, are well accepted to mediate the activation of RhoA in response to GPCRs stimulation through direct regulation of RhoGEFs (29,32,72-74). Interestingly, the data presented here reveal that only $G\alpha_{13}$, but not $G\alpha_{12}$, knockdown significantly attenuates S1P-induced RhoA

activation and that $G\alpha_{12}$ cannot subsume this role in cardiomyocytes. These data indicates that $G\alpha_{13}$ plays a critical role in transducing $S1P_3$ receptor stimulation to RhoA activation (Figure 6 and 8A). Although we cannot rule out the possibility that $G\alpha_{12}$ knockdown achieved by siRNA is insufficient to observe the contribution of $G\alpha_{12}$ to S1P-mediated RhoA activation in NRVMs, it is increasingly clear that $G\alpha_{12}$ and $G\alpha_{13}$ have distinct signaling effects. Strikingly $G\alpha_{13}$ knockout mice die during embryonic development, while $G\alpha_{12}$ knockout mice are viable, fertile, and without obvious phenotype (75,76). Indeed, a previous study using $G\alpha_{12}$ and $G\alpha_{13}$ antibody microinjection in Swiss 3T3 cells showed that lysophosphatidic acid (LPA) induced stress fiber formation, a RhoA-mediated response, through $G\alpha_{13}$ but not $G\alpha_{12}$ (77). Studies using overexpression of $G\alpha_{12}$ and $G\alpha_{13}$ constructs in 293 cells have also demonstrated that thrombin and LPA selectively couples to $G\alpha_{12}$ and $G\alpha_{13}$ respectively to activate RhoA (30).

Coupling of $G\alpha_{12}$ and $G\alpha_{13}$ to RhoGEFs has also been reported to be different. The regulator of G protein signaling homology (RH) 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 $G\alpha_{13}$. Interestingly it has been reported that several structural aspects of the their interaction with RhoGEFs differ and thus $G\alpha_{12}$ and $G\alpha_{13}$ engage different RhoGEFs (33). 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 (31,72). Leukemia-associated RhoGEF (LARG) is activated by $G\alpha_{12}$ only when this GEF is phosphorylated by tyrosine-kinase while $G\alpha_{13}$ activates it with or without phosphorylation (35). Taken together, our data using siRNA mediated

knockdown provide support at the levels of endogenous molecules suggesting the distinct regulation and roles of $G\alpha_{12}$ and $G\alpha_{13}$ in GPCRs mediated RhoA activation.

S1P receptor subtypes and Rho signaling

There are five S1P receptor subtypes, $S1P_1$-$S1P_5$ and the expression of these subtypes varies depending on cell type. Previous studies including work from our laboratory showed that $S1P_1$, $S1P_2$, and $S1P_3$ are the major subtypes expressed in the heart, with $S1P_1$ as the predominant subtype (48-50,65). Our results obtained using siRNA-mediated receptor knockdown in cardiomyocytes reveal that $S1P_3$, although a more minor receptor subtype, plays a major role in S1P-mediated RhoA activation (Figure 8A). The role of $S1P_3$ in RhoA activation was also supported by our experiments with $S1P_3$ knockout hearts (Figure 8B).

The relative roles of $S1P_3$ versus $S1P_2$ in RhoA activation are less consistent. For example, our previous studies using $S1P_2$ and $S1P_3$ KO MEF cells showed that deletion of $S1P_2$, but not $S1P_3$, significantly diminishes RhoA activation induced by S1P (65,78). A study using $S1P_2$ KO macrophages also suggested a responsible role for $S1P_2$ in RhoA activation (79). On the other hand, pharmacological studies in human coronary artery smooth muscle cells showed RhoA activation to be inhibited by either $S1P_2$ or $S1P_3$ antagonism (80).

