TGF-β/Smad3 pathway enhances the cardio-protection of S1R/SIPR1 in *in vitro* ischemia-reperfusion myocardial cell model

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Abstract. Ischemia-reperfusion (IR) injury is usually associated with a high risk of cardiomyocyte death in patients with acute myocardial infarction. Sphingosine 1-phosphate (S1P) and transforming growth factor (TGF)-β are thought to be involved in the protection of cardiomyocyte and heart function following IR-induced injury. However, the possible association of S1P and S1P receptor 1 (S1PR1) with the TGF-β/Smad3 pathway as the potential protective mechanism has remained to be investigated. In the present study, an *in vitro* ischemia/reperfusion injury model was established and evaluated by analysis of apoptosis, lactate dehydrogenase (LDH) release and caspase3 activity. The mRNA and protein levels of S1PR1, TGF-β and Smad3 after treatment with 1 μM S1P alone or combined with 0.4 μM W146 (a specific S1PR1 antagonist) were assessed. The mRNA expression of five S1PRs (S1PR1-5) and the protein levels of S1PR1 were also assayed following treatment with 1 ng/ml TGF-β for 0, 4 or 24 h. The mRNA expression of S1PR1 and the levels of S1P were further assessed following exposure to 10 μM SB4 (TGFβR1 inhibitor) plus 1 ng/ml TGF-β and 2 μM SIS3 (Smad3 inhibitor) plus 1 ng/ml TGF-β. The results indicated that apoptosis, LDH release and caspase3 activity were all increased in the established IR model. Exogenous S1P increased the mRNA and protein levels of S1PR1, TGF-β and Smad3, which was abolished by addition of W146. Extraneous TGF-β resulted in the stimulation of several S1PRs, most prominently of S1PR1, while supplementation with SB4 and SIS3 offset the stimulation by TGF-β. These results suggested that the TGF-β/Smad3 pathway was closely associated with S1P/S1PR1 in the protection of myocardial cells from IR injury.

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Introduction

Acute myocardial infarction (AMI) is one of the most common cardiovascular diseases and attributed to occlusion of the epicardial coronary artery, ultimately leading to progressive chronic heart failure. The therapeutic approaches available for AMI injury and myocardial infarction are timely myocardial reperfusion using either thrombolytic therapy or primary percutaneous coronary intervention (1). However, reperfusion to rescue ischemic myocardium may bring a high risk of cardiomyocyte death, namely myocardial ischemia-reperfusion (IR) injury, which includes the following forms: Reperfusion-induced arrhythmias, myocardial stunning, microvascular obstruction and lethal myocardial reperfusion injury (2-7).

Sphingosine 1-phosphate (S1P) is a lysophospholipid mediating a series of cell functions, including cell motility, cell proliferation and differentiation, immune system surveillance, vascular permeability, cytoskeletal organization and viral infections (8). S1P receptors (S1PRs) are a group of G-protein coupled receptors responsible for S1P functions that are referred to as S1PR1-5. However, the five S1PRs differ in their distribution. S1PR1, S1PR2 and S1PR3 are widely expressed, whereas S1PR4 and S1PR5 mainly exist in the immune and nervous systems (9-11). S1P has been reported to have a critical role in the protection of cardiomyocytes and heart function from IR injury *in vitro* and *in vivo* (12). For instance, high-density lipoprotein and its lipid component S1P are known to attenuate IR injury (13). S1P has been demonstrated to protect cardiomyocytes of neonatal rats and the heart from ischemic damage in perfused rabbits and mice (14-16). Furthermore, the S1P/S1PR1 pathway was reported to induce hypertrophy of cardiomyocytes and reduce mortality of hypoxic cardiomyocytes *in vitro* (17,18).

TGF-β is a group of structurally associated proteins regulating a number of critical cellular processes, including apoptosis, tumor occurrence and IR (19-21). The biological functions of TGF-β are initiated by binding to two types of transmembrane receptor: TGF-β receptor type I (TGFβR1) and TGFβR2. Activation of TGFBR2 leads to phosphorylation of TGFBR1, which triggers activation of Smad3 and forces Smad3 to translocate into the nucleus (22,23). In a previous study, Vivar *et al* (24) revealed that TGF-β blocked the IR-induced

apoptosis of cardiac fibroblasts through the Smad3, extracellular signal-regulated kinase (ERK)1/2 and Akt signaling pathways. Furthermore, TGF-β participated in the cross-talk of ERK1/2 and Akt with the Smad2/3 signaling pathway; however, these signaling pathways appear to have independent roles.

