Morphine prevents the mitochondrial permeability transition pore opening through NO/cGMP/PKG/Zn^{2+}/GSK-3β signal pathway in cardiomyocytes

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Xi J, Tian W, Zhang L, Jin Y, Xu Z. Morphine prevents the mitochondrial permeability transition pore opening through NO/cGMP/PKG/Zn^{2+}/GSK-3β signal pathway in cardiomyocytes. Am J Physiol Heart Circ Physiol 298: H601–H607, 2010. First published December 4, 2009; doi:10.1152/ajpheart.00453.2009—The aim of this study was to test whether morphine prevents the mitochondrial permeability transition pore (mPTP) opening through Zn^{2+} and cGMP is involved in the action of morphine. The effect of morphine on Zn^{2+} release was abolished by KT5823, a specific inhibitor of protein kinase G (PKG). Morphine prevented oxidant-induced loss of mPTP (4) through inactivation of glycogen synthase kinase 3β (GSK-3β). In support of this, morphine mobilizes Zn^{2+} via NO. Morphine rapidly produced NO, ODQ and NS2028, the inhibitors of guanylyl cyclase, prevented Zn^{2+} release by morphine, implying that cGMP is involved in the action of morphine. The effect of morphine on Zn^{2+} release was also abolished by KT5823, a specific inhibitor of protein kinase G (PKG). Morphine prevented oxidant-induced loss of mPTP. The action of morphine on the mPTP was reversed by KT5823 and the Zn^{2+} chelator N,N,N′,N′-tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN). The action of morphine on the mPTP was lost in cells transfected with the constitutively active GSK-3β mutant, suggesting that morphine may prevent the mPTP opening by inactivating GSK-3β. In support of this, morphine significantly enhanced phosphorylation of GSK-3β at Ser21, and this was blocked by TPEN. GSK-3β small interfering RNA prevented the pore opening in the control cardiomyocytes but failed to enhance the effect of morphine on the mPTP opening. In conclusion, morphine mobilizes intracellular Zn^{2+} through the NO/cGMP/PKG signaling pathway and prevents the mPTP opening by inactivating GSK-3β through Zn^{2+}.

Nitric oxide; protein kinase G; guanosine 3′,5′-cyclic monophosphate; glycogen synthase kinase 3β

Both endogenous and exogenous opioids can induce acute or delayed preconditioning (15). The first evidence addressing the important role of opioids in early preconditioning was reported by Schultz et al. (36). In the rat heart, they found that naloxone, a nonselective opioid receptor antagonist, completely blocked the anti-infarct effect of preconditioning administered either before or after preconditioning episodes, suggesting that endogenous opioids are crucial in both triggering and mediating preconditioning. The cardioprotective effects of opioids have been attributed to the activation of δ (18, 35, 37) or κ (43, 44) opioid receptors. Many studies have documented that protein kinase C (7, 30), mitochondria ATP-sensitive K⁺ channels (18, 26), tyrosine kinase (10), and mitogen-activated protein kinase (8, 9) contribute to the mechanism of opioid-induced acute cardioprotection. In these studies, the cardioprotective effects of opioids have been obtained when given before the onset of ischemia. Since pretreatments are seldom possible in the clinical settings of acute cardiac infarction, it is desirable that opioids can protect the myocardium when given after onset of ischemia or at reperfusion. Recently, Gross’s group (14) has demonstrated that opioids can reduce infarct size when administered just before reperfusion, an effect that was similar to that observed when given before ischemia, suggesting that opioids can prevent reperfusion injury.

The mitochondrial permeability transition pore (mPTP) opening has been proposed to play a critical role in myocardial ischemia-reperfusion injury (40). The mPTP remains closed during ischemia but opens at the onset of reperfusion (13), and suppression of the mPTP opening at early reperfusion leads to cardioprotection against reperfusion injury (16, 17). Postconditioning has been demonstrated to protect the heart from reperfusion injury by targeting the mPTP through activation of δ-opioid receptors in rat hearts (21). However, the precise signaling events that link opioid receptor activation and the inhibition of mPTP opening remain unclear.

