

κ-Opioid Receptors Mediate Cardioprotection by Remote Preconditioning

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Background: **Remote preconditioning is known to be cardioprotective, but the exact mechanism has not been fully elucidated. The objective of the current study was to investigate the role of κ-opioid receptors in cardioprotection by remote preconditioning and reveal possible underlying mechanisms.**

Methods: **Remote preconditioning was induced in anesthetized male Sprague-Dawley rats by three cycles of 5 min of right femoral artery occlusion followed by 5 min of reperfusion. Myocardial ischemia–reperfusion was achieved by ligation of the left anterior descending coronary artery for 30 min and then reperfusion for 120 min. Infarct size was determined by 2,3,5-triphenyltetrazolium chloride staining. Levels of lactate dehydrogenase, dynorphin, and met-enkephalin in plasma were measured. The opening of the mitochondrial permeability transition pore was monitored with fluorescent calcein in isolated ventricular myocytes.**

Results: **Both remote preconditioning and U-50,488H (10 mg/kg intravenous), a κ-opioid receptor agonist, significantly decreased the infarct size and plasma lactate dehydrogenase level induced by ischemia–reperfusion, and these effects were attenuated by nor-binaltorphimine (10 mg/kg intravenous), a κ-opioid receptor antagonist, and atractyloside (5 mg/kg intravenous), a mitochondrial permeability transition pore activator. However, administration of naltrindole (5 mg/kg), a δ-opioid receptor antagonist, had no effect on the cardioprotection by remote preconditioning. The dynorphin plasma level was increased after remote preconditioning treatment, but the met-enkephalin level did not change. In isolated ventricular myocytes loaded with calcein, U-50,488H (300 μM) decreased the mitochondrial permeability transition pore opening induced by calcium (200 μM), and this effect was attenuated by cotreatment with nor-binaltorphimine (5 μM) or atractyloside (20 μM).**

Conclusion: **Activation of cardiac κ-opioid receptors is involved in the cardioprotection induced by remote preconditioning, and the mitochondrial permeability transition pore may participate in the postreceptor pathway.**

IN 1993, Przyklenk *et al.*[1] observed that brief occlusion of the circumflex coronary artery extended its cardioprotection from myocardium perfused by that artery to myocardium perfused by the left anterior descending artery; this was called "remote preconditioning" (RPC). Subsequently, it was found that RPC by brief ischemia of other distant organs, such as intestine,[2] kidney,[3] and limb,[4,5] also provides cardioprotection as effective as the classic preconditioning described by Murry *et al.*[6]

Although classic preconditioning has been used clinically, such as in percutaneous transluminal coronary angioplasty,[7] the invasive procedure and the need for a second operation limits this application. In comparison, RPC *via* a limb is an ideal noninvasive means of inducing cardioprotection and is more easily performed than classic preconditioning or other RPC models, such as with kidney or mesenteric tissues. However, the exact mechanism by which RPC of a limb evokes cardioprotection is still not fully understood.

Recently, opioid receptors, which play an important part in classic preconditioning,[8] were also found to be involved in RPC by mesenteric artery occlusion.[9] Binding studies have identified κ- and δ-opioid receptors in myocardium,[10–12] and both are involved in the cardioprotective effect of ischemic preconditioning.[13] Weinbrenner *et al.* reported that activation of the δ_1-opioid receptor may mediate the cardioprotective effect in RPC initiated by infrarenal artery occlusion.[3] However, whether κ-opioid receptors contribute to the beneficial cardiac effect of RPC remains unknown.

Therefore, in the current study, we focused on the role of the κ-opioid receptor in the cardioprotection induced by RPC of a limb and investigated the underlying mechanism.

