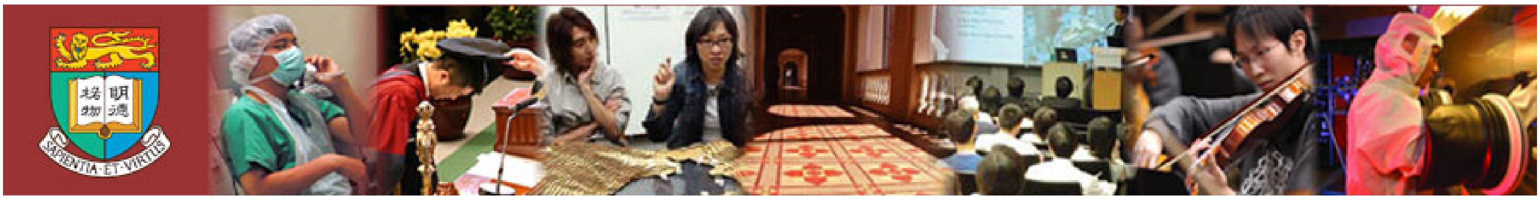

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Intrathecal Morphine Remotely Preconditions the Heart Via a Neural Pathway

Gordon Tin Chun Wong, MBBS,* Lu Yao, MD,*† Zhengyuan Xia, MD, PhD,* and
AU1 Michael G. Irwin, MD*

Abstract: Central opioid receptor activation triggers cardioprotection against ischemia reperfusion injury, independent of peripheral opioid receptor activity. Using a rodent model of myocardial ischemia reperfusion injury with infarct size as the primary outcome, we tested the hypothesis that spinal opioids confer this beneficial effect via a neural pathway. Intrathecal morphine reduced the infarct size compared with control (23% ± 7% vs. 58% ± 3%, respectively, $P < 0.01$). Prior antagonism of the autonomic pathway, and the receptors for bradykinin, calcitonin gene–related peptide, and the K_{ATP} channel, respectively, abolished this cardioprotection (54% ± 13%, 52% ± 10%, 56% ± 9%, and 49% ± 8%, respectively, $P < 0.05$). In a second set of experiments, we demonstrated that the increased expression of myocardial phosphorylated-Akt and endothelial nitric oxide synthase induced by intrathecal morphine was blocked by prior administration of hexamethonium. These findings support the notion that spinal opioid receptors stimulate a neural pathway that uses nonopioid neurotransmitters to confer cardioprotection from ischemia reperfusion injury. The use of intrathecal morphine for this purpose has potential clinical application, and it is already being used in the perioperative period to provide prolonged analgesia.

Key Words: opioids, intrathecyal morphine, cardiac protection, preconditioning, ischemia reperfusion injury AU3

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INTRODUCTION

Remote triggers of cardioprotection have been the subject of great research interest as access to the coronary circulation is not necessary to confer benefits. The protective maneuver may be effective when applied before, during, and immediately after the ischemic event and include triggers such as intermittent ischemia applied to organs remote to the heart or administration of pharmacological agents.[1,2] Fortuitously, some of the agents capable of cardioprotection are already used clinically for other reasons, such as opioid analgesics. We have previously demonstrated that the activation of spinal opioid receptors by morphine is an effective means of remotely protecting the heart.[3,4] This protection occurs independent of activation of peripheral opioid receptors. More recently, Gross et al[5] confirmed the importance of central mu opioid receptors in cardioprotection. This mode of remote preconditioning is of potential significance in the perioperative setting as neuroaxial anesthesia is often performed with the administration of intrathecal morphine to provide postoperative analgesia.

Bradykinin produced by sympathetic nerve endings[6] is released during myocardial ischemia.[7,8] This kinin has previously been implicated in the mechanism of remote ischemic cardiac preconditioning involving a neural pathway.[9] It also causes a paracrine release of neurotransmitters such as calcitonin gene–related peptide (CGRP) from C sensory nerve endings.[10] CGRP has also been demonstrated to mediate remote ischemic preconditioning.[11] Intense research efforts have been devoted to elucidating the mechanisms of cardioprotection, with elements being identified at the subcellular levels that are common to different triggers and modes of cardioprotection. In particular, the phosphatidylinositol-3-kinase nitric oxide pathway (PI3K–Akt–eNOS) has been identified to be activated in cardioprotection triggered by insulin,[12] corticosteroids,[13] and bradykinin,[14] among others. This pathway also has a role in ischemic postconditioning,[15] and in mediating the antiapoptotic effects of hypoxic preconditioning in cardiomyocytes.[16] This and other pathways may converge on the mitochondrial K_{ATP} channels and the mitochondrial permeability transition pore for its final effect.[17]

