

Inducible Nitric Oxide Synthase Mediates Delayed Cardioprotection Induced by Morphine In Vivo

Evidence from Pharmacologic Inhibition and Gene-knockout Mice

Xiaojing Jiang, M.D., Enyi Shi, M.D.,† Yoshiki Nakajima, M.D., Ph.D.,‡ Shigehito Sato, M.D.§*

Background: **It is not known whether morphine induces delayed cardioprotection against ischemia and reperfusion. The authors measured the delayed preconditioning induced by morphine and determined the role of inducible nitric oxide synthase (iNOS) in mediating this effect using a pharmacological inhibitor and iNOS gene-knockout mice.**

Methods: **Adult male wild-type and iNOS gene-knockout (B6, 129) mice were treated with morphine (0.3 or 0.1 mg/kg intraperitoneal) or saline. Twenty-four hours later, mice were subjected to 45 min of coronary artery occlusion followed by 120 min of reperfusion. *S*-methylthiourea sulfate (3 mg/kg, intraperitoneal) was given 30 min before the occlusion to block iNOS. Infarct size as a percentage of the area at risk was determined by triphenyltetrazolium chloride staining. iNOS and endothelial nitric oxide synthase expression were measured by Western blot.**

Results: **Infarct size was significantly reduced in wild-type mice from 43.1 ± 5.3% in the saline group to 22.4 ± 4.4% in the higher-dose morphine group (0.3 mg/kg) ($P < 0.05$). This cardioprotective effect was abolished by *S*-methylthiourea sulfate (43.3 ± 3.9%) and was absent in iNOS gene-knockout mice (42.3 ± 4.7%). Pretreatment with the lower dose of morphine (0.1 mg/kg) did not reduce infarct size (41.1 ± 5.4%). A significant increase in myocardial iNOS expression was observed 24 h after morphine administration (0.3 mg/kg but not 0.1 mg/kg; $P < 0.05$), whereas endothelial nitric oxide synthase remained unchanged.**

Conclusions: **Pretreatment with morphine induces delayed cardioprotection in mice. The authors demonstrated an obligatory role for iNOS in mediating morphine-induced delayed cardioprotection.**

CARDIOPROTECTION induced by ischemic preconditioning is a biphasic event. The acute or early phase occurs immediately after the ischemic preconditioning stimulus and lasts for 1–3 h,[1] whereas the delayed or late phase is seen 12–24 h after the initial stimulus and lasts up to 72 h.[2] Opioids have been shown to confer both the early and the late phase of cardioprotection, similar to ischemic preconditioning. It was first reported by Schultz *et al.*[3] that opioid receptor stimulation results in a reduction in infarct size in an *in vivo* rat model. When administered 24–48 h before a lethal ischemic injury, δ-opioid receptor agonists were demonstrated to produce a delayed cardioprotective effect.[4–6] As one of the most widely used opioids for the treatment of pain, morphine has been shown to induce the early phase cardioprotection in cardiac myocytes,[7] isolated working hearts,[8] and *in vivo* models.[3,9] In contrast, no work has been conducted regarding the delayed cardioprotection induced by morphine.

Nitric oxide is an essential modulator of biologic systems, including the cardiovascular system. It is critical in signal transduction of the ischemic myocardium.[10] The synthesis of nitric oxide is catalyzed by three isoforms of nitric oxide synthase (NOS), nNOS (neuronal), iNOS (inducible), and eNOS (endothelial). There is mounting evidence suggesting that iNOS is an essential trigger and mediator of the late phase of myocardial preconditioning induced by ischemia preconditioning[11] or by a number of pharmacologic agents.[12–14] eNOS may contribute to the early[15,16] as well as the late cardioprotection.[17] In isolated myocytes, nitric oxide is produced as a result of δ-opioid receptor stimulation and mediates the opioid-induced cardioprotection.[18] However, nothing is known about the effects of NOS in morphine-induced cardioprotection. Although no work has been performed regarding the role of morphine on nitric oxide activities in myocardium, it has been shown that morphine facilitates nitric oxide release in human monocytes, neutrophils, and endothelial cells and stimulates iNOS expression in macrophages.[19,20]

The current study sought to examine the possibility of morphine-induced delayed cardioprotection in a murine model of coronary artery occlusion and reperfusion injury and to determine the potential involvement of iNOS in this protection using a pharmacologic inhibitor and gene-knockout mice.