Materials and Methods

Surgical Procedures

All procedures in this study were approved by the Ethics Committee for the Use of Experimental Animals in Zhejiang University, Hangzhou, Zhejiang, China. Male Sprague-Dawley rats weighing 230–260 g were anesthetized with chloral hydrate (0.4 g/kg intraperitoneal) supplemented with additional doses (0.016 g/kg intraperitoneal) every 30 min to maintain effective anesthesia. The rats were tracheotomized and ventilated with room air enriched with oxygen (tidal volume, 2 ml/stroke; rate, 70 strokes/min[14]), a condition that maintains arterial pH, partial pressure of carbon dioxide, and oxygen within the normal physiologic range, as confirmed in our preliminary experiments. Body temperature was maintained at 37°C.

The left carotid artery was cannulated to permit measurement of blood pressure and heart rate *via* a pressure transducer connected to a data acquisition system (Med-

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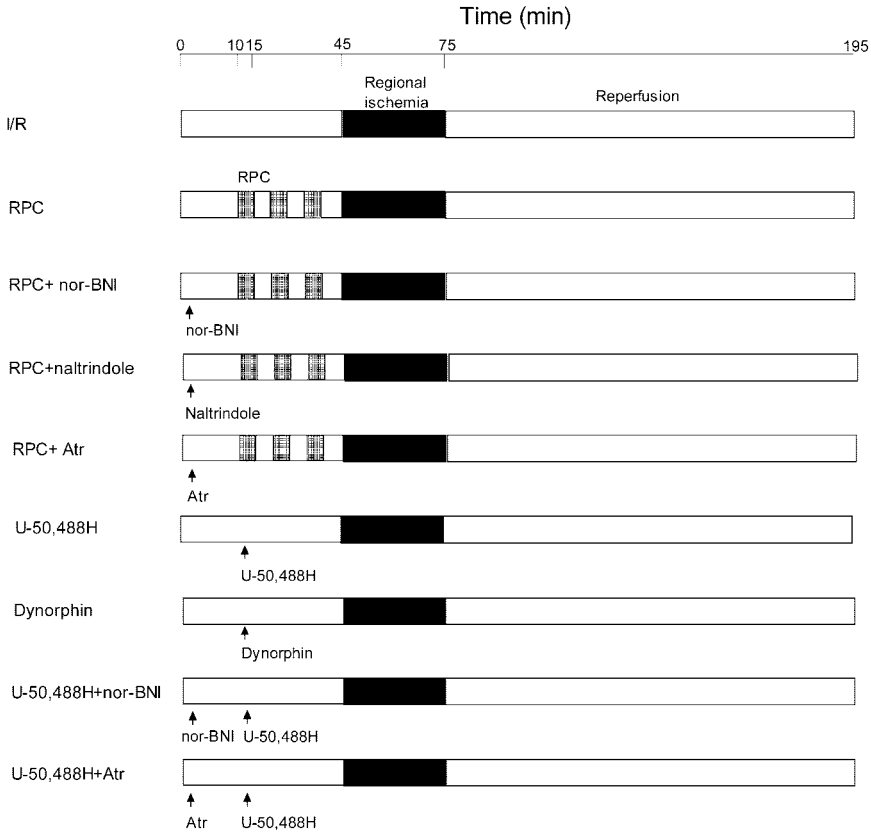

Fig. 1. Experimental protocols used for *in vivo* experiments. Atr = atractyloside; I/R = ischemia–reperfusion; nor-BNI = nor-binaltorphimine; RPC = remote preconditioning.

Lab, Nanjing, China). The left femoral vein was cannulated for administration of drugs and/or compensation for fluid loss.

Through a left thoracotomy in the fourth intercostal space, the pericardium was opened, and a 5-0 suture was passed below the left descending coronary artery 2–3 mm from its origin. The suture ends were passed through a polytetrafluoroethylene tube, and pulling these occluded the coronary artery. The occlusion was confirmed by epicardial cyanosis and subsequent decrease in blood pressure, while reperfusion was verified by epicardial hyperemia.

