Original Paper

Remote Ischaemic Preconditioning and Sevoflurane Postconditioning Synergistically Protect Rats from Myocardial Injury Induced by Ischemia and Reperfusion Partly via Inhibition TLR4/MyD88/NF- κ B Signaling Pathway

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Key Words

Sevoflurane postconditioning • Remote ischemic preconditioning • Ischemia and reperfusion injury • Inflammation • Apoptosis

Abstract

Background/Aims: A combination sevoflurane postconditioning (SPC) and remote ischemic preconditioning (RIPC) is proved effective in an *ex vivo* rat heart perfusion model. However, the combined effect of those two interventions is not tested in rat myocardial ischemia/reperfusion (I/R) model, and the underlying mechanisms remain to be elucidated. This study aimed to investigate the effect *in vivo* using a rat myocardial I/R model and illuminate the related mechanisms. **Methods:** Forty male Sprague-Dawley rats were randomly divided into the following 5 groups: i) sham-operated control; ii) I/R; iii) I/R + RIPC; iv) I/R + SPC; v) I/R + RIPC + SPC. The hemodynamic parameters were recorded at the end of reperfusion. The histological changes including the infarct size were assessed using Triphenyltetrazolium chloride (TTC) staining and H&E staining. In addition, the circulating levels of cardiac enzymes (CK-MB, hs-cTnT, and cTnT) inflammatory cytokines (IL-6, IL-8, and TNF- α) were detected. The expression levels of apoptosis-related proteins (C-Caspase 3, PARP, Bax, and Bcl-2), proinflammatory factors (TLR4, HMGB-1, MyD88, and p65), and I κ B- α were measured by Western blot analysis. Real-time PCR was performed to detect mRNA levels of the proinflammatory factors. **Results:** Both SPC and RIPC significantly reduced the infarct size, cardiac enzyme release, inflammatory cytokine secretion, and proinflammatory factor expression, and increased I κ B- α expression. JR. Zhang, J. Zhang and P. Yu contributed equally to this work.

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compared to I/R group. Furthermore, the combination of those two strategies had synergic infarct size limiting and anti-inflammatory effects. **Conclusions:** The finding of this study suggested that the combination of SPC and RIPC had a potentially cardioprotective effect through inhibiting TLR4/MyD88/NF- κ B signaling.

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Introduction

Myocardial ischemia-reperfusion (I/R) injury is a common pathophysiological feature in numerous conditions including cardiac surgery, myocardial infarction, and circulatory arrest. I/R injury decreases myocardial cell viability, causes cardiac dysfunction, may have a life-threatening consequence [1]. Different conditioning strategies is proved to be effective for prevention of I/R injury in animal models [1, 2]. However, the translating trials from bench to bedside are largely disappointing.

It is recently reported that pharmacological postconditioning, including inhalation anesthetics, may have a protective effect on I/R-induced myocardial injury [2-5]. Sevoflurane is a widely used volatile anesthetic in cardiac surgery. It is demonstrated that sevoflurane postconditioning (SPC) limits myocardial infarct size and reduces mortality in both animal models and clinical practice [3-6]. Similarly, remote ischemic preconditioning (RIPC) by applying several cycles of ischemia and reperfusion in distant tissues also presents the protective effect. The synergic effect of those two interventions is proved in an isolated rat heart perfusion model [7]. Nevertheless, the combined effect is not tested in rat myocardial ischemia/reperfusion (I/R) model.

Previous studies suggest that a variety of mechanisms may be responsible for the cardioprotection of SPC and RIPC against I/R injury, including the Reperfusion Injury Salvage Kinase pathway (RISK pathway) inhibition [8], preventing mitochondrial permeability transition pore (mPTP) from opening [9], decreasing intracellular reactive oxygen species (ROS) levels [10], and regulating the activation of autophagy or the process of autophagic flux [4, 11]. However, the precise mechanisms remain unclear.

