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Postconditioning reduces infarct size and cardiac myocyte apoptosis via the opioid receptor and JAK-STAT signaling pathway

Lu You · Li Li · Qiuling Xu · Jianghua Ren · Fan Zhang

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Abstract Brief intermittent episodes of ischemia and reperfusion could reduce infarct size, a phenomenon called "postconditioning" at the onset of reperfusion after a prolonged period of ischemia. To investigate whether the opioid receptors and signaling factor JAK-STAT might be responsible for the cardioprotection in ischemic postconditioning, and the possible molecular machinery of cardioprotection. Hundred and twenty healthy New Zealand rabbits were divided into six groups. The myocardial infarct size, cardiac myocyte apoptosis, BCL-2 and P-Stat3 protein expression were tested in the current study. The results suggested that ischemic postconditioning might increase BCL-2 protein expression by activating the opioid receptors and JAK-STAT signaling pathway, and also to reduce ischemia-reperfusion-induced cardiomyocyte apoptosis and to play a key role in myocardial protection. However, further research still needs to be done to unravel the underlying mechanisms.

Keywords Postconditioning · Infarct size · Cardiac myocyte apoptosis · Opioid receptor · JAK-STAT

L. You, L. Li are Co-first Author.

L. You · J. Ren (✉)
Department of Cardiovascular, Affiliated Zhong-Nan Hospital of Wuhan University, Wuhan 430072, China
e-mail: jhren2009@hotmail.com

L. Li · Q. Xu
Department of Physiology, Mu-Dan-Jiang Medical College, Heilongjiang 157011, China

F. Zhang (✉)
Department of Cardiovascular, Beijing Chao-Yang Hospital, Beijing 100020, China
e-mail: zhang-fan2008@hotmail.com

Introduction

Reperfusion has been demonstrated as an effective treatment for myocardial ischemia. However, reperfusion could cause additional reversible or irreversible damage to the myocardium [1, 2]. Ischemic preconditioning has been confirmed to be a strongest cardio-protective intervention that salvaged ischemic myocardium in several experiments [3–6]. However, as its name implies that preconditioning must be applied before an ischemic event to be protective, which limiting its utility [7]. Therefore, ischemic preconditioning is not beneficial for patients presenting to the hospital after onset of their myocardial infarction [1, 8–10]. In addition, lots of studies have confirmed that some factors and some drugs were protective for the myocardium, and also several transduct signaling were also involved [11–25]. Thus, an intervention is necessary that could supplement the reperfusion strategy and attenuate reperfusion injury and/or otherwise limit necrosis in the heart [7].

Recently, it has been shown that the canine heart can be effectively protected from infarction with brief cycles of ischemia and reperfusion during the early reperfusion period following a lethal ischemic insult, a phenomenon termed postconditioning [26]. Postconditioning is a novel strategy to harness nature's protection against myocardial ischemia-reperfusion injury. The cardio-protective effect invoked by postconditioning has been demonstrated to involve a reduction in infarct size, apoptosis as well as vascular injury [27]. To date, cardioprotection by postconditioning has been confirmed by several independent laboratories with kinds of species [7, 26, 28–31]. Meanwhile, it has been demonstrated that postconditioning afforded protection comparable to that of ischemic preconditioning [7, 26, 31], although there are mixed reports on whether these strategies are capable of invoking additive protection

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when combined [7, 26, 29, 31]. Substantial evidence have implicated that signal transduction mechanisms are essential components of the machinery of cardioprotection by postconditioning [32–36].

In the present study, we attempted to design the experiment to investigate whether the opioid receptors might be responsible for the cardioprotection in ischemic postconditioning. In addition, we also investigated the effect of signaling factor JAK-STAT in the molecular machinery of cardioprotection in ischemic postconditioning.

