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Involvement of Adenosine Monophosphate-Activated Protein Kinase in Morphine-induced Cardioprotection

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Background. **Adenosine monophosphate-activated protein kinase (AMPK) orchestrates the regulation of energy-generating and -consuming pathways, and protects the heart against ischemic injury and apoptosis. Recent progress shed light on various factors, including adiponectin, MIF, H11K, and metformin in the activation of AMPK. It is uncertain whether the activation of AMPK is contributed to cardioprotection of opioids. Here we show that morphine, an exogenous non-peptide opioid receptor agonist, can modulate the activation of the cardioprotective AMPK pathway during ischemia and exert anti-apoptotic effects through AMPK.**

Methods. **Isolated rat hearts were perfused on a constant pressure Langendorff system and subjected to 30min of global ischemia followed by 60min of reperfusion. The hearts received vehicles, morphine, a combination of morphine and compound C, a combination of morphine and STO609, a combination of morphine and BAPTA-AM at the onset of ischemia. Hemodynamics parameters, infarct size, release of intracellular creatine kinase, expression of AMPK, and terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling staining were analyzed.**

Results. **Morphine significantly increased phosphorylation level of Thr172 site on AMPK, left ventricular function, and reduced infarct size as a percentage of the area at risk (IS/AAR from 63% ± 7% to 40% ± 5%), release of intracellular creatine kinase (from 319 ± 46 to 156 ± 42IU/60min/gdw), apoptosis ratio (from 16% ± 2% to 5% ± 1.4%) during reperfusion in comparison with the control group. A inhibitor of AMPK, compound C abrogated phosphorylation of AMPK induced by morphine, the improvement in myocardial function, and the reduction of IS/AAR (58% ± 6%), release of intracellular creatine kinase (270 ± 40IU/60min/gdw), apoptosis ratio (13% ± 1.5%). A Ca^{2+}/calmodulin-dependent protein kinase kinase inhibitor STO609 and a chelator of intracellular Ca^{2+} stores BAPTA-AM also abolished the cardioprotection of morphine.**

Conclusions. **Morphine can ameliorate myocardial contractile dysfunction and limit infarct size following ischemia and reperfusion by a mechanism involving activation of AMPK, and activate AMPK by Ca^{2+}-CaMKKβ-dependent phosphorylation.**

Key Words: **morphine; adenosine monophosphate-activated protein kinase; cardioprotection; myocardial ischemia-reperfusion.**

INTRODUCTION

Adenosine monophosphate-activated protein kinase (AMPK) is a heterotrimeric protein kinase expressed in most mammalian tissue, including cardiac muscle [1], where it is activated by metabolic stresses and plays a pivotal role in regulating cellular metabolism for sustaining energy homeostasis under stress conditions. Once activated, AMPK increases energy supply by promoting adenosine triphosphate-generating pathways, including glucose transport, glycolysis, and fatty acid oxidation, while inhibiting energy-consuming anabolic pathways [2, 3]. Thus, AMPK has the potential to prevent myocardial injury during ischemia. Some studies have indicated that AMPK plays an important protective role in limiting damage and apoptotic activity associated with ischemia and reperfusion in the heart [4, 5], and mediates preconditioning in cardiomyocytes by regulating the activity and recruitment of sarcolemmal KATP channels [6]. Recent progress shed light on

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various factors, including adiponectin, MIF, H11 K, and metformin in the activation of AMPK [7–10], and the activation of AMPK is contributed to cardioprotection of these factors. AMPK activation depends largely on phosphorylation of the Thr-172 residue in the α–subunit, and can be positively activated by three identified upstream AMPK kinases, including LKB1, Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ) [11–13], transforming growth factor β-activated kinase 1 (TAK1)[14, 15]. Additionally, AMPK is allosterically activated by binding of AMP, and this can also promote phosphorylation of the Thr-172 residue [16]. Several studies have shown that CaMKKβ is expressed in myocardium and a rise in intracellular calcium ions can trigger activation of AMPK by Ca^{2+}-CaMKKβ-dependent pathway without detectable changes in the AMP/ATP ratio [13, 14, 17, 18], furthermore, CaMKKβ may account for early activation of AMPK during ischemia [15].

