Sphingosine-1-phosphate receptors respond differently to early myocardial ischemia and ischemia-reperfusion in vivo

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Abstract: Sphingosine-1-phosphate (S1P) has been demonstrated to be a mediator and marker of heart diseases. We hypothesized that the expression of S1P receptors is involved in the S1P-mediated cardioprotection in vivo and may serve as a biomarker of ischemic heart disease. In vivo models of myocardial ischemia (MI) and ischemia-reperfusion (IR) were established by ligation of the left anterior descending artery (LAD) of rat heart, the mRNA expressions of S1PR1–3 were detected using real time PCR at different time intervals after ischemia (LAD for 15 min, 30 min, and 1 h) and IR. The results showed that mRNA expression of S1PR3, but not S1PR1 and S1PR2, increased greatly after IR. No statistical difference was found in any of the three S1P receptors after MI within 1 h. Regarding the studies of lipid concentration changes in myocardiopathy, we conclude that S1P receptors are not early response biomarkers for MI. There are different mechanisms when S1P plays a protection role in heart during MI and IR. The cooperation of lipid content and S1P receptor expression appears to form a regulation network during MI and IR.

Key words: sphingosine-1-phosphate receptor; myocardial ischemia; ischemia-reperfusion; real time PCR

Research Paper

Sphingosine-1-phosphate (S1P) has been identified as an important biologically active lipid mediator in the regulation of a variety of cell functions, such as cell motility, cell proliferation and differentiation, immune system surveillance, vascular permeability, cytoskeleton organization, virus infections, etc. In cardiovascular system, S1P has been described as protecting mediator of cultured cardiomyocyte and heart functions from...
hypoxia and ischemia-reperfusion (IR)-induced injury in vitro and in vivo\cite{1,2}.

Generally, S1P exerts its functions via its intracellular and/or receptor-mediated pathways. S1P intracellular pathways, which have been linked to inhibition of NF-κB, class I histone deacetylases 1 and 2 in the nucleus and protection of many cells from apoptosis and regulation of inflammatory responses, are S1P receptors (S1PRs) independent\cite{3,4}. S1PRs are also known as endothelial differentiation genes (EDG), which have been revealed as a family of G-coupled receptors with high affinity with S1P or lysophosphatidic acid. The receptors with high affinity to S1P are named S1PR1/EDG-1, S1PR2/EDG-5, S1PR3/EDG-3, S1PR4/EDG-6, and S1PR5/EDG-8. S1PR1, 2 and 3 are widely expressed in cardiovascular system. S1PR4 and S1PR5 are restrained to the lymphoid and brain tissues, respectively\cite{5}. Both intracellular and receptor-mediated signaling pathways are proved to improve cell viability, but which mechanism dominates the S1P-mediated cardio-protection is under discussion. Besides the S1P-mediated signaling pathways, the content alternation of S1P and ceramide is another way to modulate heart functions. S1P content was increased immediately after coronary intervention and ischemia preconditioning (IPC), but not in IR treatment, while concentration of ceramide was reduced in IPC but increased in IR heart in vivo\cite{2}. In fact, S1P was reported as a biomarker of coronary artery disease (CAD)\cite{6}. In plasma from patients suffered from myocardial infarction, the concentration of S1P decreased by about 50% whereas the concentration of ceramide, sphinganine and sphingosine remained unchanged\cite{7}. Further, they found the plasma S1P concentration was reduced in patients with chronic systolic heart failure\cite{8}.

The biomarkers of CAD are extremely important for clinical and forensic medicine, especially during the early myocardial ischemia (MI)\cite{9}. Since the S1P content responds quickly to ischemia, we want to know whether the S1PRs respond so rapidly to ischemia or IR injury. In addition, S1PRs expression during MI and IR would help us further understanding the S1P roles in the heart.

1 MATERIALS AND METHODS

1.1 Materials

Adult male Sprague-Dawley rats (250 to 300 g) were provided by Shanxi Medical Experimental Animal Center. All procedures and animal use were approved by the Animal Care and Use Committee at Shanxi Medical University.

1.1.1 Drug source

The primers of S1PR1, S1PR2 and S1PR3 were designed using Primer Premier 5.0 and synthesized by Takara Company (Takara, Dalian, China). The sequences are listed below: S1PR1: F: 5'-CGCTTTGAGCGAGGCTGCTG-3', R: 5'-GCCGCAACTGTGTCCCCAGC-3'; S1PR2: F: 5'-ACATGGCGTAGCCGGACCT-3', R: 5'-CCCACCTCTGCAGCTGTTG-3'; S1PR3: F: 5'-GCAGGGCACCCAGCGAGAAG-3' and GAPDH: F: 5'-GGCACAGTCAAGGCTGAGAATG-3', R: 5'-ATGGTGGTGAGACGCGCAGTA-3'. RNA extraction kit (RNA iso Plus) and real-time quantitative reverse transcription kit (Prime Script RT reagent Kit Perfect Real Time; SYBR Premix Ex Taq) were purchased from Takara Company.

