Remote intrathecal morphine preconditioning confers cardioprotection via spinal cord nitric oxide/cyclic guanosine monophosphate/protein kinase G pathway

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Abstract

Background: Remote intrathecal morphine preconditioning (RMPC) induces cardioprotection, but the underlying mechanisms of this effect is unknown. The aim of this study was to investigate the role of spinal cord nitric oxide/cyclic guanosine monophosphate/protein kinase G (NO/cGMP/PKG) signal pathway in the cardioprotection of RMPC in rats.

Materials and methods: Anesthetized, open chest, male Sprague–Dawley rats were assigned to one of eight treatment groups 3 d after intrathecal catheter placement. Before ischemia and reperfusion, RMPC received intrathecal morphine (3 μg/kg) by three cycles of 5-min infusions interspersed with 5-min infusion free periods. Intrathecally administrated a nonspecific nitric oxide synthase (NOS) inhibitor Nω-Nitro-L-arginine methyl ester (30 nmol), a specific guanylate cyclase inhibitor oxadiazolo [4,3-a] quinoxalin-1-one (11 nmol) and PKG inhibitor KT-5823 (20 pmol) 10 min before RMPC was used to evaluate the role of NO/cGMP/PKG of spinal cord. Ischemia and reperfusion injury were then induced by 30 min of left coronary artery occlusion, followed by 120 min of reperfusion. Infarct size, as a percentage of the area at risk, was determined by 2,3,5-triphenyltetrazolium staining. The content of cyclic guanosine monophosphate in the thoracic spinal cord was determined by radioimmunity protocol; the contents of nitric oxide and activity of NOS in the thoracic spinal cord were determined by nitrate reductase reduction and colorimetric methods; the expression of neuronal NOS (nNOS) and PKG in the thoracic spinal cord were determined by immunohistochemical and Western blotting method; the expression of nNOS messenger RNA was determined by reverse transcription-polymerase chain reaction method.

Results: RMPC group markedly reduced the infarct size compared with the control group. However, the cardioprotection of RMPC could be abolished by pretreatment with Nω-Nitro-L-arginine methyl ester, Oxadiazolo [4,3-a] quinoxalin-1-one and KT-5823. RMPC enhanced nitric oxide, NOS, and cyclic guanosine monophosphate levels in the spinal cord. Meanwhile, RMPC increased PKG and nNOS protein or messenger RNA expression in the spinal cord.

These authors (Y.L. and J.H.) contributed equally to this study.

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1. Introduction

Opioid receptors in the central nervous system have been shown to regulate cardiovascular function [1–5]. Activation of central opioid receptors by intrathecal morphine also can produce protective effects against myocardial ischemia and reperfusion injury in rats [6,7]. Then, intrathecal or intracerebroventricular administration of morphine or fentanyl can mimic remote ischemic preconditioning effects, this manifestation is named as “remote opioids preconditioning” [5,8–10]. Nitric oxide (NO) acts as a modulator of dorsal horn spinal cord nociceptive pathways. It has been shown that activation of the NO synthase (NOS) by morphine limits spinal morphine-induced analgesia [11]. Recently it was found that only neuronal NOS (nNOS) in the thoracic spinal cord is involved in the effect of remote fentanyl preconditioning [10]. Intrathecal morphine reduces peripheral inflammatory edema via spinal cord NO/cyclic guanosine monophosphate (cGMP) pathway [12]. It has been demonstrated that spinal cord NO/cGMP signal pathway regulates blood pressure and heart rate (HR) [13]. Thus, NO/cGMP/protein kinase G (PKG) signal pathway probably mediates in remote opioids preconditioning effects. In this study, we investigate the role of spinal cord NO/cGMP/PKG signal pathway in the cardioprotection of remote intrathecal morphine preconditioning (RMPC) in rats.

2. Materials and 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 between 200 and 250 g were used for this study.

2.1. Materials

Nω-Nitro-L-arginine methyl ester (L-NAME), 1H-[1,2,4] oxadiazolo[4,3-a] quinoxalin-1-one (ODQ), KT-5823, Evan blue, and 2, 3, 5-triphenyltetrazolium were obtained from Sigma–Aldrich (St. Louis, MO). Anti-nNOS, anti-PKG, and anti-β-actin antibodies were obtained from Cell Signaling Technology Inc (MA, USA). The secondary antibodies and immunohistochemistry kit were purchased from Zhongshan Biotechnology Limited Company (Beijing, China). NO and NOS assay kits were purchased from Jiancheng Bioengineering Institute (Nanjing, China). The cGMP assay kit was purchased from Sino-uk Institute of Biological Technology (Beijing, China).

