Ischemic preconditioning and morphine attenuate myocardial apoptosis and infarction after ischemia-reperfusion in rabbits: role of δ-opioid receptor

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Okubo, Shinji, Yujirou Tanabe, Kenji Takeda, Michihiko Kitayama, Seiyu Kanemitsu, Rakesh C. Kukreja, and Noboru Takekoshi. Ischemic preconditioning and morphine attenuate myocardial apoptosis and infarction after ischemia-reperfusion in rabbits: role of δ-opioid receptor. Am J Physiol Heart Circ Physiol 287: H1786–H1791, 2004; 10.1152/ajpheart.01143.2003.—We examined whether ischemic preconditioning (IPC) attenuates ischemia-reperfusion injury, in part, by decreasing apoptosis and whether the δ-opioid receptor (DOR) plays a pivotal role in the regulation of apoptosis. Rabbits were subjected to 30-min coronary artery occlusion (CAO) and 180 min of reperfusion. IPC was elicited with four cycles of 5-min ischemia and 10-min reperfusion before CAO. Morphine (0.3 mg/kg iv) was given 15 min before CAO. Naloxone (Nal; 10 mg/kg iv) and naltrindole (Nti; 10 mg/kg iv), the respective nonselective and selective DOR antagonists were given 10 min before either morphine or IPC. Infarct size (% risk area) was reduced from 46 ± 3.8 in control to 11.6 ± 1.0 in IPC and 19.5 ± 3.8 in the morphine group (means ± SE; P < 0.001 vs. control). Nal blocked the protective effects of IPC and morphine, as shown by the increase in infarct size to 38.6 ± 7.2 and 44.5 ± 1.8, respectively. Similarly, Nti blocked IPC and morphine-induced protection. The percentage of apoptotic cells (revealed by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay) decreased in IPC (3.6 ± 1.9) and morphine groups (5.2 ± 1.2) compared with control group (12.4 ± 1.6; P < 0.001). Nti pretreatment increased apoptotic cells 11.2 ± 2.2% in IPC and 12.1 ± 0.8% in morphine groups. Nal failed to block inhibition of apoptosis in the IPC group (% of cells: 5.7 ± 1.3 vs. 3.6 ± 1.9 in IPC alone; P > 0.05). These results were also confirmed by nucleosomal DNA laddering pattern. We conclude that IPC reduces lethal injury, in part, by decreasing apoptosis after ischemia-reperfusion and activation of the DOR may play a crucial role in IPC or morphine-induced myocardial protection.

reperfusion injury

IT IS WELL KNOWN THAT APOPTOSIS plays an important role in the myocyte cell death after heart failure as well as ischemia-reperfusion (I/R). In vivo studies have demonstrated cardiomyocyte apoptosis during postnatal maturation (19), spontaneous hypertension in rats (14), rapid ventricular pacing (22), and microembolization-induced cardiac failure (35). Apoptosis has been documented in ischemic rat myocardium beginning 2 h after ischemia (18). In addition, extensive cardiomyocyte apoptosis was also observed in reperfused rabbit (12) and rat (3) hearts as well as in myocardial autopsy tissue death from acute myocardial infarction (17). Gottlieb et al. (12) and Fliss and Gattinger (5) showed that cell death during ischemia is mainly necrotic, whereas damage induced by reperfusion caused additional cell death principally through apoptosis.

Previous studies (2, 24, 30) have shown that ischemic preconditioning (IPC) markedly reduces necrosis in the heart. IPC also decreases internucleosomal DNA fragmentation in rat hearts in vivo, and there was a direct correlation between myocardial infarct size and DNA fragmentation after IPC (29). IPC has been shown to attenuate processing and activation of caspase-1 and caspase-3, and cleavage of poly(ADP-ribose) polymerase after prolonged ischemia and reperfusion in the heart (28). IPC is primarily mediated by activation of several triggers, including free radicals, adenosine, catecholamines, and bradykinin (reviewed in Ref. 26). In addition, studies from Gross’s laboratory (13) have shown that opioids play an important role in IPC against myocardial infarction. The nonselective and selective opioid receptor antagonists, naloxone (Nal) and naltrindole (Nti), respectively, have been shown to completely block the infarct-limiting effect of IPC in rats (31), rabbits (21), and pigs (34). Similarly, the nonpeptide opioid agonist morphine mimicked IPC in the rat heart (32), which was abrogated by Nal, suggesting the role of opioid receptors in cardioprotection. Because opioid receptor signaling has been identified as the integral components of the cardioprotective effect of IPC (13), we hypothesized that this pathway may also regulate apoptosis after IPC. Accordingly, the major goal of this study was to show whether the inhibition of apoptosis and necrosis after IPC is mediated by activation of the δ-opioid receptor. A second goal was to show whether pharmacological preconditioning achieved by infusion of morphine also induces anti-apoptotic effect through δ-opioid receptor in the rabbit heart after I/R.