In 1986, Murry *et al.* found that brief ischemia to the myocardium initiates a cascade of biochemical events in cardiac myocytes that protects the heart muscle during subsequent ischemic insults; this phenomenon is called ischemic preconditioning (IPC). IPC is most often assessed by observations of reduced infarct size, attenuated mechanical dysfunction, or limited ultrastructural abnormality on reperfusion after prolonged ischemia [19]. It is well known that myocardial ischemia during the perioperative period is a major cause of morbidity and mortality. As a common drug used to induce and maintain analgesia, opioids can elicit cardioprotection and represent a promising new therapeutic strategy in patients undergoing cardiac surgery, myocardial infarction, percutaneous coronary intervention, and transplantation. Some studies suggest that opioid receptors are important triggers and/or mediators of preconditioning. Nonselective opioid receptor antagonists naloxone has been shown to block ischemic preconditioning [20–22], and agonists of these same receptors, morphine, has been shown to mimic IPC in intact animals, isolated hearts, or isolated cardiomyocytes [23]. However, the signaling pathway by which morphine promotes cardiomyocytes survival has not yet been fully delineated. It is well documented that morphine could elevate intracellular free calcium in cardiomyocytes, and there is some evidence for the involvement of IP3-dependent transient rise in calcium ions in cardioprotection of opioid receptor [24, 25]. Therefore, we hypothesized that morphine, an exogenous nonpeptide opioid agonist, can activate AMPK by the Ca^{2+}-CaMKKβ-dependent pathway in preconditioning.

MATERIALS AND METHODS

All experimental protocols were approved by the Animal Care and Use Committee of Capital Medical University and were consistent with the NIH policy on the use of experimental animal. Rabbit polyclonal antibodies against AMPKα, phospho-AMPKα (Thr-172) were acquired from Cell Signaling Technologies (Beverly, MA). Morphine, compound C (a pharmacological inhibitor of AMPK), STO609 (a specific inhibitor of CaMKKβ), BAPTA-AM (a cell-permeable Ca^{2+} chelator), and other reagents, unless specified below, were purchased from Sigma-Aldrich Company (St. Louis, MO).

Isolated Heart Preparation

Male Sprague-Dawley rats weighing 200 to 250 g were heparinized (500 IU/kg intraperitoneal) and 15 min later were anesthetized with sodium pentobarbital (60 mg/kg, intraperitoneal). The heart were rapidly removed and mounted to a noncirculating Langendorff apparatus and immediately perfused retrogradely at a constant perfusion pressure of 80 mmHg with Krebs-Ringer's solution (115 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.25 mM $CaCl_2$, 25 mM $NaHCO_3$, and 11 mM glucose) gassed with 95% O_2 –5% CO_2 (pH 7.4, temperature 37°C) [26]. An incision was made in the left atrium, and a fluid-filled latex balloon was passed through the mitral valve and placed in the left ventricle. The balloon was connected to a pressure transducer, and the balloon volume was adjusted to achieve a stable left ventricular end-diastolic pressure of 5 to 10 mmHg during initial equilibration. Left developed ventricular pressure (LVDP), end-diastolic pressure (LVDEP), heart rate (HR), and maximum rate of left ventricular pressure rise and fall (±dp/dt)were monitored *via* BL-420F multi-channels physiologic signal analysis system (Taimeng Technology Company, Chengdu, China). Hearts were maintained at 37°C during the ischemic period in a water-jacketed beaker. After the initial 15 min stabilization perfusion, any hearts showing intractable arrhythmia or low LVSP (LVSP $<$ 80 mmHg) was excluded from the study. The chemicals were solved in the K-H solution perfused *via* a separate side port with or without morphine for a period of 15 min prior to ischemia, respectively. The concentrations of all chemicals used in this study were based on previous studies [27–29].

Experimental Protocol

The current study consisted of two protocols, and rat hearts were randomized into 12 groups (Fig. 1). All experimental groups began with a 15 min perfusion period to allow for stabilization of the isolated hearts. First, we studied the concentration-dependent activation of AMPK by morphine in Protocol 1, which was including morphine 0 (control) and the three morphine treatment groups received different concentrations (3×10^{-7} M, 3×10^{-6} M, 3×10^{-5} M) ($n = 6$). Under protocol 2, hearts were randomly assigned to eight groups: control, morphine, compound C, morphine+compound C, BAPTA-AM, morphine+BAPTA-AM, STO609, and morphine+STO609. Protocol 2 comprised three series of experiments: hemodynamics (n = 7) and infarct size analysis ($n = 6$), measurement of release of intracellular creatine kinase ($n = 6$), and TdT-mediated dUTP nick end labeling assessment of apoptosis of the myocardium ($n = 6$).

Determination of Myocardial Infarct Size

Infarct size was determined by 2,3,5-triphenyltetrazolium chloride (TTC) staining as previously described [30, 31]. Briefly, hearts were frozen at 20 °C for 2 h at the end of the experiment and subsequently sliced transversely from apex to base into five 2-mm cross-sections. The sections were incubated at 37 °C for 30 min in 1% TTC in 0.1 M phosphate buffer with pH adjusted to 7.4. With this technique, viable sections were stained brick red, whereas infarcted myocardium failed to stain with TTC. Slices were fixed overnight in 4% formaldehyde, digitally photographed, and planimetric analysis was performed using Image Pro Plus ver. 6.0 (Media Cybernetics, Silver Spring, MA). Because the entire left ventricle was at risk (global ischemia), infarct size was determined by dividing the total necrotic

