Original Article
Protective effects of remote ischemic preconditioning in isolated rat hearts

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Abstract: To use Langendorff model to investigate whether remote ischemic preconditioning (RIPC) attenuates post-ischemic mechanical dysfunction on isolated rat heart and to explore possible mechanisms. SD rats were randomly divided into RIPC group, RIPC + norepinephrine (NE) depletion group, RIPC + pertussis toxin (PTX) pretreatment group, ischemia/reperfusion group without treatment (ischemia group) and time control (TC) group. RIPC was achieved through interrupted occlusion of anterior mesenteric artery. Then, Langendorff model was established using routine methods. Heart function was tested; immunohistochemistry and ELISA methods were used to detect various indices related to myocardial injury. Compared with ischemia group in which the hemodynamic parameters deteriorated significantly, heart function recovered to a certain degree among the RIPC, RIPC + NE depletion, and RIPC + PTX groups (P<0.05). More apoptotic nuclei were observed in ischemia group than in the other three groups (P<0.05); more apoptotic nuclei were detected in NE depletion and PTX groups than in RIPC group (P<0.05). While, there was no significant difference between NE depletion and PTX groups. In conclusion, RIPC protection on I/R myocardium extends to the period after hearts are isolated. NE and PTX-sensitive inhibitory G protein might have a role in the protection process.

Keywords: Remote ischemic preconditioning, norepinephrine, heart transplantation, heart function

Introduction
Gho and colleagues showed that even brief ischemia in organs other than the heart was able to protect the heart [1]. This has been confirmed by others [2, 3]. This maneuver, which is now termed remote ischemic preconditioning (RIPC), features in repeated transitory ischemia-reperfusion treatment on certain organs or tissues (such as kidney, small intestine, extremities etc.) outside the heart. It has been shown that RIPC has similar protective effects on heart as traditional ischemic preconditioning [4, 5].

RIPC may find ready applicability in scheduled global cardiac ischemic/reperfusion (I/R) during transplantation, where such a maneuver is feasible before arrest. Previous studies showed that nervous and humoral factors are involved for RIPC protection effects against myocardial I/R [1, 6]. However, it is still unknown whether RIPC protection on I/R myocardium extends to the period after hearts are isolated. Furthermore, it has been established that traditional ischemia preconditioning protect myocardium via stimulation of α-adrenoceptors by endogenous catecholamines through activation of PTX-sensitive G protein which is a key step for activation of protein kinase C [7]. But it is unclear as for the role of norepinephrine and PTX-sensitive inhibitory G protein in RIPC for isolated rat heart undergoing I/R insults.

In the current study, we used a murine Langendorff model to investigate whether RIPC attenuates postischemic mechanical dysfunction on isolated rat heart and to explore possible mechanisms.

Materials and methods
Grouping, RIPC model, and preparation of Langendorff model

All animal experiments were conducted in accordance with humane animal care standards.
This study was specifically approved by the Institutional Animal Care and Use Committee (IACUC) of Fuwai Hospital and Fuwai hospital Ethics Committee (Beijing, China).