Materials and Methods

Animals

Adult male iNOS gene-knockout (−/−) B6, 129 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Wild-type (WT) mice (Jackson Laboratory) with a genetic background as close as possible to the iNOS gene-knockout mice (B6129PF2/J) were used as controls.[11,21] The mean body weight of these animals was 31.4 ± 3.0 g. The experimental protocol was approved by the Ethics Review Committee for Animal

* Graduate Student in Anesthesiology, ‡ Instructor, § Professor and Chairman, Department of Anesthesiology, † Research Fellow, First Department of Surgery, Hamamatsu University School of Medicine.

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Address reprint requests to Dr. Sato: Department of Anesthesiology, Hamamatsu University School of Medicine, 1-20-1 Handayama, Hamamatsu, 431-3192, Japan. Address electronic mail to: ssato@hama-med.ac.jp, jiangxj@hama-med.ac.jp.

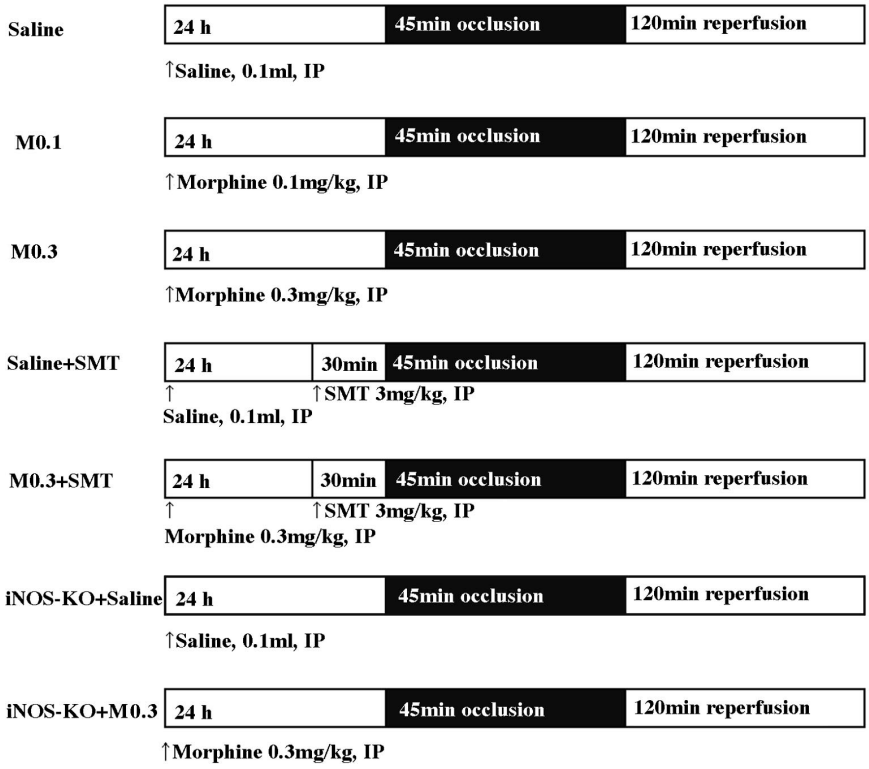

Fig. 1. Experimental groups and protocol. iNOS-KO = inducible nitric oxide synthase gene-knockout mouse; IP = intraperitoneal; M0.1 = 0.1 mg/kg morphine; 0.3 M0.3 = 0.3 mg/kg morphine; SMT = *S*-methylthiourea sulfate.

Experimentation of Hamamatsu University School of Medicine (Hamamatsu, Japan) and in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.[22]

Drugs and Chemicals

Morphine was purchased from Shionogi Co. (Osaka, Japan). *S*-methylthiourea sulfate (SMT), a selective inhibitor of iNOS, Evans blue, and triphenyltetrazolium chloride were purchased from Sigma Chemical (St. Louis, MO).

Surgical Procedures

Animals were anesthetized with pentobarbital sodium (50 mg/kg intraperitoneal), and anesthesia was maintained *via* supplemental doses of pentobarbital sodium (20 mg/kg intraperitoneal) as needed. A tracheotomy was performed, and the animals were mechanically ventilated using a rodent ventilator (SN-480-7; Shinano Manufacturing Co., Tokyo, Japan). The right carotid artery was cannulated with polyethylene-10 tubing to monitor hemodynamics. Core temperature was measured *via* a rectal temperature probe and was maintained at 36.5v–37.5°C with a heating pad. After an equilibration period of 10 min, a thoracotomy was performed. Ligation of the left anterior descending coronary artery was performed using a 7-0 silk suture with a small piece of polyethylene tubing to secure the ligature without damaging the artery. Ischemia was confirmed by visual inspection of blanching in the myocardium distal to the site of occlusion. After a period of coronary occlusion of 45 min, the 7-0 silk ligature was removed. Reperfusion was confirmed visually by the return of a red color in the region that was previously pale.

