Spinal neuronal NOS activation mediates intrathecal fentanyl preconditioning induced remote cardioprotection in rats

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A B S T R A C T

Fentanyl has been widely used in anesthesia and analgesia, especially for cardiovascular surgeries. The aim of the study was to evaluate whether remote intrathecal fentanyl preconditioning (RFPC) provides cardioprotection and the role of spinal nitric oxide synthase (NOS) system in this effect. Fentanyl (0.3 μg/kg) was administrated intrathecally during RFPC by 3 cycles of 5-minute infusions interspersed with 5-minute infusion free periods. A non-specific nitric oxide synthase (NOS) inhibitor NG-nitro-l-arginine methyl ester (L-NAME, 30 nmol) and a selective nNOS inhibitor 7-nitroindazole (7-NI, 100 nmol) were administrated intrathecally 10 min before RFPC, and were used to evaluate the involvement of the NOS system of the spinal cord. RFPC group markedly reduced the infarct size compared with control. However, the cardioprotection of RFPC could be abolished by pretreatment with L-NAME and 7-NI. RFPC merely increased the expression of nNOS and did not affect iNOS and eNOS expression. L-NAME reversed nNOS expression up-regulation induced by RFPC treatment. The current study demonstrated that RFPC effectively induced cardioprotection through activating the nNOS in the spinal cord.

1. Introduction

Intrathecal or intracerebroventricular administration of opioids induces cardiac preconditioning in a manner similar to remote ischemic preconditioning, named as remote opioid preconditioning [1–3]. However, the underline mechanism of this effect is still unclear. Opioid receptor activation in the spinal cord at least in part utilizes a neural pathway to convey its signals to the periphery for cardioprotection [2]. Administration of a single dose of intrathecal morphine can be an effective method to obtain safe and prolonged postoperative analgesia [4,5]. The analgesic action of remote opioid preconditioning may have contributed to this protective effect [3]. Nitric oxide (NO) is a neuronal messenger molecule in the central nervous system, which is involved in the mechanisms of pain generation. It has been demonstrated that the activation of the NO synthase (NOS) system by morphine limits the spinal analgesic action of this drug [6]. Intrathecal morphine can act on opioid receptors at the spinal level to produce antiedematogenic effects via the NO–cGMP pathway [7]. Spinal cord NO–cGMP pathway controls mean arterial pressure and heart rate [8]. Therefore, NO likely participates in the effect of remote opioid preconditioning. In this study, we investigate the role of the NOS system in the cardioprotection of remote intrathecal fentanyl preconditioning in rats.

2. Materials and methods

This study protocol was approved by our institution's 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. 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) (Smiths Medical International Ltd., UK) was inserted into the thoracic spinal cord through an opening in the atlanto-occipital membrane as described [1,2]. Animals demonstrating any deficits were excluded from further experimentation. Three days after intrathecal catheter placement, the rats with intrathecal catheters were re-anesthetized by intraperitoneal administration of pentobarbitone (50 mg/kg) and maintained by repeat doses of 25 mg/kg every 60–90 min as necessary. All of the animals underwent insertion, through the trachea, of a polyethylene tube connected to a respirator (Harvard Apparatus, Boston, MA) with a tidal volume of 4 ml (70 breaths/min). Body temperature was monitored and maintained at 37 ± 1 °C (mean ± SD) 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 (ML750 PowerLab/4sp with...
MLT0380 Reusable BP Transducer; AD Instruments, Colorado Springs, CO) in order to monitor the lead II ECG and heart rate. Then left thoracotomy was performed between the fourth and fifth ribs, and the left coronary artery was ligated with 6-0 silk suture using a snare occluder [1,2]. Regional ischemia was induced by pulling the snare and securing the threads with a mosquito hemostat. Ischemia was confirmed by electrophysiographic changes, a substantial decrease in mean arterial pressure and cardiac cyanosis. Rats were omitted from further data analysis if severe hypotension (arterial mean blood pressure less than 30 mm Hg) or intractable ventricular fibrillation occurred. After surgical preparation, the rats were allowed to stabilize for 15 min.

