



# Hypercholesterolemia abrogates the protective effect of ischemic postconditioning by induction of apoptosis and impairment of activation of reperfusion injury salvage kinase pathway



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## ABSTRACT

Ischemic postconditioning (IPoC) is an effective method to prevent myocardial ischemia reperfusion injury (MIRI), but its cardioprotection is usually blocked in the presence of hypercholesterolemia (HC) and the potential mechanism is still unknown. In this study, we investigated the roles of reperfusion injury salvage kinase (RISK) and apoptosis-related pathways in the attenuation of cardioprotection of IPoC in the presence of HC. The results showed that IPoC significantly decreased the infarct size and apoptosis, improved the recovery of ischemic myocardium, but these beneficial effects were reversed by high cholesterol diet-induced HC. Moreover, we also found that HC inhibited the phosphorylation of Akt and ERK1/2 which usually activated by IPoC in normal heart, induced excessive apoptosis by down-regulating Bcl-2 and up-regulating Bax, cytochrome c, caspase 9 and caspase 3 when compared with that in normal heart. Taken together, our results demonstrated that the cardioprotection of IPoC was abolished by HC, which was associated with inactivation of RISK signal pathway and dysregulation of downstream apoptosis-related pathway.

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## 1. Introduction

Ischemic heart disease is one of major causes of death worldwide [1]. Reperfusion therapy, such as coronary artery bypass grafting (CABG) and percutaneous coronary intervention (PCI), is believed to be the most effective method to salvage ischemic myocardium, especially on the treatment of acute myocardial infarction (AMI) [2]. However, the abrupt reperfusion may lead to further damage of ischemic myocardium, known as myocardial ischemia reperfusion injury (MIRI) [3]. At present, there is still lack of effective therapy for preventing MIRI [4]. Therefore, it is extremely urgent to develop an effective approach to cope with MIRI during the implement of reperfusion therapy.

Recently, much attention has been focused on the ischemic postconditioning (IPoC), repetitive cycles of brief reperfusion and

ischemia performed after sustained ischemia, which is considered as an effective method to attenuate MIRI as evidence by reduction in infarct size and accelerating recovery of cardiac function [5,6]. However, the cardioprotection of IPoC is most demonstrated in healthy animals, and several studies indicate that many risk factors of cardiovascular diseases including hyperlipidemia, diabetes, hypertension and aging may impair the protective effect of IPoC [7]. So far, the mechanism of attenuation of the cardioprotective effect of IPoC in the presence of these risk factors is not well understood.

Our previous research also confirms that hypercholesterolemia abrogates the cardioprotection of IPoC in isolated rat hearts and the abrogation of IPoC in hypercholesterolemic rat heart is involved in the impairment of anti-apoptosis effect and the excessive opening of mitochondrial permeability transition pore (mPTP) [8]. Although the cardioprotection of IPoC is involved in several signaling pathways, activation of reperfusion injury salvage kinase (RISK) signaling pathway, which subsequently acts to prevent mPTP opening and further regulate the apoptosis-related pathway, is still considered as a major mechanism of IPoC [9,10]. Therefore, we infer that the abrogation of IPoC in the presence of hypercholesterolemia is involved in inactivation of RISK signaling pathway and dysregulation of apoptosis-related pathways. In the present study,

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we investigated the roles of RISK and apoptosis-related pathways in the attenuation of IPoC in the presence of hypercholesterolemia.

## 2. Materials and methods

### 2.1. Animals and induction of experimental hypercholesterolemia

All the experimental procedures concerning animals were performed strictly in adherence to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH). The study procedure was approved by the institutional Ethics Committee of China Medical University (Shenyang, China).

