

Sphingosine-1-phosphate ameliorates the cardiac hypertrophic response through inhibiting the activity of histone deacetylase-2

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Abstract. Inhibition of histone deacetylase-2 (HDAC2), which is a prohypertrophic factor in the heart, can functionally attenuate cardiac hypertrophy. The present study aimed to investigate whether sphingosine-1-phosphate (S1P), which has recently been reported to suppress HDAC2 activity, could ameliorate the cardiac hypertrophic response and improve cardiac function in mice with transverse aortic constriction (TAC), as well as to determine the underlying mechanisms. Briefly, 8-week-old male C57BL/6 mice were randomly divided into sham, TAC and TAC + S1P groups; the results indicated that S1P treatment attenuated TAC-induced cardiac dysfunction. In addition, heart size and the expression levels of fetal cardiac genes were reduced in the TAC + S1P group compared with in the TAC group. Furthermore, in cultured H9c2 cells exposed to phenylephrine, S1P was revealed to decrease cardiomyocyte size and the exaggerated expression of fetal cardiac genes. The present study also demonstrated that S1P had no effect on HDAC2 expression, but it did suppress its activity and increase acetylation of histone H3 *in vivo* and *in vitro*. Krüppel-like factor 4 (KLF4) is an antihypertrophic transcriptional regulator, which mediates HDAC inhibitor-induced prevention of cardiac hypertrophy; in the present study, KLF4 was upregulated by S1P. Finally, the results indicated that S1P receptor 2 (S1PR2) may be involved in the antihypertrophic effects, whereas the suppressive effects of S1P on HDAC2 activity were independent of S1PR2. In conclusion, the present study demonstrated that S1P treatment may ameliorate the cardiac hypertrophic response, which may

be partly mediated by the suppression of HDAC2 activity and the upregulation of KLF4; it was suggested that S1PR2 may also be involved. Therefore, S1P may be considered a potential therapy for the treatment of heart diseases caused by cardiac hypertrophy.

Introduction

Cardiac hypertrophy refers to a complex cardiac remodeling process that is induced by various stressors, including hypertension, valve stenosis or regurgitation, and myocardial infarction, thus resulting in an increase in myocardial cell size and heart weight (HW), and ultimately cardiac dysfunction and heart failure (1,2). During this process, the arrested fetal genes that are associated with hypertrophy, including natriuretic peptide type A (*Nppa*), natriuretic peptide type B (*Nppb*) and myosin heavy polypeptide 7 (*Myh7*), are reactivated (3). Previous studies have reported that histone deacetylase-2 (HDAC2) serves an important role in cardiac hypertrophy (4,5). HDACs are post-translational modifying enzymes, which can modify the structure of chromosomes and regulate gene expression (6). HDAC2 belongs to class I HDACs and it has previously been demonstrated that inhibiting HDAC2 activity can suppress the progression of cardiac hypertrophy (7,8). Recent progress towards understanding the underlying mechanisms has revealed that the transcriptional regulator Krüppel-like factor 4 (KLF4) mediates HDAC inhibitor-induced prevention of cardiac hypertrophy (9).

Sphingosine-1-phosphate (S1P) is a lysophospholipid mediator that circulates in the blood, which has been reported to be able to inhibit HDAC2 activity, and has been proposed to protect against numerous cardiovascular disorders, including coronary artery disease, atherosclerosis, myocardial infarction and heart failure (10,11). The majority of known S1P effects are based on three specific G protein-coupled receptors (GPCRs), termed S1P receptor (R)1-S1PR3, which are expressed in the cardiovascular system (12). Nevertheless, whether S1P can function through inhibiting HDAC2 in the heart is unknown.

As aforementioned, S1P exerts cardioprotective effects and can suppress HDAC2 activity, which is closely involved in cardiac hypertrophy. However, whether S1P can alleviate cardiac hypertrophy and whether S1PRs participate in it has not been elucidated. Therefore, to further explore the role of S1P in the heart, the present study investigated the effects of S1P *in vivo* and *in vitro*. *In vivo* experiments were performed on mice under transverse aortic constriction (TAC), which

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Abbreviations: H3K9-Ac, histone H3 with lysine 9 acetylation; S1PR, sphingosine-1-phosphate receptor; siR, small interfering RNA-S1PR

Key words: sphingosine-1-phosphate, histone deacetylase-2, cardiac hypertrophy, Krüppel-like factor 4, sphingosine-1-phosphate receptor

were treated with or without S1P. *In vitro* experiments were performed with H9c2 cells to explore the effects and mechanisms of S1P.

Materials and methods

Reagents and antibodies. S1P was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Phenylephrine (PE) and fluorescein isothiocyanate (FITC)-conjugated wheat germ agglutinin were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Nuclear and Cytoplasmic Protein Extraction kit and DAPI were purchased from Wuhan Boster Biological Technology, Ltd. (Wuhan, China). Cell lysis buffer for western blotting and immunoprecipitation, and actin-Tracker Green were purchased from Beyotime Institute of Biotechnology (Shanghai, China). HDAC2 Fluorimetric Drug Discovery kit was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) for cell culture were obtained from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Small interfering (si)RNAs were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Mega Tran1.0 was purchased from OriGene Technologies, Inc. (Rockville, MD, USA). Antibodies against atrial natriuretic peptide (ANP; sc-20158), brain natriuretic peptide (BNP; sc-271185), S1PR2 (sc-25491) and GAPDH (sc-32233) were from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Antibodies against β -myosin heavy chain (β -MHC; 22280-1-AP), HDAC2 (12922-3-AP) and histone H3 (17168-1-AP) were obtained from ProteinTech Group, Inc. (Chicago, IL, USA). An antibody against KLF4 (BA3453) was purchased from Wuhan Boster Biological Technology, Ltd. Antibodies against histone H3 with lysine 9 acetylation (H3K9-Ac; ab32129), S1PR1 (ab125074) and S1PR3 (ab108370) were obtained from Abcam (Cambridge, MA, USA).

Animals and animal treatment. Male C57BL/6 mice (age, 8 weeks; weight, 23–26 g; $n=24$) used in the present study were obtained from the Experimental Animal Center of Changsha (Changsha, China). The present study was approved by the Institutional Animal Research Committee of Tongji Medical College (Wuhan, China). All animal experimental protocols complied with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (13). Mice were housed at the Animal Experimental Center of Tongji Hospital (Wuhan, China) at 25°C, under a 12-h light/dark cycle, and were allowed free access to normal mice chow and water throughout the study period.

Following 1 week acclimation, the mice were randomly divided into various treatment groups. Pressure overload by TAC was used to induce cardiac hypertrophy, as described previously (14). Briefly, a medial skin incision was made from the neck to the upper chest, and the manubrium of the sternum was opened. The transverse aorta between the right innominate artery and left carotid artery was constricted using 7-0 silk suture tied around a 27-gauge needle. Sham surgery was performed without constricting the aorta. A total of 1 week after surgery, TAC-operated mice were randomized into various cohorts, and were intraperitoneally injected with S1P (6 $\mu\text{g/g/day}$) or vehicle (saline) for a further 2 weeks (15). The mice were grouped as follows: Sham ($n=8$), TAC ($n=5$)

Table I. Hemodynamic and echocardiographic results.

