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Postconditioning Prevents Reperfusion Injury by Activating δ-Opioid Receptors

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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′-difluorofluorescein 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 antiinfarct 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.**

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BRIEF cycles of ischemia and reperfusion during the early phase of reperfusion protect the heart from infarction, a phenomenon termed *postconditioning*.[1] Studies have documented that several molecular components and pathways including nitric oxide,[2] phosphatidylinositol 3-kinase,[3,4] extracellular signal–regulated kinase,[2] protein kinase C,[5] and mitochondrial adenosine triphosphate–sensitive potassium channels[3,6] are involved in the cardioprotective effects of postconditioning. Because all of these signaling components are also implicated in ischemic preconditioning, postconditioning and preconditioning may share common mechanisms.[6]

Activation of G protein–coupled receptors such as adenosine and opioid receptors has been demonstrated to initiate preconditioning. Because postconditioning shares the protective pathways with preconditioning, G protein–coupled receptor activation may serve as an essential mechanism that triggers the protection of postconditioning. Indeed, recent reports have addressed that adenosine A_{2a}[7] and A_{2b}[6] receptors are responsible for the protection of postconditioning. It has also been reported that intermittent stimulation of bradykinin B_2 receptors triggers postconditioning through redox signaling.[8] Therefore, it is intriguing to determine whether other G protein–coupled receptor activations are also involved in postconditioning. Recently, Gross *et al.* demonstrated that opioids can reduce infarct size when administered just before reperfusion, an effect that was similar to that observed when given before ischemia.[9] Furthermore, Weihrauch *et al.*[10] reported that morphine enhanced isoflurane-induced postconditioning rabbit hearts. Therefore, it is possible that postconditioning protects the heart by activating opioid receptors at reperfusion.

If opioid receptor activation plays a role in the mechanism of postconditioning, it is important to probe the downstream signaling pathway leading to the protection. Because the mitochondria permeability transition pore (mPTP) opening has been proposed to play an essential role in reperfusion injury[11] and inhibition of mPTP opening has been reported to be an important mechanism underlying postconditioning's protection,[12] we hypothesized that the signaling pathway initiated by opioid receptor activation leads to the cardioprotective effect of postconditioning by targeting mitochondria.

In the current study, we first tested whether opioid receptor activation is responsible for the protection of postconditioning in *in vivo* and *in vitro* rat hearts. Then,

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 preformed 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 $MgSO_4$, 1.8 mM $CaCl_2$, 24.8 mM $NaHCO_3$, 1.2 mM KH_2PO_4, and 10 mM glucose, which was heated to 37°C and gassed with 95% O_2–5% CO_2. 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 coronary 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.[13] 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 $NaHCO_3$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.25 mM $CaCl_2$, and 10 mM glucose for 5 min to wash out blood. The buffer was bubbled with 95% O_2–5% CO_2. Then, the heart was perfused with a calcium-free buffer that contained all of the above components except $CaCl_2$. 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 $MgCl_2$, 1 mM $CaCl_2$, 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