Although S1P/S1PR1 and TGF-β/Smad3 were all demonstrated to be actively implicated in IR injury of myocardial cells, their association has remained to be fully elucidated. The present study reported that exogenous TGF-β significantly increased the levels of S1PR1 compared with those of S1PR2-5. In addition, the results suggested that TGF-β/Smad3 contributed to the cardioprotective effect of S1P/S1PR1 in an established *in vitro* IR model.

Materials and methods

Reagents. All treatments were administered at a concentration of 10 mM for 48 h of treatment at 37˚C. S1P, W146, SB-431542 (SB4), SIS3 and TGF-β were all purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The primary anti-S1PR1, anti-TGF-β and anti-Smad3 antibodies (Cat. no. ab72806, ab31013, ab40854, respectively) were acquired from Abcam (Cambridge, UK). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were from Hyclone (GE Healthcare, Little Chalfont, UK). The secondary antibodies conjugated to horseradish peroxidase (Cat. no. ab6789) were purchased from Zhongshan Goldenbridge Bio (Beijing, China). The enhanced chemiluminescent (ECL) kit was obtained from Thermo Fisher Scientific (Shanghai, China). For Reverse-transcription quantitative polymerase chain reaction (RT-qPCR), the MagExtractor-RNA kit and ReverTra Ace qPCR RT Master Mix with gDNA Remover kit were all from Toyobo (Cat. no. FSQ-301, Tokyo, Japan). The lactate dehydrogenase (LDH) detection activity assay kit and the commercialized caspase-3 assay kit were purchased from Sigma-Aldrich (cat. no. CASP3F-1KT, Merck KGaA) and Biovision, Inc. (cat. no. 1533-100, Milpitas, CA, USA), respectively.

Animals. Neonatal mice were purchased from SLRC Laboratory Animal (Changsha, China). Animals were provided with standard rodent chow and water *ad libitum*. All animal procedures were approved by the Institutional Animal Care and Use Committee of Jining No.1 People's Hospital (Jining, China). All protocols conformed to the National Research Council's Guide for the Care and Use of Laboratory Animals.

Isolation and cultivation of cardiomyocytes. Animals were anesthetized with ether to remove the hearts, which were put in pre-cooled D-hanks medium (Procell, Wuhan, China). The heart tissues were immersed in 0.1% trypsin (Procell) and oscillated overnight at 4˚C. Complete medium was added to terminate digestion by incubation at 37˚C for 10 min. After discarding the supernatant, the remnant was incubated with 0.08% collagenase II (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) at 37˚C for 10 min and the supernatants were pooled. The combined supernatants were centrifuged at 250 x g for 5 min, and DMEM containing 20% FBS was used to re-suspend the precipitates. The cells were then inoculated in a culture dish and incubated for 50 min at 37˚C in a humidified atmosphere containing 5% CO_2. The non-adherent cells were aspirated to be re-suspended for a second adherence culture. The final concentration of the cells was adjusted to $5x10^5$ cells/ml and 10 ml cell suspension was mixed with 100 μl bromodeoxyuridine to inhibit the proliferation and differentiation of non-fibroblasts but without any obvious effect on the proliferation of the fibroblasts (25). The cells were cultured under the abovementioned conditions and the medium was changed once every 2-3 days. The growth and morphological changes of cardiomyocytes were observed and recorded every day.

In vitro ischemia-reperfusion (IR) model. The IR model was established and evaluated as described previously with minor modifications (26). In brief, the culture medium with 20% FBS was centrifuged at 250 x g for 5 min and resuspended in D-hanks medium with a gas mixture of 95% O_2-5% CO_2 for 30 min of incubation prior to hypoxia. To simulate ischemia, the culture plate was transferred into an anoxic incubator for 2 h of incubation with a gas mixture of 95% N_2-5% CO_2. For the reperfusion process, the D-hanks medium was replaced with DMEM containing 20% FBS for 24 h of incubation at 37˚C with a gas mixture of 95% O_2-5% CO_2. The established IR model was evaluated by inspecting apoptosis, LDH release and caspase activity. For the apoptosis assay, cardiomyocytes were collected and centrifuged at 250 x g for 5 min. The cells were inoculated in 6-well flat-bottom plates and digested with 0.3 ml 1X trypsin-EDTA in PBS (37˚C). The cells were immediately stained in the dark for 30 min according to the instructions of the Annexin V-FITC/PI apoptosis detection kit (cat. no. 88-8005-72, Thermo Fisher Scientific, Inc., Waltham, MA, USA). Finally, cellular apoptosis was determined by flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA) within 1 h. The measurements of LDH release and caspase-3 activity were performed using respective commercial kits based on the manufacturer's instructions.