Since both $S1P_2$ and $S1P_3$ receptors can couple to $G\alpha_{12,}$ and $G\alpha_{13}$, the subtype predominating in RhoA regulation likely depends on receptor expression level. In mouse cardiomyocytes, we found that $S1P_1$ is the most abundant subtype followed by $S1P_3$, while $S1P_2$ is expressed at relatively lower levels (48). Maybe the difference in $S1P_3$ versus $S1P_2$ receptor expression in cardiomyocytes versus MEFs or other cell types

explains the preference for $S1P_3$ signaling to RhoA. There may also be as yet defined differences in $S1P_2$ and $S1P_3$ coupling to $G\alpha_{12,}$ versus $G\alpha_{13}$ and of $G\alpha_{12,}$ versus $G\alpha_{13}$ to endogenous RhoGEFs, as discussed previously.

S1P receptor and cardioprotection

A wide range of molecules downstream of S1P receptor activation has the potential to mediate cardioprotection. Previously, our laboratory demonstrated that S1P treatment in NRVMs leads, through RhoA and its effect on PLCε activation, to activation of PKD (20). In line with the aforementioned significant contribution of $S1P_3$ to RhoA activation, we also observed that PKD activation induced by S1P treatment was markedly reduced by $S1P_3$ knockdown. (Figure 10A). These results suggest that PKD is downstream of $S1P_3$/RhoA signaling axis. We previously demonstrated that S1P-mediated cardioprotection lost in PKD KO or PLCε KO (20). Here we demonstrate that S1P-mediated cardioprotection against I/R injury is abolished in $S1P_3$ KO hearts (Figure 9A) and that selective activation of $S1P_3$ by CYM-51736 leads to robust activation of PKD (Figure 10B) and confers protection comparable to that observed with S1P (Figure 9B). Thus, this study defines early players in the S1P signaling pathway and suggests that $S1P_3$, and its coupling to $G\alpha_{13}$, plays a major role in S1P- and RhoA-mediated cardioprotection against I/R injury, as illustrated in Scheme 2.

Our results also demonstrate that S1P, in contrast to many other GPCR agonists, does not induce hypertrophic responses in NRVMs (Figure 2A, B, C, and D) nor is S1P receptor stimulation involved in pressure-overload induced hypertrophy *in vivo* (Figure 3). With regard to G protein involvement in cardiac hypertrophy, $G\alpha_q$ has been established to be essential in development of hypertrophy induced by GPCR agonists

and by pressure-overload, presumably mediated through PLCβ activation and resultant generation of intracellular second messengers inositol triphosphate (IP_3) and diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate (PIP_2) (21,25-28). Compared to the adrenergic agonist PE, which activates $G\alpha_q$ coupled α_1 adrenergic receptors, S1P-induced PI responses are very modest and appears to be RhoA/PLCε dependent (Figure 1A, B, and C). These results suggest that although $S1P_2$ and $S1P_3$ have an ability to activate $G\alpha_q$ signaling in non-cardiomyocyte cells (55,56), recruitment of $G\alpha_q$ signaling through $S1P_2$ and $S1P_3$ in cardiomyocytes is limited and not sufficient to drive hypertrophic responses in the heart. This 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* (Figure 3).

All three S1P receptors expressed in the heart have been shown to couple to $G\alpha_i$, which negatively regulates adenylate cyclase activity, counteracting the action of $G\alpha_s$. S1P exerts a negative inotropic effect against β-adrenergic receptor stimulation induced by $G\alpha_s$ activation, and we and others have suggested that $S1P_1$, the predominant S1P receptor in the heart and one exclusively coupled to $G\alpha_i$, is responsible for $G\alpha_i$-mediated inhibition of adenylate cyclase activity (decreasing cAMP) and resultant negative inotropic effect (24,51,52). Notably, while MAPK and Akt activation induced by S1P in adult mouse ventricular myocytes (AMVMs) is also sensitive to pertussis toxin and thus mediated through $G\alpha_i$, these responses are attenuated by gene deletion of $S1P_2$ or $S1P_3$ and abolished by that of both S1P receptor subtypes (19). Thus $G\alpha_i$ activation by $S1P_2$ and $S1P_3$ stimulation is important in regulation of MAPK and Akt, and

this could also contribute, along with the $S1P_3$-mediated RhoA activation shown here, to the cardioprotective effect of S1P.