Nitric oxide (NO) has been reported to prevent the mPTP opening (42), and morphine was shown to produce NO by activating δ-opioid receptors in rat cardiomyocytes (21). Our laboratory has demonstrated that NO prevents oxidant-induced mPTP opening by mobilizing intracellular Zn^{2+} (20) and that exogenous Zn^{2+} prevents reperfusion injury by targeting the mPTP (4) through inactivation of glycogen synthase kinase 3β (GSK-3β). GSK-3β inactivation is involved in the mechanism by which opioids protect the heart at reperfusion (14) and plays a central role in modulation of the mPTP (22). Therefore, it is reasonable to hypothesize that opioids may prevent the mPTP opening by inactivating GSK-3β through mobilization of Zn^{2+}.

In the present study, we first examined whether morphine could mobilize intracellular Zn^{2+} via a NO-dependent mechanism in isolated rat cardiomyocytes. We then tested whether morphine could inactivate GSK-3β. Finally, we determined whether morphine prevents the mPTP opening via Zn^{2+} and GSK-3β.

MATERIALS AND METHODS

This study conforms to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1996). All protocols for the experiments using animals were...
approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill.

**Chemicals and antibodies.** Morphine was purchased from Sigma, and inhibitors were obtained from EMD Biosciences (La Jolla, CA). Fluorescence dyes were purchased from Molecular Probes (Eugene, OR). Antibodies were purchased from Cell Signaling Technology (Beverly, MA).

**Isolation of adult rat cardiomyocytes.** Rat cardiomyocytes were isolated enzymatically (45). Male Wistar rats weighing 250–350 g were anesthetized with thiobutabarbitual sodium (100 mg/kg ip). 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 (in mM) 118 NaCl, 25 NaHCO₃, 4.7 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 1.25 CaCl₂, and 10 glucose for 20 min. Morphine was given 10 min before exposure to H₂O₂. Inhibitors were given 10 min before the exposure to morphine. To measure intracellular NO concentrations, cardiomyocytes were loaded with 2 μM 4-aminophenyl-2,7'-difluorofluorescein (DAF-FM) for 20 min. The green fluorescence was excited at 488 nm and imaged through a 525-nm long-path filter.

**Confocal imaging of intracellular Zn²⁺.** Intracellular Zn²⁺ was detected with Newport Green Dichlorofluorescein (DCF) (20). Cardiomyocytes cultured in a specific temperature-controlled culture dish were incubated with 2 μM Newport Green DCF diacetate in standard Tyrode solution containing (in mM) 140 NaCl, 6 KCl, 1 MgCl₂, 1 CaCl₂, 5 HEPES, and 5.8 glucose (pH 7.4) for 20 min. Cells were then mounted on the stage of an Olympus FV 500 laser scanning confocal microscope. The green fluorescence was excited with the 488-nm line of argon-krypton laser and imaged through a 525-nm long-path filter. Temperature was maintained at 37°C with Delta T Open Dish Systems (Biopotech, Butler, PA). The images recorded on a computer were quantified using ImageJ.

Confocal imaging of mitochondrial membrane potential. Mitochondrial membrane potential (∆Ψₘ) was measured using confocal microscopy as reported previously (45). Briefly, cells were incubated with tetramethylrhodamine ethyl ester (TMRE; 100 nM) in standard Tyrode solution for 20 min. The red fluorescence was excited with a 543-nm line of argon-krypton laser and imaged through a 560-nm long-path filter.