2.2. Surgical procedures

Rats were anesthetized by an intraperitoneal injection of pentobarbitone (50 mg/kg). After sterile preparation of the posterior neck, a small polyethylene-10 catheter (4 cm) was inserted into the thoracic spinal cord through an opening in the atlanto-occipital membrane as mentioned previously. The wound was closed deep followed by cutaneous interrupted sutures. After recovery, these animals were examined for gross motor or sensory deficits. Animals demonstrating any deficits were excluded from further experimentation. In addition, Evan blue dye was injected through the intrathecal catheter to determine catheter location and any damage to the spinal cord after finishing the experiment [10].

Three days after intrathecal catheter placement, the rats with intrathecal catheters were reanesthetized by intraperitoneal administration of pentobarbitone (50 mg/kg) and maintained by repeat doses of 25 mg/kg every 60–90 min as necessary. All the animals underwent tracheotomy and tracheal intubation. Mechanical ventilation was provided with a Harvard Apparatus Rodent Respirator, and the rats were ventilated with room air at 70–80 breaths/min. Body temperature was monitored and maintained at 37 ± 1 °C (mean ± standard deviation) using a heating pad. The right femoral artery was cannulated for direct blood pressure monitoring via a pressure transducer and the right femoral vein was cannulated for saline infusion. Subcutaneous stainless steel electrodes were connected to a PowerLab monitoring system to monitor the lead II electrocardiogram and HR. A left thoracotomy was performed to expose the heart at the fifth intercostal space, a 6-0 Prolene loop, along with a snare occluder, was placed at the origin of the left coronary artery. Regional ischemia was induced by pulling the snare and securing the threads with a mosquito hemostat. Ischemia was confirmed by electrocardiographic changes, a substantial decrease in mean arterial pressure and cardiac cyanosis. Rats were omitted from further data analysis if severe hypotension (mean arterial blood pressure less than 30 mm Hg) or intratable ventricular fibrillation occurred. After surgical preparation, the rats were allowed to stabilize for 15 min [10].

2.3. Protocols

Rats were randomly assigned to receive 1 of 8 groups (Fig. 1). All animals were subjected to 30 min of ischemia by occlusion of the left coronary artery followed by 2 h of reperfusion by release of the occlusion: the RMPC group was received intrathecal morphine (3 μg/kg), which was administered by three cycles of consecutive 5-min infusions interspersed with 5-min infusion free periods. This pattern of alternating drug administration with a drug-free period was done to mimic the pattern of remote ischemic preconditioning. L-NAME (a nonspecific NOS inhibitor, 30 nmol) [12], ODQ (a specific guanylcylicase inhibitor, 11 nmol) [12], and KT-5823 (a specific PKG inhibitor, 20 pmol) [13] were used to determine the involvement of spinal cord NO-cGMP-PKG signal pathway. Intrathecal administration L-NAME, ODQ, or KT-5823 10 min before RMPC (L-NAME + RMPC, ODQ + RMPC, and KT-5823 + RMPC). Sole administration of L-NAME, ODQ, or KT-5823 intrathecally was also performed to exclude any
cardioprotective effect (L-NAME, ODQ, and KT-5823). As negative control (CON), a group merely received ischemia and reperfusion injury.

2.4. Infarct size determination

At the end of reperfusion, hearts were excised and transferred to a Langendorff apparatus on completion of the reperfusion period and immediately perfused with normal saline for 1 min at a pressure of 100 cm H2O to flush out residual blood. The snare was securely retightened and 0.25% Evan blue dye was injected to stain the normally perfused region of the heart. This procedure allowed visualization of the normal, non-ischemia region, and the area at risk (AAR). The hearts were then frozen and cut into 2 mm slices. Thereafter, the slices were stained by incubation at 37°C for 20 min in 1% 2,3,5-triphenyltetrazolium in phosphate buffer at pH 7.4. This was followed by immersion in 10% formalin for 20 min to enhance the contrast of the stain. The areas of infarct and risk zone for each slice was traced and digitized using a computerized planimetry technique (SigmaScan 4.0, Systat Software Inc, Richmond, CA). The volumes of the left ventricles, infarct size (IS), and AAR were calculated by multiplying each area with slice thickness and summing the product. The IS was expressed as a percentage of the AAR (IS/AAR), and this ratio was used to compare the differences among the groups.

