European Review for Medical and Pharmacological Sciences 2018; 22: 4975-4986

Role of Janus kinase 2/signal transducer and activator of transcription 3 signaling pathway in cardioprotection of exercise preconditioning

X.-J. SUN[1], J.-R. MAO[2]

[1]Department of Physical Education, Xianyang Normal University, Xianyang, China
[2]Department of Pathology, Shaanxi Provincial Institute of Traditional Chinese Medicine, Xi'an, China

Abstract. – OBJECTIVE: To investigate the role of Janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) signaling pathway in the cardioprotective mechanisms of exercise preconditioning (EP).

MATERIALS AND METHODS: Eighty male Sprague-Dawley (SD) rats were randomized into the Group control, Group EE, Group EP+EE, Group EP+EE+AG, and Group EE+AG. By using 3 days of intermittent treadmill exercise, this study established the EP animal model. Rats were subjected to run to exhaustion on the treadmill at 30 m/min with 0% grade as an exhaustive exercise (EE) protocol. The myocardial injury induced by exhaustive exercise was produced 24 h after EP. JAK2 inhibitor (AG490, 3 mg/kg) was injected before EP. Serum cardiac troponin I (cTnI) levels and hematoxylin basic fuchsine picric acid (HBFP) staining were used to observe the degree of myocardial ischemia. TUNEL, Bcl2, and cleaved caspase-3 levels were used to evaluate the change of myocardial apoptosis. Moreover, the phosphorylations of JAK2 and STAT3 were analyzed as possible mechanisms that might explain the EP-induced cardioprotection.

RESULTS: EP significantly attenuated the exhaustive exercise-induced myocardial ischemia injury, associated with lower serum cTnI levels, decreased myocardial infarct area, reduced myocardial apoptosis, increased Bcl2 level, decreased cleaved caspase-3 level, and the increased phosphorylations of JAK2 and STAT3. Treatment with AG490 abolished the cardioprotective effects and the enhanced phosphorylations of JAK2 and STAT3 induced by EP.

CONCLUSIONS: EP plays its cardioprotective role via activating the JAK2/STAT3 signaling pathway, reducing the apoptosis of myocardial cells and alleviating myocardial ischemia injury.

Key Words:
Exercise preconditioning, JAK2/STAT3 pathway, Cardioprotection, Myocardial apoptosis, Rat.

Introduction

Brief episodes of exercise enhance the tolerance of ischemic hearts to subsequent ischemia. Such exercise-induced cardioprotection and improved tolerance for myocardial ischemia are called exercise preconditioning (EP)[1,2]. Researchers[3-5] have used EP in different myocardial injury models, such as the *in vivo* ligation of the coronary artery, *in vitro* ischemia/reperfusion, injection of isoproterenol, and exhaustive exercise, proving the cardioprotective effects of EP. However, the cardioprotection mechanism of EP remains unknown.

The Janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) signaling pathway is implicated in the signal transduction pathway of cells and participates in mediating various physiological processes, such as cell stress, growth, proliferation, differentiation, and apoptosis[6,7]. Numerous studies[8,9] have revealed that JAK2/STAT3 signaling pathway plays an important role in cardioprotection induced by ischemic preconditioning (IP) and pharmacologic preconditioning. The phosphorylation and activation of JAK2/STAT3 signaling pathway confer cardioprotection via prosurvival signaling cascades or the inhibition of proapoptotic factors. The putative JAK2 inhibitor AG490 abrogated IP-induced acute cardioprotection after ischemia-reperfusion injury[10]. Considering an important role of JAK2/STAT3 signaling pathway in preconditioning and cardioprotection, we suspect that this signaling pathway may participate in EP-induced protection.

In the present work, we observed the cardioprotective effect of EP against exhaustive exercise-induced myocardial injury according to the evaluation of serum cTnI, HBFP stain-

Corresponding Author: Xiaojuan Sun, MD; e-mail: 565489181@qq.com

ing, TUNEL, Bcl2, and cleaved caspase-3 levels. In addition, we studied the phosphorylations of JAK2 and STAT3 according to the immunohistochemistry and Western blot. To confirm the role of JAK2/STAT3 signaling pathway in EP cardioprotection, a JAK2 inhibitor AG490 was used.

Materials and Methods

Animals

Adult male Sprague-Dawley (SD; 220 g to 250 g) rats that are 12 weeks old were obtained from the Fourth Military Medical University Laboratory Animal Center (Xi'an, China). These rats were housed in a constant temperature room (23°C) on a 12:12 h light-dark photoperiod, with a standard laboratory food and water provided ad libitum. All of the animal care and experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Ethics Committee for Science Research of the Xianyang Normal University.

Experimental Protocol

All rats were habituated to treadmill running (10-20 min/day) at 15 m/min for 5 consecutive days. Then, after 2 days of rest, these rats were randomly assigned to one of five experimental groups.

Group control: rats were placed on a nonmoving treadmill no experimental treatment.

Group EE, exhaustive exercise: rats performed treadmill running at 30 m/min with 0% grade till exhausted. Exhaustion was defined as the rat being unable to upright itself when placed on its back.

Group EP+EE, exercise preconditioning plus exhaustive exercise: rats performed 3 consecutive days of treadmill running for 60 min per day at 28-30 m/min, 0% grade. During the 60 min of daily exercise, the rats were provided up to three separate 5-min rest periods during the exercise bout. To verify that EP training protocol resulted in cardioprotection against myocardial injury induced by exhaustive exercise, 24 h after the final exercise bout, rats underwent exhaustive exercise protocol similar to Group EE.

Group EP+EE+AG, AG490 plus exercise preconditioning plus exhaustive exercise: similar to Group EP+EE, but the JAK2 inhibitor AG490 (dissolved in 0.1% of dimethyl sulfoxide (DMSO) solution and 3 mg/kg of body weight) was administered intraperitoneally 10 min prior to daily exercise.

