Morphine Postconditioning Protects against Reperfusion Injury via Inhibiting JNK/p38 MAPK and Mitochondrial Permeability Transition Pores Signaling Pathways

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Key Words
Morphine • Postconditioning • Reperfusion Injury • JNK • p38 MAPKs • Mitochondrial permeability transition pores

Abstract
Background: The purpose of this study was to determine whether c-jun NH\textsubscript{2}-amino-terminal kinases (JNK) and p38 mitogen-activated protein kinases (MAPK) were involved in morphine postconditioning (MpostC).

Methods: The isolated rat hearts were randomly assigned into one of the following groups. Hearts in the time control (TC) group were constantly perfused for 105 min. Hearts in the ischemia-reperfusion (I/R) group were subjected to 45 min of ischemia followed by 1 h of reperfusion. MpostC was induced by 10 min of morphine administration at the onset of reperfusion. Anisomycin (an activator of JNK/p38 kinases) was administered with or without morphine during the first 10 min of reperfusion following the 45 min of ischemia. Mitochondria and cytosolic proteins were prepared to detect mitochondrial permeability transition (MPT) and cytochrome C (Cyt-c) respectively.

Results: MpostC markedly reduced infarct size (IS/AAR), CK-MB release, and improved cardiac function recovery. However, these protective effects were partly abolished in the presence of anisomycin. I/R significantly increased the phosphorylation of JNK and p38 kinases, mitochondrial permeability transition (MPT) opening and Cyt-c release, while these effects were partly abolished by MpostC. The inhibitory effects of MpostC on the phosphorylation of JNK/p38 kinases, MPT opening and Cyt-c release were totally reversed by Anisocycin, which, used individually, did not show any influence on perfusion injury.

Conclusions: These findings suggest that MpostC protects isolated rat hearts against reperfusion injury via inhibiting JNK/p38 MAPKs and mitochondrial permeability transition pores signaling pathways.
Introduction

Postconditioning (postC) is a novel strategy of attaining cardioprotection, and it was first described by Zhao and colleagues [1], in which brief intermittent repetitive ischemia at the onset of reperfusion following a prolonged period of ischemia reduced myocardial injury to an extent comparable to ischemic preconditioning (preC), and the latter is a classic strategy of cardioprotection. Because most ischemic events are unpredictable in clinical practice, ischemic postC is of great clinical interest in that it is performed during the onset of reperfusion period. However, it might be difficult to routinely apply intermittent episodes of myocardial ischemia-reperfusion at the early moment of reperfusion in clinical practice. Pharmacological postC, which only require a drug given as adjunctive treatment to be present and active at the time of early reperfusion, could be simply applied and is particularly promising [2].

Our previous study demonstrated that administration of morphine immediately at the onset of reperfusion, which was named MpostC, reduced the infarct size to an extent similar to morphine preC via activating κ-opioid receptor in isolated rat hearts [3]. Subsequent study (unpublished) implied protein kinase C isozyme epsilon (PKCε) - extracellular signal-regulated kinase1-2 (ERK1/2) pathway, one of reperfusion injury survival kinases (RISK) pathways, is involved in MpostC. However, whether cardioprotection conferred by MpostC is mediated by inhibiting death kinases pathways has yet to be ascribed.

One family of signaling proteins commonly linked to the modulation of ischemia-reperfusion injury is mitogen-activated protein kinases (MAPK), which consists of three main subtypes: c-junNH2 amino-terminal kinases (JNK), p38 MAPK and ERK1/2. Although the findings are not consistent, it is generally accepted that JNK and p38 MAPK are death kinases while ERK1/2 are classified as survival kinases [4]. Evidence demonstrated that ischemic and acid-induced postC confer cardioprotection via inhibiting JNK and p38 MAPK phosphorylation [4, 5], while Lemoine and colleagues showed that p38 MAPK phosphorylation is involved in desflurane postC-induced cardioprotection [6] and p38 MAPK per se activation can induce postC [7]. In addition, the potential downstream targets of JNK/p38 MAPK remain to be further defined. Although it is well known that mPTP plays a paramount role in reperfusion injury [8], few studies have yet to demonstrate the direct interaction between death kinases and mPTP in postC at present.

