Ischemic preconditioning protects against myocardial ischemia-reperfusion injury through inhibiting toll-like receptor 4/NF-κB signaling pathway in rats

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Abstract: Objective To investigate whether the protection of ischemic preconditioning (IPC) against myocardial ischemia/reperfusion (I/R) injury is mediated by toll-like receptor 4 (TLR4)/NF-κB pathway, and whether these effects are related to the release of calcitonin gene-related peptide (CGRP). **Methods** Sprague-Dawley rats were subjected to 60 min of ligation of the left anterior descending coronary artery followed by 3 h of reperfusion to induce I/R injury. IPC was performed by 4 cycles of 3-min left coronary artery occlusion followed by 5-min reperfusion before the I/R. The expression of *TLR4* mRNA was determined by RT-PCR. TLR4 and NF-κB protein expression were analyzed by immunohistochemistry. Myocardial infarct size, CGRP concentration in plasma and activity of creatine kinase in serum were also measured. **Results** IPC significantly reduced the infarct size and creatine kinase activity concomitantly with the increase in plasma CGRP concentration. The expressions of TLR4 protein and mRNA and NF-κB protein were increased by myocardial I/R injury, and dramatically inhibited by IPC. **Conclusion** IPC protects against myocardial I/R injury by inhibition of TLR4/NF-κB pathway. These effects are related to the increased the release of CGRP.

Key words: toll-like receptor 4; ischemia/reperfusion injury; ischemic preconditioning; heart; rats

缺血预适应通过抑制 TLR4/NF-κB 信号通路保护大鼠心肌缺血再灌注损伤

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[摘要] **目的:**研究缺血预适应对大鼠心肌缺血再灌注损伤的保护作用是否由 toll 样受体 4 (TLR4)/NF-κB 途径所介导,以及是否与促进降钙素基因相关肽(CGRP)释放有关。**方法:**结扎 Sprague-Dawley 大鼠左冠状动脉前降支 60 min, 复灌 3 h 造成心肌缺血再灌注损伤。缺血预适应为结扎大鼠左冠状动脉前降支

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5 min, 复灌 5 min, 共 4 个周期。RT-PCR 分析心肌 *TLR4* mRNA 表达。免疫组织化学法分析心肌 TLR4 和 NF-κB 蛋白表达。同时,测定心肌梗死面积、血浆 CGRP 浓度和血清肌酸激酶活性。**结果**:缺血预适应显著减少心肌梗死面积,降低肌酸激酶活性,增高血浆 CGRP 水平。心肌缺血再灌注可显著上调 TLR4 和 NF-κB 表达,缺血预适应可抑制其作用。**结论**:缺血预适应通过抑制 TLR4/NF-κB 信号通路保护大鼠心肌缺血再灌注损伤,其作用与促进 CGRP 释放有关。

[**关键词**] Toll 样受体 4; 缺血再灌注损伤; 缺血预适应; 心脏; 大鼠
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Toll-like receptors (TLRs) are a family of pattern recognition receptors that serve as a key part of the innate immune system[1]. There is growing evidence that TLRs play an important role in cardiovascular pathologies, such as myocardial ischemia/reperfusion (I/R) injury and atherosclerosis[2]. Toll-like receptor 4 (TLR4) is a key signaling receptor in innate immune responses[1]. TLR4 is expressed not only in immune cells but also in the cardiomyocytes and endothelial cells[3]. During myocardial I/R in vivo, TLR4-mediated MyD88-dependent signaling pathway was activated by the rapid activation of IκB kinase-beta (IKKβ) and increase of IκBα phosphorylation and degradation, which resulted in myocardial nuclear factor-κB (NF-κB) activation[4]. TLR4-mediated MyD88-dependent NF-κB pathways have been documented to play a crucial role in the innate immunity and inflammatory responses[5]. It has been shown that TLR4-deficient mice have smaller infarct size and exhibit less inflammation after myocardial I/R injury[6], and TLR4 antagonist (eritoran, E-5564) given in advance reduce inflammatory response in I/R injury[7]. These findings suggested that TLR4 played a pro-inflammatory role in myocardial I/R injury[8].