Experimental Protocols

Mice were pretreated with morphine or an equal volume of saline 24 h before a 45-min period of coronary occlusion followed by 120 min of reperfusion. Seven groups were enrolled (n = 8 or 9 mice/group), as shown in figure 1. WT mice were randomly divided into the following five groups: (1) saline: pretreated with 0.1 ml intraperitoneal normal saline; (2) M0.1: pretreated with 0.1 mg/kg intraperitoneal morphine; (3) M0.3: pretreated with 0.3 mg/kg intraperitoneal morphine; (4) saline + SMT: pretreated with 0.1 ml intraperitoneal saline and 3 mg/kg intraperitoneal SMT, which was administered 30 min before coronary occlusion; and (5) M0.3 + SMT: pretreated with 0.3 mg/kg intraperitoneal morphine and 3 mg/kg intraperitoneal SMT, which was administered 30 min before coronary occlusion. iNOS gene-knockout mice were assigned randomly to another two groups: (6) iNOS-KO + saline: pretreated with 0.1 ml intraperitoneal saline; and (7) iNOS-KO + M0.3: pretreated with 0.3 mg/kg intraperitoneal morphine.

Determination of Risk and Infarct Sizes

At the end of the 120-min reperfusion period, the left anterior descending coronary artery was occluded again, and 1.0% Evans blue (1.5 ml) was infused into the carotid

artery catheter in a retrograde manner to delineate the ischemic (area at risk [AAR]) from the nonischemic zones. The heart was then excised and cut into five transverse slices of approximately equal thickness (approximately 1 mm). Incubation of sections in 1% triphenyltetrazolium chloride for 5 min at 37°C allowed differentiation between the viable (brick red) and necrotic (pale) areas of the previously rendered ischemic myocardium. All atrial and right ventricular tissues were excised. After fixation in a 10% formalin solution, each slice was weighed and photographed. The left ventricular area, AAR (absence of blue dye), and area of infarction (pale) for each slice were then determined by computer planimetry using National Institutes of Health Image software (Bethesda, MD). The weight of the myocardial infarct was determined as $(A_1 \times W_1) + (A_2 \times W_2) + (A_3 \times W_3) + (A_4 \times W_4) + (A_5 \times W_5)$, where A is the percent area of infarction obtained by planimetry from subscripted numbers 1–5 representing sections, and W is the weight of the corresponding numbered sections. The weight of the AAR was calculated in a similar fashion. The AAR was expressed as a percentage of the weight of left ventricle, and the infarct size (IS) was expressed as a percentage of the AAR.[15,23]

Western Blot Analysis

In a parallel series of experiments, ventricular tissue samples were collected from the saline, M0.1, M0.3, iNOS-KO + saline, and iNOS-KO + M0.3 groups (n = 3–7/group). To determine the time course of iNOS expression, WT mice hearts were removed at 0.5, 1, 2, 6, 12, 18, and 24 h after 0.3 mg/kg morphine administration (n = 3/group). Three additional nontreated hearts were used as controls. Samples were homogenized in 1 ml ice-cold RIPA buffer. The homogenate was centrifuged at 4°C for 30 min at 15,000 rpm, and the supernatant was recovered as the total cellular protein. All protein extracts were analyzed simultaneously for protein concentration to ensure equal protein loading between samples. Protein extracts were separated on 8% sodium dodecyl sulfate–polyacrylamide gels (60 μg protein/lane) and then transferred onto a nitrocellulose membrane. Blots were blocked for 1 h at room temperature with 5% nonfat dry milk in Tris-buffered saline and 0.1% Tween-20 and were then washed in Tween-20. The membrane was incubated with a polyclonal rabbit antibody against mouse iNOS or eNOS (dilution, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. After a washing with Tween-20, the membrane was incubated with an anti-rabbit horseradish peroxidase–linked antibody (dilution, 1:5000; Nacalai Tesque, Kyoto, Japan) for 1 h. The membranes were developed using enhanced chemiluminescence and exposed to x-ray film for the appropriate duration. The optical density for each band on the Western blot was quantified using the National Institutes of Health Image program and was normalized against the background density for each gel.[24] Each protein signal was also normalized to the corresponding Ponceau stain signal.