2.5. Measurements of spinal cord NO and NOS level

Spinal cord tissue (T4 and T5 segments) samples were collected after reperfusion. The collected samples (100 mg) were dissolved in 0.6 mL 10% trichloroacetic acid buffer. Then, they were centrifuged to remove precipitated proteins, and the supernatant fractions were extracted with 1 mL of water-saturated ether three times. The cGMP level was determined by radioimmunoassay performing with a cGMP assay kit according to the manufacturer’s instructions.

2.6. Radioimmunoassay

Spinal cord tissue (T4 and T5 segments) samples were collected after reperfusion for evaluation of nNOS and PKG 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 (SDS), and 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 (50 μg) from each group were separated by 10% sodium dodecyl sulfate-polycrylamide gel electrophoresis and transferred to the polyvinylidene fluoride (PVDF) membrane for 1 h. The membranes then were blocked in 5% skim milk for 1 h and incubated in 1:1000 dilution of anti-nNOS, anti-nPKG, and anti-β-actin antibodies and incubated overnight at 4°C. The membranes were then washed and incubated with anti-rabbit IgG conjugated to horseradish peroxidase (1:5000) for 1 h. Protein bands were detected by a standard
Table 1 – Hemodynamic parameters in rats for different groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Baseline</th>
<th>Treatment</th>
<th>30-min ischemia</th>
<th>2-h reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (beats/min)</td>
<td>CON</td>
<td>387 ± 50</td>
<td>393 ± 21</td>
<td>357 ± 38</td>
<td>349 ± 51</td>
</tr>
<tr>
<td></td>
<td>RMPC</td>
<td>384 ± 52</td>
<td>421 ± 30</td>
<td>337 ± 23</td>
<td>341 ± 43</td>
</tr>
<tr>
<td></td>
<td>L-NAME</td>
<td>376 ± 35</td>
<td>383 ± 41</td>
<td>383 ± 21</td>
<td>315 ± 98</td>
</tr>
<tr>
<td></td>
<td>ODQ</td>
<td>372 ± 16</td>
<td>386 ± 33</td>
<td>399 ± 38</td>
<td>333 ± 49</td>
</tr>
<tr>
<td></td>
<td>KT-5823</td>
<td>374 ± 30</td>
<td>383 ± 40</td>
<td>368 ± 26</td>
<td>336 ± 71</td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
<td>CON</td>
<td>82 ± 13</td>
<td>87 ± 20</td>
<td>57 ± 11</td>
<td>66 ± 13</td>
</tr>
<tr>
<td></td>
<td>RMPC</td>
<td>85 ± 19</td>
<td>69 ± 9</td>
<td>59 ± 14</td>
<td>82 ± 16</td>
</tr>
<tr>
<td></td>
<td>L-NAME</td>
<td>88 ± 24</td>
<td>93 ± 14</td>
<td>72 ± 10</td>
<td>70 ± 23</td>
</tr>
<tr>
<td></td>
<td>ODQ</td>
<td>85 ± 9</td>
<td>90 ± 13</td>
<td>59 ± 8</td>
<td>66 ± 13</td>
</tr>
<tr>
<td></td>
<td>KT-5823</td>
<td>85 ± 15</td>
<td>87 ± 8</td>
<td>68 ± 13</td>
<td>60 ± 23</td>
</tr>
<tr>
<td></td>
<td>L-NAME + RMPC</td>
<td>85 ± 16</td>
<td>80 ± 7</td>
<td>64 ± 14</td>
<td>65 ± 15</td>
</tr>
<tr>
<td></td>
<td>ODQ + RMPC</td>
<td>82 ± 14</td>
<td>87 ± 9</td>
<td>55 ± 13</td>
<td>65 ± 16</td>
</tr>
<tr>
<td></td>
<td>KT-5823 + RMPC</td>
<td>86 ± 18</td>
<td>76 ± 10</td>
<td>56 ± 15</td>
<td>61 ± 13</td>
</tr>
<tr>
<td>RPP (mm Hg·beats/min per 1000)</td>
<td>CON</td>
<td>32 ± 9</td>
<td>34 ± 9</td>
<td>20 ± 4</td>
<td>23 ± 6</td>
</tr>
<tr>
<td></td>
<td>RMPC</td>
<td>33 ± 11</td>
<td>29 ± 5</td>
<td>20 ± 5</td>
<td>23 ± 8</td>
</tr>
<tr>
<td></td>
<td>L-NAME</td>
<td>34 ± 13</td>
<td>36 ± 8</td>
<td>28 ± 4</td>
<td>23 ± 12</td>
</tr>
<tr>
<td></td>
<td>ODQ</td>
<td>32 ± 5</td>
<td>35 ± 6</td>
<td>23 ± 4</td>
<td>22 ± 6</td>
</tr>
<tr>
<td></td>
<td>KT-5823</td>
<td>32 ± 8</td>
<td>33 ± 3</td>
<td>25 ± 5</td>
<td>20 ± 9</td>
</tr>
<tr>
<td></td>
<td>L-NAME + RMPC</td>
<td>33 ± 10</td>
<td>34 ± 9</td>
<td>24 ± 4</td>
<td>30 ± 5</td>
</tr>
<tr>
<td></td>
<td>ODQ + RMPC</td>
<td>32 ± 7</td>
<td>33 ± 3</td>
<td>22 ± 7</td>
<td>25 ± 7</td>
</tr>
<tr>
<td></td>
<td>KT-5823 + RMPC</td>
<td>32 ± 9</td>
<td>24 ± 6</td>
<td>22 ± 7</td>
<td>22 ± 4</td>
</tr>
</tbody>
</table>