Group EE+AG, AG490 plus exhaustive exercise: similar to Group EE, but the JAK2 inhibitor AG490 (dissolved in 0.1% of DMSO solution and 3 mg/kg of body weight) was administered intraperitoneally 10 min prior to exhaustive exercise.

Tissue Preparation

All rats were sacrificed at 0.5 h following the completion of exercise protocols. The rats were anesthetized via an intraperitoneal injection of sodium pentobarbital (60 mg/kg). Following a midline skin incision, the abdominal cavity was opened, and blood samples were obtained from inferior vena cava. For histochemical assessment, the heart was fixed in 4% formalin and processed in paraffin. For Western blot analysis, the heart was rapidly removed, snap frozen and stored at -80°C.

Serum cTnI Concentration

The levels of cTnI in serum were quantitatively determined by electrochemiluminescence immunoanalyser (Beckman Coulter Instruments, Brea, CA, USA). This assay is a single-step sandwich immunoassay for direct cardiac injury detection. The labeling antibodies consist of the solid phase and 2 monoclonal antibodies against human. The measuring range was 0.03 to 50 ng/ml.

HBFP Staining and Analysis

Hearts were fixed in 4% paraformaldehyde and embedded in paraffin-blocks. Sections were stained with the hematoxylin basic fuchsin picric acid (HBFP) method. The result was examined with a light microscope (CX-31, Olympus, Tokyo, Japan). Twenty-five different fields were randomly selected and the positive area in each field was measured using the CellSens Entry analysis software (Olympus, Tokyo, Japan).

Apoptosis Assay

Hearts designated for terminal deoxynucleotidyl transferase-mediated dUTPX nick-end labeling (TUNEL) staining. Tissue sections were analyzed for TUNEL using a histochemical detection

kit (Roche, Basel, Switzerland). TUNEL-positive nuclei were counted under blinded conditions and the apoptotic index was expressed as the number of TUNEL-positive cell nuclei/total number of cell nuclei × 400.

Immunohistochemistry

Tissue slides were fixed in paraformaldehyde and embedded in paraffin. After the sections were blocked with serum, primary antibodies were added (p-JAK2 and p-STAT3 from Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight, followed by detection using the streptavidin-biotin complex kit (Zhongshan Jinqiao Biotechnology, Beijing, China) with diaminobenzidine/peroxidase substrate to produce a brown stain. The sections incubated without primary antibodies were used as negative controls.

Western Blot

The protein extracts (50 μg) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) techniques and transferred to nitrocellulose membrane. The membranes were incubated overnight with primary antibodies (Bcl2, cleaved caspase-3, p-JAK2, JAK2, p-STAT3, and STAT3 from Cell Signaling Technology, Danvers, MA, USA). The membrane was washed and incubated with horseradish peroxidase (HRP) conjugated secondary antibodies (Boshide Biotechnology, Wuhan, China). Results were evaluated using the Image J imaging system (NIH, Bethesda, MD, USA).

Statistical Analysis

All measures for each group were presented as means ± SD and analyzed by utilizing SPSS software 19.0 (SPSS IBM, Armonk, NY, USA). Our data were analyzed by one-way ANOVA, subsequent comparisons were made using Tukey's post-hoc analyses. Differences between two groups were analyzed by the Student's *t*-test. Significance was established *a priori* at $p < 0.05$.

Results

The Mean Running Distance to Exhaustion of Each Group

The mean running distance to exhaustion for Group EE, Group EP+EE, Group EP+EE+AG, and Group EE+AG were 2516.76 ± 717.18 m, 5193.65 ± 1126.85 m, 2739.46 ± 782.07 m, and 2613.54 ± 756.36 m, respectively. The mean running distance to exhaustion was significantly longer in Group EP+EE when compared to Group EE ($p < 0.05$). Treatment with JAK2 inhibitor, AG490 reduced this increase of running distance by EP treatment in Group EP+EE+AG, as compared with Group EP+EE ($p < 0.05$). However, AG490 alone did not have a significant influence on the mean running distance to exhaustion in Group EE+AG, as compared with Group EE.

Alterations of Serum cTnI Levels

Compared to Group control, the serum cTnI levels in Group EE was significantly increased ($p < 0.05$). EP treatment significantly reduced the serum cTnI levels in Group EP+EE when compared with Group EE ($p < 0.05$). By the treatment with JAK2 inhibitor, AG490 inhibited this decrease of serum cTnI levels induced by EP treatment in Group EP+EE+AG, as compared with Group EP+EE ($p < 0.05$). However, AG490 alone had no effect on the serum cTnI levels without EP treatment in Group EE+AG, as compared with Group EE. These results indicate that the decrease of serum cTnI levels is induced by EP treatment (Figure 1).

HBFP Staining of Cardiomyocytes and Positive Area Analysis

In Group control, cardiomyocytes stained a brown color; no red color case was seen. In Group EE, the red positive stain was found in wide range of cases. In Group EP+EE, only

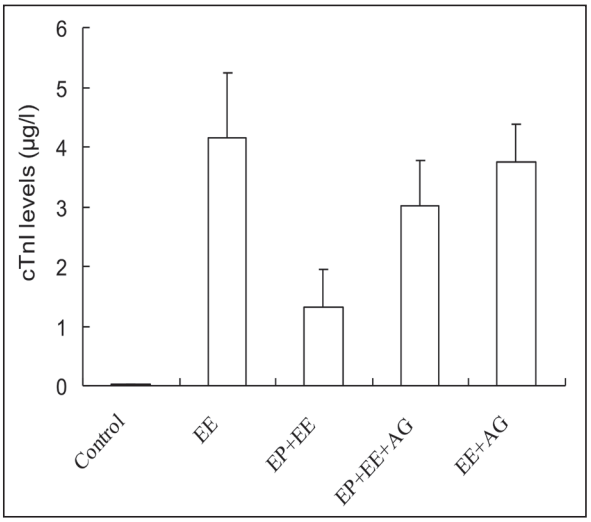

Figure 1. Alterations of serum cTnI levels among different groups. Values are mean ± SD; $^{*}p < 0.05$ *vs.* Group Control; $^{\#}p < 0.05$ *vs.* Group EE; $^{\&}p < 0.05$ *vs.* Group EP+EE.

