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ORIGINAL ARTICLE

Involvement of sphingosine-1-phosphate receptors 2/3 in IR-induced sudden cardiac death

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Abstract

It has been demonstrated that S1P receptors affect heart ischaemia–reperfusion (IR) induced injury. However, whether S1P receptors affect IR-induced cardiac death has not been investigated. The aim of this paper is to demonstrate the role of S1P receptors in IR-induced cardiac death. Healthy adult male Sprague–Dawley rats were assigned to the following groups: non-operation control group, sham operation group, IR group, IR group pretreated with DMSO, IR group pretreated with S1P3 agonist, IR group pretreated with an antagonist of S1P3, IR group pretreated with S1P2 and S1P3 antagonists, IR group pretreated with heptanol and antagonists of S1P2/3, and IR group pretreated with Gap26 and antagonists of S1P2/3 (heptanol acts as a Cx43 uncoupler and the mimic peptide Gap26 as Cx43 blocker). The groups with S1P2 or S1P3 agonist application before reperfusion were used to assess whether these can be used for therapy of IR. The haemodynamics, electrocardiograms (ECG), infarction area, and mortality rates were recorded. Immunohistological connexin 43 (Cx43) expression in the heart was detected in each group. Blocking S1P2/3 receptors with specific antagonists resulted in an increment of IR-induced mortality, increased infarction size, redistribution of Cx43 expression, as well as affecting the heart function. The infarction size, heart function, and mortality were totally or partially restored in the S1P2, S1P3 agonist-pretreated IR group, and the heptanol/Gap26-treated S1P2/3-blocked IR group. The S1P receptor S1P2/3 and Cx43 are involved in the IR-induced cardiac death.

Keywords S1P receptor 2 (S1P2) · S1P receptor 3 (S1P3) · Connexin 43 (Cx43) · ischaemia–reperfusion (IR) · Sudden cardiac death (SCD)

Abbreviations

SCD	Sudden cardiac death
AMI	Acute myocardial infarction
IR	Ischaemia–reperfusion
IRI	Ischaemia–reperfusion injury
S1P	Sphingosine 1-phosphate
HDL	High-density lipoprotein
ECG	Electrocardiogram
VT	Ventricular tachycardia
VF	Ventricular fibrillation
CV	Conduction velocity
CHD	Coronary heart disease
GJ	Gap junction
Cx43	Connexin 43
EF	Ejection fraction
FS	Fractional shortening
PKC	Protein kinase C
CAD	Coronary artery disease

Xiaojia Zhang and Deqing Chen contributed equally to this work.

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Introduction

Acute myocardial infarction (AMI) disease remains a major cause of sudden cardiac death (SCD), morbidity, and mortality worldwide. Early reperfusion after coronary occlusion is the most effective means of treating myocardial ischaemia and saving lives. However, reperfusion will lead to further complications, such as ischaemia–reperfusion injury (IRI) and even SCD [1–3]. The known IRI mechanisms include

injury via oxygen free radicals injury, calcium overload, energy metabolism dysfunction, apoptosis, leukocyte-mediated inflammatory, and microcirculatory dysfunction, etc [4, 5]. Ischaemia–reperfusion (IR)-induced SCD has not been addressed much in forensic medicine.

High-density lipoproteins (HDL) is well known to possess additional salutary effects, including anti-inflammatory, antioxidant, antiapoptotic, antithrombotic actions, vasorelaxant, antidiabetic, and infarct size reducing properties [6]. HDL mainly contains cholesteryl esters as well as other lipids, such as free cholesterol, triglycerides, and phospholipids. Phospholipids quantitatively predominate in the HDL liposome that accounting for 20–30% of total HDL mass [7]. For decades, studies have shown that the biologically active HDL compound sphingosine-1-phosphate (S1P) is responsible for the beneficial effect of HDL on the myocardium. S1P, a small lipid molecule, has been identified as an important protector against AMI and IRI, both in vivo and in vitro [6, 8–11]. Lower S1P concentration in the HDL particle leads to HDL dysfunction, and S1P loading completely corrects the functional impairment of coronary artery disease HDL [12–14]. In cardiac cells, S1P binds to three G protein-coupled receptors: S1P1, S1P2, and S1P3. Myocytes derived from isolated heart or cardiac myocytes showed that the unbalanced expression results of S1P1 > S1P3 > S1P2 and cardiac fibroblasts S1P3> S1P1 > S1P2 [15]. We showed the different S1P receptors expression patterns during myocardial infarction (MI) and IR in vivo [16]. S1P receptors lead to the activation of several intracellular signaling pathways potentially implicated in cardioprotection [17].

Connexin 43 (Cx43) is one of the main components of the gap junction (GJ) of the ventricle [18, 19]. Cx43 is reported to be involved in ischaemia preconditioning by protein kinase C (PKC), resulting in reduction of infarct size and suppression of ischaemia-associated arrhythmias [20]. Alterations in Cx43 expression and distribution were observed in myocardium disease, i.e., hypertrophic cardiomyopathy, heart failure, and ischaemia [21–23]. Mice with cardiac-specific loss of Cx43 have normal heart structure and contractile function, developing sudden cardiac death from spontaneous ventricular arrhythmias [24]. Here, we aim to determine whether S1P has a protective role against IR-induced SCD via its receptors and downstream signaling. Heptanol, a long carbon chain *n*-alkanol capable of modifying the gap junctional intercellular communication was used as an unspecific GJ inhibitor in vivo and in vitro [25]. Heptanol inhibited cell-to-cell propagation of hypercontracture and altered the spatial continuity areas of contraction band necrosis in reperfused myocardium [26]. Gap26 is a mimic peptide corresponding to specific sequences within the extracellular loops of Cx43. It has been indicated that Gap26 blocks unposed hemichannels, reduced Cx43 hemichannel currents, disruptes the docking of the hemichannels, and, therefore, reduces the assembly of functional GJs [25]. Using rat heart IR models and Cx43 uncouplers, we aimed to prove that the S1P receptor S1P2/3 were involved in the IR-induced cardiac death and we also tried to identify the putative mechanism.

