Sphingosine 1 phosphate receptor-1 (S1PR1) signaling protects cardiac function by inhibiting cardiomyocyte autophagy

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Abstract

Objective To investigate the role of sphingosine-1-phosphate (S1P) and its receptors in cardiomyocyte autophagy, cardiomyocyte hypertrophy and cardiac function. Methods Cardiomyocytes were isolated from neonatal Vista rats. Autophagy and hypertrophy of cardiomyocytes were induced via starvation culture and phenylephrine (PE), respectively, and S1P was used to treat the cardiomyocytes. The effect of S1P on cardiomyocyte autophagy was evaluated by the number of autophagosomes, the expression of autophagy-related proteins and autophagic marker genes in cardiomyocytes. The effect of S1P on cardiomyocyte hypertrophy was evaluated by examining the surface area of cardiomyocytes and the expression of hypertrophic genes. Subsequently, different small interfering RNAs (siRNAs) were used to knockdown the expression of the three types of S1P receptors on cardiomyocytes and to analyze the type of receptor that mediates S1P signaling in cardiomyocytes. Finally, sphingosine 1 phosphate receptor-1 (S1PR1) was knocked out in the mouse cardiomyocytes using the Cas9 technique. The effect of S1PR1 on cardiac autophagy and cardiac hypertrophy was examined by assessing cardiomyocyte autophagy, cardiomyocyte hypertrophy and cardiac function. Results Starvation-induced cardiomyocyte autophagy and PE-induced cardiomyocyte hypertrophy were significantly attenuated by S1P. The results showed that the formation of autophagosomes was decreased, the autophagy-associated protein LC3 II / I and the expression of autophagic marker genes Atg5, Atg12, Beclin1 and LC3B decreased after S1P treatment. The surface area of the cardiomyocytes was decreased, and the expression of hypertrophic genes, including atrial natriuretic factor (ANF), skeletal muscle and cardiac actin (SKA), myosin heavy chain (β-MHC) and brain natriuretic peptide (BNP) were all decreased after S1P treatment. The autophagy and hypertrophy of cardiomyocytes in the S1PR1 knocked-down group were significantly increased compared to those in the control group, the S1PR2 and the S1PR3 knocked-down groups. In vivo, the knockout of S1PR1 in cardiomyocytes exacerbated stress-induced cardiac autophagy, cardiac hypertrophy and the impairment of cardiac function. Conclusion S1P could inhibit cardiomyocyte autophagy, thereby inhibiting cardiomyocyte hypertrophy and protecting cardiac function by activating S1PR1 in pressure-overloaded cardiomyocytes in mice.

Keywords: Autophagy; Cardiomyocyte; Hypertrophy; S1PR1

1 Introduction

Cardiac hypertrophy is a compensatory response of the heart to various mechanical or neurohormonal stimuli manifested by an increase in heart size and myocardial thickening. It is one common pathological feature of many cardiovascular diseases, and the extent of cardiac hypertrophy is an important predictor of progressive heart diseases and is negatively correlated with the patients’ outcomes. Pathological cardiac hypertrophy will eventually develop into heart failure and becomes an important cause of death.[1] There are a variety of mechanisms involved in the development of cardiac hypertrophy. In recent years, studies have shown that an abnormal increase in autophagy in cardiomyocytes is an important initiator of cardiac hypertrophy.[2–3]

Sphingosine 1 phosphate (S1P) is a sphingolipid metabolite that is produced by sphingosine phosphorylation via the ceramide/sphingomyelin metabolic pathway. It is not only an integral part of the eukaryotic membrane but is an important biological signal molecule.[4–5] S1P activates the downstream signaling pathways by coupling to its membrane protein receptors, the sphingosine 1 phosphate receptors (S1PRs), and regulates various biological processes such as cell motility, differentiation, survival, inflammation, angiogenesis, calcium homeostasis, and immunity.[6–7] S1P also plays an important protective role in the development
of cardiac hypertrophy. Clinically, there is a negative correlation between the plasma S1P concentration and the extent of left ventricular ejection fraction (LVEF) reduction, and S1P inhibits the pathogenesis of heart failure.[8] The mechanism by which S1P inhibits cardiac hypertrophy may be as follows: (1) S1P could inhibit the activation of histone deacetylase-2 (HDAC2);[9] (2) S1P could reduce the number of mononuclear phagocytes invading the myocardium;[10] and (3) S1P could down-regulate the β1-adrenoceptor.[11] In addition, S1P can inhibit a variety of cell autophagy by inhibiting the synthesis of phosphatidyethanolamine, inhibiting the transition of LC3-I to its active form LC3-II[12] and activating the extracellular signal-regulated kinase / mammalian target of rapamycin (ERK/mTOR) signaling pathway.[13–15]