Sixty male Wistar rats weighing  $200 \pm 10$  g were randomly divided into hypercholesterolemia (HC) group and normocholesterolemia (NC) group. Animals in HC group were fed with a diet including 1.5% cholesterol, 5% egg yolk powder, 10% lard, 0.5% sodium cholate, 3% sugar, and 80% normal feedstuff for 8 weeks, and this formula was based on our previous report [8], whereas animals in NC group received a normal diet (Qian Ming Experimental animal feed factory, Shenyang, China) for the same period. At the end of 8-week feeding period, blood samples were collected from the rats' vena caudalis for determination of serum levels of total cholesterol (TC), high density lipoprotein (HDL) and low density lipoprotein (LDL) using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

### 2.2. Heart preparation

Rats were anesthetized through an intraperitoneal injection of 10% chloral hydrate (4 ml/kg). Heparin (1500 IU/kg) was administered intravenously prior to the surgery to prevent intracoronary clot formation. After opening the chest, the heart was rapidly excised and immediately immersed in ice-cold heparinized Krebs–Henseleit solution (KH solution: 127 mmol/l NaCl, 17.7 mmol/l  $\text{NaHCO}_3$ , 5.1 mmol/l KCl, 1.5 mmol/l  $\text{CaCl}_2$ , 1.26 mmol/l  $\text{MgCl}_2$ , 11 mmol/l D-glucose, pH = 7.4) for trimming. Then the heart was mounted on a Langendorff-perfusion apparatus and retrogradely perfused through the aorta with KH solution saturated with 95%  $\text{O}_2$ –5%  $\text{CO}_2$  under a constant pressure of 75 mmHg at 37 °C. The fluid-filled latex balloon was inserted in the left ventricle via the left atrium for pressure measurement. The balloon was connected to a pressure transducer and the hemodynamic parameters including heart rate (HR), left ventricular developed pressure (LVDP), positive first order derivative of ventricular pressure (+dp/dt) and negative first order derivative of ventricular pressure (–dp/dt) were continuously recorded and digitally processed via a hemodynamic system (BIOPAC MP150, USA).

### 2.3. Experimental protocol

Rats in NC and HC groups were further assigned to two subgroups: (i) ischemia reperfusion (IR) group: the isolated rat hearts were allowed for 20 min of stabilization, and then subjected to 30 min of global ischemia and 120 min of reperfusion; (ii) ischemic postconditioning (IPoC) group: the isolated rat hearts were also allowed for 20 min of stabilization and subjected to 30 min of global ischemia. At the onset of long time of reperfusion, six cycles of IPoC ( $6 \times 10$  s) were conducted, and then followed by 120 min of reperfusion.

### 2.4. Measurement of infarct size

Measurement of infarct size was previously described [11]. Briefly, at the end of reperfusion, the heart was harvested and

stored at  $-20$  °C for 1 h. The whole heart was sectioned from apex to base into 1–2 mm sections, and incubated in 1% triphenyltetrazolium chloride (TTC) solution for 20 min at 37 °C, fixed by 4% paraformaldehyde for 24 h, and then photographed by a digital camera. The infarct myocardium tissues were unstained and turned into white, whereas viable myocardium tissues were stained and presented red.

### 2.5. Determination of myocardial apoptosis

The specific procedure for determination of myocardial apoptosis was previously described [12]. Briefly, myocardial apoptosis was detected using In Situ Cell Death Detection Kit (Roche, USA) according to the manufacturer's instructions. The apoptotic nuclei were stained dark brown. In contrast, the normal nuclei were presented blue. Three sections from each myocardial sample were randomly selected and 10 microscopic fields (Olympus BX51 microscope) per section were evaluated by two independent blind observers. For each field, the number of nuclei was counted and the percentage of TUNEL-positive nuclei was calculated.