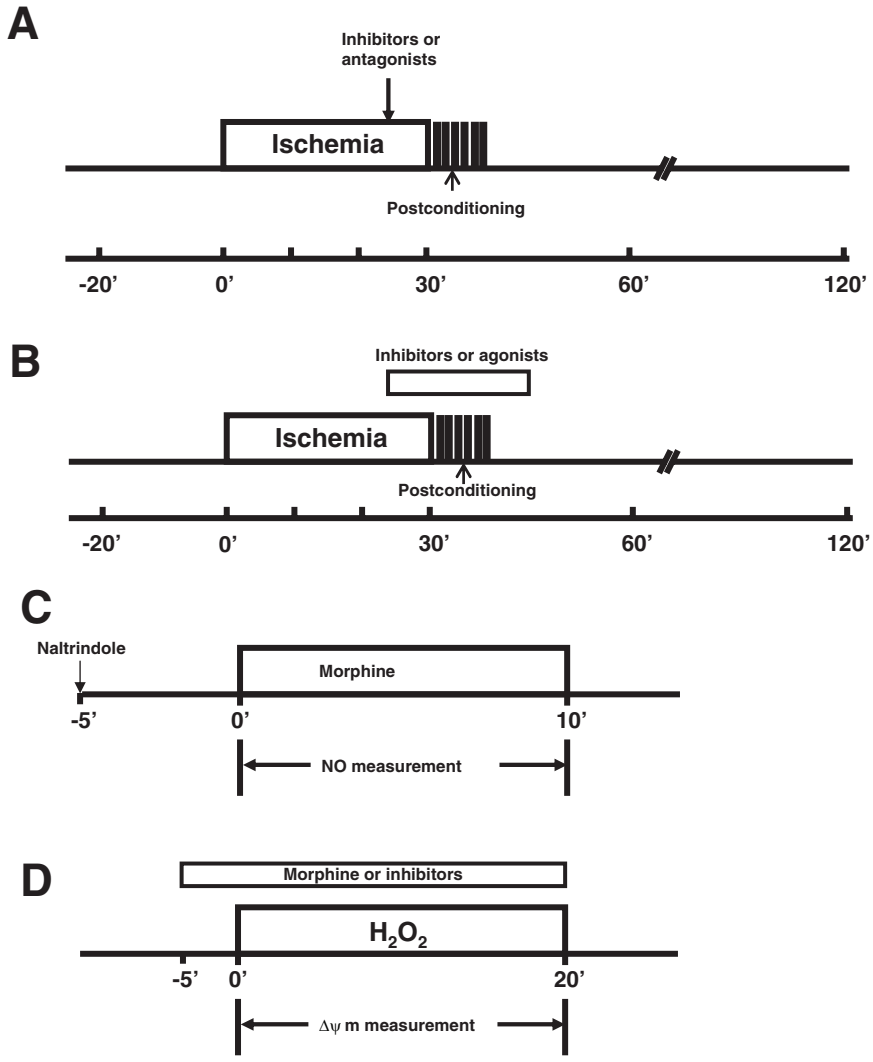

Fig. 1. Experimental protocols. (*A*) *In situ* study. (*B*) *In vitro* study. (*C*) Measurement of nitric oxide in cardiomyocytes. (*D*) Measurement of mitochondrial membrane potential ($\Delta\Psi_m$) in cardiomyocytes. H_2O_2 = hydrogen peroxide; NO = nitric oxide.

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.[13] 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_2O_2) for 20 min, and morphine was given 5 min before exposure to H_2O_2 for 25 min. In

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

		Baseline		Ischemia 30 min		Reperfusion 2 h	
	n	HR, beats/min	MAP, mmHg	HR, beats/min	MAP, mmHg	HR, beats/min	MAP, mmHg
Control	8	330 ± 8	107 ± 3	341 ± 9	90 ± 6	306 ± 10	62 ± 7
Postcon	10	338 ± 6	105 ± 3	325 ± 6	89 ± 7	310 ± 7	67 ± 6
Postcon + NTD	6	352 ± 5	110 ± 2	347 ± 8	93 ± 6	309 ± 8	59 ± 3
Postcon + NAL	7	340 ± 9	101 ± 2	352 ± 3	88 ± 5	293 ± 12	60 ± 6
Postcon + KT5823	6	343 ± 8	102 ± 2	349 ± 8	95 ± 3	298 ± 11	54 ± 8
Postcon + Atr	6	344 ± 6	107 ± 3	348 ± 7	95 ± 6	307 ± 12	51 ± 5

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

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 30.7 ± 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 δ-opioid receptor antagonist naltrindole (5 mg/kg; 26.9 ± 2.8% of risk zone, n = 6), indicating that activation of δ-opioid receptor accounts for the antiinfarct effect of postconditioning. The infarct-sparing effect of postconditioning was abolished by the mPTP opener atractyloside (5 mg/kg; 30.8 ± 4.4% of risk zone, n = 6). The antiinfarct effect of postconditioning was also blocked by the protein kinase G (PKG) inhibitor KT5823 (1 mg/kg; 32.7 ± 3.5% 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). To

