Combination of remote ischemic perconditioning and remote ischemic postconditioning fails to increase protection against myocardial ischemia/reperfusion injury, compared with either alone

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Abstract. Remote ischemic perconditioning (RIPerC) and remote ischemic postconditioning (RIPostC) have been previously demonstrated to protect the myocardium against ischemia/reperfusion (IR) injury. However, their combined effects remain to be fully elucidated. In order to investigate this, the present study used an *in vivo* rat model to assess whether synergistic effects are produced when RIPerC is combined with RIPostC. The rats were randomly assigned to the following groups: Sham, IR, RIPerC, RIPostC and RIPerC + RIPostC groups. The IR model was established by performing 40 min of left coronary artery occlusion, followed by 2 h of reperfusion. RIPerC and RIPostC were induced via four cycles of 5 min occlusion and 5 min reperfusion of the hindlimbs, either during or subsequent to myocardial ischemia. On measurement of infarct sizes, compared with the IR group (49.45±6.59%), the infarct sizes were significantly reduced in the RIPerC (34.36±5.87%) and RIPostC (36.04±6.16%) groups ($P<0.05$). However, no further reduction in infarct size was observed in the RIPerC + RIPostC group (31.43±5.43%; $P>0.05$), compared with the groups treated with either RIPerC or RIPostC alone. Activation of the reperfusion injury salvage kinase (RISK) Akt, extracellular signal-regulated kinase 1/2 and glycogen synthase kinase-3β, and survivor activating factor enhancement (SAFE) signal transducer and activator of transcription-3 pathways were enhanced in the RIPerC, RIPostC and the RIPerC + RIPostC groups, compared with the IR group, with no difference among the three groups. Therefore, whereas RIPerC and RIPostC were equally effective in providing protection against myocardial IR injury, the combination of RIPerC and RIPostC failed to provide further protection than treatment with either alone. The cardioprotective effects were found to be associated with increased activation of the RISK and SAFE pathways.

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Abbreviations: RIPerC, remote ischemic perconditioning; RIPostC, remote ischemic postconditioning; LV, left ventricular; LCA, left coronary artery; IS, infarct size; AAR, area at risk; AN, area of necrosis; SAFE, survivor activating factor enhancement; RISK, reperfusion injury salvage kinase

Key words: myocardial ischemia/reperfusion injury, remote preconditioning, remote postconditioning, survival kinase, heart

Introduction

In acute coronary events, the establishment of early and successful myocardial reperfusion is the most effective strategy to limit infarct size (IS) and improve clinical outcomes. However, reperfusion may induce further damage to the myocardium itself (1). Remote ischemic conditioning describes an innate cardioprotective mechanism, in which brief periods of sublethal ischemia and reperfusion are applied to a remote organ in order to protect the myocardium against the detrimental effects of prolonged reperfusion injury (2). This was first identified by Przyklenk *et al* (3) in a canine model; in which it was demonstrated that brief episodes of ischemia in the circumflex branch protected remote virgin myocardium from subsequent sustained left coronary artery ischemia. Since then, the use of this procedure has been extended in a series of experiments, demonstrating that intermittent ischemia of several different remote organs induces protection against subsequent myocardial ischemia/reperfusion (IR) injury (4,5). The fact that remote ischemic conditioning can be performed noninvasively using a blood pressure cuff on the upper/lower limb made it more clinically feasible, compared with conventional local ischemic conditioning (6). In addition, unlike local ischemic conditioning, remote ischemic conditioning can be applied during all three windows of IR, including prior to (remote ischemic preconditioning; RIPC), during (remote ischemic perconditioning; RIPerC) and following (remote

ischemic postconditioning; RIPostC) myocardial ischemia. Considering the unpredictable nature of myocardial ischemic events, RIPerC and RIPostC appear to be more practical than RIPC in clinical settings, however, neither are as effective as local ischemic preconditioning in terms of the ability to limit IS (7). Our previous study demonstrated that the combination of RIPerC and local ischemic postconditioning (IPostC) produces synergistic effects and reinforces the cardioprotective activities of local ischemic preconditioning. However, RIPostC remains an invasive procedure and has a limited time frame of use (8). Thus, it may be beneficial to investigate the combination of two non-invasive procedures, RIPerC and RIPostC, and determine whether these result in an additive effect in the protection against myocardial IR injury. To investigate this hypothesis, the present study analyzed the protective efficacy of the combined use of RIPerC and RIPostC against myocardial IR injury using an *in vivo* rat IR model, and the results were compared with the use of either RIPerC or RIPostC alone.

Materials and methods

Animals. A total of 90 male Sprague-Dawley rats (8-week-old), weighing between 250 and 280 g (Experimental Animal Center, Fudan University, Shanghai, China) were used in the present study. All rats were housed at a controlled temperature (25˚C) under a 12-h light/dark cycle with *ad libitum* access to food and water. The animal investigation protocol used was in compliance with the Guide for the Care of Use of Laboratory Animals published by the National Institutes of Health (NIH Publication no. 85-23, revised 1996) (9) and approved by the Animal Care Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital, (Shanghai, China). All rats were housed for 2 weeks to provide an acclimatization period prior to the experiments.

