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Original Article

# Ischemic preconditioning activates prosurvival kinases and reduces myocardial apoptosis

Chang-Chi Lai [a,b,c], Chia-Yu Tang [a,b], Shu-Chiung Chiang [d], Kuo-Wei Tseng [c], Cheng-Hsiung Huang [a,*]

[a] *Division of Cardiovascular Surgery, Department of Surgery, Taipei Veterans General Hospital, and National Yang-Ming University School of Medicine, Taipei, Taiwan, ROC*

[b] *Institute of Clinical Medicine, National Yang-Ming University School of Medicine, Taipei, Taiwan, ROC*

[c] *Department of Exercise and Health Sciences, University of Taipei, Taipei, Taiwan, ROC*

[d] *Institute of Hospital and Health Care Administration, National Yang-Ming University School of Medicine, Taipei, Taiwan, ROC*

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**Abstract**

*Background*: Ischemic preconditioning has been reported to protect the myocardium against ischemia and reperfusion injury. The underlying mechanisms have been extensively investigated but are not fully elucidated. In this study, we investigated the role of apoptosis in ischemic preconditioning protection and the signal pathways involved.

*Methods*: Myocardial ischemia and reperfusion were induced in anesthetized male Sprague–Dawley rats by a 40-minute occlusion and a 3-hour reperfusion of the left anterior descending coronary artery. Ischemic preconditioning was elicited by two 10-minute coronary artery occlusions and two 10-minute reperfusions.

*Results*: The myocardial infarct size, expressed as the percentage of area at risk, was significantly decreased in the ischemic preconditioning group (16.8 ± 2.0% and 27.9 ± 2.7% in the ischemia and reperfusion groups, respectively, $p < 0.001$). Additionally, ischemic preconditioning significantly reduced apoptosis, as evidenced by the decrease in the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling-positive nuclei, DNA laddering, and caspase-3 activation. Western blot analysis revealed that ischemic preconditioning significantly reduced myocardial tumor necrosis factor-α levels. Bcl-2 was increased, whereas Bax was decreased in the myocardium. Phosphorylation of the pro-survival kinases, including Akt and extracellular signal-regulated kinases 1 and 2, was significantly increased. Hemodynamics, area at risk, and mortality did not differ significantly among the groups.

*Conclusion*: Ischemic preconditioning reduces apoptosis induced by myocardial ischemia and reperfusion. The underlying mechanisms might be related to inhibition of both the extrinsic and the intrinsic apoptotic pathway via inhibition of production of tumor necrosis factor-α, modulation of expression of Bcl-2 and Bax, and activation of the prosurvival kinases.



*Keywords:* apoptosis; ischemia and reperfusion injury; ischemic preconditioning; myocardial infarction; prosurvival kinases

## 1. Introduction

Ischemic preconditioning is a phenomenon in which brief, reversible episodes of myocardial ischemia protect the myocardium against subsequent prolonged ischemic insult.[1] Such preconditioning protects the myocardium against ischemia and reperfusion injury, by reduction of myocardial

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* Corresponding author. Dr. Cheng-Hsiung Huang, Division of Cardiovascular Surgery, Taipei Veterans General Hospital, 201, Section 2, Shih-Pai Road, Taipei 112, Taiwan, ROC.

*E-mail address:* chhuang@vghtpe.gov.tw (C.-H. Huang).

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infarct (MI) size,[1,2] improved recovery from myocardial stunning,[3] and attenuation of postischemic arrhythmia.[4] It is a universal phenomenon found in a variety of animals, such as dogs,[1] rats,[5] rabbits,[2,6] and pigs.[3] This laboratory finding has also been used to develop strategies to protect human myocardium during cardiac surgery.[7,8]

The underlying mechanisms of ischemic preconditioning have been extensively investigated. Activation of adenosine receptors,[9] protein kinase C,[10] adenosine triphosphate-sensitive potassium channels,[11] and stretch-activated ion channels plays an important role in the protective effects of ischemic preconditioning.[6,12] Inhibition of apoptosis has also been reported to contribute to ischemic preconditioning protection.[10,13] Myocardial ischemia and reperfusion produce proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1, and interleukin-6. TNF-α is capable of inducing apoptosis via activation of the extrinsic apoptotic pathway.[14,15] Ischemic preconditioning has been shown to protect the myocardium via inhibition of TNF-α release.[16] In addition, it has been reported to upregulate antiapoptotic Bcl-2 protein, downregulate proapoptotic Bax protein, and activate the prosurvival kinases, including Akt and extracellular signal-regulated kinase 1 and 2 (ERK1/2).[17,18] Significantly reduced apoptosis has also been found.[18]

Myocardial ischemia and reperfusion occur in acute MI and cardiac surgery. These result in mortality, morbidity, prolonged hospital stays, and increased medical costs.[19] In this study, we investigated whether ischemic preconditioning reduces apoptosis induced by ischemia and reperfusion. The roles of TNF-α, Bcl-2, Bax, and the prosurvival kinases, including Akt and ERK1/2, were also examined. Our findings might serve to elucidate the mechanisms through which ischemic preconditioning protects the myocardium against ischemia and reperfusion injury, and provide a rationale for the development of therapeutic strategies to reduce myocardial ischemia and reperfusion injury.