### 2.6. Western blotting

Proteins were extracted from the myocardial tissues using a protein extraction reagent (Beyotime, Shanghai, China) and protein concentration was measured using the BCA Protein Assay kit (Beyotime, Shanghai, China). 50  $\mu\text{g}$  protein of each sample was separated by electrophoresis on SDS-PAGE and transferred onto polyvinylidene difluoride-plus membrane. The membranes were blocked with 5% skim milk followed by incubated overnight at 4 °C with the antibodies: Akt (1:500; Santa Cruz, California, USA); phospho-Akt (at Ser473, 1:500; Santa Cruz, California, USA); ERK1/2 (1:500; Santa Cruz, California, USA); phospho-ERK1/2 (at Thr 202 and Tyr 204, 1:500; Santa Cruz, California, USA); Bcl-2 (1:1000; Abcam, Hongkong); Bax (1:1000; Abcam, Hongkong); cytochrome c (1:1000; Abcam, Hongkong), caspase 9 (1:1000; Abcam, Hongkong) and caspase 3 (1:1000; Abcam, Hongkong). After incubation, the membranes were washed three times with 0.1% Tween-20 for 15 min and incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:2000; Santa Cruz, California, USA) at 37 °C for 2 h. Detection was performed by enhanced chemi-luminescence (ECL) using a Western blotting luminological reagent (Santa Cruz, California, USA) according to the manufacturer's instructions. The levels of phosphorylated proteins were normalized to their total protein levels. Relative densitometry was performed using a computerized software package (NIH Image 1.63 software).

### 2.7. Statistical analysis

The data were expressed as mean  $\pm$  SD values. Statistical analysis was performed by using Sigma Stat software version 3.5 (Systat software). Differences between groups were evaluated using one-way analysis of variance (ANOVA), followed by Student–Newman–Keuls post hoc test. All P values less than 0.05 were considered statistically significant.

## 3. Results

### 3.1. The levels of serum lipids

The values for serum lipids of rats in NC and HC groups were listed in Table 1. The levels of TC and LDL were significantly increased in HC group compared with NC group ( $P < 0.05$ ).

**Table 1**  
Serum lipid analysis.

	Total cholesterol (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)
Normocholesterolemia group	58.3 ± 6.8	21.2 ± 7.8	27.9 ± 8.3
Hypercholesterolemia group	205.6 ± 11.2*	26.8 ± 10.6	162.5 ± 13.3*

Data were expressed as mean ± SD, n = 20 for each group. \* indicates  $P < 0.05$ , hypercholesterolemia group vs. normocholesterolemia group.

However, no significant difference was showed in the level of HDL between NC group and HC group ( $P > 0.05$ ).

### 3.2. Hemodynamic changes

All changes of the hemodynamic parameters before and during reperfusion were summarized in Table 2. None of significant statistical differences in all hemodynamic parameters were observed in all experimental groups at baseline. A significant improvement in the values of HR, LVDP, +dp/dt and -dp/dt was displayed in IPoC-NC group as compared with IR-NC group during 10 min, 30 min and 120 min reperfusion ( $P < 0.05$ ). However, there was no significant difference between IR-HC group and IPoC-HC group at the same time of reperfusion ( $P > 0.05$ ).

### 3.3. Infarct size

As shown in Fig. 1A, a remarkable reduction in infarct size was noted in IPoC-NC group as compared with IR-NC group ( $28.4 \pm 4.6\%$  vs.  $51.3 \pm 7.1\%$ ,  $P < 0.05$ ). However, no significant differences were detected in the infarct size between IR-HC group and IPoC-HC group ( $52.6 \pm 7.1\%$  vs.  $48.5 \pm 6.9\%$ ,  $P > 0.05$ ).

### 3.4. TUNEL staining for apoptosis

The ratios of apoptosis among all the experimental groups were shown in Fig. 1B. A significant decrease in the ratio of apoptosis was shown in IPoC-NC group as compared with IR-NC group ( $15.5 \pm 3.7\%$  vs.  $27.8 \pm 4.5\%$ ,  $P < 0.05$ ), but the ratio of apoptosis did not differ between IR-HC group and IPoC-HC group ( $29.5 \pm 4.7\%$  vs.  $26.1 \pm 4.8\%$ ,  $P > 0.05$ ).