We hypothesized that intrathecal morphine produces its cardioprotective properties via the activation of a neural pathway, requiring the activation of bradykinin and CGRP receptors and the involvement of the PI3K–Akt–eNOS pathway and the K_{ATP} channel.

METHODS

This study protocol was approved by our institutional animal ethics committee, and the procedures were conducted in accordance with the NIH Animal Research Advisory Committee guidelines. Male Sprague–Dawley rats, weighing 300 ± 25 g were used. They were given free access to food and water, exposed to 12-hour light and dark cycles, and were AU4 housed in separate cages before experimentation (Fig. 1). F1

Surgical Procedures

The rats were anesthetized by an intraperitoneal injection of pentobarbitone (50 mg/kg of body weight). After sterile

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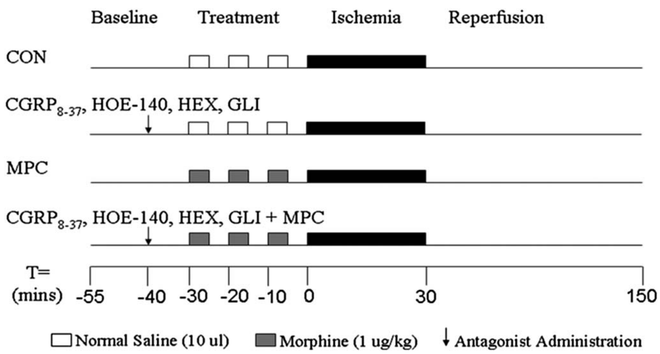

FIGURE 1. Treatment protocols. $CGRP_{8–37}$, a selective CGRP receptor antagonist; CON, control; GLI, glibenclamide, a nonselective K_{ATP} channel blocker; HEX, hexamethonium, an autonomic ganglion blocker; HOE-140, a selective bradykinin B2 receptor antagonist; MPC, intrathecal morphine preconditioning.

preparation of the posterior neck with 70% ethanol, a small polyethylene-10 catheter (4 cm; Smiths Medical International Ltd, United Kingdom) was inserted through an opening in the atlanto-occipital membrane to the thoracic spinal cord according to the method of Yaksh and Rudy.[18] The wound was closed with deep, followed by cutaneous, interrupted sutures. After emergence from anesthesia, these animals were examined for any gross motor or sensory deficits. They were provided with analgesia for the 3 days after surgery with meloxicam 2 mg/kg orally. Those animals demonstrating any neurological deficits were excluded from further experimentation. In addition, after finishing the experiment, Evan blue dye was injected through the intrathecal catheter to determine catheter location and any damage to the spinal cord.

After a minimum of 3 days after intrathecal catheter placement, the rats were reanesthetized by intraperitoneal administration of pentobarbitone (50 mg/kg of body weight) maintained by repeat doses of 25 mg/kg every 60–90 minutes as necessary. All the animals underwent tracheotomy and tracheal intubation. Mechanical ventilation was provided with a Harvard Apparatus Rodent Respirator (Harvard Apparatus, Boston, MA), and the rats were ventilated with room air at 70–80 breaths per minute. Body temperature was monitored and maintained at 37 ± 1°C (mean ± SD) using a heating pad. The femoral artery was cannulated for direct blood pressure monitoring via a pressure transducer and a lead-II electrocardiogram monitored heart rate (HR) via subcutaneous stainless steel electrodes connected to a PowerLab monitoring system (ML750 PowerLab/4sp with MLT0380 Reusable BP Transducer; AD Instruments, Colorado Springs, CO). Hemodynamic values including HR and mean arterial blood pressure (MAP) were recorded at baseline, at the end of the treatment period, at the end of the ischemic and reperfusion periods, respectively, for comparison. The right femoral vein was cannulated for saline infusion. A left thoracotomy was performed to expose the heart at the fifth intercostal space. After removing the pericardium, a 6-0 Prolene loop, along with a snare occluder, was encircled around the origin of the left coronary artery in preparation for inducing ischemia reperfusion injury. After surgical preparation, the rats were allowed to stabilize for 15 minutes before drug administration.