## 2. Methods

This study was approved by the Animal Experiment Committee of Taipei Veterans General Hospital, Taipei, Taiwan. Animals were treated humanely in accordance with the "Guide for the Care and Use of Laboratory Animals".[40]

### 2.1. Animal preparation

The techniques for animal preparation utilized in this study have been reported previously.[20] Briefly, male Sprague–Dawley rats (250–300 g) were anesthetized with intraperitoneal pentobarbiturate (40 mg/kg). After tracheotomy, each rat was intubated and ventilated. The descending aorta was cannulated for arterial pressure monitoring. Electrocardiography leads were placed on limbs. After median sternotomy, a 4-0 silk suture was passed around the proximal left anterior descending coronary artery. The ends of the silk suture were threaded through a small vinyl tube to form a snare. The body temperature was monitored using a rectal thermometer and maintained at 37°C with heating pads throughout the experiments.

### 2.2. Experimental protocol

After achieving hemodynamic stability for 20 minutes, the rats were divided randomly into three groups. Group 1 (the sham group) received the same surgical procedures without any pretreatment, coronary artery occlusion (CAO) or coronary artery reperfusion. Group 2 (the ischemia and reperfusion group) also did not receive any pretreatment. Group 3 (the ischemic preconditioning group) received ischemic preconditioning by two episodes of 10-minute CAO. Ten minutes after the above treatments, rats in Groups 2 and 3 received a 40-minute CAO, which was induced by pulling of the snare around the proximal left anterior descending coronary artery. Successful occlusions were verified by observing the development of ST-segment elevation and changes in the QRS complex on the electrocardiograms and cyanotic changes in the myocardium in the occluded area. After the 40-minute CAO, the snare was released for reperfusion for 3 hours. Reperfusion was confirmed by refilling the coronary artery and visualizing a reactive hyperemic response. Arterial pressure, heart rate, and electrocardiography were recorded simultaneously and continuously throughout the experiment.

### 2.3. Determination of area at risk and MI size

The methods for determination of area at risk (AAR) and MI size used in this study have been reported previously.[21] Briefly, 2000 units of heparin were intravenously administered at the end of the experiment. The heart was excised and placed on a perfusion apparatus. The proximal left anterior descending coronary artery was ligated at the site of the previous occlusion. The ascending aorta was perfused with 1% Evans blue dye (Sigma-Aldrich, St. Louis, MO, USA). The left ventricle plus septum was cut into six to seven transverse slices, which were incubated at 37°C in 1% triphenyl tetrazolium chloride solution for 20 minutes. The slices were weighed and fixed in 10% formalin solution for 24 hours. The basal surfaces were photographed. Images were traced with a digitizer. The AAR and MI area of each heart slice were calculated by planimetry. The percentage of area of the AAR and MI in each heart slice was multiplied by the weight of each slice. The total weight of the AAR and MI was calculated and summed. The AAR was reported as the percentage of the left ventricle plus septum, with MI size reported as a percentage of the AAR.

### 2.4. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling staining

At the end of the experiments, the hearts were excised, washed of blood, and sectioned at 3–4 μm. Tissue slides were fixed in acetone. Endogenous peroxide activity was quenched by incubation in 3% hydrogen peroxide in methanol. Slides were rinsed with phosphate-buffered saline-blocking solution and incubated with a permeabilization

solution (0.1% Triton X-100 in 0.1% sodium citrate). Samples were incubated with terminal deoxynucleotidyl transferase and detection buffer conjugated with horseradish peroxidase in a humid chamber at 37°C for 60 minutes. A diaminobenzidin chromogen (Boehringer, Mannheim, Germany) was used. Counterstaining with hematoxylin was performed. The number of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive myocyte nuclei was counted in randomly selected areas and expressed as a percentage of the total number of myocyte nuclei at a magnification of 200×.

### 2.5. DNA fragmentation using agarose gel electrophoresis

At the end of the experiment, the hearts were excised, washed of blood, frozen in liquid nitrogen, and ground to powder. The powdered tissue, transferred to a 50-mL centrifuge tube with ~10 vol. extraction buffer [10mM Tris-HCl (pH = 8.0), 0.1M EDTA (pH = 8.0), 0.5% sodium dodecyl sulfate (SDS), and 20 μg/mL pancreatic RNAase], was incubated and digested with proteinase K at 50°C overnight. An equal volume of phenol equilibrated with 1M Tris buffer (pH = 8.0) was added. After centrifugation at 5000g for 30 minutes at room temperature, the aqueous phase was transferred to a new 50-mL tube. The DNA was precipitated by the addition of 0.1 vol. 3M sodium acetate and 2 vol. 100% ethanol. The concentration of DNA was measured by spectrophotometry (260 nm). Ten microgram of each sample of DNA was electrophoretically fractionated on 2% agarose gel with 0.5 μg/mL ethidium bromide. The DNA in the gel was photographed under UV light. A qualitative analysis of DNA fragmentation was performed by analyzing the pattern of low-molecular-weight DNA (~180-bp multiples).