Reverse-transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cardiomyocytes treated with 1 μM S1P alone or in combination with 0.4 μM W146 using a MagExtractor-RNA kit. The extracted total RNA was reverse-transcribed into complementary DNA using ReverTra Ace qPCR RT Master Mix with gDNA Remover kit. The primers, which were synthesized by Sangon Biotech (Shanghai, China), had the following sequences: S1PR1 forward, 5'-AACTACACAACGGCAGCAAC-3' and reverse, 5'-GCAGGCAATGAAGACACTCA-3'; S1PR2 forward, 5'-GGCTCTGTTCCCTGTATTG-3' and reverse, 5'-GGG CTCACTTTGCTCCTC-3'; S1PR3 forward, 5'-AAATGG CTGCCTTGGAC-3' and reverse, 5'-CCCATCGGTTTGGTG CT-3'; S1PR4 forward, 5'-ACGATAGGTGCTGTTAGT-3' and reverse, 5'-CAGATATGCTGCTTCTTT-3'; S1PR5 forward, 5'-TGGTGGTCCTCATCGTCG-3' and reverse, 5'-GGA GAAGGTGGCAGTGGTAA-3'; TGF-β forward, 5'-GAC TACTACGCCAAGGAGGTC-3' and reverse, 5'-GAGAGC AACACGGGTTCAG-3'; Smad3 forward, 5'-TGTTGGTGG AGGGTGTAG-3' and reverse, 5'-AGCAGCAGTGAAGGT GAG-3'; β-actin forward, 5'-ACTCTTCCAGCCTTCCTTC-3' and reverse, 5'-ATCTCCTTCTGCATCCTGTC-3'. RT-qPCR was performed on an ABI7000 fluorescent quantitative PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.)