One hundred 3-4 month-old Sprague-Dawley (SD) rats (body weight: 250-280 g), provided by the Experimental Animal Center of Fuwai Hospital, Chinese Academy of Medical Sciences, were randomly divided into RIPC group (n=20), RIPC + norepinephrine (NE) depletion group (n=20), RIPC + pertussis toxin (PTX) pretreatment group without treatment (ischemia group, n=20) and time control (TC) group (n=20). In RIPC + NE depletion group, reserpine was administered as a 0.5 mg/kg IP dose 24 hours before study to deplete endogenous norepinephrine stores [8]; in RIPC + PTX pretreatment group, PTX was administered as a dose of 25 μg/kg IP 48 hours before study to block PTX-sensitive G proteins and prevent the protective effects of RIPC as previously described [8, 9]. During RIPC, heparin (1000 U/kg) was administered 10 minutes before anesthesia. As for anesthetic induction, 2.5% pentobarbital of sodium with a dose of 50 mg/kg IP 48 hours before study to block PTX-sensitive G proteins and prevent the protective effects of RIPC as previously described [8]; in RIPC + PTX pretreatment group, PTX was administered as a dose of 25 μg/kg IP 48 hours before study to block PTX-sensitive G proteins and prevent the protective effects of RIPC as previously described [8, 9]. During RIPC, heparin (1000 U/kg) was administered 10 minutes before anesthesia. As for anesthetic induction, 2.5% pentobarbital of sodium with a dose of 50 mg/kg IP 48 hours before study to block PTX-sensitive G proteins and prevent the protective effects of RIPC as previously described [8]. Ischemia/reperfusion group without treatment (ischemia group, n=20) and time control (TC) group (n=20). In RIPC + NE depletion group, reserpine was administered as a 0.5 mg/kg IP dose 24 hours before study to deplete endogenous norepinephrine stores [8]; in RIPC + PTX pretreatment group, PTX was administered as a dose of 25 μg/kg IP 48 hours before study to block PTX-sensitive G proteins and prevent the protective effects of RIPC as previously described [8, 9]. During RIPC, heparin (1000 U/kg) was administered 10 minutes before anesthesia. As for anesthetic induction, 2.5% pentobarbital of sodium with a dose of 50 mg/kg IP 48 hours before study to block PTX-sensitive G proteins and prevent the protective effects of RIPC as previously described [8]. During RIPC, heparin (1000 U/kg) was administered 10 minutes before anesthesia. As for anesthetic induction, 2.5% pentobarbital of sodium with a dose of 50 mg/kg IP 48 hours before study to block PTX-sensitive G proteins and prevent the protective effects of RIPC as previously described [8]. During RIPC, heparin (1000 U/kg) was administered 10 minutes before anesthesia. As for anesthetic induction, 2.5% pentobarbital of sodium with a dose of 50 mg/kg IP 48 hours before study to block PTX-sensitive G proteins and prevent the protective effects of RIPC as previously described [8].

Heart function examination

Twenty isolated hearts in each group underwent heart function examination. A small latex balloon connected to a pressure transducer was inserted through the left atrium and pushed through the mitral valve into the left ventricle. The balloon was filled with saline water to achieve an end-diastolic pressure for 0-10 mmHg. Then, the balloon volume remained unchanged during the following experiment. All signals were recorded with Labchart 7.0 (AD Instruments Inc, Colorado Springs, CO, USA) by a computer (Lenovo, Beijing, China). The left ventricular developed pressure (LVDP), left ventricular end-diastolic pressure (LVEDP), heart rate (HR), and maximal/minimal first derivatives of left ventricular pressure (+dp/dt) were analyzed by the Labchart software (AD Instruments Inc, Colorado Springs, CO, USA).

Myocardial injury examination

Myocardial infarct size (IS) was determined by 2,3,5-TTC staining. At the end of the experiments, hearts were frozen at -20°C for 2 hours and subsequently cut into five cross-section
RIPC in isolated hearts

<table>
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<th></th>
<th>TC (n=20)</th>
<th>Ischemia (n=20)</th>
<th>RIPC alone (n=20)</th>
<th>RIPC + NE depletion (n=20)</th>
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<td>LVDP (mmHg)</td>
<td>119.0±11.0</td>
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<td>62.6±9.7*</td>
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<td>LVEDP (mmHg)</td>
<td>6.0±0.7</td>
<td>6.3±1.0</td>
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<td>42.5±7.7*</td>
<td>27.0±12.1*,*§</td>
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<td>+dp/dt (mmHg/s)</td>
<td>3236.0±257.0</td>
<td>3241.0±310.0</td>
<td>3185.6±396.0</td>
<td>1650.5±439.2*</td>
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<td>-dp/dt (mmHg/s)</td>
<td>2439.0±273.0</td>
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<td>Heart rate (1/min)</td>
<td>293.0±20.2</td>
<td>299.0±11.2</td>
<td>315.4±25.9</td>
<td>161.9±20.2*</td>
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<td>312.4±29.5</td>
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Data are presented as mean ± SD. *P<0.05 vs baseline; §P<0.05 vs TC group; †P<0.05 vs RIPC group. TC= time control; RIPC= remote ischemic preconditioning; I/R= ischemia/reperfusion; NE= norepinephrine; PTX= pertussis toxin; LVDP= left ventricular developed pressure; LVEDP= left ventricular end-diastolic pressure; +dp/dt= maximal first derivatives of left ventricular pressure; -dp/dt= minimal first derivatives of left ventricular pressure.
slices. Heart slices were incubated in a 1% TTC in 0.1 mol/L phosphate buffer solution (pH 7.4) at 37°C for 20 minutes, and fixed overnight in 10% formaldehyde. Then, infarcted myocardial areas (pale color) can be differentiated from viable ones (brick-red color). Slices were photographed by digital camera, and computerized planimetry using Image J 1.37 software was performed. IS was determined by dividing the total necrotic area of the left ventricle by the total left ventricle slice area. For IS determination, ten hearts (n=10) were assessed in each experimental group.