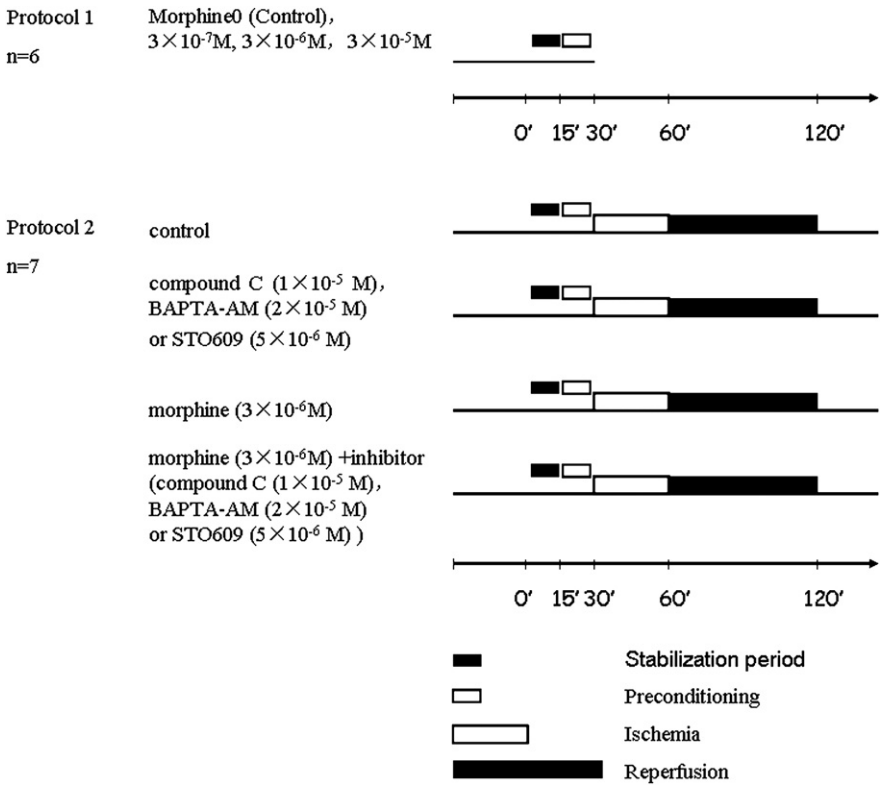

FIG. 1. Schematic diagram of experimental protocol, Protocol 1. All hearts were subjected to 15 min stabilization period and 15 min drug preconditioning period. After drug preconditioning period, the hearts were freeze-clamped and stored at –80 °C for further analysis. Protocol 2. All hearts were subjected to 30 min global ischemia and 60 min of reperfusion after 15 min stabilization period and 15 min drug preconditioning period.

area of the left ventricle by the total left ventricular slice area (IS/AAR).

Determination of Myocardial Injury *via* Creatine Kinase (CK) Efflux

Total perfusate over the 60 min of reperfusion period was collected to measure the release of creatine kinase (CK), and CK was measured by standard enzymatic methods. The hearts were stored for 2 d at 70°C for determination of dry weight. Total amount of CK released in the perfusate are expressed as IU/g dry weight of ventricle.

Western Blot Analysis for AMPKα Phosphorylation

Proteins of hearts were extracted with modified Radio Immuno Precipitation Assay (RIPA) buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 1 mM PMSF, 1 μg/mL of pepstatin, leupeptin, aprotinin, 1 mM $NaVO_4$, and 1 mM NaF, and 0.1% DS). After complete homogenization on ice rotator, samples were centrifuged at 12,000 rpm for 30 min at 4°C to precipitate cell debris. The supernatants were transferred into fresh tubes and protein concentrations were determined by BCA method. Proteins were fractionated by 10% SDS-PAGE and electro-transferred onto a polyvinylidene fluoride membrane. After blocking with Tris buffered saline (TBS) containing 5% non-fat milk, the membranes were probed with anti-phospho-AMPKα primary antibodies and anti-AMPKα primary antibodies (diluted 1:1000) at 4°C over night. Horseradish peroxidase-conjugated secondary antibody was used for luminochemical detection. Band intensities were quantified by densitometry. The results were normalized to anti-AMPKα.

TdT-mediated dUTP Nick End Labeling Assessment of Apoptosis

The apoptosis of myocardium were evaluated by a terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) procedure. The TUNEL assay was performed using a DeadEnd Fluorometric TUNEL System (Promega Corporation, Madison, WI). Briefly, the formaldehyde-fixed left ventricles were embedded in paraffin and cut into transverse sections (4 μm thick) and deparaffinized with xylene and a graded series of ethanol solutions. The slide was covered with 100 μL of equilibration buffer and equilibrated at room temperature for 5–10 min. Following that, the equilibration buffer was removed and 50 μL of rTdT incubation buffer was added to the cells on a 5 cm^2 area. The slides were incubated at 37°C for 60 min inside a dark, humidified chamber to allow tailing reaction to occur. The reactions were terminated by immersing the slides in terminal buffer 2 × SSC for 15 min and counterstained with blue fluorescence stain Hoechst. Images were captured using fluorescent microscopy. TUNEL-positive myocytes were determined by randomly counting five fields at 20 × 10 magnification from the region of myocardium at risk by a blinded observer, and were expressed as a percentage of the total cardiac myocyte population.