### 2.6. Western blot analysis for activated caspase-3, TNF-α, Bcl-2, Bax, Akt, and ERK1/2

At the end of the experiments, the hearts were excised, washed of blood, and homogenized in buffer at 4°C. After centrifugation, protein concentrations were determined using a modified Bradford assay. Equivalent amounts of protein samples were loaded and separated on 15% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. After blocking with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBST), membranes were incubated with anti-caspase-3, anti-TNF-α, anti-Bcl-2, anti-Bax, anti-Akt, anti-phospho-Akt (Ser473), anti-ERK1/2, and anti-phospho-ERK1/2 (Thr202/Thr204) antibodies [1:1000 (v/v) dilution] in 5% nonfat dry milk. The membranes were incubated in 5% nonfat dry milk in TBST containing secondary antibody conjugated to horseradish peroxidase. Peroxidase activity was visualized by an enhanced chemiluminescence substrate system, followed by exposure to hyperfilms. β-Actin (1:2000 dilution) was detected as a loading control for protein quantity. Optical density for each band was determined using NIH Image 1.6 and normalized against background density for each gel.

### 2.7. Statistical analysis

All values were expressed as mean value ± standard deviation. A computer program (SPSS 17.0; SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Hemodynamic variables were analyzed by a two-way analysis of variance with repeated measures. Multiple comparisons of repeated measures were tested by using within-subject contrasts. The data were analyzed with the Student *t* test when comparing means between two groups, or with one-way analysis of variance and the Bonferroni *post hoc* multiple comparison test when comparing multiple groups. Differences were considered significant at $p < 0.05$.

## 3. Results

### 3.1. Mortality and exclusions

Seventy-two rats were used in this study (Table 1). One rat suffered from ventricular fibrillation and died. One rat died of heart failure, which was defined as a progressive decrease of systolic arterial pressure to <50 mmHg with global left ventricular dilatation and poor contraction. These two rats were excluded. The mortality rates for the three groups were not significantly different ($p = 0.710$).

### 3.2. Hemodynamic changes during the experiments

The baseline mean arterial pressures and heart rates for the three groups were not significantly different (Table 2). Ischemic preconditioning did not cause any significant hemodynamic changes. There were no significant changes in the arterial pressures and heart rates throughout the experiments. The hemodynamic variables were not significantly different among the groups during the experiments.

### 3.3. AAR and MI size analysis

The AAR values for the three groups, expressed as the percentage of the left ventricle, were not significantly different (49.4 ± 1.6% in Group 2 vs. 50.4 ± 3.0% in Group 3, $p = 0.456$; Fig. 1A). The MI size, expressed as a percentage of AAR, was 27.9 ± 2.7% in Group 2. Ischemic preconditioning significantly reduced the MI size in Group 3 (16.8 ± 2.0%, $p < 0.001$; Fig. 1B).

Table 1
Mortality rates and exclusions for the three groups.[a]

| Group | Treatment | Number | Mortality | | No. included |
|---|---|---|---|---|---|
| | | | VF | HF | |
| 1 | Sham | 18 | 0 | 0 | 18 |
| 2 | I/R | 27 | 0 | 1 | 26 |
| 3 | IPC | 27 | 0 | 1 | 26 |

HF = heart failure; IPC = ischemic preconditioning; I/R = ischemia and reperfusion; VF = ventricular fibrillation.

[a] No significant differences in the mortality rate were observed among the groups. Pearson's Chi-square $p = 0.710$.

Table 2
Hemodynamic changes during the experiments.[a]

| Group | Treatment Protocol | Number | Baseline 1 | Baseline 2 | CAO | | CAR | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | 20 min | 40 min | 1 h | 2 h | 3 h |
| MAP (mmHg) | | | | | | | | | |
| 1 | Sham | 18 | 79 ± 9 | 80 ± 8 | 78 ± 9 | 75 ± 5 | 78 ± 7 | 76 ± 4 | 78 ± 6 |
| 2 | I/R | 26 | 80 ± 9 | 79 ± 10 | 73 ± 5 | 74 ± 8 | 75 ± 7 | 74 ± 4 | 74 ± 6 |
| 3 | IPC | 26 | 79 ± 11 | 77 ± 8 | 76 ± 6 | 78 ± 6 | 76 ± 11 | 75 ± 5 | 74 ± 6 |
| HR (beats/min) | | | | | | | | | |
| 1 | Sham | 18 | 420 ± 42 | 435 ± 53 | 431 ± 39 | 426 ± 43 | 423 ± 43 | 424 ± 49 | 414 ± 55 |
| 2 | I/R | 26 | 431 ± 32 | 451 ± 49 | 443 ± 47 | 448 ± 34 | 439 ± 29 | 451 ± 34 | 433 ± 53 |
| 3 | IPC | 26 | 428 ± 45 | 418 ± 53 | 423 ± 37 | 433 ± 50 | 431 ± 49 | 425 ± 43 | 423 ± 46 |
| PRP/1000 (mmHg*beats/min) | | | | | | | | | |
| 1 | Sham | 18 | 33.31 ± 5.14 | 34.80 ± 5.59 | 33.71 ± 4.84 | 32.00 ± 4.19 | 32.71 ± 4.24 | 32.34 ± 3.81 | 31.86 ± 3.11 |
| 2 | I/R | 26 | 34.61 ± 5.50 | 35.82 ± 7.34 | 32.48 ± 4.50 | 33.22 ± 5.76 | 33.07 ± 5.03 | 33.62 ± 3.91 | 32.31 ± 5.98 |
| 3 | IPC | 26 | 34.08 ± 6.59 | 32.39 ± 6.75 | 32.30 ± 4.13 | 33.88 ± 5.16 | 32.91 ± 6.33 | 31.78 ± 3.22 | 31.14 ± 4.01 |

Data are presented as mean ± standard deviation.
Baseline 1 = baseline prior to ischemic preconditioning; Baseline 2 = baseline after ischemic preconditioning; CAO = coronary artery occlusion; CAR = coronary artery reperfusion; HR = heart rate; IPC = ischemic preconditioning; I/R = ischemia and reperfusion; MAP = mean arterial pressure; PRP = mean arterial pressure–heart rate product.
[a] Rats in Group 1 did not receive any pretreatment, coronary artery occlusion, or reperfusion. The hemodynamic data shown for Group 1 were observed at the same time points as those of the other two groups. There were no significant changes in MAP and HR throughout the experiments. There were no significant differences in hemodynamic variables during the experiments.

