Spinal nociceptin mediates electroacupuncture-related modulation of visceral sympathoexcitatory reflex responses in rats

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THE ACTIVATION OF SYMPATHETIC afferents during myocardial ischemia evokes excitatory cardiovascular reflexes including hypertensive responses and tachyarrhythmias, which can result in significant mortality (29, 32). These cardiovascular conditions can be potentially improved by acupuncture (5, 22, 40). Our previous studies have demonstrated that electroacupuncture (EA) at P 5–6 (Jianshi-Neiguan) acupoints, which refer to the pericardial meridian, overlying the median nerve in cats, reduces the extent of myocardial ischemia during reflex increases in arterial blood pressure (BP) caused by a stimulation of chemosensitive sensory nerve endings in the gallbladder (5, 22) and attenuates the reflex pressor responses to gastric distension in rats (9, 23, 41, 42). EA, through virtually identical pathways and neurotransmitter systems in the central nervous system (CNS), exerts very similar effects in rat and cat models during the activation of sympathoexcitatory reflexes. EA reduces the sympathoexcitatory response through an opioid mechanism involving μ- and δ-opioid receptors in the rostral ventrolateral medulla (rVLM), an important region of cardiovascular regulation that controls sympathetic outflow (24). In addition, δ- and κ-opioid receptors in the spinal cord have been documented to play a role in mediating the somatic nerve stimulation-induced modulation of cardiovascular reflex responses (42).

The spinal cord processes somatic and visceral reflexes as well as outputs from the CNS to effector organs involved in cardiovascular reflex regulation (26). Nociceptin, a recently discovered ligand for the nociceptin/orphanin FQ peptide receptor (NOP) antagonist, is located in a number of sites in the CNS, including the rVLM and the spinal cord (1, 13, 17, 31). EA inhibits sympathoexcitatory reflex responses to the gastric distension through the activation of NOP receptors in the rVLM (9). Nociceptin has been shown to be located in the dorsal horn, ventral horn, and intermediolateral column of the spinal cord (1, 13). However, the roles of spinal nociceptin and its neural pathway in EA-related inhibition during visceral sympathoexcitatory reflexes have not been defined. Thus the aim of this study was to elucidate the mechanisms underlying the role of spinal nociceptin in the acupuncture-related regulation of cardiovascular sympathoexcitatory reflexes using a combined physiological and pharmacological approach. We hypothesized that nociceptin, acting through NOP receptors in the thoracic spinal cord, is responsible, in part, for the inhibitory influence of EA on cardiovascular visceral reflex responses.

METHODS

Experimental preparations and protocols were reviewed and approved by the Animal Care and Use Committees of the University of California (Irvine and Los Angeles, CA). The study conformed to the American Physiological Society’s “Guiding Principles for Research Involving Animals and Human Beings.” Studies were performed on adult Sprague-Dawley rats (400–600 g). After an overnight fast (18 h), anesthesia was induced with ketamine (100 mg/kg im) and xylazine (10 mg/kg im). The right jugular vein was cannulated for the administration of sodium bicarbonate and saline. The trachea was intubated, and respiration was controlled with a small animal ventilator (SAR-830/P, CWE). A 3-Fr pressure catheter was inserted into the right or left carotid artery (SPR-524, Millar Instruments) to monitor systemic BP. Heart rate (HR) was derived from the pulsatile BP signal. Arterial blood gases and pH were measured periodically with a blood gas analyzer (IRMA TRUPoint, ITC) and were kept within normal physiological limits (PCO2, 30–40 Torr; and Po2, >100 Torr) by adjusting the ventilatory rate or volume and enriching the inspired O2 supply. Arterial pH was maintained between 7.35 and 7.43 by an infusion of a solution of 8% sodium bicarbonate. Body temperature was kept between 36° and 38°C with a heating pad.