MATERIALS AND METHODS

All procedures were performed in conformance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1985).

Experimental groups and protocol. Ninety-two male New Zealand White rabbits (2.5–3.2 kg body wt) were used in this study. All rabbits underwent 30-min coronary artery occlusion (CAO) and 180 min of I/R, as shown in Fig. 1. The animals were randomized into the following eight groups: 1) control (n = 12), in which no treatment was given; 2) Nal control (n = 10), in which rabbits were treated with a nonspecific blocker of opioid receptor Nal (10 mg/kg iv) 10 min before I/R; 3) Nti control (n = 10), where rabbits were treated with a selective blocker of the δ-opioid receptor Nti (10 mg/kg iv) 10 min before I/R; 4) the preconditioning group (IPC; n = 12), where the IPC
protocol consisted of four 5-min occlusions, each separated by 10-min reperfusion; 5) morphine (Mor, 0.3 mg/kg, n = 12) was injected at a dose of 0.3 mg/kg iv 15 min before sustained ischemia; 6) Nal+IPC (n = 12), where rabbits were treated with Nal (10 mg/kg iv) 10 min before IPC protocol (see group 4); 7) Nti+IPC (n = 12), where animals were given Nti (10 mg/kg iv) 10 min before IPC; and 8) Nti+Mor (n = 12). Nti (10 mg/kg iv) was given 10 min before morphine, which was injected (0.3 mg/kg iv) 15 min before sustained ischemia. In each group, four rabbits were used for evaluation of apoptosis.

**Surgical preparation and measurement of infarct size.** The rabbit model of the I/R protocol has been described previously (25, 27). After the animals were anesthetized with ketamine HCl (35 mg/kg) and xylazine (5 mg/kg), a left thoracotomy was performed to expose the heart. Myocardial ischemia was induced by occlusion of coronary artery, followed by reperfusion. At the end of each experiment, the coronary artery was reoccluded and 50% Unisperse blue dye solution was injected into the right jugular vein until the eyes turned blue. This was done to distinguish between perfused and nonperfused (risk area) regions of the myocardium. The animal was then euthanized and the heart was removed. The heart slices were incubated at room temperature for 20 min in 1% solution of triphenyltetrazolium chloride. After being stained, the infarct area (triphenyltetrazolium chloride negative) and risk area (area nonstained by Unisperse blue) were determined by computer morphometry with the use of Bioquant imaging software and the percentage of infarcted and risk areas were calculated.

**Measurement of hemodynamics.** The hemodynamic measurements included heart rate and mean arterial pressures. The rate-pressure product was determined as the product of the heart rate and peak arterial pressure. These parameters were continuously measured throughout the duration of the experimental protocol (Table 1).

**In situ identification of nuclear DNA fragmentation.** Four rabbits from each group were euthanized, and tissues were sampled from the ischemic area of the left ventricle. ApoAlert DNA fragmentation assay kit (Clontech) was used, which detects apoptosis-induced nuclear DNA fragmentation via a fluorescence assay. The assay is based on terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL). After deparaffinization, the tissue sections were stained according to the manufacturer’s instructions and nuclear DNA fragmentation was visualized. Apoptotic cells exhibited strong nuclear green fluorescence with a standard fluorescence filter set (520 ± 20 nm). All cells stained with propidium iodide exhibited strong red cytoplasmic fluorescence when viewed at >620 nm. The percentage of TUNEL-positive cardiomyocyte nuclei was determined using an eyepiece grid (×200 magnification). Five microscopic fields per section were selected from within the ischemic region. In each field, cells were counted and the percentage of apoptotic cardiomyocytes was calculated.