### 3.4. TUNEL staining

TUNEL staining localizes DNA fragmentation in the nuclei of apoptotic myocytes. The reaction product is dark brown.[13] There were few stained nuclei in the myocardia of rats that received the sham operation (Fig. 2A). Numerous nuclei in the AAR of Group 2 rats contained the dark brown reaction product, while only scattered nuclei in the AAR of Group 3 rats showed the dark brown reaction product. The TUNEL-positive nuclei, expressed as the percentage of total nuclei, were significantly increased in the AAR of Group 2, as compared with their presence in the myocardia of Group 1 rats (22.7 ± 3.3% vs. 0.8 ± 0.2%, $p < 0.001$; Fig. 2A). Ischemic preconditioning significantly reduced the number of TUNEL-positive nuclei (7.1 ± 1.5%, $p < 0.001$ vs. Group 2).

### 3.5. DNA fragmentation using agarose gel electrophoresis

Consistent with the findings of TUNEL staining, no DNA fragmentation was detected in the myocardia of Group 1 rats (Fig. 2B, lane 2). A clearly detected electrophoretic pattern characterized by mononucleosomal and oligonucleosomal DNA fragmentation (laddering) was found in the AAR of Group 2 rats (Fig. 2B, lane 3). Little or no DNA laddering was found in the AAR of Group 3 rats (Fig. 2B, lane 4).

### 3.6. Assay of activated caspase-3

Caspase-3 is constitutively expressed in cells as an inactive precursor (32 kDa). The precursor is cleaved to an active p17

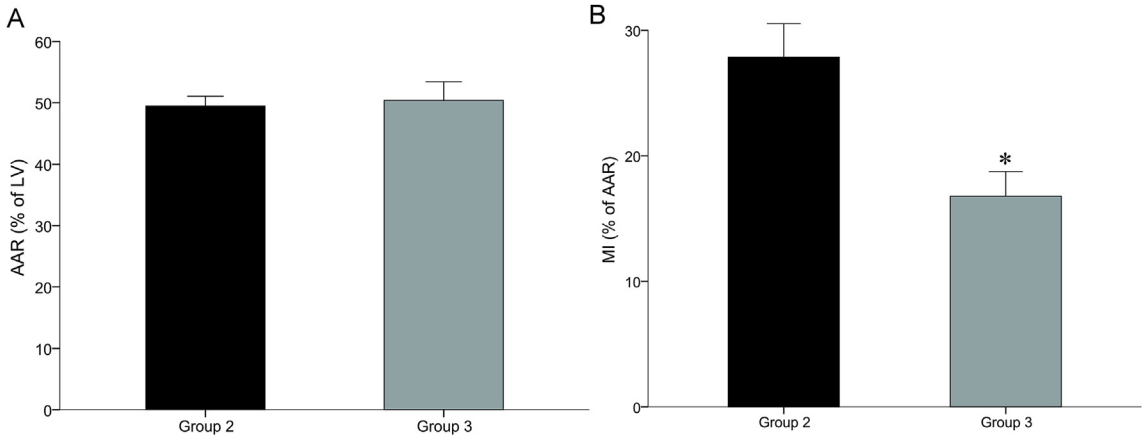


Fig. 1. (A) Size of AAR, expressed as a percentage of the LV. (B) Size of MI, expressed as a percentage of the AAR. * $p < 0.001$ versus Group 2 ($N = 8$). AAR = area at risk; LV = left ventricle; MI = myocardial infarct.