As additional markers of myocardial injury, LDH and CK-MB were determined from collected effluent in all three groups by an automatic biochemistry analyzer (Hitachi 7600, Tokyo, Japan) using commercial LDH and CK-MB assay kits (Roche Diagnostics, Mannheim, Germany). cTnI levels were also assayed by ACS: 180 automated chemiluminescence system (Bayer Corp., Tarrytown, NY, USA) with commercial kits (Bayer Corp.). For these purposes, coronary effluent (1 ml) was spot collected from hearts during equilibration and at the end of experiments.

**Apoptosis indices**

mPTP opening determination: After 15 minutes of reperfusion, nicotinamide adenine dinucleotide (NAD+) was extracted from left ventricular tissue (n=10) using the Klingenberg method [11]. NAD+ concentrations were then determined fluorometrically using ADH at a wavelength of 340 nm (DU 640, Beckman Coulter, Fullerton, CA, USA).

Apoptosis assay: Double staining was employed using the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) assay and DAPI staining [12]. Cardiomyocyte apoptosis was assessed by TUNEL assay. Briefly, apoptotic cells were identified using an in situ cell death detection kit (Roche, Mannheim) following the manufacturer’s instructions. For each group, twenty randomly selected fields were assessed.

**Figure 2.** Indices of post I/R myocardial injury among groups (Bars represent mean ± SD). A. LDH level in the coronary effluent. B. CK-MB level in the coronary effluent. C. cTnI level in the coronary effluent. *P<0.05 vs TC group; §P<0.05 vs Ischemia group.

**Figure 3.** mPTP opening testing by NAD+ level measurements among groups (Bars represent mean ± SD). *P<0.05 vs TC group; §P<0.05 vs Ischemia group; †P<0.05 vs RIPC group.
Figure 4. Apoptosis assay. Time control group was not included in the figure because no significant apoptosis staining was identified. A. RIPC alone. B. Ischemia group. C. RIPC + NE depletion. D. RIPC + PTX. E. Apoptosis index. Data are presented as mean ± SD. *$P<0.05$ vs Ischemia group; §$P<0.05$ vs RIPC group.
(10 hearts per group, four fields per heart) were observed. And apoptotic index (AI), or the percentage of apoptotic nuclei (TUNEL-positive) vs. total number of nuclei, was determined.

### Statistical method

Statistical analysis was conducted using SPSS15.0 statistical software, measurement data were represented using mean ± standard deviation (SD), repeated measurement ANOVA was used for inter-group comparison. All tests are two-tailed and statistical differences with $P<0.05$ were considered significant.

### Results

#### Heart function comparison after I/R insults among the four groups

As is shown in Table 1, hemodynamic variables at baseline were not significantly different among groups. Compared with ischemia group in which the hemodynamic parameters deteriorated significantly, heart function parameters such as LVEDP recovered to a certain degree among the RIPC, RIPC + NE depletion, and RIPC + PTX groups (Ischemia vs RIPC, RIPC + NE, RIPC + PTX=42.5±7.7 vs 27.0±12.1, 35.1±10.6, 35.7±11.6, $P<0.05$). As for LVDP, ±dp/dt, and heart rate, the recovered indices were significantly higher in RIPC group than in the RIPC + NE depletion or RIPC + PTX groups ($P<0.05$) but there was of no significant differences between RIPC + NE depletion and RIPC + PTX groups.

#### Myocardial infarct size

As is shown in Figure 5, RIPC provided significant protection in the hearts resulting in a lower infarct size ($P<0.05$), the volume of infarction was also decreased in NE depletion and

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**Figure 5.** Myocardial infarction size in Ischemia, RIPC, NE depletion, and PTX groups (Bars present mean ± SD). Time control group was not included in the figure because no significant necrotic area was identified. *$P<0.05$ vs Ischemia group; $\$P<0.05$ vs RIPC group.
PTX group (P<0.05), however, there was of no significant difference between NE and PTX groups.