Materials and Methods

Animal preparation

The study was approved by the Ethical Committee of Shanxi Medical University. All surgery was performed under 20% urethane anesthesia, and all efforts were made to minimize animal suffering. The healthy adult male Sprague–Dawley rats (weighing between 280 and 350 g) were provided by Shanxi Medical University Experimental Animal Center. The rats were anesthetized with an intraperitoneal injection of 20% urethane (0.7 ml/100g) and were placed in a supine position. Subdermal electrodes were placed to allow the determination of a lead II electrocardiogram (ECG). Each animal was endotracheally intubated and ventilated with a tidal volume of 5 ml at a rate of 80 strokes/min using a rodent respirator (HX101E, Chengdu Techman Software Co. Ltd, China). Cardiac function was recorded by inserting a tube through the right jugular artery to the left ventricular (BL-420S, Chengdu Techman Software Co., Ltd, China). Occlusion and reperfusion of the coronary artery were performed as previously reported [16]. Briefly, after left thoracotomy, a 6-0 surgical suture was passed underneath the left anterior descending coronary artery (LAD) at a position 2 mm from the tip of the left auricle. A pad of cotton was placed under the ligation knot to avoid tissue injury around the LAD. Myocardial ischaemia was verified by blanching of the left ventricle (LV) and by changing the ST elevation in the ECG. Blood flow was restored after 40 min of occlusion by removing the ligature. During the whole process of 40 min of ischemia and 2 h of reperfusion, the ECGs and death rates were recorded. S1P2 antagonist JTE-013, S1P2 agonist CYM5520, S1P3 antagonist CAY10444, and S1P3 agonist CYM5541 were dissolved in DMSO and applied via intraperitoneal injection with final concentrations of 142 μg/100 g, 11.6 μg/100 g, 100 μg/100 g, and 11.05 μg/100 g, respectively. All agonists and antagonists were from the company of Cayman Chemical, USA. Gap26 was from APExBIO, USA. The animals were assigned as 16 groups: (1) non-operation control group ($n = 5$), (2) sham operation group ($n = 11$), (3) IR group ($n = 34$), (4) DMSO-treated IR group which was set as a control for IR ($n = 18$), (5) S1P3 agonist-pretreated IR group ($n = 14$), (6) S1P3 antagonist-pretreated IR group ($n = 31$), (7) S1P2/3 antagonist-pretreated IR

group ($n=20$), (8) heptanol-pretreated IR group ($n=6$), (9) heptanol and S1P2/3 antagonist-pretreated IR group ($n=14$), (10) Gap26-pretreated IR group ($n=14$), (11) Gap 26 and S1P2/3 antagonists-pretreated IR group ($n=17$), (12) S1P2 agonist-pretreated IR group ($n=7$), (13) S1P2 antagonist-pretreated IR group ($n=10$), (14) S1P2/3 agonist-pretreated IR group ($n=9$), (15) administration of S1P2 agonist before reperfusion ($n=10$), and (16) administration of S1P3 agonist before reperfusion ($n=9$). The S1P receptor agonist or antagonist was injected intraperitoneally, 3 h before the IR surgery. Group 15 and 16 were intended to assess the therapeutic effects against IR of different administration time points, compared with group 5 and 12. Heptanol (0.6 mg/kg) and Gap26 (1 μg/kg) were administrated by injection via the tail vein before beginning reperfusion where necessary. The surgical procedure can be seen in Fig. S1. The rats that died in the process of ischaemia and reperfusion were accounted for in the death rates.

Evaluation of ECG by scale of arrhythmia

The occurrence of cardiac arrhythmia was determined in accordance with the Lambeth Conventions, and scores were tabulated for the entire IR period using criteria previously described [27]. The Lambeth score and the method used to scale the score are shown in Table 1.

Echocardiographic study

Rats underwent baseline echocardiography before the commencement of experimental protocols. Echocardiographic studies were repeated after the ischaemia of 40 min and reperfusion of 2 h using a Vivid 7 Dimension system (GE Vingmed Ultrasound AS) equipped with a 12-MHz electronic transducer. Images were obtained from the left parasternal short-axis views of the left ventricle (LV) at the level of papillary muscles, to define wall thicknesses and internal diameters during systole and diastole, together with regional wall motion abnormality in the LAD region.

Table 1 Lambeth conventions

Score	Conventions
0	Ventricular premature beats(VPB)<50 ventricular premature beats
1	VPB ≥ 50 to 499
2	VPB ≥ 500 or 1 episode of < 1 min of ventricular tachycardia (VT) or ventricular fibrillation (VF)
3	Episodes of VT or VF≥ 2 and totalling time < 1 min combined
4	Total VT or VF≥ 1 to 2 min
5	Total VT or VF> 2 min

Measurement of infarct size

At the end of the experiment, the coronary ligation was tightened, and 2% Evan's Blue was injected into the postcava until the tongue was stained blue. The heart was removed, sectioned into five equal slices, and immersed in 0.1% 2,3,5-triphenyltetrazolium chloride (TTC) at 37 °C for 10 min. The risk areas and infarct sizes were assessed using the Image J software (https://imagej.nih.gov/ij/index.html).

Real-time polymerase chain reaction

To assess Cx43 mRNA expression, total RNA was isolated from heart divided into the noninfarction zone, the risk zone, and the infarction zone, showing blue, red, and white colors after the double staining with Evan's Blue and TTC. Transcription to cDNA was conducted with the M5 Super qPCR RT kit (Mei5 Biotechnology Co., Ltd. China), primers sequence were: Cx43: F:5′-AGCAAGCTAGCGAGCAAAAC; R:5′-GAGTTCATGTCCAGCAGCAA [28]; GAPDH: F:5′-TCGTGGAGTCTACTGGCGTCTT, R:5′- CATTGCTGACAATCTTGAGGGAG.