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 mice 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 therapeutic benefits in ischemic heart disease without the undesirable effects of global activation of other cardiac S1P receptors.

REFERENCES

1. Finegold, J. A., Asaria, P., and Francis, D. P. (2013) Mortality from ischaemic heart disease by country, region, and age: statistics from World Health Organisation and United Nations. *Int J Cardiol* 168, 934-945

2. Carden, D. L., and Granger, D. N. (2000) Pathophysiology of ischaemia-reperfusion injury. *J Pathol* 190, 255-266

3. Hausenloy, D. J., and Yellon, D. M. (2013) Myocardial ischemia-reperfusion injury: a neglected therapeutic target. *The Journal of clinical investigation* 123, 92-100

4. Braunwald, E., and Kloner, R. A. (1985) Myocardial reperfusion: a double-edged sword? *The Journal of clinical investigation* 76, 1713-1719

5. Galagudza, M., Kurapeev, D., Minasian, S., Valen, G., and Vaage, J. (2004) Ischemic postconditioning: brief ischemia during reperfusion converts persistent ventricular fibrillation into regular rhythm. *Eur J Cardiothorac Surg* 25, 1006-1010

6. Zhao, Z. Q., Corvera, J. S., Halkos, M. E., Kerendi, F., Wang, N. P., Guyton, R. A., and Vinten-Johansen, J. (2003) Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. *American journal of physiology. Heart and circulatory physiology* 285, H579-588

7. O'Neill, W. W., Martin, J. L., Dixon, S. R., Bartorelli, A. L., Trabattoni, D., Oemrawsingh, P. V., Atsma, D. E., Chang, M., Marquardt, W., Oh, J. K., Krucoff, M. W., Gibbons, R. J., Spears, J. R., and Investigators, A. (2007) Acute Myocardial Infarction with Hyperoxemic Therapy (AMIHOT): a prospective, randomized trial of intracoronary hyperoxemic reperfusion after percutaneous coronary intervention. *J Am Coll Cardiol* 50, 397-405

8. Stone, G. W., Martin, J. L., de Boer, M. J., Margheri, M., Bramucci, E., Blankenship, J. C., Metzger, D. C., Gibbons, R. J., Lindsay, B. S., Weiner, B. H., Lansky, A. J., Krucoff, M. W., Fahy, M., Boscardin, W. J., and Investigators, A.-I. T. (2009) Effect of supersaturated oxygen delivery on infarct size after percutaneous coronary intervention in acute myocardial infarction. *Circ Cardiovasc Interv* 2, 366-375

9. Hearse, D. J., Humphrey, S. M., and Chain, E. B. (1973) Abrupt reoxygenation of the anoxic potassium-arrested perfused rat heart: a study of myocardial enzyme release. *Journal of molecular and cellular cardiology* 5, 395-407

10. Zweier, J. L., Flaherty, J. T., and Weisfeldt, M. L. (1987) Direct measurement of free radical generation following reperfusion of ischemic myocardium. *Proceedings of the National Academy of Sciences of the United States of America* 84, 1404-1407

11. Herzog, W. R., Vogel, R. A., Schlossberg, M. L., Edenbaum, L. R., Scott, H. J., and Serebruany, V. L. (1997) Short-term low dose intracoronary diltiazem administered at the onset of reperfusion reduces myocardial infarct size. *Int J Cardiol* 59, 21-27

12. Miyamae, M., Camacho, S. A., Weiner, M. W., and Figueredo, V. M. (1996) Attenuation of postischemic reperfusion injury is related to prevention of [Ca2+]m overload in rat hearts. *Am J Physiol* 271, H2145-2153