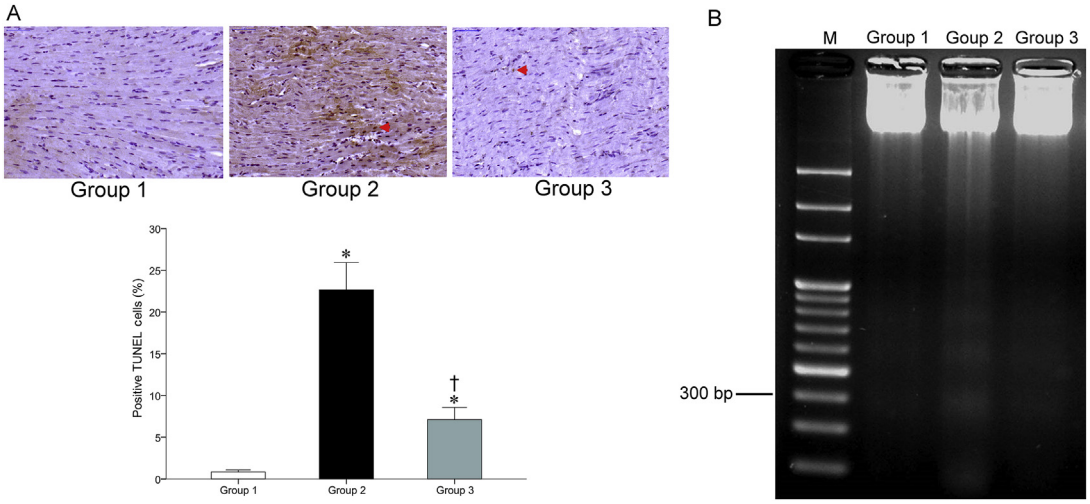


Fig. 2. (A) Detection of DNA fragmentation in apoptotic cell nuclei in the myocardium using TUNEL staining. A representative photomicrograph is shown (200× magnification). Dark-brown staining (red arrowhead) indicates TUNEL-positive nuclei (upper panel). The percentage of TUNEL-positive nuclei in each group is shown (lower panel). (B) Detection of DNA fragmentation in the myocardium using agarose gel electrophoresis. No DNA fragmentation was detected in the myocardium of Group 1 rats (lane 2). DNA fragmentation was clearly detected in the area at risk of Group 2 rats (lane 3). Little or no DNA fragmentation was found in the area at risk of Group 3 rats (lane 4). * $p < 0.001$ versus Group 1 ($N = 6$). † $p < 0.001$ versus Group 2 ($N = 6$). bp = base pair; M = molecular marker; TUNEL = terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

subunit (17 kDa) when apoptosis is induced.[22] Western blot analysis revealed that the band of activated caspase-3 was faintly detectable in the myocardia of Group 1 rats (Fig. 3A, lane 1). Myocardial ischemia and reperfusion significantly increased the generation of activated caspase-3 in the AAR of Group 2 rats, as evidenced by the increased density of the p17 subunit band ($p < 0.001$ vs. Group 1; Fig. 3A, lane 2). Ischemic preconditioning significantly reduced the increase of activated caspase-3 in Group 3 rats ($p < 0.01$ vs. Group 2; Fig. 3A, lane 3).

### 3.7. Assay of TNF-α

TNF-α may induce apoptosis via activation of the extrinsic apoptotic pathway.[14,15] We analyzed the TNF-α production by western blot analysis and found that myocardial ischemia and reperfusion significantly increased TNF-α in the AAR of Group 2 rats, as demonstrated by the increased density of the TNF-α band ($p < 0.001$ vs. Group 1; Fig. 3B, lane 2). Ischemic preconditioning significantly inhibited the increase of TNF-α in Group 3 rats ($p < 0.01$ vs. Group 2; Fig. 3B, lane 4).

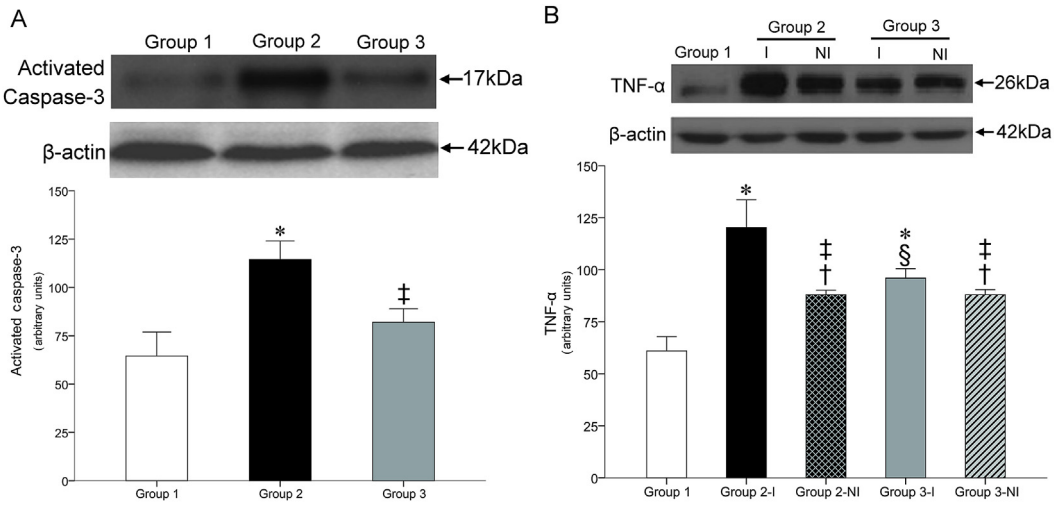


Fig. 3. (A) Caspase-3 activation assay. (B) TNF-α assay. Representative Western blots of activated caspase-3 and TNF-α are shown (upper panels). The densities of activated caspase-3 and TNF-α band were analyzed by arbitrary units (lower panels). * $p < 0.001$ versus Group 1 ($N = 4$). † $p < 0.01$ versus Group 1 ($N = 4$). ‡ $p < 0.001$ versus Group 2 (A) or Group 2-I (B) ($N = 4$). § $p < 0.01$ versus Group 2-I ($N = 4$). I = ischemic myocardium; NI = nonischemic myocardium; TNF-α = tumor necrosis factor-α.