Real-time PCR was performed using MyGoPro (Gene Company Ltd., China), with a total volume of 20 μl containing 2 × real-time PCR Super mix 10 μl, 10 μM each primer 0.5 μl, cDNA 2 μl, dH_2O 7 μl. The PCR cycling conditions were as follows: 15 s at 95 °C, 15 s at 60 °C, and 30 s at 72 °C (40 cycles). Relative gene expression was calculated by the $2^{\Delta\Delta CT}$ method and normalized to GAPDH.

Immunohistochemistry staining and measurement of Cx43

The hearts were embedded in paraffin ($n=3$ for each group). The tissue section was deparaffinized, rehydrated, washed, and immersed in 3% hydrogen peroxide. After blocking of unspecific binding sites by bovine serum albumin, the section was incubated with rabbit anti-rat Cx43 antibodies (1:100, Boster Biological Engineering, Wuhan, China) at 4 °C overnight. Then, Cx43 was visualized after staining with the antibody-affiliated peroxidase using a horseradish peroxidase complex kit (1:200, goat anti-rabbit IgG, Boster Biological Engineering, Wuhan, China). Images were captured using microscopy (DM4 B, Leica, Germany). The integral optical density (IOD) of the immunohistochemistry and the area of positive Cx43 on the confocal micrographs were analyzed using Image-Pro Plus 6.0 software program. The area of positive Cx43 labeling was defined as the number of pixels with Cx43 signal intensity exceeding the threshold of 30 on the 0–255 Gy intensity scale [29].

Statistical analysis

The data were presented as the mean ± SD. All statistical calculations were performed with the statistical software SPSS 16.0. Statistical analysis was performed using an unpaired *t* test for two groups, paired *t* tests before and after the IR operation for each group, and a one-way ANOVA followed by Dunnett's test for three or more groups. Death rates were compared using the Chi-square test. A difference was considered significant at $p < 0.05$.

Results

S1P2/3 were involved in IR-induced cardiac death

No death event happened in the non-operation group or the sham operation group. The IR surgery caused a death rate of 21% during reperfusion, which is comparable to the results of other studies [30, 31]. The application of a comparable amount of DMSO solvent caused no change in mortality. When S1P2/3 receptors were blocked, the IR-induced mortality increased to more than twice that of the IR group (50% vs 21%, Fig. 1). Application of S1P3 agonist (CYM5541) and antagonist (CAY10444) before surgery changed the death rates to 7% and 39%, respectively. Application of S1P2 agonist (CYM5520) and antagonist (JTE-013) changed the IR-induced death to 14% and 30%, respectively. These results showed that S1P2 or S1P3 agonists and antagonists had a tendency to alter the mortality.

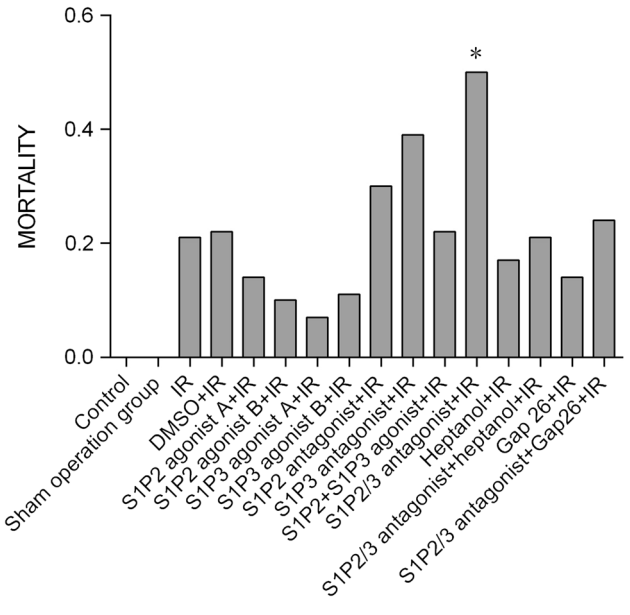

Fig. 1 The mortality in each group. *Indicates $p < 0.05$ compared with the IR group. Control and sham operation groups were not available for statistics their zero value

However, rats treated with S1P2/3 agonists had a mortality of 22%, showing no significant difference from the IR group. Administration of heptanol reduced the mortality of S1P2/3 antagonist-pretreated IR rats from 50% to 21%. Administration of Gap26 reduced the mortality of S1P2/3 antagonists-pretreated IR rats from 50% to 24% (Fig. 1).

Haemodynamic and echocardiographic measurements

We set four time points for recording the haemodynamic changes. The haemodynamic data were recorded at times T0–T4 (T0: before surgery, T1: 30 min after occlusion of LAD, T2: 30 min after reperfusion, T3: 1 h after reperfusion, and T4: 2 h after reperfusion) as presented in Table S1. Using merely pretreatment with DMSO solvent, S1P receptor agonists and antagonists over 3 days led to no significant differences among each group for haemodynamics with the following parameters: LVSP, LVEDP, and ± *d*p/*d*t max. The IR groups with application of S1P2 agonist and S1P2/3 agonists showed increased heart rate from T0 to T4. Correspondingly, the IR group pretreated with S1P2/3 antagonists showed decreased heart rate during the reperfusion period compared with the IR group (153.25 ± 24.66 beats/min for the S1P2/3-blocked group vs 222.83 ± 35.03 beats/min for the IR group at the end of reperfusion, $p < 0.05$). An elevation of LVEDP in the S1P2/3-blocked IR group was found at the end of the reperfusion period (10.97 ± 4.75 mmHg vs 5.73 ± 3.02 mmHg for the IR group at the end of reperfusion). No significant changes were found in LVSP and ± *d*p/*d*t max (Table S1).

Echocardiographic measurements revealing the LV function were assessed by ejection fraction (EF) and fractional shortening (FS). Compared with the IR group, application of S1P2 agonist increased the IR-mediated EF reduction, whether it took place before the reperfusion or before the surgery. Compared with IR, administration S1P3 of agonist before surgery greatly increased EF compared with giving drugs before reperfusion. Correspondingly, the IR group pretreated with S1P2/3 antagonists exacerbated the cardiac function impairment induced by IR, and this effect was partially restored by pretreatment with Gap26 and heptanol, both of which affect the activation of Cx43 (Table 2). FS was used to calculate systolic volume, and IR impaired the heart function by reducing the FS value to 33.68%. IR groups pretreated with S1P2 or S1P3 agonist exerted a potentially protective effect on FS, showing a FS value comparable to its normal magnitude. S1P2/3 antagonists' application aggravated the FS by about 20%, indicating the cardiac function damage. The results are summarized in Table 3.