Variable	Sham	TAC	TAC + S1P
Hemodynamics			
HR (bpm)	409 \pm 33	375 \pm 9	432 \pm 20
dp/dt _{max} (mmHg/sec)	5,489 \pm 599	2,356 \pm 390 ^a	6,330 \pm 1241 ^b
dp/dt _{min} (mmHg/sec)	-4,427 \pm 562	-1,620 \pm 369 ^a	-4,997 \pm 782 ^b
Echocardiography			
EF (%)	51.5 \pm 0.9	38.4 \pm 2.5 ^a	55.0 \pm 2.9 ^b
FS (%)	26.1 \pm 0.5	18.7 \pm 1.4 ^a	28.2 \pm 1.8 ^b
LVID (d) (mm)	4.30 \pm 0.13	4.77 \pm 0.10 ^a	3.81 \pm 0.14 ^b
LVID (s) (mm)	3.22 \pm 0.13	3.85 \pm 0.11 ^a	2.84 \pm 0.11 ^b

Values are presented as the means \pm standard error of the mean; ^a $P<0.05$ vs. Sham group; ^b $P<0.05$ vs. TAC group; $n\geq 5$ /group. dp/dt_{max}, maximal slope of systolic pressure increment; dp/dt_{min}, minimal slope of diastolic pressure decrement; EF, ejection fraction; FS, fractional shortening; HR, heart rate; LVID (d), left ventricular internal dimension in diastole; LVID (s), left ventricular internal dimension in systole; S1P, sphingosine-1-phosphate; TAC, transverse aortic constriction.

and TAC + S1P ($n=6$) groups. Originally, there were 8 mice in each group; however, following TAC surgery, 3 mice died in the TAC group and 2 died in the TAC + S1P group. This may be the result of the impact of TAC and the specificity of different mice. Hypertrophic responses at the end of the treatment were analyzed by echocardiography, and hemodynamic, histological and biochemical analyses.

Sampling method. Following treatment with S1P for 2 weeks, mice were sacrificed by exsanguination through the carotid artery under pentobarbital (100 mg/kg) anesthesia; euthanasia was performed in the same way for all of the groups. Subsequently, the whole heart was collected, including the aorta, using ophthalmic scissors to cut along the backbone. The root of the aorta was removed and the heart was separated, weighed and washed with ice-cold saline; one part was stored in liquid nitrogen at -80°C for western blotting and HDAC2 activity assays. The other part of the heart was maintained in 10% formalin solution for histochemical analysis.

Hemodynamic measurements. Ventricular hemodynamic measurements were performed using a Millar Catheter system (Millar, Inc., Houston, TX, USA) via the right carotid artery under intraperitoneal injection of 100 mg/kg pentobarbital, as described previously (16). Briefly, a pressure-volume catheter (Millar 1.4F, SPR 835; Millar, Inc.) was inserted into the right carotid artery and advanced into the left ventricle to measure heart rate and instantaneous intraventricular pressure.

Analysis of cardiac function by echocardiography. Cardiac function was assessed by echocardiography, using a high-resolution imaging system equipped with a 30-MHz high frequency scanhead (VisualSonics Vevo770; VisualSonics, Inc., Toronto, ON, Canada) applied to the chest wall. Ventricular dimensions, ejection fraction (EF) and fractional shortening (FS) were examined as described previously (17).

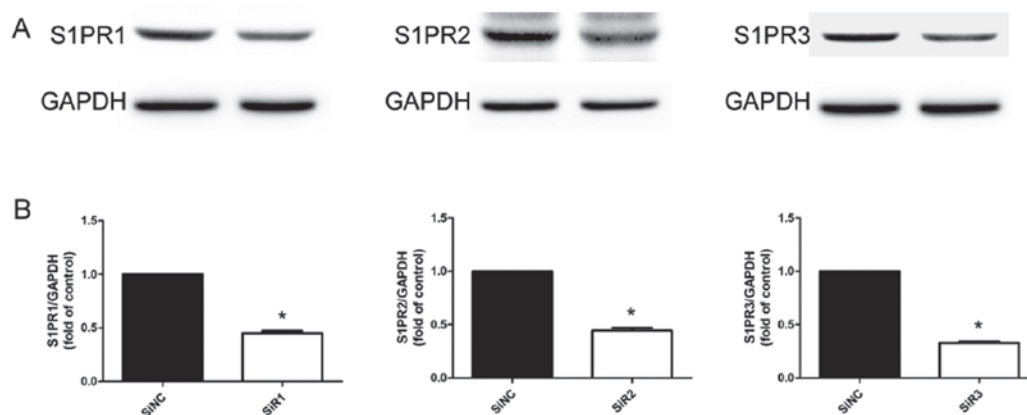


Figure 1. Effects of siRNAs targeting S1PR1-3 in H9c2 cells. (A) Representative immunoblots for S1PR1, S1PR2 and S1PR3 and (B) semi-quantification of S1PRs protein expression in H9c2 cells. Data are presented as the means \pm standard error of the mean ($n \geq 3$ for each experiment). * $P < 0.05$ vs. siNC-transfected cells. S1PR, sphingosine-1-phosphate receptor; siNC, negative control siRNA; siR1-3, siRNA-S1PR1-3; siRNA, small interfering RNA.

Histochemical analysis. Hearts were fixed with 10% formalin solution for 24 h at room temperature. They were then embedded in paraffin and sectioned into slices (5 μ m). To measure the area of cardiomyocytes, heart sections were stained with hematoxylin and eosin (H&E) and FITC-conjugated wheat germ agglutinin, as previously described (18), and were visualized by light microscopy. Image-Pro Plus Version 6.0 (Media Cybernetics, Inc., Bethesda, MD, USA) was used to measure the area of each cell.

Cell culture and treatments. H9c2 cells, which are a subclone of the original clonal cell line derived from the heart of embryonic BDIX rats, were obtained from American Type Culture Collection (CRL-1446; ATCC, Manassas, VA, USA). The cells were cultured in DMEM supplemented with 10% FBS and penicillin-streptomycin (100 IU/ml) in a humidified atmosphere containing 95% air and 5% CO₂ at 37°C.

Cells were plated in 6-well or 12-well plates at 37°C, and were treated with 1 μ M S1P for 1 h, followed by 100 μ M PE for 24 h, after which cells were collected. For some experiments, 2×10^5 cells per well were transfected for 24 h at room temperature with small interfering (si)RNA negative control (siNC) and siRNAs targeting rat S1PR1, S1PR2 and S1PR3 (siR1, siR2 and siR3) using MegaTran 1.0, according to the manufacturer's protocol. The concentration of siRNAs used was 50 nM. A total of 24 hours post-transfection, cells were treated with S1P for 1 h and PE for 24 h.

All *in vitro* experiments were repeated three times with the same interventions. The siRNA sequences were as follows: siRNA-S1PR1, 5'-CCUCCUUGCUAUCGCCAUUdTdT-3' (sense) and 3'-dTdTGGAGGAACGAUAGCGGUAA-5' (antisense); siRNA-S1PR2, 5'-CCUUGUACGUCCGAAUCUAdTdT-3' (sense) and 3'-dTdTGGACAUGCAGGCUUAGAU-5' (antisense); siRNA-S1PR3, 5'-GCCACUCUCCAAAGGUCAAdTdT-3' (sense) and 3'-dTdTTCGGUGAGAGGUUUCAGUU-5' (antisense). Successful transfection was confirmed by western blotting (Fig. 1).