Baseline = 15 min before surgery; 30 min occlusion = 30 min after regional ischemia; 2 h reperfusion = 2 h after reperfusion; MAP = mean arterial blood pressure (mm Hg); RPP = rate-pressure product (mm Hg·beats/min per 1000). CON = three cycles of consecutive 5 min intrathecal normal saline (3 × 10 μL) infusions interspersed with 5 min infusion free periods; RMPC = three cycles of consecutive 5 min intrathecal morphine (3 × 1 μg/kg) infusions interspersed with 5 min infusion free periods; L-NAME = intrathecal nonspecific NOS inhibitor L-NAME (30 nmol, 10 μL); ODQ = intrathecal specific guanylate cyclase inhibitor ODQ (11 nmol, 10 μL); KT-5823 = intrathecal specific PKG inhibitor KT-5823 (20 pmol, 10 μL); L-NAME + RMPC, ODQ + RMPC, KT-5823 + RMPC = intrathecal L-NAME (30 nmol, 10 μL), ODQ (11 nmol, 10 μL) or KT-5823 (20 pmol, 10 μL), respectively, 10 min before RMPC.

Values are presented as mean ± standard deviation.

2.8. Immunohistochemistry

Spinal cord tissue (T4 and T5 segments) samples were collected after reperfusion. They were fixed with 4% paraformaldehyde solution. After inactivating endogenous peroxidase with 3% H2O2, and blocking cross-reactivity with normal serum for 15 min, the spinal cord sections were incubated overnight at 4°C with the rabbit anti-nNOS IgG (1:500) and anti-PKG IgG (1:500), followed by incubation with peroxidase- conjugated secondary anti-rabbit antibodies (1:100). Then, the peroxidase was visualized with diaminobenzidine. The sections were dehydrated in graded ethanol, soaked in xylene, and mounted with neutral gum. The sections were examined with a light microscope. Cytoplasm stained brown in nNOS- and PKG-positive cells. The expression of PKG and nNOS was monitored by an integrated optical density.