Materials and methods

Animal models

Hundred and twenty healthy New Zealand rabbits, weighing 1.5–2.5 kg, were provided by the Laboratory Animal Center of Mu-Dan-Jiang Medical College. The housing and treatment of the animals were in accordance with institutional guidelines and approved by the Institutional Animal Care and Use Committee. All animals were anesthetized with pentobarbital sodium (30 mg/kg) and ventilated with room air. As Argaud et al. described [37], a cannula was inserted into the right internal jugular vein for administration of drugs and fluids and into the left carotid artery for measurement of blood pressure. Limb lead II of the ECG was used to measure the heart rate. After a left thoracotomy was performed, the heart was exposed and a snare was passed around a marginal branch of the left coronary artery (LCA) to induce regional myocardial ischemia. After the surgical procedure, a 15 min stabilization period was observed. Animals were then randomly assigned to the experimental groups. All animals underwent 30 min of ischemia and 3 h of reperfusion.

Separating groups

Animals were randomly allocated to the following groups ($n = 20$ per group): (1) Sham-operated group, not to tighten the ligature after thoracotomy; (2) the Control group, no intervention either before or after LCA occlusion; (3) postconditioning group, initiated immediately at the onset of reperfusion, 3 cycles of 10 s full reperfusion and 10 s reocclusion (total intervention time of 1 min); (4) morphine (0.1 mg/kg) + postconditioning group; (5) DMSO (3 mg/kg) + postconditioning group; (6) AG490 (3 mg/kg) + postconditioning group. In those hearts that received drug treatment, antagonists were given as a bolus dose 5 min prior to reperfusion to ensure proper receptor to be blockaded.

Determination of myocardial infarct size

At the end of the final reperfusion, the coronary artery was briefly reoccluded, and 0.5 mg/kg Uniperse blue pigment (Ciba-Geigy) was injected intravenously to delineate the in vivo area at risk, as previously described [38]. The heart was excised and cut into 5–6 transverse slices, 2 mm thick, parallel to the atrioventricular groove. Each heart slice was photographed for later measurement of the area at risk. After incubation for 15 min in a 1% solution of triphenyltetrazolium chloride at 38°C to differentiate infarcted (pale) from viable (brick red) myocardial area, the slices were rephotographed. Germany OPTON VIDAS image analysis system was utilized to determinate the myocardial infarct size.

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay

Cardiac myocyte apoptosis was quantitated using the (TUNEL) assay. This specific assay uses terminal deoxynucleotidyl transferase to attach biotinylated deoxyuridine triphosphate to free 3′-OH DNA ends. All cardiac muscle tissue specimens (about 4 mm in size) were fixed in freshly prepared 4% paraformaldehyde in PBS. Tissue sections (5 μm) were prepared using a microtome and placed on glass slides. The sections were deparaffinized in xylene and dehydrated in ethanol. The sections were then incubated with 20 μg/ml proteinase K in PBS for 20 min at room temperature. After rinsing the specimen twice with PBS, the sections were stained by streptavidin–horseradish peroxidase conjugate and then counterstained with hematoxylin. The peroxidase-positive cells were identified morphometrically by brown staining nuclei. The number of TUNEL-positive cells was counted in Axioplan 2 imaging analysis system. Three heart slices were randomly selected from each group, five high power fields were randomly selected from each slice, and then the number of apoptotic cells and normal cells were counted. The cardiac myocyte apoptosis index (%) = (the number of apoptotic myocardial/the number of normal myocardial cells) × 100%.

Western blot analysis

Tissue samples were homogenized in a lysis buffer containing 10 μg/ml of aprotinin, leupeptin, and pepstatin A, 0.5 mM of phenylmethanesulfonyl fluoride and 1 mM of *p*-nitrophenyl phosphate. The protein concentration was quantified by the BCA protein assay. Equal amounts of proteins samples were separated by 12% SDS-PAGE and transferred to PVDF membranes (Amersham Biosciences, Piscataway, NJ, USA). The membranes were blocked overnight with PBS containing 0.1% Tween 20 in 5% skimmed

milk at 4°C, and subsequently probed with anti-BCL-2 and anti-P-Stat3 polyclonal antibody (1:2000) followed by a horseradish peroxidase-conjugated second antibody (Santa Cruz Biotechnology). The membrane was incubated with NBT/BCIP solution (Beyotime Institute of Biotechnology) until color development was achieved. Signal intensities were quantitated using the Scion Image software (Scion Corp., USA). β-actin was used as an internal control.