Immunocytofluorescence. Cells were initially plated in 12-well plates and were treated as aforementioned. Subsequently, cells were washed with ice-cold PBS, fixed in 4% paraformaldehyde

for 10 min and treated with 0.3% Triton X-100 for 20 min at room temperature. After blocking with 5% bovine serum albumin (BSA; Abcam) for 30 min at room temperature, cells were incubated in actin-Tracker Green at 4°C overnight. Subsequently, the cells were stained with DAPI for nuclear detection and were visualized under a Nikon DXM1200 fluorescence microscope (Nikon Corporation, Tokyo, Japan). Image-Pro Plus 6.0 (Media Cybernetics, Inc., Bethesda, MD, USA) was applied to merge images and measure the area of cells.

Immunoprecipitation. Nuclear proteins were extracted using Nuclear and Cytoplasmic Protein Extraction kit according to the manufacturer's protocol, after which they were precleared with protein A/G-agarose beads and incubated overnight with HDAC2 antibody at 4°C. Subsequently, protein A/G-agarose beads were added and incubated for 2-3 h at 4°C on a rotator. Beads were collected, washed with ice-cold PBS and used for HDAC2 activity assays. Aliquots of agarose-bound immunocomplexes were boiled in SDS-PAGE sample buffer and the released HDAC2 proteins were analyzed by western blot analysis using HDAC2 antibody.

HDAC2 activity assays. HDAC2 activity was tested using a HDAC2 Fluorimetric Drug Discovery kit according to the manufacturer's protocol. Briefly, HDAC2 was isolated by immunoprecipitation and was then subjected to an activity assay. Briefly, samples were incubated with Fluor de Lys®-Green Substrate at 37°C for 30 min. Fluor de Lys® Developer was then added and the samples were incubated for 15 min at room temperature. HDAC2 activity levels were expressed as arbitrary fluorescence units and were determined using a Synergy 2 reader (Bio-Tek Instruments, Inc., Winooski, VT, USA) by measuring fluorescence with excitation at 485 nm and emission at 528 nm.

Western blot analysis. For protein extraction, heart samples and cells were lysed with cell lysis buffer for western blotting and immunoprecipitation. Subsequently, lysates were centrifuged at 12,000 \times g for 20 min at 4°C and the supernatants were used for western blot analysis. The protein concentration was quantified using BCA Protein Assay kit. About 8.5 mg/

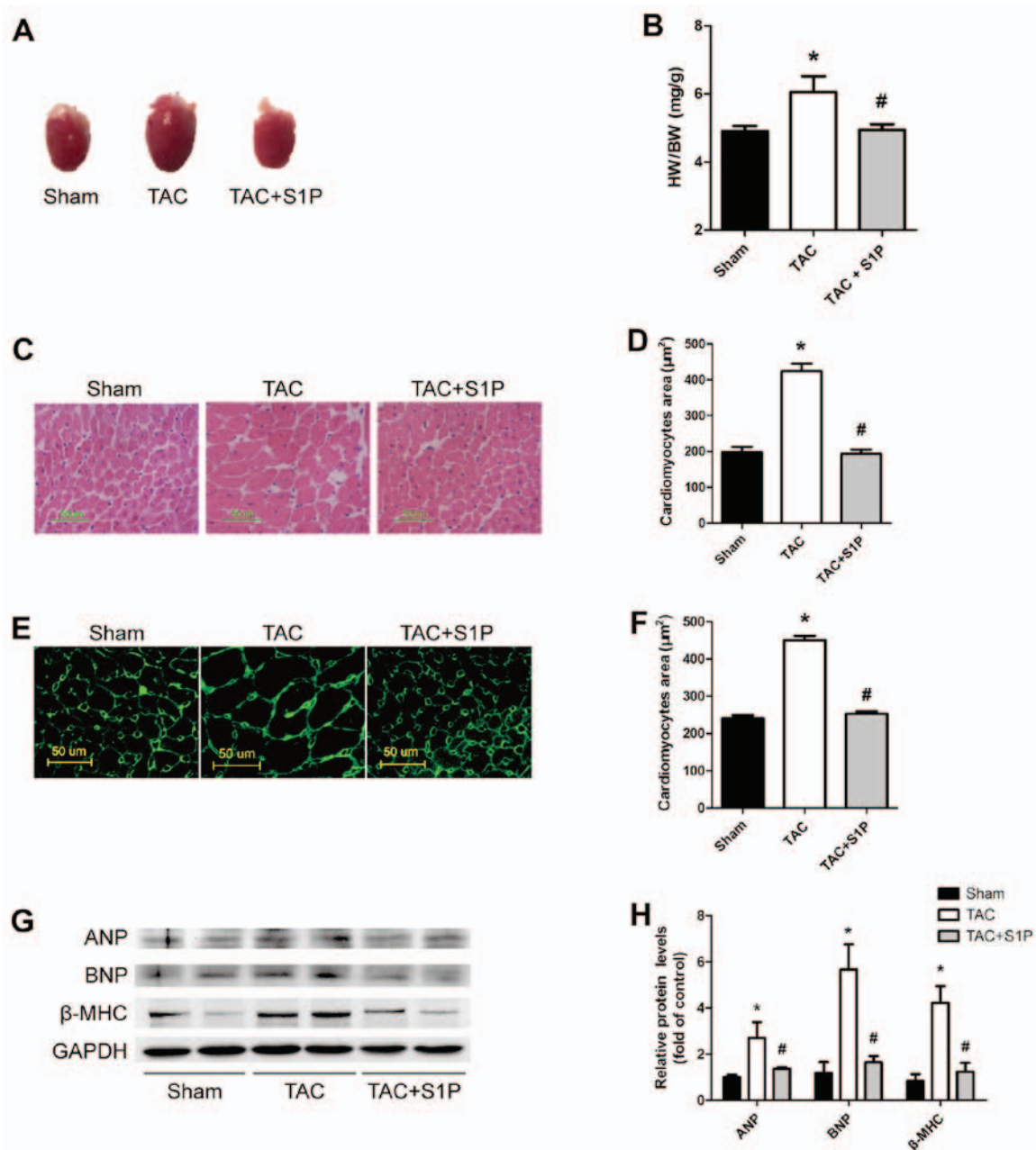


Figure 2. Treatment of mice with SIP prevents the development of TAC-induced cardiac hypertrophy. (A) Representative images of hearts and (B) HW/BW ratios. (C) Histological analysis of cardiomyocytes by hematoxylin and eosin and (D) quantification of cardiomyocyte size. (E) Fluorescein isothiocyanate-conjugated wheat germ agglutinin staining and (F) quantification of cardiomyocyte size. (G) Representative immunoblots for cardiac hypertrophic proteins (ANP, BNP and β -MHC) and (H) semi-quantification of cardiac hypertrophic proteins. Data are presented as the means \pm standard error of the mean ($n \geq 5$ for each group). * $P < 0.05$ vs. Sham group; # $P < 0.05$ vs. TAC group. ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; β -MHC, β -myosin heavy chain; BW, body weight; HW, heart weight; SIP, sphingosine-1-phosphate; TAC, transverse aortic constriction

