Postconditioning Prevents Reperfusion Injury by Activating δ-Opioid Receptors

Youngho Jang, M.D., Ph.D.,* Jinkun Xi, M.D., M.S.,† Huihua Wang, M.D.,† Robert A. Mueller, M.D., Ph.D.,‡ Edward A. Norfleet, M.D.,§ Zhelong Xu, M.D., Ph.D.¶

Background: While postconditioning has been proposed to protect the heart by targeting the mitochondrial permeability transition pore (mPTP), the detailed mechanism underlying this action is unknown. The authors hypothesized that postconditioning stimulates opioid receptors, which in turn protect the heart from reperfusion injury by targeting the mPTP.

Methods: Rat hearts (both in vivo and in vitro) were subjected to 30 min of ischemia and 2 h of reperfusion. Postconditioning was elicited by six cycles of 10-s reperfusion and 10-s ischemia. To measure nitric oxide concentration, cardiomyocytes loaded with 4-amino-5-methylamino-2',7'-difluorofluorescin were imaged using confocal microscopy. Mitochondrial membrane potential was determined by loading cardiomyocytes with tetramethylrhodamine ethyl ester.

Results: In open chest rats, postconditioning reduced infarct size, an effect that was reversed by both naloxone and naltrindole. The antinfarct effect of postconditioning was also blocked by the mPTP opener atractyloside. In isolated hearts, postconditioning reduced infarct size. Morphine mimicked postconditioning to reduce infarct size, which was abolished by both naltrindole and atractyloside. N-nitro-l-arginine methyl ester and guanylyl cyclase inhibitor 1H-[1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one blocked the action of morphine. Further experiments showed that morphine produces nitric oxide in cardiomyocytes by activating δ-opioid receptors. Moreover, morphine could prevent hydrogen peroxide–induced collapse of mitochondrial membrane potential in cardiomyocytes, which was reversed by naltrindole, N-nitro-l-arginine methyl ester, and the protein kinase G inhibitor KT5823.

Conclusions: Postconditioning protects the heart by targeting the mPTP through activation of δ-opioid receptors. The nitric oxide–cyclic guanosine monophosphate–protein kinase G pathway may account for the effect of postconditioning on the mPTP opening.

This article is accompanied by an Editorial View. Please see:


* Visiting Scholar, † Postdoctoral Fellow, ‡ Professor and Vice Chair, § Professor and Chair, ¶ Assistant Professor and Director of Cardiovascular Research.

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Address correspondence to Dr. Xu: Department of Anesthesiology, CB# 7010, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599. xzu@ãums.unc.edu. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY’s articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.
we investigated the mechanism by which opioid receptor activation leads to postconditioning’s protection.

**Materials and Methods**

All procedures were approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill and were performed in accordance with the guidelines of the Committee.

*Open Chest Rat Model (In Situ Study)*

Male Wistar rats (250–350 g) were anesthetized with thiobutabarbital sodium (100 mg/kg intraperitoneal). Rats were intubated through a tracheotomy and ventilated with 100% oxygen. Arterial pH, partial pressure of carbon dioxide, and partial pressure of oxygen were monitored and maintained throughout the experiment. Body temperature was maintained at 38°C using a heating pad. The left carotid artery was cannulated to monitor arterial blood pressure, heart rate, and blood gas. To administer chemicals, a catheter filled with saline was placed in the right jugular vein. A thoracotomy was performed at the fifth intercostal space, and a 5-0 silk suture was placed around the left anterior descending coronary artery. The ends of the suture were passed through a small piece of soft vinyl tubing to form a snare. All rats were allowed to stabilize for at least 20 min before ischemia was induced by pulling the snare and then fixing it by clamping the tubing with a small hemostat.

*Isolated Rat Heart Model (In Vitro Study)*

Male Wistar rats (250–350 g) were anesthetized with thiobutabarbital sodium (100 mg/kg intraperitoneal). The hearts were removed rapidly and mounted on a Langendorff apparatus. The hearts were perfused with Krebs-Henseleit buffer containing 118.5 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 1.8 mM CaCl₂, 24.8 mM NaHCO₃, 1.2 mM KH₂PO₄, and 10 mM glucose, which was heated to 37°C and gassed with 95% O₂–5% CO₂. A latex balloon connected to a pressure transducer was inserted into the left ventricle through the left atrium. The left ventricular pressure and heart rate were continuously recorded with a PowerLab system (ADInstruments, Mountain View, CA). A 5-0 silk suture was placed around the left carotid artery. The ends of the suture were passed through a small piece of soft vinyl tubing to form a snare. All hearts were allowed to stabilize for at least 20 min. Ischemia was induced by pulling the snare and then fixing it by clamping the tubing with a small hemostat. Total coronary artery flow was measured by timed collection of the perfusate dripping from the heart into a graduated cylinder.