2.9. RNA extraction and reverse transcription-polymerase chain reaction

Total RNAs from heart tissue were extracted using TRIzol (Invitrogen life technologies, CA) following the protocol provided by the manufacturer. Extracted RNA was reverse transcribed to complementary DNA with the SuperScript TM II RNase H Reverse Transcriptase. Synthesized complementary DNA was amplified by Platinum Taq PCRx DNA Polymerase (Invitrogen life technologies, CA). Specific primers for nNOS: nNOS forward 5’-TCTCCTCCTATGTCTGACCCAG-3’; nNOS reverse 5’-TCTCCTCCCTCTCCAGTCCTC-3’. PCR conditions for nNOS were 30 s at 95°C, 30 s at 53°C, and 40 s at 72°C for 35 cycles. The expression of a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase, served as internal control.

2.10. Data analysis

Data are expressed as mean ± standard deviation. The hemodynamic data were analyzed using two-way analysis of variance, with the Bonferroni correction applied for multiple comparisons if significant F ratios were obtained. The IS, as expressed as percentage of the AAR, (IS/AAR) were analyzed for nNOS were 30 s at 95°C, 30 s at 53°C, and 40 s at 72°C for 35 cycles. The expression of a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase, served as internal control.

3. Results

A total of 60 rats were used for the study. Six rats were excluded because of neurologic damage following intrathecal...
catheter insertion. A further six did not complete the ischemia–reperfusion protocol because of severe hypotension or ventricular fibrillation. There were two each from the L-NAME, ODQ + RMPC, and KT-5823 + RMPC. A total of 48 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.

3.1. Hemodynamic values

The hemodynamic values are shown in Table 1. There was no significant difference between each of the groups when compared with control group for each time point. As expected, there was a significant drop of mean arterial blood pressure at 30 min of ischemia and 2 h of reperfusion in all groups confirming the successful induction of ischemia and reperfusion injury model.

3.2. Infarct size

Myocardial IS as a percentage of the AAR in all groups is shown in Table 2 and Figure 2. The AAR ranged from $0.41 \pm 0.03$ cm$^3$ to $0.45 \pm 0.05$ cm$^3$ and there was no difference in AAR between the control and treatment groups. As shown in Figure 2, the IS/AAR of CON was $51.3\% \pm 5.7\%$, RMPC markedly reduced IS/AAR to $29.7\% \pm 5.0\%$ ($P < 0.05$ versus CON). Intrathecally administered with the inhibitors (L-NAME, ODQ, and KT-5823) of NO/cGMP/PKG signal pathway all blocked the reduction in myocardial IS induced by RMPC (L-NAME + RMPC 53.4\% \pm 6.1\%, ODQ + RMPC 48.9\% \pm 5.2\%, and KT-5823 + RMPC 49.8\% \pm 7.2\%; $P < 0.01$ versus RMPC), but it had no effect on myocardial IS when intrathecally administered alone (L-NAME 50.9\% \pm 5.9\%, ODQ 50.0\% \pm 2.6\%, and KT-5823 52.7\% \pm 7.9\%; $P > 0.05$ versus CON).

3.3. NO, NOS, and cGMP levels in the thoracic spinal cord

Having established that RMPC could induce cardioprotection in rats, we investigated the mechanisms underlying this protection. To determine whether NO/cGMP signal pathway underlies this protection, NO, NOS, and cGMP levels in the thoracic spinal cord were assessed for protection against myocardial ischemia and reperfusion injury. As shown in Figure 3, compared with control group, RMPC significantly enhanced NO, NOS, and cGMP levels in the thoracic spinal cord ($P < 0.01$ versus CON), suggesting that NO/cGMP signal pathway likely mediates this protective effect.