1.1.2 Instruments

To measure the quality and quantity of RNA, the OD value was detected on a UV analyzer (Ultrospec4300, GE, USA). Real time PCR was conducted on a thermocycler of Stratagene, Mx3000P.

1.2 Methods

1.2.1 Animal preparation

Surgical procedures and tissue preparation were conducted as previously described\cite{9}. Briefly, Animals were anaesthetized with 350 mg/kg Chloral Hydrate, intubated, and ventilated with a ventilator (TKR200-C, JiangXi Teli Anaesthesia & Respiration Equipment Co., Ltd., China). In MI groups, following thoracotomy, the left anterior descending coronary artery (LAD) was ligated with a 5/0 silk suture after piercing the pericardial membrane. Electrocardiogram (ECG, BL-420S data acquisition and analysis system, Chengdu TME technology Co., Ltd., China). In MI groups, following thoracotomy, the left anterior descending coronary artery (LAD) was ligated with a 5/0 silk suture after piercing the pericardial membrane. Electrocardiogram (ECG, BL-420S data acquisition and analysis system, Chengdu TME technology Co., Ltd., China) was recorded to confirm the successful ligation. ST-segment elevation (over 0.1 mV) and/or combination of ST-segment and T wave right after the ligation of LAD in II lead were considered as success of occlusion (Fig. 1). The ischemic myocardium was confirmed by injection of 1% Evans blue from inferior caval vein by the end of experiment. The time interval after ligation was used as a grouping index: 15 min, 30 min, 1 h ($n = 4–7$ for each group). The sham-operated animals ($n = 6$) were treated just as the operated animals except that they did not receive LAD ligation. The IR group was treated similar to the MI groups.
except that a plastic tube was used and tightened after suture rounding the LAD, the vessel was released after 40 min of ischemia and reperfused for another 2 h before the myocardium was harvested. For the IR group, the ischemic area was confirmed by the 2nd ligation before injection of Evan blue. The identified ischemic myocardium from left ventricle and the counterparts of sham operation group were collected, dissected, weighed and frozen immediately in liquid nitrogen and stored at −80 °C until analysis.

1.2.2 RNA extraction and real time PCR
RNA was extracted following the extraction kit protocol. Briefly, cardiac myocardium (80–100 mg) was added in 1 mL iso Plus and homogenate. After addition of 0.2 mL chloroform, the mixture was stewed for 5 min. The supernatant was removed to another tube and equal volume of isopropanol was added to mix for another 10 min. After centrifugation the precipitate was washed by 75% ethanol. RNA was resolved in RNase-free water. The total RNA was treated with DNase I (Takara, Dalian) before the quality and quantity was measured by $A_{260}/A_{280}$ ratio and evaluated by agarose gel electrophoresis.

Transcription was conducted in 20 μL volume, including 5 × PrimeScript Buffer 4 μL, RT Enzyme Mix 1 μL, Oligo dT 1 μL, Random 6mers 1 μL, RNA 2 μL, RNase-free dH2O 11 μL, and carried out in a thermo cycler at 37°C for 15 min and then 85°C for 5 s. Then, the transcription products (cDNA) was added to the total volume of 25 μL system for RT-PCR, which composed by SYBRPremix ExTaq 12.5 μL, primers (10 μmol/L) 1 μL, ROX Reference Dye or Dye II 1 μL, cDNA 2 μL, dH2O 8.5 μL. The amplification was carried out with following parameters: denature at 95 °C for 30 s; 40 cycles of 95°C 6 s, 60°C 55 s, 72°C 30 s, then 95°C 1 min, 55°C 30 s, 95°C 30 s. The levels of mRNA were expressed as ratios of experimental groups/sham groups after normalization by housekeeping GAPDH gene.

1.2.3 Data analyses
Statistical analysis was performed by two-tailed t-test (independent sample t-test, SPSS16.0). Data were presented as the mean ± SEM. $P < 0.05$ was considered significant.

2 RESULTS
A total of 46 rats were used in this study and 34 rats survived for this experiment. The ratio of $A_{260}/A_{280}$ and the integrity of 5S, 18S and 28S RNA after agarose
gel electrophoresis were used to evaluate the RNA quality. Melting curve peaks of all the test genes were narrow and pure, indicating the specificity of the PCR products. The peaks of the dissolve melting temperatures were 86.03 °C for GAPDH and 86.58 °C for S1PR1–3 respectively (Fig. 2).

2.1 No change of S1PRs in early ischemia heart
After heart ischemia, mRNA levels of S1PR2 and S1PR3 in myocardium did not change in MI groups compared with the sham operation group. At the very early stage of MI (ligation for 15 min), S1PR1 seemed increase but showed no statistical difference, as well as other time intervals. No alternation of S1PRs mRNA level was found within 1 h after the ligation of LAD (Fig. 3).