The femoral artery of the right hind limb was freed from surrounding tissue, a suture was placed below it for later occlusion with an arterial clamp, and reperfusion was initiated by removing the clamp.

Experimental Protocols

All rats received 30 min of regional ischemia by ligation of the left anterior descending artery followed by 120 min of reperfusion. RPC was elicited by three cycles of 5 min of right femoral artery occlusion interspersed with 5 min of reperfusion before 30 min of regional ischemia in the heart. The role of κ-opioid receptors in RPC was determined by activating the receptors by intravenous injection of 10 mg/kg U-50,488H[14] (U-50,488H group), a specific κ-opioid receptor agonist; by intravenous injection of 24 ng/kg dynorphin (dynorphin group), an endogenous κ-opioid receptor agonist; or by inhibiting the receptor by intravenous injection of 10 mg/kg nor-binaltorphimine[15] (RPC + nor-BNI group and U-50,488H + nor-BNI group), a specific κ-opioid receptor antagonist, delivered during and 10 min before the RPC procedure, respectively. The contribution of δ-opioid receptors was determined by intravenous injection of naltrindole, a δ-opioid receptor antagonist, at a dose (5 mg/kg) that blocks δ-opioid receptors in the myocardium.[16] The effects of mitochondrial permeability transition pore (MPTP) opening on RPC were determined by intravenous injection of 5 mg/kg atractyloside,[14] an activator of the MPTP, 10 min before the RPC procedure or U-50,488H administration (RPC + Atr group and U-50,488H + Atr group) (fig. 1).

Infarct Size Measurement

Infarct size was determined by the 2,3,5-triphenyltetrazolium chloride staining method. At the end of the 120 min of reperfusion, the heart was quickly excised and mounted on a Langendorff apparatus to wash out the blood. The coronary artery was then reoccluded, and hearts were perfused with 1% Evans blue to stain the myocardium, while the risk area remained unstained. After that, the hearts were frozen at −20°C for 2–3 h, cut into 2-mm slices, and stained with 1% TTC at 37°C for 10–15 min. Infarct (pale) and risk (red) areas were measured by planimetry using Image/J software from National Institutes of Health (Bethesda, MD). Infarct size was expressed as percentage of risk zone.

Measurement of Plasma Lactate Dehydrogenase Level

Lactate dehydrogenase levels in plasma were measured spectrophotometrically. In a preliminary study, we observed that the lactate dehydrogenase level in plasma reached its maximum at 10 min of reperfusion after myocardial ischemia, so we measured the plasma lactate dehydrogenase level at this time point to provide comparisons of cardioprotection among groups.

Radioimmunoassays for Dynorphin and Met-enkephalin in Plasma

In rats subjected to RPC, we measured plasma dynorphin and met-enkephalin levels with commercially available radioimmunoassay kits (Second Military Medical University, Shanghai, China).

Isolation of Ventricular Myocytes

Myocytes were isolated from adult male Sprague-Dawley rats by enzymatic dissociation as described previously.[17] Briefly, the hearts were perfused with 100% oxygenated, nonrecirculating Ca^{2+}-free Tyrode solution (100.0 mM NaCl, 10.0 mM KCl, 1.2 mM KH_2PO_4, 5.0 mM $MgSO_4$, 20.0 mM glucose, 10.0 mM MOPS; pH 7.2), and then the perfusion solution was switched to 100% oxygenated, recirculated low-Ca^{2+} (50 μM) Tyrode solution containing 0.03% collagenase and 1% bovine serum albumin for 10 min. The ventricles were cut, minced, and gently triturated with a pipette in the low-Ca^{2+} Tyrode solution containing bovine serum albumin at 37°C for 10 min. The cells were filtered through 200-μm nylon mesh and resuspended in Tyrode solution in which the Ca^{2+} concentration was gradually increased to 1.25 mM over 40 min. After isolation, the cardiomyocytes were allowed to stabilize for at least 60 min before experiments.