Statistical analysis

All statistical analysis were done by the computer program SPSS 11.5 (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± SD. SNK-q and one way ANOVA analysis were used to examine differences among multiple groups. P values < 0.05 were considered as statistically significant.

Results

Ischemia size and infarct size

The ischemia size and myocardial infarct size for each group of rabbits were shown in Table 1, there were no significant differences among all groups of rabbits. The infarct size of postconditioning group (19.9 ± 5.4), morphine + postconditioning group (20.1 ± 4.2), DMSO + postconditioning group (20.8 ± 5.1), were significantly decreased when compared with the control group (29.9 ± 4.6). However, there was no significant difference between the control group and AG490 + postconditioning group (27.7 ± 4.1).

Cardiac myocyte apoptosis

Cardiac myocyte apoptosis was quantitated using the TUNEL assay. The cardiac myocyte apoptosis index (%) was utilized in the present study. As is shown in Fig. 1 and Table 2, the cardiac myocyte apoptosis index of the control group (29.7 ± 7.3) was significantly higher than those in Sham-operated group (5.83 ± 2.5), postconditioning group (18.7 ± 7.2), morphine + postconditioning group (20.1 ± 6.8), DMSO + postconditioning group (19.7 ± 7.1). However, there was no significant difference between the control group and AG490 + postconditioning group (27.6 ± 6.1). Table 3

Analysis of BCL-2 and P-Stat3 protein expression by western blotting

Next, we determined whether the molecular machinery of cardioprotection by postconditioning was associated with the levels of BCL-2 and P-Stat3 protein expression. BCL-2 and P-Stat3 protein expression in the myocardium was assessed by western blotting analysis. The western blotting of BCL-2 and P-Stat3 protein expression of each groups of rabbits were shown in Fig. 2a and b, respectively. The expression of BCL-2 and P-Stat3 protein in the control group [BCL-2: (29.2 ± 4.9), P-Stat3: (26.9 ± 4.1)] were significantly increased than the Sham-operated group [BCL-2: (19.6 ± 4.1), P-Stat3: (9.2 ± 1.4)]. The expression of BCL-2 and P-Stat3 protein in the postconditioning group [BCL-2: (72.3 ± 5.2), P-Stat3: (46.7 ± 5.4)], morphine + postconditioning group [BCL-2: (68.5 ± 5.6), P-Stat3: (43.5 ± 4.8)], DMSO + postconditioning group [BCL-2: (67.1 ± 5.8), P-Stat3: (42.1 ± 3.9)] were significantly increased than the control group. However, there was no significant difference between the control group and AG490 + postconditioning group [BCL-2: (32.3 ± 4.7), P-Stat3: (25.2 ± 4.3)]. Meanwhile, there was no significant difference between the postconditioning group and the morphine + postconditioning group or DMSO + postconditioning group.

Discussion

The aim of cardiovascular research is currently the identification of a reliable cardioprotection intervention that

Table 1 The infarct size data in each group of New Zealand rabbits

Group	Weight (kg)	Ischemia size	Myocardial infarction size
Sham-operated	1.73 ± 0.17	–	–
Control	1.81 ± 0.31	33.9 ± 10.6	29.9 ± 4.6
Postconditioning	1.63 ± 0.25	31.6 ± 9.1	19.9 ± 5.4$^{\triangle}$
Morphine + postconditioning	1.93 ± 0.44	34.8 ± 7.1	20.1 ± 4.2$^{\triangle}$
DMSO + postconditioning	1.71 ± 0.21	35.2 ± 6.4	20.8 ± 5.1$^{\triangle}$
AG490 + postconditioning	1.83 ± 0.38	31.8 ± 4.9	27.7 ± 4.1$^{\#}$