Ischemic preconditioning (IPC) refers to the phenomenon whereby a brief period of ischemia protects a tissue against a subsequent severe ischemic insult[9]. IPC seems to involve a variety of signals which include activation of membrane receptors, signaling molecules and expression of many endogenous protective substances[9]. Calcitonin related-gene peptide (CGRP), a predominant neurotransmitter in capsaicin-sensitive sensory nerves, has been shown to be an endogenous myocardial protective substance which mediates the cardioprotection of IPC[10-13]. In addition, CGRP may inhibit the activities of both innate and adaptive immune cells by negative regulation of TLR responses[14]. In the present study, we therefore examined whether the protection of IPC against myocardial I/R injury is mediated by TLR4/NF-κB pathway, and whether these effects are related to the increase of CGRP.

1 MATERIALS AND METHODS

1.1 Animals and reagents

Male Sprague-Dawley rats (Laboratory Animal Center, Xiangya School of Medicine, Central South University, Changsha, China) weighing 220–290 g were randomly divided into 4 groups: a normal control group ($n=5$), a sham-operated group ($n=5$), an I/R group ($n=6$), and an I/R + IPC group ($n=7$). All animals received humane care in compliance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals published by the National Institutes of Health (Public Law 99-158, revised 1986, reprinted 2000).

Triphenyl tetrazolium chloride and Evans blue were purchased from Sigma (St. Louis, MO, USA). Immunohistochemistry kit, creatine kinase assay kits and radioimmunoassay kits for measurement of CGRP were purchased from Jingmei Bioengineering (Shenzhen, China), Beijing Zhongsheng High-tech Bioengineering (Beijing, China) and Dongya Immunity Technology Institution (Beijing, China), respectively.

1.2 Surgical preparation

Animals were anesthetized with chloral hydrate (300 mg/kg, i.p.), and then mechanically ventilated with room air using a positive pressure ventilator. The ventilation rate was maintained at 30 – 35 strokes/min with a tidal volume of approximately 15 mL/kg body weight. Electrocardiograph (ECG) leads were connected to the chest and limbs for continuous ECG monitoring throughout the experiment. A left thoracotomy was performed in the fourth intercostal space and the pericardium opened to expose the heart. A 4-0 silk suture was passed around the left coronary artery at a point two-thirds of the way between its origin near the pulmonary conus and the cardiac apex and a snare was formed by passing both

ends of the suture through a piece of polyethylene tubing. Occlusion of the coronary artery, by clamping the snare against the surface of the heart, caused an area of epicardial cyanosis with regional hypokinesis and ECG changes. Reperfusion was achieved by releasing the snare and was confirmed by conspicuous hyperaemic blushing of the previously ischemic myocardium and gradual resolution of the changes in the ECG signal. The sham-operated group underwent the same procedure but without clipping of the coronary artery.

1.3 Experimental protocols

The experiment was done with the animals randomly divided into 4 groups. All animals were subjected to 60 min of coronary artery occlusion followed by 180 min of reperfusion, except the sham-operated group. For IPC, rats were subjected to 4 cycles of 3-min left coronary artery occlusion followed by 5-min reperfusion before the I/R.

1.4 Infarct size and area at risk

At the end of 3-h reperfusion, blood samples were collected from the carotid artery. The left coronary was reoccluded, and 1 mL Evans blue (1%) was injected into the left ventricular cavity in vivo and allowed to perfuse the nonischemic portions of the heart. The entire heart was excised, weighed, rinsed of excess blue dye, trimmed of right ventricular and atrial tissue, and sliced into 1-mm thick sections from the apex to base. The slices were incubated in 1% triphenyl tetrazolium chloride solution at 37 ℃ for 20 min to stain the viable myocardium brick red. The samples were then fixed in a 10% formalin solution for 24 h. Sections were traced onto acetate sheets and photographed. The area of infarct and risk zone was determined by planimetry of the tracings (Image J 1.38). Infarct and risk area volumes were expressed as mm^3 and infarct size was calculated as a percentage of the risk zone.

1.5 Creatine kinase assay

At the end of 3-h reperfusion, the serum creatine kinase activity was measured spectrophotometrically.