Statistical Analysis

All values are reported as mean ± SD. Differences among the groups were analyzed with multiple analyses of variance for repeated measures followed by Student-Newman-Keuls *post hoc* test. Statistical significance was defined as $P < 0.05$.

Results

A total of 61 mice were enrolled in the current study. Ten mice were excluded because of death or marked hypotension (mean arterial blood pressure below 30 mmHg) during coronary occlusion and reperfusion. Another two mice were omitted as a result of technical difficulties with the experimental preparation. Complete data were obtained in the remaining 49 mice. Final numbers in the groups are as follows: saline, n = 7; M0.1, n = 6; M0.3, n = 7; saline + SMT, n = 6; M0.3 + SMT, n = 8; iNOS-KO + saline, n = 7; iNOS-KO + M0.3, n = 8.

Hemodynamics

Mean arterial blood pressure and heart rate were recorded in all groups throughout the experimental protocols. The rate–pressure product (mean arterial blood pressure × heart rate/1,000) was calculated as an index of oxygen demand. Table 1 indicates the hemodynamic values from all seven groups. No significant differences in baseline hemodynamic values were observed among these groups. Compared with the saline group, the mean arterial blood pressure was higher in the M0.3 group after 20 min of ischemia ($P < 0.05$) and was decreased in the M0.3 + SMT group after 60 min of reperfusion ($P < 0.05$). After 60 min reperfusion, the M0.3 group exhibited a lower heart rate than the saline group ($P < 0.05$). No significant differences in rate–pressure product were found among these groups at any time point. Coronary artery occlusion and reperfusion produced similar increases in heart rate and decreases in mean arterial blood pressure and rate–pressure product in all experimental groups.

Infarct Size

Figure 2 summarizes the data of the AAR and IS in all groups. No significant differences in AAR/left ventricle were observed between all groups. IS/AAR was 43.1 ± 5.3% in the saline group. Pretreatment with 0.3 mg/kg morphine markedly reduced the IS/AAR to 22.4 ± 4.4% ($P < 0.05$ *vs.* saline group); however, pretreatment with 0.1 mg/kg morphine did not reduce the infarct size (41.1 ± 5.4%; $P > 0.05$ *vs.* saline group). SMT abolished

Table 1. Hemodynamic Data

	n	Baseline	Ischemia, 20 min	Ischemia, 45 min	Reperfusion, 60 min	Reperfusion, 120 min
MABP, mmHg						
Saline	7	77 ± 7	65 ± 6*	65 ± 7*	58 ± 8*	56 ± 6*
M0.1	6	72 ± 8	65 ± 5	61 ± 6*	57 ± 7*	53 ± 6*
M0.3	7	80 ± 4	75 ± 3†	63 ± 7*	59 ± 7*	53 ± 6*
Saline + SMT	6	74 ± 11	67 ± 9	64 ± 9	53 ± 6*	53 ± 7*
M0.3 + SMT	8	76 ± 6	70 ± 7*	58 ± 4*	49 ± 4*†	52 ± 4*
iNOS-KO + saline	7	71 ± 5	66 ± 4*	62 ± 4*	57 ± 3*	54 ± 4*
iNOS-KO + M0.3	8	74 ± 2	67 ± 5*	62 ± 5*	55 ± 5*	60 ± 4*
HR, beats/min						
Saline	7	437 ± 21	455 ± 13	459 ± 14*	481 ± 17*	488 ± 18*
M0.1	6	433 ± 26	454 ± 18	453 ± 18	464 ± 22	471 ± 21*
M0.3	7	421 ± 12	443 ± 8*	452 ± 11*	464 ± 9*†	470 ± 16*
Saline + SMT	6	444 ± 14	458 ± 13	467 ± 19	491 ± 17*	500 ± 23*
M0.3 + SMT	8	433 ± 11	464 ± 12*	469 ± 11*	486 ± 6*	484 ± 13*
iNOS-KO + saline	7	428 ± 17	453 ± 10*	464 ± 4*	479 ± 7*	491 ± 9*
iNOS-KO + M0.3	8	446 ± 19	442 ± 16	468 ± 15*	476 ± 14*	474 ± 16*
RPP, mmHg · min^{-1} · 1,000^{-1}						
Saline	7	33.7 ± 3.5	29.4 ± 3.1	30.0 ± 3.5	28.2 ± 4.5*	27.3 ± 3.3*
M0.1	6	31.3 ± 3.8	29.6 ± 3.0	27.4 ± 3.3	26.5 ± 3.6	25.1 ± 2.7*
M0.3	7	33.5 ± 1.8	33.1 ± 1.5	28.7 ± 3.7*	27.4 ± 3.3*	24.9 ± 3.1*
Saline + SMT	6	32.8 ± 4.9	30.6 ± 4.7	29.7 ± 4.8	25.9 ± 3.6	26.7 ± 4.2
M0.3 + SMT	8	32.8 ± 2.9	32.3 ± 2.5	27.1 ± 2.3*	23.8 ± 1.9*	25.0 ± 2.5*
iNOS-KO + saline	7	30.4 ± 3.1	29.9 ± 2.4	28.7 ± 1.8	27.2 ± 1.2*	26.3 ± 1.9*
iNOS-KO + M0.3	8	32.9 ± 2.2	29.7 ± 2.8*	29.0 ± 2.3*	26.4 ± 2.8*	28.6 ± 2.3*

