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Limb remote ischemia per-conditioning protects heart against
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Running title: Cardiac protection of per-conditioning

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Abstract: Remote ischemia per-conditioning (RPerC) has been demonstrated to have cardiac protection, but the underlying mechanism remains unclear. This study aimed to investigate the mechanism underlying cardiac protection of RPerC. Adult male Sprague-Dawley rats were used in this study. Cardiac ischemia/reperfusion (I/R) was induced by 30-min occlusion and 3-h reperfusion of left anterior descending coronary artery. RPerC were performed by 5-min occlusion of right femoral artery followed by 5-min reperfusion for three times during cardiac ischemia. Hemodynamics, left ventricular function, arrhythmia, and infarct area were measured. Protein expression levels of eNOS, iNOS, PKC ϵ , and PKC δ in myocardium were assayed. During I/R, systolic artery pressure and left ventricular function were decreased, infarct area was increased, and arrhythmia score was increased ($P<0.05$). However, changes of the above parameters were significantly attenuated in RPerC-treated rats compared with control rats ($P<0.05$). The cardiac protective effects of RPerC were prevented by naloxone or glibenclamide. Also, RPerC increased the protein expression levels of eNOS, iNOS, PKC ϵ , and PKC δ in myocardium compared with control rats. These effects were blocked by naloxone, an opioid receptor antagonist, and glibenclamide, an ATP-sensitive K⁺ channel blocker (K_{ATP}). In summary, this study suggests that RPerC protects heart against I/R injury through activation of opioid receptors and NO-PKC-K_{ATP} channel signaling pathways.

Key words: Remote ischemic per-conditioning, cardiac protection, ischemia/reperfusion, opioid receptors, ATP-sensitive K⁺ channels.

Introduction

Coronary heart disease (CHD) is the leading cause of death all over the world. The major consequence of CHD is acute ischemia/reperfusion (I/R) injury in cardiac myocardium. Thus, development of innovative strategies to protect myocardium against I/R injury is necessary for patients with CHD.

Ischemic pre-conditioning (IPC) protects heart from cardiac tissue injury during prolonged ischemia through applying a short period of sub-lethal ischemia conducted in the heart (Murry et al. 1986). Ischemic post-conditioning (PostC), a modified schedule of reperfusion, also protects the heart from intermittent restoration of blood flow after a prolonged episode of ischemia (Zhao et al. 2003). Myocardial protection against I/R can also be induced by ischemia in organs and tissues which are distant from the heart, a process called remote ischemic pre-conditioning (RIPC) (Aimo et al. 2015). RIPC is induced by a brief period of ischemia in distant organs such as limb (Birnbaum et al. 1997), kidney (Pell et al. 1998), or mesentery (Gho et al. 1996) to reduce myocardial infarction and protect against I/R injury in porcine, rabbit, rat, as well as human (Kharbanda et al. 2002). RIPC is more attractive than traditional cardiac pre-conditioning or post-conditioning for its simple application in clinical practice and being the same efficiency as IPC or PostC. Like traditional cardiac conditioning, RIPC can be used before I/R. Also, it has been shown that several short periods of limb ischemia administered at the time of myocardial ischemia, termed as remote ischemia per-conditioning (RPerC), produce significant cardiac protection in pigs (Schmidt et al. 2007). Recently, Zhu et al compared three remote conditioning strategies on the cardiac protection in rats and found that RPerC produced more prevention of arrhythmia during I/R than that produced by RIPC or RIPostC (Zhu et al. 2013). The mechanism, however, for this phenomenon was not fully elucidated.

The opioid receptors including μ , δ , and κ subtypes and their endogenous ligands play important role in cardiac protection (Headrick et al. 2015). Many studies demonstrated that opioid receptors play important roles in the cardiac protection during IPC and PostC (Fraessdorf et al. 2015; Liu 2007; Lu et al. 2011). Also, opioid receptors are involved in the cardiac protection of RIPC (Rentoukas et al. 2010; Surendra et al. 2013; Wever et al. 2013; Wong et al. 2012). In this study, we determine whether opioid receptors are involved in the cardiac protection of RPerC.