ml protein was loaded onto gels. Proteins were subjected to 10% SDS-PAGE and were transferred onto polyvinylidene fluoride membranes, after which they were incubated at room temperature for 2 h with blocking solution (5% BSA), and were then incubated with the indicated antibodies overnight at 4°C. Rabbit anti-ANP (1:1,000 dilution), rabbit anti-BNP (1:1,000 dilution), rabbit anti- β -MHC (1:2,000 dilution), mouse anti-GAPDH (1:2,000 dilution), rabbit anti-HDAC2 (1:1,000 dilution), rabbit anti-H3K9-Ac (1:500 dilution), rabbit anti-H3-total (1:2,000 dilution), rabbit anti-KLF4 (1:200 dilution), rabbit anti-S1PR1 (1:5,000 dilution), rabbit anti-S1PR2 (1:1,000 dilution) and rabbit anti-S1PR3 (1:5,000 dilution)

were used. Subsequently, these membranes were incubated with a secondary antibody conjugated to horseradish peroxidase (1:5,000 dilution; cat. nos. ab6734 and ab131368; Abcam) at room temperature for ~2 h. Following incubation with each antibody, the membranes were washed five times with Tris-buffered saline-Tween containing 10 mM Tris-Cl (pH 7.5), 100 mM NaCl, and 0.1% Tween-20 at room temperature. Immunoreactive bands were examined with enhanced chemiluminescence solution (Thermo Fisher Scientific, Inc.), and were semi-quantified by densitometry and normalized to GAPDH or histone H3 expression. All groups were then normalized to their respective controls.

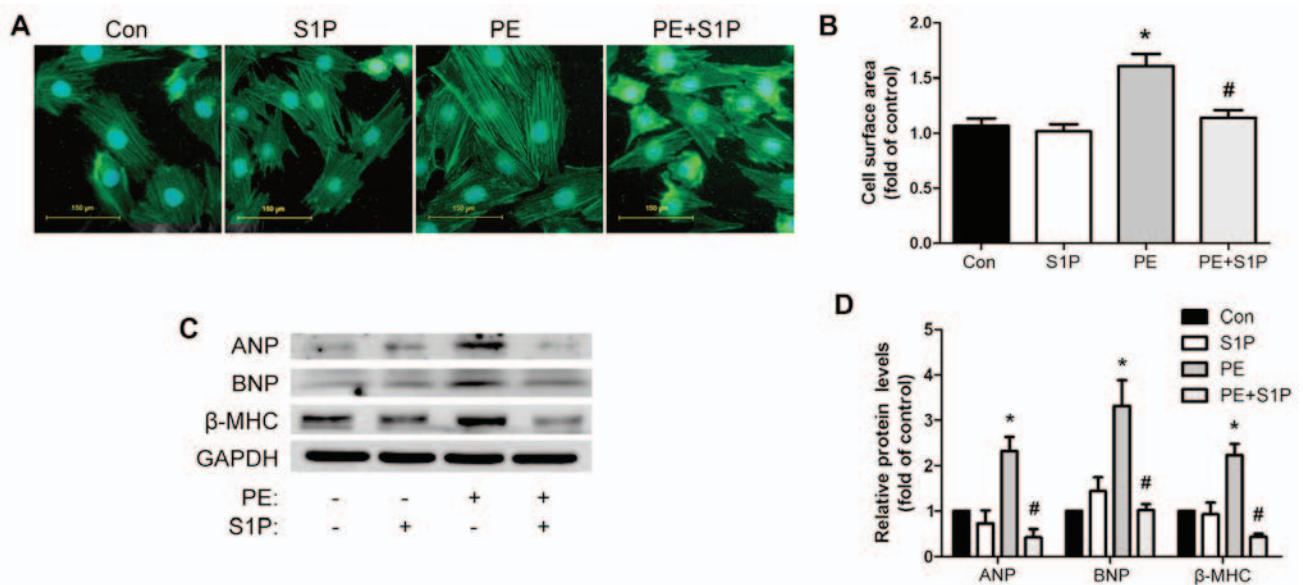


Figure 3. S1P administration inhibits the PE-induced cardiac hypertrophic response *in vitro*. (A and B) Measurement and quantification of the surface area of H9c2 cells following various treatments (magnification, x400). (C and D) Representative immunoblots and semi-quantification of cardiac hypertrophy-associated proteins (ANP, BNP and β -MHC). Data are presented as the means \pm standard error of the mean ($n \geq 3$ for each experiment). * $P < 0.05$ vs. Con group; # $P < 0.05$ vs. PE group. ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; β -MHC, β -myosin heavy chain; Con, control; PE, phenylephrine; S1P, sphingosine-1-phosphate.

Statistical analysis. All continuous data were expressed as the means \pm standard error of the mean. the densitometry was realized using Gel-Pro 32 analyzer and the statistical software used was GraphPad Prism5. Differences between groups were evaluated using unpaired Student's t-test or one-way analysis of variance (ANOVA) and Bonferroni post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

S1P attenuates TAC-induced cardiac dysfunction in mice. To determine the effects of S1P on cardiac performance, heart tissues obtained from the various groups underwent hemodynamic and echocardiographic assessments. The results demonstrated that, compared with in the TAC group, maximal slope of systolic pressure increment and minimal slope of diastolic pressure decrement were consistently increased in the TAC + S1P group; there were no significant alterations in heart rate between the groups (Table I). Enlargement of ventricular chambers at end-systole and end-diastole, and decreased EF and FS were observed following TAC, thus indicating impaired cardiac function. Conversely, compromised cardiac function was significantly improved following S1P treatment (Table I). These data indicated that S1P may ameliorate TAC-induced cardiac function deterioration.

S1P treatment prevents the development of TAC-induced cardiac hypertrophy and reduces the expression of cardiac hypertrophic proteins in mice. To determine the effects of S1P on cardiac hypertrophy, heart weight (HW)/body weight (BW) ratios and cardiac morphology were compared in mice under various treatment conditions. Heart size and HW/BW ratios were markedly increased in the TAC group; however,

in TAC mice treated with S1P, these parameters were reduced to normal levels (Fig. 2A and B). Consistent findings were observed when paraffin-embedded heart sections were stained with H&E and FITC-conjugated wheat germ agglutinin to evaluate cardiac hypertrophy. The TAC group exhibited larger sizes of cardiomyocytes compared with in the sham group, whereas the TAC + S1P group exhibited similar sizes as the sham group (Fig. 2C-F).

To determine whether TAC induced upregulation of cardiac hypertrophic proteins, western blot analysis was performed to examine the expression levels of ANP, BNP and β -MHC. The results revealed that TAC increased the expression of these proteins; however, S1P treatment inhibited the expression of these proteins (Fig. 2G and H).

These findings suggested that S1P treatment may prevent the development of TAC-induced cardiac hypertrophy in mice.