**Genomic DNA extraction and agarose gel electrophoresis.** For determination of genomic DNA fragmentation, hearts were rapidly removed after I/R and tissue samples from the ischemic region were homogenized. DNAs were isolated by lysis in digestion buffer (75 mM NaCl, 25 mM EDTA, 10 mM Tris, pH 8.0, 1% SDS, and 0.4 mg/ml freshly added proteinase K). After overnight digestion, DNAs were phenol/chloroform extracted, precipitated, spooled onto glass rods, dried, and dissolved in 10 mM Tris and 1 mM EDTA, pH 8.0, and quantified with the use of a spectrophotometer. For determination of DNA fragmentation, we used ApoAlert LM-PCR Ladder assay kit (Clontech) according to a previously described method (36). Briefly, 6 μg genomic DNA was mixed with 1 nmol 24 base pairs (bp) and 1 nmol 12-bp unphosphorylated oligonucleotides, annealed, and ligated. Ligations were stored at −20°C until PCR was performed. PCR reactions used ~150-ng
ligated DNA and used the 24-bp oligonucleotide as a primer. Samples were amplified for 25 cycles of PCR, and PCR products were analyzed by electrophoresis on 3% agarose gel (16).

Exclusion criteria. Animals were excluded from the study due to the following reasons: 1) CAO did not produce adequate ischemia (the area at risk was <20% of the left ventricle); 2) intractable ventricular fibrillation occurred during ischemia and/or reperfusion; and 3) the animal died during the surgical procedure, so the protocol was not completed.

Statistical analysis. Values are expressed as means ± SE. The statistical significance was determined by ANOVA with repeated measurements. If a significant F value was found, Scheffe’s test for multiple comparisons was used to identify differences among groups. Comparisons of means were made by using the Student’s t-test for unpaired values. Statistical differences were considered significant if the P value was <0.05.

RESULTS

Infarct size. The risk areas ranged from 50% to 56% with no significant differences between all the groups (Fig. 2A; P > 0.05). These data suggest that changes in the size of infarcts observed between various groups in our experiments were not related to the percentage of area of the left ventricle occluded by our technique. Infarct size (expressed as a percentage of risk area) was 11.6 ± 1.0 in the IPC group and 19.5 ± 3.8 in the morphine-treated group, both of which were significantly lower compared with the infarct size of 46 ± 3.8 in the control group (P < 0.001, means ± SE; Fig. 2B). This reduction in infarct size by IPC was abolished by pretreatment with the nonselective opioid receptor antagonist Nal (38.6 ± 7.2%) and the δ-opioid-specific antagonist Nti (44.5 ± 1.8%). The infarct-limiting effect of morphine was also abolished by pretreatment with Nti (Nti+Mor: 49.3 ± 1.7% vs. 19.5 ± 3.8% for morphine; P < 0.001). Treatment of control rabbits with Nti or Nal had infarct size of 41.5 ± 3.6% and 42 ± 4.5%, respectively, which were not different compared with the infarct size in the untreated control group (P > 0.05).

Cardiomyocyte apoptosis. Figure 3A shows representative TUNEL-positive cardiomyocyte nuclei at ×200 and ×1,000 magnification.
magnifications. Quantitative analysis of DNA fragmentation was performed by using the TUNEL method at the single-cell level. The mean percentage of TUNEL-positive cardiomyocyte nuclei was 3.6 ± 1.9 in the IPC group, 5.2 ± 1.2 in the morphine group, which was significantly <12.4 ± 1.6, 12.9 ± 2.5, and 10.5 ± 1.9 of apoptotic cardiomyocytes in the control, Nal, and Nti-treated I/R hearts, respectively (P < 0.001; Fig. 3B). Nti pretreatment significantly increased TUNEL-positive cardiomyocytes in the IPC group (11.2 ± 2.2%) as well as the morphine-preconditioned group (12.1 ± 0.8%). Curiously, Nal failed to block the antiapoptotic effect of IPC, as shown by the percentage of apoptotic cells, which were 5.7 ± 1.3 in the Nal+IPC group and 5.2 ± 1.2 in the Nal+Mor-treated groups compared with the untreated control as well as Nal- and Nti-treated controls.