**RESULTS**

Morphine (1 μM) significantly enhanced Newport Green DCF fluorescence intensity (155.9 ± 7.2% of baseline) compared with the control (109.8 ± 2.4% of baseline), an effect that was abolished by the NO synthase (NOS) inhibitor N’G, nitro-l-arginine methyl ester (l-NNAME), suggesting that morphine mobilizes intracellular Zn²⁺ by producing NO in isolated rat cardiomyocytes (Fig. 1). H₂O₂ and l-NNAME did not alter the fluorescence intensity. To confirm the above observation that morphine releases Zn²⁺ via NO, we then tested whether morphine could produce NO in cardiomyocytes. Figure 2 shows that morphine markedly enhanced DAF-FM fluorescence intensity (154.9 ± 19.7% of baseline) 10 min after the treatment compared with the control (102.2 ± 2.4% of baseline), suggesting that morphine indeed produces NO in cardiomyocytes. The effect of morphine on NO generation was reversed by the NOS inhibitor l-NNAME (99.6 ± 4.3% of baseline), indicating that morphine produces NO via NOS. Figure 3 shows that the effect of morphine (155.9 ± 7.2% of baseline) on Newport Green DCF fluorescence was blocked by
ODQ (119.3 ± 6.3% of baseline), a potent and selective inhibitor of NO-sensitive guanylyl cyclase, implying that cGMP is responsible for the Zn$^{2+}$-releasing effect of morphine. The involvement of cGMP in the action of morphine was confirmed by further experiments in which the action of morphine was reversed by another specific and irreversible inhibitor of guanylyl cyclase: NS2028 (105.8 ± 4.1% of baseline; Fig. 3). Figure 3 further shows that KT5823, a highly specific cell-permeable inhibitor of protein kinase G (PKG), nullified the action of morphine (106.0 ± 3.3% of baseline), suggesting that PKG contributes to the action of morphine.

To examine whether morphine prevents the mPTP opening by the cGMP/PKG/Zn$^{2+}$ pathway, we tested the effect of morphine on the oxidant-induced loss of ΔΨ$m$ in rat cardiomyocytes. Treatment of cardiomyocytes with 100 μM H$_2$O$_2$ dramatically decreased TMRE fluorescence (45.1 ± 8.2 of baseline in the control), indicating that oxidant stress caused loss of ΔΨ$m$ (Fig. 4). Because the loss of ΔΨ$m$ is caused by the mPTP opening (6), this result may indicate that oxidant stress caused the mPTP opening. Treatment of cells with 1 μM morphine prevented the loss of TMRE fluorescence (90.0 ± 2.0% of baseline), indicating that morphine can modulate the mPTP opening. This effect of morphine was abolished by the PKG inhibitor KT5823 (51.1 ± 4.8% of baseline). Further experiments showed that morphine was not able to preserve TMRE fluorescence in cells pretreated with the Zn$^{2+}$ chelator N,N,N’,N’-tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN) (57.5 ± 2.0% of baseline), indicating that morphine prevent the mPTP opening by mobilizing intracellular Zn$^{2+}$. ZnCl$_2$ (1 μM) in the presence of the zinc ionophore pyrithione mimicked the action of morphine to preserve TMRE fluorescence (Fig. 4). The recognized closer of cyclosporine A (0.2 μM) also prevented the loss of TMRE fluorescence, confirming that morphine and zinc moderate the pore opening in cardiomyo-
cytes. KT5823, ODQ, and TPEN did not alter TMRE fluorescence.

To define the role of GSK-3β in the protective action of morphine, we determined the effect of morphine on GSK-3β activity by detecting its phosphorylation at Ser9 10 min after exposure to morphine. As shown in Fig. 5, morphine significantly enhanced GSK-3β phosphorylation (174.3 ± 16.2% of control), an effect that was reversed by TPEN (114.2 ± 13.6% of control), implying that morphine inactivates GSK-3β via Zn²⁺ in cardiomyocytes. The effect of morphine on GSK-3β phosphorylation was further reversed by L-NAME (118.2 ± 9.4% of control), ODQ (110.3 ± 7.8% of control), and KT5823 (105.3 ± 9.9% of control). H₂O₂ (100 μM) reduced GSK-3β phosphorylation (76.2 ± 5.8% of control).