Surgical preparation. The IR model was performed, as previously described (10). In brief, the rats were anesthetized by intraperitoneal injection with 1.2% pentobarbital sodium (Sigma-Aldrich, St. Louis, MO, USA), at a dose of 50 mg/kg. The left coronary artery (LCA) was ligated using a 6-0 Prolene suture immediately distal to its first branch, and cardiac ischemia was confirmed by the formation of a pale area below the suture, which gradually became cyanotic. After 40 min, the suture was released, and reperfusion was characterized by the rapid disappearance of cyanosis, followed by vascular blush. Following 120 min of reperfusion, the rats were sacrificed with an overdose of pentobarbital sodium (150 mg/kg) and the hearts were harvested for further assessment. For rats undergoing sham surgery, a suture was placed in a corresponding location without ligation.

RIPerC and RIPostC were delivered via non-invasive occlusion of both lower limbs using tourniquets, which was validated by the disappearance of Doppler blood flow of the femoral artery (5-10 MHz; M-Turbo System L38X; SonoSite, Inc., Bothell, WA, USA). Both RIPerC and RIPostC consisted of four cycles of 5-min limb ischemia/reperfusion, with RIPerC initiated at the onset of coronary, while RIPostC initiated at the onset of coronary reperfusion, as shown in Fig. 1.

Experimental protocols. The rats were randomly assigned to the following experimental groups: i) Sham group (n=6; rats underwent sham surgery); ii) IR group (n=6; rats underwent 40 min left anterior descending artery occlusion followed by 2 h reperfusion) iii) RIPerC group (n=6; as in the IR group, in addition to four cycles of 5 min bilateral hindlimb occlusion followed by 5 min reperfusion during myocardial ischemia); iv) RIPostC group (n=6; as in the IR group, with four cycles of 5 min bilateral hindlimb occlusion followed by 5 min reperfusion at the onset of coronary reperfusion); v) RIPerC + RIPostC group (n=6; RIPerC combined with RIPostC. Following 2 h of subsequent reperfusion, all rats were sacrificed for IS quantification (Fig. 1).

An additional 30 Sprague-Dawley rats (six in each group) underwent the procedures described above and were also sacrificed 2 h following reperfusion. In these rats, a cross section of the left ventricular (LV) myocardium (~5 mm thick) at the papillary muscle level was obtained from each rat for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. The remaining myocardium from the area at risk (AAR) was collected for quantification of the protein expression levels of B cell lymphoma (Bcl)-2 and Bcl-2-associated X protein (Bax).

In addition, a further six rats from each group underwent the same procedure, were sacrificed following 40 min of reperfusion, and the myocardium from the AAR was obtained for western blot analysis. All tissues were snap-frozen in liquid nitrogen (Shanghai Jiangnan Gas Co., Ltd., Shanghai, China) and stored in a freezer at -80˚C.

AAR and IS determination. Following 2 h of reperfusion, the LCA was re-occluded, and 2% Evans blue dye (Sigma-Aldrich) was retrogradely injected into the ascending aorta to delineate the AAR. The heart was removed and sliced transversely from the base to the apex into five sections (2-3 mm), which were incubated for 15 min at 37˚C in a phosphate-buffered 1% 2,3,5-triphenyltetrazolium chloride solution (Sigma-Aldrich) to determine the infarcted area. All slices were then fixed in 10% formalin (Goodbio, Shanghai, China), and the extent of the area of necrosis was quantified by computerized planimetry using ImagePro Plus software, version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA) and corrected for the weight of the tissue slices. IS is expressed as the percentage of total weight of the LV AAR.

Determination of serum cardiac troponin I (cTnI) and inflammatory cytokines. Subsequent to 2 h of reperfusion, blood samples were collected into tubes containing microscopic silica particles and rested for 30 min. Following centrifugation at 2,500 x g for 10 min at 25˚C, the supernatants were collected and stored at -80˚C until required for future analysis. The serum levels of cTnI, tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β) were assessed using Cardiac Troponin-I enzyme-linked immunosorbent assay (ELISA) (cat. no. CTNI-HS), Rat TNF-alpha Platinum ELISA (cat. no. BMS622), and Rat IL-1 beta Platinum ELISA (cat. no. BMS630) kits, according to the manufacturer's instructions (cTnI, Life Diagnostics, Inc., West Chester, PA, USA; TNF-α and IL-1β, eBioscience, Inc., San Diego, CA, USA). Levels of cTnI were expressed as ng/ml, whereas levels of cytokines were expressed as pg/ml.

Assessment of LV function. The right carotid artery was cannulated using a 1.6F Pressure Catheter (Transonic Scisense, Inc., London, ON, Canada) for measuring the hemodynamic parameters. The catheter was passed retrogradely into the LV, and LV pressure tracings were digitized using a PowerLab Physiological Recorder (ADInstruments Pty, Ltd., Bella Vista, Australia) and stored for later analysis. The LV end-diastolic pressure (LVEDP), maximum/minimum first derivative of LV pressure over time (± dP/dt_{max}) and mean arterial pressure (MAP) were analyzed in a blinded-manner using LabChart software, version 8 (ADInstruments Pty Ltd, Oxford, UK).

TUNEL staining. TUNEL staining was performed using a commercially available kit (In Situ Cell Death Detection kit; Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's protocol, on heart tissue slices randomly selected from each group (n=6 tissue slices/group). A minimum of 100 cells from the peri-infarct area were counted using a microscope (magnification, x400; Q500MC; Leica Microsystems GmbH, Wetzlar, Germany) in 10 fields for each sample. The peri-infarct area was predetermined using hematoxylin and eosin (Goodbio) staining, which was performed on the adjacent tissue slide. The percentages of cells positive for TUNEL staining were calculated as follows: Number of apoptotic cells / total number of cells x 100%.