2.2. Drug protocols

Rats were randomly assigned to receive 1 of 6 groups, 8 rats each group (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: control group (CON) received intrathecal administration of 30 μl normal saline; the remote intrathecal fentanyl preconditioning (RFPC) group received intrathecal fentanyl (0.3 μg/kg) [9], which was administered by 3 cycles of 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 remote ischemic preconditioning. A non-selective NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME), and a selective nNOS inhibitor 7-nitroindazole (7-NI) were respectively used to evaluate the involvement of NOS of the spinal cord. 30 nmol of L-NAME [7] or 100 nmol of 7-NI [10] 10 min before RFPC (L-NAME + RFPC, 7-NI + RFPC) was intrathecally administered. Sole administration of L-NAME or 7-NI was also intrathecally performed to exclude any cardioprotective effect (L-NAME, 7-NI).

2.3. Infarct size determination

The hearts were excised and transferred to a Langendorff apparatus upon 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% Evans blue dye was injected to stain the normally perfused region of the heart. This procedure allowed visualization of the normal, non-ischemic region and the area at risk (AAR). The hearts were then frozen and cut into 2 mm slices. Thereafter, myocardial infarct size (IS) was measured using TTC (1% 2, 3, 5-triphenyltetrazolium chloride) staining as described [1,2]. 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 IS was expressed as a percentage of the AAR (IS/AAR) and this ratio was used to compare the differences among the groups.

2.4. Western blotting

Spinal cord tissue (T4 and T5 segments) samples were collected after reperfusion for evaluation of eNOS, iNOS and nNOS contents. 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 EGTA (ethylene glycol tetraacetic acid), 2 mM EDTA (ethylenediaminetetraacetic acid), 10 mM NaF, 1 mM PMSF (phenylmethylsulfonyl fluoride), 25 mg/ml leupeptin, 2 mg/ml aprotinin, 1% NP40, 0.1% SDS (sodium dodecyl sulfate), and 0.1% deoxycholic acid for protein extraction. The homogenate was centrifuged at 10,000 g to separate debris and supernatant. Protein concentrations were determined using the Bradford assay (Bio-Rad, USA). Samples containing equal amounts were separated on a 10% SDS-polyacrylamide gel, and then proteins were transferred to a PVDF membrane overnight at 4 °C. The membranes were blocked in 5% skim milk for 1 h and incubated in 1:1000 dilution of anti-eNOS, anti-iNOS, anti-nNOS and anti-GAPDH (glyceraldehyde 3-phosphate dehydrogenase) antibodies (Cell Signaling Technology Inc., MA, USA) and incubated overnight at 4 °C. After washing with Tris buffered saline–TWEEN (TBST) three times for 30 min, membranes were then incubated with anti-rabbit IgG (Cell Signaling Technology Inc., MA, USA) conjugated to horseradish peroxidase (1:10000) for 1 h. Protein bands were detected by a standard ECL method and images were measured by a densitometer with analysis software.

2.5. Statistical 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 area at risk (IS/AAR) was analyzed between groups using one way analysis of variance with analysis software.

Fig. 1. Bar graphs depicting the experimental protocol. CON = 3 cycles of consecutive 5-minute intrathecal normal saline (3 × 10 μl) infusions interspersed with 5-minute infusion free periods; RFPC = 3 cycles of consecutive 5-minute intrathecal fentanyl (3 × 0.1 μg/kg) infusions interspersed with 5-minute infusion free periods; L-NAME or 7-NI = intrathecal non-selective NOS inhibitor NG-nitro L-arginine methyl ester (30 nmol, 10 μl) or selective nNOS inhibitor 7-nitroindazole (100 nmol, 10 μl); L-NAME + RFPC or 7-NI + RFPC = intrathecal NG-nitro-L-arginine methyl ester (30 nmol, 10 μl) or 7-nitroindazole (100 nmol, 10 μl) 10 min before RFPC.
3. Results

3.1. Hemodynamic values

Table 1 shows the heart rate (HR), mean arterial blood pressure (MAP) and rate–pressure product (RPP) at baseline, after treatment, 30 min after left coronary artery occlusion, and 2 h after reperfusion. There were no significant differences between each of the groups when compared with the CON for each time point. Then, there was a significant drop of MAP and RPP 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. Myocardial infarct size