*Measurement of Infarct Size*

At the end of each experiment, the coronary artery was reoccluded, and fluorescent polymer microspheres (diameter, 2–9 μm) were infused to demarcate the risk zone as the tissue without fluorescence. The hearts were weighed, frozen, and cut into 1-mm slices. The slices were incubated in 1% triphenyltetrazolium chloride in sodium phosphate buffer at 37°C for 20 min. The slices were immersed in 10% formalin to enhance the contrast between stained (viable) and unstained (necrotic) tissue and then squeezed between glass plates spaced exactly 1 mm apart. The myocardium at risk was identified by illuminating the slices with ultraviolet light. The infarcted and risk zone regions were traced on a clear acetate sheet and quantified with ImageTool (San Antonio, TX). The areas were converted into volumes by multiplying the areas by slice thickness. Infarct size is expressed as a percentage of the risk zone.

*Isolation of Adult Rat Cardiomyocytes*

Rat cardiomyocytes were isolated enzymatically. Male Wistar rats weighing 250–350 g were anesthetized with thiobutabarbital sodium (100 mg/kg intraperitoneal). A midline thoracotomy was performed, and the heart was removed and rapidly mounted on a Langendorff apparatus. The heart was perfused in a nonrecirculating mode with Krebs-Henseleit buffer (37°C) containing 118 mM NaCl, 25 mM NaHCO₃, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 1.25 mM CaCl₂, and 10 mM glucose for 5 min to wash out blood. The buffer was bubbled with 95% O₂–5% CO₂. Then, the heart was perfused with a calcium-free buffer that contained all of the above components except CaCl₂. After 5 min of perfusion, collagenase (type II) was added to the buffer (0.1%), and the heart was perfused in a recirculating mode for approximately 15 min. The heart was removed from the apparatus, and the ventricles were placed into a beaker containing the calcium-free buffer. The ventricles were agitated in a shaking bath (37°C) at a rate of 50 cycles/min until individual cells were released. The released cells were suspended in an incubation buffer containing all of the components of the calcium-free buffer, 1% bovine serum albumin, 30 mM HEPES, 60 mM taurine, 20 mM creatine, and amino acid supplements at 37°C. Calcium was gradually added to the buffer containing the cells to a final concentration of 1.2 mM. The cells were filtered through nylon mesh and centrifuged briefly. Finally, the cells were suspended in culture medium M199 for 4 h before experiments.

*Confocal Imaging of DAF-FM*

To measure intracellular nitric oxide concentration, cardiomyocytes cultured in a specific temperature-controlled culture dish were incubated with 2 μM 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) diacetate in standard Tyrode solution containing 140 mM NaCl, 6 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 5 mM HEPES, and 5.8 mM glucose (pH 7.4) for 20 min. The cells were then mounted on the stage of an Olympus FV500 laser
scanning confocal microscope (Olympus America Inc., Center Valley, PA). The cells were randomly selected and imaged. The green fluorescence was excited at 488 nm and imaged through a 525-nm long-path filter. Temperature was maintained at 37°C with Delta T Open Dish Systems (Bioptechs, Butler, PA). The images recorded on a computer were quantified using Image J (National Institutes of Health, Bethesda, MD).

**Confocal Imaging of $\Delta \Psi_m$**

Mitochondrial membrane potential ($\Delta \Psi_m$) was measured using confocal microscopy as reported previously. Briefly, cardiomyocytes cultured in a specific temperature-controlled culture dish were incubated with tetramethylrhodamine ethyl ester (100 nM) in standard Tyrode solution containing 140 mM NaCl, 6 mM KCl, 1 mM MgCl$_2$, 1 mM CaCl$_2$, 5 mM HEPES, and 5.8 mM glucose (pH 7.4) for 20 min. Cells were then mounted on the stage of an Olympus FV 500 laser scanning confocal microscope. The red fluorescence was excited with the 543-nm line of an argon–krypton laser and imaged through a 560-nm long-path filter. Temperature was maintained at 37°C with Delta T Open Dish Systems. The images recorded on a computer were quantified using Image J.