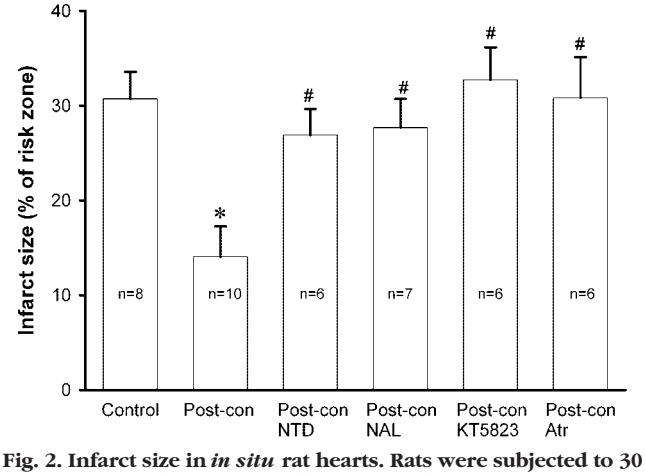

Fig. 2. Infarct size in *in situ* rat hearts. Rats were subjected to 30 min of regional ischemia followed by 2 h of reperfusion. Six postconditioning (Postcon) cycles reduced infarct size. Naltrindole (NTD, 5 mg/kg), naloxone (NAL, 3 mg/kg), KT5823 (0.1 mg/kg), and atractyloside (Atr, 5 mg/kg) blocked the antiinfarct effect of postconditioning. * $P < 0.05$ *versus* control. # $P < 0.05$ *versus* postconditioning.

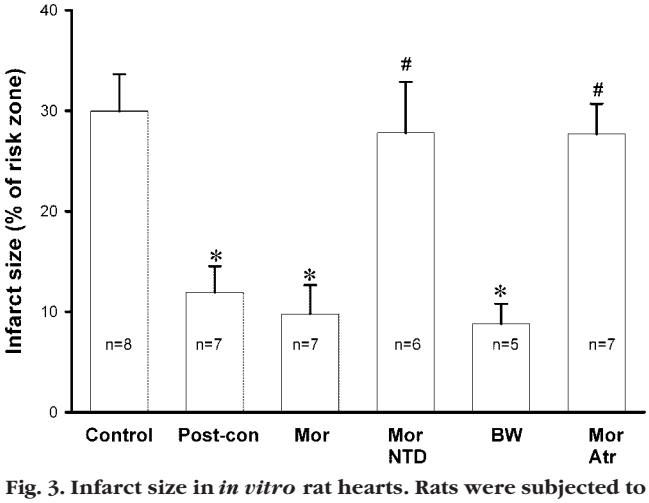

Fig. 3. Infarct size in *in vitro* rat hearts. Rats were subjected to 30 min of regional ischemia followed by 2 h of reperfusion. Postconditioning (Postcon), morphine (Mor, 1 μM), and BW373U86 (BW, 1 μM) reduced infarct size. The infarct-sparing effect of morphine was reversed by atractyloside (Atr, 60 μM). * $P < 0.05$ *versus* control. # $P < 0.05$ *versus* morphine. NTD = naltrindole.

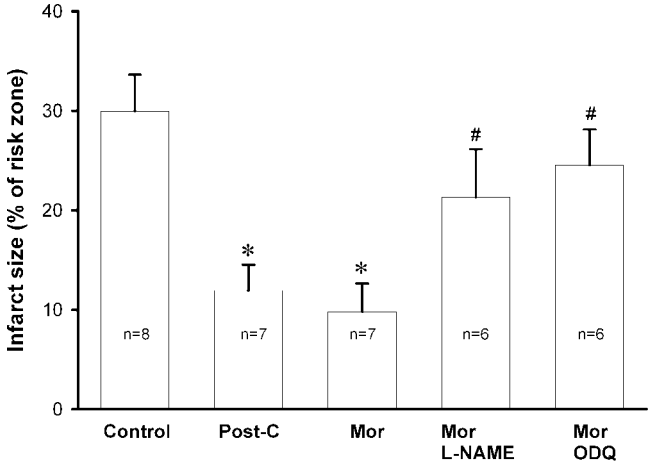

Fig. 4. Infarct size in *in vitro* rat hearts. Rats were subjected to 30 min of regional ischemia followed by 2 h of reperfusion. Postconditioning (Post-C) and morphine (Mor, 1 μM) reduced infarct size. The infarct-sparing effect of morphine was reversed by both *N*-nitro-L-arginine methyl ester (L-NAME, 20 μM) and 1H-[1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one (ODQ, 10 μM). * *P* < 0.05 *versus* control. # *P* < 0.05 *versus* morphine.

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 (24.5 ± 3.6% of risk zone, n = 6).