Values are given as mean ± SD.

* $P < 0.05$ compared with baseline. † $P < 0.05$ compared with the respective value in saline group.

HR = heart rate; iNOS-KO = inducible nitric oxide synthase gene–knockout mouse; M0.3 = 0.3 mg/kg morphine; M0.1 = 0.1 mg/kg morphine; MABP = mean arterial blood pressure; n = number of animals; RPP = rate–pressure product (MABP × HR/1,000); SMT = *S*-methylthiourea sulfate.

this cardioprotective effect, as indicated by an increase of IS/AAR to 43.3 ± 3.9% ($P > 0.05$ *vs.* saline group). SMT by itself did not affect the infarct size. Morphine (0.3 mg/kg) pretreatment in iNOS knockout mice did not reduce the infarct size (42.3 ± 4.7%; $P > 0.05$ *vs.* iNOS-KO + saline group). No significant differences in IS/AAR were observed between the saline group and iNOS-KO + saline group, as reported previously.[12,25]

Effect of Morphine on Induction of iNOS Protein

Densitometric analysis of the Western blot images revealed that pretreatment with 0.3 mg/kg but not 0.1 mg/kg morphine resulted in significantly enhanced expression of myocardial iNOS compared with the saline-treated hearts. Consistent with the knockout status of the mice, no iNOS protein was expressed in either of the iNOS-KO + saline or iNOS-KO + M0.3 groups (fig. 3). A representative photograph of a Western blot from hearts harvested at different time points after 0.3 mg/kg morphine pretreatment and controls is shown in figure 4. Densitometric analysis indicated that the induction of iNOS reached significance 18 and 24 h after morphine administration, compared with controls.

eNOS Expression

Endothelial nitric oxide synthase was constitutively expressed in both WT and iNOS gene-knockout mouse hearts. The higher dose (0.3 mg/kg) of morphine caused a slight increase, whereas the lower dose (0.1 mg/kg) caused a slight decrease in eNOS expression; however, these differences were not significant ($P > 0.05$). No significant differences in eNOS expression was found between the iNOS-KO + saline and iNOS-KO + M0.3 groups ($P > 0.05$). A representative Western blot image and the results of the densitometric analysis are depicted in figure 5.

Discussion

The most important findings of this study are summarized as follows: (1) Higher doses of morphine (0.3 mg/kg), when administered 24 h before ischemia and reperfusion, resulted in a significant reduction of myocardial infarct size. (2) This cardioprotective effect was abolished by previous pharmacologic inhibition of iNOS and was absent in mice with targeted ablation of the iNOS gene. (3) The cardioprotective dose of morphine pretreatment significantly enhanced the expression of iNOS in myocardium. In summary, these data demonstrate that high-dose morphine induces a significant delayed cardioprotection in mice and that iNOS mediates this effect.