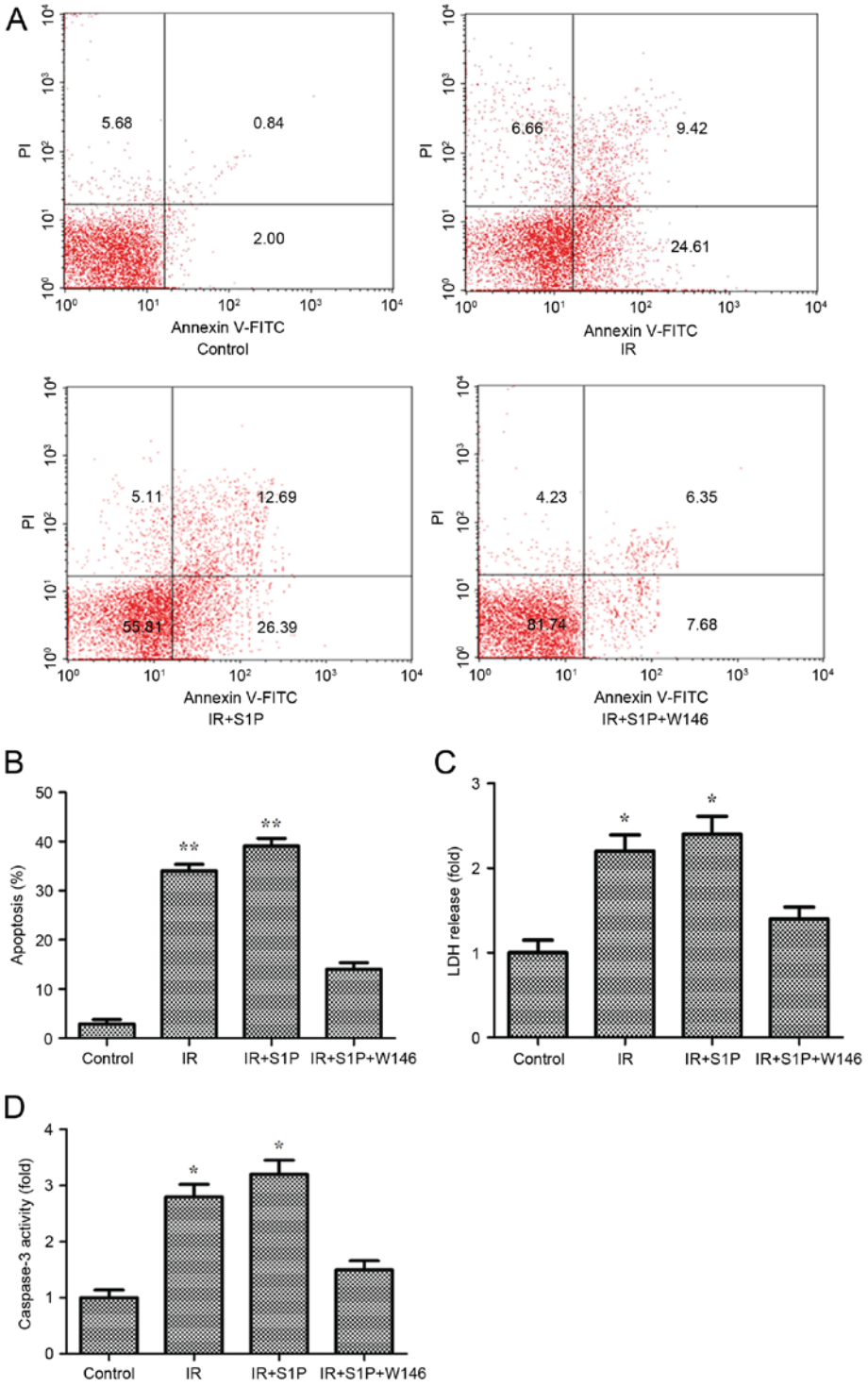

Figure 1. An *in vitro* ischemia-reperfusion model was established and evaluated in isolated cardiomyocytes. The IR-treated cells were incubated with 1 μM S1P alone and in combination with 0.4 μM W146 for 4 h. (A) Flow cytometric analyses after the staining with Annexin V-FITC and PI. (B) Apoptotic rates obtained by quantification of A (percentage in right upper + and right lower quadrant). (C) LDH release. (D) Caspase-3 activity. *P<0.05; **P<0.01, compared with the control. LDH, lactate dehydrogenase; IR, ischemia-reperfusion; PI, propidium iodide; FITC, fluorescein isothiocyanate; S1P, sphingosine 1-phosphate.

with the following thermocycling procedure: 95˚C for 60 sec, followed by 40 cycles of 95˚C for 15 sec, 55˚C for 15 sec and 72˚C for 45 sec. All data were normalized to the housekeeping gene β-actin used as a reference. The relative expression of the target genes was calculated using the $2^{-\Delta\Delta Cq}$ method (27).

Western blot analysis. Cardiomyocytes were incubated with 200 ml lysis buffer (25 mM $MgCl_2$, 5 mM KCl, 20 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, 0.5% (v/v) complete protease inhibitor and Triton X-100). Protein concentrations were determined using a BCA Protein Quantification kit (Vazyma, Nanjing, China) according to the manufacturer's instructions. Cellular protein (50 mg) was separated using 12% SDS-PAGE prior to transfer onto a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). The protein bands were blocked for 1 h in blocking buffer at

room temperature. Antibodies (monoclonal rabbit anti-S1PR1, TGF-β and Smad3; 1:500 dilution) were incubated with the membranes overnight at 4˚C. Secondary antibodies conjugated to horseradish peroxidase (1:2,000 dilution) were incubated with the membranes for 1 h at room temperature, followed by an ECL assay. The bands were imaged with the ChemiDoc™ XRS Gel image system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and their intensities were measured using Quantity one v4.62 software (Bio-Rad Laboratories, Inc.).

S1P measurement by liquid chromatography tandem mass spectrometry (LC-MS/MS). The S1P content was measured by LC-MS/MS according to previously described procedures (28).