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A 3-cm (unstressed dimension) latex balloon was attached to a polyurethane tube (3 mm diameter) that was inserted into the stomach through the mouth and esophagus. A syringe was attached to the cannula to inflate and deflate the balloon with air. Distension pressures were selected to fall within the range that a rat normally experiences during ingestion of food and fluids in a single meal (2, 10, 11). Within 5–10 s of inflation, we noted an increase in systemic arterial BP. To evaluate the action of nociceptin at the spinal cord level, we inserted a polyethylene tube (PE-10) into the spinal subarachnoid space at the level of T1–2 through an incision made on the atlanto-occipital membrane in the anesthetized rats. We chose an intrathecal injection at T1–2 because the sympathetic preganglionic neurons that provide innervation to the heart are located in the IML of the thoracic cord at T1–3 (37). In addition, afferents from the median nerve, which wereinnervation to the heart are located in the IML of the thoracic cord at T1–2 because the sympathetic preganglionic neurons that provide innervation to the heart are located in the IML of the thoracic cord at T1–3 (37). In addition, afferents from the median nerve, which were innervation to the heart are located in the IML of the thoracic cord at T1–2 because the sympathetic preganglionic neurons that provide innervation to the heart are located in the IML of the thoracic cord at T1–3 (37). In addition, afferents from the median nerve, which were innervation to the heart are located in the IML of the thoracic cord at T1–2 because the sympathetic preganglionic neurons that provide innervation to the heart are located in the IML of the thoracic cord at T1–3 (37). In addition, afferents from the median nerve, which were innervation to the heart are located in the IML of the thoracic cord at T1–2 because the sympathetic preganglionic neurons that provide innervation to the heart are located in the IML of the thoracic cord at T1–3 (37). In addition, afferents from the median nerve, which were isolated and without the IML at T1 (9, 44). A stimulation current (0.5-ms pulse, 10 Hz, 0.4 mA) was sufficient to produce an increase in BP (>25 mmHg). The pontamine blue dye mixed with the nociceptin antagonist was microinjected to mark the microinjection sites in the dorsal horn, IML, and rVLM. The spinal cord and brain stem were removed and fixed in 4% paraformaldehyde and 20% sucrose. They were cut in 40-μm coronal sections with a freezing microtome and then mounted on slides. The sections in the brain stem where the current produced the increases in arterial BP were marked by a single electrode track. Sites were plotted on coronal sections. The areas of electrical stimulation in the rVLM and microinjections in the spinal cord were identified by the atlas of Paxinos and Watson (33).

**Experimental Protocols**

After the surgical procedures, we allowed a 30-min period of stabilization before beginning the experimental protocols. The balloon was inflated every 10 min throughout each experiment by injecting 5–10 ml of air for 30 s, a volume that induced a distension pressure of ~20 mmHg (41). The volume of air used for distension was maintained constant for each animal throughout the protocol. Ten-minute intervals between inflations prevented tachyphylaxis of the cardiovascular responses (23, 41). After the maximal cardiovascular pressor response was observed, air was withdrawn from the balloon. BP and HR responses were recorded and analyzed off-line with data acquisition software PowerLab (ADInstruments). The stomach was distended repeatedly every 10 min throughout the protocol.

All experimental drugs were purchased from Sigma-Aldrich (St. Louis, MO). Drugs were dissolved in normal saline at room temperature to an initial concentration of 1 mg/ml. Stock solutions were stored in a freezer and used within 2 wk, after which a fresh stock solution was prepared. On day of the experiment, appropriate serial dilutions were made to obtain the desired concentrations. The concentration of naloxone, nociceptin, and the nociceptin antagonist [N-Phe1]nociceptin1-13 NH2 was 10 nM. The volumes for intrathecal injection and microinjections into the dorsal horn and IML were 10 μl and 30 nl, respectively. The 30-nl injection was administered over a period of 10 s. The coordinates for the IML at T1 were 0.8–1 mm lateral to the midline and 0.8–1 mm deep from the dorsal surface of the spinal cord. The doses and volumes were based on preliminary studies demonstrating consistent responses. We did not observe a dye spread from the dorsal horn to the IML or vice versa. The distance from the injection site in the superficial lamina of the dorsal horn to the IML is ~0.7 mm. Nealey and Maunsell (30) have not observed a spread of 100 nl injectate beyond 0.5 mm from the site of injection.

**Intrathecal NOP.** In eight animals, after observing two repeatable responses to gastric distension, we intrathecally injected the NOP receptor agonist nociceptin (10 μl, 10 nM) at T1–2, followed by repeated gastric distension every 10 min for an additional 70 min.

**Vehicle control.** In five rats, after two repeatable responses to gastric distension, saline (10 μl) was injected intrathecally at T1–2 as the vehicle control.

**Opioid Antagonism in Nociceptin Response**

Naloxone + NOP blockade with [N-Phe1]nociceptin1-13 NH2. Eight animals were pretreated with naloxone (10 μl, 10 nM), followed by an intrathecal injection of the NOP agonist and followed by the NOP receptor antagonist [N-Phe1]nociceptin1-13 NH2 (10 μl, 10 nM) (3, 4). [N-Phe1]nociceptin1-13 NH2 is the first truly selective and competitive nociceptin receptor antagonist devoid of any residual agonist activity (4).