**Experimental Protocol**

All the hearts were subjected to 30 min ischemia followed by 2 h of reperfusion. Postconditioning was induced by six cycles of 10-s reperfusion and 10-s occlusion started immediately after release of the index ischemia. In open chest rats (fig. 1A), antagonists and inhibitors were given 5 min before the onset of reperfusion (bolus injection). In isolated rat hearts (fig. 1B), chemicals (agonists and inhibitors) were given starting 5 min before reperfusion and continued for 15 min. In the experiments measuring nitric oxide generation (fig. 1C), morphine was applied immediately after baseline measurements, whereas naltrindole was given 5 min before the application of morphine. In the $\Delta \Psi_m$ measurement study (fig. 1D), cardiomyocytes were exposed to 100 µM hydrogen peroxide (H$_2$O$_2$) for 20 min, and morphine was given 5 min before exposure to H$_2$O$_2$ for 25 min. In
another series of experiments, cardiomyocytes were exposed to the simulated ischemia solution (glucose-free Tyrode solution containing 10 mM 2-deoxy-D-glucose and 10 mM sodium dithionite\(^{14}\)) for 10 min, followed by 20 min of reperfusion with normal Tyrode solution.

**Statistical Analysis**

Data are expressed as mean ± SEM and were obtained from 6–11 separate experiments. Statistical significance was determined using the Student t test or one-way analysis of variance followed by Tukey test (SigmaStat; Systat Software, Inc., San Jose, CA). A value of \( P < 0.05 \) was considered as statistically significant.

**Results**

**Open Chest Rat Study**

Baseline heart rate and mean arterial pressure were not different among the groups (table 1). As shown in figure 2, infarct sizes in the control hearts were 29.7 ± 2.9% of risk zone (\( n = 8 \)). It should be mentioned that infarct sizes measured in this study was smaller than those reported by others.\(^9,15\) Although the exact reason for this discrepancy is unknown, differences in the risk and infarct determination may account for it. Postconditioning significantly reduced infarct size to 14.0 ± 3.2% of risk zone (\( n = 12 \)), an effect that was abrogated by both the nonselective opioid receptor antagonist naloxone (3 mg/kg; 25.5 ± 3.9% of risk zone, \( n = 7 \)) and the selective \( \delta \)-opioid receptor antagonist naltrindole (5 mg/kg; 26.9 ± 2.8% of risk zone, \( n = 6 \)), indicating that activation of \( \delta \)-opioid receptor accounts for the antiinfarct effect of postconditioning. The infarct-sparing effect of postconditioning was abolished by the mPTP opener atracyloside (5 mg/kg; 30.8 ± 4.4% of risk zone, \( n = 6 \)).

**Isolated Heart Study**

Baseline heart rate, developed pressure, and coronary flow were not different among the experimental groups. In control hearts, infarct size was 29.9 ± 3.7% of risk zone (\( n = 8 \)). Infarct size was significantly reduced by postconditioning to 11.9 ± 2.5% of risk zone (\( n = 7 \)).

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**Table 1. Hemodynamic Data for the In Vivo Studies**

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Ischemia 30 min</th>
<th>Reperfusion 2 h</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>HR, beats/min</td>
<td>MAP, mmHg</td>
<td>HR, beats/min</td>
</tr>
<tr>
<td>Control</td>
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<td>330 ± 8</td>
<td>107 ± 3</td>
</tr>
<tr>
<td>Postcon</td>
<td>10</td>
<td>338 ± 6</td>
<td>105 ± 3</td>
</tr>
<tr>
<td>Postcon + NTD</td>
<td>6</td>
<td>352 ± 5</td>
<td>110 ± 2</td>
</tr>
<tr>
<td>Postcon + NAL</td>
<td>7</td>
<td>340 ± 9</td>
<td>101 ± 2</td>
</tr>
<tr>
<td>Postcon + KTS823</td>
<td>6</td>
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<td>102 ± 2</td>
</tr>
<tr>
<td>Postcon + Atr</td>
<td>6</td>
<td>344 ± 6</td>
<td>107 ± 3</td>
</tr>
</tbody>
</table>

Atr = atracyloside; HR = heart rate; MAP = mean arterial pressure; NAL = naloxone; NTD = naltrindole; Postcon = postconditioning.

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Fig. 2. Infarct size in *in situ* rat hearts. Rats were subjected to 30 min of regional ischemia followed by 2 h of reperfusion. Six postconditioning (Postcon) cycles reduced infarct size. Naltrindole (NTD, 5 mg/kg), naloxone (NAL, 3 mg/kg), KTS823 (0.1 mg/kg), and atracyloside (Atr, 5 mg/kg) blocked the antiinfarct effect of postconditioning. * \( P < 0.05 \) versus control. # \( P < 0.05 \) versus postconditioning.