Imaging of Mitochondrial Permeability Transition Pore Opening

Mitochondrial permeability transition pore opening was detected by calcein fluorescence (excitation at 488 nm and emission at 505 nm). Cells were loaded with 1 μM calcein-AM for 20 min, and the cytosolic calcein was quenched by addition of 5 mM $CoCl_2$ to the solution for 60 min. Then, Co^{2+} was removed by rinsing with fresh Tyrode solution before the cells were imaged in a laser scanning confocal microscope. All drugs were added 30 min before myocyte permeabilization with 0.05% Triton X-100 for 6 min in Tyrode solution.[18] After baseline confocal images had been collected from permeabilized myocytes, Ca^{2+} (200 μM) was added to induce MPTP opening.[19,20] After that, images were collected every minute for a total of 6 min. The fluorescence intensity was integrated, and MPTP opening was indicated by a reduction in the mitochondrial calcein signal.

Chemicals

U-50,488H, nor-binaltorphimine, atractyloside, and cyclosporin A were purchased from Sigma Chemical Company. Calcein-AM was from Molecular Probes Inc. (Carlsbad, CA). Dynorphin was from Calbiochem (Darmstadt, Germany). Radioimmunoassay kits for dynorphin and met-enkephalin were from the Second Military Medical University, Shanghai.

Statistical Analysis

All values are expressed as mean ± SD. Statistical significance was determined by one-way analysis of variance with Newman-Keuls *post hoc* test. Differences of $P < 0.05$ were regarded as significant.

Results

Hemodynamics

Mean arterial blood pressure and heart rate decreased in all groups after 30 min of coronary artery occlusion and recovered to varying extents in the different groups after 120 min of reperfusion. No differences were observed among groups (table 1).

Myocardial Infarct Size and Lactate Dehydrogenase Levels in Plasma

Remote preconditioning of a limb before 30 min of regional heart ischemia significantly decreased the myocardial infarct size (fig. 2) and plasma lactate dehydrogenase level (fig. 3) induced by ischemia and reperfusion, and these effects were attenuated by pretreatment with nor-binaltorphimine (10 mg/kg), a specific κ-opioid receptor antagonist. Pretreatment with the δ-opioid receptor antagonist naltrindole (5 mg/kg) did not change the effect of RPC of a limb. Intravenous administration of U-50,488H (10 mg/kg), a specific κ-opioid receptor agonist, had effects similar to those of RPC, which were abolished by nor-binaltorphimine (10 mg/kg). Administration of atractyloside (5 mg/kg), a specific activator of the MPTP, attenuated the effects of RPC and U-50,488H.

Intravenous administration of dynorphin (24 ng/kg), the endogenous κ-opioid receptor agonist, significantly reduced the myocardial infarct size and plasma lactate dehydrogenase level induced by heart ischemia and reperfusion (figs. 2 and 3).

Plasma Dynorphin and Met-enkephalin Levels after RPC

To investigate whether endogenous opioids are released during RPC, we measured the plasma levels of dynorphin and met-enkephalin by radioimmunoassay 5, 15, 30, 60, and 120 min after RPC. The results showed that RPC significantly increased plasma dynorphin levels during the 120-min period; however, the met-enkephalin levels did not differ from baseline (fig. 4).