$^{\triangle}$ Compared with the control group, $P < 0.05$

$^{\#}$ Compared with the control group, $P > 0.05$

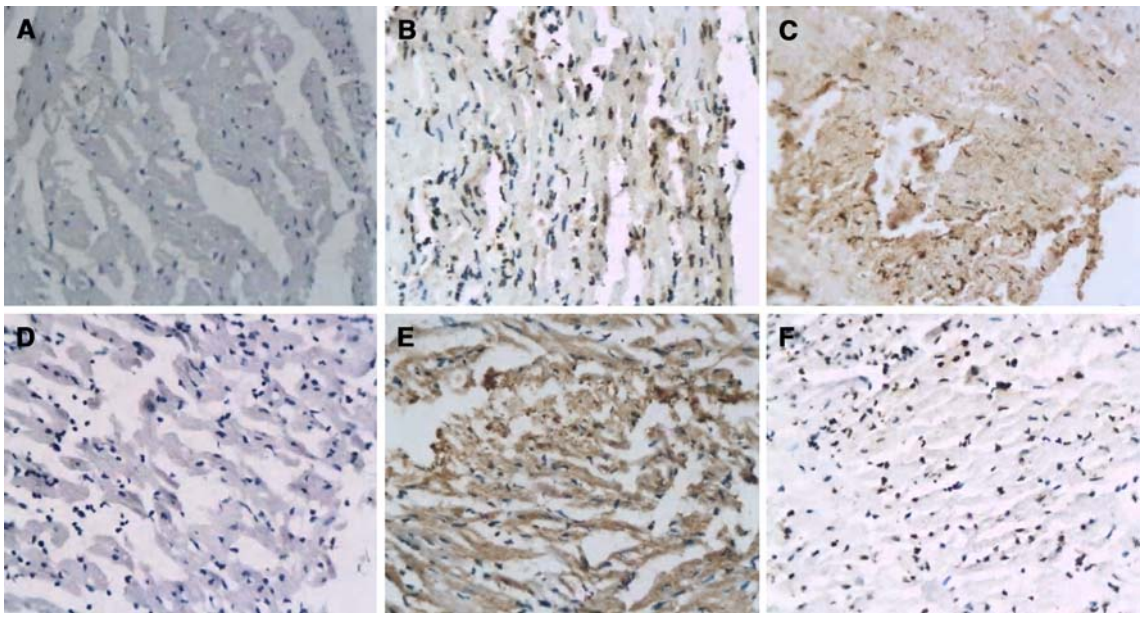

Fig. 1 TUNEL assay of the myocardium. **a** Sham-operated group, not to tighten the ligature after thoracotomy. **b** The control group, no intervention either before or after LCA occlusion. **c** Postconditioning group, initiated immediately at the onset of reperfusion, three cycles of 10 s full reperfusion and 10 s reocclusion (total intervention time of 1 min). **d** Morphine (0.1 mg/kg) + postconditioning group. **e** DMSO (3 mg/kg) + postconditioning group. **f** AG490 (3 mg/kg) + postconditioning group. The *brown* is the apoptotic nucleus with TUNEL

Table 2 The test results of cardiac myocyte apoptosis index in each group of New Zealand rabbits

Group	Sham-operated	Control	Postconditioning	Morphine + postconditioning	DMSO + postconditioning	AG490 + postconditioning
Cardiac myocyte apoptosis index (%)	$5.83 \pm 2.5^{\triangle}$	29.7 ± 7.3	$18.7 \pm 7.2^{\triangle}$	$20.1 \pm 6.8^{\triangle}$	$19.7 \pm 7.1^{\triangle}$	$27.6 \pm 6.1^{\#}$

$^{\triangle}$ Compared with the control group, $P < 0.05$

$^{\#}$ Compared with the control group, $P > 0.05$

Table 3 The results of BCL-2 and P-Stat3 protein expression in each group of New Zealand rabbits

Group	BCL-2	P-Stat3
Sham-operated	$20.6 \pm 4.3^{\triangle}$	$10.3 \pm 2.6^{\triangle}$
Control	31.2 ± 6.9	27.6 ± 5.3
Postconditioning	$74.5 \pm 11.0^{\triangle}$	$47.0 \pm 4.8^{\triangle}$
Morphine + postconditioning	$70.2 \pm 9.8^{\triangle}$*	$45.8 \pm 4.3^{\triangle}$*
DMSO + postconditioning	$68.3 \pm 10.4^{\triangle}$*	$44.2 \pm 5.1^{\triangle}$*
AG490 + postconditioning	$33.5 \pm 7.1^{\#}$	$26.8 \pm 5.0^{\#}$