Western blotting. Western blotting was performed on the myocardium from the AAR obtained following 40 min of reperfusion for quantification of the levels of total and phosphorylated (p-) STAT-3, Akt, extracellular signal-related kinase (ERK) 1/2 and glycogen synthase kinase (GSK) 3β, and following 120 min of reperfusion for Bcl-2 and Bax. Briefly, freshly frozen myocardial tissue samples were ground into small pieces (~1x1x1 mm) in liquid nitrogen. The samples were then transferred to microcentrifuge tubes containing radioimmunoprecipitation lysis buffer (~150 μl per 10 mg tissue; Beyotime Institute of Biotechnology, Shanghai, China) and 1 mM phenylmethylsulfonyl fluoride. The samples were thoroughly homogenized and kept on ice for 1 h, vortexing every 10 min. The samples were subsequently centrifuged at 20,000 x g for 30 min at 4˚C, and the supernatants were transferred into fresh tubes and kept on ice. Following protein quantification using a Bicinchoninic Acid assay (Beyotime Institute of Biotechnology), equal quantities of protein (50 μg) were separated on 10% Tris-glycine sodium dodecyl sulfate gels (Beyotime Institute of Biotechnology), and transferred onto a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). Subsequent to blocking with 5% non-fat milk for 2 h and washing twice with Tris-buffered saline containing 0.05% Tween (TBST; 5 min/wash; Goodbio), the membranes were incubated overnight at 4˚C with the following primary antibodies: Rabbit monoclonal anti-Bcl-2 (1:1,000; cat. no. 2870; Cell Signaling Technology, Inc., Danvers, MA, USA) and rabbit polyclonal anti-Bax (1:300; cat. no. sc-493; Santa Cruz Biotechnology, Inc., Dallas TX, USA) to quantify apoptotic signaling, rabbit monoclonal anti-^{705}Tyr-p-STAT-3 (1:1,000; cat. no. 9145), rabbit polyclonal anti-total STAT-3 (1:1,000; cat. no. 9132), rabbit monoclonal anti-^{473}Ser-p-Akt (1:1,000; cat. no. 4060), rabbit polyclonal anti-total Akt (1:800; cat. no. 9272), rabbit monoclonal anti-$^{202/204}$Tyr-p-ERK1/2 (1:1,000; cat. no. 4370), rabbit polyclonal anti-total ERK1/2 (1:1,000; cat. no. 9102), rabbit monoclonal anti-^{9}Ser-p-GSK-3β (1:2,000; cat. no. 5558) and rabbit monoclonal anti-total GSK-3β (1:1,000; cat. no. 9315) (Cell Signaling Technology, Inc.) to quantify salvage signaling pathways. Mouse monoclonal anti-β-actin (1:3,000; cat. no. A1978; Sigma-Aldrich) was used as a loading control. Following primary antibody incubation, the membranes were washed five times with TBST (5 min/wash) and incubated with the horseradish peroxidase-conjugated goat anti-rabbit (1:5,000; cat. no. sc-2004) or goat anti-mouse (1:3,000; cat. no. sc-2005) IgG secondary antibodies (Santa Cruz Biotechnology, Inc.). Subsequently, the membranes were washed five times with TBST (5 min/wash), and the bands were detected using Western Blotting Luminol reagent (cat. no. sc-2048; Santa Cruz Biotechnology, Inc.) and quantified by densitometric analysis of digitized autoradiograms using Quantity One version 4.6.2 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Each immunoblotting experiment was repeated three times, and the averages of the results were calculated.

Statistical analysis. All values are expressed as the mean ± standard deviation. All data analyses were performed using SPSS statistical software, version 17.0 (SPSS, Inc., Chicago, IL, USA). Differences in the hemodynamic indexes were compared within groups using repeated-measures analysis of variance, and between groups using two-way analysis of variance followed by a least significant difference (LSD) corrected multiple comparisons test. Differences in other variables between groups were evaluated using one-way analysis of variance, followed by Fisher's post-hoc LSD-corrected multiple comparisons test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Myocardial IS. The AAR was similar among the groups (data not shown). Compared with the IR group (49.45±6.59%), IS was significantly reduced in the RIPerC (34.36±5.87%) group and RIPostC (36.04±6.16%) group ($P<0.05$; Fig. 2). However, no further reductions in IS were observed in the RIPerC + RIPostC group, compared with either the group exposed to RIPerC alone or the group exposed to RIPostC alone (31.43±5.43%; Fig. 2).

Myocardial apoptosis. As shown in Fig. 3A and B, the results of the TUNEL staining demonstrated that the percentages of positively-stained cells were lower in the RIPerC (22.35±4.22%) and RIPostC (24.63±4.44%) groups, compared with the IR group (35.81±5.27%; $P<0.05$). The combination of RIPerC and RIPostC did not further reduce the percentage of apoptotic cells, compared with the percentages of apoptosis in either of the groups exposed to RIPerC or RIPostC alone (20.33±3.67%). Similarly, the protein expression ratio of Bcl-2/Bax was found to be higher in all the conditioning groups, compared with the IR group ($P<0.05$), with no significant differences identified among the groups (Fig. 3C and D).