the red spot stain was seen in a small fraction of cardiomyocytes. In Group EP+EE+AG and Group EE+AG, the red spot stain was scattered across the cardiomyocytes (Figure 2). The HBFP staining of cardiomyocytes in Group control had no positive area. Compared with Group control, the positive area of HBFP staining in Group EE was significantly increased ($p < 0.05$). EP treatment significantly reduced the positive area of HBFP staining in Group EP+EE when

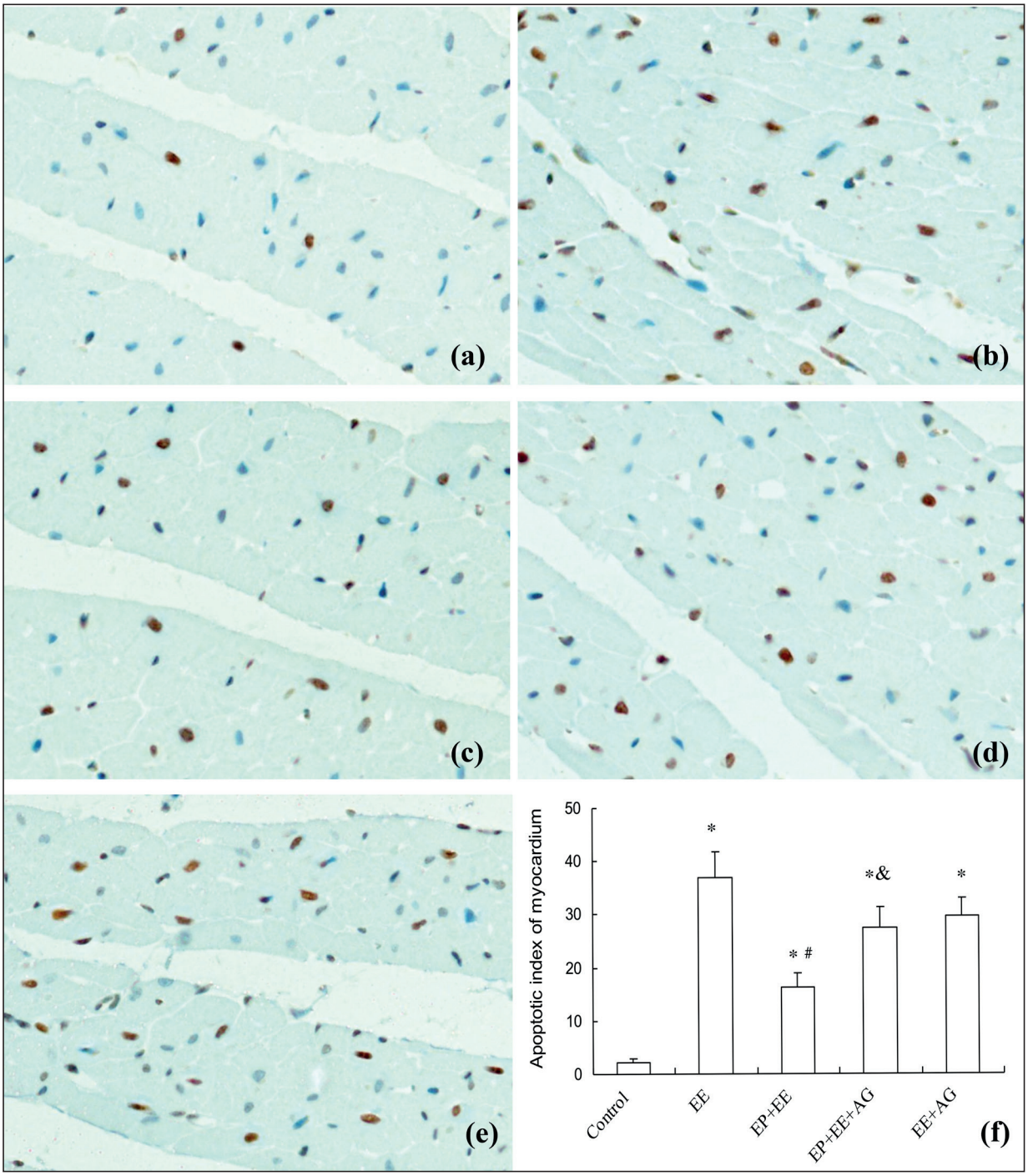

Figure 2. Myocardial apoptotic staining and apoptotic index analysis. **(a)** Group Control; **(b)** Group EE; **(c)** Group EP+EE; **(d)** Group EP+EE+AG; **(e)** Group EE+AG. Original magnification = ×400. **(f)** Apoptotic index analysis of myocardium. Values are mean ± SD; $*p < 0.05$ *vs.* Group Control; $^{\#}p < 0.05$ *vs.* Group EE; $^{\&}p < 0.05$ *vs.* Group EP+EE.

compared with Group EE ($p < 0.05$). Treatment with AG490 inhibited this decrease of positive area of HBFP staining induced by EP treatment in Group EP+EE+AG, as compared with Group EP+EE ($p < 0.05$). However, AG490 alone had no effect on the positive area of HBFP staining without EP treatment in Group EE+AG, as compared with Group EE. These results indicate that the decrease of positive area of HBFP staining is induced by EP treatment (Figure 3).