Using selective δ-opioid receptor agonists, the biphasic nature of the opioid-induced cardioprotection against myocardial infarction has been demonstrated in several mammalian species[4–6] and even human myocytes.[26] δ-Opioid agonist mediated delayed cardioprotection has

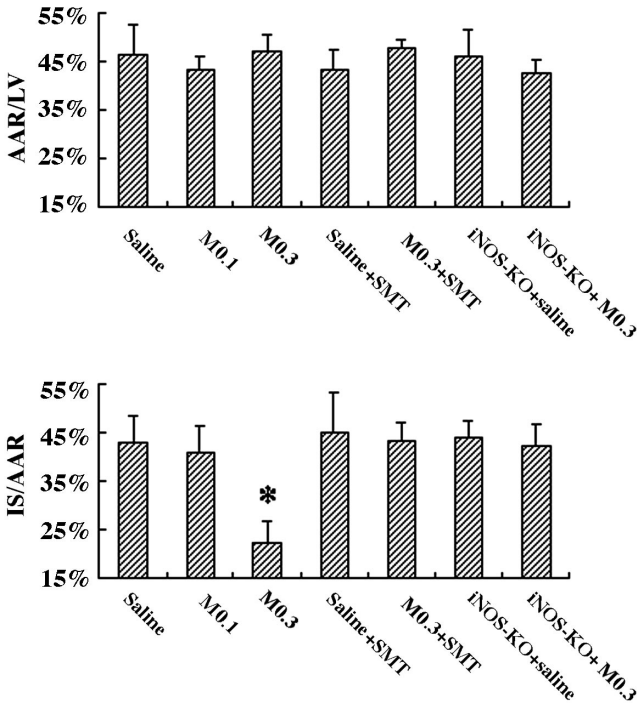

Fig. 2. Area at risk (AAR) expressed as a percentage of the left ventricle (LV) and myocardial infarct size (IS) expressed as a percentage of AAR in all experimental groups. Saline: mice received saline 0.1 ml 24 h before coronary occlusion. M0.1 or M0.3: mice received 0.1 or 0.3 mg/kg morphine, respectively, 24 h before coronary occlusion. *S*-methylthiourea sulfate (SMT) was given 30 min before coronary occlusion. Inducible nitric oxide synthase gene-knockout mice (iNOS-KO) were pretreated with saline or morphine. * $P < 0.05$ *versus* saline group.

been also found to be only partially dependent on δ-opioid receptor stimulation and *via* a free radical mechanism.[27] Morphine, a μ-opioid receptor agonist with δ-opioid receptor properties,[28] has been shown to induce acute cardioprotection by a large number of *in vivo* and *in vitro* studies.[3,7–9] However, it remains unknown whether morphine could also induce delayed cardioprotection. The current investigation demonstrates that morphine induced late cardioprotective effects in a dose-dependent manner. A higher dose (0.3 mg/kg) of morphine was necessary to achieve significant reduction of the infarct size, whereas the lower dose (0.1 mg/kg) was ineffective. These data are consistent with the findings of a recent study in which a higher dose of morphine, but not a lower dose, induced acute cardioprotection in the rat heart.[9]

In recent investigations, iNOS has received considerable attention as the trigger and mediator of delayed cardioprotection induced by divergent pathophysiologic stimuli or pharmacologic agents. iNOS induction has been shown to be necessary for the development of delayed protection conferred by ischemic preconditioning in rabbit models of myocardial infarction[29] and stunning.[30] The late phase of ischemic preconditioning is abrogated by targeted disruption of the iNOS gene.[11]

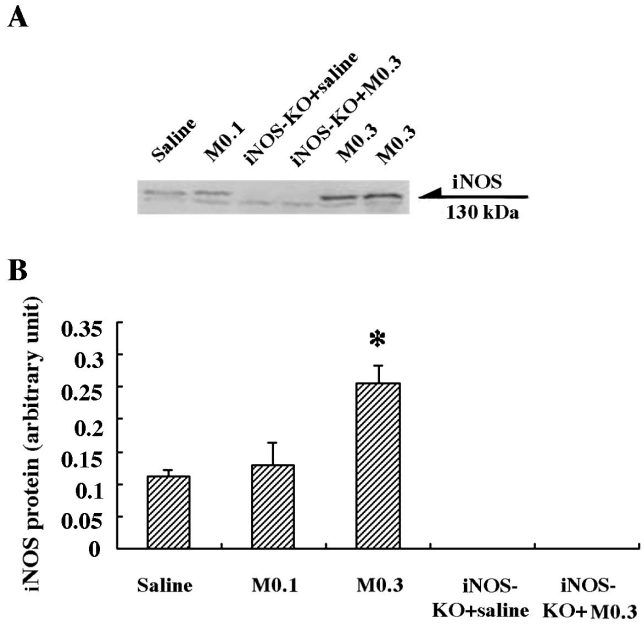

Fig. 3. Myocardial inducible nitric oxide synthase (iNOS) expression in wild-type or iNOS gene-knockout mice (iNOS-KO) pretreated with saline or morphine (M0.1 = 0.1 mg/kg; M0.3 = 0.3 mg/kg) 24 h before death. (*A*) Representative Western blot picture showing iNOS expression. (*B*) Densitometric quantification of iNOS expression (arbitrary units). * $P < 0.05$ *versus* saline group.