**Effect of EA on Pressor Response to Gastric Distension: Influence of Nociceptin Antagonism**

EA effect. In seven rats, after observing two repeatable responses to gastric distension, we induced 30 min of EA (2 Hz, 2–4 mA, 0.5-ms duration) at P 5–6 followed by repeated gastric distension.

EA + NOP blockade. Eight rats were subjected to an identical protocol with the exception that the NOP antagonist was intrathecally injected at T1–2 20 min after beginning EA, followed by repeated gastric distension every 10 min for an additional 50 min.

EA + NOP blockade + naloxone. In seven rats, the NOP antagonist and naloxone were injected intrathecally at T1–2 20 min after beginning EA followed by repeated gastric distension every 10 min for an additional 50 min.

**Spinal Nociceptin System in Cardiovascular Responses to Electrical Stimulation of rVLM**

rVLM stimulation + NOP. An intrathecal injection of nociceptin at T1–2 was performed in seven animals followed by a electrical stimulation of the rVLM.

**Spinal Neural Pathway of Nociceptin in EA-modulation of Cardiovascular Reflex**

EA + NOP blockade in dorsal horn. In seven rats, the NOP antagonist (30 nl, 10 μM) was microinjected bilaterally into the dorsal horn at T1 20 min after the initiation of EA, followed by repeated gastric distension every 10 min.

EA + NOP blockade in IML. In six rats, the NOP antagonist was microinjected bilaterally into the IML at T1 20 min after the start of EA, followed by repeated gastric distension every 10 min.

**Statistical Analysis**

Data are presented as means ± SE. Mean arterial pressures at rest were compared over time using repeated-measures ANOVA, followed post hoc by the Tukey test. Gastric distension responses were also assessed by a one-way repeated-measure ANOVA, followed by the Tukey test, to compare BP responses before, during, and after EA in...
each group. Statistical calculations were performed with SigmaStat software (Jandel Scientific Software, San Rafael, CA). Differences were considered significant when \( P < 0.05 \).

RESULTS

Gastric Distension Response to Nociceptin in the Spinal Cord

The gastric distension-induced pressor response of 26 ± 4 mmHg was reduced to 17 ± 3 mmHg by nociceptin administered intrathecally at T1–2, representing a 35% change. The original BP tracings are displayed in Fig. 1A. The inhibition lasted for 50 min before returning to 24 ± 3 mmHg (Fig. 1B). The intrathecal injection of nociceptin did not alter HR or resting BP before gastric distension. The intrathecal injection of saline did not alter the pressor responses to gastric distension (n = 5).

Opioid Antagonism in Nociceptin Response

The magnitude of the pressor response induced by gastric distension was unaltered by intrathecal injection of naloxone at T1–2. However, in the presence of opioid receptor blockade, spinal nociceptin promptly reduced the pressor response by 32% (Fig. 1C) with inhibition lasting for 50 min. The intrathecal injection of the nociceptin antagonist \([N\text{-Phe}^1]\text{n}ociceptin_{1-13}\text{NH}_2\) reversed the nociceptin-induced inhibition for 20 min. Resting arterial BP and HR remained constant throughout this protocol.

Effect of EA on Pressor Response to Gastric Distension: Influence of Nociceptin Antagonism

The inhibitory effect of low-frequency EA at P 5–6 was evaluated in seven animals (Fig. 2A). Baseline BP and HR were not significantly altered during the period of the experiment. Thirty minutes of EA attenuated the gastric distension-induced pressor reflex from 23 ± 3 to 15 ± 4 mmHg (35%), a response that persisted for 30 min after the termination of EA. The original BP tracings of an individual animal demonstrate the inhibitory modulation by EA of the pressor reflex as well as the effect of nociceptin antagonism during EA (Fig. 2B).

The intrathecal injection of the nociceptin antagonist at T1–2 immediately following 20 min EA partially reversed the EA-related modulation of the visceral reflex from 15 ± 2 to 21 ± 3 mmHg (Fig. 2B), responses that were not different from those observed during the pre-EA control period but which were significantly higher than the responses during EA. A combination of nociceptin and classical opioid receptor antagonism with \([N\text{-Phe}^1]\text{n}ociceptin_{1-13}\text{NH}_2\) and naloxone completely abolished the EA inhibitory effect. Resting BP and HR were unchanged throughout this protocol (Fig. 2C).