### 3.5. Expression of Akt and ERK1/2 proteins

As shown in Fig. 2B and C, Akt and ERK1/2 phosphorylation were enhanced in IPoC-NC group as compared with IR-NC group ( $0.89 \pm 0.16$  vs.  $0.48 \pm 0.17$  in Akt phosphorylation,  $P < 0.05$ ; and  $1.49 \pm 0.13$  vs.  $0.94 \pm 0.16$  in ERK1/2 phosphorylation,  $P < 0.05$ ), but none of significant differences in Akt and ERK1/2 were shown between IR-HC group and IPoC-HC group. Furthermore, there was no significant change in the expression of total Akt and ERK1/2 among all groups (Fig. 2D and E).

### 3.6. Expression of Bcl-2 and Bax proteins

A significant increase in the expression of Bcl-2 protein and a decrease in the expression of Bcl-2 protein were presented in IPoC-NC group as compared with IR-NC group ( $0.62 \pm 0.10$  vs.  $0.29 \pm 0.12$  in Bcl-2 protein expression,  $P < 0.05$ ; and  $0.19 \pm 0.06$  vs.  $0.32 \pm 0.04$  in Bax protein expression,  $P < 0.05$ ), but no significant changes either in the expression Bcl-2 or Bax protein were found between IR-HC group and IPoC-HC group (Fig. 3C and D). Moreover, the ratio of Bcl-2/Bax was also significantly increased in IPoC-NC group as

compared with IR-NC group ( $3.34 \pm 0.19$  vs.  $0.90 \pm 0.22$ ,  $P < 0.05$ ) (Fig. 3B).

### 3.7. Expression of cytochrome c, caspase 9 and caspase 3 proteins

A notable reduction in the expression of cytochrome c, caspase 9 and caspase 3 proteins was found in IPoC-NC group as compared with IR-NC group ( $0.24 \pm 0.08$  vs.  $0.55 \pm 0.12$  in cytochrome c protein expression,  $P < 0.05$ ;  $0.27 \pm 0.05$  vs.  $0.44 \pm 0.06$  in caspase 9 protein expression,  $P < 0.05$ ; and  $0.24 \pm 0.07$  vs.  $0.40 \pm 0.09$  in caspase 3 protein expression,  $P < 0.05$ ). However, no significant differences in the expression of cytochrome c, caspase 9 and caspase 3 proteins were shown between IR-HC group and IPoC-HC group. These results were illustrated in Fig. 4.