Toll-like receptor 4 (TLR4) is a well-known upstream sensor of multiple intracellular signaling pathways such as TLR4/NF- κ B signaling [12]. Previous finding confirmed that TLR4 was a detrimental regulatory factor in myocardial I/R injury. Mice deficient in TLR4 presented suppressed NF- κ B activation, reduced pro-inflammatory cytokine expression, and less myocardial infarction compared to their wild-type littermates [13, 14]. Similar results were also observed after administration of a specific TLR4 antagonist [15].

Though the potential cardioprotective effect of a combined conditioning of SPC and RIPC is proved in an isolated rat heart model, it is not tested in a rat I/R myocardial injury model. In this study, we tried to clarify whether a combination of SPC and RIPC also had an additional protection against I/R injury in a rat myocardial infarct model and elucidate the underlying mechanism with the focus on TLR4/NF- κ B signaling.

Materials and Methods

Animals and surgical preparations

A total of 40 adult male Sprague-Dawley rats (250-300g) were housed in individual cages with a 12-h cycle of light and dark and temperature at 22-24°C. The rats were allowed free access to food and water. The rats were anesthetized by intraperitoneal injection of a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg). Then, left thoracotomies were performed under artificial ventilation and the hearts were exposed in the third intercostals space. The left anterior descending artery was ligated with a 6-0# silk suture to induce myocardial ischemia. After 30 min of ischemia, untie the knot to allow reperfusion for 2 hr. The same surgical procedures without ligation were performed on rats in sham group. Remote ischemic preconditioning (RIPC) was induced by 4 cycles of 5-min ischemia and 5-min reperfusion in hind limbs by

wrapping tourniquets on proximal place of the limbs. A Powerlab data-acquisition system (ADInstruments) was used to record the hemodynamic parameters. Rats were sacrificed at the end of reperfusion, and hearts and blood samples collected for further analysis. All experiments were approved by the Animal Care and Use Committee of Jiangnan University.

Fig. 1. Experimental procedures.

Experimental procedures

The rats were randomly allocated into five groups (n = 8 for each group): Control group, I/R group, RIPC group, SPC group, and SPC + RIPC group. In Control group, rats underwent a sham operation without intervention. In I/R group, rats were subjected to 30 min of ischemia followed by 2 hr of reperfusion without intervention. In RIPC group, rats received RIPC before I/R. In SPC group, rats were treated with sevoflurane for 2 min in parallel with reperfusion onset. In SPC + RIPC group, rats were preconditioned with RIPC before ischemia and postconditioned with sevoflurane on the onset of reperfusion. The experimental procedures and groups were shown in Fig. 1.

Assessment of myocardial infarct size

Triphenyltetrazolium chloride (TTC, Sigma-Aldrich) staining was performed for determination of myocardial infarct sizes. Briefly, excised hearts were firstly perfused with saline to wash blood out from the coronary vasculature. The hearts were then sectioned horizontally into 2-mm slices. Sliced hearts were then incubated in 1% TTC in Tris buffer at pH 7.8 for 15 min at 37°C. The region of infarcted myocardium appeared pale white for absence of TTC staining. The infarct area was measured with Image J software (NIH). The ratio of infarcted ventricle area was calculated and presented as a percentage.

Histology

Heart samples were excised and fixed in 10% neutral buffered formalin. Fixed hearts were embedded in paraffin and sectioned at 5 µm. Paraffin sections were stained with hematoxylin and eosin (Beyotime, China). Pictures were taken by a Leica IX71 microscope.