Spinal Nociceptin System in Cardiovascular Responses to Electrical Stimulation of rVLM

Unilateral electrical stimulation of the rVLM elicited pressor responses averaging 24 ± 3 mmHg. The intrathecal injection of nociceptin attenuated the pressor responses by 45% to 13 ± 2 mmHg (Fig. 3A). The examination of the rat brain slices revealed that the electrical stimulation sites were within the rVLM (9, 44). The stimulation sites were confined to an area that was 2.0–3.0 mm caudal to interaural line, 1.6–2.1 mm lateral to the midline, 0.2–1.0 mm from the ventral surface, lateral to the inferior olive nucleus and the pyramidal tracts, as well as ventral and medial to the facial and retrolimbic nuclei (Fig. 3B).

Spinal Neural Pathway of Nociceptin in EA-Modulation of Cardiovascular Reflex

The bilateral microinjection of the nociceptin antagonist into either the dorsal horn or the IML at T1 partially reversed the inhibitory effect of EA on the excitatory cardiovascular reflex responses to gastric distension (Fig. 4, A and B). The composite anatomical map shows the location of the microinjection sites in the dorsal horn and the IML in the high thoracic region (Fig. 4C).
DISCUSSION

In this study we investigated the role of the opioid-like peptide nociceptin in the spinal cord with respect to its contribution to the inhibitory effects of EA on cardiovascular function. We made several novel observations. First, the intrathecal injection of nociceptin at T1–2 attenuated the gastric distension-induced reflex pressor responses, very similar to the influence of EA at P 5–6 on reflex sympathoexcitatory responses. Second, the intrathecal injection of the NOP antagonist \([\text{N-Phe}^1]\text{nociceptin}_{1-13} \text{NH}_2\) partially reversed the EA response. Third, the pretreatment with the opioid receptor antagonist naloxone did not alter the EA-like inhibitory influence of nociceptin on the pressor reflex, whereas a combination of naloxone and nociceptin receptor antagonist completely abolished the EA effect. Fourth, the intrathecal injection of nociceptin attenuated the pressor responses to electrical stimulation of the rVLM, suggesting that nociceptin can act on sympathetic outflow. Fifth, the bilateral microinjection of NOP antagonist into either the superficial lamina (I–III) of the dorsal horn or the IML of the spinal cord at T1 partially reversed the EA inhibitory effect. Taken together, these results suggest that nociceptin and its associated NOP receptor in the spinal cord contribute to the inhibitory effect of EA on the reflex autonomic responses during mechanical stimulation of the stomach, through a mechanism that is independent of the classical opioid system.

Our previous studies in experimental models and in human subjects have shown that acupuncture does not significantly alter baseline BP but that it is capable of lowering elevated BP (21, 41–43). In this regard, reflex excitatory cardiovascular responses are significantly reduced when low-current and low-frequency EA (0.3–0.5 mA, 2 Hz) stimulation is applied at P 5–6 acupoints in rats (23, 41, 42). As such, in the present study...

![Fig. 2. Influence of opioid antagonism on nociceptin response. A: effect of electroacupuncture (EA) on reflex blood pressure responses. B: blood pressure responses to intrathecal injection of nociceptin receptor antagonist after onset of EA. Labels a–d on the bars correspond to the tracings shown above the bars. C: blood pressure responses to intrathecal injection of both nociceptin and opioid receptor antagonist during EA. \(\ast P < 0.05\), significant difference compared with pre-EA. N, number of animals.](http://ajpheart.physiology.org/)

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![Fig. 3. Spinal nociceptin system in cardiovascular responses to electrical stimulation in the rostral ventrolateral medulla (rVLM). A: effect of intrathecal injection of nociceptin on the BP responses to electrical stimulation of the rVLM (bars). \(\ast P < 0.05\), significant difference after intrathecal injection of nociceptin. B: composite map displaying sites of insertion of stimulation electrode (*) in the rVLM. All insertions were unilateral (side chosen randomly). Sections are 2.0–2.5 and 2.5–3.0 mm caudal to the interaural line (Ref. 33). Py, pyramidal tract; Amb, ambiguous nucleus; 7, facial nucleus; Sp5, spinal trigeminal nucleus; ION, inferior olivary nucleus; N, number of animals.](http://ajpheart.physiology.org/)
we stimulated an abdominal visceral reflex to elevate BP. The stimulation of mechano- or chemosensitive receptors in a number of abdominal visceral organs like the stomach activates the reflex responses in the cardiovascular system (27, 28). In particular, the stimulation of mechanosensitive C and Aδ fiber spinally projecting afferents in the stomach reflexly increases sympathetic tone to the heart, blood vessels, and adrenal medulla (27). Thus either a passive gastric distension within the physiological range or an application of capsaicin that stimulates C fibers in a cat significantly increases BP, HR, and myocardial contractility (27, 28). The distension of the rat’s stomach likewise increases BP (23, 41) and thus provides an appropriate model to study the influence of acupuncture on the cardiovascular responses to reflex activation.