Furthermore, a direct cause-and-effect relation of iNOS in adenosine-induced late cardioprotection was demonstrated by increased iNOS expression, which occurred concomitant with the lack of protective effect of aden-

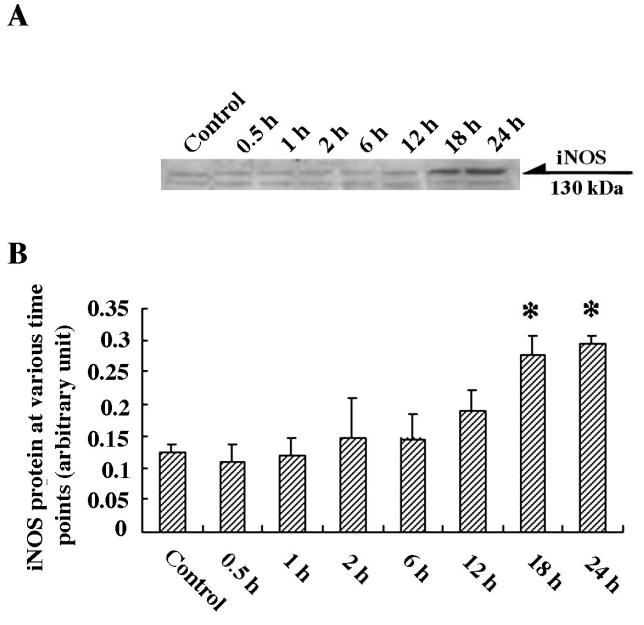

Fig. 4. Myocardial inducible nitric oxide synthase (iNOS) expression in wild-type mice at different time points after morphine (0.3 mg/kg) administration. Control: normal mice without any intervention. (*A*) Representative Western blot picture showing iNOS expression. (*B*) Densitometric quantification of iNOS expression (arbitrary units). * $P < 0.05$ *versus* control.

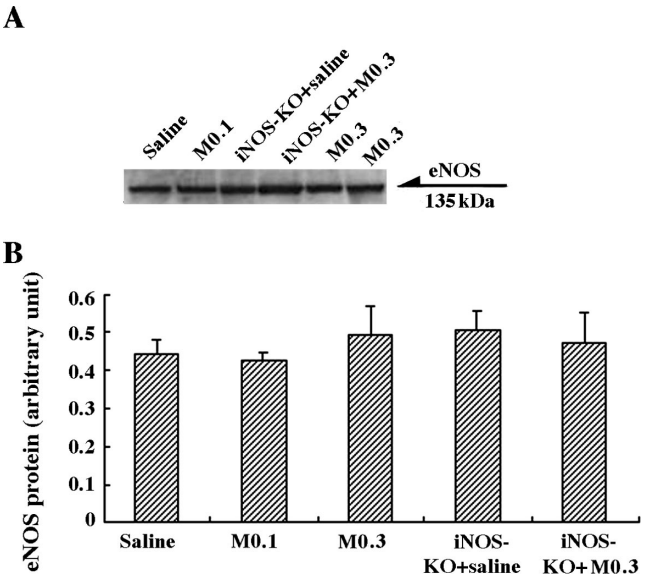

Fig. 5. Myocardial endothelial nitric oxide synthase (eNOS) expression 24 h after saline or morphine (M0.1 = 0.1 mg/kg; M0.3 = 0.3 mg/kg) preconditioning in wild-type or inducible nitric oxide synthase gene-knockout mice (iNOS-KO). (*A*) Representative Western blot picture showing eNOS expression. (*B*) Densitometric quantification of eNOS expression (arbitrary units). There was no significant difference among the five groups.