Table 2 EF of rats before and after surgery

Groups	Before surgery	After surgery	Ratio (%)
IR	88.00 ± 2.73%	67.20 ± 13.18%[a]	− 23.86
S1P2 agonist (A) + IR	81.67 ± 5.51%	75.67 ± 10.06%	− 7.31
S1P2 agonist (B) + IR	87.25 ± 2.75%	89.25 ± 2.50%[b]	2.30
S1P3 agonist (A) + IR	87.00 ± 6.89%	89.50 ± 3.00%[b]	2.27
S1P3 agonist (B) + IR	85.33 ± 2.31%	75.33 ± 2.08%	− 11.76
S1P2 antagonist + IR	83.25 ± 7.41%	57.25 ± 9.03%[a]	− 31.33
S1PR3 antagonist + IR	85.60 ± 3.58%	55.40 ± 8.02%[ab]	− 36.05
S1P2/3 agonist + IR	91.75 ± 2.06%	78.25 ± 7.50%[a]	− 15.22
S1P2/3 antagonist + IR	88.00 ± 2.00%	52.67 ± 8.15%[ab]	− 39.77
S1P2/3 antagonist + heptanol + IR	85.00 ± 8.54%	69.00 ± 6.43%	− 18.82
S1P2/3 antagonist + Gap26 + IR	86.00 ± 6.01%	73.00 ± 8.89%	− 15.12

Ratio = (after surgery–before surgery)/before surgery. Significant changes for groups before surgery vs after IR are indicated by a, and b indicates $p < 0.05$ for each group after surgery compared with IR group. Values are presented with mean ± SD

A Drug application before IR, *B* drug application before reperfusion

Table 3 Fractional shortening (FS) of rats before and after myocardial IR

	Before surgery	After surgery
IR	52.80 ± 3.42%	33.60 ± 9.46%*
S1P2 agonist (A) + IR	57.33 ± 25.81%	52.00 ± 28.93%
S1P2 agonist (B) + IR	51.50 ± 3.70%	53.50 ± 3.70%
S1P3 agonist (A) + IR	53.50 ± 4.95%	55.00 ± 4.24%
S1P3 agonist (B) + IR	48.50 ± 3.11%	42.25 ± 7.27%
S1P2 antagonist + IR	47.25 ± 8.62%	26.25 ± 5.80%*
S1P3 antagonist + IR	50.00 ± 4.42%	25.20 ± 4.92%**
S1P2/3 agonist + IR	58.00 ± 3.46%	44.33 ± 7.09%
S1P2/3 antagonist + IR	53.00 ± 3.00%	23.33 ± 4.93%*
S1P2/3 antagonist + heptanol + IR	50.00 ± 11.14%	34.33 ± 8.02%
S1P2/3 antagonist + Gap26 + IR	50.00 ± 8.72%	37.33 ± 7.37%

A Drug application before surgery, *B* drug application before reperfusion

*Before surgery vs after surgery, $p < 0.05$

**Before surgery vs after surgery $p < 0.000$. Values are presented with mean ± SD

ECG alternation

Representative ECG recordings are shown in Fig. S2. Compared with the IR group, the trend was for the occurrence and duration of ventricular tachycardia (VT) and ventricular fibrillation (VF) to increase, during the ischaemia interval when the S1P2/3 receptors were blocked before reperfusion (Fig. S3), though no statistically significant difference was detected. The occurrence of arrhythmia was scaled using the Lambeth score (Table 4). Both heptanol and Gap 26 alleviated the arrhythmia during IR, and reduced the arrhythmia caused by pretreatment with S1P2/3 antagonists (Fig. S3). Another ECG alteration was atrioventricular block, which was not included in the Lambeth scaling system. Notably, atrioventricular block was detected in 40% of the S1P2/3 antagonist-pretreated IR rats, 33.33% of the S1P2 antagonist-pretreated IR rats, and 8.33% of the S1P3 antagonist-pretreated IR rats before death but was not found in other groups (Table 5).

S1P2/3 reduced the IR-induced infarction size

The infarct area reflects the cardiac injury scale. There was no difference between the IR group (28.75 ± 3.20%) and the DMSO group (27.66 ± 3.52%), which suggested that DMSO, as an organic solvent, had no impact on infarct size. Administration of S1P3 agonist alone before IR reduced the infarct area to 18.62 ± 2.17%, while treating with S1P2 agonist alone before IR did not change the infarct area significantly (25.35 ± 4.31%). Blocking S1P3 receptor or S1P2/3 receptors enlarged the infarct area to 54.66 ± 10.23% and 50.06 ± 8.09%, respectively (Table 6 and Fig. S4). No further increment of infarction size was observed on addition of S1P2 antagonist. The Cx43 uncoupler heptanol and Gap26 groups reduced infarct size to 18.78 ± 2.17% and 10.17 ± 2.76%, respectively. These data indicated that S1P3 played a determining role in decreasing the infarction size during IR.