### Table 2 – Morphometrics in rat hearts for different groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>LV + RV volume/cm$^3$</th>
<th>ARR volume/cm$^3$</th>
<th>IS/AAR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>$0.84 \pm 0.06$</td>
<td>$0.42 \pm 0.03$</td>
<td>$51.3 \pm 5.7$</td>
</tr>
<tr>
<td>RMPC</td>
<td>$0.74 \pm 0.07$</td>
<td>$0.41 \pm 0.03$</td>
<td>$29.7 \pm 5.9$</td>
</tr>
<tr>
<td>L-NAME</td>
<td>$0.85 \pm 0.09$</td>
<td>$0.44 \pm 0.02$</td>
<td>$50.9 \pm 5.9$</td>
</tr>
<tr>
<td>ODQ</td>
<td>$0.85 \pm 0.10$</td>
<td>$0.43 \pm 0.06$</td>
<td>$50.0 \pm 2.6$</td>
</tr>
<tr>
<td>KT-5823</td>
<td>$0.83 \pm 0.04$</td>
<td>$0.42 \pm 0.04$</td>
<td>$52.7 \pm 7.9$</td>
</tr>
<tr>
<td>L-NAME + RMPC</td>
<td>$0.86 \pm 0.05$</td>
<td>$0.42 \pm 0.04$</td>
<td>$53.4 \pm 6.1$</td>
</tr>
<tr>
<td>ODQ + RMPC</td>
<td>$0.79 \pm 0.09$</td>
<td>$0.44 \pm 0.03$</td>
<td>$48.9 \pm 5.2$</td>
</tr>
<tr>
<td>KT-5823 + RMPC</td>
<td>$0.81 \pm 0.04$</td>
<td>$0.45 \pm 0.05$</td>
<td>$49.8 \pm 7.2$</td>
</tr>
</tbody>
</table>

LV = left ventricle volume; RV = right ventricle volume. CON = three cycles of consecutive 5-min intrathecal normal saline (3 × 10 μL) infusions interspersed with 5-min infusion free periods; RMPC = three cycles of consecutive 5-min intrathecal morphine (3 × 1 μg/kg) infusions interspersed with 5-min infusion free periods; L-NAME = intrathecal nonspecific NOS inhibitor L-NAME (30 nmol, 10 μL); ODQ = intrathecal specific guanylate cyclase inhibitor ODQ (11 nmol, 10 μL); KT-5823 = intrathecal specific PKG inhibitor KT-5823 (20 pmol, 10 μL); L-NAME + RMPC, ODQ + RMPC, KT-5823 + RMPC = intrathecal L-NAME (30 nmol, 10 μL), ODQ (11 nmol, 10 μL) or KT-5823 (20 pmol, 10 μL), respectively, 10-min before RMPC. Values are presented as mean ± standard deviation. *$P < 0.01$ versus CON; †$P < 0.01$ versus RRIMC.
3.4. nNOS and PKG protein expression in the thoracic spinal cord

Despite having showed NO/cGMP signal pathway as the potential mechanism underlying cardioprotection induced by RMPC, signaling pathways within the NOS on which it acts and the downstream to produce this effect is unknown. Hence, we determined the nNOS and PKG protein expression in the thoracic spinal cord by the methods of Western blotting and immunohistochemistry. As shown in Figure 4 and Figure 5, RMPC obviously increased the nNOS and PKG protein expression in the thoracic spinal cord (P < 0.01 versus CON), suggesting that nNOS is important in the cardioprotection induced by RMPC, and PKG is probably the downstream target of the NO/cGMP signal pathway.

Fig. 3 – The levels of NO, NOS, and cGMP in the thoracic spinal cord (T4 and T5 segments) after reperfusion. CON = three cycles of consecutive 5-min intrathecal normal saline (3 × 10 μL) infusions interspersed with 5-min infusion free periods; RMPC = three cycles of consecutive 5-min intrathecal morphine (3 × 1 μg/kg) infusions interspersed with 5-min infusion free periods. Values are presented as mean ± standard deviation. *P < 0.05; †P < 0.01 versus CON.

Fig. 4 – The nNOS and PKG protein expression in the thoracic spinal cord (T4 and T5 segments) after reperfusion. CON = three cycles of consecutive 5-min intrathecal normal saline (3 × 10 μL) infusions interspersed with 5-min infusion free periods; RMPC = three cycles of consecutive 5-min intrathecal morphine (3 × 1 μg/kg) infusions interspersed with 5-min infusion free periods. Values are presented as mean ± standard deviation. *P < 0.01 versus CON.
3.5. nNOS messenger RNA expression in the thoracic spinal cord

Having established that nNOS likely mediates the cardioprotection of RMPC, we further demonstrated the role of nNOS in RMPC effects by the use of reverse transcription-polymerase chain reaction technology. As shown in Figure 6, RMPC significantly increased the nNOS messenger RNA expression in the thoracic spinal cord (P < 0.01 versus CON), indicating the key role of the nNOS in this effect.