The ATP-sensitive K^+ channels (K_{ATP}) are activated by low concentration of intracellular ATP and are important K^+ channels that regulate myocardial function. K_{ATP} channels are categorized into membrane K_{ATP} channels and mitochondrion K_{ATP} channels (mito K_{ATP}). K_{ATP} , especially mito K_{ATP} channels, have been demonstrated to be involved in short-term cardiac protection such as ischemia pre-conditioning. In this regard, opening of the mito K_{ATP} channels by diazoxide induces cardiac protection in nitric oxide (NO)-dependent manner (Wang et al. 2001b). Furthermore, Rakhit et al reported that the exogenous NO donor induces an early IPC through a cGMP-dependent mechanism and a delayed IPC by a cGMP-independent mechanism, which is mediated by activation of PKC and mito K_{ATP} channels (Rakhit et al. 2000). Thus, the cardiac protection of ischemia pre-conditioning is related with K_{ATP} , NO, and PKC. The aim of present study is to determine if RPerC protects heart against I/R injury through activation of opioid receptors via NO-PKC-mito K_{ATP} signaling pathway.

Materials and methods

Animals

All animal experiments were conducted in compliance with *the 1996 Guide for the*

Care and Use of Laboratory Animals (National Research Council, Bethesda, MD) and all procedures were reviewed and approved by the Ethics committee for the Use of Experiment Animals at Hebei Medical University.

Adult male Sprague-Dawley rats (250-280g in body weight) were purchased from the experimental animal center of Hebei Medical University. These rats were divided randomly into five groups: control group, pre-conditioning group, per-conditioning group, naloxone group, and glibenclamide group. All rats were housed at room temperature with a natural light-dark cycle and had free access to standard rodent diet and tap water.

Hemodynamics and left ventricular function measurement

The rats were anesthetized with urethane ($1\text{g}\cdot\text{kg}^{-1}$, i.p.). After the trachea being cannulated, the rats were ventilated mechanically through rodent respirator (1.0~1.5 ml/100 g, 60~120 strokes/min, model 680A, Harvard Apparatus, Holliston, MA). The blood pressure (BP) was monitored through a pressure transducer with a tubing cannulated into the left femoral artery. The BP signals were fed into a PowerLab data acquisition system (AD Instrument, Australia). The right femoral artery was isolated for I/R conditioning and right femoral vein was cannulated for administration of drugs. A cannula was placed into the left cardiac ventricle through the right carotid artery to record left ventricular systolic pressure (LVSP). The maximal rate of rise of left ventricular systolic pressure ($dp/dt_{\max}$) and the maximal rate of decline of left ventricular systolic pressure ($dp/dt_{\min}$) were recorded by differentiating LVSP. LVSP, $dp/dt_{\max}$ and $dp/dt_{\min}$ were used to assess the function of left ventricle. Rectal temperature was measured and kept at 37°C by using a servo-controlled heat blanket. Needle electrode was inserted subcutaneously in the legs and the standard lead II electrocardiography (ECG) was recorded throughout the experiment. The heart rate

(HR) was calculated from ECG signal.

Induction of myocardial I/R

Myocardial ischemia was induced as described previously (Zhou et al. 2015). In brief, after thoracotomy and pericardiotomy, a thin silk suture (6.0 metric) was placed superficially into the myocardium around the left anterior descending (LAD) coronary artery 2 mm proximal from its origin in the atrio-ventricular sulcus. The suture was passed through small diameter tubing to form a snare, which was used to occlude the LAD coronary artery when it was tightened. The presence of myocardial ischemia was confirmed by elevation of the S-T segment in ECG and an immediate fall in blood pressure of 15-30mmHg. For reperfusion, the snare was released, and reperfusion was visually confirmed by epicardial blushing, restoration of S-T segment in ECG gradually returned to the baseline level. A total of 30-min ischemia and 3-h reperfusion was performed. The changes of hemodynamics, left ventricular function and cardiac arrhythmia were recorded during I/R. The arrhythmia score was used to evaluate the degree of arrhythmia (Johnston et al. 1983; Walker et al. 1988).