Drug Protocols

The rats were assigned to 1 of 10 treatment groups according to a computer generated randomized sequence: control group (CON) received intrathecal administration of 30 μL normal saline (saline vehicle); the morphine group (MPC) received intrathecal morphine at a total dose of 3 μg/kg (morphine sulfate injection BP, David Bull Laboratory, Hong Kong). This morphine dose was chosen on the basis of a previous dose response study.[19] The solutions were administered by way of 3 consecutive 5-minute infusions interspersed with 5-minute infusion free periods. This pattern of alternating drug administration with a drug-free period was done to mimic the pattern of ischemic preconditioning. Fifteen minutes before intrathecal morphine infusions, different antagonists were given via the intravenous route in respective groups to evaluate the effects that blocking the autonomic pathway, K_{ATP} channels, CGRP receptors or bradykinin receptors, have on morphine preconditioning. Similarly, the same chemicals were given 15 minutes before intrathecal saline infusions to evaluate for any intrinsic effects that each of the chemicals may have had on infarct size (IS). Therefore, groups of animals (n = 6 per group) were given, respectively, 0.01 nmole/kg of CGRP fragment 8–37,[11] 300 μg/kg of the bradykinin B_2 antagonist HOE-140,[9] 20 mg/kg of the nicotinic receptor blocker hexamethonium bromide (HEX)[9,20] or 0.3 mg/kg of glibenclamide (GLI)[21] before either saline or morphine infusions. The doses chosen were sufficient to overcome the effects of remote preconditioning in previous studies. Immediately after the drug protocol, 30 minutes of ischemia was induced in the territory supplied by the left coronary artery by pulling the snare tight and securing the threads with a mosquito hemostat. Ischemia was confirmed by electrocardiographic changes, a substantial decrease in mean arterial pressure, and cardiac cyanosis. The rats were omitted from further data analysis if severe hypotension (arterial mean blood pressure $<$30 mm Hg) or intractable ventricular fibrillation occurred. Where this occurred, the subsequent rat received the same treatment as the deceased rat and thereafter the computer sequence was followed. The ischemia period was followed by 120 minutes of reperfusion. The hearts were then excised, and the IS as a percentage of the area at risk (IS/AAR) was determined by triphenyltetrazolium and Evan Blue staining.

Infarct Size Determination

The hearts were excised and transferred to a Langendorff apparatus on completion of the reperfusion period and immediately perfused with normal saline for 1 minute at a pressure of 100 cm H_2O to flush out residual blood. The snare was securely retightened and 0.25% Evan blue dye injected to stain the normally perfused region of the heart. This procedure allowed visualization of the normal, nonischemia region, and the AAR. The hearts were then frozen and cut into 2-mm slices. Thereafter, the slices were stained by incubation at 37°C for 20 minutes in 1% 2,3,5-triphenyltetrazolium (Sigma Chemical Co) in phosphate buffer at pH 7.4. This was followed by immersion in 10% formalin for 20 minutes to enhance the contrast of the stain. The areas of infarct and risk zone for each slice were traced and digitized using a computerized planimetry technique (SigmaScan 4.0, Systat Software Inc, Richmond, CA). The volumes of the left ventricles, IS, and AAR were calculated by multiplying each area with slice thickness and summing the product. The infarct was expressed as a percentage of the AAR (IS/AAR) and this ratio was used to compare the differences among the groups.

Tunel Staining for Apoptosis

Heart samples were collected from the left ventricular ischemic area in 3 separate groups immediately after reperfusion for tunel staining, using a proprietary kit (In Situ Cell Death Detection Kit, Roche Biochemicals, Mannheim, Germany). The 3 groups consisted of a sham group that underwent all surgical procedures and infusion of saline but without the induction of ischemia reperfusion injury; a CON that received saline and ischemia reperfusion injury, a morphine preconditioning group (n = 3). The tissue was fixed with formalin, embedded in paraffin, and 4-μm thick sections were incubated with proteinase K (20 mg/mL) and then with terminal deoxynucleotidyl transferase. After washing, antidigoxigenin conjugate and peroxidase substrate were applied to the sections. Finally, they were counterstained with hematoxylin. For each section, 10 random fields were examined under a Nikon light microscope (×400 magnification).