Expression of Cx43

Figure 2 represents the real-time PCR results of myocardial Cx43. IR reduced the Cx43 mRNA level in infarcted tissue of the heart, but not in the non-ischaemic tissue or the area at risk (AAR). Compared with IR group, the IR group pretreated with S1P2 agonist before surgery showed increased

Table 4 Arrhythmia and Lambeth score for each group

Groups	Occurrence of PVB (frequency)		Lambeth score	
	Ischemia	Reperfusion	Ischemia	Reperfusion
Control	3.6 ± 4.98		0	0
Sham	9.67 ± 12.66		0	0
IR	99.5 ± 100.42	40.16 ± 71.12	2.38 ± 1.06	2.25 ± 3.54
DMSO + IR	65.00 ± 35.77	58.05 ± 85.62	2.33 ± 1.97	2.40 ± 3.72
S1P3 agonist + IR	27.88 ± 12.92	39.24 ± 73.91	0.88 ± 1.13	0.75 ± 1.04
S1P3 antagonist + IR	115.25 ± 87.93	43.23 ± 49.65	1.00 ± 0.50	0.93 ± 0.53
S1P2/3 antagonist + IR	175.7 ± 155.57	30.93 ± 31.45	4.10 ± 3.21	1.10 ± 0.83
Heptanol + IR	39.14 ± 56.58	29.85 ± 95.04	1.43 ± 1.51	0.57 ± 1.13
S1P2/3 antagonist/heptanol + IR	116.09 ± 131.06	7.21 ± 22.32	3.28 ± 3.25	0.14 ± 0.37
Gap26 + IR	21.5 ± 8.61	4.33 ± 3.82	1.67 ± 1.25	0
S1P2/3 antagonist/Gap26 + IR	69.67 ± 69.94	7.18 ± 12.12	6.10 ± 4.41	0

Values presented as mean ± SD

Table 5 Atrioventricular block in each group

	Death (*N*)	Atrioventricular block[a] (*N*)
IR	7	0 (0.00%)
DMSO + IR	4	0 (0.00%)
S1P2 agonist A + IR	1	0 (0.00%)
S1P2 agonist B + IR	1	0 (0.00%)
S1P3 agonist A + IR	1	0 (0.00%)
S1P3 agonist B + IR	1	0 (0.00%)
S1P2 antagonist + IR	3	1 (33.33%)
S1P3 antagonist + IR	12	1 (8.33%)
S1P2/3 agonist + IR	2	0 (0.00%)
S1P2/3 antagonist + IR	10	4 (40.00%)
S1P2/3 antagonist + heptanol + IR	3	1 (33.33%)
S1P2/3 antagonist + Gap26 + IR	4	0 (0.00%)

Values are numbers of dead rats, and percentages are based on the number of dead rat subjects in each group

Table 6 Infarction area in each group

Groups	Infarction area
IR	28.75 ± 3.20%
DMSO + IR	27.66 ± 3.52%
S1P2 agonist (A) + IR	25.35 ± 4.31%
S1P3 agonist (B) + IR	29.93 ± 5.79%
S1P3 agonist (A) + IR	18.62 ± 4.87%[abc]
S1P3 agonist (B) + IR	27.01 ± 9.04%
S1P2/3 agonist + IR	22.64 ± 4.68%
S1P2 antagonist + IR	40.05 ± 5.24%
S1P3 antagonist + IR	54.66 ± 10.23%[a]
S1P2/3 antagonist + IR	50.06 ± 8.09%[a]
Heptanol + IR	18.78 ± 2.17%[abc]
S1P2/3 antagonist + heptanol + IR	39.27 ± 4.79%[abc]
Gap 26 + IR	10.17 ± 2.76% [abc]
S1P2/3 antagonist + Gap + IR	25.18 ± 3.70% [bc]

A: Drug application before surgery, B: drug application before reperfusion; a indicates $p < 0.05$ compared with the IR group and DMSO + IR group, b indicates $p < 0.05$ compared with the S1P2/3 antagonist + IR group, c indicates $p < 0.05$ compared with the S1P3 antagonist + IR group. Values are presented as mean ± SD

Cx43 mRNA levels in the whole heart. Intriguingly, the IR group pretreated with S1PR2/3 antagonists also improved Cx43 mRNA. Compared with the IR group, no significant difference in the Cx43 mRNA level in myocardium was found in the other groups.

Compared with the sham operation group, the IR group showed Cx43 redistribution to the lateral myocardial cells instead of the intercalated disks (Fig. 3b). Application of S1P3 receptor antagonist or S1P2/3 antagonists greatly enhanced the lateral distribution of Cx43 in a heterogeneous manner, whereas Cx43 was hardly observed at all at the intercalate disks (Fig. 3f, h). Pretreatment with S1P3 agonist before IR suppressed this redistribution, i.e., the Cx43 was located mainly at the intercalated disks (Fig. 3e). The above-mentioned changes in Cx43 occurred in the ventricular region subjected to IR, while, in the area distant from this region, the Cx43 presented normally at the intercalated disks. These results reminded us that S1P3 and/or S1P2 were involved in the regulation of Cx43 expression and distribution.

Integrated optical density (IOD) of Cx43 in the infarct area of the heart showed a significant decrease both in the IR group and the S1P receptor antagonist application groups, compared with the control group. Application of S1P2 or S1P3 agonist alone increased the Cx43 expression and expression area. All data are shown in Table 7.