It is unclear whether S1P can inhibit cardiomyocyte autophagy.[16] There are three types of S1PRs on the myocardium, namely, S1PR1, S1PR2 and S1PR3.[17] There is no consensus on the subtype of S1PR and the mechanism involved in the protection of the myocardium.[18] Therefore, it is very important to clarify the role of S1P in myocardial autophagy and cardiac hypertrophy, to identify the subtype of S1PR that contributes to its role and to elucidate the mechanism of its protective effect on the cardiomyocytes.

Our study shows that S1P can inhibit starvation-induced cardiomyocyte autophagy and phenylephrine (PE) -induced cardiomyocyte hypertrophy; the inhibitory effect of S1P on cardiomyocyte autophagy and hypertrophy was mainly mediated by S1PR1. We established a mouse model of S1PR1 knockout in cardiomyocytes and found that the knockout of S1PR1 in cardiomyocytes aggravates cardiomyocyte autophagy, cardiomyocyte hypertrophy, and the decrease in cardiac function induced by the pressure overload, which further confirmed the inhibitory effect of S1PR1 in cardiac autophagy and cardiac hypertrophy.

2 Methods

2.1 Isolation, culture, and treatment of cardiomyocytes

Neonatal ventricular myocytes were isolated from 2-day-old Vista rats, cultured, and treated as described.[19] Briefly, a central thoracotomy was performed after the neonatal rats were deeply anesthetized with 1.0% isoflurane. The hearts of the rats were washed and minced in sodium bicarbonate-, Ca²⁺-, and Mg²⁺-free Hanks balanced salt solution (D-Hanks). Tissues were then dispersed in a series of incubations at 37 °C in D-Hanks buffered solution containing 1.2 mg/mL pancreatin and 0.14 mg/mL collagenase (Worthington, USA). The cells were centrifuged and then suspended in Dulbecco's modified Eagle medium/F-12 (GIBCO, USA) containing 10% heat-inactivated fetal bovine serum, 100 U/mL of penicillin, 100 mg/mL of streptomycin, and 0.1 mmol/L of bromodeoxyuridine. The dissociated cells were pre-plated at 37 °C for 1 hour to separate the cardiomyocytes via the adherence of cardiac fibroblasts. The cardiomyocytes were collected and diluted to 1 × 10⁶ cells/mL and plated in 1% gelatin-coated different culture dishes. Neonatal cardiomyocytes were incubated at 37°C and 5% CO₂ in a humidified chamber. After 48 hours of culture, the various treatments were performed.

2.2 Small interfering RNA (siRNA), Ad-GFP-LC3B and the luciferase assay

siRNA targeting S1PR1, S1PR2 and S1PR3 (siS1PR1, 5'-GCUGCUUGAUCUCCUAGTT-3' and 5'-UCUAG GAUGAUAAGCAGCTT-3'; siS1PR2, 5'-CCUGUCAC CCGUGCUCAAATT-3' and 5'-UUAGGACGCCAAGGU GCAGGTT-3'; and siS1PR3, 5'-CCUGUAGCUUCAUCG UCUUTT-3’ and 5'-AAGACGAGUAAGCAGTACAGGTT-3') and negative control siRNA (siNC) were synthesized. Ad-GFP-LC3B (adenovirus-expressing GFP-LC3B fusion protein) was purchased from Beijing BioLabo Technology Co., Ltd. and used according to the protocol. Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) was mixed with siRNA or Ad-GFP-LC3B solution in serum-free medium for 20–30 min before adding to the cells.