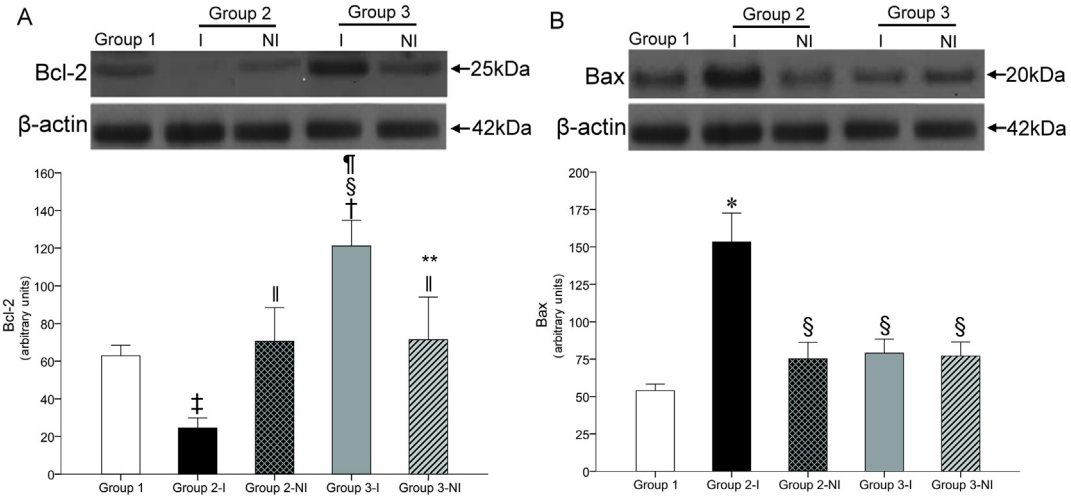


Fig. 4. (A) Bcl-2 assay. (B) Bax assay. Representative western blots of Bcl-2 and Bax are shown (upper panel). The densities of Bcl-2 and Bax bands were analyzed by arbitrary units (lower panel). * $p < 0.001$ versus Group 1 ($N = 4$). † $p < 0.01$ versus Group 1 ($N = 4$). ‡ $p < 0.05$ versus Group 1 ($N = 4$). § $p < 0.001$ versus Group 2-I ($N = 4$). ‖ $p < 0.01$ versus Group 2-I ($N = 4$). ¶ $p < 0.01$ versus Group 2-NI ($N = 4$). ** $p < 0.01$ versus Group 3-I ($N = 4$). I = ischemic myocardium; NI = nonischemic myocardium.

### 3.8. Assay of Bcl-2 and Bax

Western blot analysis showed that myocardial ischemia and reperfusion significantly decreased Bcl-2 in the AAR of Group 2 rats, as demonstrated by the attenuated densities of the Bcl-2 bands ($p < 0.05$; Fig. 4A, lane 2). Ischemic preconditioning significantly prevented the decrease of Bcl-2 in Group 3 rats ($p < 0.001$ vs. Group 2; Fig. 4A, lane 4).

Compared with the sham group, myocardial ischemia and reperfusion significantly increased the density of Bax bands in the AAR of Group 2 rats ($p < 0.001$; Fig. 4B, lane 2), indicating increased tissue abundance of Bax. Ischemic preconditioning significantly inhibited the increase of Bax in the AAR of Group 3 rats ($p < 0.001$ vs. Group 2; Fig. 4B, lane 4).

### 3.9. Assay of Akt and ERK1/2

Akt and ERK1/2 were activated by phosphorylation to protect cells and inhibit apoptosis.[17,18] Western blot analysis showed that myocardial ischemia and reperfusion significantly increased phospho-Akt in the AAR of Group 2 rats ($p < 0.01$ vs. Group 1; Fig. 5A, lane 2). Ischemic preconditioning further