Protein Extraction and Western Blotting Analysis

The involvement of total Akt, total eNOS, phosphorylated-Akt (p-Akt) and phosphorylated-eNOS (p-eNOS) was evaluated in a second set of experiment consisting of 5 groups (n = 3): a sham group that underwent all surgical procedures and infusion of saline but without the induction of ischemia reperfusion injury; a CON that received saline and ischemia reperfusion injury, a morphine preconditioning group, a group receiving hexamethonium and 1 group receiving hexamethonium before intrathecal morphine. Heart tissue samples were collected after reperfusion, respectively, from the ischemic left ventricle regions for evaluation of total eNOS, total Akt, p-Akt, and p-eNOS content. The collected samples were immediately frozen at −80°C until further processing. The frozen tissues in groups were powdered and homogenized in lysis buffer containing 20 mM Tris–HCl (pH 7.5), 50 mM 2-mercaptoethanol, 5 mM ethylene glycol tetraacetic acid, 2 mM ethylenediaminetetraacetic acid, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 25 mg/mL leupeptin, 2 mg/mL aprotinin, 0.1% NP40, 0.1% sodium dodecyl sulfate, 0.1% deoxycholic acid for protein extraction. The homogenate was centrifuged at 10,000*g* to separate debris and supernatant, and then the concentrations of proteins in the supernatant were determined by Bradford protein assay. Equal amounts of protein (60 μg) from each group were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to PVDF membrane for AU5 1 hour. The membranes then were blocked in 5% skimmed milk for 1 hour and incubated in 1:1000 dilution of anti-p-Akt, anti-eNOS, and anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibodies (Cell Signaling Technology Inc, MA) and incubated overnight at 4°C. The membranes were then washed and incubated with antirabbit IgG (Cell Signaling Technology Inc) conjugated to horseradish peroxidase (1:10,000) for 1 hour. Protein bands were detected by a standard ECL method and images were measured by means of a densitometer with analysis software.

Statistics

Previous work performed in our laboratory using the same model of cardiac ischemia reperfusion injury[3,19] indicated the expected IS/AAR of the CON to be between 50% and 65% and the magnitude of IS/AAR reduction to be at least 50%. Therefore, 5 animals per group would be required to give a power of 80% and a *P* value of 0.05. Data are expressed as mean ± SD and data analysis was performed with a personal computer statistical software package (Prism v4.0; GraphPad Software, San Diego, CA). Data were analyzed between groups using analysis of variance with a Student–Newman–Keuls post hoc test for multiple comparisons. For the hemodynamic data, a 2-factor mixed design analysis of variance with repeated measure on time was used for analysis performed by SPSS v16 for Windows. Statistical differences were considered significant if the *P* value was <0.05.

RESULTS

A total of 94 rats were used for the study. Three rats were excluded because of neurological damage after intrathecal catheter insertion. A further 7 did not complete the ischemia reperfusion protocol becomes of severe hypotension or ventricular fibrillation. There was 1 each from the CON, GLI, MPC, $CGRP_{8-37}$ + MPC, HEX + MPC and 2 from HEX group. A total of 84 rats completed the study, all had the correct position of the intrathecal catheters confirmed at necropsy, and none had obvious macroscopic damage to the spinal cord.

The hemodynamic parameters are shown in Table 1. T1 There were no significant differences between each of the groups when compared with the CON for each time point. As expected, myocardial ischemia resulted in a substantial drop in MAP across all the groups.