Serum levels of cTnI. Following 2 h of reperfusion, the serum levels of cTnI were significantly increased in the IR group,

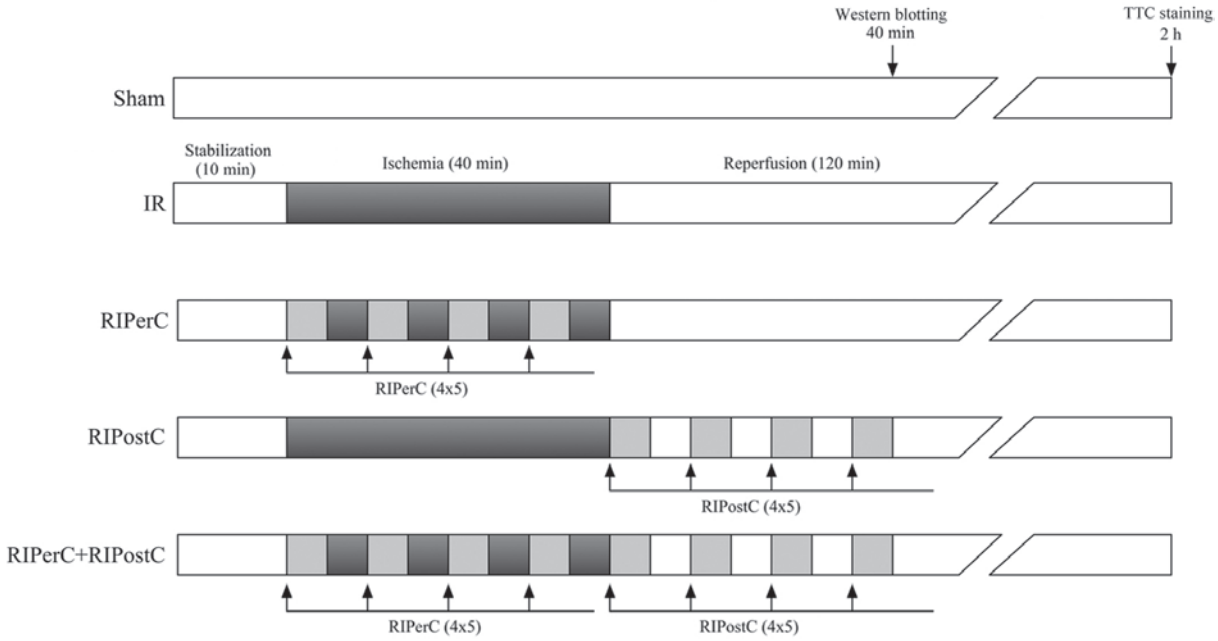

Figure 1. Experimental protocols. Sprague-Dawley rats underwent open-chest surgery. Subsequent to a 10 min stabilization period, they were randomly assigned into five groups: i) Sham group (rats underwent sham surgery); ii) IR (rats underwent 40 min left anterior descending artery occlusion, followed by 2 h reperfusion); iii) RIPerC group (rats were treated as in the IR group, with four cycles of 5 min hindlimb ischemia followed by 5 min reperfusion at the initiation of cardiac ischemia); iv) RIPostC group (rats were treated as in the RIPerC group, with remote conditioning was applied at the initiation of cardiac reperfusion; v) RIPerC + RIPostC group (rats received the RIPostC and RIPerC treatments, described above). IR, ischemia/reperfusion, RIPerC, remote ischemic perconditioning; RIPostC, remote ischemic postconditioning.

compared with the sham group. However, the increase in the levels of cTnI were significantly attenuated in the RIPerC, RIPostC and RIPerC + RIPostC groups (P<0.05, vs. IR group), with no significant differences observed among the three groups (Table I).

Levels of serum inflammatory cytokines. Compared with the sham group, the serum levels of TNF-α and IL-1β following 2 h of reperfusion were significantly increased in the IR group (P<0.05, vs. sham group), and were significantly attenuated by RIPerC, RIPostC and RIPerC + RIPostC (P<0.05, vs. IR group). However, no differences were observed between the groups (Table II).

LV functions. The heart rates were observed to be consistent among the groups at all time points (data not shown). During the periods of ischemia and reperfusion, there were increases in LVEDP, and a significant reduction in MAP, $+dP/dt_{max}$ and $-dP/dt_{max}$ in each group, compared with the data at baseline (P<0.05, vs. baseline). However, compared with the IR group, none of the conditioning methods had a significant effect on MAP, LVEDP, $+dP/dt_{max}$ or $-dP/dt_{max}$ at any given time point. Individual group data are presented in Table III.

Reperfusion injury salvage kinase (RISK) and survivor activating factor enhancement (SAFE) pathways. The protein levels of total Akt, ERK1/2, GSK-3β (RISK pathway) and signal transducer and activator of transcription (STAT) 3 (SAFE pathway) were found to be similar among the groups. The levels of p-Akt, p-ERK1/2, p-GSK-3β and p-STAT-3 are expressed as densitometric levels, normalized by levels of total protein.

In the IR group, the phosphorylation levels of Akt, ERK1/2, GSK-3β and STAT-3 were significantly increased following 40 min of reperfusion, compared with the sham group (P<0.05, vs. sham group; Fig. 4). In the RIPerC, RIPostC and RIPerC + RIPostC groups, further increases in the phosphorylation levels of STAT-3, Akt, ERK1/2 and GSK-3β were detected (P<0.05, vs. IR group; Fig. 4), however, no significant differences were observed among the three groups.