13. Hayward, R., Campbell, B., Shin, Y. K., Scalia, R., and Lefer, A. M. (1999) Recombinant soluble P-selectin glycoprotein ligand-1 protects against myocardial ischemic reperfusion injury in cats. *Cardiovascular research* 41, 65-76

14. Ma, X. L., Tsao, P. S., and Lefer, A. M. (1991) Antibody to CD-18 exerts endothelial and cardiac protective effects in myocardial ischemia and reperfusion. *The Journal of clinical investigation* 88, 1237-1243

15. Vinten-Johansen, J. (2004) Involvement of neutrophils in the pathogenesis of lethal myocardial reperfusion injury. *Cardiovascular research* 61, 481-497

16. Karliner, J. S., Honbo, N., Summers, K., Gray, M. O., and Goetzl, E. J. (2001) The lysophospholipids sphingosine-1-phosphate and lysophosphatidic acid enhance survival during hypoxia in neonatal rat cardiac myocytes. *Journal of molecular and cellular cardiology* 33, 1713-1717

17. Vessey, D. A., Li, L., Honbo, N., and Karliner, J. S. (2009) Sphingosine 1-phosphate is an important endogenous cardioprotectant released by ischemic pre- and postconditioning. *American journal of physiology. Heart and circulatory physiology* 297, H1429-1435

18. Theilmeier, G., Schmidt, C., Herrmann, J., Keul, P., Schafers, M., Herrgott, I., Mersmann, J., Larmann, J., Hermann, S., Stypmann, J., Schober, O., Hildebrand, R., Schulz, R., Heusch, G., Haude, M., von Wnuck Lipinski, K., Herzog, C., Schmitz, M., Erbel, R., Chun, J., and Levkau, B. (2006) High-density lipoproteins and their constituent, sphingosine-1-phosphate, directly protect the heart against ischemia/reperfusion injury in vivo via the S1P3 lysophospholipid receptor. *Circulation* 114, 1403-1409

19. Means, C. K., Xiao, C. Y., Li, Z., Zhang, T., Omens, J. H., Ishii, I., Chun, J., and Brown, J. H. (2007) Sphingosine 1-phosphate S1P2 and S1P3 receptor-mediated Akt activation protects against in vivo myocardial ischemia-reperfusion injury. *American journal of physiology. Heart and circulatory physiology* 292, H2944-2951

20. Xiang, S. Y., Ouyang, K., Yung, B. S., Miyamoto, S., Smrcka, A. V., Chen, J., and Heller Brown, J. (2013) PLCepsilon, PKD1, and SSH1L transduce RhoA signaling to protect mitochondria from oxidative stress in the heart. *Science signaling* 6, ra108

21. Salazar, N. C., Chen, J., and Rockman, H. A. (2007) Cardiac GPCRs: GPCR signaling in healthy and failing hearts. *Biochimica et biophysica acta* 1768, 1006-1018

22. Kang, M., Chung, K. Y., and Walker, J. W. (2007) G-protein coupled receptor signaling in myocardium: not for the faint of heart. *Physiology (Bethesda)* 22, 174-184

23. Wettschureck, N., and Offermanns, S. (2005) Mammalian G proteins and their cell type specific functions. *Physiol Rev* 85, 1159-1204

24. Means, C. K., Miyamoto, S., Chun, J., and Brown, J. H. (2008) S1P1 receptor localization confers selectivity for Gi-mediated cAMP and contractile responses. *The Journal of biological chemistry* 283, 11954-11963

25. Keys, J. R., Greene, E. A., Koch, W. J., and Eckhart, A. D. (2002) Gq-coupled receptor agonists mediate cardiac hypertrophy via the vasculature. *Hypertension* 40, 660-666