The IS in the CON was (58% ± 3%) The sole administration of the antagonist compounds before ischemia reperfusion did not alter ISs compared with the CON (Fig. 2). Intrathecal F2 morphine significantly reduced the IS compared with control (23% ± 7%, $P < 0.01$). However, this effect was abolished with the prior administration of $CGRP_{8-37}$, HOE-140, hexamethonium or GLI. Intrathecal morphine also reduced the number of apoptotic cells compared with control (Fig. 3). F3

Phosphorylated-Akt (pAkt) expression was comparable between the sham, control, and morphine preconditioning groups at reperfusion. Intrathecal morphine preconditioning significantly increased the level of pAkt expressed after reperfusion (Fig. 4). The levels of eNOS decreased after F4 ischemia reperfusion injury in the CON compared with the sham but morphine preconditioning was able to attenuate this decrease, restoring the levels to that of the sham group (Fig. 5). F5

DISCUSSION

In this study, we delineated the major aspects of intrathecal morphine mediated cardioprotection. In addition to demonstrating the antiinfarct effects of intrathecal morphine preconditioning, these results support our hypothesis

TABLE 1. Hemodynamic Parameters (Mean ± SD)

Group	n	Baseline		Treatment		Ischemia		Reperfusion	
		HR	MAP	HR	MAP	HR	MAP	HR	MAP
CON	6	399 ± 28	111 ± 16	390 ± 19	103 ± 17	360 ± 26	81 ± 16	355 ± 23	89 ± 9
$CGRP_{8–37}$	6	393 ± 31	99 ± 19	381 ± 27	93 ± 17	355 ± 16	69 ± 17	355 ± 21	87 ± 15
HOE-140	6	397 ± 22	107 ± 14	385 ± 17	93 ± 11	356 ± 18	73 ± 10	347 ± 16	86 ± 7
HEX	6	403 ± 26	103 ± 23	384 ± 22	82 ± 21	356 ± 17	80 ± 15	357 ± 14	86 ± 13
GLI	6	389 ± 46	102 ± 21	376 ± 41	99 ± 18	341 ± 39	71 ± 10	337 ± 33	85 ± 9
MPC	6	388 ± 23	103 ± 24	380 ± 20	96 ± 19	343 ± 29	73 ± 11	352 ± 32	86 ± 16
$CGRP_{8–37}$ + MPC	6	388 ± 18	96 ± 28	380 ± 19	92 ± 22	348 ± 31	68 ± 14	343 ± 32	84 ± 11
HOE-140 + MPC	6	394 ± 30	102 ± 13	391 ± 36	86 ± 15	354 ± 37	70 ± 11	343 ± 34	82 ± 9
HEX + MPC	6	397 ± 23	108 ± 16	371 ± 22	80 ± 12	361 ± 11	73 ± 11	361 ± 17	87 ± 9
GLI + MPC	6	386 ± 26	98 ± 21	374 ± 24	96 ± 17	348 ± 19	77 ± 6	342 ± 28	85 ± 7

$CGRP_{8–37}$, a selective CGRP receptor antagonist; GLI = glibenclamide, a nonselective K_{ATP} channel blocker; HEX, hexamethonium, an autonomic ganglion blocker; HOE-140, a selective bradykinin B2 receptor antagonist; MPC = intrathecal morphine preconditioning.

that spinal opioid receptor activation, at least in part, uses a neural pathway to confer cardioprotection. We further demonstrated that this pathway involves bradykinin and CGRP receptors and invokes the PI3K–AKT–eNOS pathway and, consequently, the opening of the K_{ATP} channels. Ultimately this chain of events results in a reduction of cardiac myocyte apoptosis. This process may be abolished with the prior administration of hexamethonium with changes in eNos and pAkt expression similar to control.

Pharmacological modes of cardioprotection, such as remote cardioprotection from ischemic preconditioning or postconditioning, circumvent the limitation of having to access the coronary circulation to induce protection. Systemic and intrathecal opioids are known to be cardioprotective in experimental models,[22,23] with the latter mode of delivery being a form of remote cardioprotection.[3,4] Endothelial nitric oxide synthase has been shown to be associated with myocardium protection triggered by a number of different ligands.[24] In particular, it is the increased phosphorylation of the eNos–Ser[1177] site that accounts for the beneficial effects.[12] The K_{ATP} channel have long been suggested as one of the end effectors of ischemic preconditioning, with its opening during ischemia conferring benefits to the ischemic cell.[25] Therefore, although not novel, the data