Discussion

In the present study, RIPerC was combined with RIPostC in an *in vivo* rat IR model, and its protective efficacy was compared with RIPerC and RIPostC alone. The results demonstrated that RIPerC and RIPostC were equally effective in providing protection against myocardial IR injury, however, the combination of RIPerC and RIPostC did not produce additive protective effects, compared with either treatment alone. Additionally, the protective activities were found to be associated with activation of the RISK and SAFE pathways.

Remote ischemic conditioning confers potent protective effects against myocardial IR injury by conducting brief periods of IR to a remote organ (2), and RIPerC and RIPostC have been reported to be beneficial in animal investigations (11,12) and randomized clinical trials (13,14). However, not all results are consistent (15,16). In the present study, the efficacy of RIperC and RIPostC was investigated by performing four cycles of 5 min occlusion and 5 min reperfusion to hindlimbs, either during or subsequent to myocardial ischemia. The results suggested that RIPerC and RIPostC were equally effective in protecting against myocardial IR injury in

Table I. Serum levels of CTnI following 2 h of reperfusion.

Group	CTnI (ng/ml)
Sham	3.31±0.75[a]
IR	139.85±21.18
RIPerC	54.02±9.60[a]
RIPostC	50.90±10.95[a]
RIPerC + RIPostC	46.96±8.81[a]

All data are expressed as the mean ± standard deviation (n=6 in each group). [a]P<0.05, vs. IR group. CTnI, cardiac troponin I; IR, ischemia/reperfusion; RIPerC; remote ischemic perconditioning; RIPostC, remote ischemic postconditioning.

Table II. Serum levels of TNF-α and IL-1β 2 h post-reperfusion.

Group	TNF-α (pg/ml)	IL-1β (pg/ml)
Sham	10.50±2.74[a]	10.17±2.32[a]
IR	215.67±41.80	148.67±20.16
RIPerC	173.33±32.72[a]	128.83±17.99[a]
RIPostC	180.17±30.30[a]	126.00±16.99[a]
RIPerC + RIPostC	167.33±25.44[a]	120.17±14.35[a]

All data are expressed as the mean ± standard deviation (n=6 in each group). [a]P<0.05, vs. IR group. TNF-α, tumor necrosis factor α; IL-1β, interleukin 1β; IR, ischemia/reperfusion; RIPerC; remote ischemic perconditioning; RIPostC, remote ischemic postconditioning.

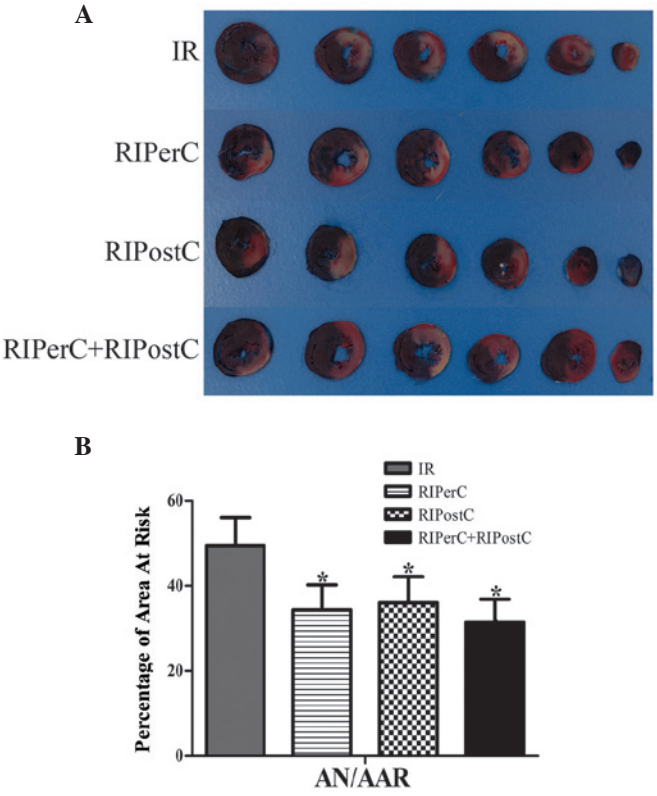

Figure 2. Myocardial infarct size 2 h post-reperfusion. (A) Representative sequential LV slices from each group, indicating the AAR, delineated with Evans blue staining, in the normal heart tissue and the area of necrosis, determined using 2,3,5-triphenyltetrazolium chloride staining (pale area = infarcted tissue). (B) Percentages of LV weights in the AN/AAR. Data are expressed as the mean ± standard deviation. [a]P<0.05, vs. IR group. LV, left ventricular; AN, area of necrosis; AAR, area at risk; IR, ischemia/reperfusion; RIPerC, remote ischemic perconditioning; RIPostC, remote ischemic postconditioning.

terms of myocardial IS, cell apoptosis, serum troponin I levels and inflammatory responses, which is consistent with previous studies demonstrating all three remote conditioning strategies (RIPC, RIPerC and RIPostC) have similar therapeutic potential for cardiac IR injury (17).