Western blotting

The heart tissues were homogenized in RIPA buffer (50 mM Tris pH7.4, 150 mM NaCl, 1% Triton X-100) with freshly added PMSF (0.1%) protease inhibitor (Sigma-Aldrich). Cell lysates were obtained by centrifuging homogenate at 12,000g for 10 min at 4°C. Protein concentration was determined by standard BCA assay. Proteins (20 µg) were loading to a 10% sodium dodecyl sulfate polyacrylamide gel. The proteins were transferred to polyvinylidene fluoride membranes (Millipore) after electrophoresis. The membranes were then blocked in Tris-buffered saline with Tween-20 (TBST, 20 mM Tris, 137 mM NaCl, 0.1% Tween-20) containing 5% non-fat milk for 1 hr. The PVDF membranes were incubated with primary antibodies against Cleaved-Caspase 3 (1:1000, mouse, Santa Cruz), PARP (1:1000, mouse, Santa Cruz), Bax (1:1000, mouse, Santa Cruz), Bcl-2 (1:1000, rabbit, Santa Cruz), TLR4 (1:500, mouse, Santa Cruz), HMGB-1 (1:1000, rabbit, Cell Signaling Technology), MyD88 (1:500, mouse, Santa Cruz), NF-κB (1:1000, rabbit, Cell Signaling Technology), IKB-α (1:1000, mouse, Cell Signaling Technology), and Tubulin (1:1500, mouse, Beyotime Biotechnology) overnight at 4°C.

After being washed with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hr at room temperature. Subsequently, the washed membranes were analyzed with an enhanced chemiluminescence detection kit (BeyoECL Plus, Beyotime, China). The band intensities were quantified using ImageJ software (NIH).

RNA extraction and real-time PCR

Total RNA was isolated with Trizol reagent (Invitrogen). Reverse transcription reactions were performed using a PrimeScript™ RT reagent Kit (TaKaRa). The cDNA was amplified with SYBR Fast qPCR Mix (TaKaRa) on an ABI Prism 7500 system. The amplification was carried out with initial enzyme activation

at 95°C for 15 min, followed by 40 cycles of 95°C for 15 sec and then 60°C for 1 min. The relative gene expression data was analyzed with the $2^{-\Delta\Delta CT}$ method. The primers used for real-time PCR were listed as follows:

TLR4-RT(RAT)-F:GCTTTCAGCTTTGCCTTCAT
TLR4-RT(RAT)-R:TACACCAACGGCTCTGGATA
HMGB-1-RT(RAT)-F:TCAGATACAAGGAAAGCGGAT
HMGB-1-RT(RAT)-R:AAATTGCCAAATTGTTCCCT
MyD88-RT(RAT)-F:TGGTGGTTGTTTCTGACGAT
MyD88-RT(RAT)-R:GATCAGTCGCTTCTGTTGGA
IKB- α -RT(RAT)-F:CAAGTACCCGGATACAGCAG
IKB- α -RT(RAT)-F:ACACAGTCATCGTAGGGCAA
NF- κ B-RT(RAT)-F:GCTACACAGAGGCCATTGAA
NF- κ B-RT(RAT)-R:ATGTGCTGTCTTGTGGAGGA

Enzyme-linked Immunosorbent Assay (ELISA)

Serum cardiac enzymes (CK-MB, hs-cTnT and cTnT) and inflammatory cytokines (IL-8, IL-6 and TNF- α) were measured by enzyme-linked immunosorbent assay (ELISA). And the ELISA was performed with commercial kits according to manufacturer's instructions.

Statistical analysis

Data were expressed as mean $\pm$ standard error of mean (SEM) and analyzed using one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons with a SPSS package. *P* values less than 0.05 were considered statistically significant.

Results

Cardiac hemodynamic parameters

In comparison to control group, I/R group markedly decreased in LVDP, $\pm$ dp/dt, and HR ($P < 0.05$). The hearts of SPC + RIPC group exhibited more significant improvement in LVDP, $\pm$ dp/dt, and HR than that of SPC or RIPC groups ($P < 0.05$, Table 1).

Myocardial infarct size and cardiac injury

Postconditioning of sevoflurane or preconditioning of RIPC led to reduction in myocardial infarct size caused by I/R. Combination of these two interventions further decreased the infarct size (Fig. 2). Serum cardiac enzymes such as CK-MB, hs-cTnT and cTnT considered for the present protocol, are canonical markers of myocardial injury [13]. Their serum levels dramatically increased in I/R group, compared to control group ($P < 0.01$, Fig. 3). The enzyme levels significantly reduced in serum from rats treated with sevoflurane or RIPC alone ($P < 0.01$, Fig. 3). The release of myocardial enzymes further reduced in SPC + RIPC group ($P < 0.01$, vs. I/R group).