1.6 Semiquantitative reverse transcription-polymerase chain reaction

Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA integrity was confirmed by agarose gel electrophoresis, and RNA was quantified by spectrophotometric analysis. cDNA was synthesized from 4 μg of the total RNA using RevertAid™ first strand cDNA Synthesis kit(MBI) in a total reaction volume of 20 μL. Synthetic oligonucleotide primers based on the cDNA sequences of rat *TLR4* (GenBank Accession No. NM_019178) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; GenBank Accession No. NM_017008) were prepared: 5′-CAGCTCTCAACCTTGGTACT-3′ and 5′-GATGGACATGTAAACCAGTC-3′ (*TLR4*); 5′-AACTCCCTCAAGATTGTCAGC-3′ and 5′-GGGAGTTGCTGTTGAAGTCACA-3′ (*GAPDH*). After initial denaturation of the cDNA at 94 ℃ for 5 min, 37 amplification cycles (denaturation at 94 ℃ for 45 s, annealing at 64 ℃ for 30 s, and polymerization at 72 ℃ for 1 min) were performed, followed by a final extension at 72 ℃ for 10 min. For *GAPDH*, annealing temperature was 58 ℃, and 25 cycles were performed. PCR products were analyzed by electrophoresis on a 1% agarose gel, stained with ethidium bromide and photographed under UV light. The intensity of the amplified bands was analyzed by Gray-scale image analysis system and gene level was expressed by *TLR4/GAPDH* ratio.

1.7 Immunohistochemistry

Hearts were perfused with 4% paraformaldehyde, fixed with 4% paraformaldehyde at 4 ℃ for 24 h, embeded in paraffin and then cut into 8 μm sections. Prior to visualization of proteins, the sections were treated with xylene to remove the paraffin and rehydrated using ethanol. Sections were stained with hematoxylin and eosin to assess morphology and evidence of injury. To quench endogenous peroxidase activity, sections were incubated in 3% hydrogen peroxide for 10 min. The following primary antibodys were used, and applied to the sections for 24 h at 4 ℃: rabbit anti-rat TLR4 antibody (1∶100, SC-30002, Santa Cruz Biotechnology Inc., USA) and rabbit anti-rat NF-κB antibody (1∶100, 1559-1, Epitomics). Subsequently, biotinylated anti-rabbit antibodies were incubated with the sections for 15 min, followed by incubation with horseradish peroxidase and development with 3, 3′-diaminobenzidine substrate. To exclude unspecific staining of the HRP-labeled polymer, antibody diluents without primary antibody were used as negative control. Sections were counterstained with hematoxylin, dehydrated with increasing concentrations of ethanol, rinsed in xylene and mounted with neutral resin. Finally, the staining were examined with light microscopy,

and analyzed with microarray image analysis system. The results were expressed by average optical density.

1.8 Measurement of plasma CGRP concentration

Blood samples were collected from the carotid artery, and placed in tubes containing 10% Na_2EDTA 30 μL and aprotinin 400 mU/L. Plasma was obtained by centrifugation at 1 300 g for 20 min (4 ℃). Plasma concentration of CGRP-like immunoreactivity (CGRP-LI) was determined by radioimmuoassay kits using antisera raised against rat CGRP, ^{125}I-labelled CGRP and rat CGRP standard. CGRP concentration (pg/mL) was calculated according to the standard curve.

1.9 Statistical analysis

Data were expressed as means ± standard error and were analyzed with SPSS12.0 software. All values were analyzed by using one-way analysis of variance (ANOVA) and the Student-Newman-Keuls test. The significance level was $P < 0.05$.

2 RESULTS

2.1 Infarct size

There were no difference in heart wet weights and risk zone, indicating that the size of the risk zone was comparable in all groups. I/R caused a (40.39 ± 2.40) % necrosis in the area at risk. IPC significantly reduced infarct size (Tab. 1). Sham operation had no effect on infarct size.

Tab. 1 Comparison of body weight, heart wet weight, area at risk and infarct size

Groups	*n*	Body weight/g	Heart wet weight/g	Area at risk/mm^3	Infarct volume/mm^3	Infarct size/%
I/R group	6	255.4 ± 3.3	0.85 ± 0.01	293.0 ± 16.6	114.3 ± 14.3	40.4 ± 2.4
I/R + IPC group	7	251.2 ± 5.1	0.85 ± 0.02	381.5 ± 65.7	83.3 ± 19.9 *	21.8 ± 1.8 *

Compared with the I/R group, * $P < 0.05$.

2.2 Creatine kinase release

I/R caused a significant increase in the serum level of creatine kinase. IPC significantly reduced the release of creatine kinase (Fig. 1). Sham operation had no effect on the release of creatine kinase.

