Morphine mimics the antiapoptotic effect of preconditioning via an Ins(1,4,5)P₃ signaling pathway in rat ventricular myocytes

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Barrère-Lemaire, Stéphanie, Nicolas Combes, Catherine Sportouch-Dukhan, Sylvain Richard, Joël Nargeot, and Christophe Piot. Morphine mimics the antiapoptotic effect of preconditioning via an Ins(1,4,5)P₃ signaling pathway in rat ventricular myocytes. Am J Physiol Heart Circ Physiol 288: H83–H88, 2005. First published September 2, 2004; doi:10.1152/ajpheart.00881.2003.—Morphine has cardioprotective effects against ischemic-reperfusion injuries. This study investigates whether morphine could mimic the antiapoptotic effect of preconditioning using a model of cultured neonatal rat cardiomyocytes subjected to metabolic inhibition (MI). To quantify MI-induced apoptosis, DNA fragmentation and mitochondrial cytochrome c release levels were measured by ELISA. MI-dependent DNA fragmentation was prevented by both Z-VAD-fmk (20 μM), a pan-caspase inhibitor, and cyclosporine A (CsA; 5 μM), a mitochondrial pore transition blocker, added during MI (36% and 54% decrease, respectively). MI-dependent cytochrome c release was not blocked by Z-VAD-fmk but was decreased (38%) by CsA during MI. Metabolic preconditioning (MIP) and preconditioning with morphine (1 μM) were also assessed. MI-dependent DNA fragmentation and cytochrome c release were prevented by MIP (40% and 45% decrease, respectively) and morphine (34% and 45%, respectively). The antiapoptotic effect of morphine was abolished by naloxone (10 nM), a nonselective opioid receptor antagonist, or xestospongion C (XeC, 400 nM), an inhibitor of inositol (1,4,5)-trisphosphate [Ins(1,4,5)P₃]-mediated Ca²⁺ release. Ca²⁺ preconditioning, induced by increasing extracellular Ca²⁺ from 1.8 to 3.3 mM, mimicked the antiapoptotic effect of morphine on DNA fragmentation (24% decrease) and cytochrome c release (57% decrease). This effect mediated by extracellular Ca²⁺ was also abolished by XeC. Measurements of intracellular Ca²⁺ concentration using fura-2 microspectrofluorimetry showed that morphine induces Ins(1,4,5)P₃-dependent Ca²⁺ transients abolished by 2-aminoethoxydiphenyl borate (2-APB), a cell-permeable Ins(1,4,5)P₃ antagonist. These results suggest that morphine preconditioning prevents simulated ischemia-reperfusion-induced apoptosis via an Ins(1,4,5)P₃ signaling pathway in rat ventricular myocytes.

opiod; cardioprotection; calcium

Murry and co-workers (18) first demonstrated that brief periods of acute myocardial ischemia protect the heart against subsequent episodes of prolonged ischemia by delaying lethal cell injury. This phenomenon, termed ischemic preconditioning, is recognized as the strongest form of in vivo protection against myocardial ischemia-reperfusion injury (13). Recent studies have suggested that endogenous opioids can act as triggers of cardioprotection (for a review, see Ref. 9). The nonselective opioid receptor antagonist naloxone and selective pharmacological antagonists of δ- or the κ-opioid receptors have been shown to antagonize the beneficial effects of acute ischemic preconditioning in rat hearts (26, 27, 30).

Morphine, an exogenous nonpeptide opioid agonist, mimics the cardioprotective effect of ischemic preconditioning in the intact rat heart (25). The infarct size reduction achieved by morphine is blocked both by naloxone and glibenclamide, a nonselective ATP-sensitive K⁺ (KATP) channel antagonist. Beneficial effects of morphine are also observed in vitro. Indeed, morphine was shown to produce concentration-dependent cardioprotective effects equivalent to those observed during hypoxic preconditioning in an embryonic chick myocyte model of hypoxia/reoxygenation injury (15). In addition, this effect of morphine was shown to be blocked by naloxone and the selective δ₁-opioid receptor antagonist 7-benzylindene naltrexone (BNTX), as reported by McPherson and Yao (16). However, the signaling pathway by which morphine promotes cardiomyocyte survival remains unclear. Both mitochondrial KATP channel activation and modulation of intracellular Ca²⁺ have been suggested to mediate this effect (15, 16, 23, 32).