Histopathological results

H&E stained section showed that the hearts from I/R group had aberrant and irregular myocardial fibers presented, unclear or disordered transverse striation, Intracytoplasmic vacuoles and edema cardiomyocytes

Table 1. Cardiac hemodynamic parameters (means $\pm$ SD) Note: LVSP, left ventricular systolic pressure; LVDEP, left ventricular end-diastolic pressure; +dp/dtmax, maximal rate of left ventricular systolic pressure; -dp/dtmax, maximal rate of left ventricular diastolic pressure; HR, heart rate. * $P < 0.05$ vs. Con group; # $P < 0.05$ vs. I/R group; Δ $P < 0.05$ vs. RIPC group; & $P < 0.05$ vs. SPC group

Groups	LVDP (mmHg)	+dp/dt max(mmHg/s)	-dp/dt max (mmHg/s)	HR (beat/min)
Con	72.5 $\pm$ 4.9	2119.5 $\pm$ 133.6	1456.5 $\pm$ 129.4	297.5 $\pm$ 14.8
I/R	34.0 $\pm$ 2.8*	1496.0 $\pm$ 39.6*	612.5 $\pm$ 6.4*	241.5 $\pm$ 6.4*
RIPC	42.5 $\pm$ 2.1*#	1602 $\pm$ 8.5*#	763 $\pm$ 15.7*#	257.5 $\pm$ 6.4*#
SPC	53.5 $\pm$ 3.5*#	1897.5 $\pm$ 14.8*#	829.0 $\pm$ 18.4*#	268.5 $\pm$ 10.6*#
SPC+RIPC	66 $\pm$ 1.4*# Δ &	2000 $\pm$ 19.8*# Δ &	1120.5 $\pm$ 74.2*# Δ &	281.5 $\pm$ 2.1*# Δ &

Fig. 2. Effects of SPC and RIPC on myocardial infarct size after cardiac I/R injury. The columns represent means $\pm$ SEM of six independent experiments. * $P < 0.05$ vs. Con group; # $P < 0.05$ vs. I/R group; $\Delta P < 0.05$ vs. RIPC group; & $P < 0.05$ vs. SPC group.

Fig. 3. Effects of SPC and RIPC on serum cardiac enzymes: CK-MB, hs-cTnT and cTnT in the different states. The columns represent means $\pm$ SEM of three independent experiments. * $P < 0.05$ vs. Con group; # $P < 0.05$ vs. I/R group; $\Delta P < 0.05$ vs. RIPC group; & $P < 0.05$ vs. SPC group.

(Fig. 4). Relieved myocardium injury was presented in the hearts of SPC or RIPC groups compared that in I/R group (Fig. 4). The cardioprotective effect was potentiated by co-treating with RIPC and sevoflurane (Fig. 4).

Expression of inflammatory cytokines

The circulating levels of inflammatory cytokines, including IL-6, IL-8, and TNF- α largely increased after I/R injury ($P < 0.01$ vs. control group, Fig. 5). Their secretion mildly decreased in both SPC group and RIPC group (Fig. 5). The inflammatory response was significantly suppressed by combining SPC and RIPC ($P < 0.01$ vs. I/R group, Fig. 5).

Activation of inflammatory signaling pathway

We further investigated the mechanism underlying the anti-inflammatory effect of combined conditioning of sevoflurane and RIPC. TLR4 is a key initiator of inflammatory response induced by I/R in heart [13]. To confirm whether TLR4 signaling pathway is disrupted by sevoflurane or/and RIPC, we detected the expression levels of several key factors in the signaling pathway. I/R induced increased expression of TLR4, HMGB-1, MyD88, and NF- κ B (p65) (Fig. 6 a,b). In contrast, IKB- α , an inhibitor of NF- κ B signaling pathway, was down-regulated by I/R injury (Fig. 6a, b). The up-regulation of TLR4, HMGB-1, MyD88 and NF- κ B (p65) and the down-regulation of IKB- α were partially abolished by sevoflurane

Fig. 4. Effects of SPC and RIPC on myocardial histology after I/R injury. Relieved myocardium injury was presented in the hearts of SPC or RIPC groups, compared that of I/R group. ($\times 200$, scar bars: 50 μm).