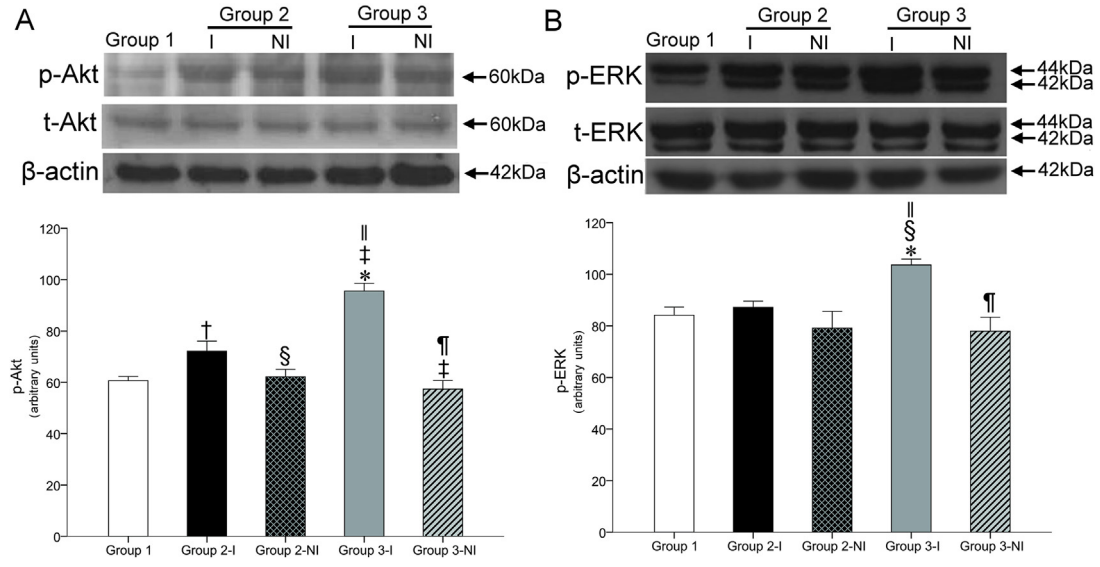


Fig. 5. (A) Phospho-Akt and t-Akt assays. (B) Phosphoextracellular signal-regulated kinases and t-ERK assay. Representative western blots of p-Akt, t-Akt, p-ERK, and t-ERK are shown (upper panel). The densities of p-Akt and p-ERK bands were analyzed by arbitrary units (lower panel). * $p < 0.001$ versus Group 1 ($N = 4$). † $p < 0.01$ versus Group 1 ($N = 4$). ‡ $p < 0.001$ versus Group 2-I ($N = 4$). § $p < 0.01$ versus Group 2-I ($N = 4$). ‖ $p < 0.001$ versus Group 2-NI ($N = 4$). ¶ $p < 0.001$ versus Group 3-I ($N = 4$). I = ischemic myocardium; NI = nonischemic myocardium; p-Akt = phospho-Akt; p-ERK = phosphoextracellular signal-regulated kinases; t-Akt = total Akt; t-ERK = total extracellular signal-regulated kinases.

increased phospho-Akt in the AAR of Group 3 rats ($p < 0.001$ vs. Group 2; Fig. 5A, lane 4). The total Akt was not significantly changed and was not significantly different among the three groups.

Compared to the sham group, myocardial ischemia and reperfusion did not significantly change the phospho-ERK1/2 in the AAR of Group 2 rats ($p > 0.99$ vs. Group 1; Fig. 5B, lane 2). Ischemic preconditioning significantly increased the phospho-ERK1/2 in the AAR of Group 3 rats ($p < 0.01$ vs. Group 2; Fig. 5B, lane 4). The total ERK1/2 was not significantly changed and was not significantly different among the three groups.

## 4. Discussion

In the current study, we demonstrated that ischemic preconditioning significantly reduced apoptosis in anesthetized rats. Apoptosis and necrosis are two distinct forms of cell death induced by ischemia and reperfusion. Ischemic preconditioning has been reported to reduce MI size.[1] Inhibition of apoptosis has been shown to contribute to ischemic preconditioning protection.[10,13] Zhao et al[23] reported that inhibition of apoptosis was associated with reduction of necrosis. Piot et al[13] reported that ischemic preconditioning significantly reduced MI size, DNA laddering, and TUNEL-stained nuclei. They suggested that ischemic preconditioning might limit irreversible injury by reducing apoptosis. There were no significant hemodynamic changes during the experiments. The reduction of apoptosis found in this study could not be ascribed to hemodynamic changes. Although the underlying mechanisms are not certain, we demonstrated that ischemic preconditioning significantly reduced TNF-α, increased Bcl-2, and decreased Bax. Phosphorylation of the prosurvival kinases, including Akt and ERK1/2, was significantly increased by ischemic preconditioning. Therefore, the underlying mechanism for reduction of apoptosis by ischemic preconditioning might be related to inhibition of both the extrinsic and the intrinsic apoptotic pathway via regulation of the prosurvival and proapoptotic signals.

Activated caspase-3 is a universal effector of apoptosis.[22] Activation of both the extrinsic and the intrinsic apoptotic pathway results in cleavage of caspase-3, which activates the execution phase of apoptosis.[22,24,25] Yaoita et al[26] reported that administration of a caspase inhibitor significantly decreased MI size and apoptosis. Piot et al[27] demonstrated that ischemic preconditioning attenuated caspase-3 activation. They suggested that prevention of caspase activation contributed to myocardial protection.

It has been reported that TNF-α may bind with TNF-α receptors and activate the extrinsic apoptotic pathway to induce apoptosis.[14,15] In this study, we demonstrated that myocardial ischemia and reperfusion significantly increased the TNF-α level in the myocardium, and ischemic preconditioning limited the increase of TNF-α. Inhibition of TNF-α release and apoptosis has been reported to protect the myocardium against ischemia and reperfusion injury.[16,28]

Bcl-2 family proteins are important regulatory proteins of the intrinsic apoptotic pathway. The ratio of antiapoptotic Bcl-2 and proapoptotic Bax regulates the apoptotic pathways and determines the death or survival of cells.[24,25,29] Ischemic preconditioning has been reported to protect the myocardium by preventing the decrease of Bcl-2 and increase of Bax induced by ischemia and reperfusion.[24,30]

Akt is a member of the prosurvival phosphatidylinositol-3 kinase pathway, which reduces apoptosis and leads to cell