To determine whether morphine prevents the mPTP opening by inactivating GSK-3β, cardiac H9c2 cells were transfected with the constitutively active GSK-3β mutant (GSK-3β-S9A) plasmid and then treated with morphine. As shown in Fig. 6 (left), 600 μM H₂O₂ caused a dramatic decrease in TMRE fluorescence (38.8 ± 2.0% of baseline), indicating the mPTP opening by oxidant stress. The treatment of cells with 1 μM morphine prevented the loss of TMRE fluorescence (73.5 ± 4.9% of baseline), suggesting that morphine can modulate the mPTP opening in H9c2 cells. In contrast, morphine failed to preserve TMRE fluorescence in cells transfected with GSK-3β-S9A (37.9 ± 2.9% of baseline), suggesting that GSK-3β is critical for the action of morphine on the mPTP opening. To confirm the role of GSK-3β in the action of morphine in rat cardiomyocyte, we suppressed GSK-3β protein expression silencing GSK-3β RNA. GSK-3β silencing prevented the loss of TMRE fluorescence in control hearts, indicating that the suppression of GSK-3β protein levels leads to modulation of the mPTP opening (Fig. 6, right). In contrast, siRNA failed to further increase TMRE fluorescence in the hearts treated with morphine, implying that the preventive effect of morphine on the pore opening was attributable to inactivation of GSK-3β.

**Fig. 3.** Newport green DCF fluorescence intensity 10 min after exposure to morphine expressed as a percentage of baseline in isolated rat cardiomyocytes. Morphine (1 μM) markedly increased the fluorescence intensity compared with the control, which was reversed by the guanylyl cyclase inhibitor ODQ (5 μM) and NS2028 (NS, 1 μM) and protein kinase G (PKG) inhibitor KT5823 (KT; 1 μM). *P < 0.05 vs. control; #P < 0.05 vs. morphine.

**Fig. 4.** Tetramethylrhodamine ethyl ester (TMRE) fluorescence intensity 20 min after exposure to 100 μM H₂O₂ expressed as a percentage of baseline in isolated rat cardiomyocytes. When compared with the control, morphine (Morp; 1 μM) prevented oxidant-induced TMRE fluorescence reduction. The action of morphine was blocked by the guanylyl cyclase inhibitor ODQ; the PKG inhibitor KT5823 (KT; 1 μM), and the Zn²⁺ chelator TPEN (10 μM). ZnCl₂ (1 μM) and cyclosporin A (CsA; 0.2 μM) mimicked the effect of morphine by preserving TMRE fluorescence. *P < 0.05 vs. control; #P < 0.05 vs. morphine.

**Fig. 5.** Western blot analysis of glycogen synthase kinase 3β (GSK-3β) phosphorylation at Ser9 in cardiomyocytes. Cells were treated with 1 μM morphine for 10 min. Morphine significantly increased GSK-3β phosphorylation, an effect that was blocked by TPEN. *P < 0.05 vs. control; #P < 0.05 vs. morphine.

**Fig. 6.** Left panel: Correlation between the fluorescence intensity of TMRE and the mPTP opening in cardiac H9c2 cells treated with various concentrations of H₂O₂ (0, 50, 100 μM) and 1 μM morphine. *P < 0.05 vs. control; #P < 0.05 vs. morphine. Right panel: Western blot analysis of GSK-3β expression in rat cardiomyocytes transfected with GSK-3β-S9A plasmid and treated with or without morphine (1 μM) and H₂O₂ (100 μM) for 20 min. *P < 0.05 vs. control; #P < 0.05 vs. morphine.
DISCUSSION

In this study, we have demonstrated that morphine mobilizes intracellular Zn\(^{2+}\) via the NO/cGMP/PKG signaling pathway in cardiomyocytes. Zn\(^{2+}\) mediates the inhibitory effect of morphine on the mPTP opening by inactivating GSK-3β.