**Genomic DNA fragmentation.** For detection and qualitative evaluation of DNA fragmentation, we examined genomic DNA extracted from ischemic hearts that produced a typical “ladder” pattern. As shown in Fig. 4, ischemic left ventricular regions obtained from control, Nti-, and Nal-treated controls, Nti-pretreated IPC, and morphine groups subjected to I/R showed a typical DNA electrophoretic laddering pattern characterized by mononucleosomal and oligonucleosomal DNA fragmentation. However, DNA fragmentation (laddering pattern) was not observed in the tissue samples from animals in the IPC and Nal-treated preconditioned (IPC) as well as morphine-preconditioned rabbits showing protection from apoptosis.

**Systemic hemodynamics.** Heart rate, mean arterial blood pressure, and rate pressure product are shown in Table 1. Except as indicated, no significant differences in the baseline levels of these parameters were observed between each group. In addition, heart rate and mean arterial pressure remained reasonably stable throughout the reperfusion period, although these parameters decreased gradually at most of the data points in all the groups. Mean values were not significantly different between the groups at any time point for all the groups.

**DISCUSSION.**

The major findings of this study are summarized as follows. First, the infarct-limiting effect of IPC and morphine is significantly blocked by Nal and Nti, the nonselective and selective δ-opioid receptor antagonists, respectively. Second, both IPC and morphine caused a significant decrease in the TUNEL-positive cardiomyocytes, inhibited the fragmentation of genomic DNA in the ischemic zone, and reduced infarct size compared with the control hearts. Third, Nti treatment before IPC and morphine reversed the anti-apoptotic and infarct-limiting effect of IPC and morphine. On the other hand, Nal failed to block the antiapoptotic effect of IPC as well as morphine. Both Nal and Nti had no effect on infarct size or apoptosis in the control rabbits subjected to I/R. The hemodynamics remained largely unchanged among the groups during the I/R protocol. For the first time, our results show an impressive anti-apoptotic effect of IPC, which is selectively mediated by δ-opioid receptor in the intact rabbit heart.

Many previous studies (12, 18) support the evidence that apoptosis may be the predominant form of ischemia-related cell death in the heart. Also, it has been shown that internucleosomal DNA fragmentation is restricted in the infarcted area after prolonged I/R in rat hearts in vivo. Both DNA fragmentation and infarct size were significantly decreased after prolonged ischemia and reperfusion in the preconditioned rat heart. 

![Fig. 4. Ligation-mediated PCR analysis of DNA from the ischemic zone. Genomic DNAs were prepared from various experimental groups, mixed with unphosphorylated oligonucleotides, annealed, and ligated. The DNA samples were amplified and PCR products were analyzed by electrophoresis on 3% agarose gel. The data shown are representative of 5 separate experiments. M, marker lane. The treatment abbreviations are same as in the experimental protocol and described in detail in MATERIALS AND METHODS.](http://ajpheart.physiology.org/)

![Fig. 3. A: light photomicrographs of heart sections from control group of rabbits subjected to 30 min of ischemia and 3 h of reperfusion. The bright green dots (indicated by arrows) correspond to a representative terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL)-positive (fluorescent) nucleus. B: percentage of TUNEL-positive cells in heart cells. Data shown are means ± SE of four independent experiments quantified from five fields. The treatment groups and abbreviations are described in detail in MATERIALS AND METHODS. *P < 0.001.](http://ajpheart.physiology.org/)
hearts in vivo (29). Moreover, a direct correlation between myocardial infarct size and internucleosomal DNA fragmentation was also reported (29). Consistent with these studies, our data also showed significant reduction of apoptotic cardiomyocytes after IPC in the ischemic zone, which was correlated with reduction of infarct size in the heart. An interesting observation in these studies is that selective δ-opioid receptor antagonist completely abolished the anti-apoptotic protection of IPC, suggesting an important role of this receptor in preventing cell death. Moreover, nonpeptide opioid, morphine also mimicked the cardioprotective effect of IPC, both in terms of infarct size reduction and attenuation of apoptosis. Morphine is considered a classic μ-opioid receptor agonist, although there is evidence that it also has important effects on the δ- and κ-opioid receptors and that cross-talk between μ- and δ-receptors can occur (4). The observation that morphine-mediated inhibition of apoptosis was also abolished by pretreatment with Nti further confirms the role of δ-opioid receptor in protecting against cardiomyocyte death. Interestingly, whereas Nal abolished the infarct-limiting effect of IPC, the drug failed to block anti-apoptotic effect of IPC or morphine. The reason for these discordant results remains unclear, but it is possible that Nal could selectively block the necrotic rather than the antiapoptotic pathway of acute IPC in the rabbit heart. Alternatively, perhaps different opioid receptors have opposing effects on apoptosis, thus blocking both by Nal may result in no significant effect.