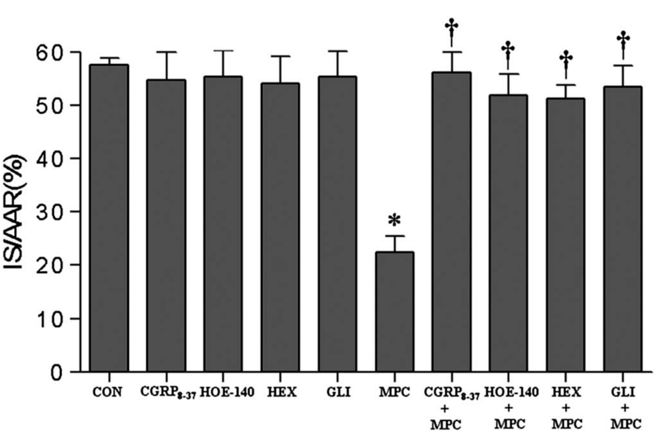

FIGURE 2. Infarct sizes. $CGRP_{8–37}$, a selective CGRP receptor antagonist; GLI = glibenclamide, a nonselective K_{ATP} channel blocker; HEX = hexamethonium, an autonomic ganglion blocker; HOE-140, a selective bradykinin B2 receptor antagonist; MPC = intrathecal morphine preconditioning. $*P < 0.05$ versus control; $†P < 0.05$ versus MPC.

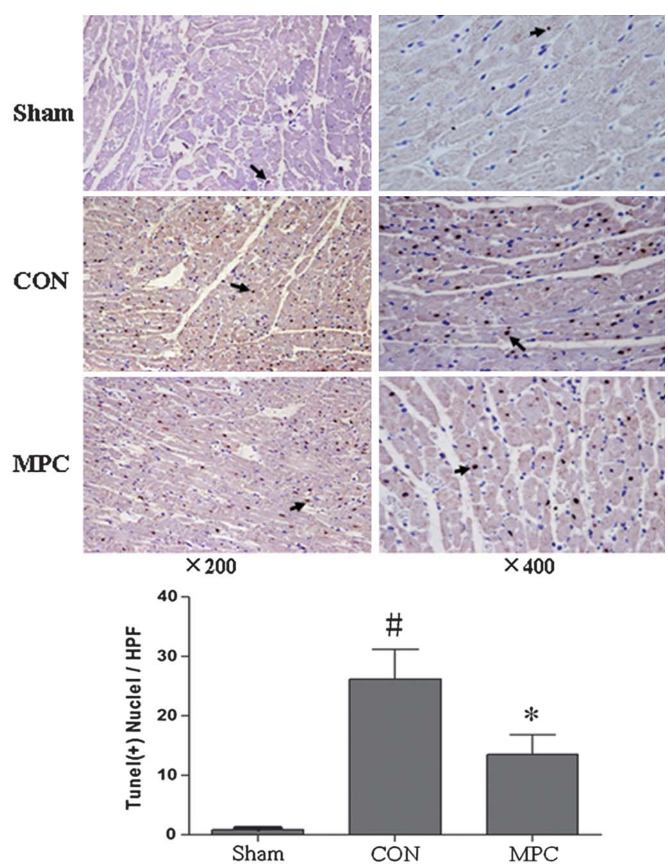

FIGURE 3. Antiapoptotic effect of intrathecal morphine. Top panel: representative pictures for tunel stain of sections. Bottom panel: tunel cell positive counts per high power field; MPC = intrathecal morphine preconditioning; $\#P < 0.01$ versus Sham; $*P < 0.01$ versus CON.