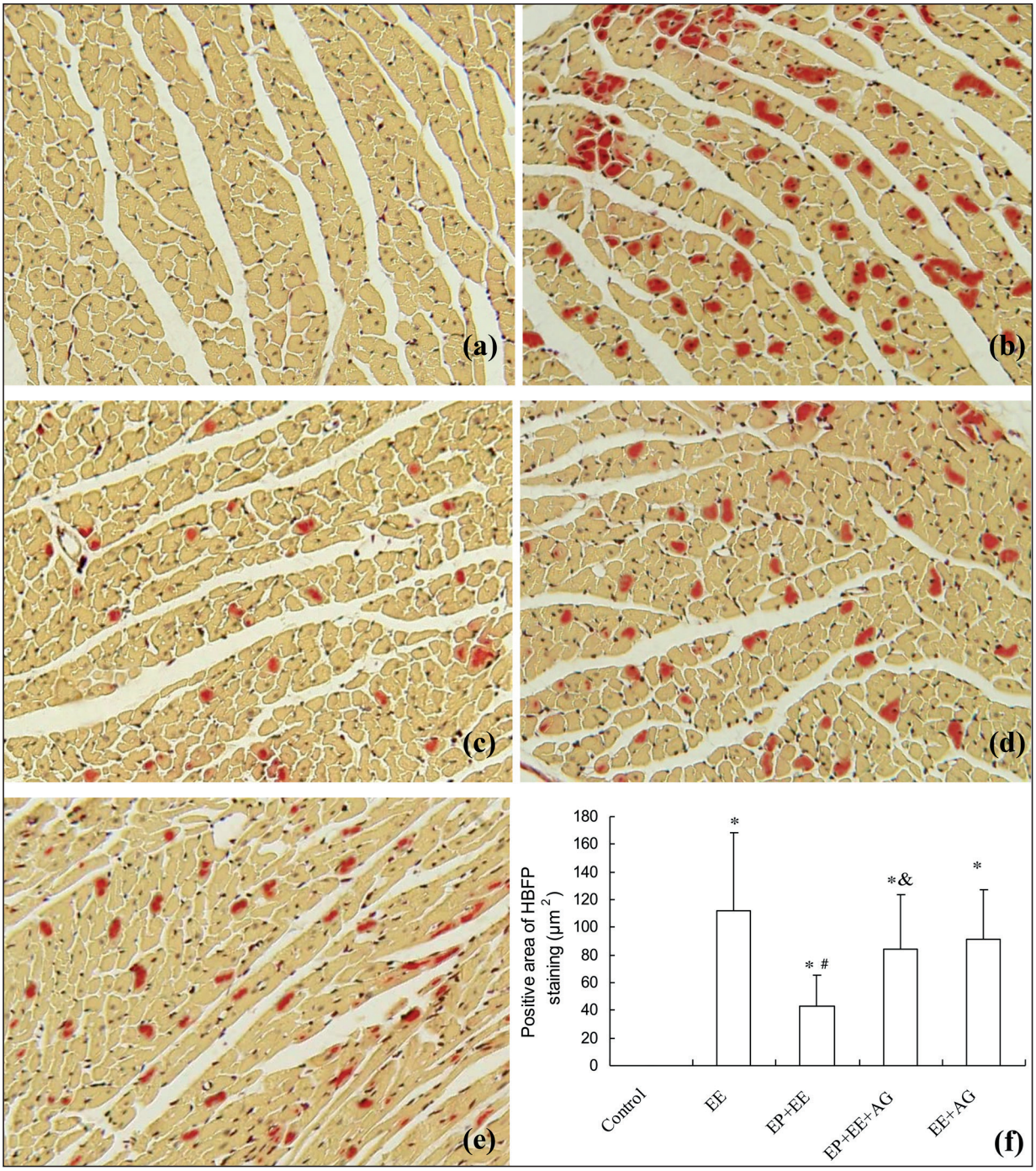

Figure 3. HBFP staining of cardiomyocytes and image analysis. **(a)** Group Control; **(b)** Group EE; **(c)** Group EP+EE; **(d)** Group EP+EE+AG; **(e)** Group EE+AG. Original magnification = ×400. **(f)** Positive area analysis of HBFP staining. Values are mean ± SD; *$p < 0.05$ *vs.* Group Control; $^{\#}p < 0.05$ *vs.* Group EE; $^{\&}p < 0.05$ *vs.* Group EP+EE.

TUNEL Staining of Cardiomyocytes and Apoptotic Index Analysis

The immunostaining of apoptotic cardiomyocytes was mainly expressed in the nuclei of cardiomyocytes in Group control. A large number of apoptotic cells were visible in the hearts of Group EE. Decreased apoptotic cells were found in Group EP+EE. AG490 treatment significantly increased the apoptotic cells in Group EP+EE+AG and Group EE+AG (Figure 4). Compared with Group control, the number of apoptotic cells in Group EE was significantly increased ($p < 0.05$). EP treatment significantly reduced the apoptotic cells in Group EP+EE when compared with Group EE ($p < 0.05$). Treatment with AG490 inhibited the antiapoptotic effects induced by EP treatment, and the number of apoptotic cells was significantly increased in Group EP+EE+AG, as compared with Group EP+EE ($p < 0.05$). However, AG490 alone did not change the number of apoptotic cells without EP treatment in Group EE+AG, as compared with Group EE. These results indicate that the decrease of apoptotic cells is induced by EP treatment (Figure 2).