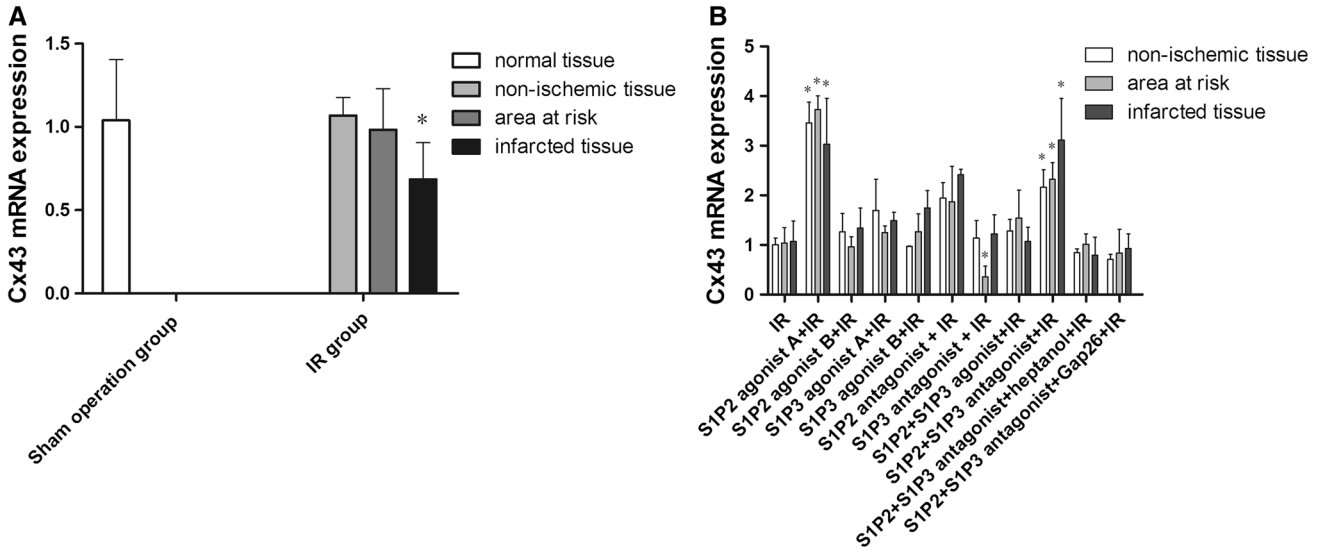

Fig. 2 Cx43 mRNA expression. **a** *Indicates $p < 0.05$ compared with the sham operation group. Each rat heart with IR was divided into non-ischemic tissue, area at risk and infarcted tissue. **b** *Indicates $p < 0.05$ compared with the IR group. Data are shown in mean ± SD

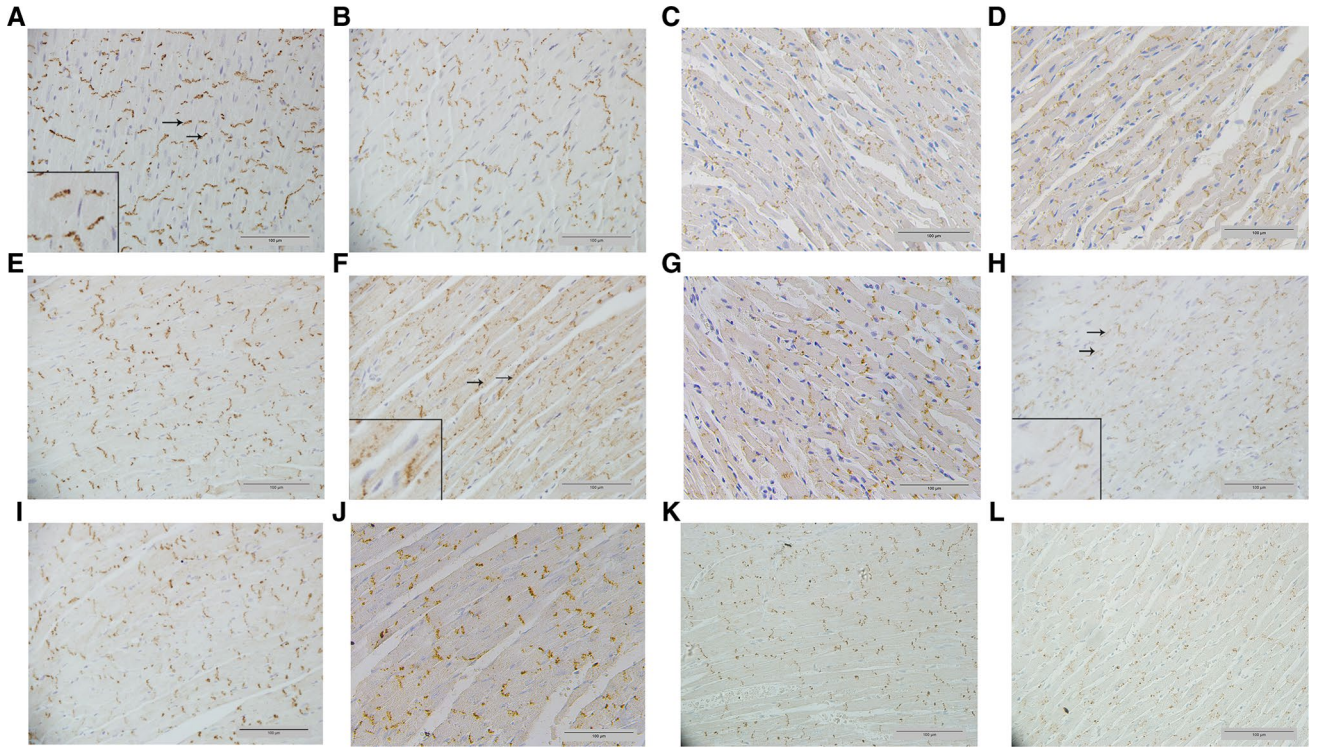

Fig. 3 Representative immunohistochemistry staining of Cx43 in rat heart. **a** Normal myocardium, **b** IR surgery group, **c** S1PR2 agonist-pretreated IR group, **d** S1PR2 antagonist-pretreated IR group, **e** S1PR3 agonist-pretreated IR group, **f** S1PR3 antagonist-pretreated IR group, **g** S1PR2/3 agonist-pretreated IR group, **h** S1PR2/3 antagonist-pretreated IR group, **i** Gap26-pretreated IR group, **j** S1PR2/3 antagonists and Gap26-pretreated IR group, **k** heptanol-pretreated IR group, and **l** S1PR2/3 antagonists and heptanol-pretreated IR group. Black arrows in **a** show the Cx43 distribution in intercalated disks, black arrows in **f**, and **h** show the Cx43 lateral redistribution. The lateral distribution of Cx43 with barely in intercalated disks was observed in S1P3 and S1P2/3 antagonist-pretreated IR hearts. The arrows indicate the lateral redistribution of Cx43. The S1P3 agonist group (**e**), heptanol (**k**), and Gap26 (**i**) pretreatment groups showed Cx43 remaining in the intercalated disks