2.3 Cell immunostaining, photo capture and image analysis

Cardiomyocytes were fixed with 4% formaldehyde for 30 min at 4 °C and then treated with 0.5% Triton-X 100 in phosphate buffer saline (PBS) for 5 min at room temperature. The cells were then incubated with primary antibody against α-actinin (Sigma, USA, Lot No. A7811) at 4 °C overnight, followed by incubation with fluorescence-conjugated secondary antibody. Photo capture was performed using a Nikon laser microscope (Eclipse E600, Nikon Instruments Inc., Japan). For each sample, more than five fields covering the whole slide were chosen and counted.[20] The image analysis was performed using Imagepro Plus software.

2.4 Western blot

Western blots were performed on myocardial extracts as described.[20] Proteins (30 µg) were electrophoresed on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto poly vinylidene fluoride (PVDF) membranes. Immunoblotting was performed according to the manufacturer's instructions using the following antibodies: P-62, phospho protein kinase B (P-AKT),
total protein kinase B (T-AKT), phospho ERK (P-ERK), total ERK (T-ERK), phospho mTOR (P-mTor), and LC3B (Cell Signaling, Beverly, MA, USA), as well as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz, Dallas, TX, USA). The analysis of densitometry of the bands was performed using Gel-Pro Application software.

### 2.5 Real-time Polymerase Chain Reaction (PCR)

Total RNA was isolated from heart tissues and cardiomyocytes using TRIzol Reagent (Invitrogen, USA). cDNA was synthesized using SuperRT One Step RT–PCR Kit (CWBio, China) and subjected to real-time PCR using SYBR Green Real-time PCR Master Mix (TOYOBO, Japan) and subjected to real-time PCR using Applied Biosystems, USA). GAPDH was used as a reference gene. The following primers were used: GAPDH, 5′-TGCCCCAGAC ATCATCCT-3′ and 5′-GGTCTCTAGTGTAGCCCAAG-3′; atrial natriuretic factor (ANF), 5′-GGCGGTAAGAGAT GAGGCTCA-3′ and 5′-GGGCCAATCTGTGCAAATC-3′; skeletal muscle and cardiac actin (SKA), 5′-GGTCTCCAG CACCATGAAGA-3′ and 5′-CAGCAAGAGGATCGTATGTT-3′; myosin heavy chain (β-MHC), 5′-GTCAGAAGGGC ATGAGGAAGAGT-3′ and 5′-AGGCTTCACCTCTAGC TG-3′; brain natriuretic peptide (BNP), 5′-GCTTCTGAA GGCCAAGGCCCTGCAC-3′ and 5′-GATCCGATCCGCTG TCT-3′; total protein kinase B (T-AKT), phospho ERK (P-ERK), total mTOR (T-mTOR), and LC3B (Cell Signaling, Beverly, MA, USA).

### 2.6 Transverse aortic constriction (TAC) surgery and the sacrifice of mice

TAC surgeries were performed in 8-week-old male ROSA26-LSP-CAS9-EGFP mice. The mice were anesthetized with 2.0% isoflurane (v/v) and an oxygen flow rate of 45 mL/min. After thoracotomy, the transverse thoracic aorta was dissected, and a 6–0 silk suture was tied around the aorta against a 26-gauge needle. The sham groups underwent an operation involving thoracotomy and aortic dissection without constriction of the aorta. The mice were sacrificed by cervical dislocation, and the hearts were harvested for analysis. The ROSA26-LSP-CAS9-EGFP mice used in the study were provided by the Institute of Biotechnology, Academy of Military Medical Sciences, Beijing, China. The experiments were performed according to the protocols approved by the Animal Experiment Committee of the Chinese People’s Liberation Army General Hospital (No. S2016-072-02) and conformed to the US National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### 2.7 Cas9-mediated generation of S1PR1 knockout mice

Two single-guide RNAs (sgRNA) were designed based on the sgRNA targets of the mouse S1PR1 exons. The sequences were sgRNA1 (AAGGACGAAACACCGGTGT CCACGTAGCATCCCGGGTTTCTAGCTAGAA) and sgRNA2 (AAGGACGAAACACCGGTGAACATCGGGCG GAGAGTTTTAGAGCTAGAA). The double-stranded oligonucleotide chains of sgRNA1 and sgRNA2 were synthesized by Gene Pharma (Suzhou, China). The vector that expresses S1PR1-sgRNA was constructed. After sequenced, it was ligated into the adenoviral vector, and the adenovirus was amplified. TAC surgeries were performed on male ROSA26-LSP-CAS9-EGFP mice (8-weeks-old) as described. Treatments started 1 week after TAC, and the mice received 0.2 mL of adenovirus (6 injections, 30 mg per kg of body weight, and 3 injections per week) via tail vein injections.