Statistical Analysis

All values were expressed as mean±SD. Hemodynamics data were analyzed with repeated measures ANOVA, with Bonferroni's multiple comparison test. For all other data, one-way analysis of variance (ANOVA) with Student-Newman-Keuls test for multiple comparisons was used. All P values were two-tailed, and statistical differences were considered significant when probabilities were $P < 0.05$. Statistical analysis was performed using SPSS software (Chicago, IL).

RESULTS

Morphine Activates AMPK in Heart

To examine whether morphine can activate AMPK in heart, we used Langendorff apparatus to perfuse four groups of hearts respectively with Krebs-Ringer solution containing 0 (control group), 3×10^{-7} M, 3×10^{-6} M, and 3×10^{-5} M morphine 15 min, and then the hearts were freeze-clamped and stored at –80°C (Protocol 1). AMPK activation depends largely on phosphorylation of the Thr-172 residue in the activating domain of the α-catalytic subunit. So we analyzed the phosphorylation of AMPKα by Western blot assay, total AMPK expression was as the internal control. As shown in Fig. 2, AMPK was almost not activated in control group, however morphine elevated the phosphor-AMPK protein level in a concentration-dependent manner. These data indicate that morphine activates AMPK in rat heart.

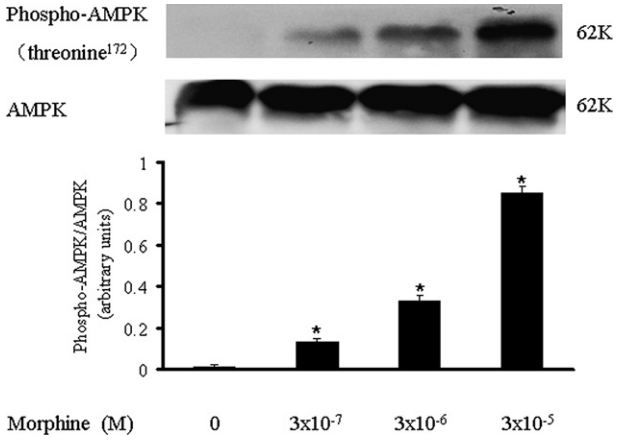

FIG. 2. Morphine-induced AMPK activation in a dose-dependent manner. Isolated hearts were perfused for 15 min stabilization period and 15 min different concentrations morphine preconditioning period, as described in Protocol 1. Data are mean±SD ($n = 6$ per group). *$P < 0.05$ *versus* untreated controls.

The Roles of AMPK in the Cardioprotective Actions of Morphine

To investigate the role of AMPK in the cardioprotection of morphine, we have designed the Protocol 2. All hearts were subjected to 15 min stabilization and 15 min drug preconditioning following 30 min global ischemia and 60 min of reperfusion in the Langendorff model. There was no significant difference in heart rate, LVDP, dp/dt, or –dp/dt between the groups before ischemia (Tables 1-4). However, the postischemic recoveries of heart rate, LVDP, dp/dt, or –dp/dt were significantly better in the morphine group than in the control group ($P < 0.05$; Tables 1-4), and administration of compound C, BAPTA-AM, or STO609 reversed the beneficial effect of morphine on LVDP, dp/dt, or –dp/dt ($P < 0.05$; Tables 1-4). Heart rate decreased in all groups during reperfusion with no significant differences between the groups. The release of the intracellular creatine kinase and infarct size (percentage of infarct *versus* total area at risk) was significantly higher in the control hearts compared with the hearts treated by morphine. Administration of compound C, BAPTA-AM, or STO609, respectively, reversed the beneficial effect of morphine.

Meanwhile, we analyzed the phosphorylation level of AMPK by Western blot assay under the same treatment. We found that morphine preconditioning significantly ($P < 0.05$ *versus* control) increased the phosphorylation level of AMPK concomitant with the improvement of myocardial contractile function and the reduction of the intracellular creatine kinase release and infarct size, while compound C (Fig. 6), STO-609 (Fig. 7), or BAPTA-AM (Fig. 8) blocked the morphine's effect on phospho-AMPK level accompanied by the reversion of cardioprotection of morphine.