Table 1. Hemodynamic Data in Rats

Group	n	Baseline	CAO 30 min	Reperfusion 60 min	Reperfusion 120 min
MAP, mmHg					
I/R	6	110 ± 8	88 ± 2†	96 ± 4†	89 ± 6†
RPC	6	118 ± 11	90 ± 15†	94 ± 9†	95 ± 9†
RPC + nor-BNI	4	114 ± 3	87 ± 19*	93 ± 16*	88 ± 16*
RPC + naltrindole	5	104 ± 8	81 ± 6†	85 ± 11†	93 ± 4*
RPC + Atr	6	110 ± 11	83 ± 10*	87 ± 17*	90 ± 20*
U-50,488H	5	111 ± 12	89 ± 11†	91 ± 11†	98 ± 9*
Dynorphin	5	108 ± 11	85 ± 10†	93 ± 11†	95 ± 8*
U-50,488H + nor-BNI	5	101 ± 10	82 ± 11*	87 ± 7*	88 ± 6*
U-50,488H + Atr	5	104 ± 3	86 ± 19†	88 ± 15*	89 ± 16*
Heart rate, beats/min					
I/R	6	450 ± 46	337 ± 18†	355 ± 32†	351 ± 34†
RPC	6	409 ± 37	352 ± 17*	362 ± 20*	357 ± 51*
RPC + nor-BNI	4	390 ± 23	315 ± 23†	325 ± 31†	340 ± 35†
RPC + naltrindole	5	390 ± 12	346 ± 9†	358 ± 13†	369 ± 17*
RPC + Atr	6	404 ± 46	346 ± 31	359 ± 40	338 ± 42
U-50,488H	5	395 ± 34	327 ± 39†	341 ± 28*	349 ± 27*
Dynorphin	5	410 ± 33	354 ± 14*	365 ± 18*	354 ± 31*
U-50,488H + nor-BNI	5	398 ± 15	328 ± 27†	334 ± 23†	351 ± 13†
U-50,488H + Atr	5	408 ± 26	368 ± 31	376 ± 38	345 ± 32

Mean arterial blood pressure (MAP) and heart rate during the experimental protocols.

* $P < 0.05$, † $P < 0.01$ *vs.* baseline.

Atr = atractyloside; CAO = coronary artery occlusion; I/R = ischemia–reperfusion; nor-BNI = nor-binaltorphimine; RPC = remote preconditioning.

MPTP Opening

To support the findings of the *in vivo* study, in which the κ-opioid receptor and the MPTP were found to be linked, we investigated this linkage in ventricular myocytes isolated from normal heart. Pretreatment of calcein-loaded myocytes with U-50,488H (300 μM) or cyclosporin A (0.1 μM), the specific inhibitor of the MPTP, attenuated the reduction in mitochondrial calcein fluorescence induced by Ca^{2+} (200 μM). The effect of U-50,488H was attenuated by copretreatment with nor-binaltorphimine (5 μM), a concentration known to block κ-opioid receptors,[21] or by copretreatment with atractyloside (20 μM) (fig. 5).

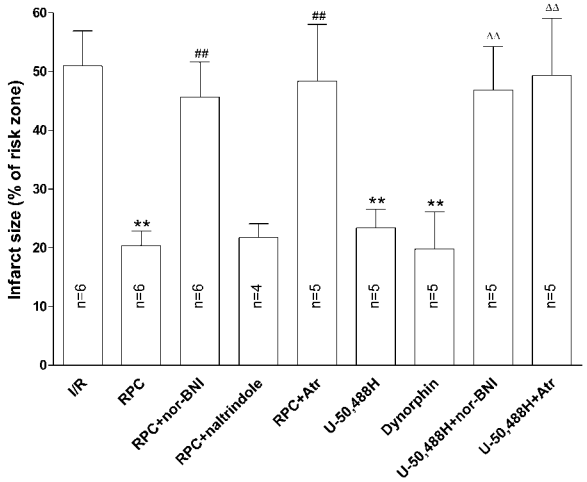

Fig. 2. Infarct size analysis. Remote preconditioning (RPC) and U-50,488H (10 mg/kg intravenous) greatly reduced the infarct size induced by ischemia–reperfusion (I/R), an effect that was abolished by nor-binaltorphimine (nor-BNI, 10 mg/kg intravenous), a specific κ-opioid receptor antagonist, and atractyloside (Atr, 5 mg/kg intravenous), a mitochondrial permeability transition pore activator. Naltrindole (5 mg/kg intravenous), a δ-opioid receptor antagonist, did not attenuate the effect of RPC. Administration of dynorphin (24 ng/kg intravenous), the endogenous κ-opioid receptor agonist, induced effects similar to those of U-50,488H and RPC. Infarct size is expressed as percentage of risk zone. Values are mean ± SD. n values are indicated on the columns. ** $P < 0.01$ *versus* I/R. ## $P < 0.01$ *versus* RPC. ΔΔ $P < 0.01$ *versus* U-50,488H.