## 4. Discussion

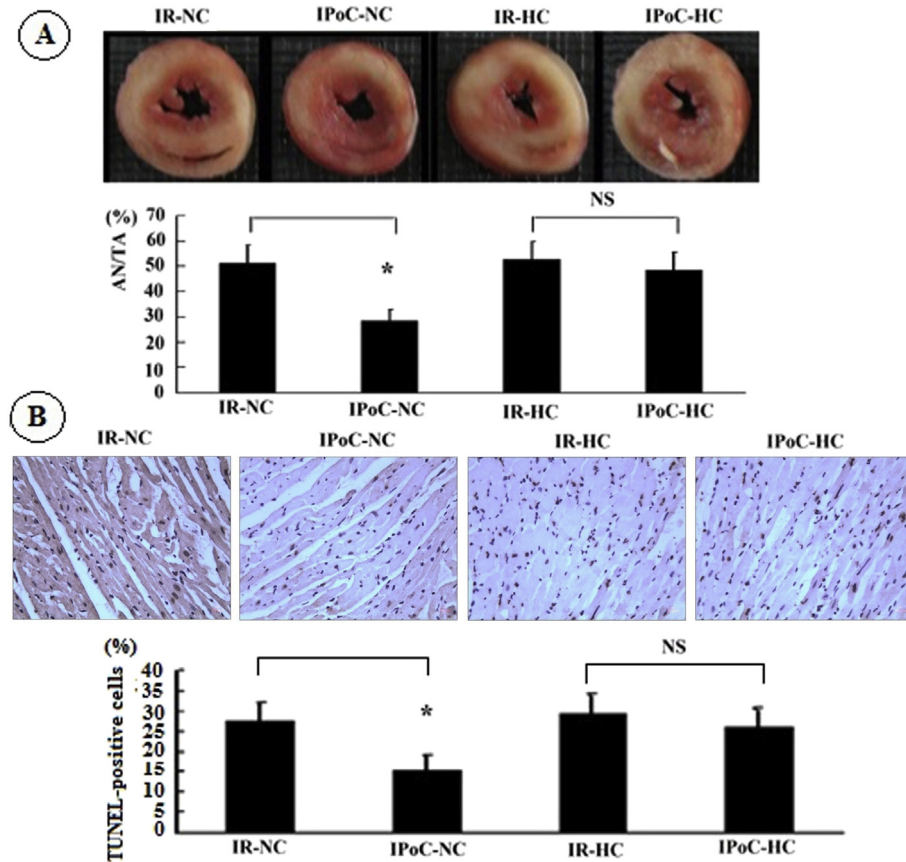
Ischemic preconditioning (IPC) and IPoC are two similar protective methods against MIRI. One of major limitation of IPC is that it should be applied before the occurrence of ischemic events, which is hard to predict in advance in clinical practice. In contrast, IPoC can be applied after sustained ischemia and overcome the limitation of IPC [13]. Therefore, IPoC is more important to prevent MIRI in clinical practice. Recently, several reports from large scale randomized controlled trial and meta-analysis have shown that IPoC did not improve myocardial reperfusion in patients with ST-segment-elevation myocardial infarction undergoing primary PCI [14–16]. The reason may be that several cardiovascular risk factors that are common in myocardial infarction patients compromise the cardioprotection of IPoC [7].

Hyperlipidemia, especially hypercholesterolemia, is one of major risk factor for myocardial infarction. In the present study, our result suggested that hypercholesterolemia impaired the infarct size limiting effect of IPoC, which was consistent with most of studies and our previous research [8,17–22]. However, two studies still suggested that IPoC was still effective in hyperlipidemic rats and rabbits [23,24]. Furthermore, our study found that hypercholesterolemia abrogated the effect of IPoC on the recovery of

**Table 2**  
Hemodynamic parameters changes before and during reperfusion.

Time	Baseline	R-10	R-30	R-120
<b>HR</b>				
IR-NC	246 ± 13	145 ± 12	168 ± 13	150 ± 14
IPoC-NC	252 ± 16	163 ± 11*	183 ± 12*	167 ± 12*
IR-HC	247 ± 11	141 ± 16	162 ± 20	149 ± 16
IPoC-HC	250 ± 12	149 ± 20	171 ± 22	156 ± 15
<b>LVDP (mm Hg)</b>				
IR-NC	98 ± 6.4	35 ± 4.4	39 ± 3.8	34 ± 4.0
IPoC-NC	96 ± 6.9	43 ± 3.5*	49 ± 3.2*	45 ± 4.3*
IR-HC	100 ± 4.4	35 ± 5.6	38 ± 4.8	33 ± 5.8
IPoC-HC	97 ± 7.3	33 ± 6.8	40 ± 6.2	34 ± 3.3
<b>+dp/dt(mm Hg/s)</b>				
IR-NC	2322 ± 230	1563 ± 205	1632 ± 188	1482 ± 178
IPoC-NC	2308 ± 275	1754 ± 218*	1840 ± 175*	1690 ± 168*
IR-HC	2380 ± 244	1506 ± 197	1608 ± 256	1540 ± 193
IPoC-HC	2335 ± 262	1589 ± 211	1675 ± 205	1530 ± 238
<b>-dp/dt(mm Hg/s)</b>				
IR-NC	1586 ± 202	1088 ± 138	1314 ± 124	1158 ± 152
IPoC-NC	1606 ± 188	1255 ± 148*	1450 ± 145*	1282 ± 126*
IR-HC	1598 ± 213	1109 ± 202	1282 ± 186	1188 ± 184
IPoC-HC	1625 ± 195	1012 ± 186	1294 ± 230	1164 ± 182