TABLE 1

Changes of Heart Rate (bpm) During Myocardial Ischemia-Reperfusion

				Reperfusion (min)	
	Baseline	Intervention	Before ischemia	30	60
Control	316 ± 15	310 ± 15	309 ± 13	247 ± 15*	241 ± 11*
Morphine	332 ± 12	330 ± 12	328 ± 14	280 ± 17*,†	272 ± 14*,†
Compound C	322 ± 16	317 ± 17	317 ± 15	257 ± 17*	247 ± 17*
Morphine+compound C	321 ± 11	320 ± 11	318 ± 12	246 ± 16*	231 ± 16*
BAPTA-AM	324 ± 11	323 ± 11	320 ± 12	245 ± 14*	242 ± 14*
Morphine+BAPTA-AM	323 ± 11	319 ± 11	318 ± 11	250 ± 16*	238 ± 17*
STO609	316 ± 11	311 ± 11	310 ± 11	256 ± 15*	235 ± 15*
Morphine+STO609	321 ± 13	319 ± 13	319 ± 13	261 ± 15*	258 ± 15*

Data are presented as mean±SD ($n = 7$ per group) and compared by repeated measures ANOVA, with Bonferroni's multiple comparison test. Baseline = 15 min after stabilization.

*Significantly ($P < 0.05$) different from baseline (intragroup comparison).

†Significantly ($P < 0.05$) different from value in control (intergroup comparison).

TABLE 2

Changes of LVDP (mmHg) During Myocardial Ischemia-Reperfusion

				Reperfusion (min)	
	Baseline	Intervention	Before ischemia	30	60
Control	92 ± 7	93 ± 7	93 ± 8	37 ± 9*	32 ± 7*
Morphine	99 ± 11	94 ± 10	95 ± 11	55 ± 8*,†	53 ± 10*,†
Compound C	97 ± 9	96 ± 9	94 ± 10	36 ± 7*	30 ± 7*
Morphine+compound C	102 ± 11	102 ± 11	100 ± 11	38 ± 10*	38 ± 9*
BAPTA-AM	100 ± 8	100 ± 8	101 ± 8	33 ± 8*	32 ± 8*
Morphine+BAPTA-AM	104 ± 11	104 ± 11	103 ± 11	37 ± 7*	39 ± 10*
STO609	95 ± 10	94 ± 9	93 ± 9	37 ± 7*	36 ± 7 *
Morphine+STO609	97 ± 10	95 ± 10	95 ± 10	35 ± 8*	39 ± 8*

Data are presented as mean±SD ($n = 7$ per group) and compared by repeated measures ANOVA, with Bonferroni's multiple comparison test. Baseline = 15 min after stabilization.

*Significantly ($P < 0.05$) different from baseline (intragroup comparison).

†Significantly ($P < 0.05$) different from value in control (intergroup comparison).

The Roles of AMPK in the Anti-Apoptosis Effect of Morphine

The anti-apoptosis effect is involved in the cardioprotection of morphine [24]. Some recent studies showed that activation of AMPK reduced apoptosis during ischemia-reperfusion [4, 7]. To determine the role of AMPK in the anti-apoptosis effect of morphine preconditioning, we used the TUNEL assay to detect cells undergoing apoptosis. The results showed that pharmacologic blocking of AMPK by compound C led to increasing apoptosis ratio following the cardial ischemia/reperfusion (Fig. 5). This indicate that AMPK mediated the anti-apoptosis effect of morphine.

The Role of CaMKKβ and Ca^{2+} in Morphine Stimulated Activation of AMPK

To determine whether CaMKKβ and Ca^{2+} were the upstream kinase mediating activation of AMPK by Thr-172 phosphorylation, morphine stimulations of heart were performed in the presence and absence of inhibitor of CaMKKβ STO609 (5×10^{-6} M, 15 min) and intracellular Ca^{2+} chelator BAPTA-AM (2×10^{-5} M, 15 min). STO609 and BAPTA, respectively, led to an inhibition of morphine-stimulated AMPK phosphorylation (Fig. 7 and 8) and cardioprotection of AMPK (Tables 1-4, Figs. 3 and 4).

DISCUSSION

There were several observations in this study. First, using the rat Langendorff-perfused isolated heart model, we found morphine was able to activate AMPK by concentration-dependent way. Second, the protective effect of morphine against myocardial contractile dysfunction, release of the intracellular creatine kinase, infarct size, and apoptosis ratio following ischemia/reperfusion was inhibited by compound C, a pharmacologic blockade of AMPK, with phosphorylation of

TABLE 3

Changes of dP/dtmax (mm Hg/s) During Myocardial Ischemia-Reperfusion

				Reperfusion (min)	
	Baseline	Intervention	Before ischemia	30	60
Control	2993 ± 208	2925 ± 200	2925 ± 205	923 ± 160*	917 ± 170*
Morphine	3024 ± 142	3000 ± 140	3024 ± 142	1600 ± 207*,†	1615 ± 177*,†
Compound C	3055 ± 178	3000 ± 180	2984 ± 186	880 ± 200*	851 ± 198*
Morphine+compound C	3002 ± 154	2992 ± 143	2995 ± 150	960 ± 152*	991 ± 178*
BAPTA-AM	3025 ± 173	2925 ± 173	2825 ± 162	937 ± 149*	937 ± 149*
Morphine+BAPTA-AM	2900 ± 162	2980 ± 155	2988 ± 152	860 ± 190*	1034 ± 181*
STO609	2984 ± 175	2954 ± 165	2900 ± 165	874 ± 178*	864 ± 143*
Morphine+STO609	2994 ± 159	2984 ± 160	2902 ± 170	980 ± 180*	1150 ± 157*

Data are presented as mean±SD ($n = 7$ per group) and compared by repeated measures ANOVA, with Bonferroni's multiple comparison test. Baseline = 15 min after stabilization; dP/dt_{max} = maximum rate of left ventricular pressure rise.