Assessment of myocardial infarction

At the end of reperfusion, the heart was removed quickly and frozen at -20°C. The frozen heart was cut into 1.5-mm thin slices, which were perpendicular to the septum from the apex to the base. The myocardial slices were stained as described previously (Vivaldi et al. 1985). In brief, after 3-h reperfusion, 500 IU heparin was administered through the jugular vein to remove any clot in the heart. The LAD coronary artery was re-occluded, and 2 ml of 1.5% Evans Blue (Sigma, USA) was injected through the jugular vein to distinguish the non-ischemic area from area at risk (AAR). The heart was then excised, and the left ventricle was cut transversely into slices at 1-mm thickness and incubated at 37°C with TTC (triphenyltetrazolium

chloride; SD Fine Chemicals, Mumbai, India) solution (1% in phosphate buffer, pH 7.4) for 30 min. In the presence of dehydrogenase enzymes in the viable myocytes, NADH reacts with TTC to stain deep red while infarcted myocardium remains pale owing to the absence of dehydrogenases. The slices were fixed in 10% formalin for 6 h to obtain 3 colors: blue (non-ischemic area), deep and pale red (AAR as a whole), and unstained pale, representing the infarcted area (IA). The slices were sandwiched between 2 transparent slides and captured by using a scanner. The images were saved and analyzed in a blinded manner by computerized planimetry. Infarct size was expressed as the percentage of IA relative to AAR.

Performance of pre-conditioning and per-conditioning

RIPC was achieved by 5-min occlusion of right femoral artery followed by 5-min reperfusion and the occlusion-reperfusion was repeated for three times before LAD coronary artery occlusion. RPerC was performed during myocardial ischemia and before reperfusion. The femoral artery was exposed in the hind limb muscle where it joins the abdomen and isolated from the nerve and tissue in the pelvic region distal to any branch and occluded with a clip. During the ischemic period, the skin of the leg became purple and the temperature decreased. Reperfusion was performed by removing the clip and was confirmed when the skin color returned to rose color and temperature increased to the level before ischemia.

Western blot assay

Western blotting was performed as described previously (Xi et al. 2002). Tissue samples from the myocardium were homogenized in ice-cold lysis buffer. The total proteins were extracted. After protein quantification, 50 µg of total protein extracts were electrophoresed through polyacrylamide SDS gels and transferred by electroblotting onto PVDF membranes. Proteins were blocked with nonfat milk and

incubated with primary rabbit polyclonal antibody against iNOS (dilution 1:500; Abcam), eNOS (dilution 1:2000; Abcam), PKC ϵ (dilution 1:500; Abcam), and PKC δ (dilution 1:250; Abcam) at 4°C overnight. The membranes were then incubated with a secondary horseradish peroxidase-conjugated anti-rabbit or anti-goat IgG (1:1000 or 2000 dilution; Amersham) for 1 h at room temperature. The blots were developed using the ECL system (immobilion TM Western, Millipore) and were analyzed by Quantity One Software (Bio-Rad, USA). Protein contents were normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Proteintech) level. All experiments were repeated three times.

Research protocols

- I. Rats in control group were subjected to 30-min ischemia (occlusion of LAD coronary artery) followed by 3-h reperfusion (re-opening the LAD coronary artery).
- II. Rats in pre-conditioning group were subjected to remote pre-conditioning procedure: 5-min femoral artery occlusion followed by 1-min re-opening of the artery, which was repeated for three times before LAD coronary artery occlusion.
- III. Rats in per-conditioning group were subjected to remote per-conditioning procedure: 5-min femoral artery occlusion followed by 1-min re-opening of the artery, which was repeated for three times during LAD coronary artery occlusion.
- IV. Rats in naloxone group were received the same treatment as per-conditioning procedure and an injection of naloxone ($1.0 \text{ mg} \cdot \text{kg}^{-1}$, Sigma), an antagonist of opioid receptors, 5 min before per-conditioning procedure.
- V. Rats in glibenclamide group were received the same treatment as per-conditioning procedure and an injection of glibenclamide ($1.0 \text{ mg} \cdot \text{kg}^{-1}$, Sigma), an antagonist of K_{ATP} channels, 5 min before per-conditioning procedure.

Statistics analysis

All data were expressed as mean $\pm$ SEM. Differences in infarct sizes, biochemical values, and hemodynamics were compared by one-way ANOVA followed by Tukey-Kramer *post hoc* test for multiple comparisons. The comparison between same groups was made by student *t* test. Differences were considered significant at *P* values less than 0.05.