$^{\triangle}$ Compared with the control group, $P < 0.05$

$^{\#}$ Compared with the control group, $P > 0.05$

* Compared with the postconditioning group, $P > 0.05$

could salvage ischemic myocardium. Preconditioning was first introduced in 1986 [39]. Although ischemic preconditioning is a powerful anti-infarct intervention, it has not been possible to apply it in clinical patients with acute myocardial infarction because of the requirement for pre-treatment [39, 40]. In addition, restoration of blood flow to ischemic myocardium, a requisite for long-term survival of tissue, appears to hasten the transition from ischemic but viable to necrotic myocardium [8–10, 41]. The phenomenon of ischemic postconditioning was first described by Vinten-Johansen's group [10], in which brief intermittent repetitive interruptions to reperfusion at the onset of reperfusion after a prolonged period of ischemia reduced myocardial injury to an extent comparable to ischemic preconditioning, provides a novel approach to myocardial protection. Potential mechanisms of the protection include a reduction in neutrophil accumulation and decreased endothelial dysfunction, attenuation of oxidative stress, a reduction in apoptotic cell death, and attenuation of mitochondrial calcium accumulation [26, 28, 31, 42, 43]. However, the mechanism of this newly described form of cardioprotection has not been completely understood.

The JAK-STAT signaling pathway involves two protein families, the JAKs and the STATs. Upon tyrosine receptor phosphorylation, JAKs are activated and, in turn,

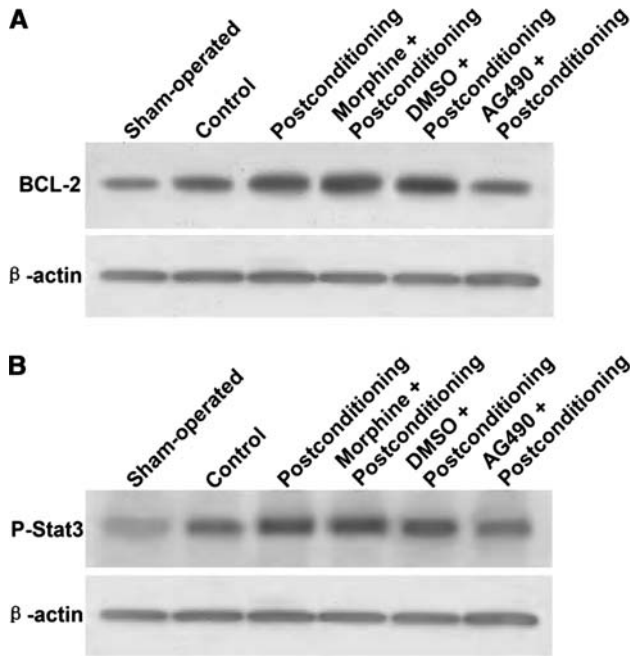

Fig. 2 Analysis of BCL-2 (**a**), and P-Stat3 (**b**). Protein expression by western blotting. The expression of BCL-2 and P-Stat3 protein in the control group were significantly increased than the Sham-operated group. The expression of BCL-2 and P-Stat3 protein in the postconditioning group, morphine + postconditioning group, DMSO + postconditioning group was significantly increased than the control group. However, there was no significant difference between the control group and AG490 + postconditioning group. Meanwhile, there was no significant difference between the postconditioning group and the morphine + postconditioning group or DMSO + postconditioning group