*Significantly ($P < 0.05$) different from baseline (intragroup comparison).

†Significantly ($P < 0.05$) different from value in control (intergroup comparison).

TABLE 4

Changes of -dP/dtmax (mm Hg/s) During Myocardial Ischemia-Reperfusion

				Reperfusion (min)	
	Baseline	Intervention	Before ischemia	30	60
Control	2166 ± 160	2161 ± 160	2161 ± 160	696 ± 178*	689 ± 173*
Morphine	2177 ± 169	2167 ± 167	2178 ± 166	1051 ± 169*,†	1000 ± 173*,†
Compound C	2160 ± 182	2159 ± 179	2159 ± 178	671 ± 165*	665 ± 164*
Morphine+compound C	2098 ± 180	2094 ± 174	2096 ± 172	661 ± 152*	648 ± 151*
BAPTA-AM	2140 ± 173	2136 ± 170	2138 ± 172	670 ± 167*	666 ± 160*
Morphine+BAPTA-AM	2179 ± 164	2173 ± 161	2175 ± 161	699 ± 165*	689 ± 162*
STO609	2136 ± 170	2138 ± 168	2134 ± 169	707 ± 175*	636 ± 170*
Morphine+STO609	2116 ± 180	2116 ± 181	2110 ± 177	737 ± 175*	686 ± 171*

Data are presented as mean±SD ($n = 7$ per group) and compared by repeated measures ANOVA, with Bonferroni's multiple comparison test. Baseline = 15 min after stabilization; -dP/dtmax = maximum rate of left ventricular pressure fall.

*Significantly ($P < 0.05$) different from baseline (intragroup comparison).

†Significantly ($P < 0.05$) different from value in control (intergroup comparison).

AMPK repressed. Third, cardioprotection of AMPK was inhibited, respectively, by STO609, a specific inhibitor of CaMKKβ and BAPTA-AM, a cell-permeable Ca^{2+} chelator. Finally, anti-apoptotic effect of AMPK was blocked by compound C, STO609, and BAPTA-AM. Taken together, these findings suggest that AMPK has a critical role in mediating the metabolic and functional responses of the heart to pharmacologic preconditioning with morphine.

Although the cardioprotection of AMPK against ischemia and reperfusion injury has been investigated extensively, and multiple mechanisms by which AMPK activation protects ischemic heart have been identified, few studies have directly linked alterations in AMPK activity with alterations in cardiac function [32, 33]. Studies in isolated perfused heart model suggested that loss of AMPK function significantly impaired recovery of postischemic heart contractile function [4, 34]. Recently, using AMPK-deficient cardiac models, Wang *et al.* demonstrated that AMPK activation was beneficial to functional recovery during reperfusion *in vivo* [5]. Presently, in the rat Langendorff-perfused isolated heart model, our results demonstrate that AMPK activation contributes to the cardioprotection of morphine by a meliorating recovery of left ventricular contractile function during ischemia/reperfusion *in vitro*. Additionally, our data showed that when AMPK activation was blocked, the release of the intracellular creatine kinase and degree of myocardial infarct size during reperfusion were increased, indicating that AMPK activated by morphine prevents myocardium from membrane damage and myocardial necrosis.

The role of AMPK in both anti-apoptotic and pro-apoptotic actions has been reported. But in the heart, the majority of studies have suggested that AMPK activation is anti-apoptotic. The higher energy demand in the intact heart may explain these differences. During ischemia, energy starvation is an important trigger of apoptosis, and the energy generating actions of AMPK in the intact heart may provide an anti-apoptotic action that can overcome the potential effects of AMPK