IR, ischemia reperfusion; IPoC, ischemic postconditioning; NC, normocholesterolemia; HC, hypercholesterolemia; HR, heart rate; LVDP, left ventricular developed pressure; +dp/dt, positive first order derivative of ventricular pressure; -dp/dt, negative first order derivative of ventricular pressure. Values are means ± SD. \* $P < 0.05$  vs. IR-NC (n = 8–10).

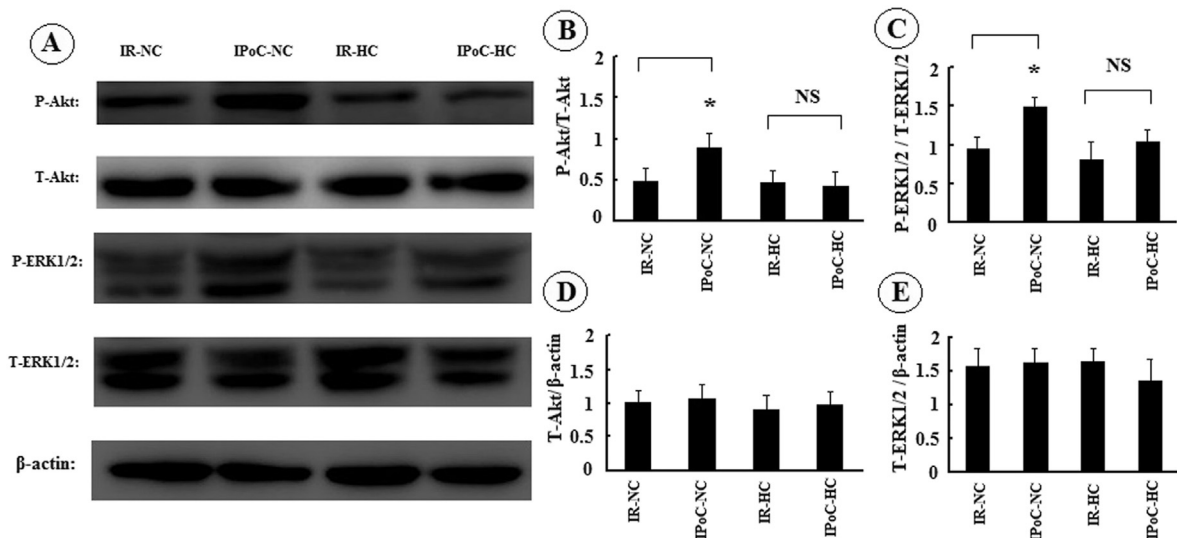


**Fig. 1.** Measurement of infarct size and apoptosis. (A) Infarct size was expressed as a percentage of area of necrosis (AN) to total area (TA). (B) Apoptosis was expressed as the percentage of TUNEL-positive cells. IR, ischemia reperfusion; IPoC, ischemic postconditioning; NC, normocholesterolemia; HC, hypercholesterolemia; NS, no significant difference. Values are means  $\pm$  SD. \* $P < 0.05$  vs. IR-NC,  $n = 6$  for each group.

ischemic myocardium which was similar to Zhao et al. founding [18], whereas Lauzier et al. suggested that hypercholesterolemia induced by high-cholesterol diet led to improved recovery of the myocardium after the ischemia-reperfusion [20]. These discrepancies may ascribe to the substantial differences in hyperlipidemic