Fig. 3. Infarct size in *in vitro* rat hearts. Rats were subjected to 30 min of regional ischemia followed by 2 h of reperfusion. Postconditioning (Postcon), morphine (Mor, 1 \( \mu \)g), and BW373U86 (BW, 1 \( \mu \)g) reduced infarct size. The infarct-sparing effect of morphine was reversed by atracyloside (Atr, 60 \( \mu \)g). * \( P < 0.05 \) versus control. # \( P < 0.05 \) versus morphine. NTD = naltrindole.
confirm the involvement of opioid receptor activation in postconditioning, we examined whether direct activation of opioid receptors with morphine at reperfusion could mimic the antiinfarct effect of postconditioning. As shown in figure 3, morphine (1 μM) given at reperfusion markedly reduced infarct size (9.8 ± 2.9% of risk zone, n = 7). Similar to the observation from our in vivo study, the δ-opioid receptor antagonist naltrindole (100 μM) aborted the antiinfarct effect of morphine at reperfusion (27.8 ± 5.1% of risk zone, n = 6), whereas BW373U86, an agonist of δ-opioid receptor, mimicked the effect of morphine by reducing infarct size (8.8 ± 2.0% of risk zone, n = 6). Figure 4 shows that the infarct-sparing effect of morphine was blocked by both the nitric oxide synthase inhibitor N-nitro-l-arginine methyl ester (21.3 ± 4.9% of risk zone, n = 6) and the guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo [4,3-a] quinoxalin-1-one (ODQ, 10 μM). *P < 0.05 versus control. #P < 0.05 versus morphine.

Study with Cardiomyocytes
To confirm the role of nitric oxide in the opioid-mediated postconditioning, we determined whether opioid receptor activation with morphine could produce nitric oxide by loading cardiomyocytes with the nitric oxide-specific fluorescence dye DAF-FM diacetate. DAF-FM has been demonstrated to be useful for detection of nitric oxide in living cells. As shown in figure 5, morphine (1 μM) significantly enhanced DAF-FM fluorescence intensity (151.4 ± 18.9% of baseline, n = 12) compared with the control (102.0 ± 2.4% of baseline, n = 11), an effect that was reversed by the δ-opioid receptor antagonist naltrindole (110.2 ± 9.6% of baseline, n = 7), indicating that morphine can produce nitric oxide by stimulating δ-opioid receptors in cardiomyocytes. Cells exposed to 100 μM H2O2 for 20 min revealed a marked decrease in tetramethylrhodamine ethyl ester fluorescence intensity (45.1 ± 8.2% of baseline, n = 8; fig. 6A), implying that oxidant stress causes loss of ΔΨm. In contrast, cells treated with morphine showed much smaller decrease in tetramethylrhodamine ethyl ester fluorescence intensity (90.0 ± 2.0% of baseline, n = 8), suggesting that activation of opioid receptors can modulate the mPTP opening. This effect of morphine was reversed by naltrindole (58.2 ± 3.5% of baseline, n = 7). Moreover, N-nitro-l-arginine methyl ester (55.0 ± 6.8% of baseline, n = 8) and the PKG inhibitor KT5823 (51.1 ± 4.8% of baseline, n = 6) aborted the preventive effect of morphine on ΔΨm. Further experiments revealed that morphine was also able to prevent simulated ischemia-reperfusion-induced loss of ΔΨm in cardiomyocytes (56.8 ± 6.3% of baseline in control and 82.1 ± 5.3% of baseline in morphine; fig. 6B).

Discussion
In this study, we have demonstrated that activation of δ-opioid receptors plays an important role in postcondi-
tioning. Postconditioning may protect the heart at reperfusion by modulating the mPTP opening via a signaling cascade involving δ-opioid receptors, nitric oxide, and PKG.