The mechanisms responsible for the cardioprotective effects of remote ischemic conditioning remain to be fully elucidated. Current evidence suggests that the majority of mechanisms identified for conventional local ischemic conditioning are also applicable to remote ischemic conditioning. The well-described RISK and SAFE pathways have been reported to be involved in remote ischemic conditioning (18,19). The present study demonstrated that RIPerC and RIPostC increased the phosphorylation of Akt, Erk1/2, GSK-3β and STAT-3, with no significant differences between the two procedures, suggesting that the protective effects of RIPerC and RIPostC are associated with activation of the RISK and SAFE pathways, is consistent with previous studies (10,19).

Ischemic conditioning was originally described to be an 'all or nothing' event (20,21). Subsequent studies have reported that the protective effect of ischemic conditioning varies with the strength of the stimulus, optimized by the number of cycles and duration, suggesting a dose-dependent response (22,23). A previous study combined different doses of RIPerC with IPostC, and the results demonstrated that the combination of the optimized dose of RIPerC and IPostC offered higher protective potential against myocardial IR injury, compared with either treatment alone (10). The present study aimed to investigate whether the combination of RIPerC and RIPostC, which can be induced non-invasively using standard blood pressure cuffs, provide additive protection against myocardial IR injury. However, the results demonstrated that the combination RIPerC and RIPostC failed to produce further protective effects against myocardial IR injury, compared with either alone, as indicated by similar myocardial IS values, levels of cell apoptosis, serum levels of troponin I and LV function.

A previous study suggested that the additive protection induced by the combination of RIPerC and IPostC was associated with the additional phosphorylation of Akt and ERK1/2 (10), whereas Tamareille *et al* (19) reported that the enhanced protective effects observed with RIPerC + IPostC were accompanied by increased levels of p-STAT-3. However, in the present study, none of the above-mentioned kinases were found to be further activated by the combination of RIPerC and RIPostC. Taken together, the presents study hypothesized that there is a certain mechanistic aspect of IPostC, which is not shared by RIPostC, and that the difference may lie upstream of the RISK and SAFE pathways, and that the 'passive' effects of IPostC may be involved (24). The immediate full-flow reperfusion of the coronary artery following lethal ischemia

Table III. Left ventricular functions at different stages of IR.

Time point	MAP (mmHg)	LVEDP (mmHg)	+dP/dt$_{max}$ (mmHg/sec)	-dP/dt$_{max}$ (mmHg/sec)
Baseline				
Sham	113.2±5.4	4.0±0.5	9,985.2±957.7	5,927.7±519.2
IR	114.9±6.1	4.1±0.5	10,128.9±1,044.2	5,987.4±488.5
RIPerC	116.4±6.3	4.2±0.5	10,207.3±1,068.6	6,036.2±594.1
RIPostC	113.7±5.9	4.1±0.6	10,041.1±914.5	5,960.1±566.8
RIPerC + RIPostC	115.4±6.2	4.2±0.5	10,157.3±1,054.1	6,020.6±598.3
20 min post-ischemia				
Sham	110.3±6.1[b]	4.0±0.6[a]	9,897.3±952.1[a]	5,892.5±590.4
IR	103.3±10.7[b]	5.1±0.6[b]	8,308.5±885.2[b]	5,257.3±620.9[b]
RIPerC	106.8±12.4[b]	5.1±0.8[b]	8,392.3±876.8[b]	5,327.0±721.1[b]
RIPostC	104.1±9.8[b]	5.1±0.8[b]	8,283.7±951.4[b]	5,239.2±619.2[b]
RIPerC + RIPostC	105.6±11.3[b]	5.1±0.8[b]	8,377.4±1,087.2[b]	5,324.1±545.8[b]
40 min post-ischemia				
Sham	107.7±6.2[a,b]	4.1±0.6[a]	9,798.6±941.8[a]	5,854.4±507.6[a]
IR	95.3±8.5[b]	5.7±0.8[b]	7,006.2±815.2[b]	4,860.7±544.5[b]
RIPerC	98.7±9.7[b]	5.6±0.9[b]	7,114.5±875.1[b]	4,972.3±749.3[b]
RIPostC	95.5±7.2[b]	5.7±0.9[b]	6,982.9±703.4[b]	4,830.3±668.9[b]
RIPerC + RIPostC	98.4±9.0[b]	5.6±0.9[b]	7,103.7±858.7[b]	4,968.9±596.1[b]
30 min post-reperfusion				
Sham	104.9±5.3[a,b]	4.1±0.6[a]	9,607.5±936.7[a,b]	5,756.3±547.1[a,b]
IR	86.8±9.2[b]	6.6±0.9[b]	5,276.2±679.4[b]	4,255.1±486.6[b]
RIPerC	89.1±8.8[b]	6.5±0.9[b]	5,414.1±751.2[b]	4,401.2±623.4[b]
RIPostC	88.7±8.3[b]	6.5±0.9[b]	5,417.8±610.8[b]	4,384.7±556.5[b]
RIPerC + RIPostC	90.6±8.4[b]	6.4±0.9[b]	5,422.4±660.3[b]	4,404.9±538.2[b]
1 h post-reperfusion				
Sham	104.3±5.7[a,b]	4.1±0.6[a]	9,401.9±965.5[a,b]	5,650.2±491.7[a,b]
IR	84.4±8.1[b]	6.3±0.8[b]	5,286.2±606.3[b]	4,278.8±514.2[b]
RIPerC	85.9±8.3[b]	6.1±0.8[b]	5,452.3±694.7[b]	4,473.1±616.1[b]
RIPostC	85.2±6.9[b]	6.2±0.8[b]	5,432.8±609.1[b]	4,450.5±576.8[b]
RIPerC + RIPostC	87.6±8.3[b]	6.1±0.8[b]	5,474.6±723.4[b]	4,480.4±518.4[b]
2 h post-reperfusion				
Sham	103.2±6.1[a,b]	4.1±0.6[a]	9,238.7±928.5[a,b]	5,507.3±517.4[a,b]
IR	85.5±8.2[b]	5.9±0.8[b]	5,307.3±655.1[b]	4,320.1±535.6[b]
RIPerC	88.8±7.8[b]	5.7±0.7[b]	5,603.3±731.4[b]	4,561.5±633.4[b]
RIPostC	87.4±7.9[b]	5.8±0.8[b]	5,587.6±704.3[b]	4,538.6±591.7[b]
RIPerC + RIPostC	90.1±8.4[b]	5.7±0.8[b]	5,634.1±727.0[b]	4,575.3±540.8[b]