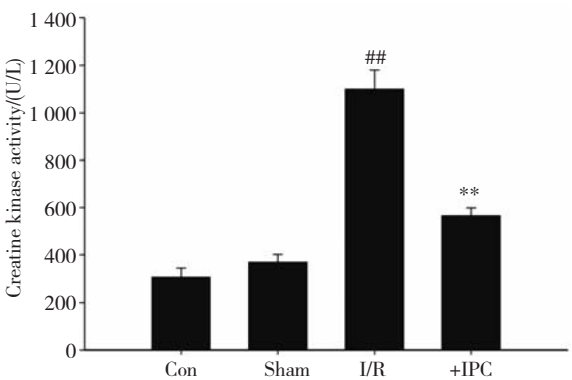

Fig. 1 Effect of cardiac ischemic preconditioning on creatine kinase activity. Con: Control group; Sham: Sham-operated group; I/R: Ischemia/reperfusion group; + IPC: I/R with ischemic preconditioning group. Compared with the sham group, ## $P < 0.01$; compared with the I/R group, * * $P < 0.01$.

2.3 Inflammatory response

In the normal control and sham-operated groups, left ventricular fibers were regularly arranged, the cell border was integrated, and no inflammatory cells infiltration was found. I/R caused myocardial fibers necrosis, twist and broken, myocardial cells swelling and degeneration, cell nuclear concentration or dissolution, and neutrophil infiltration, and the effect was attenuated by IPC (Fig. 2).

2.4 TLR4 mRNA and protein expression

Compared with the normal control group, sham operation had no effect on *TLR4* mRNA expression. I/R significantly up-regulated *TLR4* mRNA expression ($P < 0.05$, Fig. 3). IPC reversed up-regulation of *TLR4* mRNA expression caused by I/R.

Consistent with Fig. 3, immunohistochemistry staining showed that I/R also significantly up-regulated TLR4 protein expression ($P < 0.05$, Fig. 4), and the effect was reversed by IPC.

2.5 NF-κB protein expression

Compared with the normal control group, sham operation had no effect on NF-κB protein expression. In contrast, I/R significantly increased NF-κB protein expression, and IPC remarkably reduced NF-

κB protein expression caused by I/R (the optical density: 0.56 ± 0.02 vs. 0.33 ± 0.03, $P < 0.01$; Fig. 5).

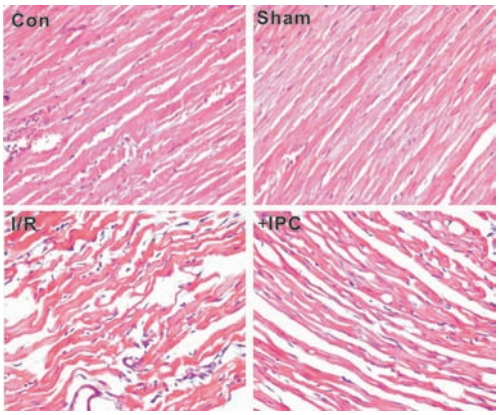

Fig. 2 Myocardial HE staining in rats (×400). Con: Control group; Sham: Sham-operated group; I/R: Ischemia/reperfusion group; + IPC: I/R with ischemic preconditioning group.

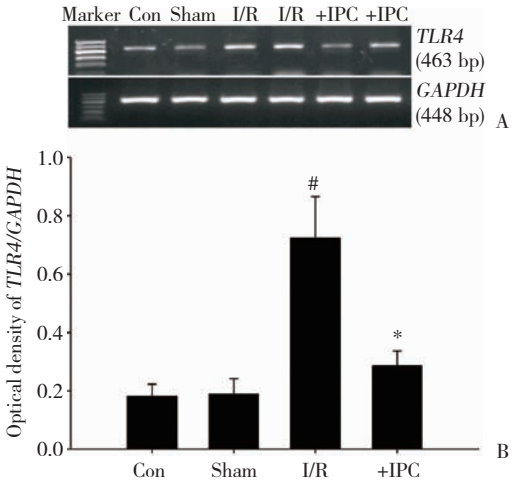

Fig. 3 Effect of ischemic preconditioning on *TLR4* mRNA expression. A: RT-PCR products of *TLR4* and *GAPDH*. B: Optical density of *TLR4/GAPDH*. Con: Control group; Sham: Sham-operated group; I/R: Ischemia/reperfusion group; + IPC: I/R with ischemic preconditioning group. Compared with the sham group, #$P < 0.05$; compared with the I/R group, *$P < 0.05$.