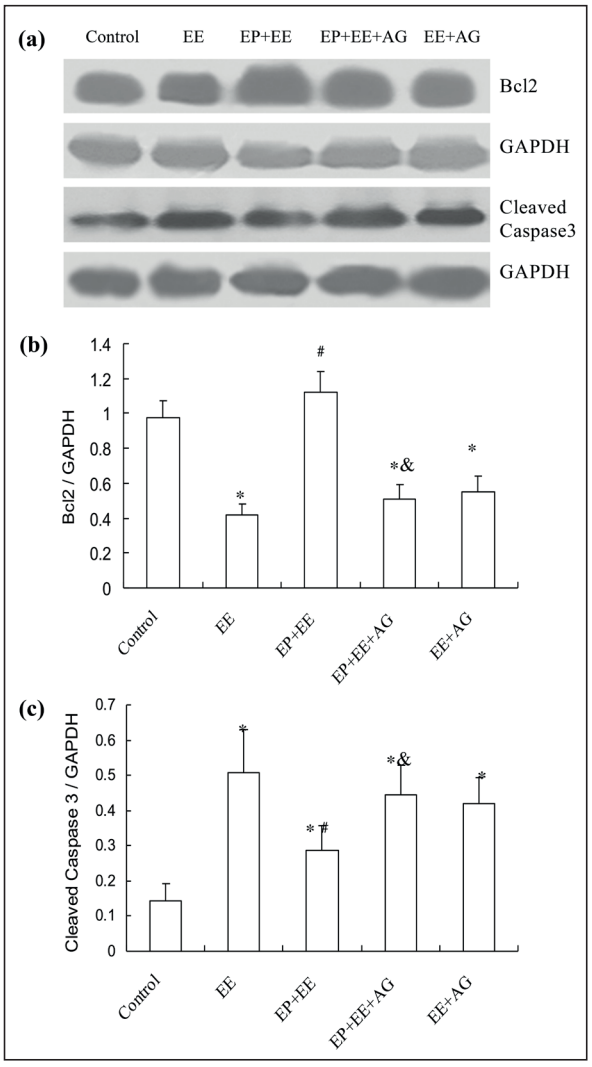

Figure 4. The expressions of Bcl2 and cleaved caspase 3 in cardiomyocytes under Western blot analysis. **(a)** Representative immunoblots of Bcl2 and cleaved caspase 3. **(b)** Densitometry analysis for the ratio of Bcl2/GAPDH. **(c)** Densitometry analysis for the ratio of cleaved caspase 3/GAPDH. Values are mean ± SD; $^{*}p < 0.05$ *vs.* Group Control; $^{\#}p < 0.05$ *vs.* Group EE; $^{\&}p < 0.05$ *vs.* Group EP+EE.

Expressions of Bcl2 and Cleaved Caspase-3 in Cardiomyocytes by Western Blot

Compared with Group control, the expression of Bcl2 in Group EE was significantly decreased ($p < 0.05$). EP treatment significantly increased the expression of Bcl2 in Group EP+EE when compared with Group EE ($p < 0.05$). Treatment with AG490 inhibited this increase of Bcl2 induced by EP treatment in Group EP+EE+AG, as compared with Group EP+EE ($p < 0.05$). However, AG490 alone did not change the expression of Bcl2 without EP treatment in Group EE+AG, as compared with Group EE (Figure 4).

In contrast, compared with Group control, the expression of cleaved caspase-3 in Group EE was significantly increased ($p < 0.05$). EP treatment significantly decreased the expression of cleaved caspase-3 in Group EP+EE when compared with Group EE ($p < 0.05$). Treatment with AG490 inhibited this decrease of cleaved caspase-3 induced by EP treatment in Group EP+EE+AG, as compared with Group EP+EE ($p < 0.05$). However, AG490 alone had no effect on the expression of cleaved caspase-3 without EP treatment in Group EE+AG, as compared with Group EE (Figure 4).

Immunostaining of p-JAK2 and p-STAT3 Proteins in Cardiomyocytes

The immunostaining of p-JAK2 and p-STAT3 proteins were mainly expressed in the nuclei of cardiomyocytes in Group control. The increased p-JAK2 and p-STAT3 positive nuclei were found in Group EE. The immunostaining showed a large number of p-JAK2 and p-STAT3 positive nuclei in Group EP+EE. AG490 treatment significantly decreased the numbers of p-JAK2 and p-STAT3 positive nuclei in Group EP+EE+AG and Group EE+AG (Figures 5 and 6).