Table 7 Integrated optical density of Cx43 in each group

	Area	IOD
Control	1,447,855.44 ± 268,177.71	139,054.66 ± 8723.56
IR	240,730.44 ± 171,789.07[a]	26,439.70 ± 6184.33[a]
S1P2 agonist (A) + IR	1,003,173.44 ± 174,717.79[ab]	109,353.75 ± 35,356.75[b]
S1P2 agonist (B) + IR	424,205.24 ± 269,861.37[a]	37,541.51 ± 33,291.11[a]
S1P3 agonist (A) + IR	256,462.80 ± 185,120.24[a]	17,347.08 ± 5897.48[a]
S1P3 agonist (B) + IR	894,605.00 ± 177,021.65[ab]	72,621.45 ± 5693.96[ab]
S1P2/3 agonist + IR	710,620.86 ± 318,715.60[a]	64,352.91 ± 28,163.60[a]
S1P2 antagonist + IR	320,500.69 ± 74,120.13[a]	38,378.01 ± 9568.47[a]
S1P3 antagonist + IR	516,397.53 ± 313,423.71[a]	32,307.32 ± 21,521.98[a]
S1P2/3 antagonist + IR	323,868.15 ± 118,982.46[a]	30,233.66 ± 6784.50[a]

A Drug application before surgery, *B* Drug application before reperfusion

[a] $p < 0.05$ compared with the sham operation group

[b] $p < 0.05$ compared with the IR group. Values presented as mean ± SD

Application of Cx43 uncoupler or mimic peptides ameliorated the S1P2/3-mediated IR mortality

To investigated whether S1P2/3-Cx43 is involved in the pathway-regulating IR-induced SCD, we employed heptanol, an uncoupler of Cx43 which has been used to stabilize Cx43 in many studies [32–34]. Our results showed that administration of heptanol reduced the death rate of the S1P2/3 antagonist-pretreated IR group from 50% to 21%, a similar mortality rate to the IR group. Application of heptanol alone did not change the mortality rate compared with the IR group (17% vs 21%). However, heptanol significantly reduced the IR-induced infarct area compared with the IR group (18.78 ± 2.17% vs 28.75 ± 3.20%, $p < 0.05$). Application of heptanol did not ameliorate the infarct size in the S1P2/3-pretreated IR group (Table 6).

Compared with the IR group, the redistribution of Cx43 was not aggravated in the heptanol treatment group (Fig. 3k). Moreover, heptanol treatment repressed the redistribution of Cx43 when S1P2/3 were blocked (Fig. 3l). Our results indicated that Cx43, whose distribution and function were affected by the application of heptanol, was involved in IR-S1P2/3-mediated cardiac death.

To confirm the above observation, we utilized Gap26, a structural mimetic peptides of the Cx43 selective blocker of Cx43. Hawat et al. found that infarct size reductions did not vary significantly ($p > 0.05$) when using 1 and 10 μg/kg of Gap26 [35, 36]. In this study, application of 1 μg/kg of Gap26 resulted in a reduction in mortality of the S1P2/3-pretreated IR group from 50% to 24%. Our results also showed the ability of Gap26 to ameliorate the infarct size in the S1P2/3-pretreated IR group.

In summary, S1P2/3-mediated changes in IR mortality rate, Cx43 redistribution, infarct size, and heart functions were wholly or partially mitigated by application of heptanol or Gap26.

Discussion

For years, the SCD has been a mysterious threat to human health and its mechanism has been illusive in forensic and clinical medicine. Many researchers have shown that heart function, serum biomarkers, and Cx43 expression are linked to the occurrence of SCD [24, 37, 38]. About 30% of SCDs may be due to the genetic disorders considering the whole genome sequence [39]. A hypoxia/ischaemia event remains the main cause of SCD, especially in the patients with coronary artery disease (CAD) [40]. Due to the lack of appropriate SCD models, research on the mechanism of SCD makes slow progress. Here, we found that modulation of S1P receptors could increase/reduce the IR-induced mortality, and we supposed that this could occur via signaling to Cx43. IR is a typical process in CAD patients, and our finding is important for improving survival opportunities. Since forensic data showed that the infarct size and heart function are markers for SCD, we also assessed these factors. In this study, we found that blocking of S1P2/3 receptors was sufficient to change the IR-related mortality, myocardial infarct size, heart function, and Cx43 redistribution. Furthermore, we found that S1P2/3 mediated IR-induced SCD could be partially reduced via the intervention of Cx43.

Using an IR model, we found that blocking of S1P2/3 increased IR-induced mortality. However, in vivo hemodynamic measurements showed no significant changes except in the heart rate and LVEDP at the end of the reperfusion. Our results showed that heart rate remained stable after pretreatment with S1P3 receptor agonist and antagonist administered 24 h, or even 3 days, prior to the IR surgery. These results were consistent with those of Morel et al., who also found that the myocardial function was similar in WT mice during basal conditions [41]. In our study, although no significant difference in Lambeth score was seen in the S1P2/3-pretreated IR group compared with the IR group,

we found the occurrence of atrioventricular block in 40.00% of the S1P2/3 antagonist-pretreated, 33.33% of the S1P2 antagonist-pretreated, and 8.33% of the S1P3 antagonist-pretreated IR rats before death. The IR group pretreated with S1P2/3 antagonists showed decreased heart rate during the reperfusion period compared with the IR group. Researchers found that fingolimod (FTY720), which is an agonist for S1P1/3/4/5, attenuated both ischaemia- and reperfusion-induced AVN rhythmic disturbance, stabilized ischaemia/IR-induced arrythmia through Pak1/Akt signaling, and inhibited both Na^+ and K^+ channels in an ex vivo rat heart model [42]. Bonz's study showed that FTY720 application before reperfusion increased mortality due to induction of fatal ventricular tachyarrhythmia. Pretreatment with FTY720 before ischemia abrogated the deleterious pro-arrhythmic effects [31]. The exact mechanism of how FTY720-mediated arrhythmia is unknown. It may be related to its direct (acute) agonist effect at the S1P receptor or potentially owing to down-regulation of the S1P receptors [43].