2.2 Increase in expression of S1PR3 after IR
S1PR3 mRNA level in myocardium was greatly enhanced to 5.4 ± 1.3 \((P < 0.05)\) folds after IR compared...
with the sham operation group. S1PR1 and S1PR2 expression levels showed no difference after IR compared with sham operation group (Fig. 4).

3 DISCUSSION

We show here the totally different S1PRs expression patterns during MI and IR in vivo, which imply the discrepancy of S1P-mediated protection role on heart suffering from MI and IR. It is believed that S1PR1 mRNA level is the highest among the three receptors in human myocardium. Our data showed the same results based on the rough evaluation of Ct number (data not shown). The stable mRNA levels of S1PRs during early MI indicate they do not qualify as a potential biomarker of early myocardial infarction, which are highly addressed for diagnosis of sudden cardiac death. Since the dominating receptor in myocardium is S1PR1, it is usually expected as the main protecting receptor of S1P during MI and IR. The results that all receptors were negative for MI and the S1PR3 was positive response to IR are out of our expectation. This may be explained as followed: (1) S1PRs’ mRNA regulation may not be involved in MI in vivo, although S1P content would change during MI and IPC. (2) S1PR3 responds rapidly during IR, and this may be one mechanism of S1P-mediated cardioprotection. As contrast, ceramide increment, but not S1P, was found in the rat hearts after IR. (3) S1PRs are not early response genes during MI, and they are excluded as candidates of MI markers.

There is now broad consensus that S1P plays a critical role in maintaining cardiac cell survival and function in vitro and in vivo. However, the conclusion of which receptor mediates such a role is controversy in different models. Tao’s study showed the S1PRs were essential for intracellular generated S1P to exert cardioprotection role in a model of cultured cardiomyocytes from SK1 null mouse, while they did not refer to any specific receptor. In vitro study by S1PR1 agonist and antagonist suggested the pivotal role of S1PR1 to enhance the mouse ventricular myocytes viability after hypoxia. Although no S1PRs alternations were reported, S1PR1 was considered as the mediator in agonist/antagonist study in the cultured cardiomyocyte. Neither in vitro nor in vivo studies on S1PRs expression changes after MI are reported by now. Reports from other groups using gene deficiency mouse indicated S1PR3 and/or S1PR2 rather than S1PR1 mediated the protective role of S1P during IR. The conflicting data may be caused by using different models and reflect the complex of the heart organ. Our data indicate that S1PRs respond to IR but not MI. With intensively time intervals during early MI, we did not find any receptor mRNA changes after the onset of LAD ligation. Such results indicate that mRNA levels of S1PRs are not targets for S1P function after MI in vivo. Regarding the protection role of S1P mentioned by other studies, our data may be explained as follows: (1) S1PRs expression may not be involved in MI in vivo although studies showed the S1P content changes rapidly during IPC and MI. So the protection role of S1P during ischemia may be caused by S1P concentration alternations or by the intracellular pathways in vivo or, (2) the protection role of S1P during MI may be caused by other cells, such as vascular endothelial cells and immune cells. Due to the minority number of these cells, the S1PRs changes would not be significant enough to be detected in our in vivo model. (3) Time limitation: we focused on the mRNA change in the early ischemia and did not detect the mRNA levels in prolonged time intervals, which might be expected as different results. Anyway, in contrast to rapid S1P content alternations, S1PRs remain unchanged during MI.

Unlike S1P, the content of ceramide, the precursor of S1P, increased in vivo after IR in the hearts in rats and rabbits when the S1P remained stable. The increment of S1PR3 and stable expression of S1PR1 and 2 in our study imply the involvement of S1PR3 in IR injury. These results might be explained by Theilmieier’s study, which showed the protection role of S1PR3 during IR injury. It was an interesting conflicting result that S1PR1 and S1PR3 mRNA expressions were extremely enhanced in a kidney injury subjected to IR treatment. The early involvement of S1PR3 mRNA during IR may be responsible for cardioprotection and the side effects after IR. Several studies have shown that stimulation of S1PR1 and/or S1PR3 resulted in bradycardia in both mice and humans. S1PR1 stimulation exaggerated IR injury by prolonged duration of ventricular tachycardia and ventricular fibrillation.

Many studies reported that S1PR2 played opposite effects in some cell functions, such as cell migration and permeability. We did not observe significant mRNA changes of S1PR2 during MI and IR. The function of S1PR2 needs to be investigated in further studies. The balance of lipid concentration, S1P intracellular pathway and the balance of S1PRs expression patterns during MI and IR in vivo.
constitute the network in regulation of heart functions. S1P and ceramide have been linked with MI and IR injury, respectively. Here we further indicate that S1PRs respond to IR but not MI. The picture of cooperation of lipid content and S1PRs in the regulation of heart function during MI and IR is expected.

REFERENCES