Fig. 5. Effects of SPC and RIPC on serum levels of inflammatory cytokines (IL-8, IL-6 and TNF- α). The columns represent means $\pm$ SEM of three independent experiments. * $P < 0.05$ vs. Con group; # $P < 0.05$ vs. I/R group; $\Delta P < 0.05$ vs. RIPC group; & $P < 0.05$ vs. SPC group.

or RIPC conditionings. All these trends were more obvious in SPC + RIPC group and were confirmed by mRNA expression levels (Fig. 6c).

Cardiac apoptosis

We detected apoptotic cardiomyocytes with TUNEL staining (Fig. 7a, b). Apoptosis index of the cardiomyocytes was significantly lower in SPC or RIPC groups compared with I/R group. In addition, the apoptotic cardiomyocytes were further decreased by combined conditioned with those two strategies in SPC + RIPC group. Next, we examined the expression of apoptosis related proteins. The I/R-induced levels of C-Caspase 3 and cleaved PARP, and Bax/Bcl-2 ratio markedly decreased in both SPC and RIPC groups, and further reduced in SPC + RIPC group (Fig. 7c, d).

Discussion

In present study, we employed a rat model of myocardial infarction to determine whether the combination of SPC and RIPC offered a potential cardioprotective effect. Our results suggested that combination of SPC and RIPC provided an enhanced protective effect compared with SPC or RIPC alone. Especially, combination of SPC and RIPC had stronger inhibitory effect on the inflammatory response, smaller myocardial infarction area and better

Fig. 6. Effects of SPC and RIPC on TLR4, HMGB-1, MyD88, IKB-α and NF-κB protein and mRNA expression. (A) Representative Western blot images in the hearts after I/R injury. (B) Quantitative protein analysis of TLR4, HMGB-1, MyD88, IKB-α and NF-κB expression. The columns and errors bars represent means $\pm$ SEM of three independent experiments. (C) The mRNA levels of TLR4, HMGB-1, MyD88, IKB-α and NF-κB were measured using quantitative real-time PCR in different groups. The columns and errors bars represent means $\pm$ SEM of six independent experiments. The columns and errors bars represent means $\pm$ SEM of six independent experiments. * $P < 0.05$ vs. Con group; # $P < 0.05$ vs. I/R group; Δ $P < 0.05$ vs. RIPC group; & $P < 0.05$ vs. SPC group.

myocardial compliance through activation of TLR4/NF-κB signaling pathway potential than separate treatment.

Several interventions in animal models were developed to protect against myocardial I/R injury. However, these interventions were *in vitro* animal model. Although these ways were confirmed and could attain better effects, these *in vitro* animal models were relatively far distance with clinical patients. Therefore, in order to make the study closer to clinical conditions, some interventions in the experiment were designed to carry out *in vivo* myocardial ischemia model. It was an innovation of this experiment.

Volatile anaesthetics were widely used in cardiac surgery and exerted protective effects on myocardial I/R injury both in clinical [16] and animal models [17]. Postconditioning with sevoflurane reduced myocardial apoptosis induced by I/R *via* multiple mechanisms, such as preventing activation of caspase 3/9 [18], and inhibiting the opening of mitochondrial permeability transition pore (mPTP) [9]. Clinical research uncovered that the molecular mechanisms of sevoflurane protection include its anti-inflammatory effects [19], which was consistent with our present finding.