S1P administration inhibits the PE-induced cardiac hypertrophic response in cultured H9c2 cells. The present study further investigated the effects of S1P on the PE-induced hypertrophic response in H9c2 cells. Consistent with the *in vivo* findings, PE (100 μ M) increased cardiomyocyte size, as detected by cell surface area measurement. Notably, pretreatment with S1P (1 μ M) restricted this effect (Fig. 3A and B). Furthermore, western blot analysis demonstrated that S1P suppressed the expression of PE-induced markers of cardiac hypertrophy, including ANP, BNP and β -MHC (Fig. 3C and D). S1P treatment alone had no effect on cardiac hypertrophy compared with in the control group (Fig. 3). These data indicated that S1P could attenuate the cardiac hypertrophic response *in vitro*.

S1P regulates the cardiac hypertrophic response through inhibiting HDAC2 activity, but not by influencing HDAC2 expression. To test the hypothesis that S1P may regulate the cardiac hypertrophic response by inhibiting HDAC2, the

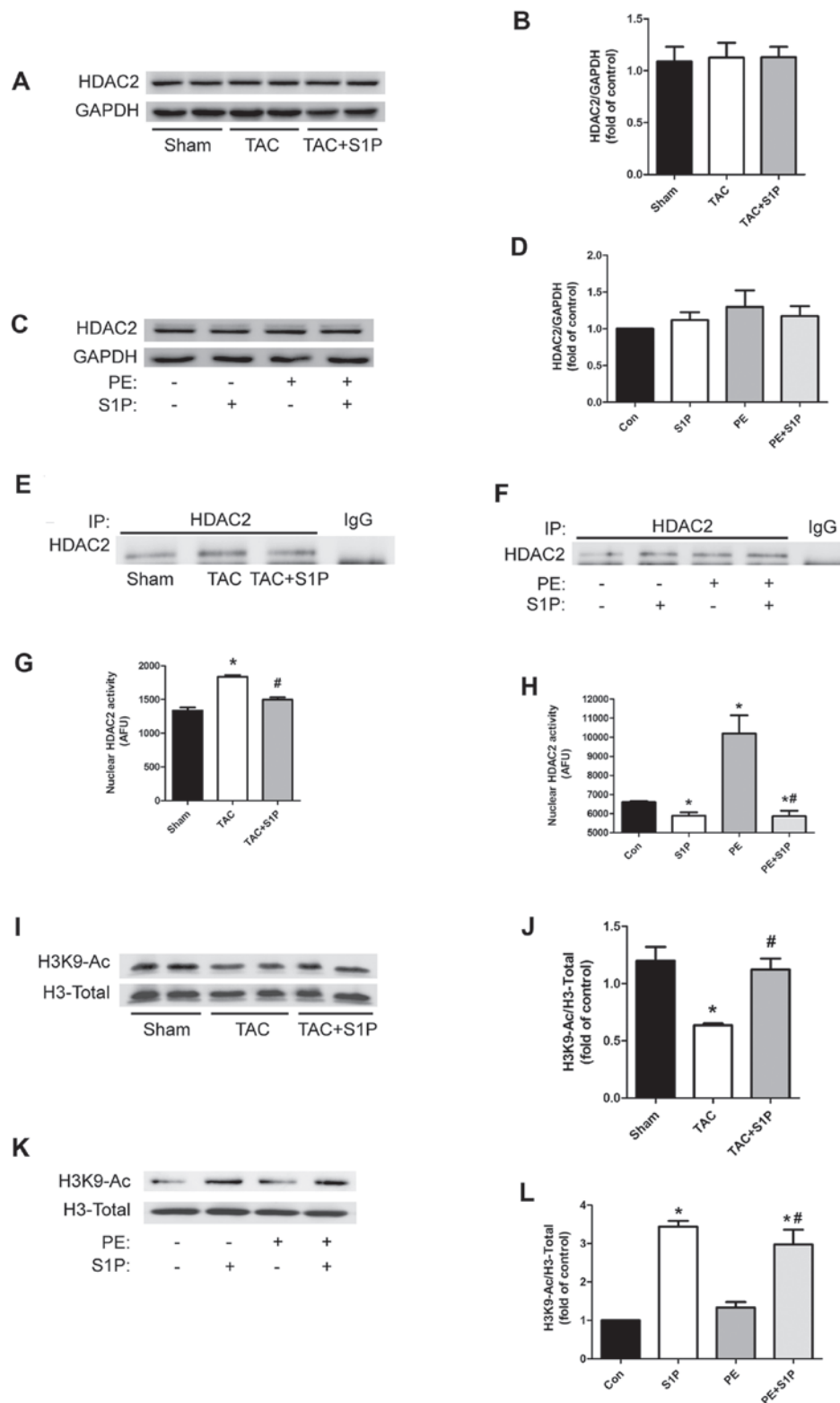


Figure 4. S1P regulates cardiac hypertrophic response through inhibiting HDAC2 activity, but not by affecting HDAC2 expression. (A-D) Representative immunoblots and semi-quantification of HDAC2 expression in heart samples and H9c2 cells. (E and F) IP of HDAC2 in nuclear proteins from various groups. (G and H) Nuclear HDAC2 activity was determined in duplicate cardiac samples and H9c2 cells. (I-L) H3K9-Ac expression was determined by western blotting with the indicated antibodies. Data are presented as the means \pm standard error of the mean. *In vivo* experiments, $n \geq 5$ for each group; * $P < 0.05$ vs. Sham group; # $P < 0.05$ vs. TAC group; *in vitro* experiments, $n \geq 3$ for each experiment; * $P < 0.05$ vs. Con group; # $P < 0.05$ vs. PE group. Con, control; H3K9-Ac, histone H3 with lysine 9 acetylation; HDAC2, histone deacetylase-2; IP, immunoprecipitation; PE, phenylephrine; S1P, sphingosine-1-phosphate; TAC, transverse aortic constriction.

expression levels of HDAC2 were detected in heart samples and H9c2 cells; S1P was revealed to have no effect on HDAC2 expression levels (Fig 4A-D). Therefore, the present

study investigated whether S1P could affect HDAC2 activity. HDAC2 was initially extracted from nuclear proteins by immunoprecipitation (Fig. 4E and F) and underwent HDAC2