Results

Effect of RPerC on hemodynamics

The baseline systolic artery pressure (SAP), diastolic artery pressure (DAP) and heart rate (HR) showed no differences among groups. Cardiac ischemia decreased SAP significantly ($P < 0.05-0.01$, Table 1). However, pre-conditioning procedure attenuated the depressor effect induced by ischemia. In the following reperfusion phase, SAP still remained at lower level. Pre-conditioning procedure and per-conditioning procedure restored the SAP to baseline level from I/R-induced depressor response ($P < 0.05-0.01$). After 3-h reperfusion, SAP was significantly higher in pre-conditioning rats than in control rats ($P < 0.05$, Table 1). DAP was not changed during whole process of I/R in all groups ($P > 0.05$, Table 2).

There was no significant difference of HR during ischemia among all groups ($P > 0.05$). During reperfusion phase, HR was significantly decreased in control, naloxone, and glibenclamide rats compared with pre-conditioning and per-conditioning rats, respectively ($P < 0.05$, Table 3).

Effect of RPerC on left ventricular function

The parameters of left ventricular function were similar at basal condition among groups ($P > 0.05$). Ischemia procedure caused significant decreases in LVSP, $dp/dt_{\max}$

and dp/dt_{min} . Per-conditioning or pre-conditioning treatment significantly attenuated ischemia-induced reduction of LVSP, dp/dt_{max} and dp/dt_{min} . However, naloxone or glibenclamide injection eliminated the effects of per-conditioning and preconditioning treatment on these parameters. During reperfusion phase, LVSP, dp/dt_{max} and dp/dt_{min} were partly recovered gradually. The degree of recovery for LVSP, dp/dt_{max} and dp/dt_{min} was higher in per-conditioning and pre-conditioning groups than those in control, naloxone, or glibenclamide groups ($P<0.05-0.01$). In addition, the left ventricular function during I/R did not differ between control, naloxone or glibenclamide-treated rats ($P>0.05$, Figure 1).

Effect of RPerC on cardiac infarct size

After cardiac I/R, the ratios of infarct area to risk area were 42.0, 12.2, 11.5, 31.4 and 45.6% in control, pre-conditioning, per-conditioning, naloxone and glibenclamide rats, respectively. The ratio in per-conditioning rats was significantly smaller than in control, naloxone, or glibenclamide rats ($P<0.05-0.01$), but did not differ compared with pre-conditioning rats ($P>0.05$, Figure 2).

Effect of RPerC on ventricular arrhythmia

Arrhythmia including ventricular premature heartbeat, tachycardia, and fibrillation was induced by I/R procedure. The arrhythmia scores were 2.9, 1.5, 1.4, 2.1, and 3.0 in control, pre-conditioning, per-conditioning, naloxone and glibenclamide rats during ischemia and 4.1, 1.6, 2.0, 2.8, and 3.4 during reperfusion. The scores in pre-conditioning rats were significantly smaller than those in control, naloxone, and glibenclamide rats ($P<0.05$, Figure 3), but did not differ compared with per-conditioning rats during I/R ($P>0.05$, Figure 3). These results suggest that per-conditioning and pre-conditioning protected heart against I/R injury, and the protective effect was eliminated by pretreatment with naloxone or glibenclamide.

Effect of RPerC on expression of NOS in myocardium

Compared with control rats, the expression of iNOS and eNOS in per-conditioning rats was increased significantly ($P < 0.01$). The expression of iNOS was higher in naloxone or glibenclamide rats than in control rats, but was lower than in per-conditioning rats ($P < 0.05$). The expression levels of eNOS did not differ among control, naloxone, or glibenclamide rats ($P > 0.05$, Figure 4).

Effect of RPerC on expression of PKC in myocardium

Compared with control rat, PKC ϵ and PKC δ expression levels in per-conditioning rats were increased significantly ($P < 0.01$). The expression of PKC ϵ was similar among control, naloxone, or glibenclamide rats ($P > 0.05$). The expression of PKC δ in naloxone rats was increased compared with control rats, but decreased compared with per-conditioning rats ($P < 0.05$, Figure 5).