### 2.8 Transthoracic echocardiography measurement

Cardiac function was assessed by echocardiography using the Vevo 770TM Imaging System (Visual Sonics Inc., Toronto, Canada) equipped with a 30-megahertz (MHz) microprobe. Animals were anesthetized with 1.5% isoflurane allowing spontaneous breathing.[21]

### 2.9 Histology and staining

Heart tissues were fixed in 4% Paraformaldehyde (PFA) at 4 °C overnight, embedded in paraffin, and cut into 5-μm sections. Sections were stained with hematoxylin and eosin (H&E) and Masson trichrome staining.

### 2.10 Statistical analysis

All statistical analyses were performed using SPSS software. The results are expressed as the means ± SEM. Statistical differences between two groups were determined by Student’s t-test, and statistical differences among more than two groups were determined by ANOVA followed by SNK-q method. P-values of < 0.05 were considered significant.

### 3 Results

#### 3.1 S1P inhibits starvation-induced cardiomyocyte autophagy

Wild-type neonatal Vista rat cardiomyocytes were iso-
lated and cultured for 48 hours. Then they were transferred into normal or starvation conditions (serum-free and sugar-free medium to induce cardiomyocyte autophagy). S1P of 100 nmol/L final concentration or blank control treatment was administered concurrently. The cardiomyocytes were cultured for another 48 hours and then transfected with or without Ad-GFP-LC3B. The myocardial cells were fixed and observed using confocal fluorescence microscopy, 24 hours after transfection with Ad-GFP-LC3B (Figure 1-A). The results show that starvation could effectively induce autophagy of the cardiomyocytes, and S1P could significantly decrease the number of autophagosomes in the starvation-cultured cardiomyocytes (Figure 1-B). Cells that were not transfected with Ad-GFP-LC3B were also harvested at the 120th hour, and RNA and protein were extracted. Real-time PCR showed that the expression of important autophagic marker genes Atg5, Atg12, Beclin1 and LC3B were significantly lower in the S1P-treated group than that in the control group (Figure 1-C). LC3 is an important protein of autophagy. During autophagy, the cyto-

Figure 1. S1P inhibited starvation-induced cardiomyocyte autophagy. (A): The strategy of S1P treat. (B): Representative images and quantification of GFP-LC3B puncta (green) in control and S1P treated cardiomyocytes under normal or starved conditions (n = 30 cells/per condition; n = 4 independent experiments). The scale bar represents 50 μm; P < 0.05 relative to the Control group. Cardiomyocytes were stained for α-actinin (red) and Hoechst (blue). S1P significantly decreased the number of autophagosomes in starvation-cultured cardiomyocytes. (C): Real-time PCR shows that the expression of important autophagic marker genes Atg5, Atg12, Beclin1 and LC3B was significantly lower in the S1P-treated group vs. that in the Control group; n = 4 independent experiments; P < 0.05 relative to the Control group. (D): Western blotting shows that LC3 II / I was significantly decreased and P62 was significantly increased after S1P treatment in starvation-cultured cardiomyocytes. The phosphorylation of ERK1/2 and mTOR was significantly increased after S1P treatment in starvation-cultured cardiomyocytes. Quantification of the results is shown down. n = 4 independent experiments; P < 0.05 relative to the Control group. The extracellular S1P concentration was 100 nmol/L in the experiment. Ad-GFP-LC3B: adenovirus-expressing GFP-LC3B fusion protein; KD: Kilo Dalton; P-Akt: phosphorylated Akt; PCR: polymerase chain reaction; P-ERK: phosphorylated ERK; P-mTOR: phosphorylated mTOR; S1P: sphingosine 1 phosphate; T-Akt: total Akt; T-ERK: total ERK.