26. Akhter, S. A., Luttrell, L. M., Rockman, H. A., Iaccarino, G., Lefkowitz, R. J., and Koch, W. J. (1998) Targeting the receptor-Gq interface to inhibit in vivo pressure overload myocardial hypertrophy. *Science* 280, 574-577

27. Filtz, T. M., Grubb, D. R., McLeod-Dryden, T. J., Luo, J., and Woodcock, E. A. (2009) Gq-initiated cardiomyocyte hypertrophy is mediated by phospholipase Cbeta1b. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 23, 3564-3570

28. Wettschureck, N., Rutten, H., Zywietz, A., Gehring, D., Wilkie, T. M., Chen, J., Chien, K. R., and Offermanns, S. (2001) Absence of pressure overload induced myocardial hypertrophy after conditional inactivation of Galphaq/Galpha11 in cardiomyocytes. *Nat Med* 7, 1236-1240

29. Kozasa, T., Jiang, X., Hart, M. J., Sternweis, P. M., Singer, W. D., Gilman, A. G., Bollag, G., and Sternweis, P. C. (1998) p115 RhoGEF, a GTPase activating protein for Galpha12 and Galpha13. *Science* 280, 2109-2111

30. Yamaguchi, Y., Katoh, H., and Negishi, M. (2003) N-terminal short sequences of alpha subunits of the G12 family determine selective coupling to receptors. *The Journal of biological chemistry* 278, 14936-14939

31. Tanabe, S., Kreutz, B., Suzuki, N., and Kozasa, T. (2004) Regulation of RGS-RhoGEFs by Galpha12 and Galpha13 proteins. *Methods in enzymology* 390, 285-294

32. Xiang, S. Y., Dusaban, S. S., and Brown, J. H. (2013) Lysophospholipid receptor activation of RhoA and lipid signaling pathways. *Biochimica et biophysica acta* 1831, 213-222

33. Ritchie, B. J., Smolski, W. C., Montgomery, E. R., Fisher, E. S., Choi, T. Y., Olson, C. M., Foster, L. A., and Meigs, T. E. (2013) Determinants at the N- and C-termini of Galpha12 required for activation of Rho-mediated signaling. *Journal of molecular signaling* 8, 3

34. Bian, D., Mahanivong, C., Yu, J., Frisch, S. M., Pan, Z. K., Ye, R. D., and Huang, S. (2006) The G12/13-RhoA signaling pathway contributes to efficient lysophosphatidic acid-stimulated cell migration. *Oncogene* 25, 2234-2244

35. Suzuki, N., Nakamura, S., Mano, H., and Kozasa, T. (2003) Galpha 12 activates Rho GTPase through tyrosine-phosphorylated leukemia-associated RhoGEF. *Proceedings of the National Academy of Sciences of the United States of America* 100, 733-738

36. Jaffe, A. B., and Hall, A. (2005) Rho GTPases: biochemistry and biology. *Annu Rev Cell Dev Biol* 21, 247-269

37. Amano, M., Chihara, K., Kimura, K., Fukata, Y., Nakamura, N., Matsuura, Y., and Kaibuchi, K. (1997) Formation of actin stress fibers and focal adhesions enhanced by Rho-kinase. *Science* 275, 1308-1311

38. Chrzanowska-Wodnicka, M., and Burridge, K. (1996) Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. *J Cell Biol* 133, 1403-1415

39. Radeff-Huang, J., Seasholtz, T. M., Matteo, R. G., and Brown, J. H. (2004) G protein mediated signaling pathways in lysophospholipid induced cell proliferation and survival. *Journal of cellular biochemistry* 92, 949-966

40. Hill, C. S., Wynne, J., and Treisman, R. (1995) The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF. *Cell* 81, 1159-1170

41. Xiang, S. Y., Vanhoutte, D., Del Re, D. P., Purcell, N. H., Ling, H., Banerjee, I., Bossuyt, J., Lang, R. A., Zheng, Y., Matkovich, S. J., Miyamoto, S., Molkentin, J. D., Dorn, G. W., 2nd, and Brown, J. H. (2011) RhoA protects the mouse heart against ischemia/reperfusion injury. *The Journal of clinical investigation* 121, 3269-3276