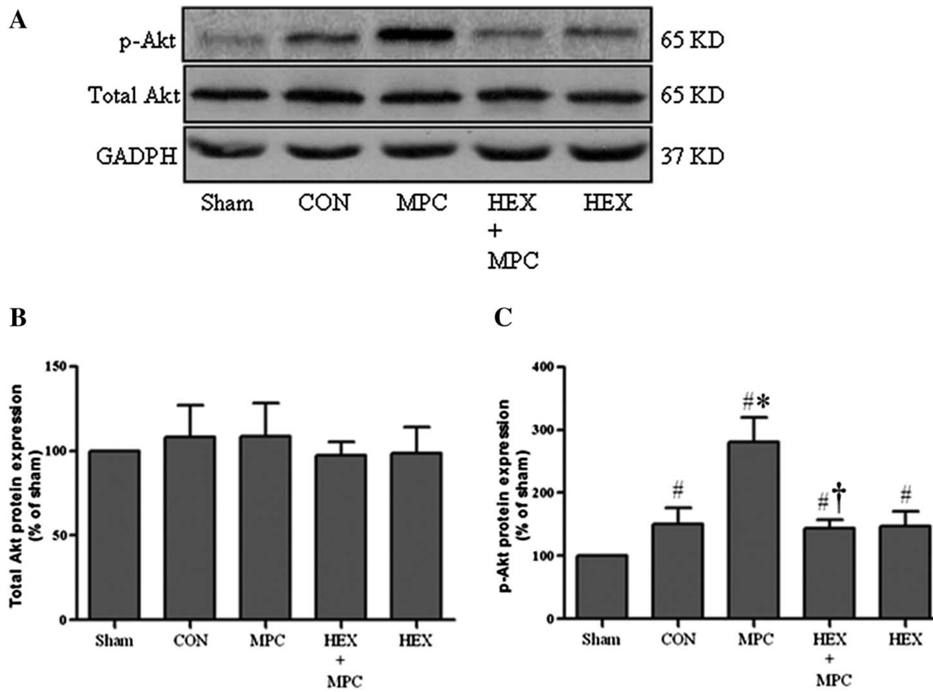

FIGURE 4. Total Akt and p-Akt protein expression. A, Representative Western Blot images. B, Total Akt expression as a percentage of sham. C, Phosphorylated-Akt as percentage of sham. Akt, = serine/threonine protein kinase (also known as protein kinase B); GADPH, glyceraldehyde-3-phosphate dehydrogenase; HEX = Hexamethonium; MPC, intrathecal morphine preconditioning; pAkt = phosphorylated Akt; #$P < 0.05$ versus Sham; *$P < 0.01$ versus CON; †$P < 0.01$ versus MPC.

nevertheless show that pharmacological manipulation of spinal opioid receptors can activate these salvage mechanisms in the myocyte. Of particular interest are the implications of this to the understanding of remote preconditioning signaling.

In support of a neural pathway of transmission, Gho et al[26] have demonstrated that remote ischemic preconditioning can be abolished with the use of hexamethonium. More recently, a novel mode of remote preconditioning has been demonstrated whereby stimulation of small pain fibers resulted in cardioprotection, a phenomenon that can be abolished by transection of the spinal cord. In that study, the authors proposed that the nociceptive signals trigger a dorsal root reflex and activation of the cardiac sympathetic nervous system that ultimately leads to cardioprotection.[20] Interestingly, this neural circuit can be interrupted by the administration of bradykinin and CGRP antagonists. Our results, when interpreted in the light of this study, seem to suggest that activation of central receptors by intrathecal morphine triggers a neural response that similarly involves CGRP and bradykinin receptors. Moreover, it is possible that intrathecal morphine activates the same