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 H_2O_2 for 20 min revealed a marked decrease in tetramethylrhodamine ethyl ester fluorescence (45.1 ± 8.2% of baseline, n = 8; fig. 6A), implying that oxidant stress causes loss of $\Delta\Psi_m$. In contrast, cells treated with morphine showed a 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 $\Delta\Psi_m$. Further experiments revealed that morphine was also able to prevent simulated ischemia-reperfusion–induced loss of $\Delta\Psi_m$ 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-

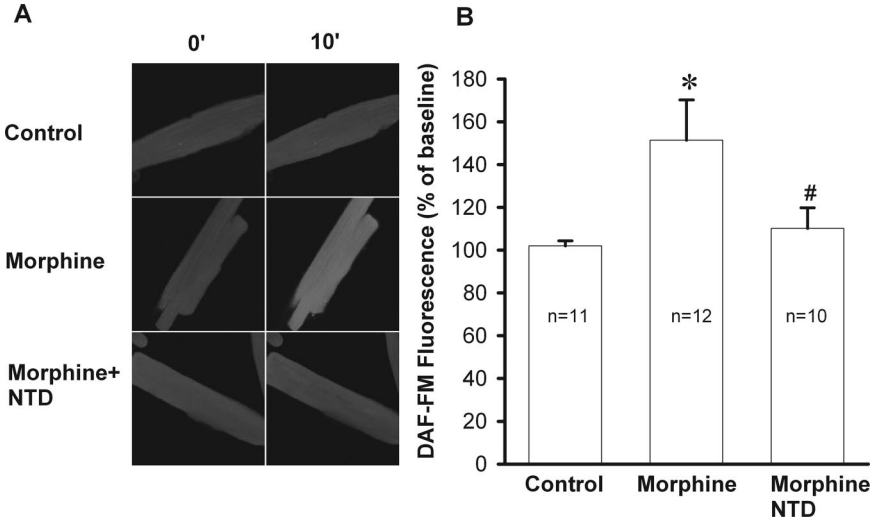

Fig. 5. (*A*) Confocal fluorescence images of 4-Amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) at the baseline and 10 min after exposure to morphine in rat cardiomyocytes. Morphine (1 μM) increased nitric oxide production in cardiomyocytes, which was blocked by naltrindole (NTD, 5 μM). (*B*) Summarized data for DAF-FM fluorescence intensity 10 min after exposure to morphine expressed as a percentage of the baseline. * *P* < 0.05 *versus* control. # *P* < 0.05 *versus* morphine.