Therefore, the objective of the present study was to determine (1) whether MpostC protects against reperfusion injury via inhibiting JNK and/or p38 MAPK signaling pathways, and (2) whether mitochondrial permeability transition pores (mPTP) is the potential downstream target of the activated JNK and/or p38 MAPK.

Materials and Methods

All experimental procedures and protocols used in this investigation were reviewed and approved by the Institutional Animal Care and Use Committee of the Affiliated Hospital of Qingdao University and were performed in accordance with the Guide for the Care and Use of Laboratory Animals from the Institute of Laboratory Animals Resources. All reagents, unless specified, were obtained from Sigma Chemicals (St. Louis, MO).

Isolated Heart Preparation

These methods were similar to those used in previous experiments [3]. Briefly, male Sprague-Dawley rats weighing 180–200 g were anesthetized with an intraperitoneal injection of 40 mg/kg sodium pentobarbital and decapitated. The hearts were removed rapidly and mounted on a non-circulating Langendorff apparatus and underwent retrograde perfusion at 100 cm H_2O with Krebs-Ringer’s solution (115 mM NaCl, 5 mM KCl, 1.2 mM MgSO_4, 1.2 mM KHPO_4, 1.25 mM CaCl_2, 25 mM NaHCO_3, and 11 mM glucose) gassed with 95% O_2-5% CO_2 (pH 7.4, temperature 37°C). An incision was made in the left atrium, and a fluid-filled latex balloon was passed through the mitral orifice and placed in the left ventricle. The
balloon was connected to a pressure transducer for continuous monitoring of left ventricular developed pressure (LVDP), left ventricular end-diastolic pressure (LVEDP) and heart rate (HR) via PowerLab Systems (PowerLab/8sp, AD Instruments, Australia). In the first 15 min of perfusion, the heart was allowed to stabilize, and any heart showing intractable arrhythmia or low left ventricular systolic pressure (LVSP) < 50 mmHg was excluded from the study.

**Experimental Protocols**

Following a 15-min stabilization period, all hearts were randomly divided into 1 of 5 groups (n = 8, respectively) (Fig. 1). 1) TC group (time control): the hearts were constantly perfused with K-H buffer for 105 min; 2) I/R group (ischemic control): the hearts were subjected to 45 min of ischemia followed by 1 h of reperfusion; 3) MpostC group: the hearts firstly undertook MpostC induced by morphine (3.0 μmol/L) administered for 10 min at the onset of reperfusion, and then were followed by 50 min reperfusion; 4) MpostC + Aniso group: anisomycin (an activator of JNK/p38 kinases, 1.0 μmol/L) was administered with morphine for the first 10 min reperfusion period following 45 min ischemia, then the hearts were followed by 50 min reperfusion; 5) Aniso group: anisomycin (1.0 μmol/L) was administered alone for the first 10-min reperfusion period following 45 min ischemia, then the hearts were followed by 50 min reperfusion. Additional hearts were used 20 min after reperfusion of the above 5 groups (n = 5, respectively) to detect the expression of JNK and p38 kinases, and also mitochondrial permeability transition (MPT) and the level of cytochrome C (Cyt-c) in cytosol. The concentrations used in this study were based on previous studies [3, 4].

**Determination of myocardial infarct size and myocardial injury**

Myocardial infarct size and myocardial injury were measured in accordance to previous research [3]. In short, infarct size was determined by 2, 3, 5-triphenyltetrazolium chloride (TTC) staining method, and infarct size was determined by dividing the total necrotic area of the left ventricle by the total left ventricular slice area (IS/AAR). Additionally, the CK-MB release was measured by collecting total coronary effluent over the 60 min reperfusion period, and expressed as U/h/g.

**Detection of the JNK and p38 expression**

The expression of JNK and p38 was detected as described by Zhang et al. [9] with Western blotting analysis. In brief, left ventricular myocardium was homogenized in ice-cold lysis buffer. The proteins were loaded on SDS-polyacrylamide gel and transferred to a nitrocellulose membrane and incubated with antibodies against phosphorylated JNK (p-JNK), JNK, phosphorylated p38 MAPK (p-p38 MAPK), p38 MAPK and β-actin (all were mouse polyclonal antibodies diluted 1:200, obtained from Santa Cruz Biotechnology.)
USA) for 6 hours followed by a peroxidase-conjugated secondary antibody. The signals were detected by an enhanced chemo-luminescent detection (ECL) system (Amersham Pharmacia Biotech), and the density of each band was measured by NIH image analysis software. The phosphorylation levels of JNK and p38 MAPK were all expressed as a percentage of respectively total levels, and then were translated into a percentage of TC.