osine A(1) receptor activation in iNOS gene-knockout mice.[12] iNOS has also been found to mediate the delayed cardioprotective effects induced by systemic hypoxia,[24] endotoxin derivatives,[13] and whole body hyperthermia.[31] In the isolated perfused myocardium, Rebrova *et al.*[32] reported that through activation of NOS, stimulation of the δ-opioid receptor increases the tolerance of the heart to oxidative stress. However, no studies are available that demonstrate a link between morphine and activation of a NOS-dependent signaling cascade. In the current study, the morphine-induced late cardioprotection was abolished by previous administration of SMT, a selective iNOS antagonist. SMT is a non–amino acid analog of L-arginine, which has been reported to be 10- to 30-fold more potent than aminoguanidine and the L-arginine analogs at inhibiting iNOS activity.[33] Targeted disruption of iNOS in the gene-knockout mice also eliminated the delayed protection induced by morphine, whereas morphine was successful in preconditioning hearts from WT mice. Morphine has been found to stimulate iNOS expression in human macrophages.[20] In the current study, morphine pretreatment significantly up-regulated the iNOS protein content in the myocardium 18–24 h after administration. Taken together, these data provide the unique observation that iNOS is a mediator of the morphine-induced late cardioprotection.

It has also been reported that eNOS mediates delayed cardioprotection.[17] In the current study, no significant increase of eNOS expression was detected 24 h after morphine pretreatment compared with the control groups. Furthermore, the morphine-induced cardioprotection was completely blocked by administration of an iNOS antagonist and was absent in the iNOS gene-knockout mice. These data refute the role of eNOS as a mediator of the morphine-induced delayed protection. However, the role of eNOS during the trigger phase remains unclear. Nitric oxide derived from eNOS at the time of morphine treatment may initiate the signaling cascade leading to the resulting increase of iNOS expression.[34,35]

The opening of a mitochondrial adenosine triphosphate–sensitive potassium (mitoK_{ATP}) channel or a mitochondrial site of action seems to be an important trigger for the mediation of cardioprotection. Both ischemic and pharmacologic preconditioning may be dependent on the activation of mitoK_{ATP} channels. Numerous studies have shown that morphine-induced and opioid-induced cardioprotection were mediated by opening of K_{ATP} channels.[3,4,7–9,36] However, the mediator of the opening of the K_{ATP} channel after preconditioning is not fully known. In the current investigation, we observed that iNOS expression was enhanced by morphine pretreatment, which would consequently lead to increased nitric oxide production. Morphine causes the release of nitric oxide in human neutrophils, monocytes, immunocytes,[19,37] and macrophages.[20] Opioids have also been found to increase nitric oxide synthesis from vascular endothelial cells and monocytes.[38] Furthermore, in isolated myocytes, δ-opioid receptor stimulation has been reported to produce nitric oxide, which mediates the cardioprotection.[18] Nitric oxide may modulate K_{ATP} channels *via* the second messenger cyclic guanosine monophosphate. Nitric oxide produced by iNOS could potentially activate guanylate cyclase and stimulate cyclic guanosine monophosphate formation. Protein kinase G may be activated by the enhanced cyclic guanosine monophosphate, which can subsequently open mitoK_{ATP} channels, resulting in the cardioprotection.[39] Sasaki *et al.*[40] have reported that nitric oxide directly activates mitoK_{ATP} channels in rabbit ventricular myocytes. Functioning as an endogenous mitoK_{ATP} channel opener, nitric oxide may titrate the coupling level of the mitochondria to an optimum that blunts mitochondrial calcium overload, without significantly undermining adenosine triphosphate synthetic capacity. Although enhanced iNOS expression could lead to greater nitric oxide production, increasing evidence suggests that iNOS could also generate superoxide rather than nitric oxide under pathophysiologic conditions, such as ischemia, in which L-arginine and tetrahydrobiopterin may be depleted.[41,42] Therefore, the exact mechanism by which iNOS exerts its effect in morphine-induced protection necessitates further examination.

In conclusion, the current study has demonstrated that morphine induced a delayed cardioprotection against myocardial infarction in mice in a dose-dependent manner. This cardioprotective effect was associated with

enhanced expression of iNOS protein in myocardium and was completely abrogated by either selective pharmacologic inhibition of iNOS or targeted disruption the iNOS gene. To the best of our knowledge, this is the first study showing delayed cardioprotection induced by morphine and providing a direct link between morphine preconditioning and the induction of iNOS that subsequently results in delayed cardioprotection. Further investigations are necessary to elucidate the upstream and downstream signaling cascades that lead to the activation of iNOS and whether additional opioid agents are also capable of inducing late preconditioning *via* signal transduction pathways analogous to those of morphine.

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