Discussion

In the current study, we found that RIPC had protective effects on isolated heart in murine Langendorff model. The depletion of endogenous NE mitigated the protective effects. PTX administration mimicked the NE depletion phenomenon, in which RIPC protective effects were partly abolished but not completely. In parallel with the above observation, apoptosis were depressed in RIPC group and partly depressed in NE depletion and PTX groups.

I/R insults represent a major clinical challenge. Under heart transplantation scenario, the donor hearts undergo typical global I/R process. The contemporary process of transplantation all over the world requires the exposure of the donor heart to periods of cold ischemia as well as a period of warm ischemia. It is documented that this I/R not only decreases graft quality but also increases immunogenicity, thus leading to increased probability of catastrophe [13]. As a result, various protocols were developed aiming at mitigating I/R injury during heart transplantation [14, 15]. RIPC is an intriguing maneuver for the control of I/R injury to myocardium in that the pretreatment protocols don’t necessarily involve the heart, and therefore is more feasible in clinical settings.

Previous studies suggested that the sympathetic nervous system may have a role in cardiac preconditioning. For example, through stimulation of the left stellate cardiac nerve, Iwamoto and co-workers observed preconditioning phenomenon in the heart [16]. However, in the current study, the heart was still under RIPC protection even after taken from donor, indicating that substances endogenous to the heart may be involved in the protection process.

Several studies suggested that cardiac preconditioning can be achieved by increasing endogenous catecholamine release [17]. Banerjee A and colleagues indicated in a rat model that NE release increased after local transient ischemia [18]. In the present study, we observed the depletion of endogenous NE also blocked the protective effects, which was in support of the above studies. However, the actual role of endogenous NE in RIPC precondition was somewhat conflicting in the literature [19]. Different animal models or multiple and parallel mechanism involved may confound the results. Half life and critical time window mismatches between mimetic agents and preconditioning reaction may also contributed to the conflicting outcomes.

Stimulation of α-adrenoceptors by endogenous catecholamines can exert preconditioning effects through the activation of protein kinase C (PKC) via such G protein. The latter was demonstrated to be activated by α1-adrenoceptor agonists, and may play a central role in ischemic preconditioning mediated by PKC across a broad range of species [20, 21]. In the current study, PTX treated subjects presented abolishing effects, indicating that in RIPC process, α-adrenoceptors might also be an important pathway for endogenous NE to take protective effects. However, we also found that PTX didn’t block the RIPC effects completely at the presence of NE. Other pathways, such as β-adrenoceptor which involves in classical ischemic preconditioning [22], might have a role in RIPC.

In parallel with the abolished protective effects in NE depletion and PTX groups, we found decreased tissue content of NAD+ and increased apoptosis index when compared with RIPC alone. Putative mechanism is the extracellular signal regulated protein kinase (ERK) [23], pathway through activation of α-adrenoceptors by endogenous NE. PTX administration partly blocked this theoretical pathway, suggesting the involvement of G protein and PKC. These findings were in contrast to previous studies which indicated positive associations between NE and myocytes apoptosis. However, apoptosis was induced by excessive NE dose or in heart failure models [24, 25]. What’s more, Lai and colleagues suggested mechanisms different from adrenoceptors in the process of NE induced apoptosis in cultured cardiac fibroblast [25].

The major limitation of the current study underlines the generalization of results from Langendorff model to in vivo settings. Langendorff model is not a real heart transplantation model, but this model can more directly reflect the influence of I/R and eliminate the effects of immunological rejection. We will establish car-
RIPC in isolated hearts

diac transplantation model to explore this question in the future. Another limitation was the lack of complete data to the assessment of heart function, the severe injury produced a dramatic increase in LVEDP might limits the evaluation of systolic elastance. Moreover, although the intervention improved post-ischemic function, we don’t established the mechanisms involved, further studies in underlying mechanism are needed to confirm the results of the present study.

In conclusion, RIPC protection on I/R myocardium extends to the period after hearts are isolated. NE and PTX-sensitive inhibitory G protein might have a role in the protection process. RIPC may find ready applicability in scheduled global cardiac I/R such as transplantation. However, the mechanisms remain to be further clarified.

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Disclosure of conflict of interest

None.

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References

RIPC in isolated hearts