Detection of mPTP opening and Cyt-c release in cytosol

The preparation of mitochondria and cytosolic fractions was obtained by the method described by Li et al [10] with some modifications. Briefly, left ventricular myocardium was minced and homogenized in ice-cold isolation medium. Large cell debris was pelleted by centrifuging the homogenate twice for 5 min at 600 g. The obtained supernatant was centrifuged at 10 000 g for 10 min at 4 °C. The pellet was resuspended in isolation buffer and washed twice by recentrifugation at 10 000 g for 10 min at 4 °C. The final pellet was resuspended in isolation buffer as a mitochondrial preparation. The supernatant was centrifuged for 1 h at 100,000 g to obtain cytosolic fraction. The protein concentrations were measured by BCA protein assay kit (Pierce, Rockford, IL) and using bovine serum albumin standards.

Isolated mitochondria (1mg protein) were resuspended in swelling buffer (71 mmol/L sucrose, 215 mmol/L mannitol and 10 mmol/L succinate in 3 mmol/L HEPES, pH 7.4) to a final volume of 2 ml, incubated at 25°C for 2 min. Mitochondrial permeability transition (MPT) due to opening of mPTP, which was induced by 2, 20 and 200 μmol/L CaCl₂ resulted in mitochondrial swelling and was measured spectrophotometrically (DU800; Beckman Coulter, USA) as a reduction in the optical density at 540nm (OD540) during 5 min (ΔOD/min) [11].

After SDS-PAGE, cytosolic proteins were transferred to a nitrocellulose membrane and incubated with anti-Cytochrome c and β-actin antibodies (mouse polyclonal antibodies diluted 1:200, Santa Cruz Biotechnology, USA) for 6 hours followed by a peroxidase-conjugated secondary antibody. The signal was detected and measured as ascribed above. The results were presented as a percentage of TC.

Statistical analysis

Statistical analysis of infarct size and p-JNK, p-p38 MAPK and Cyt-c measurement was performed by Student’s t test with Bonferroni’s correction for multiple comparisons. Hemodynamics was analyzed using two-way repeated measures analysis of variance for time and treatment effects. If an overall significance was found, comparisons between groups were done for each time point using one-way analysis of variance, followed by Tukey post hoc testing. Time effects within each group were analyzed using repeated-measures analysis of variance, followed by Dunnett post hoc testing, with the baseline value as the reference time point. Statistical differences were considered significant when P < 0.05. Data are expressed as mean ± SD.

Results

A total of 65 Sprague-Dawley rats were included in the study and all the hearts were successfully obtained and perfused.

Hemodynamic parameters

The baseline hemodynamics were similar in all experimental groups (P = 0.25), and all variables remained constant in the TC group during the experiment (Table 1). Compared to baseline, the cardiac function in the other four groups deteriorated, showing an obvious reduction in CF, HR, and LVDP, and a significant increase in LVEDP at 10, 30, 60 min of reperfusion (P < 0.05). All hemodynamic variables were better in the MpostC group in the I/R group (P < 0.05). However, the functional improvements elicited by MpostC were partly reversed by coadministration of anisomycin, which did not influence cardiac recovery when used alone (P = 0.08, P = 0.12, P = 0.10 and P = 0.10, respectively) (Table 1).

Infarct size and CK-MB release

Compared to the I/R group, MpostC markedly reduced IS/AAR (P < 0.05) (Fig. 2A) and CK-MB (P < 0.05) (Fig. 2B). However, the protective effects offered by morphine were partly reversed by coadministration with anisomycin (P < 0.05), which, when given alone, showed no influence on reperfusion injury (P = 0.10, P = 0.08, respectively) (Fig. 2).
Expression of p-JNK and p-p38MAPK
Compared to the TC hearts, the phosphorylation of JNK and p38MAPK was significantly increased in I/R group. MpostC markedly reduced the levels of JNK and p38MAPK phosphorylation as compared to I/R group (*P < 0.05) (Fig. 3A and B). In addition, anisomycin coadministered with morphine at the start of reperfusion, reversed the inhibitory effects observed with MpostC, while it had no influence on the phosphorylation of JNK and p38MAPK.