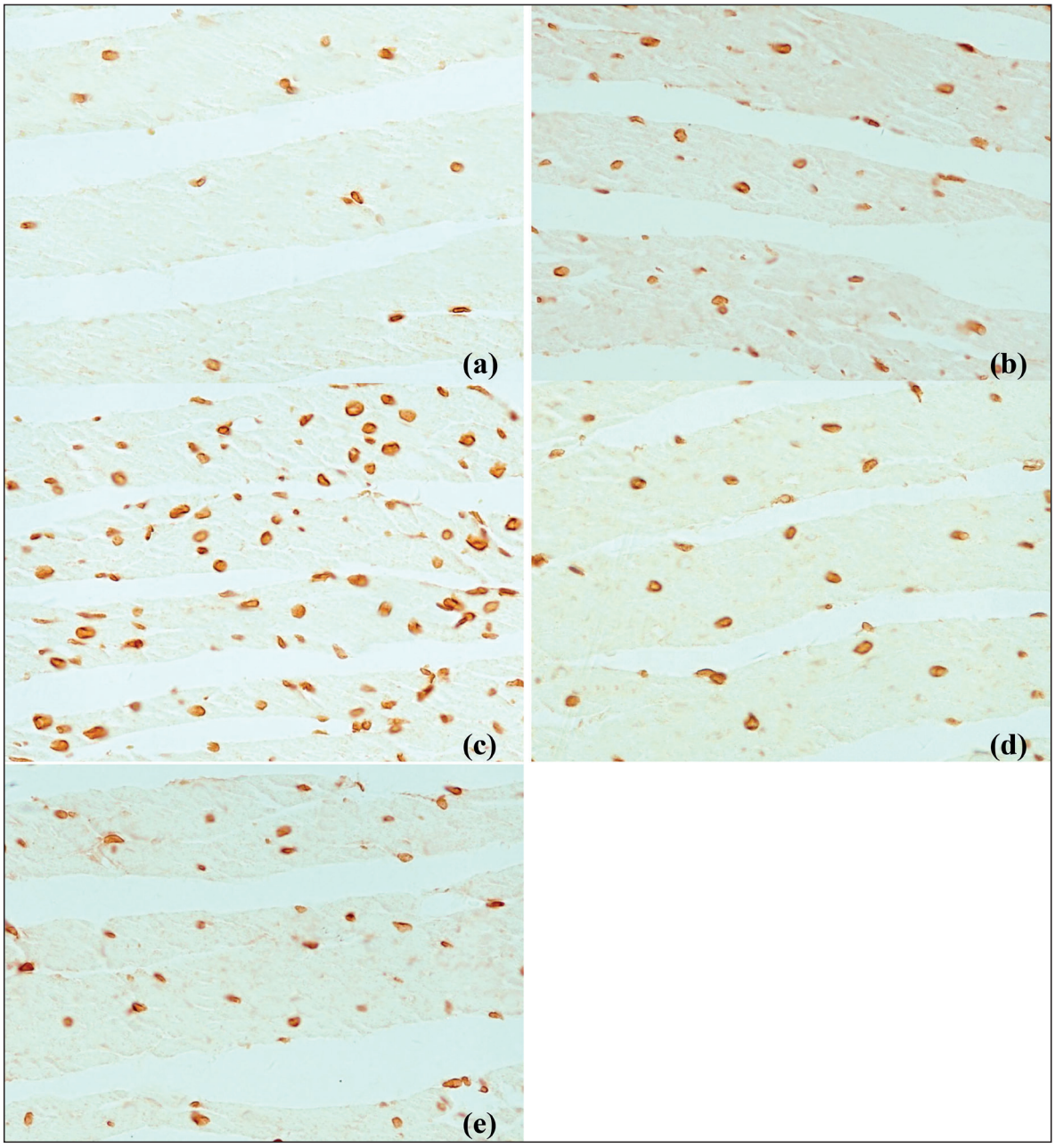

Figure 5. Immunostaining of p-JAK2 protein in cardiomyocytes. **(a)** Group Control; **(b)** Group EE; **(c)** Group EP+EE; **(d)** Group EP+EE+AG; **(e)** Group EE+AG. Original magnification = ×400.

Phosphorylations of JAK2 and STAT3 under Western Blot Analysis

In contrast to Group control, exhaustive exercise induced the phosphorylations of JAK2 and STAT3 in Group EE ($p < 0.05$). Exercise preconditioning further increased the phosphorylations of JAK2 and STAT3 in Group EP+EE when compared with Group EE ($p < 0.05$). Treatment with AG490 inhibited the increased phosphorylations of JAK2 and STAT3 induced by EP treatment in Group EP+EE+AG, as compared with Group EP+EE ($p < 0.05$). However, AG490 alone had no effect on the phosphorylations of JAK2 and STAT3 without EP treatment in Group EE+AG, as compared with Group EE. These results indicate that the increase of phosphorylations of JAK2 and STAT3 are induced by EP treatment (Figure 7).