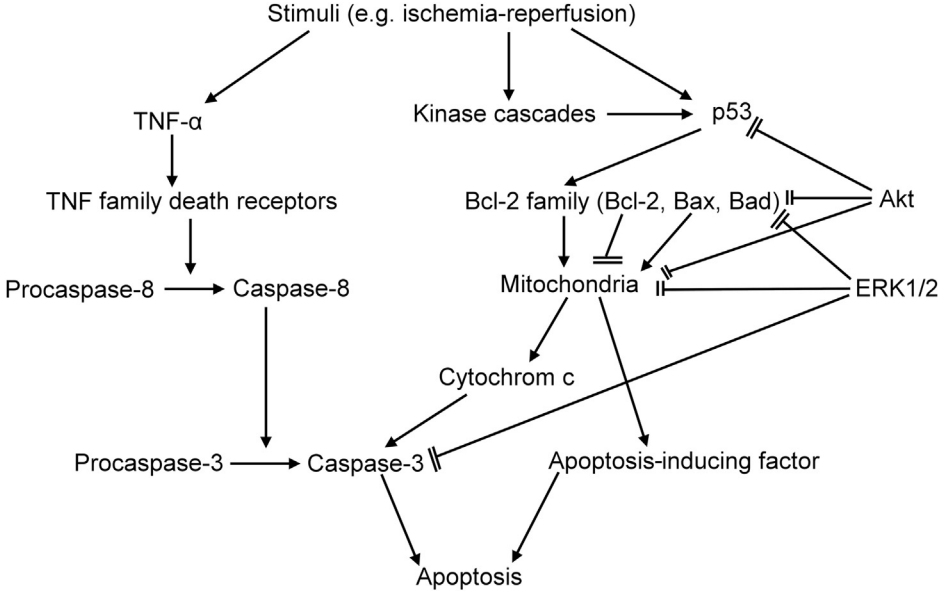


Fig. 6. Schematic presentation of the extrinsic (left) and intrinsic (right) pathways of apoptosis. Myocardial ischemia and reperfusion induces apoptosis by production of TNF-α and stimulation of TNF family death receptors. The activation of the Bcl-2 family causes release of cytochrome C from the mitochondria, leading to activation of caspase cascades. Ischemic preconditioning reduces apoptosis via inhibition of TNF-α production. Activation of prosurvival kinases, including Akt and ERK1/2, reduces apoptosis via modulation of expression of Bcl-2 and Bax. ERK1/2 = extracellular signal-regulated kinases 1 and 2; TNF-α = tumor necrosis factor-α.

survival upon phosphorylation.[31] Hausenloy et al[17] reported that ischemic preconditioning significantly increased phosphorylation of Akt and reduced MI size. The effects on apoptosis, however, were not investigated in their study. The Akt-involved pathways leading to antiapoptotic effects are complex. Akt may phosphorylate the proapoptotic protein Bad and prevent Bad from binding to antiapoptotic Bcl-2 and Bcl-xL, releasing them for a prosurvival response.[31] Additionally, Akt may prevent apoptosis by inhibiting the activity of the proapoptotic transcription factor p53.[32,33]

ERK1/2 are members of the mitogen-activated protein kinases. It has been shown that activation of ERK1/2 by phosphorylation protects cells against necrosis and apoptosis.[34] Activation of ERK1/2 plays a significant role in myocardial protection. Hausenloy et al[17] reported that ischemic preconditioning significantly increased phosphorylation of ERK1/2 and reduced MI size. Administration of an ERK1/2 inhibitor significantly decreased the phosphorylation of ERK1/2 and the limitation of MI size induced by ischemic preconditioning. ERK1/2 activation has been reported to reduce apoptosis by downregulating Bad and upregulating Bcl-2.[35] Activation of the prosurvival pathways involving Akt and ERK1/2 has been demonstrated to reduce apoptosis by inhibiting the opening of the mitochondrial permeability transition pore.[36]

In a report by Hausenloy et al,[17] ischemic preconditioning has been shown to significantly increase phosphorylation of Akt and ERK1/2. MI size was significantly reduced. However, the effects on apoptosis were not investigated in their study. Lazou et al[37] reported that ischemic preconditioning activated ERK1/2 and attenuated apoptosis in rabbits. The role of Akt was not determined in their study. In the current study, the roles of both Akt and ERK1/2 were investigated. The protective effects of ischemic preconditioning on both reduction of MI size and inhibition of apoptosis were observed. Additionally, we demonstrated that ischemic preconditioning reduced TNF-α, Bcl-2, and Bax in the myocardium. The effects of ischemic preconditioning on both the extrinsic and the intrinsic apoptotic pathway were seen in this study (Fig. 6).

Our study has limitations. MI size is affected by the size of AAR and the extent of coronary collateral circulation. In the current study, the sizes of the AAR, expressed as the percentage of the left ventricle weight, were not significantly different among the groups. No definite myocardial blood flow data were collected in our study. However, rats have limited collateral coronary circulation,[38] and our model is comparable to that widely used in experiments on myocardial ischemia and preconditioning.[5,10,13,26,27,33] TUNEL staining is a sensitive but not a specific method for detection of apoptosis. DNA fragmentation in the noncardiomyocytes might be stained. DNA fragmentation on agarose gel electrophoresis is specific but not quantitative. In this study, we conducted assays of caspase-3 activation, which provide specific and quantitative analysis of apoptosis.[39]

In conclusion, our study demonstrated that ischemic preconditioning reduced apoptosis. The underlying mechanisms might be related to inhibition of both the extrinsic and the intrinsic apoptotic pathway via inhibition of TNF-α production, modulation of expression of Bcl-2 and Bax, and activation of the prosurvival kinases, including Akt and ERK1/2. Our findings disclose the protective effects and underlying mechanisms of ischemic preconditioning. Further studies are required to investigate other signal elements involved.

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