Several previous studies by Gross and co-workers (see Ref. 13 for review) suggest the role of opioids in early IPC. These investigators demonstrated that the nonselective opioid receptor antagonist Nal completely antagonized the ability of IPC to reduce infarct size, whether administered before IPC stimulus or after the IPC stimulus just before the index ischemia. Subsequent studies by Gross’s group showed that selective δ-1 antagonist 7-benzylidenenaltrexone blocked IPC-mediated protection in a dose-dependent fashion (33). Conversely, the selective δ-1 opioid receptor agonist TAN-67 produced a potent cardioprotective effect in chick embryonic myocytes, which was blocked by 7-benzylidenenaltrexone (15). In the present study, the dose of Nal or Nti (10 mg/kg) used in this study was higher compared with the one used in other studies used to block preconditioning (31, 32). However, none of these doses of the drugs had a significant effect on myocardial infarct size or apoptosis (in terms of TUNEL-positive nuclei or DNA fragmentation) compared with the nontreated control animals.

In the present study, we assessed apoptosis after a relatively short period of reperfusion (i.e., 3 h) by TUNEL and DNA fragmentation, which are known to measure the later stages of cell death. It has been reported (38) that the extent of necrosis peaks at 24 h of reperfusion, and remains constant thereafter. On the other hand, the appearance of apoptotic cells in the perinecrotic area progressively increases up to 72 h, suggesting that necrosis and apoptosis occur simultaneously during reperfusion, with a rapidly developing necrotic cell death during the early phase of reperfusion, followed by a slower appearance of apoptosis during the late phase of reperfusion. The number of apoptotic cells in the perinecrotic myocardium progressively increases during the extended period of reperfusion, which suggests the role of apoptosis in the development of infarction (39). Considering the reported delayed preconditioning effect of opioids (6), it would not be surprising if morphine or IPC also delay the extent of apoptosis in the survival animal models. This is an interesting question, which warrants investigations in the future.

It has been reported that the protective effect of opioids involves selective activation of signaling pathways, including PKC-δ (10), non-Src-dependent tyrosine kinase and ERK-1/2 (8, 9, 11). There are studies (7) that suggest the δ-1-opioid receptor reduced infarct size through the opening of mitochondrial ATP-sensitive K⁺ channels, the proposed effectors of IPC. Moreover, the pharmacological opening of mitochondrial ATP-sensitive K⁺ channels by diazoxide has been shown to preserve mitochondrial integrity and suppress the markers of apoptosis (1). It remains to be seen whether a similar signaling pathway also follows the δ-opioid receptor-mediated antiapoptotic effect of morphine and IPC. A recent study (20) showed that morphine protected against the death of primary rat neonatal astrocytes by nitric oxide and peroxynitrite. Pretreatment of astrocytes with phosphatidylinositol 3-kinase (PI3-kinase) inhibitors, including wortmannin and LY-294002, abrogated the effect of morphine on nitric oxide-induced cell death, indicating that the effects of morphine were, in part, mediated by PI3-kinase. It is possible that activation of δ-opioid receptor stimulation by IPC or morphine potentially activate the PI3-kinase pathway that may ultimately be responsible for the antiapoptotic effect of this receptor. There is currently no data in support of this contention, although the role of PI3-kinase in the acute phase of IPC against lethal ischemia-reperfusion injury has been well documented by recent published studies (23, 37).

In summary, our results provide the first direct evidence that both IPC and morphine attenuate cardiomyocyte apoptosis through the activation of δ-opioid receptors. These observations raise the interesting possibility that novel agonists of δ-opioid receptors may be clinically useful in preventing cell death during I/R injury and heart failure and could potentially be used for treatment directed at these lethal disorders.

GRANTS

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