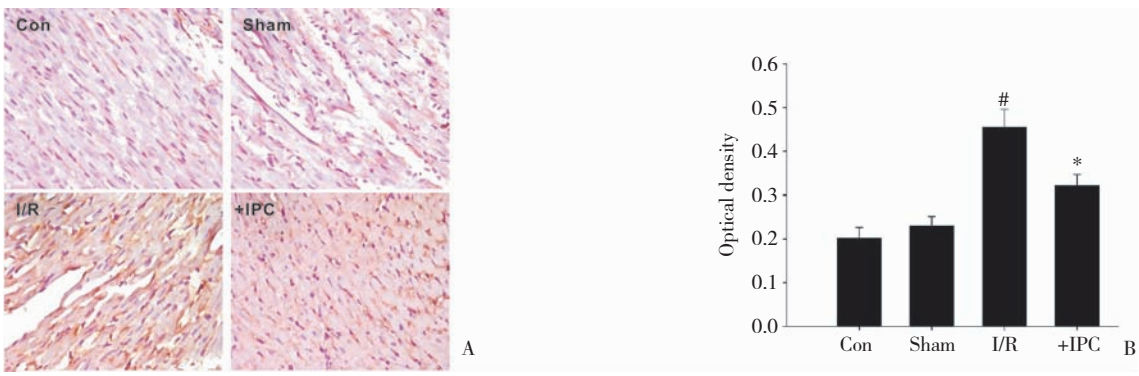

Fig. 4 Immunohistochemistry analysis of myocardial TLR4 protein in rats. A: The positive expression of TLR4 (brown) was localized in cell membrane and cytoplasm (×400); B: Optical density. Con: Control group; Sham: Sham-operated group; I/R: Ischemia/reperfusion group; + IPC: I/R with ischemic preconditioning group. Compared with the sham group, #$P < 0.05$; compared with the I/R group, *$P < 0.05$.

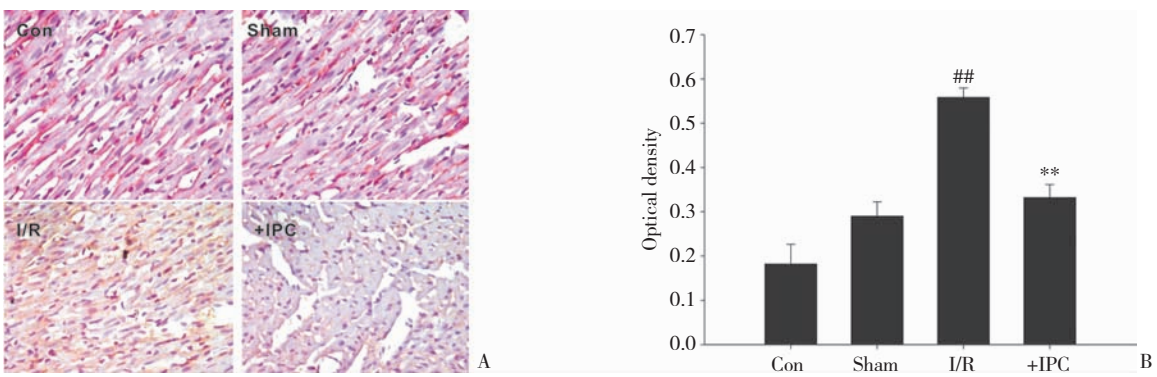

Fig. 5 Immunohistochemistry analysis of myocardial NF-κB protein in rats. A: The positive expression of NF-κB (brown) was localized in cell nucleus and cytoplasm (×400); B: Optical density. Con: Control group; Sham: Sham-operated group; I/R: Ischemia/reperfusion group; + IPC: I/R with ischemic preconditioning group. Compared with the sham group, ##$P < 0.01$; compared with the I/R group, **$P < 0.01$.

2.6 Plasma concentrations of CGRP-LI

Compared with the normal control group, sham operation and I/R had no effect on plasma concentrations of CGRP-LI. Importantly, IPC markedly increased plasma concentrations of CGRP-LI ($P < 0.01$, Fig. 6).