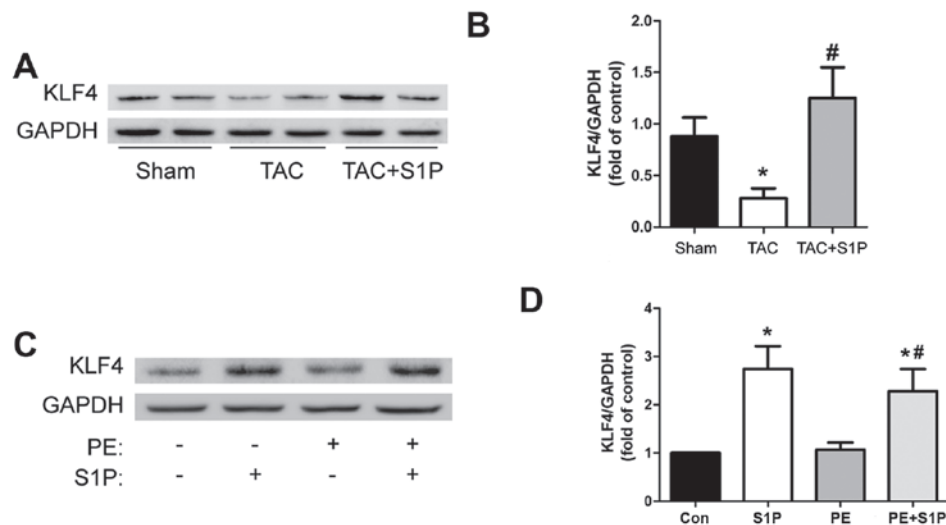


Figure 5. S1P upregulates KLF4, an anti-hypertrophic factor, *in vivo* and *in vitro*. (A and B) Representative immunoblots and semi-quantification of KLF4 in heart samples. Data are presented as the means \pm standard error of the mean ($n \geq 5$ for each group). * $P < 0.05$ vs. Sham group; # $P < 0.05$ vs. TAC group. (C and D) Representative immunoblots and semi-quantification of KLF4 in H9c2 cells. Data are presented as the means \pm standard error of the mean ($n \geq 3$ for each experiment). * $P < 0.05$ vs. Con group; # $P < 0.05$ vs. PE group. Con, control; KLF4, Krüppel-like factor 4; PE, phenylephrine; S1P, sphingosine-1-phosphate; TAC, transverse aortic constriction

activity assays. The results indicated that *in vivo* and *in vitro*, HDAC2 activity was upregulated under hypertrophic conditions, whereas it was significantly suppressed in groups treated with S1P (Fig. 4G and H). To further verify these findings, the expression of H3K9-Ac was detected; treatment with S1P was shown to increase acetylation of H3K9 (Fig. 4I-L).

KLF4, an antihypertrophic factor, is upregulated by S1P in vivo and in vitro. To determine whether S1P, like other HDAC inhibitors, such as trichostatin A, may upregulate KLF4 to prevent the progression of cardiac hypertrophy, the present study measured the expression levels of KLF4 in heart samples. The results demonstrated that KLF4 was significantly upregulated by treatment with S1P compared with in the TAC group (Fig. 5A and B). This increase in KLF4 expression was confirmed in H9c2 cells (Fig. 5C and D).

S1PR expression is altered following TAC, and S1PR2 may be involved in the antihypertrophic effects of S1P. Since S1PRs have been reported to serve a role in mediating cardioprotection, the present study aimed to determine whether they participate in the antihypertrophic effects of S1P. Initially, the expression levels of S1PR1, 2 and 3 were analyzed, which have been suggested to be expressed in the cardiovascular system of mice (12,26). The results indicated that the expression of all three S1PRs was altered following TAC; S1PR1 and S1PR3 were upregulated, whereas S1PR2 was downregulated, thus suggesting that S1PRs may be involved in cardiac hypertrophy (Fig. 6A and B). Subsequently, S1PR-specific siRNAs were used to investigate whether S1P attenuated cardiac hypertrophic responses through S1PR in H9c2 cells. The results demonstrated that the representative hypertrophic marker, β -MHC, was upregulated by PE; however, expression was decreased by S1P and this protective effect was partially suppressed by siR2, but not by siR1 or siR3 (Fig. 6C-H). These findings suggested that S1PR2 may be involved in the antihypertrophic effects of S1P.

Suppressive effects of S1P on HDAC2 activity are independent of S1PR2. Since S1PR2 may be involved in the antihypertrophic effects of S1P, the present study aimed to determine whether inhibition of the effects of S1P on HDAC2 activity was S1PR2-dependent. HDAC2 was extracted from nuclear proteins by immunoprecipitation and its activity was detected following siR2 transfection. The results indicated that S1P significantly inhibited PE-induced upregulation of HDAC2 activity; however, siR2 did not suppress this effect (Fig. 7A). Furthermore, the downstream factor of HDAC2, KLF4, was investigated. The results demonstrated that siR2 did not affect the S1P-induced upregulation of KLF4 (Fig. 7B and C). These findings suggested that the suppressive effects of S1P on HDAC2 activity were independent of S1PR2.

Discussion

In the present study, a mouse model of TAC-induced cardiac hypertrophy was generated, as well as a PE-induced H9c2 cardiac hypertrophy cell model. Mice and cells were administered S1P in order to investigate its effects on the cardiac hypertrophic response and the underlying mechanisms. The results indicated that S1P could attenuate the progression of cardiac hypertrophy, and this may be mediated by inhibiting HDAC2 activity, and upregulating KLF4 independently of S1PRs. The results demonstrated that administration of S1P ameliorated TAC-induced cardiac function deterioration, and reduced the hypertrophic response in TAC-treated mice and PE-treated H9c2 cells. Furthermore, the expression levels and activity of HDAC2 were detected; the results suggested that S1P treatment did not affect HDAC2 expression but it did decrease its activity. In addition, the downstream antihypertrophic factor of HDAC2, KLF4, was examined and the results indicated that S1P upregulated KLF4, which may be associated with the suppression of HDAC2 activity. S1PR2 was revealed to be potentially involved in the anti-hypertro-