Statistical analysis. Each experiment was performed in triplicate on three independent occasions. Values are expressed as the mean ± standard deviation and analyzed using one-way analysis of variance with the Least Significant Difference post hoc test. The statistical analyses were performed using SPSS 11.5 software (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Establishment and evaluation of in vitro IR injury model. The isolated myocardial cells were used for the establishment of an *in vitro* IR injury model. In the established IR model, the apoptotic rate was ~34% (P<0.01 vs. control). Addition of S1P resulted in a further increase of cell apoptosis up to ~39% (P<0.01). However, exogenous W146 inhibited the increase of cell apoptosis caused by S1P, resulting in an apoptotic rate that was significantly different from that of the control (Fig. 1A and B). The LDH levels and caspase-3 activity were also measured due to the stability of LDH in dead cells and the critical role of caspase-3 in apoptosis. The results demonstrated that in IR-treated cells, LDH levels and caspase3 activity increased by 2.2- and 2.8-fold, respectively (P<0.05), and were further enhanced in IR+S1P-treated cells, resulting in 2.4- and 3.2-fold increases, respectively, compared with the control (P<0.05). However, introduction of W146 inhibited the increases of LDH levels and caspase3 activity caused by S1P with the resulting values being not significantly different from those in the control group (Fig. 1C and D).

S1P increases the mRNA and protein levels of S1PR1, TGF-β and Smad3 in an in vitro IR model. After IR injury, the mRNA and protein levels of S1PR1, TGF-β and Smad3 were significantly increased (P<0.01). Exogenous S1P further increased the mRNA and protein expression of S1PR1, TGF-β and Smad3 (P<0.001). In comparison, W146 abolished the stimulatory effects of S1P on S1PR1, TGF-β and Smad3 mRNA and protein expression, resulting in levels that were comparable to those of the control group (Fig. 2).

TGF-β/Smad3 pathway activation stimulates S1P/S1PR1 in IR injury model. Induction of IR resulted in upregulation of S1PR1-3 in myocardial cells (P<0.05 or P<0.01). Pretreatment with TGF-β caused a significant increase of S1PR1 mRNA at 4 h (P<0.05). After 24 h of treatment with TGF-β, the levels of S1PR1-3 mRNA were all significantly stimulated (P<0.05 or P<0.01). The expression of S1PR5 mRNA was not detectable,

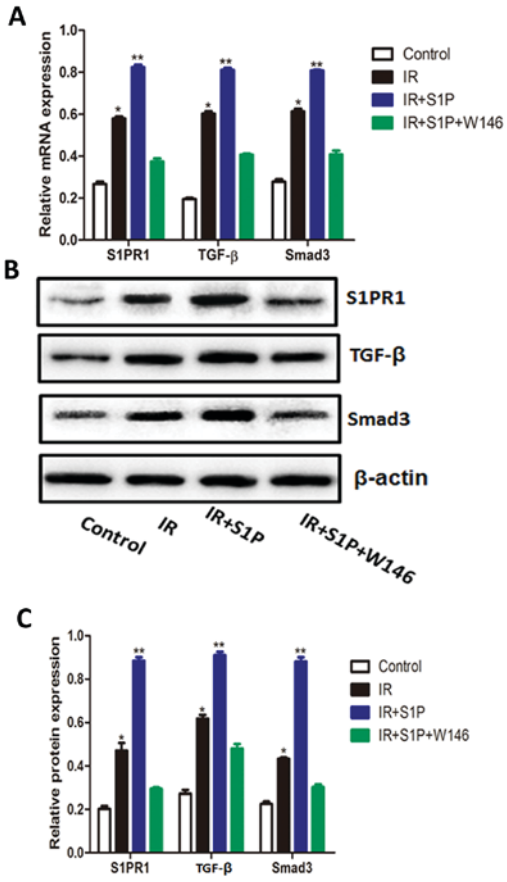

Figure 2. S1P and its inhibitor affected the levels of S1PR1, TGF-β and Smad3. The IR-treated cells were incubated with 1 μM S1P alone and in combination with 0.4 μM W146 for 4 h. The control group remained untreated. (A) mRNA expression levels of S1PR1, TGF-β and Smad3 assessed by reverse-transcription quantitative polymerase chain reaction. (B and C) Representative images and relative quantitation measurements of S1PR1, TGF-β and Smad3 proteins by western blotting. *P<0.05; **P<0.01, compared with the control. S1PR1, sphingosine 1-phosphate receptor 1; IR, ischemia-reperfusion; TGF, transforming growth factor.

while S1PR4 mRNA appeared to not be significantly affected by TGF-β (Fig. 3A). The protein levels of S1PR1 were also increased by pretreatment with TGF-β for 0, 4 and 24 h (P<0.05 or P<0.01; Fig. 3B). These results suggested that S1PR1 mRNA was more affected by TGF-β than S1PR2 and S1PR3 mRNA. By using TGFβR1 inhibitor SB4 and Smad3 inhibitor SIS3, the stimulatory effects of TGF-β on S1PR1 and S1P were abolished (Fig. 4A and B).