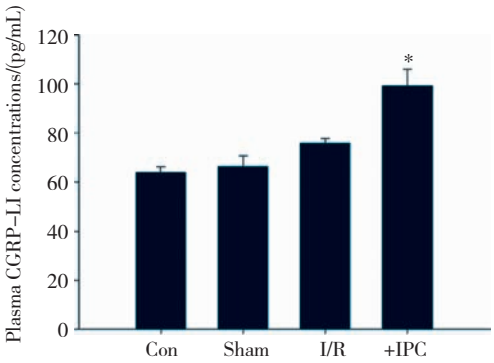

Fig. 6 Effect of ischemic preconditioning on plasma CGRP-LI concentration. Con: Control group; Sham: Sham-operated group; I/R: Ischemia/reperfusion group; + IPC: I/R with ischemic preconditioning group. Compared with the I/R group, $*P < 0.05$.

3 DISCUSSION

TLR4 is a key signaling receptor in innate immune responses, and could be a key link between the innate and adaptive immune system[1]. In addition to being expressed in immune cells, TLR4 are expressed in cardiovascular system, such as cardiomyocytes and endothelial cells[3]. There is an innate immune system with functional integrity in myocardium[15]. TLR4 mediated MyD88-dependent NF-κB pathways play a crucial role in the innate immunity and inflammatory responses[5, 15]. In the resting state, NF-κB is inactive in the cytoplasm with its inhibitory protein—IκB. While IκB can be phosphorylated and degraded by stimulus, NF-κB can translocate from cytoplasm to nucleus; then several inflammatory mediator genes are transcribed. In coronary artery endothelial cells, TLR4 activation induces the production of TNF-α, IL-1, VCAM-1, and ICAM-1[16]. In macrophages, TLR4 siRNA inhibits LPS-induced the release of inflammatory cytokines[17]. The promoters of these inflammatory cytokines contain NF-κB binding sites. NF-κB can be activated by different stimuli. When hearts were subjected to I/R, myocardial and endothelial cells expressed several inflammatory cytokines, such as IL-1, IL-6, IL-10, and TNF-α[3]. Finally, local inflammatory reactions were started and regulated to induce cardiac impairment[3]. Moreover, activity of NF-κB and expression of inflammatory factors are increased in myocardial I/R injury models, and NF-κB subunit p50 knockout mice were lower sensitive to I/R injury[18]. These suggested that TLR4/NF-κB-mediated inflammatory responses played an important role in myocardial I/R injury.

IPC is the most effective means in the prevention of myocardial I/R injury[9]. The main cardioprotective effect of IPC is to limit infarct size. IPC can not entirely prevent the development of myocardial ischemic necrosis, and also can not substitute reperfusion therapy which remains the only treatment to terminate ischemic injury. IPC is believed to be a preventive and prophylactic approach[9, 19]. The mechanisms responsible for IPC are viewed as a complex signaling cascade, which may involve several G-protein coupled cell surface receptors, second messengers, specific Ser-Thr-protein kinase-C isoforms, mitogen-activated protein kinases, and production of endogenous protective substances[20].

CGRP, a major transmitter of capsaicin-sensitive sensory nerves, has been shown to play an important role in mediation of the preconditioning induced by brief ischemia or hyperthermia or by some drugs[10-13]. The cardioprotection afforded by CGRP-mediated preconditioning is due to inhibition of cardiac TNF-α production[13]. Interestingly, CGRP has been found to inhibit the activation of the immune cells by negative regulation of TLR responses[14]. Whether the protection of IPC against myocardial I/R injury is mediated by TLR4/NF-κB pathway remains unclear. In the present study, we found that IPC increased CGRP release, down-regulated TLR4 and NF-κB expression, and resultantly limited myocardial infarct size and reduced creatine kinase release. Neutrophils have been implicated as primary and secondary mediators of lethal injury after reperfusion to coronary vascular endothelium and cardiomyocytes[21]. The present study showed that IPC also concomitantly decreased neutrophil infiltration. These results suggested that the cardioprotection afforded by IPC was related to inhibiting TLR4/NF-κB-mediated inflammatory responses.

In summary, TLR4/NF-κB-mediated inflammatory responses are involved myocardial I/R injury,

and IPC protects against myocardial I/R injury by inhibiting TLR4/NF-κB pathway. These effects are related to the increased release of CGRP.

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