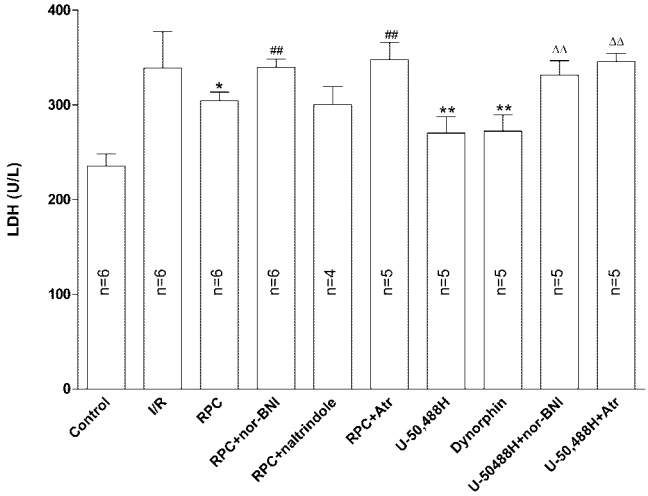

Fig. 3. Effect of remote preconditioning (RPC), nor-binaltorphimine (nor-BNI), U-50,488H, dynorphin, naltrindole, and atractyloside (Atr) on lactate dehydrogenase (LDH) levels in plasma. LDH levels in plasma at 10 min of reperfusion after 30 min myocardial ischemia in rats subjected to different treatments (see details in Material and Methods). n values are indicated on the columns. Values are mean ± SD. * $P < 0.05$, ** $P < 0.01$ *versus* I/R. ## $P < 0.01$ *versus* RPC. ΔΔ $P < 0.01$ *versus* U-50,488H. I/R = ischemia–reperfusion; RPC = remote preconditioning.