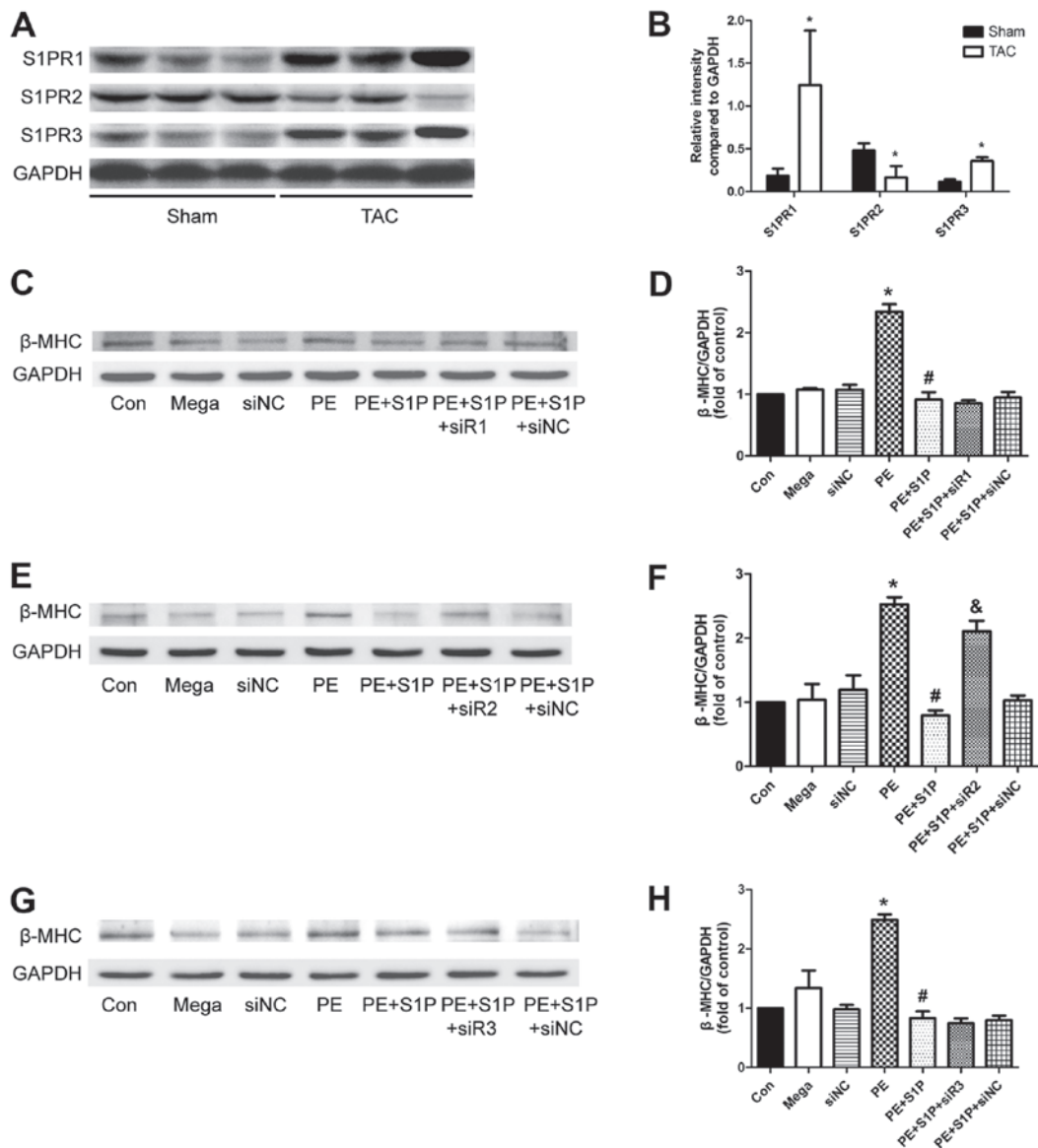


Figure 6. S1PR expression is altered following TAC; S1PR2 may be involved in the antihypertrophic effects of S1P. (A and B) Representative immunoblots and semi-quantification of S1PRs in the hearts of mice in the sham and TAC groups. Data are presented as the means \pm standard error of the mean ($n \geq 5$ for each group). * $P < 0.05$ vs. the Sham group. (C-H) Representative immunoblots and semi-quantification of β -MHC in H9c2 cells following various treatments. Data are presented as the means \pm standard error of the mean ($n \geq 3$ for each experiment). * $P < 0.05$ vs. Con group; # $P < 0.05$ vs. PE group; & $P < 0.05$ vs. PE + S1P group. β -MHC, β -myosin heavy chain; Con, control; PE, phenylephrine; S1P, sphingosine-1-phosphate; siNC, negative control siRNA; siR1-3, siRNA-S1P receptors 1-3; siRNA, small interfering RNA; TAC, transverse aortic constriction.

phic effects of S1P, whereas S1P functioned independently of it. These findings are summarized in the schematic diagram presented in Fig. 8.

Cardiac hypertrophy is a common pathological characteristic of numerous heart diseases and usually occurs in response to pressure overload, volume overload or myocardial infarction (19). S1P is a lipid mediator formed by metabolism of sphingomyelin, which regulates important functions in cardiac and vascular homeostasis (20). It can increase viability of cardiomyocytes incubated under hypoxic conditions and reduce infarct size in isolated, perfused rat hearts following ischemia/reperfusion (21,22). Furthermore, it participates in the regulation of vascular tone and permeability of vessels (12,23-25). The majority of these effects are mediated by the activation of S1PRs. It has previously been reported

that S1P has five specific GPCRs, S1PR1-S1PR5; however, only S1PR1, S1PR2 and S1PR3 are mainly expressed in the heart (12,26). Binding of S1P to each of these receptors activates distinct intracellular signals. A previous study indicated that S1PR signaling is important in heart development, and influences the migration, differentiation and survival of embryonic cardiomyocytes (27). In the present study, the expression levels of S1PRs were markedly altered in hypertrophic hearts; S1PR1 and S1PR3 were upregulated, whereas S1PR2 was downregulated, thus suggesting an association between S1PRs and cardiac hypertrophy.

Hait *et al* previously reported that S1P could function independently of S1PRs, through binding HDAC2 and inhibiting its enzymatic activity (11). HDACs are post-translational modifying enzymes that can remove acetyl functional groups

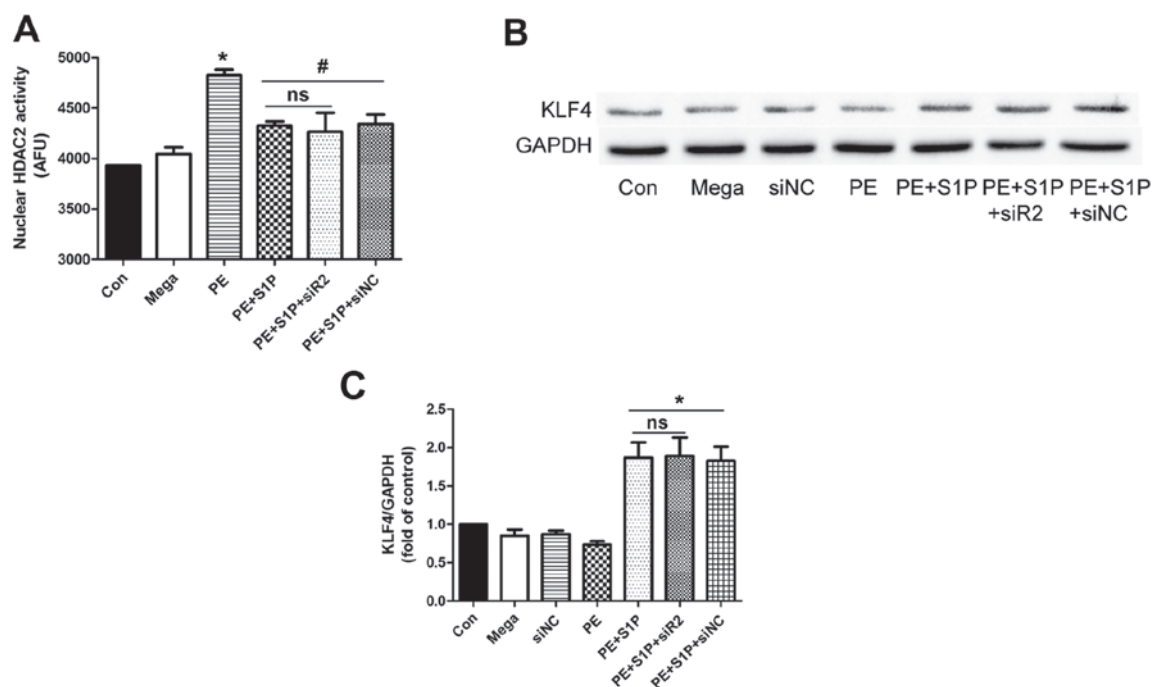


Figure 7. Suppressive effects of S1P on HDAC2 activity are independent of S1PR2. (A) Nuclear HDAC2 activity was determined in H9c2 cells following various treatments. (B and C) Representative immunoblots and semi-quantification of KLF4 expression in H9c2 cells following various treatments. Data are presented as the means \pm standard error of the mean ($n \geq 3$ for each experiment). * $P < 0.05$ vs. Con group; # $P < 0.05$ vs. PE group; ns, not significant. Con, control; HDAC2, histone deacetylase-2; KLF4, Krüppel-like factor 4; PE, phenylephrine; S1P, sphingosine-1-phosphate; siNC, negative control siRNA; siR2, siRNA-S1PR2; siRNA, small interfering RNA