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plasmic LC3 (i.e., LC3-I) is enzymatically cleaved off a small segment of polypeptide and converted to a (autophagosomes) membrane type (i.e., LC3-II). Therefore, the LC3-II/I ratio is widely used as a measure of cellular autophagy. As a substrate for autophagy, the level of P62 is negatively correlated with the degree of autophagy and is often used for the detection of autophagy. LC3-II/I and P62 were detected using the Western blot method, and the results showed that LC3 II / I was significantly decreased and that P62 was significantly increased after S1P treatment in starvation-cultured cardiomyocytes. The ERK1/2-mTOR pathway is an important cell signaling pathway that inhibits autophagy. We found that the phosphorylation of ERK1/2 and mTOR was significantly increased after S1P treatment in the starvation-cultured cardiomyocytes. This indicates that S1P can inhibit starvation-induced myocardial autophagy by activating the ERK1/2-mTOR signaling pathway (Figure 1D).

3.2 S1P inhibits PE-induced hypertrophy of cardiomyocytes

After isolated and cultured for 48 hours, the cardiomyocytes were transferred into normal medium or medium with 100 μmol/L PE. S1P of 100 nmol/L final concentration or blank control was added to the medium concurrently to investigate the effect of S1P on cardiomyocyte morphology. 72 hours later, the cells were immunostained with α-actinin (green) and Hoechst (blue), observed and photographed under a microscope, and the size of the cardiomyocytes was determined using Imagepro Plus software (Figure 2A). The

Figure 2. S1P inhibited PE-induced hypertrophy of cardiomyocytes. (A): The strategy of S1P treat. (B): Representative images and quantification of the cell surface area of α-actinin-immunostained cardiomyocytes (n = 250 cells per condition; n = 4 independent experiments). The scale bar represents 50 μm. *P < 0.05 relative to the Control group. Cardiomyocytes were stained for α-actinin (green) and Hoechst (blue). S1P could significantly inhibit PE-induced hypertrophy of the cardiomyocytes. (C): ANF, SKA, β-MHC, and BNP transcripts in different groups were assessed using real-time PCR; n = 5 independent experiments; *P < 0.05 relative to the Control group. S1P could significantly reduce the expression of these genes in PE-treated cardiomyocytes. The extracellular S1P concentration was 100 nmol/L and PE concentration was 100 μmol/L in the experiment. ANF: atrial natriuretic factor; BNP: brain natriuretic peptide; PCR: polymerase chain reaction; PE: phenylephrine; S1P: sphingosine 1 phosphate; SKA: skeletal muscle and cardiac actin; β-MHC: myosin heavy chain.
results showed that PE could effectively stimulate hypertrophy of cardiomyocytes, and S1P could significantly inhibit PE-induced hypertrophy of cardiomyocytes (Figure 2B). Cardiomyocyte hypertrophy often accompanies the reactivation of embryonic genes. We examined the expression of four embryonic genes including ANF, SKA, β-MHC, BNP. The results showed that S1P significantly reduced the expression of these genes in PE-treated cardiomyocytes (Figure 2C).

### 3.3 The inhibitory effect of S1P on cardiomyocyte autophagy and hypertrophy was mainly mediated by S1PR1.

It has been previously reported that the expression of the three types of S1P receptors on cardiomyocytes is as follows: S1PR1 >> S1PR3 > S1PR2.[22] We confirmed this conclusion using real-time PCR and Western blot analysis of the expression of S1PR1, S1PR2 and S1PR3 in ventricular myocytes (Figure 3A). Subsequently, we designed siRNAs respectively targeting S1PR1, S1PR2, and S1PR3 of cardiomyocytes and successfully knocked down the expression of these 3 receptors in cardiomyocytes (Figure 3B). Subsequently, starvation-induced autophagy and PE-induced hypertrophy were performed on the S1PRs knocked-down cardiomyocytes. We found that the autophagy and hypertrophy of the cardiomyocytes was significantly increased in the S1PR1 knocked-down group compared to that in the control group, however, there was no significant change in S1PR2 and S1PR3 knocked-down groups (Figure 4 and Figure 5).

### 3.4 In vivo, the knockout of S1PR1 in cardiomyocytes aggravates stress-induced cardiac autophagy, hypertrophy and cardiac function decrease.