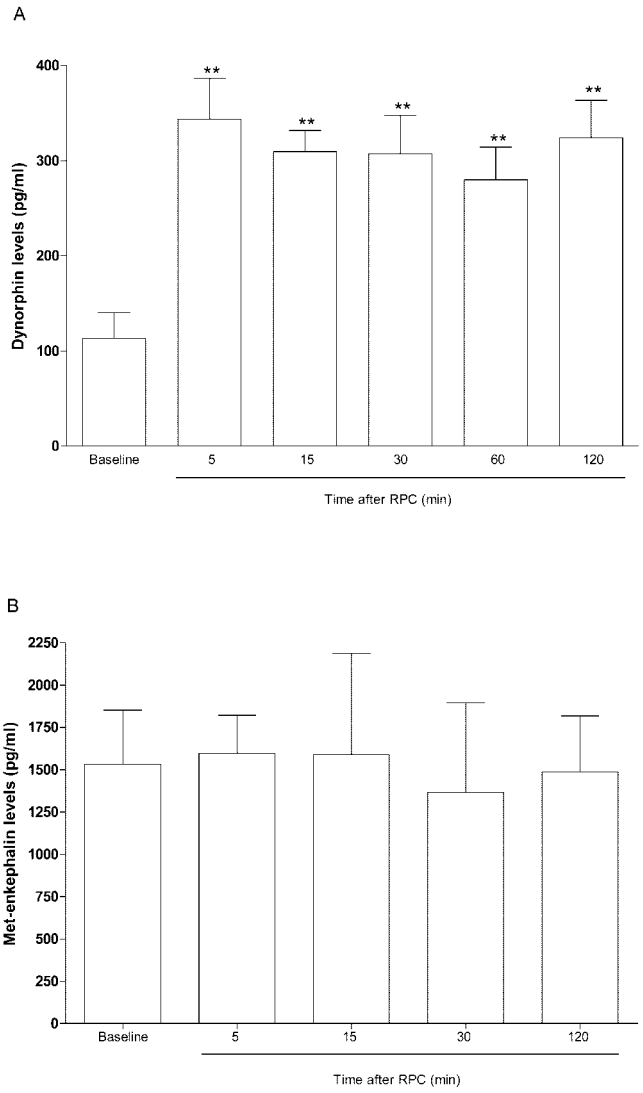

Fig. 4. Effect of remote preconditioning (RPC) on plasma dynorphin (*A*) and met-enkephalin (*B*) levels. The levels of dynorphin and met-enkephalin in plasma were measured by radioimmunoassay. Values are mean ± SD. n = 6 and 5 for dynorphin and met-enkephalin measurements, respectively. ** $P < 0.01$ *versus* baseline.

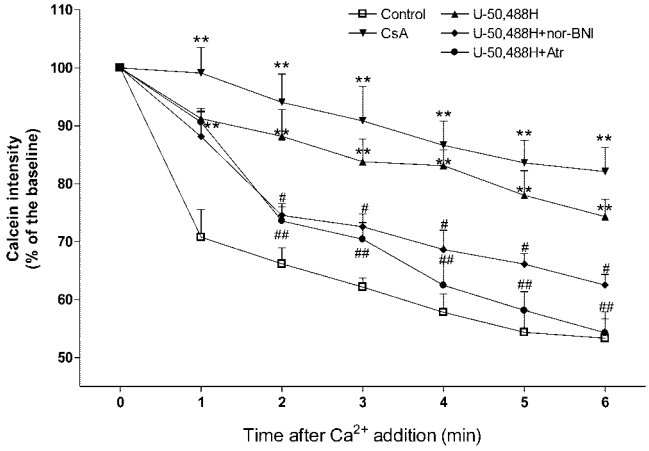

Fig. 5. Effects of U-50,488H, cyclosporin A (CsA), nor-binaltorphimine (nor-BNI), or atractyloside (Atr) on mitochondrial fluorescence changes in permeabilized myocytes loaded with calcein. Drugs were added 30 min before permeabilization of myocytes with Triton X-100. After baseline confocal images were collected from permeabilized myocytes, Ca^{2+} (200 μM) was added to induce opening of the mitochondrial permeability transition pore. After that, the images were collected every minute for 6 min. In the absence of CsA, Ca^{2+} addition caused a marked decrease in mitochondrial calcein fluorescence. In the presence of CsA, the decrease in mitochondrial calcein fluorescence after Ca^{2+} addition was small. U-50,488H had an effect similar to that of CsA, and this effect was attenuated by nor-BNI, an antagonist of the κ-opioid receptor, or atractyloside, an activator of the mitochondrial permeability transition pore. The plot shows the time course of fluorescence changes induced by Ca^{2+} addition. Values are mean ± SD. n = 5/group. ** $P < 0.01$ *versus* control. # $P < 0.05$, ## $P < 0.01$ *versus* U-50,488H.

Discussion

The opioid receptor family is divided into three primary subgroups, μ, κ, and δ, all of which have been cloned.[22] Furthermore, both κ- and δ-opioid receptors are localized in the myocardium of rat[10–12] and are involved in cardioprotection by ischemic preconditioning.[8,23] As for the μ-opioid receptors, most previous studies showed their absence from the adult rat myocardium[12,24]; however, a recent study by Head *et al.*[25] showed the existence of μ-opioid receptors in adult rat myocardium. It is known that the nonspecific opioid receptor antagonist naloxone blocks the cardioprotection conferred by RPC by mesenteric artery occlusion,[9] which suggests the involvement of opioid receptors in this phenomenon. However, which subtype of opioid receptor participates in RPC is still not fully elucidated.