4. Discussion

The present study confirms our previous observations that RMPC produces myocardial protection against ischemia and reperfusion injury in open chest anesthetized rats. In our present study, the cardioprotection of RMPC could be abolished by pretreatment with L-NAME, ODQ, and KT-5823. RMPC enhanced the NO, NOS, and cGMP levels in the spinal cord. Also, RMPC increased PKG and nNOS protein or messenger RNA expression in the spinal cord. It is the first time to...
indicate that RMPC-induced IS reduction occurs through activating spinal cord NO/cGMP/PKG signal pathway.

Opioids are a commonly used analgesic of choice in acute myocardial infarction. Intrathecal opioids delivery is an effective pain management option for patients after surgery. The delivery of opioids into the intrathecal space provides superior analgesia with smaller doses of analgesics to minimize side effects while significantly improving quality of life. Morphine was the first clinically used opioid shown to be cardioprotective [14], but the intravenous dose required limited its use clinically. Spinal dorsal horn plays a key role in the nociceptive transmission and modulation. Jones et al. [15] found that nociceptive stimulation of sensory nerves in the skin of the abdomen, which initiating remote preconditioning of trauma, triggers a neurogenic signal that is transmitted via nerve fibers, and causes a dorsal root reflex that activates cardiac sympathetic nervous system, ultimately leading to cardioprotection. Degeneration of capsaicin sensitive afferent nerves can enhance the myocardial injury of acute myocardial ischemia, possibly due to reduction of endogenous calcitonin gene related peptide (CGRP) and substance P [16]. Remote preconditioning of intracerebroventricular morphine inducing CGRP release, one of the mediators of remote preconditioning, contributes to this cardioprotective effect [9].

NO acts as a modulator of dorsal horn spinal cord nociceptive pathways. Activation of NO by intrathecal administration with morphine limits the spinal analgesic action of this drug [11]. It is well known that morphine shows antinociceptive effects and that inhibitors of the NO/cGMP pathway attenuate the analgesic effect of morphine or other opioids [17,18]. Intrathecal morphine administration is known to reduce peripheral inflammatory edema involvement of NO/cGMP pathway [12]. nNOS is likely an important role mediating in this anti-inflammatory effect [19]. RMPC may confer cardioprotection by inducing anti-inflammatory effect via activating the NO/cGMP pathway of the spinal cord [10]. Sachs et al. [20] found that opening of adenosine triphosphate-sensitive potassium channel via an NO/cGMP/PKG signal pathway induces antinociceptive effect of morphine. Recently, we have shown that remote intrathecal fentanyl preconditioning (RFPC) only enhanced the expression of nNOS, not iNOS and eNOS in the spinal cord. Then, L-NAME, a nonspecific NOS inhibitor, abolished the RFPC effects. That study indicated that RFPC induces the cardioprotection via activating the nNOS in the thoracic spinal cord [10]. Our previous study shows that intrathecal lidocaine and remote morphine preconditioning both can induce cardioprotective effects against ischemia and reperfusion injury in rats [21]. It also has been demonstrated that RMPC is ineffective in the presence of neuraxial blockade with lidocaine [21]. From that study, we populate that the potential mechanism of RMPC is more than just analgesic factors. NO/cGMP/PKG plays a key role in nociceptive transmission in the pathologic pain states [22]. It has been found that activation of central receptors by intrathecal morphine triggers a neural response that releases CGRP and bradykinin [5]. Spinal cord NO/cGMP/PKG pathway can increase the release of glutamate and CGRP [23]. Stable blood pressure at the end of reperfusion might contribute to the cardioprotection of intrathecal morphine preconditioning [24]. Spinal cord NO/cGMP signal pathway regulates blood pressure and HR [13]. Therefore, it is feasible that the increment of nNOS and PKG expression in the thoracic spinal cord induced by RMPC as observed in the present study would be advantageous in terms of protection against myocardial ischemia and reperfusion injury.

5. Conclusions

In conclusion, as shown in Figure 7, the present study has clearly established that spinal cord NO/cGMP/PKG signaling pathway mediates RMPC-induced cardioprotective effect.

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Disclosure

The authors declare that they have no conflict interests.

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