Data are expressed as the mean ± standard deviation (n=6 in each group). [a]P<0.05, vs. IR group; [b]P<0.05, vs. baseline. MAP, mean arterial pressure; LVEDP, left ventricular end-diastolic pressure; +dP/dt$_{max}$, maximal rate of increase in intraventricular pressure; -dP/dt$_{max}$, maximal rate of reduction in intraventricular pressure; IR, ischemia/reperfusion; RIPerC; remote ischemic perconditioning; RIPostC, remote ischemic postconditioning.

has been found to lead to sudden changes in the extracellular environment, including altered osmolarity, ion concentrations and pH, which may lead to intracellular edema, opening of the mitochondrial permeability transition pore and myocardial cell death (25). Local IPostC at the onset of reperfusion may function as a type of gradual reperfusion, to reduce the severity of these changes, thereby protecting the myocardium against the potentially lethal consequences (26,27). This hypothesis is supported by the fact that IPostC is only effective in the first ~3 mins of reperfusion (26,28), whereas RIPostC has a wider time frame. However, further investigations are required to in order to clarify the mechanistic differences between local and remote ischemic conditioning.

It has been suggested that the inflammatory response is also important in the mechanism of myocardial IR injury, and local and remote ischemic conditioning have been identified to exhibit anti-inflammatory effects (29,30). The present study demonstrated that RIPerC, RIPostC and the two in

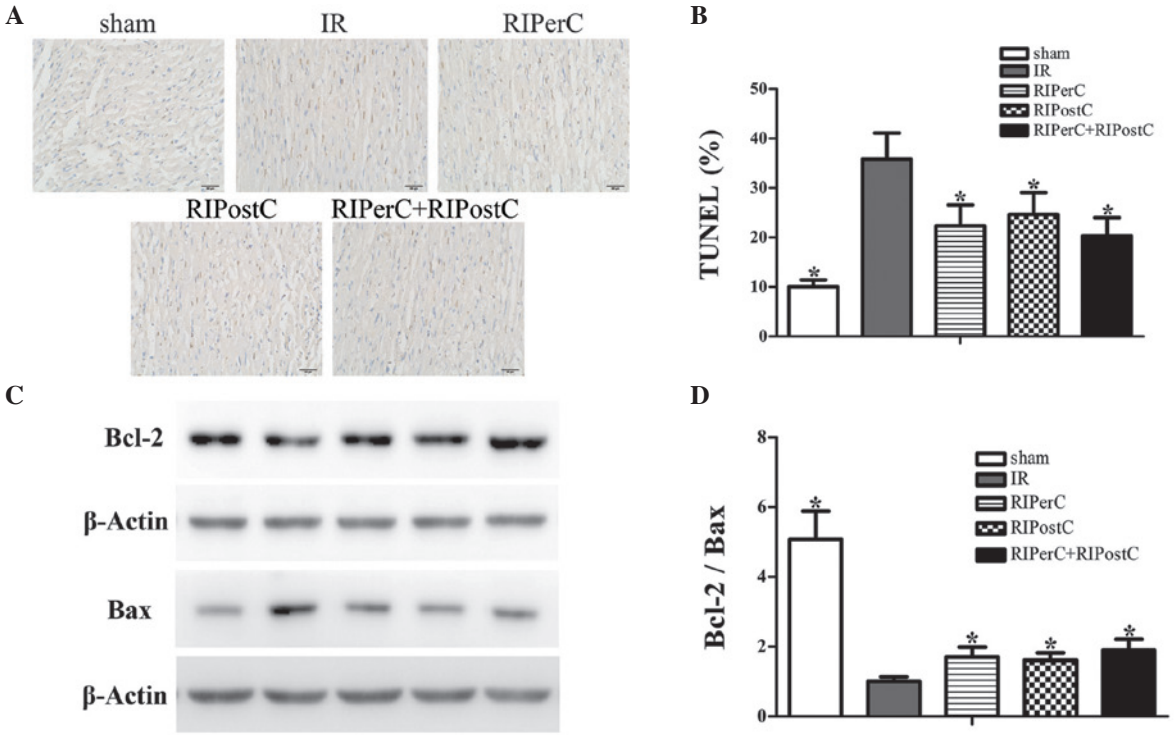

Figure 3. Myocardial apoptosis 2 h post-reperfusion. (A) Representative TUNEL staining at the peri-infarct area of the left ventricle in each group (magnification, 400x). TUNEL-positive cells were stained brown. (B) Quantification of the percentages of positively-stained cells in each group. (C) Representative western blotting for the protein expression levels of Bax and Bcl-2 in each group. β-Actin was used as a protein loading control. (D) Quantification of the protein expression ratios of Bcl-2 to Bax. Data are expressed as the mean ± standard deviation. *P<0.05, vs. IR group. TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; IR, ischemia/reperfusion; RIPerC, remote ischemic perconditioning; RIPostC, remote ischemic postconditioning; Bcl-2, B cell lymphoma-2; Bax, Bcl-2-associated X protein.