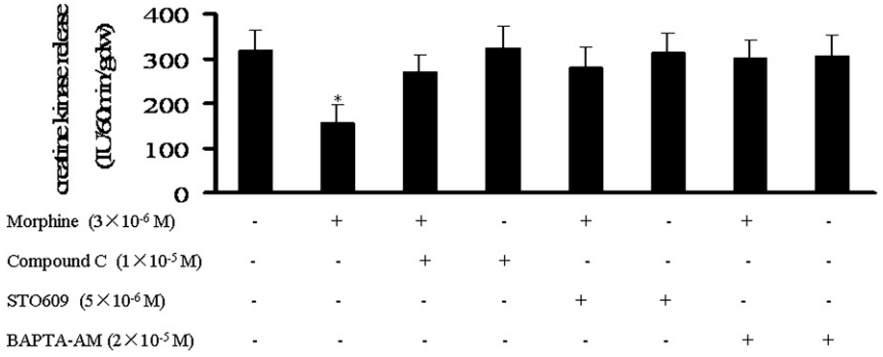

FIG. 3. Release of myocardial creatine kinase during the 60 min reperfusion period per gram heart dry weight (gdw) for the eight different groups. Hearts were subjected to 30 min global ischemia and 60 min of reperfusion after 15 min stabilization period and 15 min drug preconditioning period. Data are mean±SD ($n = 6$ per group). *$P < 0.05$ *versus* untreated controls.

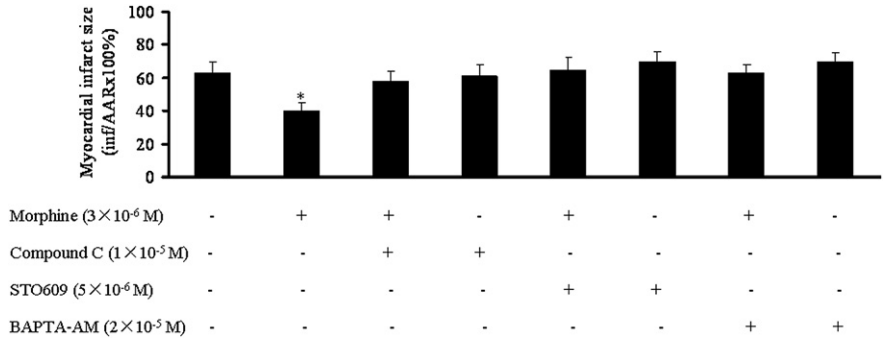

FIG. 4. Myocardial Infarct size (IS/AAR, as a percentage of infarct in area at risk) was determined by 2,3,5-triphenyltetrazolium staining. Hearts were subjected to 30 min global ischemia and 60 min of reperfusion after 15 min stabilization period and 15 min drug preconditioning period. Data are mean±SD ($n = 6$ per group). *$P < 0.05$ *versus* untreated controls.

activation on pro-apoptotic pathways such as p53 activation or mitochondrial Bax translocation [35]. AMPK activation reducing apoptosis was found in intact animals [5], isolated hearts [4], or cultured cardiomyocytes [36] during ischemia-reperfusion. Recently, a study indicated that in the ischemic heart, AMPK up-regulated spermidine/spermine acetyl-transferase (SSAT), a catabolic enzyme involved in polyamine metabolism, which limited activation of pro-apoptotic pathways [37]. Additionally, AMPK also mediated the anti-apoptotic effects of adiponectin in both mice and isolated cardiomyocytes during ischemia/reperfusion [7]. To test whether AMPK signaling was involved in the anti-apoptotic actions of morphine, we pretreated heart, respectively, with a combination of morphine and compound C. The results showed that the inhibitors reversed the inhibitory effects of morphine on apoptosis under conditions of ischemia-reperfusion. These results are consistent with the former findings, and suggest that AMPK mediates the anti-apoptotic effect of morphine and contributes to the cardioprotection of morphine. Our present study supports the notion that AMPK plays an anti-apoptotic role during myocardium ischemia-reperfusion.

The ability of morphine to elevate intracellular free calcium has been well documented in cardiomyocytes. The signaling mechanism responsible for the cardioprotection of morphine involves activation of the inositol phosphate pathway and Ca^{2+} release from intracellular stores [38]. More recent studies suggested that thrombin, bradykinin, oxytocin, and caffeine all stimulated Ca^{2+} release from intracellular stores and activated AMPK *via* Ca^{2+}-CaMKKβ-dependent pathway [28, 39–41)]. To determine whether calcium transient are required for the activation of AMPK by morphine, BAPTA-AM (a cell-permeable Ca^{2+} chelator) was used to block calcium signal induced by morphine. Our data showed that BAPTA-AM significantly inhibited phosphorylation level of Thr172 site on AMPK and abrogated the cardioprotection of morphine. Furthermore, another inhibitor, STO609, a cell permeable selective inhibitor of CaMKKβ, also blocked morphine-stimulated AMPK phosphorylation and myocardioprotection. These data indicate that Ca^{2+}-CaMKKβ-dependent pathway mediates AMPK activation by morphine. This is consistent with the well-described myocardioprotection role of

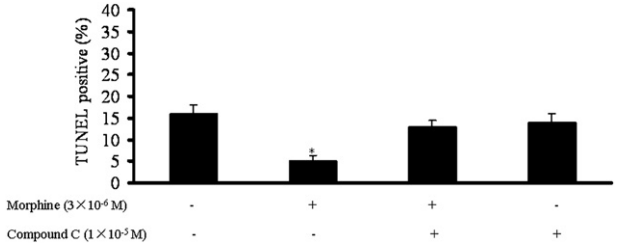

FIG. 5. Cardiomyocyte apoptosis determined by TUNEL staining. Hearts were subjected to 30 min global ischemia and 60 min of reperfusion after 15 min stabilization period and 15 min drug preconditioning period. Data are mean±SD ($n = 6$ per group). *$P < 0.05$ *versus* untreated controls.