Table 1. Effects of various treatments on hemodynamic parameters. Baseline, 15 min after stabilization; CF, coronary flow; Aniso, anisomycin; I/R, ischemia reperfusion; HR, heart rate; LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; MpostC, morphine postconditioning. Data were presented as mean ± SD (n = 8/group). *P<0.05 versus baseline (intragroup comparison), †P<0.05 versus respective value in TC group (intergroup comparison), ‡P<0.05 versus respective value in I/R group (intergroup comparison).

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>10min</th>
<th>30min</th>
<th>60min</th>
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<tbody>
<tr>
<td>CF, ml/min</td>
<td></td>
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<tr>
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<td>Aniso</td>
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<tr>
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Fig. 2. Effects of various treatments on infarct size (IS/AAR) (A) and CK-MB release (B). The upper panel shows representative images of TTC stained hearts and infarct size was expressed as a percentage of the area at risk (IS/AAR). TC group was not included because no obvious necrotic area was measured. TC, time control; I/R, ischemia reperfusion; MpostC, morphine postconditioning (3.0 μmol/L); aniso, anisomycin (1.0 μmol/L). Values were presented as mean ± SD (n = 8/group). *P < 0.05 versus TC, †P < 0.05 versus I/R, ‡P < 0.05 versus MpostC.
when used alone \((P < 0.05)\) (Fig. 3A and B). The total levels of JNK and p38MAPK had no difference between the groups \((P = 0.20)\).

**Ca\(^{2+}\) induced mPTP opening**

\(\Delta OD/min\) in the I/R group was higher than that of the TC group \((P < 0.05)\) (Fig. 4). Hearts in the MpostC group have a lower \(\Delta OD/min\) than that of the I/R group, and anisomycin abolished the inhibitory effect observed with MpostC \((P < 0.05)\). In addition, anisomycin had no influence on the \(\Delta OD/min\) when used alone \((P = 0.11)\).

**Cytosolic levels of Cyt-c release**

Compared to the TC group, the expression of Cyt-c in cytosol was markedly increased in the I/R group \((P < 0.05)\) (Fig. 5). Relative to R/I group, MpostC significantly reduced the expression of Cyt-c in the cytosol \((P < 0.05)\). In addition, Anisomycin reversed the inhibitory effect observed with MpostC \((P < 0.05)\), while it had no influence on the expression of Cyt-c in cytosol when used alone \((P = 0.15)\).
In the present study, we investigated the role of JNK and p38MAPK in morphine-induced postC in the rat heart in vitro. The new findings were summarized as follows: (1) MpostC markedly protected against myocardial reperfusion injury by reducing IS/AAR and CK-MB release, improving functional recovery in the isolated rat hearts. (2) Because anisomycin, an stimulator of JNK and p38MAPK, partly abolished the protective effects of MpostC on reperfusion injury, it indicated that JNK and/or p38MAPK were involved in MpostC; (3) Subsequent Western blot analysis showed that MpostC significantly inhibited JNK and p38MAPK phosphorylation, Cyt-c release in cytosol and CaCl$_2$-induced MPT (ΔOD/min), and these effects induced by MpostC were completely reversed by anisomycin. Taken together, the present study shows MpostC protects against myocardial reperfusion injury, potentially mediated by inhibiting JNK/p38 MAPK signaling pathways, further more, mitochondrial permeability transition pores (mPTP) might be the downstream target.