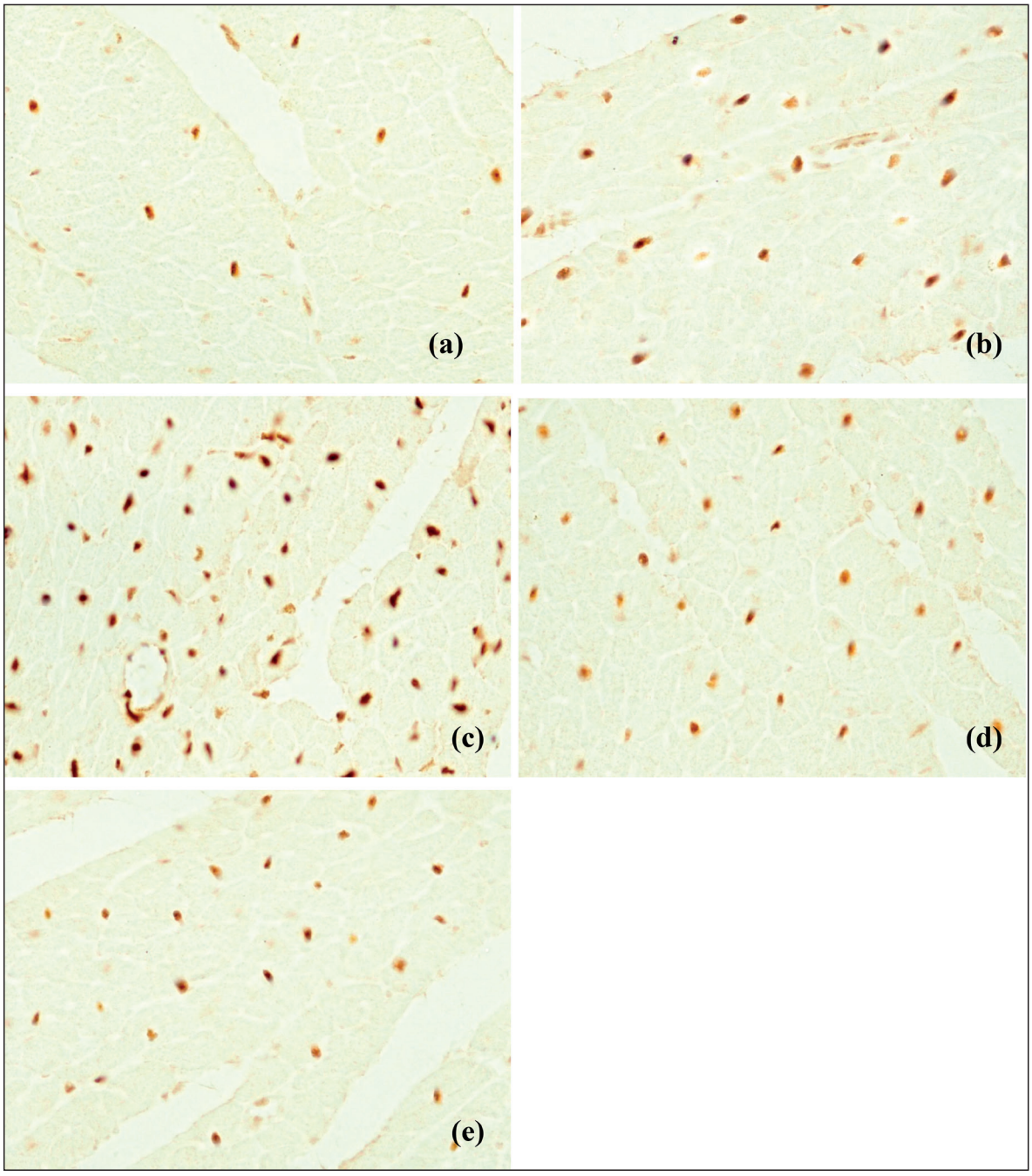

Figure 6. Immunostaining of p-STAT3 protein in cardiomyocytes. **(a)** Group Control; **(b)** Group EE; **(c)** Group EP+EE; **(d)** Group EP+EE+AG; **(e)** Group EE+AG. Original magnification = ×400.

Discussion

Cell apoptosis is an exceptional form of cell death. This process refers to the gene-regulated initiative cell death process under certain physiological or pathological conditions[11,12]. Apoptosis plays an essential role in myocardial injury induced by high-intensity exercises in rat hearts[13-15]. This condition causes myocardial injury similar to that caused by ischemia/reperfusion injury[16,17]. Researches[18-20] has proved that EP decreases the myocardial infarct area, reduces myocardial stunning, accelerates myocardial functional recovery, and decreases arrhythmia. Furthermore, EP is

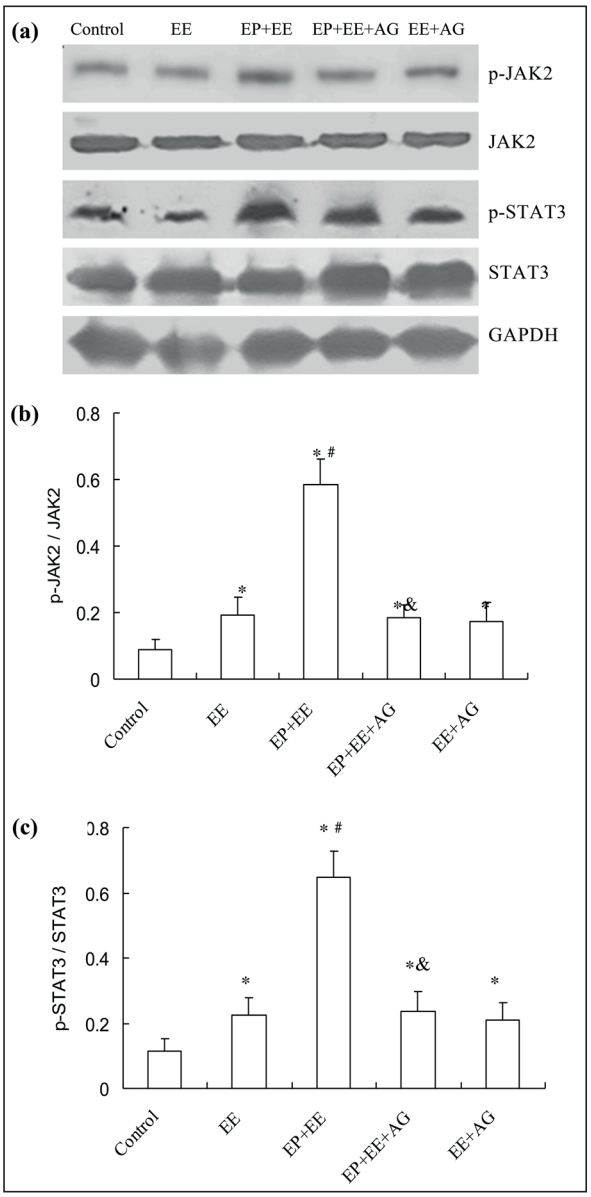

Figure 7. Phosphorylations of JAK2 and STAT3 under Western blot analysis. **(a)** Representative immunoblots of p-JAK2, JAK2, p-STAT3 and STAT3. **(b)** Densitometry analysis for the ratio of p-JAK2/JAK2. **(c)** Densitometry analysis for the ratio of p-STAT3/STAT3. Values are mean ± SD; $^{*}p < 0.05$ *vs*. Group Control; $^{\#}p < 0.05$ *vs*. Group EE; $^{\&}p < 0.05$ *vs*. Group EP+EE.

also implicated in myocardial apoptosis. Quindry et al[21] reported that subjecting rats to 60 min treadmill exercise for 3 days, EP significantly decreased infarction size after ischemia/reperfusion and reduced myocardial apoptosis number and caspase-3 expression. These data indicate that EP can reduce myocardial apoptosis by inhibiting caspase-3 expression to protect the heart. Chen et al[22] studied the cardioprotective effect of EP on intermittent hypoxia-induced myocardial apoptosis in rats. Results demonstrated that 5 consecutive days of treadmill exercise can reduce the myocardial apoptosis index and caspase-3 expression and increase Bcl2/Bax ratios, indicating that EP provides cardioprotective effects by attenuating hypoxia-induced myocardial apoptosis. In line with previous studies, we showed that EP significantly attenuated the exhaustive exercise-induced myocardial ischemia injury, associated with lower serum cTnI levels, a decreased myocardial infarct size, a reduced number of myocardial apoptosis, upregulation of anti-apoptotic Bcl2, and downregulation of pro-apoptotic cleaved caspase-3. These results indicated that EP provides cardioprotective effects against myocardial injury and apoptosis.