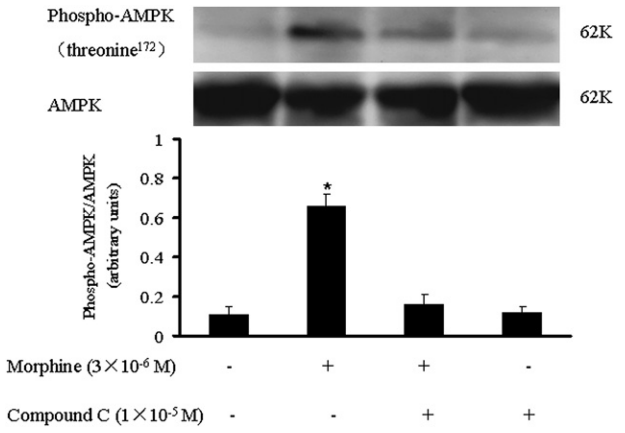

FIG. 6. Compound C inhibits AMPK activation. Hearts were subjected to 30 min global ischemia and 60 min of reperfusion after 15 min stabilization period and 15 min drug preconditioning period. Data are mean±SD ($n = 6$ per group). *$P < 0.05$ *versus* untreated controls.

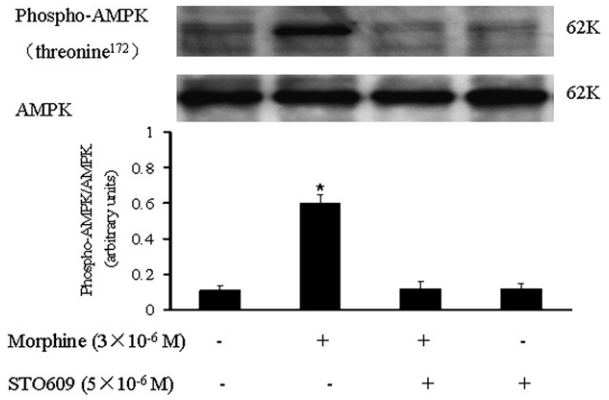

FIG. 7. Morphine-induced AMPK activation is inhibited by STO609,a specific inhibitor of CaMKKβ. Hearts were subjected to 30 min global ischemia and 60 min of reperfusion after 15 min stabilization period and 15 min drug preconditioning period. Data are mean±SD ($n = 6$ per group). *$P < 0.05$ *versus* untreated controls.

intracellular calcium transient rise in cardiomyocytes caused by morphine [24].

Of course, there are several limitations in our study. First, in order to further verify our finding, genetic altered animal model should be used. Secondly, this work lacks detailed molecular mechanism. Further study should focus on a cell model system with biochemistry and molecular biology methods. Finally, a method combining rat coronary artery ligation model and Langendorff to study cardioprotection may reveal more detailed information about how morphine works.

In summary, our results from an isolated rat heart model provide a novel mechanism that morphine can ameliorate myocardial contractile dysfunction, reduce apoptosis, and limit infarct size following ischemia and reperfusion by a mechanism involving activation of AMPK, and activate AMPK by Ca^{2+}-CaMKKβ-dependent phosphorylation.

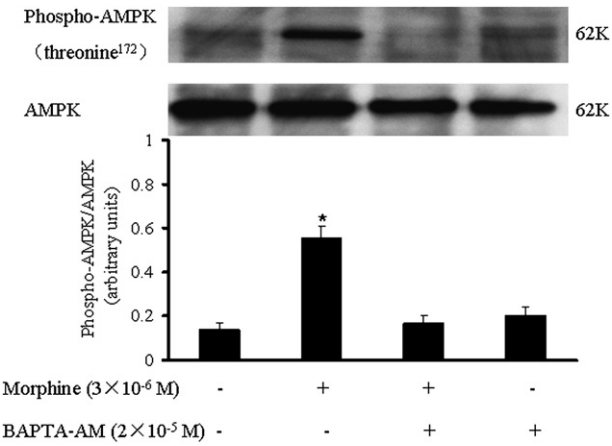

FIG. 8. Morphine-induced AMPK activation is inhibited by BAPTA-AM, a cell-permeable Ca^{2+} chelator. Hearts were subjected to 30 min global ischemia and 60 min of reperfusion after 15 min stabilization period and 15 min drug preconditioning period. Data are mean±SD ($n = 6$ per group). *$P < 0.05$ *versus* untreated controls.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jss.2009.11.007.

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