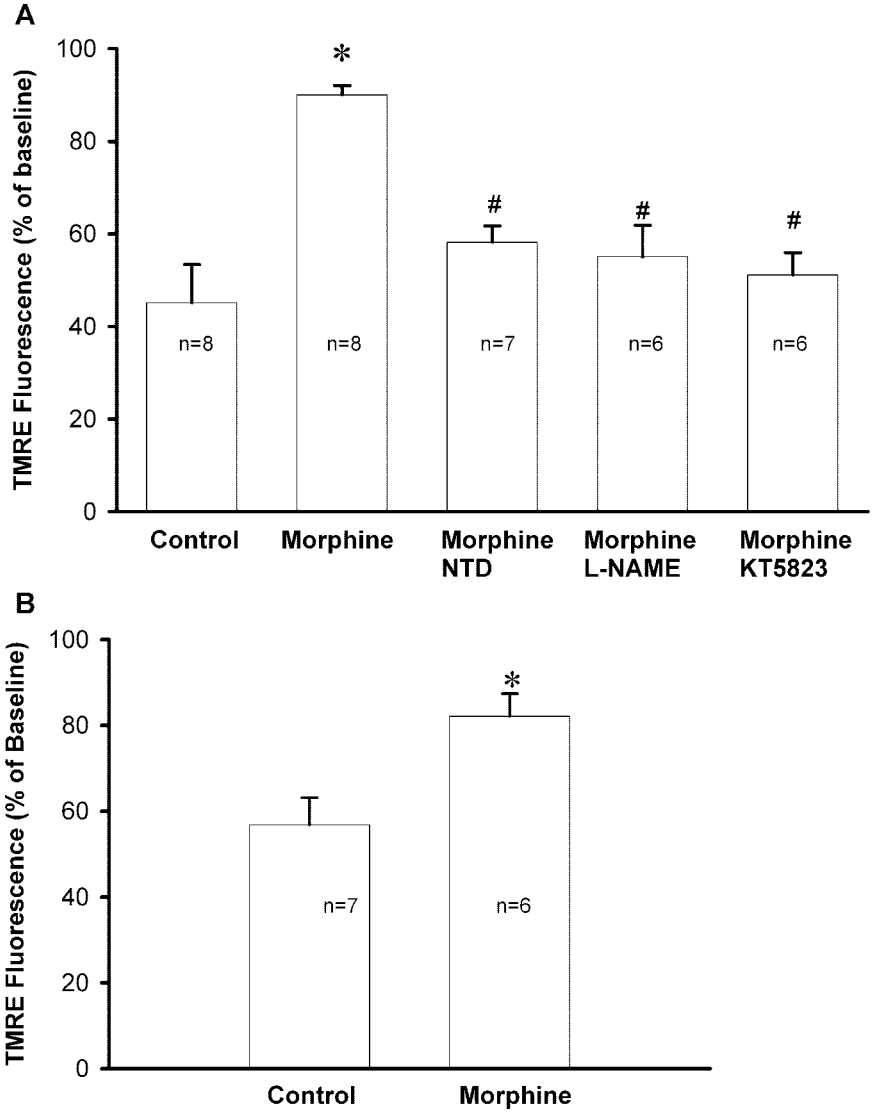

Fig. 6. (*A*) Tetramethylrhodamine ethyl ester (TMRE) fluorescence intensity 20 min after exposure to hydrogen peroxide expressed as percentage of baseline. Morphine (1 μM) prevented oxidant-induced dissipation of mitochondrial membrane potential. Naltrindole (NTD, 5 μM), *N*-nitro-L-arginine methyl 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.

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.*,[1] postconditioning has been demonstrated in various experimental models.[1–4,16,17] Although the detailed mechanism by which postconditioning protects the heart remains unknown, studies have proposed that nitric oxide,[3] protein kinase C,[5] phosphatidylinositol 3-kinase–Akt,[2,4] extracellular signal–regulated kinase,[2] and mitochondrial adenosine triphosphate–sensitive potassium channels[2] may mediate postconditioning's protection. As to endogenous ligands that initiate postconditioning, recent studies have shown that adenosine is involved in the triggering mechanism by activating either A_{2A} [7] or A_{2B} [18] receptors. A recent study has also documented that intermittent bradykinin B_2 receptor activation triggers postconditioning in isolated rat hearts.[8] Endogenous opioids are well known to trigger ischemic preconditioning by activating opioid receptors. Recently, Gross *et al.* [9] 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 antiinfarct 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.[19,20] While postconditioning has also been reported to protect the heart by

targeting the mPTP,[12] 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.[21,22] 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[13] and bradykinin[23] prevent 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[24,25] and nitric oxide is proposed to mediate the cardioprotective effect of postconditioning,[2] 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 was 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[26] and that adenosine prevents the mPTP opening by activating PKG.[13] In addition, Yang *et al.* [3] have recently shown that cyclic guanosine monophosphate plays a role in postconditioning's protection. Therefore, we tested whether this pathway plays a role in the effect of postconditioning on the mPTP opening. In this study, the antiinfarct effect of postconditioning was reversed by the PKG inhibitor KT5823, and morphine did not inhibit the mPTP opening in cardiomyocytes in the presence of KT5823. Therefore, it is likely that PKG plays a role in the effect of postconditioning on the mPTP opening, presumably as a downstream signal of nitric oxide.

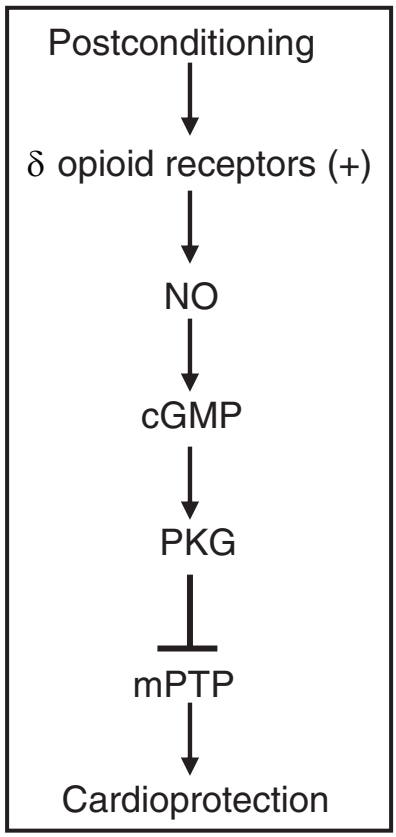

Fig. 7. Signaling pathway leading to the cardioprotection of postconditioning mediated by activation of δ-opioid receptors. cGMP = cyclic guanosine monophosphate; mPTP = mitochondrial permeability transition pore; NO = nitric oxide; PKG = protein kinase G.

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

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