Increasing evidences showed that combined treatment of sevoflurane and propofol led to enhancing the cardioprotective effects in patients undergoing aortic valve replacement

Fig 7. Effects of SPC and RIPC on cardiac apoptosis after I/R injury. (A) TUNEL staining was performed to detect the cardiac apoptosis assay in the ischemic core of heart sections. Representative images of TUNEL staining (green fluorescence) were captured by a fluorescence microscope ($\times 200$, scar bars: 50 μm). (B) Bar graph represents the quantification of TUNEL assays. The columns represent means $\pm$ SEM of four independent experiments. (C) Representative Western blot images in the hearts after I/R injury. (D) Quantitative protein analysis of C-Caspase3, PPAR, Bax, and Bcl-2 expression. The columns represent means $\pm$ SEM of three independent experiments. * $P < 0.05$ vs. Con group; # $P < 0.05$ vs. I/R group; Δ $P < 0.05$ vs. RIPC group; & $P < 0.05$ vs. SPC group.

with cardiopulmonary bypass and the concomitant regimen induced more effective anti-inflammatory responses against liver I/R injury [20, 21]. In addition, recent research found that stronger protective effect of combined treatment of hyperbaric oxygen and diltiazem against cardiac I/R injury [22]. Thus, combined treatment with different intervention drug combination could better protect against I/R injury.

RIPC is also an effective intervention to protect myocardium against I/R injury [7, 11]. However, the majority of preclinical studies are based on healthy animals, whereas myocardial ischemia disease is a complex disorder in clinical practice that may affect the efficacy of the two interventions. Combination of different conditioning protocols may provide a potential protection and overcome obstacles to clinical translation. Our data agree with those reported by Zhou and co-authors in an isolated perfused rat heart model [7]. Notably, numerous studies show that RIPC may protect multiple organ I/R injury through inhibition of inflammation in rats [23-25]. Studies confirmed that SPC and RIPC protect against I/R injury by anti-inflammation, so we speculate they can provide synergistic effects and additional protective effects. Indeed, SPC and RIPC produced synergistic protective effect evidenced by reduced cardiac enzyme release, limited infarct size, and lightened myocardial edema in a rat model of myocardial infarction. Nevertheless, the underlying mechanisms still need to be elucidated.

Myocardial inflammation is widely accepted to play a key role in the pathogenesis of myocardial I/R injury [26]. In reperfused myocardium, cardiac myocytes express various inflammatory cytokines that contribute to neutrophil infiltration into the myocardium, including chemokines and pro-inflammatory cytokines. The regulatory mechanisms in myocardial inflammation are complicated. Pattern recognition receptors, especially TLR4, are considered as the major initiator of the myocardial inflammation by recognizing damage-associated molecular patterns (DAMPs) [12-14]. HMGB-1, one of best known DAMPs, is reported to participate in the initiation of myocardial I/R injury via activating TLR4 [27]. Subsequently, activation of TLR4 can activate NF- κ B via MyD88-dependent pathway or MyD88-independent pathway leading to the production of inflammatory cytokines [14, 27]. It is demonstrated that inhibition of TLR4 signaling pathways can attenuate inflammatory response and cardiac myocyte apoptosis following myocardial I/R injury [28-30]. In our study, we found that I/R could up-regulated the mRNA levels of TLR4, HMGB-1, MyD88, and NF- κ B (p65), whereas down-regulated I κ B- α , which agrees with a previous report [31]. Combination of SPC and RIPC had a superior effect on normalizing the expression of their expression levels potential than SPC or RIPC alone. This may be ascribed to that those two interventions conduct the anti-inflammatory effect by different mechanisms. It is reported that sevoflurane can alleviate oxidative stress in I/R organ [32], which may attenuate I/R injury. Alternatively, anti-inflammatory properties of RIPC may be associated with the inhibition of the NF- κ B by suppressing its nuclear translocation [33]. Moreover, less secretion of inflammatory cytokines was observed in SPC combined RIPC group compared with those in SPC or RIPC groups. Our results suggested that combination of SPC and RIPC reinforced their anti-inflammatory effect in myocardial I/R injury through enhanced suppression of TLR4/MyD88/NF- κ B signaling pathway. In order to obtain more comprehensive information about this signaling pathway during SPC and RIPC on myocardial I/R injury, more intensive investigation will be done *in vivo* and *in vitro* in the near future.

In conclusion, our results suggested combination of SPC and RIPC provided an additional protection against myocardial I/R injury. This synergistically cardioprotective effect was associated with TLR4/MyD88/NF- κ B signaling pathway.

Acknowledgments

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Disclosure Statement

The authors have declared that no competing interests exist.

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