The activation of JNK and p38 MAPK is associated with phosphorylation [12], and the role of JNK and p38 MAPK activation in cardioprotection remains controversial at present [13]. In addition, most of the studies were conducted under preC strategy [14-17], and the role of JNK and p38 MAPK in postC needs to be well defined. Using MpostC strategy, the present study showed that both JNK and p38 MAPK were activated at 20 min during reperfusion, and MpostC markedly inhibited the activation of JNK and p38 MAPK followed by a reduction in IS/AAR. Moreover, the inhibitory effects observed with MpostC were
abolished by Anisomycin, a stimulator of JNK/ p38 MAPK. The present findings provided a new insight into MpostC- induced cardioprotection with the inhibitory effect on death kinases. These results were consistent with previous reports suggesting that inhibition of JNK/ p38 MAPK phosphorylation accounts for the cardioprotective effects of postC induced by hypoxemia or lactic acid [4, 5].

It is well known that mPTP plays a paramount role in reperfusion injury [8]. The opening of mPTP results in swelling of the mitochondrial matrix and rupture of the outer mitochondrial membrane, leading to the translocation of Cyt-c and other pro-apoptotic factors into the cytosol, and these actions rapidly produce cell death and apoptosis [18-22]. Because it appears to specifically open at the onset of reperfusion [17-21], mPTP has been strongly implicated as an end-effector in postC [23-27]. Previous studies demonstrated that MpostC modulates mitochondrial permeability transition pore (mPTP) opening via δ-opioid receptors [28] in isolated rat heart, but the post-receptor signaling mechanisms through which mPTP is regulated remain to be defined. The present findings indicated that MpostC protects against reperfusion injury by inhibiting mPTP opening, and JNK/p38MAPK might be the upstream regulators. Förster and colleagues showed that myocytes pretreated with delta-opioid receptor agonist [d-Ala (2)-D-Leu (5)]-enkephalin (DADLE) in vitro have a enhanced endurance to peroxide-induced collapse of mitochondrial membrane potential by inhibiting glycogen synthase kinase 3β [29], while the present study provide an alternative explanation for the post-receptor signaling pathways involved in regulation of mPTP.

So far, the role of activated death kinases such as JNK and p38MAPK in regulation of mitochondrial function in myocardium in postC remains to be defined. Previous studies showed ischemic postC inhibits the phosphorylation of p38 MAPK, through which it can attenuate Cyt-c release from mitochondria in rat hearts [30]. In addition, studies also demonstrated that I/R induces JNK translocation to the mitochondria by binding to the mitochondrial membrane protein Sab (SH3BP5), and blocking JNK mitochondrial translocation or JNK inhibition prevents mitochondrial dysfunction and cardiomyocyte death [31, 32]. The present study also showed that JNK/ p38MAPK activation facilitates mPTP opening during reperfusion, and MpostC inhibits JNK/ p38MAPK activation and results in a decrease in mPTP opening, Cyt-c release and IS/AAR. But, Sehwan and Sabzali demonstrated that inhibition of JNK aggravates cardiac function during reperfusion, and the detrimental effects of JNK inhibition are associated with reciprocal p38MAPK activation and mitochondrial dysfunction [33]. So, there might exist a cross-talk between JNK and p38MAPK pathways. The discrepancy might be associated with the different aspects of type of stimulate, experimental model, non-specific inhibitor, and interventional time and strategy, and it underlines the complicated role of JNK/p38MAPK in the regulation of mitochondria in heart. Studies are needed to further verify the role of JNK/p38MAPK, especially specific isoforms, in the regulation of mitochondria in myocardium in postC.

The current results must be interpreted within the constraints of several potential limitations. (1) In contrast to an in vivo model, isolated hearts have a limited long-term biologic stability and may undergo short confounding ischemic periods during the surgical procedure, which could potentially affect our observation. Therefore, it is inappropriate to directly extrapolate our results to in vivo conditions. (2) Anisomycin was coadministered with morphine in the study. Considering their different characters of pharmacodynamics, it is possible that it may not have completely responsible for the pharmacological effects on MpostC. In addition, anisomycin was also found to be a stimulator of two anti-apoptotic proteins Akt and Bcl-2 in addition to be an activator of JNK and p38MAPK [34]. So, it must be prudent to explain the present results.

In conclusion, the present study shows MpostC protects isolated rat hearts against ischemia-reperfusion injury via inhibition of JNK/p38 MAPK-mPTP signaling pathways, which provides a new insight into the post-receptor signal transduction mechanisms involved in opioid-induced postC in myocardium.
Acknowledgements

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Disclosure Statement

None.

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