It is believed that the lower EF is a disputable factor in SCD for those with cardiac diseases, although at least 70% of patients with preserved EF suffered SCD [44, 45]. Our results indicated that the treatment of IR groups with S1P2, S1P3, or S1P2/3 antagonists substantially impaired EF and led to a concordant change in the mortality rate. The EF might be an important marker of death. We did only pilot experiments give S1P2 and S1P3 agonist before reperfusion mainly for the assessment of time-dependent effect; S1P2 and S1P3 agonist groups have cardioprotection.

Blocking S1P2/3 enlarged the IR-induced infarction area, as elucidated by others [8, 9]. Our results showed that application of heptanol reduced the infarction area both in the IR group and the S1P2/3-pretreated IR group. As previously stated, heptanol itself could reduce infarction size and transduction of Lucifer yellow among cardiomyocytes [46]. Researchers further identified that the S1P-induced reduction of infarct size was lost in mice hearts with a truncated C-terminus of Cx43 [41]. Whether the infarction area enlargement leads to SCD has not been widely discussed. One can deduce that the tremendous dead cells would result in a post-infarction heart failure, thereby increasing the occurrence of SCD in the long term.

Gap junctions (GJ) mediate electrical coupling between cardiac myocytes, allowing the spreading of electrical current responsible for synchronized contraction in the heart. The most abundant GJ is Cx43, which is extensively expressed in atrial, ventricular myocytes, and parts of the ventricular conduction system [47]. Cx43 remodeling due to hypoxia and IR injury will change the velocity of impulse propagation. Cx43 becomes highly discontinuous with regard to conduction between the normal and abnormal tissue and this may set the stage for re-entrant arrhythmias [48]. Abnormality of Cx43 could be found in acquired cardiomyopathies. Reduction and redistribution of Cx43 might lead to arrhythmia by influencing conduction velocity (CV) and disease-caused fibrosis [47, 49, 50]. The lateral location of Cx43 resulted in increments of transverse velocity in myocytes in AF patients [49]. Evidence shows that the Cx43 mutant is deadly. The deletion and mutation of CX43 has been linked to cardiac death [38–41]. The Cx43 mutation, $Cx43^{D378stop}$, was reported to lead to lethal ventricular arrhythmias in mice via decrement of I_{Na} and I_{to} amplitude, as well as the down-regulation of N-cadherin and $Na_{v1.5}$, at intercalated disks [40]. In all, Cx43 alternation has been closely linked to cardiac death.

We verified that the Cx43 expression was affected by administration of S1P2/3 agonists/antagonists during IR in vivo. Compared with the sham operation group, IR surgery reduced the mRNA level of Cx43. Compared with the IR group, both IR groups that pretreated with S1P2 agonist and S1P2/3 antagonists both increased Cx43 mRNA expression. The results seemed contradictory and conflicting; therefore, there must be other pathways beyond our knowledge. At the protein level, we found the IOD changes and Cx43 redistribution when IR rats were treated with S1P receptor agonists/antagonists.

To further confirm the expression and function relations between S1P receptors and Cx43, we employed Gap26 and heptanol in the next experiments. The application of heptanol and Gap26 reversed the S1P2/3-mediated detrimental effects in heart function, infarction size, and Lambeth score, and improved the rats' survival rate after IR. The literature shows that low concentration of heptanol (0.05–1 mmol/L) before the ischaemia period is cardioprotective in rat hearts [32–34]. This may be attributed to the fact that a low concentration of heptanol induces incomplete inhibition of the gap junction channels without any effects on Na^+, K^+, or Ca^{2+} channel activity, and accelerates electrical interaction between cells [50, 51]. Another possible mechanism for the cardioprotective effect of heptanol is the mitochondrial signaling. Johansen et al. showed that heptanol reduced IR-mediated infarction size via stimulation of the mitochondrial potassium channel, reducing mitochondrial respiration and increasing the time of mitochondrial permeability transition pore (MPTP) opening [33]. Our results further showed that the application of heptanol led to a decrease in S1P2/3-mediated SCD by affecting Cx43. To further verify the conclusion, we then tested the molecule Gap26, which showed the similar results. This is in concordance with Hawat. et al., who demonstrated that injections of Cx43 mimetic peptides can successfully confer protection on adult rat hearts against ischaemic injury and reduce the resulting infarct size significantly by up to ~ 65% compared to animals treated with saline only [35].

Recently, the S1P-S1P2/3-PKC-Cx43 phosphorylation pathway has been suggested as a mechanism for decreased

infarct size [18, 41]. It is reported that S1P3 activation initiated an increase of $[Ca^{2+}]_i$, Rho activation, and coronary flow[52]. Since $[Ca^{2+}]_i$ is responsible for the open/close of hemichannels. RhoA activity also affects GJ without major cellular redistribution of junctional plaques or changes in the Cx43 phosphorylation pattern [53]. It is rationale to deduce that $[Ca^{2+}]_i$ and RhoA may be involved in linkage signaling of S1P3 and Cx43 in IR and SCD. Such pathways remained to be tested.

As mentioned above, the mechanism of SCD is still a great challenge. In this study, we found that blocking S1P2/3 induced a decrease and redistribution of overall Cx43 in IR rat hearts. In addition, the IR-S1P2/3-induced lethality could be reduced by applying the Cx43 uncouplers heptanol and Gap26. Therefore, the IR-S1P2/3-Cx43-SCD pathway could be involved in this process. Since SCD could have many causes, whether other resources could invoke this pathway has not yet been investigated. These results also remind the clinical physician that administration of Cx43 stabilizer may help to reduce the clinical death rate during IR. Whether it will help to reduce heart failure-induced cardiac death needs to be investigated in the future.

Limitation of study

In this paper, we paid much attention to the function of S1P2/3 on the heart and in SCD, and neglected the function of S1P1. S1P1 is indispensable for normal cardiac function, ion homeostasis, activity of the Na^+/H^+ exchanger NHE-1, and myofibrillar Ca^{2+} sensitivity. S1P1 signaling by ischaemic preconditioning is involved in cardioprotection [54]. The relationship and function of S1P1 should also be studied in IR-induced SCD.

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Conflict of interest The authors declare that they have no conflict of interest.

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