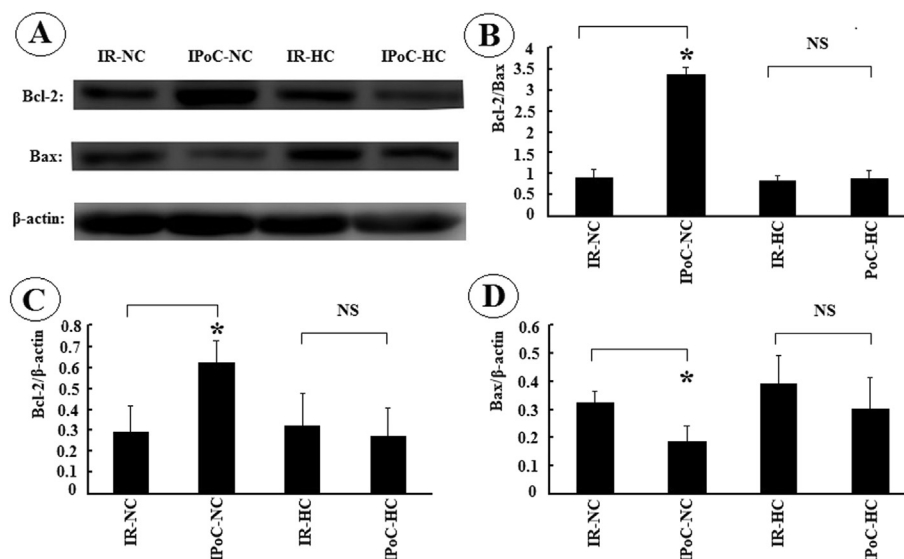
models (such as species, duration of hyperlipidemia diet, and the presence/absence of significant coronary sclerosis) [8].

In addition, the results suggested that anti-apoptosis effect of IPoC was blocked by hypercholesterolemia induced by 8-weeks high-cholesterol diet, which confirmed our previous founding [8].



**Fig. 2.** Measurement of Akt and ERK1/2 protein expressions. (A) Phosphorylation of Akt and ERK1/2 (P-Akt and P-ERK1/2) and total expression of Akt and ERK1/2 (T-Akt and T-ERK1/2) were detected by Western blot. (B) Quantitative analysis of P-Akt protein expression. (C) Quantitative analysis of P-ERK1/2 protein expression. (D) Quantitative analysis of T-Akt protein expression. (E) Quantitative analysis of T-ERK1/2 protein expression. IR, ischemia reperfusion; IPoC, ischemic postconditioning; NC, normocholesterolemia; HC, hypercholesterolemia; NS, no significant difference. Values are means  $\pm$  SD. \* $P < 0.05$  vs. IR-NC,  $n = 3$  for each group.