42. Keul, P., Sattler, K., and Levkau, B. (2007) HDL and its sphingosine-1-phosphate content in cardioprotection. *Heart Fail Rev* 12, 301-306

43. Zhang, H., Desai, N. N., Olivera, A., Seki, T., Brooker, G., and Spiegel, S. (1991) Sphingosine-1-phosphate, a novel lipid, involved in cellular proliferation. *J Cell Biol* 114, 155-167

44. Olivera, A., and Spiegel, S. (1993) Sphingosine-1-phosphate as second messenger in cell proliferation induced by PDGF and FCS mitogens. *Nature* 365, 557-560

45. Kupperman, E., An, S., Osborne, N., Waldron, S., and Stainier, D. Y. (2000) A sphingosine-1-phosphate receptor regulates cell migration during vertebrate heart development. *Nature* 406, 192-195

46. Wendler, C. C., and Rivkees, S. A. (2006) Sphingosine-1-phosphate inhibits cell migration and endothelial to mesenchymal cell transformation during cardiac development. *Dev Biol* 291, 264-277

47. Cuvillier, O., Pirianov, G., Kleuser, B., Vanek, P. G., Coso, O. A., Gutkind, S., and Spiegel, S. (1996) Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate. *Nature* 381, 800-803

48. Means, C. K., and Brown, J. H. (2009) Sphingosine-1-phosphate receptor signalling in the heart. *Cardiovascular research* 82, 193-200

49. Alewijnse, A. E., Peters, S. L., and Michel, M. C. (2004) Cardiovascular effects of sphingosine-1-phosphate and other sphingomyelin metabolites. *Br J Pharmacol* 143, 666-684

50. Zhang, D. X., Zou, A. P., and Li, P. L. (2001) Ceramide reduces endothelium-dependent vasodilation by increasing superoxide production in small bovine coronary arteries. *Circulation research* 88, 824-831

51. Windh, R. T., Lee, M. J., Hla, T., An, S., Barr, A. J., and Manning, D. R. (1999) Differential coupling of the sphingosine 1-phosphate receptors Edg-1, Edg-3, and H218/Edg-5 to the G(i), G(q), and G(12) families of heterotrimeric G proteins. *The Journal of biological chemistry* 274, 27351-27358

52. Lee, M. J., Evans, M., and Hla, T. (1996) The inducible G protein-coupled receptor edg-1 signals via the G(i)/mitogen-activated protein kinase pathway. *The Journal of biological chemistry* 271, 11272-11279

53. Kluk, M. J., and Hla, T. (2002) Signaling of sphingosine-1-phosphate via the S1P/EDG-family of G-protein-coupled receptors. *Biochimica et biophysica acta* 1582, 72-80

54. Adams, J. W., and Brown, J. H. (2001) G-proteins in growth and apoptosis: lessons from the heart. *Oncogene* 20, 1626-1634

55. Takashima, S., Sugimoto, N., Takuwa, N., Okamoto, Y., Yoshioka, K., Takamura, M., Takata, S., Kaneko, S., and Takuwa, Y. (2008) G12/13 and Gq mediate S1P2-induced inhibition of Rac and migration in vascular smooth muscle in a manner dependent on Rho but not Rho kinase. *Cardiovascular research* 79, 689-697

56. Nussbaum, C., Bannenberg, S., Keul, P., Graler, M. H., Goncalves-de-Albuquerque, C. F., Korhonen, H., von Wnuck Lipinski, K., Heusch, G., de Castro Faria Neto, H. C., Rohwedder, I., Gothert, J. R., Prasad, V. P., Haufe, G., Lange-Sperandio, B., Offermanns, S., Sperandio, M., and Levkau, B. (2015) Sphingosine-1-phosphate receptor 3 promotes leukocyte rolling by mobilizing endothelial P-selectin. *Nat Commun* 6, 6416