In the current study, administration of κ-opioid receptor agonists, U-50,488H or dynorphin, mimicked the infarct size-limiting effect of RPC induced by femoral occlusion, and the effects of RPC and κ-opioid receptor agonists were attenuated by nor-binaltorphimine, indicating that the κ-opioid receptor is involved in this cardioprotection. The limitation of infarct size by κ-opioid receptor activation in our experiments is consistent with the study by Wang *et al.*[26] These results demonstrate that activation of cardiac κ-opioid receptors by RPC or an exogenous agonist during ischemia and reperfusion is beneficial, although Aitchison *et al.*[27] found the contrary effect.

To further test our hypothesis that RPC may facilitate the release of endogenous opioids, we measured the plasma levels of dynorphin, an endogenous κ-opioid receptor agonist, in rats subjected to RPC, and found that the plasma dynorphin level was significantly increased and remained high during the reperfusion period. To determine the cardiac effect of dynorphin at a similar concentration *in vivo*, dynorphin (24 ng/kg) was intravenously administered, and this mimicked the cardioprotection by RPC. Although the source of increased plasma dynorphin after RPC procedure is not yet known,

the results suggest that the endogenous κ-opioid receptor agonist dynorphin is involved in the mechanism of cardioprotection by remote limb preconditioning.

It has been reported that activation of another member of the family, the δ-opioid receptor, is cardioprotective.[28] In the current study, we measured met-enkephalin plasma levels before and after RPC and found that it did not change. In pharmacologic experiments *in vivo*, we blocked the δ-opioid receptor with its specific antagonist naltrindole (5 mg/kg intravenous) before RPC induced by femoral occlusion and found that this had no effect on cardioprotection. These results suggest that RPC did not induce the release of endogenous δ-opioid receptor agonist met-enkephalin and that δ-opioid receptors may not participate in this form of cardioprotection. This finding is incompatible with the results of Weinbrenner *et al.*,[3] who found that the δ_1-opioid receptor is involved in the cardioprotection by RPC by infrarenal aortic occlusion. However, Weinbrenner *et al.* used a different RPC model from that adopted in our experiments, so different mechanisms of cardioprotection may be induced by RPC in different organs. For example, the calcitonin gene–related peptide is involved in the mechanism of RPC by mesenteric artery occlusion.[29]

The MPTP is a nonspecific pore in the inner membrane of mitochondria. It plays an important role in modulating cell and mitochondrial volume, the mitochondrial membrane potential, and calcium homeostasis.[30–32] When open, it can lead to mitochondrial swelling, cytochrome *c* release from the mitochondria, dissipation of the mitochondrial membrane potential, and ultimately apoptosis and cell death. Because studies showed that the MPTP plays an important role in classic preconditioning,[33,34] we hypothesized that it may participate in the cardioprotection induced by RPC downstream from activation of the cardiac κ-opioid receptor. In the current study, administration of a specific MPTP activator, atractyloside, before initiating RPC, blocked the reduction in infarct size after RPC, and the effect of the κ-opioid receptor agonist U-50,488H was also blocked by atractyloside, indicating that MPTP inhibition may mediate the cardioprotection by κ-opioid receptor activation. To further confirm that activation of the cardiac κ-opioid receptor induces inhibition of the MPTP, we showed that U-50,488H greatly attenuated the decrease of calcein fluorescence in mitochondria in isolated cardiomyocytes, and this effect was blocked by cotreatment with nor-binaltorphimine or atractyloside. However, the mechanism underlying this sarcolemmal κ-opioid receptor modulation of mitochondrial function remains unknown.

In conclusion, under our experimental conditions, activation of the cardiac κ-opioid receptor may be involved in the cardioprotection induced by RPC of a limb, and the MPTP may lie in the postreceptor pathway.

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