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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.