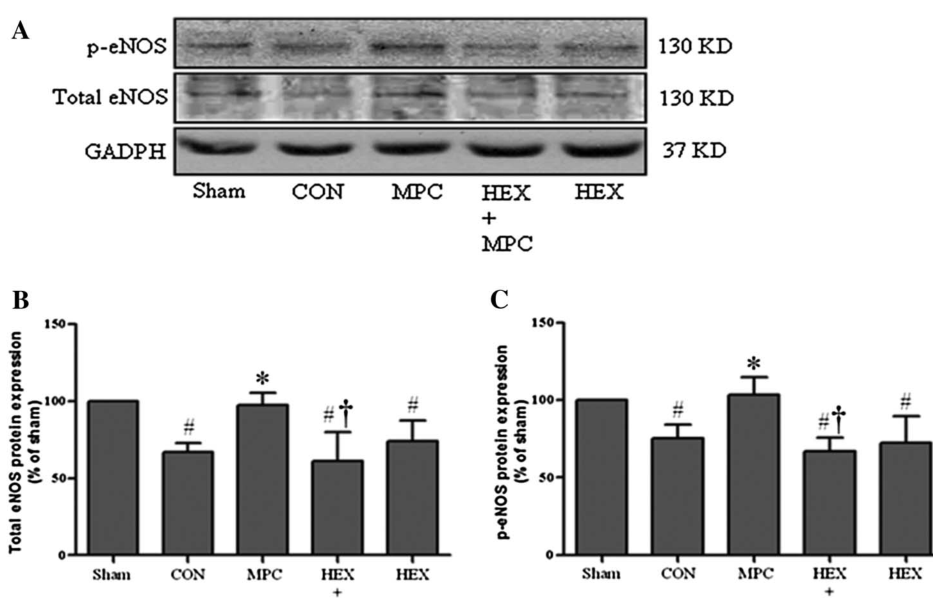

FIGURE 5. Total eNOS and p-eNOS protein expression. A, Representative Western Blot images. B, Total eNOS. C, Phosphorylated eNOS. CON = control; eNOS = endothelial nitric oxide synthase; MPC = intrathecal morphine preconditioning; GADPH = glyceraldehyde-3-phosphate dehydrogenase; p-eNOS = phosphorylated endothelial nitric oxide synthase. #$P < 0.05$ versus Sham; *$P < 0.01$ versus CON; †$P < 0.01$ versus MPC.

efferent component of the neural arc. This morphine-activated pathway similarly can be blocked by hexamethonium, implying that the signals are conveyed along autonomic fibers as is the case with remote ischemic preconditioning and remote preconditioning of trauma. The signals lead to the release of bradykinin and CGRP that triggers the cascade of intracellular events that result in the cardioprotective effect. Thus, in this study, we have demonstrated that activation of spinal opioid receptors can activate the PI3K–Akt, eNOS pathway in the myocardium similar to that produced by direct receptor activation by systemic ligands. A further implication is that the efferent impulses may not be confined to the segments that innervate the heart. It is possible that its benefits may spread beyond that of cardioprotection. Interestingly, limb ischemia has been shown in to be protective for both the heart and lung in an animal model of cardiopulmonary bypass.[27]

Results from laboratory studies into signaling pathways of remote preconditioning tend to promote one mode of signal transmission as being responsible to the exclusion of other modes. However, evidence exists to support multiple means via which the message is conveyed to the heart in remote ischemic preconditioning, including neural and humoral pathways.[28] Remote ischemic preconditioning may also induce systemic anti-inflammatory and antiapoptotic responses that may contribute to the protection.[29,30] Although this study demonstrated a neural component to the transmission of signals from intrathecal morphine, other mechanisms may concurrently contribute to the cardioprotective effects. Intrathecal morphine administration is known to reduce peripheral inflammatory edema in a nitric oxide dependent mechanism.[31] It is possible that such anti-inflammatory effect may contribute to the infarct sparing effect of intrathecal morphine in addition to inducing myocardial adaptation to ischemia.

There are several methodological limitations to this study that may have implications on the interpretation of the findings. First, we did not provide direct evidence of neural involvement by measuring specific nerve activity or by physically interrupting parts of the nervous system. One of the difficulties of performing the former is the need to identify specific nerve fibers responsible, which at this stage is not known. Further, interrupting autonomic fibers can cause hemodynamic instability, which could confound the results. Therefore, we have only provided indirect evidence of neural involvement. Second, the antagonists used in this study may have off target effects that may possibly influence the degree of myocardial damage. For example, GLI may reduce basal coronary blood flow[32] and promotes insulin release from pancreatic beta cells and may cause hypoglycemia. Together these may adversely effects on myocardial ischemia reperfusion injury. On the other hand, $CGRP_{8-37}$ is not known to worsen ischemic injury[33] and HOE 140 may even have a favorable effect.[34] Nevertheless, the ISs of the groups receiving only the antagonists were not different from the saline CON so any potential increases in IS from these chemicals may not be significant. Lastly, we did not rule out any diffusible factor that may have contributed to the protective effects. Efferent nerve signals may release substances that may have beneficial effects not only from nerve terminals but from other organs.

CONCLUSIONS

Myocardial ischemia and or infarction are not uncommon in the perioperative period[35] and, therefore, preconditioning in anticipation of myocardial insult is reasonable. The advantage of using intrathecal morphine is that it is already in clinically use, with a well-known risk benefit ratio that is familiar to clinicians. It has the added advantage of providing analgesia that may favorably alter the myocardial oxygen supply and demand balance. We have provided strong indirect evidence that opioid receptor activation in the spinal cord at least in part uses a neural pathway to convey its signals to the periphery for cardioprotection. Intrathecal morphine can activate similar cellular salvage mechanisms as other experimental ligands and is, therefore, worthy of further study to determine any clinical benefit.

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