The adenovirus carrying the S1PR1-sgRNA sequence was injected into the tail vein of ROSA26-LSP-CAS9-EGFP mice at 1 week after TAC surgery. After 2 weeks of injections, the cardiac proteins, genes, morphology and function of the mice were analyzed (Figure 6A). The results showed that the expression of S1PR1 in mouse cardiomyocytes was successfully knocked down (Figure 6). Compared with the control group, stress-induced cardiac autophagy was aggravated in the S1PR1 knockout mice as evidenced by a significant increase in the expression of LC II/I and the autophagic marker genes and a significant decrease in the expression of P62, as shown in Figure 7. Hypertrophy as evidenced by a significant increase in the size of the heart, the size of the individual cardiomyocytes, and the left ven-
Figure 4. The inhibitory effect of S1P on starvation-induced cardiomyocyte autophagy was mainly mediated by S1PR1. (A): The strategy of siRNA and S1P treat. (B): Representative images and quantification of GFP-LC3B puncta (green) in control and S1PRs knocked-down cardiomyocytes under starvation conditions (n = 30 cells/per condition; n = 4 independent experiments) are shown. The scale bar represents 50 μm; *P < 0.05 relative to the other groups. Cardiomyocytes were stained for α-actinin (red) and Hoechst (blue). The number of autophagosomes in the S1PR1 knocked-down group was significantly increased compared to that in the control group. (C): Western blotting shows LC3 / was significantly increased, and P62 was significantly decreased in the S1PR1 knocked-down cardiomyocytes under starvation-cultured conditions. Quantification of the results is shown down. n = 4 independent experiments. *P < 0.05 relative to the other groups. The extracellular S1P concentration was 100 nmol/L in the experiment. GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GFP-LC3B: green fluorescent protein GFP-LC3B fusion protein; KD: Kilo Dalton; S1P: sphingosine 1 phosphate; S1PR: sphingosine 1-phosphate receptor; siRNA: small interfering RNA.

tricular mass/body weight, significant thickening of the left ventricle, and a significant increase in the expression of ANF, BNP, β-MHC and SKA, as shown in Figure 8, was also aggravated in the S1PR1 knockout mice as well as the decrease in cardiac function (Figure 9).

4 Discussion

As an important biological signal molecule, S1P plays an important protective role in the progression of cardiac hypertrophy and heart failure, but there are still many mechanisms that remain unclear. In this study, we investigated the role of S1P and its receptors in the development of myocardial hypertrophy. Our study shows the following: (1) S1P can inhibit cardiomyocyte autophagy induced by starvation; (2) S1P can inhibit cardiomyocyte hypertrophy induced by PE; (3) the inhibitory effect of S1P on myocardial cell autophagy and myocardial hypertrophy is mainly mediated by S1PR1; and (4) in vivo, the knockout of S1PR1 in cardiomyocytes could aggravate cardiac autophagy, myocardial hypertrophy and cardiac dysfunction induced by pressure overload.

The inhibitory effect of S1P on cardiac myocyte hypertrophy has been reported previously, and our study confirmed the previous conclusion. Autophagy, also known as type II cell death, is the process of the degradation of damaged organelles and macromolecules by lysosomes under the regulation of autophagy-related gene. It participates in the regulation of the metabolic balance of the synthesis, degradation and reutilization of cell substances, which affects all aspects of biological processes.[23] S1P inhibits the autophagy of a variety of cells, but it is not clear whether it could inhibit the autophagy of cardiomyocytes. The inhibitory effect of S1P on the autophagy of cardiomyocytes was
Figure 5. The inhibitory effect of S1P on PE-induced cardiomyocyte hypertrophy was mainly mediated by S1PR1. (A): The strategy of siRNA and S1P treat. (B): Representative images and quantification of the cell surface area of α-actinin (green)- and Hoechst (blue)-immunostained cardiomyocytes (n = 250 cells per condition; n = 4 independent experiments). The scale bar represents 50 μm; *P < 0.05 relative to the other groups. The cell surface area of the siRNA-S1PR1 group was significantly larger than that of the other groups. (C): ANF, SKA, β-MHC, and BNP transcripts in different groups were assessed using real-time PCR; n = 5 independent experiments; *P < 0.05 relative to the other groups. The expression of ANF, SKA, β-MHC and BNP was significantly increased in the S1PR1 knocked-down group compared to that in the other groups. The extracellular S1P concentration was 100 nmol/L and PE concentration was 100 μmol/L in the experiment. ANF: atrial natriuretic factor; BNP: brain natriuretic peptide; PCR: polymerase chain reaction; PE: phenylephrine; S1P: sphingosine 1 phosphate; SKA: skeletal muscle and cardiac actin; siRNA-S1PR: small interfering RNA targeting S1P receptor; β-MHC: myosin heavy chain.