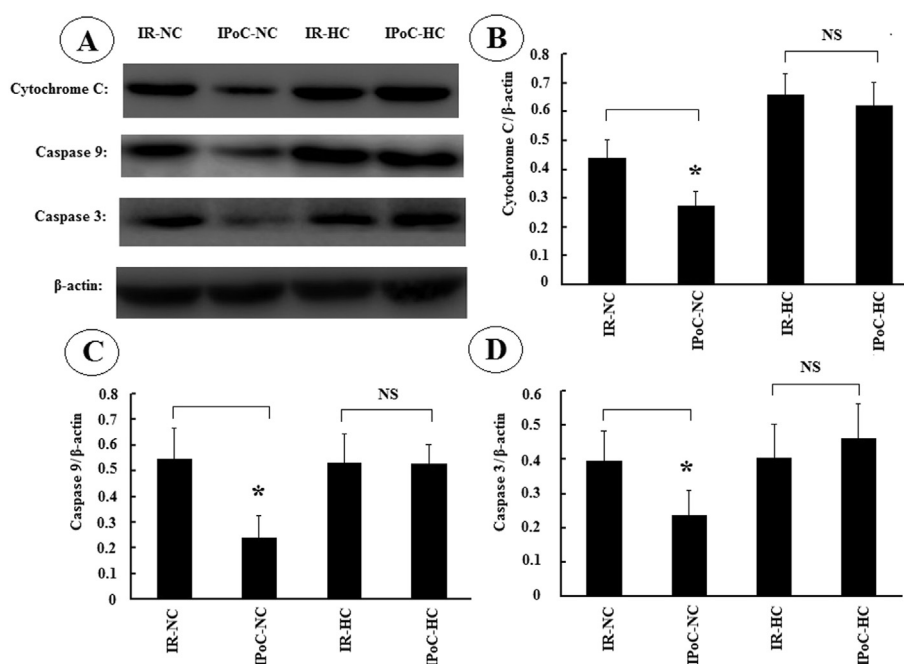




**Fig. 3.** Measurement of Bcl-2 and Bax protein expressions. (A) Expressions of Bcl-2 and Bax were detected by Western blot. (B) Quantitative analysis of the ratio of Bcl-2/Bax. (C) Quantitative analysis of Bcl-2 protein expression. (D) Quantitative analysis of Bax protein expression. IR, ischemia reperfusion; IPoC, ischemic postconditioning; NC, normocholesterolemia; HC, hypercholesterolemia; NS, no significant difference. Values are means  $\pm$  SD. \* $P$  < 0.05 vs. IR-NC,  $n$  = 3 for each group.

In order to elucidate the potential mechanism, we further explore whether hypercholesterolemia had influence on the apoptosis-related signal pathways. The results also indicated that hypercholesterolemia down-regulated the expression of anti-apoptosis protein (Bcl-2), and meanwhile, hypercholesterolemia also up-regulated the expression of pro-apoptotic proteins (Bax, cytochrome c, caspase 9 and caspase 3) in IPoC myocardium, which was similar to Osipov et al. finding that hypercholesterolemia is associated with induction of apoptosis in the ischemic territory in the setting of MIRI [25].

RISK (Akt and ERK1/2) signal pathway is considered as one of major prosurvival pathways activated by IPoC in the setting MIRI and is also the upstream signal of GSK-3 $\beta$ , Bcl-2, Bax and caspase 9 [9,26]. Our previous study showed hypercholesterolemia impaired phosphorylation of GSK-3 $\beta$  in IPoC myocardium and the present study also demonstrated that hypercholesterolemia had influence on the expression of Bcl-2, Bax and caspase 9 [8]. However, whether hypercholesterolemia had influence on the activation of RISK signaling pathway was still unknown. Therefore, we further compared the phosphorylation of Akt and ERK1/2 in ischemic



**Fig. 4.** Measurement of cytochrome c, caspase 9 and caspase 3 protein expressions. (A) Expressions of cytochrome c, caspase 9 and caspase 3 were detected by Western blot. (B) Quantitative analysis of cytochrome c protein expression. (C) Quantitative analysis of caspase 9 protein expression. (D) Quantitative analysis of caspase 3 protein expression. IR, ischemia reperfusion; IPoC, ischemic postconditioning; NC, normocholesterolemia; HC, hypercholesterolemia; NS, no significant difference. Values are means  $\pm$  SD. \* $P$  < 0.05 vs. IR-NC,  $n$  = 3 for each group.

myocardium between IPoC-NC group and IPoC-HC group and the results showed IPoC significantly up-regulate the phosphorylation of Akt and ERK1/2, but this effect was blocked by hypercholesterolemia, which indicating that the abrogation of IPoC in the presence of hypercholesterolemia was involved in inactivation of RISK signal pathway.

In conclusion, we demonstrated that the cardioprotection of IPoC was abolished by hypercholesterolemia, which was associated with inactivation of RISK signal pathway and dysregulation of downstream apoptosis-related pathway.

### Conflict of interest

The authors declare that they have no conflict of interests.

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### Transparency document

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