Our main objective was to determine whether morphine could mimic the antiapoptotic effect of preconditioning using a validated in vitro model of cultured neonatal rat cardiomyocytes subjected to metabolic inhibition (MI) (32). Our results show that morphine exerts antiapoptotic effects through an insitol (1,4,5)-trisphosphate [Ins(1,4,5)P₃]-dependent Ca²⁺ release.

METHODS

Isolation and culture of rat ventricular myocytes. Cardiomyocytes were isolated from hearts of newborn (1–4 days after birth) Wistar Kyoto rats after enzymatic dissociation with collagenase (10). Per dissocation, 12–16 animals were killed by decapitation, and a total of 24 dissociations was performed. The investigation conformed with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996) and according to European directives (86/609/CEE). Cells were plated at the density of 5 × 10⁵ cells/35-mm dish (Primaria Easy Grip Falcon, VWR) in a culture medium containing 50% DMEM, 50% medium 199, 10% horse serum, 5% fetal calf serum, 2 mM L-glutamine, and 1% penicillin-streptomycin. The medium was replaced 24 h after cells were plated. Cells were allowed to stabilize 40 h before the experiment was started. Only contracting cells (assessed by microscopic...
examination) were subjected to the experimental protocol. Cultures with evident necrosis area were discarded before the experiment.

Experimental protocol of metabolic inhibition. Serum and glucose deprivation was performed to simulate ischemia. Cardiomyocytes were incubated for 3 h in a MI buffer, which was glucose and serum free, containing (in mM) 125 NaCl, 4.9 KCl, 1.2 NaH2PO4, 1.2 MgSO4, 8 NaHCO3, 1.8 CaCl2, 20 HEPES, and 5 NaCN, pH adjusted to 6.6 ± 0.05 with HCl. The reperfusion was obtained by replacing the cells in the culture medium for 16 h. The protocols used to achieve a preconditioning are described in Fig. 1. For metabolic preconditioning (MIP), the cells were incubated for 5 min with MI solution 10 min before the sustained MI (5 min of reperfusion with the culture medium). Preconditioning with 1 μM morphine (morphine chlorhydrate, Lavosier) or 3.3 mM calcium was performed by pretreating the cells in the culture medium for 5 min, followed by 5 min of reperfusion before sustained MI.

To evaluate the implication of the apoptotic pathway in this model, we used a pharmacological approach. Z-VAD-fmk (R&D Systems), a pan-caspase inhibitor, was used at 20 μM during sustained MI. Cyclosporine A (CsA; Novartis-Pharma), a potent inhibitor of mitochondrial pore transition (MPT), was used during sustained MI at the concentration of 5 μM. Naloxone (Narcan, DuPont-Pharma), a non-selective opioid receptor antagonist, was prepared as a stock solution (1 mM in H2O), stored at (−20°C), and subsequently diluted to 10 nM in test solutions. Xestospongin C (XeC; Calbiochem; San Diego, CA) was used as a membrane-permeable antagonist of Ins(1,4,5)P3-mediated Ca2+ release (IC50 = 358 nM) and was prepared as a 5 mg/ml stock in DMSO, stored at −20°C, and diluted to a working concentration of 400 nM. Each change of incubation medium was preceded by a washout performed with 2 ml of PBS (Dulbecco’s Phosphate-Buffered Saline, Eurobio).

Quantification of DNA fragmentation. Fragmentation of DNA into mono- and oligonucleosomes was quantified by an ELISA specific for cytosolic histone-bound DNA (Cell Death detection ELISAPLUS Roche Diagnostic). After 16 h of reperfusion, the culture medium was removed, and cells were scraped and incubated with lysis buffer. After centrifugation (200 g for 10 min), the supernatant was incubated with specific antibodies against histones and DNA. A colorimetric agent (ABTS; Roche Applied Science) was used to visualize the reaction. Measurement of optical density (OD) was performed for quantification.