Discussion

In the present study, an *in vitro* IR model was successfully established in myocardial cells and evaluated by analysis of apoptosis, LDH release and caspase-3 activity. It was observed that extraneous TGF-β induced the most significant increase of S1PR1 among the five S1P receptors (S1PR1-5) within 24 h. External S1P caused elevated S1PR1, TGF-β and Smad3, while W146, a specific S1PR1 antagonist (29), abolished the effects of S1P. It was also revealed that SB4 (TGFβR1 inhibitor) and SIS3 (Smad3 inhibitor) offset the stimulatory effect of TGF-β on the levels of S1PR1 mRNA and S1P. These results suggested an intimate association of S1P/S1PR1 with TGF-β/Smad3.

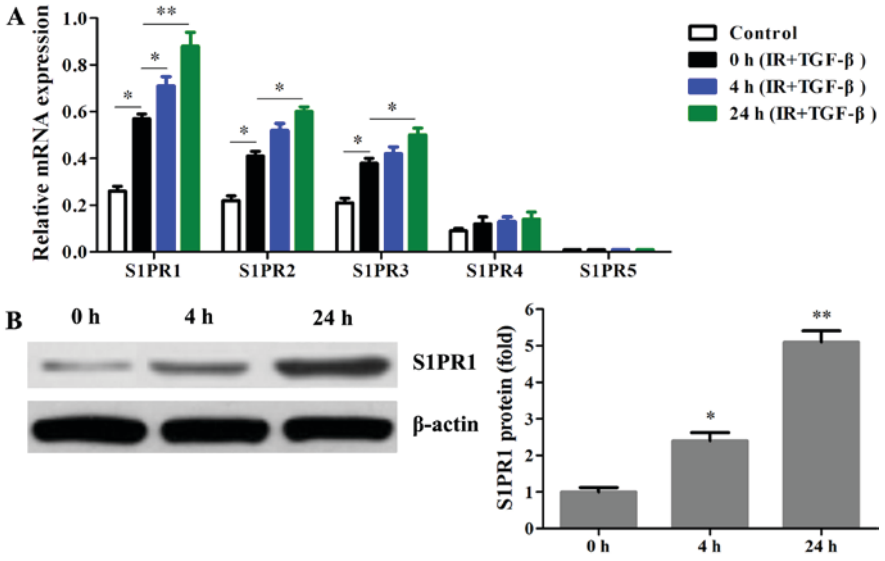

Figure 3. Exogenous TGF-β increases the mRNA and protein levels of S1PR1. The IR-treated cells were incubated with 1 ng/ml TGF-β for 0, 4 or 24 h. The control group remained untreated. (A) mRNA expression levels of S1PR1, S1PR2, S1PR3, S1PR4 and S1PR5 were assessed by reverse-transcription quantitative polymerase chain reaction. (B) Representative images and relative quantitation measurements of S1PR1 proteins by western blotting. *P<0.05; **P<0.01, compared with the control. S1PR1, sphingosine 1-phosphate receptor 1; IR, ischemia-reperfusion; TGF, transforming growth factor.