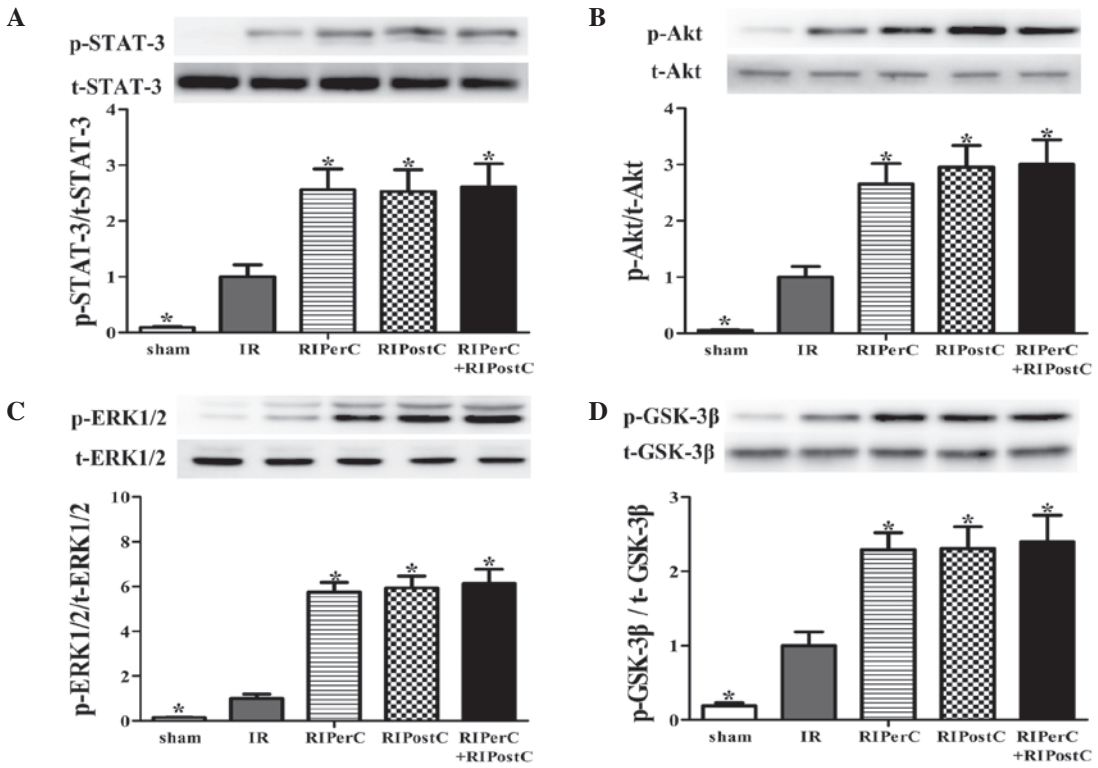

Figure 4. Reperfusion injury salvage kinase (Akt, ERK1/2 and GSK-3β) and survivor activating factor enhancement (STAT-3) pathway activation following 40 min reperfusion. Western blot analysis of total and phosphorylated (A) STAT-3, (B) Akt, (C) ERK1/2 and (D) GSK-3β proteins in rat hearts in left ventricular homogenates of heart tissues subjected to IR, and the expression ratios of phosphorylated/total protein are shown. All expression levels were normalized to that of β-actin. Data are expressed as the mean ± standard deviation. *P<0.05, vs. IR group. ERK1/2, extracellular signal-related kinase 1/2; GSK-3β, glycogen synthase kinase 3; STAT-3, signal transducer and activator of transcription 3; IR, ischemia/reperfusion; RIPerC, remote ischemic perconditioning; RIPostC, remote ischemic postconditioning; p-, phosphorylated; t-, total.

combination significantly alleviated the systemic inflammatory response induced by myocardial IR injury, as indicated by reduced serum levels of TNF-α and IL-1β, with no significant difference among the groups, consistent with previous studies (31,32).

In the present study, an optimized dose (number of cycles and duration) of the RIPerC stimulus was determined according to a previous study (10), and the same algorithm was used for RIPostC for comparison purposes. However, whether additional cycles or a longer duration of RIPostC stimulation enhances the cardioprotective effects of optimized RIPerC remain to be elucidated. Of note, the lack of further protection by combining RIPerC and RIPostC can only be interpreted in light of the ischemic duration and animal model selected in the present study.

In conclusion, the present study demonstrated that RIPerC and RIPostC were equally effective in protecting against myocardial IR injury, and that the combination of RIPerC and RIPostC failed to provide additional protection, compared with either alone. These cardioprotective effects were found to be associated with increased activation of the RISK and SAFE pathways.

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