Quantification of cytochrome c release. ELISA testing was used to measure mitochondrial cytochrome c release (Quantikine rat/mouse cytochrome c, R&D Systems). The samples were collected as described for DNA quantification. Cell lysates were centrifuged at 16,000 g for 10 min. The supernatant was incubated with specific antibodies, and OD quantification was performed.

Measurements of intracellular Ca2+ concentration. The dual-excitation ratiometric Ca2+-sensitive dye fura-2 was used for intracellular Ca2+ concentration ([Ca2+]i) imaging studies in single cells with an Olympus-LSR system (MERLIN, Life Science Resources; Cambridge, UK). The variations in [Ca2+]i, were detected by a digital charge-coupled device camera. Briefly, cells were loaded by incubation with 2.5 μM fura-2 AM and 0.02% Pluronic F-127 (Molecular Probes; Eugene, OR) for 40 min at room temperature in Locke buffer containing (in mM) 140 NaCl, 5 KCl, 1.2 KH2PO4, 1.2 MgSO4, 1.8 CaCl2, 10 glucose, and 10 HEPES; pH 7.2. Cells were then rinsed and reincubated with Locke buffer at 37°C for 20 min. Cells were illuminated via a LSR SpectraMASTER monochromator coupled to the microscope fitted with a UV transparent oil, objective (Uapo/340 40X/1.35) of an inverted microscope (Olympus IX70). The image was detected with an LSR Astrocum 12/14-bit frame transfer digital camera. The MERLIN system controlled the illuminator and camera and performed image ratioing and analysis. The intensity of fluorescent light emission at wavelength = 510 nm, using excitation at 340 and 380 nm, was monitored from each single fura-2-loaded cell. The ratio of fluorescence emission when excited at 340 nm (the absorbance peak of fura bound to Ca2+) to 380 nm (the absorbance peak of free fura) was used as an index of [Ca2+]i, with an increase in the ratio signifying an increase in intracellular free calcium.

Acute (1–2 min) extracellular application of morphine and control solutions was achieved using a multiple capillary perfusion system (200 μm inner diameter; 100 μl/min) placed in close proximity (<0.5 mm) to each cell evaluated, whereas more prolonged incubations were carried out in a 500 μl Locke buffer bath. After each application, cells were washed with Locke buffer. All the test solutions were prepared in Locke buffer. To test the implication of the Ins(1,4,5)P3 pathway in the morphine response, cells were preincubated during 1 h with 10 μM 2-aminooethoxydiphenyl borate (2-APB; Sigma; St. Louis, MO) dissolved in the culture medium before Ca2+ recordings.

Statistical analysis. Measurements of DNA fragmentation and cytochrome c release were standardized by taking into account the dispersion of cells density plating among cultures (ratio of OD test to OD control for each culture). Data (means ± SE) were normalized by taking MI values as references. The number of experiments (n) is indicated in each figure and reflects the number of dishes used for each experimental condition. The drug effect was analyzed by ANOVA, followed by the Tukey’s post test for multiple comparisons. For Ca2+ measurements, experiments were performed on individual cells (n reflects the number of cells). For comparison between two treatment groups, unpaired Student’s t-tests were used. Probability values <0.05 were accepted as statistically significant, and the P values are noted with asterisks. The statistical analysis was performed with GraphPad Prism (GraphPad Software; San Diego, CA).
RESULTS