Postconditioning protects the heart from reperfusion injury by targeting the mPTP through activation of δ-opioid receptors and that the opioid receptor agonist morphine mimicked the effect of postconditioning by modulating oxidant-induced mPTP opening (21). Similarly, a recent study by Obame et al. (32) has also shown that morphine delayed the mPTP opening induced by anoxia/reoxygenation in rat cardiomyocytes. These observations indicate that opioid receptor activation protects the heart from ischemia-reperfusion injury by targeting the mPTP. Although inactivation of GSK-3β was proposed to be responsible for the preventive effect of morphine on the mPTP opening (32), the exact mechanism for the action of morphine on the mPTP remains unclear. We hypothesized that morphine mobilizes intracellular Zn\(^{2+}\) through the NO/cGMP/PKG signaling pathway and Zn\(^{2+}\) modulates the mPTP opening by inactivating GSK-3β. This hypothesis was based on the following observations. First, exogenous NO (11) mobilizes intracellular Zn\(^{2+}\) through the cGMP/PKG pathway and morphine can produce NO in cardiomyocytes (20). Second, Zn\(^{2+}\) inhibits the mPTP opening by inactivating GSK-3β in cardiac cells (4).

It is well known that the major intracellular Zn\(^{2+}\) binding protein is metallothionein (46). Nitrosylation of metallothionein has been proposed to be the mechanism by which NO at high doses (2 mM SNO or 0.2 and 2 mM DETA/NO) releases Zn\(^{2+}\) (3, 25, 38, 39). In a recent study, we have demonstrated that exogenous NO at a low dose mobilizes intracellular Zn\(^{2+}\) via the cGMP/PKG signaling pathway in cardiomyocytes (20). In the present study, we found that morphine mobilizes intracellular Zn\(^{2+}\) through the same signaling pathway. This was evidenced by the observation that the effect of morphine on Newport Green DCF fluorescence was blocked by the NOS inhibitor L-NAME, the guanylyl cyclase inhibitor ODQ, and the PKG inhibitor KT5823. In addition, morphine was also able to produce NO in cardiomyocytes. It is, therefore, reasonable to propose that morphine produces NO, which in turn mobilizes intracellular Zn\(^{2+}\) through the cGMP/PKG pathway. Since the cGMP/PKG pathway plays an important role in cardioprotection (5), the release of Zn\(^{2+}\) by morphine through this pathway may serve as an important mechanism for cardioprotection against ischemia-reperfusion injury.

Zn\(^{2+}\) has been demonstrated to prevent apoptosis both in vitro and in vivo models (28, 41). Recently, it has been reported that the treatment of isolated rat hearts at reperfusion with Zn\(^{2+}\) ionophore pyrithione induces cardioprotection against ischemia-reperfusion injury (23). Recent studies from our laboratory have shown that Zn\(^{2+}\) modulates oxidant-induced mPTP opening in isolated rat cardiomyocytes (20) and prevents reperfusion injury by targeting mPTP in cardiac H9c2 cells (4). In this study, the inhibitory effect of morphine on the mPTP was partially but significantly blocked by both the PKG inhibitor KT5823 and the Zn\(^{2+}\) chelator TPEN, suggesting that morphine protects mitochondria by mobilizing Zn\(^{2+}\) via the cGMP/PKG pathway. Although we report the role of zinc in the preventive effect of morphine on the mPTP opening in the setting of oxidant stress in this study, the release of zinc by morphine may play an important role in cardioprotection against reperfusion injury. We have recently demonstrated that...
intracellular free zinc levels were markedly decreased upon reperfusion. The adenosine receptor agonist 5’-(N-ethylcarboxamido) adenosine (NECA) given at reperfusion reversed the decrease in free zinc levels by mobilizing intracellular zinc, and this contributed to the anti-infarct effect of NECA (manuscript under review). Similarly, Karagulova et al. (23) have also demonstrated decreased levels of intracellular free zinc in isolated rat hearts subjected to ischemia-reperfusion. Thus the zinc release by morphine may also prevent the decrease in free zinc levels in the setting of ischemia-reperfusion, which may account in part for the cardioprotective effect of morphine at reperfusion. Mobilization of intracellular zinc at reperfusion may serve as a useful approach to reduce reperfusion injury.