The JAK/STAT signaling pathway is an important intracellular signal transduction pathway, which involves various physiological and pathological processes, such as mechanical stress, ischemia, bacterial inflammation, or cytotoxic agents[23-26]. It is well documented that JAK/STAT pathway plays a role in the cardioprotection of IP. Xuan et al[8] found that IP induced the phosphorylations of JAK1, JAK2, STAT1, and STAT3. Pretreatment with the JAK inhibitor AG490 blocked the enhanced tyrosine phosphorylation of JAKs and STATs. Meanwhile, IP-induced cardioprotection is cancelled completely, indicating that the JAK/STAT pathway plays an essential role in the development of IP. Furthermore, recent studies have been focused on the essential role of JAK/STAT pathway in the pharmacologic preconditioning. Yu et al[9], for example, have shown that the preconditioning of PCI2 cells with 100 μmol/L of H_2O_2 for 90 min can significantly inhibit cell apoptosis caused by 300 μmol/L of H_2O_2 for 12 h, and upregulate the phosphorylations of JAK2, STAT1, and STAT3. AG490 blocked the phosphorylations of JAK2, STAT1, and STAT3. At the same time, the cardioprotection of H_2O_2 preconditioning was inhibited, indicating that the JAK2/STAT3 signaling pathway plays an important role in cytoprotection induced by H_2O_2 preconditioning. Das et al[27] and Gross et al[28] also demonstrated that JAK/STAT signaling pathway is essential to the cardioprotective effects induced by rapamycin preconditioning or opioid preconditioning.

Thus, considering an important role of JAK/STAT signaling pathway in preconditioning and cardioprotection, it seems plausible that EP cardi-

oprotection may be related with the JAK2/STAT3 signaling pathway. We found that EP significantly increased the expression of phosphorylated forms of JAK2 and STAT3, and ameliorated the myocardial ischemia injury following exhaustive exercise. Our results indicated that EP cardioprotection may depend on the activation of the JAK2/STAT3 signaling pathway.

The activation of the JAK2/STAT3 pathway in response to IP confer cardioprotection via prosurvival signaling cascades or the inhibition of proapoptotic factors. Hattori et al[29] and Negoro et al[30] reported that IP can quickly induce the phosphorylations of JAK2 and STAT3, up-regulate the antiapoptosis gene Bcl-2, and down-regulate the apoptosis-promoting gene Bax. AG490 can attenuate the anti-apoptotic effect of IP, indicating that IP may afford an anti-apoptotic effect via a JAK2/STAT3 signaling pathway. In another investigation by Duan et al[10], curcumin preconditioning can attenuate ischemia/reperfusion injury through the activation of the JAK2/STAT3 signaling pathway. AG490 before preconditioning can significantly inhibit the phosphorylations of JAK2 and STAT3. Meanwhile, curcumin-induced cardioprotection can be inhibited. Constitutive cardiomyocyte-restricted deletion of STAT3 has been shown to increase apoptosis and infarct area and cause loss of cardioprotection during IP and pharmacological preconditioning[31,32]. We used AG490 to investigate the role of the JAK2/STAT3 pathway in mediating the EP cardioprotection against myocardial apoptosis. Our results showed that the EP-induced inactivation of JAK2/STAT3 was observed with a reduced number of myocardial apoptosis, an increase in anti-apoptotic Bcl2 and a decrease in pro-apoptotic Cleaved caspase-3. Furthermore, AG490 treatment significantly inhibited the phosphorylations of JAK2 and STAT3, while also abolishing the increase of Bcl2 and decrease of cleaved caspase-3 induced by EP, which indicated a close connection of the JAK2/STAT3 pathway and EP anti-apoptotic effect.

Studies[28,33] have shown that JAK2/STAT3 signaling pathway can play cardioprotective roles with the help of downstream correlation factors, such as GSK-3β, NF-κB, and mTOR. Furthermore, IP activates the JAK2/STAT3 signaling pathway and up-regulates NOS, COX-2, and HSP70 in favor of cardioprotection[34,35]. However, the downstream-specific protective mechanism that EP activates the JAK2/STAT3 signaling pathway and the problems with other family members of JAKs and STATs in EP cardioprotection should be further studied.

Conclusions

We observed that EP significantly attenuated the exhaustive exercise-induced myocardial ischemia injury, associated with lower serum cTnI levels, decreased myocardial infarct size, reduced myocardial apoptosis, upregulation of anti-apoptotic Bcl2 protein, and downregulation of pro-apoptotic cleaved caspase-3. EP cardioprotection activates JAK2/STAT3 signaling pathway and protects myocardium from ischemic injury. The inhibition of JAK2/STAT3 pathway by AG490 abolished the myocardial protection of EP and affected the anti-apoptotic pathways, therefore, suggesting that the JAK2/STAT3 signaling pathway plays an essential role in the EP cardioprotection against myocardial ischemia injury induced by exhaustive exercise. EP cardioprotection is a noninvasive manner of applying ischemic preconditioning. This cardioprotection mechanism is beneficial and strongly endogenous and has important theoretical significance and clinical values. Therefore, the cardioprotection of EP is a promising research prospect.

Acknowledgements

This study was supported by grants from Scientific Research Program of Shaanxi Provincial Education Department, China (Program No. 16JK1830).

Conflict of Interest

The Authors declare that they have no conflict of interests.

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