Figure 6. S1PR1 expression was successfully knocked down in mouse cardiomyocytes. (A): The strategy of TAC surgery and Ad-S1PR1 injection. (B): Real-time PCR shows that the expression of S1PR1 RNA was significantly decreased in the S1PR1 knockout cardiomyocytes; n = 5 independent experiments; *P < 0.05 relative to the Control group. (C): Western blotting shows that S1PR1 was significantly decreased in the S1PR knockout cardiomyocytes. Quantification of the results is shown down; n = 4 independent experiments; *P < 0.05 relative to the Control group. Ad-S1PR1: adenovirus carrying the S1PR1-sgRNA sequence; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; KD: Kilo Dalton; PCR: polymerase chain reaction; RNA: ribonucleic acid; S1P: sphingosine 1 phosphate; TAC: transverse aortic constriction.
Figure 7. Knockout of S1PR1 exacerbates cardiomyocyte autophagy in the TAC mouse model. (A): Western blotting shows that LC3 II/1 was significantly increased and P62 was significantly decreased in the cardiomyocytes of S1PR1 knockout mice. Quantification of the results is shown right; n = 4 independent experiments. *P < 0.05 relative to Control group. (B): Real-time PCR shows that the expression of important autophagic marker genes Atg5, Atg12, Beclin1 and LC3B was significantly increased in the cardiomyocytes of S1PR1 knockout mice; n = 4 independent experiments; *P < 0.05 relative to Control group. Ad: adenovirus carrying the S1PR1-sgRNA sequence; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; KD: Kilo Dalton; TAC: transverse aortic constriction.

Figure 8. Knockout of S1PR1 exacerbates cardiac hypertrophy in the TAC mouse model. (A): The gross morphology of the hearts from different groups (The scale bar represents 5 mm) and the histological analysis of the hearts using H&E (the scale bar represents 2 mm) and Masson staining (The scale bar represents 50 μm) are shown. n = 4 independent experiments. (B): Measurements of the left ventricular mass/body weight (LVM/BW, mg/g) in the different groups is shown. Data are expressed as the means ± SEM; n = 4 per condition; *P < 0.05 vs. the TAC + Ad group and *P < 0.05 vs. the Sham group. (C): Cross-sectional areas were analyzed after Masson immunostaining (n = 400 cells per condition; n=4 independent experiments). *P < 0.05 vs. the TAC + Ad group and *P < 0.05 vs. the Sham group. (D): Analysis of the transcripts for ANF, BNP, β-MHC, and SKA using real-time PCR is shown. Data are expressed as the means ± SEM; n = 4 per condition; *P < 0.05 vs. the TAC + Ad group and *P < 0.05 vs. the Sham group. Ad: adenovirus carrying the S1PR1-sgRNA sequence; ANF: atrial natriuretic factor; BNP: brain natriuretic peptide; H&E: hematoxylin and eosin; LVB/BM: left ventricular mass/body weight; S1PR1: Sphingosine 1 phosphate receptor-1; SKA: skeletal muscle and cardiac actin; TAC: transverse aortic constriction; β-MHC: myosin heavy chain.
Figure 9. Cardiac function was significantly reduced in the S1PR1 knockout mice. (A): Representative M-mode images of the S1PR1 knockout mice and littermate controls are shown. (B-E): Measurements of the left ventricular posterior and anterior wall thickness in diastole (LVPWd and LVAWd) and in systole (LVPWs and LVAWs) are shown. (F-G): Measurements of the left ventricular volume in diastole (LVVd) and in systole (LVVs) are shown. (H-I): The quantifications of the ejection fraction (EF) and fractional shortening (FS) are shown. The mean values ± SEM were determined by echocardiography. Sham mice: n = 8; Sham + S1PR1 knockout mice: n = 9; TAC mice: n = 7; TAC + S1PR1 knockout mice: n = 8; * P < 0.05 vs. the TAC + Control mice and * P < 0.05 vs. the Sham + Control mice. Ad: adenovirus carrying the S1PR1-sgRNA sequence; LVAWd: left ventricular anterior wall thickness in diastole; LVAWs: left ventricular anterior wall thickness in systole; LVPWd: left ventricular posterior wall thickness in diastole; LVPWs: left ventricular posterior wall thickness in systole; LVVd: left ventricular volume in diastole; LVVs: left ventricular volume in systole; S1PR1: Sphingosine 1 phosphate receptor-1; TAC: transverse aortic constriction.