Discussion

In the present study, we examined the cardiac protective effect of RPerC during I/R. We also compared the protective effect of RPerC with that generated by RIPC. We found that RPerC, like RIPC, induced cardiac protection against I/R injury effectively. RPerC or RIPC procedure significantly improved the recovery of left ventricular function and reduced I/R-induced myocardial infarction. Our findings demonstrated that RPerC has significant cardiac protection against I/R injury. Unlike Zhu's report that RPerC induces much better cardiac protection than RIPC or RIPCpostC does (Zhu et al. 2013), our study demonstrated that RPerC produced the same level of protective effects against cardiac I/R injury as RIPC does. Furthermore, we found that the cardiac protective effect of RPerC was eliminated by naloxone, a blocker of opioid receptors, or glibenclamide, a blocker of K_{ATP} channels. These data suggest that opioid receptors and K_{ATP} channels are involved in the cardiac protection produced by RPerC

treatment.

Naloxone, a specific antagonist for opioid receptors, antagonizes the effect generated by both exogenous and endogenous opioids. Opioid receptors are divided into four sub-types: μ , δ , κ , and σ subtypes. The μ , δ , and κ opioid subunits are distributed in human and rodent heart tissues. Only δ and κ subunits are found in adult rat heart. It has been shown that cardiac protection of RIPC is related to opioid signaling pathways (Patel et al. 2002). Furthermore, cardiac protection of RIPC is closely related to δ opioid receptor, but not μ - or κ -opioid receptors (Schultz et al. 1998). Previous study has shown that κ -opioid receptors mediate the improvement of ischemia-induced infarction and arrhythmia through PKC and K_{ATP} pathways, while δ -opioid receptors are involved in improvement of ischemia-induced infarction (Wang et al. 2001a). It has been shown that K_{ATP} channels mediate the cardiac protection of δ -receptor. Activation of K_{ATP} channels inhibits Ca^{2+} influx to reduce Ca^{2+} overload in myocardium and leads to an alleviation of myocardium injury (Schultz et al. 1997). However, whether opioid receptor system is involved in the cardiac protection of RPerC is not previously known. In this study, we found that the cardiac protection of RPerC was abolished by naloxone, suggesting that the opioid receptors are involved in the cardiac protection of RPerC. However, we did not identify the receptor subtypes, which are involved in RIPC or RPerC. Thus, further studies are needed to identify the subtype of opioid receptors involved in cardiac protection by RIPC or RPerC (Maslov et al. 2016).

K_{ATP} channels play an important role in the cardiac protection under pathological situations. It has been reported that K_{ATP} channel openers suppress cardiac arrhythmia after ischemia reperfusion (Watanabe et al. 2011). Also, overexpression of K_{ATP} subunit increases resistance against cardiac ischemia (Du et al. 2006). Previous

studies focusing on mechanisms of remote pre- or post-conditioning have revealed that K_{ATP} channels also contribute to cardiac protection in remote ischemia conditioning (Loukogeorgakis et al. 2007). In addition, $mitoK_{ATP}$ channels play roles in cardiac protection of pre-conditioning and post-conditioning (Das and Sarkar 2005; Penna et al. 2006). Thus, it is likely that opening of K_{ATP} channels, especially $mitoK_{ATP}$ channels, serve as a common mechanism to prevent myocardium from I/R injury in variety of conditionings. Our study found that glibenclamide completely abolished the protective effect of RPerC in rats, suggesting that K_{ATP} channels in sarcolemma or mitochondria membrane participate in cardiac protection of RPerC against I/R injury.

It is well known that enhanced bioavailability of endogenous nitric oxide (NO) produced by the NO-synthases (NOS) mediates cardiac protection against I/R-induced injury. Both in vivo and in vitro studies have demonstrated that pharmacological inhibition of NO synthesis or genetic ablation of constitutive endothelial NOS (eNOS) exerts detrimental effects during myocardial infarction. On the other hand, NO donors or overexpression of eNOS or iNOS reduce myocardial injury (Jones et al. 2004; Jugdutt 2002; Kanno et al. 2000; Xuan et al. 2007). In this study, we found that the expression levels of eNOS and iNOS were increased in RPerC rats compared with control rats, suggesting that NO is involved in the cardiac protection produced by RPerC.