Sustained metabolic inhibition induces apoptosis in neonatal rat cardiomyocytes. DNA fragmentation and mitochondrial cytochrome c release were assessed to evaluate MI-induced apoptosis in our cellular model. Quantitative determinations of soluble mono- and oligonucleosomes and cytosolic cytochrome c were performed by ELISA. In serum/glucose-deprived cells treated with 20 μM of the pan-caspase inhibitor Z-VAD-fmk (see protocol, Fig. 1), which had no effect per se, DNA fragmentation was decreased by 36% (n = 13, P < 0.01; Fig. 2). In contrast, Z-VAD-fmk did not prevent the increase in cytosolic cytochrome c (n = 13, P = not significant; Fig. 2B). In cells subjected to sustained MI, CsA (5 μM), an inhibitor of MPT, reduced DNA fragmentation by 54% (n = 10, P < 0.001; Fig. 2A) and cytochrome c release by 38% (n = 10, P < 0.001; Fig. 2B).

Morphine preconditioning prevents sustained metabolic inhibition-induced apoptosis. The effects of MIP on DNA fragmentation and cytochrome c release were examined by ELISA. MIP decreased DNA fragmentation by 40% (n = 29, P < 0.001; Fig. 3A) and cytosolic cytochrome c by 45% (n = 18, P < 0.001; Fig. 3B). Similar protection was observed when 1 μM morphine was applied before sustained MI. Indeed, preconditioning with morphine reduced DNA fragmentation by 34% (n = 44, P < 0.001; Fig. 3A) and cytochrome c release by 45% (n = 24, P < 0.001; Fig. 3B). These cardioprotective effects were completely abolished in the presence of 10 nM naloxone (Fig. 3, A and B). Naloxone had no significant agonist effect on DNA fragmentation (data not shown).

Intracellular Ca\(^{2+}\) contributes to the protective effect of morphine preconditioning. Exposure of neonatal ventricular myocytes to 1 μM morphine elicited a consistent and significant transient increase in [Ca\(^{2+}\)]\(_i\) versus baseline (n = 19, P = 0.001; Fig. 4, A and B). This effect was antagonized by the application of 10 nM naloxone (Fig. 4A). This increase in [Ca\(^{2+}\)]\(_i\) was attributed to Ins(1,4,5)P\(_3\) signaling because preincubation of cells with 2-APB (10 μM) before morphine administration completely abrogated the response to morphine stimulation (n = 52, P = not significant; Fig. 4B).

The cardioprotective effect of morphine on DNA fragmentation and cytochrome c release was completely abolished by the potent inhibitor of Ins(1,4,5)P\(_3\)-mediated Ca\(^{2+}\) release XeC (400 nM; Fig. 5, A and B). Brief exogenous infusions of CaCl\(_2\) resulting in a transient increase in intracellular Ca\(^{2+}\) have been demonstrated to protect the heart against subsequent sustained ischemic insult (21). Preconditioning by Ca\(^{2+}\) reproduced the protective effect of morphine preconditioning on DNA fragmentation (24% decrease, n = 22, P < 0.05) and cytochrome c release (57% decrease, n = 13, P < 0.001) in our model (Fig. 5A) and CsA (5 μM) were applied throughout MI. Data (means ± SE) were normalized relative to MI values. The number (n) of experiment is indicated for each bar. Four to fifteen dissociations were used for each experimental condition except for MI (24) in A. MIP, metabolic preconditioning. *P < 0.05 vs. MI.

Fig. 2. Effects of Z-VAD and CsA on DNA fragmentation and cytochrome c release. Histograms represent ratio values of relative oligonucleosome amounts (A) and cytochrome c release (B) in cultured cardiomyocytes subjected to MI. Z-VAD (20 μM) and CsA (5 μM) were applied throughout MI. Data (means ± SE) were normalized relative to MI values. The number (n) of experiment is indicated for each bar. Three to four dissociations were used for each experimental condition except for MI (24) in A. *P < 0.05 vs. MI.