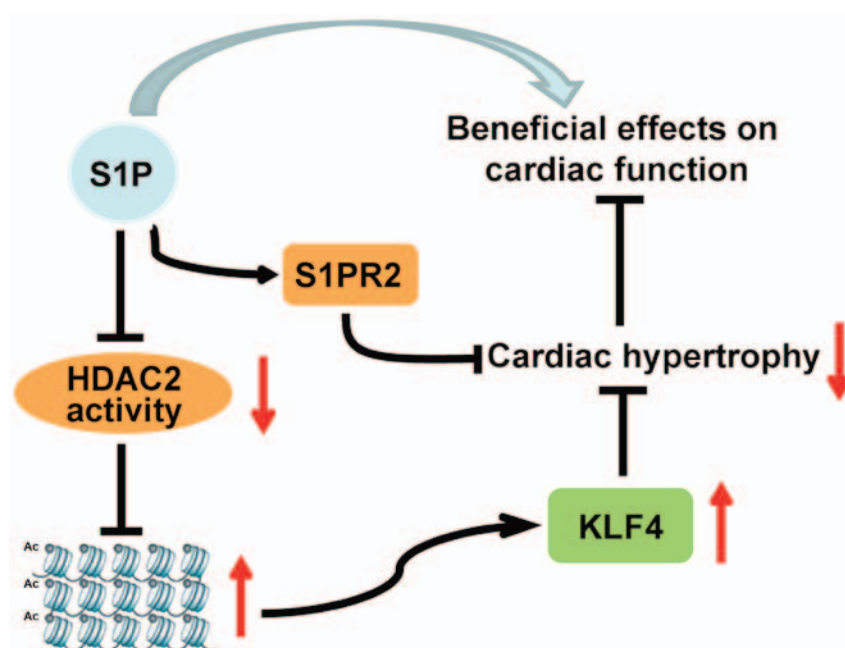


Figure 8. Model of the mechanisms underlying the ameliorative effects of S1P on cardiac hypertrophy. S1P treatment inhibited HDAC2 activity, resulting in increased histone acetylation and upregulation of KLF4, thus ameliorating cardiac hypertrophy. Cardiac hypertrophy is potentially also mediated by S1PR2. These effects may contribute to the improvement of cardiac function. HDAC2, histone deacetylase-2; KLF4, Krüppel-like factor 4; S1P, sphingosine-1-phosphate; S1PR2, S1P receptor 2.

from lysine residues of histone and nonhistone proteins (28). Previous studies regarding HDAC inhibitors have provided evidence to suggest that class I HDACs are prohypertrophic, among which HDAC2 is predominantly activated by hypertrophic stress; activated HDAC2 triggers hypertrophy through

inhibiting the signaling cascades of KLF4 (6,9). Therefore, a selective inhibitor of HDAC2 is considered an effective treatment for cardiac hypertrophy (7,8).

In the present study, the effects of S1P on cardiac hypertrophy were investigated. There are conflicting data regarding

the role of S1P in cardiac hypertrophy. A previous study performed in neonatal rat cardiomyocytes demonstrated that S1P did not induce hypertrophy, as determined by measuring ANP expression and phenylalanine incorporation, whereas the related sphingolipid, sphingosylphosphorylcholine, was able to induce hypertrophy (29). However, the underlying mechanism remained unclear. Data from another study indicated that S1P induced hypertrophy in neonatal rat cardiomyocytes, as assessed by cell size, cytoskeletal organization, phenylalanine incorporation and BNP expression; this hypertrophic response appeared to be mediated by S1PR1 (30). Nevertheless, S1P treatment has not previously been performed under hypertrophic conditions, and it should be recognized that S1P-induced cardiac hypertrophy is less robust and occurs more slowly than the canonical hypertrophic responses elicited by phenylephrine and endothelin (31).

Since Hait *et al* reported that S1P could inhibit HDAC2 activity, the effects of S1P have been studied on diseases associated with HDAC2. For example, a recent study demonstrated that S1P increased the ability of muscle cells to use fatty acids as an energy source in mice with Duchenne muscular dystrophy through inhibiting HDAC2 activity and increasing the expression of beneficial muscle genes (32). S1P has also been reported to protect the liver from lipid metabolism dysfunction in mice fed a high fat diet by decreasing HDAC2 activity and upregulating key genes encoding nuclear receptors/enzymes involved in nutrient metabolism (33). The present study demonstrated that S1P was able to prevent cardiac hypertrophy via the suppression of HDAC2 activity; this finding was in agreement with previous reports, which suggested that inhibition of HDAC2 may suppress cardiac hypertrophy (7,8).

KLF4 belongs to a large family of transcription factors named KLFs, which have common structures, including a transcriptional activation/repression domain and three Krüppel-like zinc fingers (34). Previous studies have indicated that KLF4 is a novel regulator of cardiac hypertrophy that is responsible for HDAC inhibitor-induced prevention of cardiac hypertrophy (9,35,36). It was previously revealed that KLF4 bound to the *Nppa* promoter region from -130 to ~-105 bp downregulates its expression and suppresses cardiac hypertrophy (9). The present study expanded on this established mechanism to determine how S1P regulates cardiac hypertrophy; the results demonstrated that the protective effects of S1P may be mediated by upregulating KLF4.

Notably, cardiac function in the TAC + S1P group was a little better than in the Sham group. We considered that cardiac function would be improved after one week of TAC when cardiac hypertrophy was adapted and compensatory and at that time we treated mice with S1P. Furthermore, the known beneficial effects of S1P on the cardiovascular system may be responsible for this phenomenon. H3K9-Ac and KLF4 were downregulated in mice in the TAC group compared with in the Sham group, whereas PE had no effects on H3K9-Ac and KLF4 expression in H9c2 cells. This may be due to the different *in vivo* and *in vitro* environments. In addition, their expression levels were originally low in control H9c2 cells. In cultured H9c2 cells, S1P upregulated H3K9-Ac and KLF4 expression; however, it had no effect on the expression of hypertrophic markers (ANP, BNP and β -MHC) compared with in the control group. The reason for this may be that the expression levels of hypertrophic markers were originally low under control condition.

In conclusion, the present study indicated that S1P attenuates cardiac hypertrophy by inhibiting HDAC2 activity, thus resulting in the upregulation of KLF4 in a S1PR2-independent manner. These findings may provide important information regarding the potential clinical applications to prevent cardiac hypertrophy. However, whether S1P directly binds to HDAC2 in cardiomyocytes and in mouse models, or whether there are other possible mechanisms underlying S1P-induced inactivation of HDAC2, requires further exploration in future studies, and the role of S1PR2 in hypertrophy should be confirmed in S1PR2-knockout mice.

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