Since its first introduction by Zhao et al., postconditioning has been demonstrated in various experimental models. Although the detailed mechanism by which postconditioning protects the heart remains unknown, studies have proposed that nitric oxide, protein kinase C, phosphatidylinositol 3-kinase-Akt, extracellular signal-regulated kinase, and mitochondrial adenosine triphosphate-sensitive potassium channels may mediate postconditioning’s protection. As to endogenous ligands that initiate postconditioning, recent studies have shown that adenosine is involved in the targeting mechanism by activating either A<sub>2A</sub> or A<sub>2B</sub> receptors. A recent study has also documented that intermittent bradykinin B<sub>2</sub> receptor activation triggers postconditioning in isolated rat hearts. Endogenous opioids are well known to trigger ischemic preconditioning by activating opioid receptors. Recently, Gross et al. demonstrated that exogenous opioid agonists (morphine and BW373U86) given just before the onset of reperfusion reduced infarct size in rat hearts, suggesting that opioid receptor (especially δ-opioid receptors) activation may elicit postconditioning. In this study, we found that in open chest rat hearts, the antinfarct effect of postconditioning was blocked by the nonselective opioid receptor antagonist naloxone and the selective δ-opioid receptor antagonist naltrindole, indicating that endogenous opioids trigger postconditioning by activating δ-opioid receptors. The importance of δ-opioid receptors in postconditioning was further supported by our in vitro study in which the infarct-sparing effect of morphine given at reperfusion was reversed by naltrindole, whereas the δ-opioid receptor agonist BW373U86 mimicked postconditioning by reducing infarct size.

Prevention of the mPTP opening has been proposed to be cardioprotective at reperfusion. While postconditioning has also been reported to protect the heart by

![Fig. 6. (A) Tetramethylrhodamine ethyl ester (TMRE) fluorescence intensity 20 min after exposure to hydrogen peroxide expressed as percentage of baseline. Morphine (1 μM) prevented oxidant-induced dissipation of mitochondrial membrane potential. Naltrindole (NTD, 5 μM), N-nitro-l-arginine methyl ester (l-NAME, 20 μM), and KT5823 (0.2 μM) blocked the effect of morphine. (B) TMRE fluorescence intensity 20 min after the onset of reperfusion as percentage of baseline. *P < 0.05 versus control. # P < 0.05 versus morphine.](image-url)
targeting the mPTP, the signaling mechanism by which postconditioning leads to inhibition of the mPTP opening is unknown. In this study, we determined whether postconditioning modulates the mPTP opening by activating opioid receptors. Our data have shown that the mPTP opener atractyloside aborted the antiinfarct effects of both postconditioning and morphine, suggesting that opioid receptor activation-triggered postconditioning may protect the heart by targeting the mPTP. In support of this intervention, oxidant-induced mPTP opening in isolated cardiomyocytes was prevented by morphine, and this prevention was abolished by naltrindole, strongly suggesting that δ-opioid receptor activation can result in modulation of the mPTP opening. Recent studies have documented that inhibition of the mPTP may involved in the mechanisms underlying κ-opioid receptor-mediated cardioprotection by preconditioning. Therefore, it would be interesting to determine the role of κ-opioid receptors in postconditioning in the future. Furthermore, it should also be mentioned that activation of other G protein–coupled receptors such as adenosine and bradykinin receptors may also contribute to inhibition of the mPTP opening by postconditioning, because our previous studies demonstrated that adenosine and bradykinin receptor activation can result in modulation of the mPTP opening. Therefore, further studies are needed to identify whether these receptors are also involved in the action of postconditioning on the mPTP opening.

Having demonstrated that postconditioning protects the heart by targeting the mPTP through activation of δ-opioid receptors, we were next interested in seeking the signaling pathway that provides a linkage between δ-opioid receptor activation and inhibition of the mPTP opening in the protection of postconditioning. Because exogenous nitric oxide has been demonstrated to protect the heart by modulating the mPTP opening and nitric oxide is proposed to mediate the cardioprotective effect of postconditioning, we speculated that nitric oxide may serve as a critical signal that mediates inhibition of the mPTP opening by postconditioning. Our data have shown that the antiinfarct effect of morphine is blocked by the nitric oxide synthase inhibitor N-nitro-L-arginine methyl ester and that morphine produces nitric oxide by activating δ-opioid receptors in cardiomyocytes. Further, the preventive effect of morphine on the mPTP opening also blocked by N-nitro-L-arginine methyl ester. These data suggest that postconditioning inhibits the mPTP opening by producing nitric oxide in cardiomyocytes through activation of δ-opioid receptors. Nitric oxide at low concentrations stimulates the synthesis of the second messenger cyclic guanosine monophosphate, which in turn regulates various cellular functions by activating downstream targets including PKG. Our previous studies have demonstrated that the cyclic guanosine monophosphate–PKG signaling pathway is responsible for the cardioprotective effect of nitric oxide.

In summary, we have demonstrated that activation of δ-opioid receptors plays a role in triggering the cardioprotective effect of postconditioning. Postconditioning protects the heat by modulating the mPTP opening via a signaling cascade involving δ-opioid receptor, nitric oxide, and PKG (fig. 7).

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