Fig. 3. Effects of metabolic and morphine preconditioning on DNA fragmentation and cytochrome c release. Histograms represent ratio values of relative oligonucleosome amounts (A) and cytochrome c release (B) in cultured cardiomyocytes subjected to MI. Morphine (1 μM) with or without naloxone (10 nM) was applied (for 5 min) 10 min before MI, followed by washout. Data (means ± SE) were normalized relative to MI values. The number (n) of experiment is indicated for each bar. Four to fifteen dissociations were used for each experimental condition except for MI (24) in A. MIP, metabolic preconditioning. *P < 0.05 vs. MI.
These cardioprotective effects were completely abolished in presence of 400 nM XeC (Fig. 5, A and B).

**DISCUSSION**

In this study, we demonstrate that pharmacological preconditioning with morphine is able to attenuate both internucleosomal DNA fragmentation and release of cytochrome c into the cytosol under conditions of sustained MI in neonatal rat cardiomyocytes. This cardioprotective effect of morphine was Ca\(^{2+}\) dependent and blocked by Ins(1,4,5)P\(_3\) receptor antagonists. These results suggest that morphine preconditioning prevents simulated ischemia-reperfusion-induced apoptosis via an Ins(1,4,5)P\(_3\) signaling pathway in rat ventricular myocytes.

**Apoptosis during MI induced by simulated ischemia-reperfusion.** Apoptosis has significant contribution to cell death in the ischemic and reperfused myocardium (for a review, see Ref. 14). We used MI to induce apoptosis in our in vitro model of simulated ischemia-reperfusion. Apoptosis was attenuated by two well-known reagents, Z-VAD-fmk and CsA, a peptidic caspase inhibitor and an MPT inhibitor, respectively. Z-VAD-fmk and CsA prevented DNA fragmentation. CsA also prevented elevation of cytosolic cytochrome c. Our findings are consistent with a previous study showing that serum and glucose deprivation activate both the mitochondrial apoptotic pathway (cytochrome c release) and caspases (3 and 9) in cultured neonatal rat cardiomyocytes (5).

**Inhibition of apoptosis by morphine preconditioning.** Brief periods of acute myocardial ischemia protect the heart against subsequent episodes of prolonged ischemia by delaying lethal cell injury (18). The mechanism(s) by which transient ischemia prevents myocyte cell death remain(s) poorly understood. In previous reports, we suggested that the cardioprotection induced by ischemic preconditioning in the intact rat heart is linked to a decrease in apoptosis (19, 20). Both DNA fragmentation and caspase processing were attenuated in this in vivo model of acute myocardial ischemia and reperfusion. In the present study, MIP had similar antiapoptotic effect in cultured neonatal rat cardiomyocytes, consistent with findings in cultured rabbit cardiomyocytes (8). MIP attenuated both DNA fragmentation and cytochrome c release, indicating that preconditioning promotes cardioprotection by inhibiting apoptosis via the mitochondrial pathway.

Previous studies have demonstrated that morphine, currently used as a potent analgesic, mimics ischemic preconditioning through opioid receptor activation in the intact rat heart (17, 24, 5, A and B).
26). We report in this study that preconditioning with morphine prevents MI-induced DNA fragmentation and cytochrome c release in neonatal rat cardiomyocytes. However, the effect of morphine on programmed cell death remains controversial. T-cell hybridoma and human peripheral blood lymphocytes treated with morphine for 2 h underwent apoptosis only when stimulated by L cells expressing Fas ligand (34). In contrast, the κ-opioid receptor agonists rapidly, but transiently, suppressed serum deprivation-induced apoptosis in a PC12 rat pheochromocytoma cell line (6). Kim et al. (12) demonstrated that morphine protected primary rat astrocytes from apoptosis mediated by nitric oxide species, a protective effect that was antagonized by naloxone. To our knowledge, the present study suggests for the first time that preconditioning with morphine promotes cardiomyocyte survival through an antiapoptotic mechanism, i.e., by inhibiting the mitochondrial apoptotic pathway.