Previous studies have shown that opioid, PKC, $mitoK_{ATP}$ channels and NO in IPC or RIPC interact (Cohen and Downey 2015; Maslov et al. 2016). Liu *et al.* reported that IPC is triggered by receptor occupancy (Liu et al. 1991). Schultz *et al.* reported that opioid, an endogenously released substances during I/R, binds to its receptors (Gprotein-coupled receptor) and the second messenger G_i protein is cleaved into active α and $\beta\gamma$ subunits which then results in activation of PKC (Schultz et al.

1995). After being activated and translocated to the cell membrane, PKC phosphorylates multiple intracellular substrates and leads to the opening of the mitoK_{ATP} channels (Downey et al. 2007; Schulz et al. 2001). The opening of mitoK_{ATP} channel is a critical step in cardiac protection signaling cascade. Opening of mitoK_{ATP} blocks the mitochondrial permeability transition pore (MPTP) and inhibits apoptosis (Costa and Garlid 2008). IPC's end-effector appears to be the MPTP and the inhibition of MPTP is considered to be the final step in the protective signal transduction pathway of pre-conditioning (Di Lisa et al. 2001; Hausenloy et al. 2002). Recent studies have shown that PKC ϵ translocation may be a downstream event of mitoK_{ATP} channels (Wang et al. 2015). It is possible that there is feedback regulation between PKC and mitoK_{ATP} channels. Studies on cardiac protection induced by IPC showed that activation of PKC leads to the opening of the mitoK_{ATP} channels which triggers the modest ROS formation and then activates PKC (Downey et al. 2007). NO is an important biological regulator and cellular signaling molecule. The eNOS has been implicated in the preconditioning pathways between Gi/o-coupled receptors and PKC (Maslov et al. 2016). Thus, we propose that opioid produced during ischemia in RPerC may activate NOS, PKC, and mitoK_{ATP} channels and inhibit MPTP to produce cardiac protection. The blockade of any elements in this signaling pathway may result in a loss of cardiac protection.

In summary, this study demonstrated for the first time that RPerC protects heart against I/R injury through activation opioid receptor system and NO-PKC-mitoK_{ATP} channel signaling pathways.

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Tables

Table 1. Effect of per-conditioning on systolic arterial pressure during I/R in rats

	Baseline (mmHg)	Ischemia (mmHg)	Reperf-15min (mmHg)	Reperf-1h (mmHg)	Reperf-2h (mmHg)	Reperf-3h (mmHg)
CON	110.7±7.4	90.6±5.7#	92.4±5.2#	91.6±7.1#	88.8±7.4#	81.3±8.6#
Pre-C	114.6±9.4	98.9±6.5	101.3±7.9	103.0±9.5	95.9±10.1	92.4±10.6
Per-C	109.6±2.9	90.5±5.7#	95.3±7.9	97.6±6.0	103.0±4.5	105.8±4.0*
Nal	117.6±3.0	97.0±7.9#	100.6±7.0#	99.9±6.4#	101.3±7.0#	101.2±6.9#
Gli	116.8±3.1	90.4±7.2##	93.3±2.9##	100.2±2.5##	100.8±4.4#	100.9±5.3#

CON: control group; Pre-C: pre-conditioning group; Per-C: per-conditioning group; Nal: Naloxone group; Gli: glibenclamide group; Reperf-: reperfusion; All data were expressed as Mean±SEM, $n = 8$ for each group, * $P < 0.05$ vs CON # $P < 0.05$ ## $P < 0.01$ vs Baseline

Table 2. Effect of per-conditioning on diastolic artery pressure during I/R in rats

	Baseline (mmHg)	Ischemia (mmHg)	Reperf-15min (mmHg)	Reperf-1h (mmHg)	Reperf-2h (mmHg)	Reperf-3h (mmHg)
CON	52.6±3.8	49.3±4.8	45.9±3.9	44.6±5.8	49.8±6.1	45.7±7.7
Pre-C	61.4±5.3	53.0±2.7	52.5±4.3	55.2±5.2	52.1±5.5	46.8±6.1
Per-C	57.2±5.1	56.8±4.5	59.2±5.6	58.2±4.0	63.4±7.4	60.4±5.8
Nal	62.0±3.5	56.3±4.7	56.2±4.9	58.1±3.7	58.9±4.1	59.8±4.2
Gli	65.4±7.4	59.3±7.9	56.2±5.5	60.1±4.9	60.4±6.8	60.2±7.4