phosphorylate the tyrosine motifs in the cytoplasmic tail of the receptor, which allows for STAT binding [44, 45]. The JAK protein family consists of four members [JAK1, JAK2, JAK3, tyrosine kinase 2 (TYK2)], whereas the STAT family consists of seven members (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, STAT6) [46]. Several studies have demonstrated that STAT3, activated via JAK2, transduces cyto- protective and survival signals in infarcted heart [47]. In vitro, researches have indicated that STAT3 potentiated anti-apoptotic signals through the induction of BCL-2 or BCL-xL genes [48]. STAT3 also showed a transduce protective signal against oxidative stress and doxorubicin-induced cardiomyopathy [49]. Hattori and his colleague [50] have demonstrated that early phase of IPC potentiated JAK-STAT signaling by activating STAT3 which transmitted a survival signal to the myocardium. In our current study, the myocardial infarction size and cardiac myocyte apoptosis index in the AG490 + postconditioning group were significantly increased than the postconditioning group, indicating that the antagonist AG490 for JAK abolished the myocardial ischemic protective effect, and also suggesting that JAK-STAT pathway may mediate the cardioprotection in ischemic postconditioning. We also found that the P-Stat3 protein expression in postconditioning group was significantly higher than the other groups, which suggesting that ischemic postconditioning is possible by inducing STAT3 phosphorylation and inhibiting the apoptosis of myocardial cells to play an important role in the process of myocardial protection. Meanwhile, our current results was in concert with the prior work on STAT3 in postconditioning [51, 52]. We thought that the different experimental conditions and other factors might led to the difference. Till now, the protective role of JAK-STAT signaling pathway is in the cardioprotection in ischemic postconditioning has been reported [51, 53, 54], this study is also confirmed that the cardioprotection in ischemic postconditioning is mediated by the JAK-STAT pathway.

Opioid receptors have been implicated in protecting several organ systems from hypoxic or ischemic events [55–58]. In this regard, Professor Schultz's and his colleague [59] have demonstrated that naloxone, a nonselective opioid receptor antagonist, abolished the protective effect of ischemic PC using an in vivo rat model of myocardial infarction. Recently, Chien and his colleague [60] have also shown that opioid receptors are involved in PC in rabbit heart. In addition, Zattas et al. [54] confirmed that the integral involvement of endogenous opioids in the induction of postconditioning. In particular, the intrinsic activation of MORs and possibly DORs by endogenous opioid agonists may be involved in infarct size reduction by postconditioning. Postconditioning might facilitate this opioid effect in part by protecting the enzymes responsible for the synthesis and processing of proenkephalin. However, at present, the mechanism by which opioid receptors produce their cardioprotective actions in ischemic postconditioning is not clear. In our present study, the myocardial infarction size and cardiac myocyte apoptosis index in the morphine + postconditioning group were significantly decreased than the postconditioning group, indicating that opioid receptor agonist morphine in post-processing to active the ischemic myocardial protective effect, and also suggesting that opioid receptors might mediate the cardioprotection in ischemic postconditioning.

Recently, compelling evidence has accumulated indicating that apoptotic cell death might also play a critical role in a variety of cardiovascular diseases, including myocardial infarction, heart failure, and atherosclerosis [61]. Because apoptosis affects the basic function of a cell, namely cell viability, several mechanisms that regulate its initiation have evolved. These include specific mechanisms to apoptosis regulation (such as, BCL-2 family proteins) and signal transduction pathways involved in other cellular functions (such as, SAPKs) [61]. Postconditioning sets in motion triggers and signals that are functionally related to

reduced cell death [62]. In the current study, we also observed that the ischemia-reperfusion could induce the expression of BCL-2 protein. In addition, ischemic postconditioning might further increase the level of BCL-2 protein, which inhibiting the myocardial cell apoptosis when compared with the ischemia-reperfusion group. At the same time, JAK antagonist AG490 in ischemic postconditioning could abolish the ischemic myocardial protective effect, while the opioid receptor agonist morphine in ischemic postconditioning could active the ischemic myocardial protective effect.

In conclusion, the results suggested that ischemic postconditioning might increase BCL-2 protein expression by activating the opioid receptors and JAK-STAT signaling pathway, to reduce ischemia-reperfusion-induced cardiomyocyte apoptosis and to play a key role in myocardial protection. However, it should be noted that our study have several limitations. However, further research still needs to be done to unravel the underlying mechanisms.

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