57. Knapp, M. (2011) Cardioprotective role of sphingosine-1-phosphate. *Journal of physiology and pharmacology : an official journal of the Polish Physiological Society* 62, 601-607

58. Vessey, D. A., Kelley, M., Li, L., Huang, Y., Zhou, H. Z., Zhu, B. Q., and Karliner, J. S. (2006) Role of sphingosine kinase activity in protection of heart against ischemia reperfusion injury. *Med Sci Monit* 12, BR318-324

59. Karliner, J. S. (2013) Sphingosine kinase and sphingosine 1-phosphate in the heart: a decade of progress. *Biochimica et biophysica acta* 1831, 203-212

60. Zhang, J., Honbo, N., Goetzl, E. J., Chatterjee, K., Karliner, J. S., and Gray, M. O. (2007) Signals from type 1 sphingosine 1-phosphate receptors enhance adult mouse cardiac myocyte survival during hypoxia. *American journal of physiology. Heart and circulatory physiology* 293, H3150-3158

61. Graler, M. H., and Goetzl, E. J. (2004) The immunosuppressant FTY720 down-regulates sphingosine 1-phosphate G-protein-coupled receptors. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 18, 551-553

62. Tsukada, Y. T., Sanna, M. G., Rosen, H., and Gottlieb, R. A. (2007) S1P1-selective agonist SEW2871 exacerbates reperfusion arrhythmias. *J Cardiovasc Pharmacol* 50, 660-669

63. Miyamoto, S., Purcell, N. H., Smith, J. M., Gao, T., Whittaker, R., Huang, K., Castillo, R., Glembotski, C. C., Sussman, M. A., Newton, A. C., and Brown, J. H. (2010) PHLPP-1 negatively regulates Akt activity and survival in the heart. *Circulation research* 107, 476-484

64. Ishii, I., Friedman, B., Ye, X., Kawamura, S., McGiffert, C., Contos, J. J., Kingsbury, M. A., Zhang, G., Brown, J. H., and Chun, J. (2001) Selective loss of sphingosine 1-phosphate signaling with no obvious phenotypic abnormality in mice lacking its G protein-coupled receptor, LP(B3)/EDG-3. *The Journal of biological chemistry* 276, 33697-33704

65. Schmittgen, T. D., and Livak, K. J. (2008) Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 3, 1101-1108

66. Maillet, M., Purcell, N. H., Sargent, M. A., York, A. J., Bueno, O. F., and Molkentin, J. D. (2008) DUSP6 (MKP3) null mice show enhanced ERK1/2 phosphorylation at baseline and increased myocyte proliferation in the heart affecting disease susceptibility. *The Journal of biological chemistry* 283, 31246-31255

67. Wilkins, B. J., Dai, Y. S., Bueno, O. F., Parsons, S. A., Xu, J., Plank, D. M., Jones, F., Kimball, T. R., and Molkentin, J. D. (2004) Calcineurin/NFAT coupling participates in pathological, but not physiological, cardiac hypertrophy. *Circulation research* 94, 110-118

68. Sagi, S. A., Seasholtz, T. M., Kobiashvili, M., Wilson, B. A., Toksoz, D., and Brown, J. H. (2001) Physical and functional interactions of Galphaq with Rho and its exchange factors. *The Journal of biological chemistry* 276, 15445-15452

69. Citro, S., Malik, S., Oestreich, E. A., Radeff-Huang, J., Kelley, G. G., Smrcka, A. V., and Brown, J. H. (2007) Phospholipase Cepsilon is a nexus for Rho and Rap-mediated G protein-coupled receptor-induced astrocyte proliferation. *Proceedings of the National Academy of Sciences of the United States of America* 104, 15543-15548

