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AUTHOR QUERIES

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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 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<sub>ATP</sub> 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, intrathecal morphine, cardiac protection, preconditioning, ischemia reperfusion injury

ORIGINAL ARTICLE

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 al5 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 endings6 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<sub>ATP</sub> 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<sub>ATP</sub> 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 housed in separate cages before experimentation (Fig. 1).

Surgical Procedures

The rats were anesthetized by an intraperitoneal injection of pentobarbitone (50 mg/kg of body weight). After sterile
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. 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 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 tracheotom 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 snares 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. Therefore, groups of animals (n = 6 per group) were given, respectively, 0.01 nmole/kg of CGRP fragment 8–37, 300 μg/kg of the bradykinin B2 antagonist HOE-140, 20 mg/kg of the nicotinic receptor blocker hexamethonium bromide (HEX) or 0.3 mg/kg of glibenclamide (GLI) 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 H2O 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

![FIGURE 1. Treatment protocols, CGRP 8–37, a selective CGRP receptor antagonist; CON, control; GLI, glibenclamide, a non-selective KATP channel blocker; HEX, hexamethonium, an autonomic ganglion blocker; HOE-140, a selective bradykinin B2 receptor antagonist; MPC, intrathecal morphine preconditioning.](image-url)
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, antidigoxygenin 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 ischemic area in 3 separate groups immediately after reperfusion protocol becomes of severe hypotension or ventricular fibrillation. The IS in the CON was (58% ± 3%) The sole administration of the antagonist compounds before ischemia reperfusion did not alter IS/AAR compared with the CON (Fig. 2). Intrathecal morphine significantly reduced the IS compared with control (23% ± 7%, P < 0.01). However, this effect was abolished with the prior administration of CGRP8-37, HOE-140, hexamethonium or GLI. Intrathecal morphine also reduced the number of apoptotic cells compared with control (Fig. 3).

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 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).

**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...
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 KATP 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–Ser1177 site that accounts for the beneficial effects.12 The KATP 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

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<td>CGRP8–37</td>
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CGRP8–37, a selective CGRP receptor antagonist; GLI = glibenclamide, a nonselective KATP channel blocker; HEX, hexamethonium, an autonomic ganglion blocker; HOE-140, a selective bradykinin B2 receptor antagonist; MPC = intrathecal morphine preconditioning.

![FIGURE 2](image1.png)

**FIGURE 2.** Infarct sizes. CGRP8–37, a selective CGRP receptor antagonist; GLI = glibenclamide, a nonselective KATP 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](image2.png)

**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.
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.\textsuperscript{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.\textsuperscript{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...
CONCLUSIONS

Myocardial ischemia and or infarction are not uncommon in the perioperative period 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.

REFERENCES