Myocardial infarct size as a percentage of the AAR in the experimental groups is shown in Fig. 2. The AAR ranged from 0.35 ± 0.02 cm³ to 0.39 ± 0.03 cm³ and there was no difference in the AAR between the control and treatment groups. As shown in Fig. 2, the IS/AAR of CON was 51.5% ± 9.1%, RFPC markedly reduced IS/AAR to 33.8% ± 8.7% when compared with the CON for each time point. Then, there was a significant drop of MAP and RPP at 30 min of ischemia and 2 h of reperfusion in all groups, confirming the successful induction of ischemia and reperfusion injury model.

3.3. Three subtypes of NOS protein expression

Results for the three subtypes of the NOS (nNOS, iNOS and eNOS) protein expression of the spinal cord are shown in Fig. 3. RFPC merely increased the expression of nNOS. Then, RFPC did not affect iNOS and eNOS expression. However, intrathecal administration of the NOS inhibitor l-NAME could prevent the increasing expression of nNOS induced by RFPC.

4. Discussion

In the present study, we showed for the first time that remote intrathecal fentanyl reduced the infarct size in open chest anesthetized rats. However, the cardioprotection of RFPC could be abolished by pretreatment with l-NAME and 7-NI. More interestingly, RFPC only enhanced the expression of nNOS, not iNOS and eNOS in the spinal cord. Then, the up-regulation of nNOS induced by RFPC was canceled by the non-selective NOS inhibitor l-NAME.

Preconditioning with intrathecal or intracerebroventricular administration of morphine has been shown to protect the heart against ischemia and reperfusion injury. This manifestation of myocardial adaptation is named as “remote morphine preconditioning” [1-3]. Fentanyl is approximately 100 times more potent than morphine and is widely used in clinical practice. In this study, we found that RFPC could induce cardioprotection.

Three isoforms of NOS forming enzymes include neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). The nNOS is known to be associated with signal transduction in the central and peripheral nervous systems [11], and NOS is concentrated in the superficial dorsal horn of the spinal cord and the dorsal root ganglia [12], indicating a key role for spinal NO in pain processing [13]. The development of hyperalgesia in the rat’s hindpaw is followed by nNOS up-regulation in the spinal cord [14]. It has been shown that NOS is associated with inhibition of peripheral inflammatory edema triggered by intrathecal morphine [15]. It is possible that such anti-inflammatory effect may contribute to the infarct sparing effect of intrathecal morphine. A novel mode of remote preconditioning has been found whereby stimulation of small pain fibers produced cardioprotection, and this effect can be abolished by transection of the spinal cord [16]. In that study, the authors suggested that the nociceptive signals trigger a dorsal root

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reflex and activation of the cardiac sympathetic nervous system that ultimately leads to a cardioprotective effect. Recently, it has been demonstrated that intrathecal morphine triggers a neural response, which results in protection against myocardial ischemia and reperfusion injury [2]. Spinal cord NO–cGMP pathway controls mean arterial pressure and heart rate [8]. It has been shown that neonatal mice lacking nNOS are less vulnerable to hypoxic and ischemic injury [17]. Moreover, nNOS is also found to play a key role in the oxygen-glucose deprivation-induced neuroprotection in cultured cortical neurons [18]. Neurons that express nNOS are notoriously resistant to many types of injury [19]. Intrathecal morphine administration is known to reduce peripheral inflammatory edema involvement of the NO–cGMP pathway [7]. Intrathecal morphine preconditioning [2] and remote ischemic preconditioning [20] both induce anti-inflammatory responses that contribute to cardioprotection. It is possible that RFPC induce anti-inflammatory via activating the NO–cGMP pathway of the spinal cord.

In conclusion, RFPC induced cardioprotection via activation of the nNOS of the spinal cord. Our findings suggest that through the new pharmaceutical activity of nNOS, it may become a potential leading drug in the therapy of myocardial ischemia.

Acknowledgments

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References