70. Dusaban, S. S., Purcell, N. H., Rockenstein, E., Masliah, E., Cho, M. K., Smrcka, A. V., and Brown, J. H. (2013) Phospholipase C epsilon links G protein-coupled receptor activation to inflammatory astrocytic responses. *Proceedings of the National Academy of Sciences of the United States of America* 110, 3609-3614

71. Mishra, S., Ling, H., Grimm, M., Zhang, T., Bers, D. M., and Brown, J. H. (2010) Cardiac hypertrophy and heart failure development through Gq and CaM kinase II signaling. *J Cardiovasc Pharmacol* 56, 598-603

72. Hart, M. J., Jiang, X., Kozasa, T., Roscoe, W., Singer, W. D., Gilman, A. G., Sternweis, P. C., and Bollag, G. (1998) Direct stimulation of the guanine nucleotide exchange activity of p115 RhoGEF by Galpha13. *Science* 280, 2112-2114

73. Siehler, S., and Manning, D. R. (2002) Pathways of transduction engaged by sphingosine 1-phosphate through G protein-coupled receptors. *Biochimica et biophysica acta* 1582, 94-99

74. Sugimoto, N., Takuwa, N., Okamoto, H., Sakurada, S., and Takuwa, Y. (2003) Inhibitory and stimulatory regulation of Rac and cell motility by the G12/13-Rho and Gi pathways integrated downstream of a single G protein-coupled sphingosine-1-phosphate receptor isoform. *Molecular and cellular biology* 23, 1534-1545

75. Ruppel, K. M., Willison, D., Kataoka, H., Wang, A., Zheng, Y. W., Cornelissen, I., Yin, L., Xu, S. M., and Coughlin, S. R. (2005) Essential role for Galpha13 in endothelial cells during embryonic development. *Proceedings of the National Academy of Sciences of the United States of America* 102, 8281-8286

76. Offermanns, S., Mancino, V., Revel, J. P., and Simon, M. I. (1997) Vascular system defects and impaired cell chemokinesis as a result of Galpha13 deficiency. *Science* 275, 533-536

77. Gohla, A., Harhammer, R., and Schultz, G. (1998) The G-protein G13 but not G12 mediates signaling from lysophosphatidic acid receptor via epidermal growth factor receptor to Rho. *The Journal of biological chemistry* 273, 4653-4659

78. Ishii, I., Ye, X., Friedman, B., Kawamura, S., Contos, J. J., Kingsbury, M. A., Yang, A. H., Zhang, G., Brown, J. H., and Chun, J. (2002) Marked perinatal lethality and cellular signaling deficits in mice null for the two sphingosine 1-phosphate (S1P) receptors, S1P(2)/LP(B2)/EDG-5 and S1P(3)/LP(B3)/EDG-3. *The Journal of biological chemistry* 277, 25152-25159

79. Wang, F., Okamoto, Y., Inoki, I., Yoshioka, K., Du, W., Qi, X., Takuwa, N., Gonda, K., Yamamoto, Y., Ohkawa, R., Nishiuchi, T., Sugimoto, N., Yatomi, Y., Mitsumori, K., Asano, M., Kinoshita, M., and Takuwa, Y. (2010) Sphingosine-1-phosphate receptor-2 deficiency leads to inhibition of macrophage proinflammatory activities and atherosclerosis in apoE-deficient mice. *The Journal of clinical investigation* 120, 3979-3995

80. Murakami, A., Takasugi, H., Ohnuma, S., Koide, Y., Sakurai, A., Takeda, S., Hasegawa, T., Sasamori, J., Konno, T., Hayashi, K., Watanabe, Y., Mori, K., Sato, Y., Takahashi, A., Mochizuki, N., and Takakura, N. (2010) Sphingosine 1-phosphate (S1P) regulates vascular contraction via S1P3 receptor: investigation based on a new S1P3 receptor antagonist. *Molecular pharmacology* 77, 704-713