Zn$^{2+}$ can regulate activities of many intracellular signaling elements including Akt (2), p70S6 kinase (24), mTOR (27), ERK (2), and GSK-3β (19). Among these signaling molecules, GSK-3β has recently been proposed to play a critical role in the modulation of the mPTP opening in cardiomyocytes. Many cardioprotective interventions protect the heart from ischemia-reperfusion injury by targeting the mPTP through inactivation of GSK-3β (12, 22, 31, 33, 34). GSK-3β activity is regulated by the phosphorylation of Ser9 and Tyr216 residues. Phosphorylation of Ser9 decreases, but Tyr216 phosphorylation increases of GSK-3β, suggesting that morphine may inactivate GSK-3β by the phosphorylation of Ser9 and Tyr216 residues. Phosphorylation of Ser9 decreases, but Tyr216 phosphorylation increases GSK-3β activity. Zn$^{2+}$ can inactivate GSK-3β (1, 19), and we have demonstrated that Zn$^{2+}$ prevents reperfusion injury by inactivating GSK-3β in cardiac cells (4). Gross et al. (14) have documented that GSK-3β inactivation accounts for the cardioprotective effect of morphine at reperfusion. In the present study, morphine markedly increased GSK-3β phosphorylation at Ser9 and this was inhibited by the Zn$^{2+}$ chelator TPEN, suggesting that morphine may inactivate GSK-3β through Zn$^{2+}$. In addition, the inhibitory effect of morphine on the mPTP opening was blocked by the Zn$^{2+}$ chelator TPEN and the action of morphine on the mPTP was lost in cells transfected with the constitutively active GSK-3β mutant (GSK-3β-S9A). Moreover, suppression of GSK-3β protein levels with siRNA could prevent the pore opening in the control but failed to enhance the protective effect of morphine. Therefore, it is reasonable to conclude that morphine modulates the mPTP opening by inactivating GSK-3β through Zn$^{2+}$. As to the mechanism by which mobilized Zn$^{2+}$ inactivates GSK-3β, we assume that Zn$^{2+}$ may inhibit GSK-3β through the Akt/phosphatidylinositol 3-kinase signaling pathway, as we documented previously (4).

In summary (Fig. 7), morphine mobilizes intracellular Zn$^{2+}$ via the NO/cGMP/PKG signaling pathway and modulates the mPTP opening by inactivating GSK-3β through Zn$^{2+}$. These findings provide new insights into the mechanism by which opioids protect the heart from ischemia-reperfusion injury.

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**DISCLOSURES**

No conflicts of interest are declared by the author(s).

**REFERENCES**

1. An WL, Bjorkdahl C, Liu R, Cowburn RF, Winblad B, Pei JJ. Mechanism of zinc-induced phosphorylation of p70 S6 kinase and glyco-

2. An WL, Pei JJ, Nishimura T, Winblad B, Cowburn RF. Zinc-induced anti-apoptotic effects in SH-SYSY neuroblastoma cells via the extracel-
16. Halseastrap AP, Clarke SJ, Javadov SA. Mitochondrial permeability transition pore opening during myocardial reperfusion—a target for car-
17. Hausenloy DJ, Duchen MR, Yellon DM. Inhibiting mitochondrial permeability transition pore opening at reperfusion protects against isch-
18. Huh J, Gross GJ, Nagase H, Liang BT. Protection of cardiac myocytes via δ-opioid receptors, protein kinase C, and mitochondrial KATP chan-
20. Jang Y, Wang H, Xi J, Mueller RA, Norfleet EA, Xu Z. NO mobilizes intracellular zinc$^{2+}$ via cGMP/PKG signaling pathway and prevents mitochon-