The exact mechanism(s) by which morphine promotes cell survival in cardiomyocytes still remain(s) speculative. However, there is some evidence for a role of mitochondrial K\textsubscript{ATP} channels as a final common effector in the ischemic preconditioning-dependent cardioprotective pathway in the rat heart (29). One attractive hypothesis is that ischemic preconditioning may prevent apoptosis through mitochondrial K\textsubscript{ATP} channel activation (1). Consistent with this hypothesis, previous reports have suggested that the preconditioning-like effect of morphine is the result of mitochondrial K\textsubscript{ATP} channel activation in both the intact rat heart and cultured ventricular myocytes from chick embryos (15, 16). By activating mitochondrial K\textsubscript{ATP} channels through opioid receptors, morphine may prevent mitochondrial pore opening and release of cytochrome c into the cytosol. However, an alternative hypothesis was that morphine protects the cells via an Ins(1,4,5)P\textsubscript{3} signaling pathway.

**Involvement of Ca\textsuperscript{2+} signaling in morphine-induced cardioprotection.** Changes in Ca\textsuperscript{2+} homeostasis might be involved in the cardioprotective effect of ischemic preconditioning (22). Preconditioning has been shown to prevent intracellular Ca\textsuperscript{2+} overload during ischemia (28). Brief infusions of CaCl\textsubscript{2}, resulting in transient [Ca\textsuperscript{2+}]	extsubscript{i} increase, protect the heart against subsequent ischemic insult (21). Gysenbergh et al. (10) also demonstrated that D-myo-Ins(1,4,5)P\textsubscript{3}, a synthetic and cell-impermeable agonist of Ins(1,4,5)P\textsubscript{3} receptors, administered before coronary artery occlusion, mimics the beneficial effect of ischemic preconditioning on the infarct size in isolated rabbit hearts. Accordingly, Bauer et al. (4) observed a twofold increase in Ins(1,4,5)P\textsubscript{3} content after 5 min of brief preconditioning ischemia. These data support the concept that ischemic preconditioning may elicit cardioprotection via an Ins(1,4,5)P\textsubscript{3} signaling pathway.

Here, we report that morphine consistently elicits a transient increase in [Ca\textsuperscript{2+}]	extsubscript{i} in rat neonatal ventricular cells. This increase depends on Ins(1,4,5)P\textsubscript{3} signaling because preincubation with 2-APB, a membrane-permeable agent that inhibits Ins(1,4,5)P\textsubscript{3}-mediated Ca\textsuperscript{2+} release with no effect on ryanodine-mediated Ca\textsuperscript{2+} release, abolished the response to morphine. We found that both DNA fragmentation and cytochrome c release were significantly attenuated by either morphine and Ca\textsuperscript{2+} preconditioning. These cardioprotective effects were abolished by XeC, a very potent, highly specific membrane-permeable antagonist of Ins(1,4,5)P\textsubscript{3}-mediated Ca\textsuperscript{2+} release (7). Therefore, these results suggest that Ins(1,4,5)P\textsubscript{3}-mediated Ca\textsuperscript{2+} release is involved in the cardioprotective effect of morphine observed here in ventricular rat cardiomyocytes.

There is emerging evidence that Ins(1,4,5)P\textsubscript{3}-mediated Ca\textsuperscript{2+} signals are involved both in cell survival and apoptotic cell death (11). Ca\textsuperscript{2+} overload in mitochondria is associated with the early phase of cell death (2), and a rise in intracellular Ca\textsuperscript{2+} might induce MPT opening (31). Recently, Argaud et al. (3) showed that ischemic preconditioning delays Ca\textsuperscript{2+}-induced MPT openings. Similarly, the Ins(1,4,5)P\textsubscript{3}-dependent transient rise in [Ca\textsuperscript{2+}]	extsubscript{i} observed here during morphine preconditioning might delay MPT opening and prevent cardiomyocyte apoptosis in agreement with the effect of Ca\textsuperscript{2+} preconditioning on MPT and apoptosis in cultured neonatal rat cardiomyocytes (33).

In summary, our results demonstrate in an in vitro model of simulated ischemia-reperfusion that the cardioprotective effect of morphine mediates an antiapoptotic effect through the activation of the Ins(1,4,5)P\textsubscript{3}-dependent Ca\textsuperscript{2+} release.

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