CON: control group; Pre-C: preconditioning group; Per-C: per-conditioning group; Nal: Naloxone group; Gli: glibenclamide group; Reperf-: reperfusion; All data were expressed as Mean±SE, $n = 8$ for each group

Table 3. Effect of per-conditioning on heart rate during I/R in rats

	Baseline	Ischemia	Reperf-15min	Reperf-1h	Reperf-2h	Reperf-3h
	(mmHg)	(mmHg)	(mmHg)	(mmHg)	(mmHg)	(mmHg)
CON	374.7±18.0	361.0±8.0	321.5±29.6	313.9±13.6#	321.6±9.4#	311.6±7.4##
Pre-C	384.2±20.8	363.3±28.5	351.5±24.8	347.7±16.4	337.4±11.3	332.7±16.0
Per-C	377.5±13.6	373.6±12.6	362.4±11.9	344.2±12.2	337.4±11.8	334.4±12.2
Nal	388.6±8.7	358.8±12.6	315.8±11.7##	334.3±10.8##	325.2±8.1##	326.4±10.0##
Gli	370.9±18.6	325.3±15.7	312.1±15.2#	308.8±10.1#	307.4±8.7##	299.9±7.1##

CON: control group; Pre-C: Preconditioning group; Per-C: per-conditioning group; Nal: Naloxone group; Gli: glibenclamide group; Reperf-: reperfusion; All data were expressed as Mean±SEM, *n* = 8 for each group, #*P*<0.05
 ##*P*<0.01 vs Baseline

Figurelegend

Figure 1. Effect of RPerC on left ventricular function. Data were expressed as Mean $\pm$ SEM, $n = 8$ for each group * $P < 0.05$ ** $P < 0.01$ vs CON # $P < 0.05$ ## $P < 0.01$ vs Pre-C + $P < 0.05$ ++ $P < 0.01$ vs Per-C. LVSP: left ventricular systolic pressure; dp/dt_{max} : maximal rate of rise of left ventricular systolic pressure; dp/dt_{min} : maximal rate of decline of left ventricular systolic pressure; CON: control group; Pre-C: pre-conditioning group; Per-C: per-conditioning group; Nal: naloxone group; Gli: glibenclamide group.

Figure 2. Effect of RPerC on myocardial infarct size. A. The representative staining of myocardium in rats. B. Myocardial infarct size in different groups. Blue stands for non-ischemic area; Deep and pale red represents area at risk; Unstained pale represents the infarcted area. Data were expressed as Mean $\pm$ SEM, $n = 4$ for each group * $P < 0.05$ ** $P < 0.01$ vs CON # $P < 0.05$ ## $P < 0.01$ vs Per-C. CON: control group; Pre-C: pre-conditioning group; Per-C: per-conditioning group; Nal: Naloxone group; Gli: glibenclamide group;

Figure 3. Effect of RPerC on arrhythmia induced by I/R in rat. Data were expressed as Mean $\pm$ SE, $n = 8$ for each group * $P < 0.05$ ** $P < 0.01$ vs CON, # $P < 0.05$ vs Per-C. AS: arrhythmia score; CON: control group; Pre-C: pre-conditioning group; Per-C: per-conditioning group; Nal: Naloxone group; Gli: glibenclamide group.

Figure 4. Effect of RPerC on expression of NOS in myocardium. Data were expressed as Mean $\pm$ SEM, $n = 4$ for each group. ** $P < 0.01$ vs CON, ## $P < 0.01$ vs Per-C. CON: control group; Pre-C: pre-conditioning group; Per-C: per-conditioning group; Nal: Naloxone group; Gli: glibenclamide group.

Figure 5. Effect of RPerC on expression of PKC in myocardium. Data were expressed as Mean $\pm$ SEM, $n = 4$ for each group ** $P < 0.01$ vs CON, ## $P < 0.01$ vs Per-C. CON: control group; Pre-C: pre-conditioning group; Per-C: per-conditioning group; Nal: Naloxone group; Gli: glibenclamide group.

A **B**

Figure 4

Figure 5