identified in our study. There are several signaling pathways that regulate cell autophagy, including AMPK-mTOR, PI3K-Akt-mTOR, p53-mTOR and ERK1/2-mTOR signaling pathways, and our research confirms that the inhibitory effect of S1P on autophagy in cardiomyocytes is mainly achieved by activating the ERK1/2-mTOR signaling pathway. The activation of the ERK1/2-mTOR signaling pathway can inhibit the expression of autophagy-related genes and inhibit the occurrence of autophagy.[24] This finding provides a research target for further research on the relationship of autophagy and hypertrophy of cardiomyocytes.

There are 3 types of S1P receptors on the myocardium, namely, S1PR1, S1PR2 and S1PR3, and the subtype of S1P receptor that mediates the protective effect of cardiomyocytes is not clear.[22–25] Our study shows that S1PR1 mediates the inhibitory effect of S1P on cardiomyocyte autophagy and hypertrophy. The simultaneous increase in cardiomyocyte autophagy and hypertrophy after S1PR1 knockdown suggests that cardiomyocyte autophagy may be the precursor to hypertrophy. The mechanism of cardiomyocyte autophagy leading to cell hypertrophy is not yet clear.[26–27] We speculate that myocardial autophagy can promote the degradation and circulation of proteins, lipids, and damaged organelles and therefore provide a source of substrates for the hypertrophic growth of cardiomyocytes.[28]

S1PR1 plays a very important role in the development of the mouse heart by mediating S1P signaling. Clay H, et al.[29] reported that the S1PR1 knockout in fertilized eggs could lead to embryonic death in animal models. In this study, S1PR1 expression was knocked out in ROSA26-LSP-CAS9-EGFP mice cardiomyocytes using CAS9 technology.[30-31] We found that the knockout of S1PR1 in car-
cardiomyocytes significantly aggravated stress-induced cardiac function decrease, which was consistent with the previous study by Cannavo A, which reported that S1PR1 protects cardiac function.\textsuperscript{[11]} The activation of S1PR1 could help maintain cardiac ejection function by inhibiting myocardial fibrosis\textsuperscript{[32]} promoting cyclic Adenosine monophosphate (cAMP) accumulation in myocardial cells and improving myocardial contractility.\textsuperscript{[17]} In addition, an abnormal increase in autophagy in cardiomyocytes in the pressure-overloaded mice may be an important initiator of cardiac structural and functional injury. First, excessive autophagy leads to non-selective degradation of proteins and organelles, which results in functional defects in cardiomyocytes and ultimately heart failure.\textsuperscript{[33]} Second, cardiomyocyte autophagy can aggravate pathological remodeling of the myocardium, resulting in decreased cardiac contractility and cardiac dysfunction.\textsuperscript{[34]} At the same time, increased autophagy leads to excessive myocardial cell apoptosis, thereby reducing the number of myocardial cells and myocardial contractility.\textsuperscript{[35]} The timely and effective inhibition of cardiac autophagy is expected to offer a new treatment to reduce myocardial damage, thereby protecting heart function.\textsuperscript{[36–37]} Our study provides a prospective site for the regulation of autophagy in cardiomyocytes.

In conclusion, in this study, we explored the protective effects of S1P and S1PR on the heart and found that S1P could inhibit cardiomyocyte autophagy and hypertrophy by activating S1PR1, and the S1PR1 signaling pathway could help maintain the cardiac ejection function in pressure-overloaded mice. It is expected that the application of S1P and its analogs or the regulation of S1PR1 expression could inhibit myocardial autophagy and delay the development of cardiac hypertrophy and may become a new treatment for delaying the progress and improving the outcome of heart failure.

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