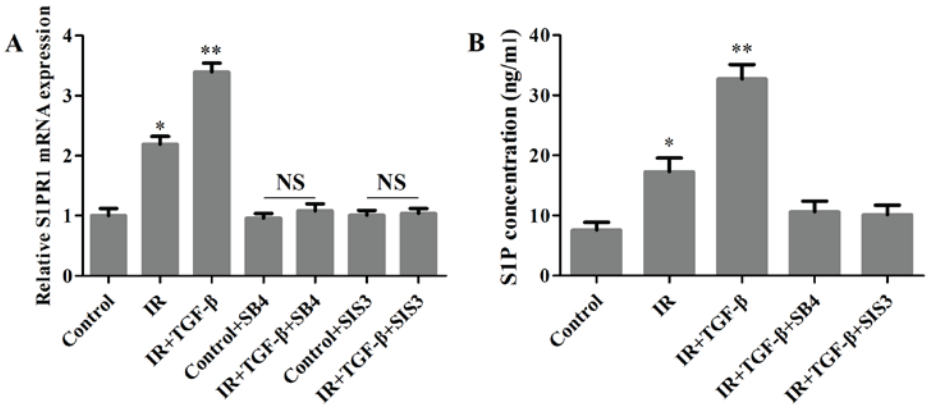

Figure 4. Extraneous SB-431542 (TGFβR1 inhibitor) and SIS3 (Smad3 inhibitor) abolished TGF-β-induced increases of (A) S1PR1 mRNA as determined by reverse-transcription quantitative polymerase chain reaction analysis and (B) S1P levels assessed by liquid chromatography tandem mass spectrometry measurement. The IR-treated cells were incubated with 10 μM SB4 and 2 μM SIS3 for 30 min following stimulation with 1 ng/ml TGF-β for 4 h. The control group remained untreated. *P<0.05; **P<0.01, compared with the control. NS, non-statistical significance; IR, ischemia-reperfusion; TGFβR1, transforming growth factor β receptor 1; SB4, SB-431542.

As mentioned above, the levels of S1P rose following IR, which was indicative of the protection of myocardial cells from IR (14-16). The inherent TGF-β levels increased when IR occurred and autoinduction or exogenous addition of TGF-β also protected the heart from IR to a large extent, suggesting a potentially cardioprotective role of TGF-β against IR in cardiomyocytes (30-33). The results of the present study were consistent with those of these previous studies on IR. The present study also observed that replenishment of S1P further promoted the mRNA and protein expression of S1PR1, TGF-β and Smad3 after IR. These results did not only suggest a protective effect of S1P against IR, but also the close association of S1P/S1PR1 with TGF-β/Smad3. The present results also demonstrated that the increases of S1PR1, TGF-β and Smad3 were almost reversed by the addition of the S1PR1 antagonist W146, which was consistent with a previous study (34). From these results, it may be deduced that the abolishment of the protective effect caused by W146 was mainly due to the disruption of the ligation between S1P and S1PR1, resulting in the interruption of the association of S1P/S1PR1 with TGF-β/Smad3.

As is known, differential expression patterns of S1PR subtypes are important for subsequent cellular responses (35). Although S1PR1-5 are widely distributed in numerous tissue types, the present results indicated that the expression of S1PR1-3 mRNA was significantly upregulated after induction of IR, while the expression of S1PR4 and 5 was generally low and not affected, suggesting that S1PR1-3 may have a more important role in cardioprotection than S1PR4 and -5. However, the relative expression of S1PR1-3 in cardiac myocytes is still under debate. For instance, Forrest *et al* (36) reported that S1PR1 was not detected in myocytes in adult rat and mouse heart sections with subtype-selective antibodies against S1PR1

and S1PR3 compared with marked staining with S1PR3 antibody, indicating that S1PR3, but not S1PR1, may be involved in the protective effect of S1P on cardiac myocytes. However, Robert *et al* (17) demonstrated that S1PR1 existed in neonatal rat heart homogenates and membranes of neonatal cardiomyocytes detected with polyclonal antibodies against a S1PR1 domain that is highly homogenous across multiple mammalian species. A similar conclusion that S1PR1 resided in human ventricular myocytes as well as coronary artery endothelial cells was also drawn using the same antibodies (37). Growing evidence appears to reach a consensus that S1PR1 levels are relatively high in cardiomyocytes throughout development (18,35,38-40).

As S1PR2 and S1PR3 mRNA are expressed in myocardial cells, their roles in TGF-β-mediated cardioprotection via S1P/S1PR1 were also investigated in the present study. It was revealed that extraneous TGF-β increased the levels of S1PR1 mRNA but not those of S1PR2 and S1PR3 mRNA after 4 h of incubation. The results also suggested that the stimulatory effect of TGF-β on the expression of S1PR1 mRNA was more prominent than on that of S1PR2 and S1PR3 mRNA, implying a more critical role of S1PR1 in the treatment of IR. The wide-spectrum use of inhibitors vastly facilitates the study of the TGF-β/Smad3 pathway (28,41,42). The present results demonstrated that either TGFβR1 inhibitor SB4 or Smad3 inhibitor SIS3 was able to abolish the enhancement of the cardioprotective effect of S1P/S1PR1 by TGF-β.

In conclusion, the present study suggested that the TGF-β/Smad3 pathway mediates the protection of myocardial cells from IR injury via the stimulation of S1P/S1PR1. However, the TGF-β pathway may be either Smad-dependent or –independent (43), and the mRNA levels of S1PR2 and S1PR3 were also significantly affected by TGF-β. Further study is required to elucidate the underlying cardioprotective mechanisms for IR treatment and the association of S1P/S1PRs with TGF-β-mediated pathways.

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