Several CNS regions, including the arcuate nucleus, periaqueductal gray, and rVLM, serve as important nuclei that process the influence of EA on the cardiovascular system (5, 9, 25, 38, 44). More specifically, EA inhibits sympathoexcitatory reflex responses to gastric distension through the activation of NOP receptors in the rVLM (9). NOP receptors are located in a number of sites in the CNS, including the rVLM and the spinal cord (1, 13, 17, 31). Nociceptin modulates sympathetic outflow at several anatomical levels. At a supraspinal level, it has been shown that a bilateral injection of nociceptin into the rVLM reduces BP and HR in rats (8). Thus nociceptin acts centrally to inhibit central sympathetic outflow, consequently producing bradycardia and hypotension (18, 36). Spinally, nociceptin-like immunoreactivity has been found to be present in nerve fibers of the dorsal horn (1, 13) as well as in the somata and fibers of the IML (1, 13), suggesting that nociceptin may participate in the regulation of sensory as well as autonomic function. We have found that enkephalin and dynorphin but not β-endorphin in the spinal cord play an important role in the magnetic somatic nerve stimulation-related modulation of cardiovascular reflex responses (42). In the present study, nociceptin receptor antagonism, even in the presence of prior blockade with naloxone, completely abolished the EA modulation of reflex excitatory responses, indicating that the nociceptin system in the spinal cord plays an important role for EA-related cardiovascular regulation, independent from any action of opioids.

As an inhibitory neurotransmitter, nociceptin plays a role in the regulation of cardiovascular responses. In anesthetized rats, an intravenous injection of nociceptin produces a transient, dose-dependent fall in systemic BP, accompanied by a reduction in HR (14). An intracerebroventricular injection of nociceptin also triggers hypotension and bradycardia (18). Interestingly, an intrathecal injection of nociceptin does not alter the resting BP and HR but significantly attenuates the pressor reflex responses to gastric distension, suggesting that spinal and systemically administered nociceptin likely regulate the cardiovascular system through actions on separate centers and/or pathways. Furthermore, these data also suggest that nociceptin in the spinal cord does not provide a tonic modulation of BP.

The spinal cord is an important integrative region of afferent and efferent pathways that participates in cardiovascular regulation. Anatomical and physiological studies indicate that the dorsal horn of the spinal cord serves as a major center for EA-induced analgesia (19, 20). Both low- and high-frequency EA at Zusanli (St 36) acupoint increase Fos immunoreactive neurons in the superficial laminae (I and II) in the dorsal horn of the spinal cord (19). Since nociceptin-like immunoreactivity is present in the spinal sympathetic nuclei (i.e., IML) (13), it is possible that EA also influences the neurotransmission between the brain stem and the IML (13, 16). In this study, we did find that nociceptin reduced the response to rVLM-induced sympathoexcitation, indicating that nociceptin can regulate sympathetic outflow. In addition, there has been a suggestion that the descending pathways from the brain stem (presumably to the dorsal horn of the spinal cord) may influence the segmental processing of somatic inputs during EA (15, 34, 35). Afferent stimulation can modulate sympathetic activity through the inhibition of excitatory interneurons (39). In addition, somatic stimulation can elicit excitatory and inhibitory responses in both IML and dorsal horn interneurons, depending on the dermatome stimulated (6). These interneurons appear to form important links in the spinal cord circuitry involved in autonomic control (12). In the present study, we observed that the bilateral microinjection of the NOP antagonist in the superficial lamina (I to III) of the dorsal horn or the
IML at T1 partially reversed the EA modulation of the visceral excitatory cardiovascular reflex. We speculate that nociceptin serves a role in the processing of spinal cord interneuron activity in the EA response. However, spinal circuits controlling the cardiovascular visceral reflex responses during EA require further elucidation.

In conclusion, these data provide the first documentation that the endogenous nociceptinergic system in the spinal cord contributes to the inhibitory actions of EA on the excitatory reflexes elicited by mechanical distension of the stomach. In this regard, the antagonism of the action of nociceptin in the spinal cord during EA reverses the inhibitory action of EA on the pressor reflex during visceral afferent stimulation. Additionally, in the absence of EA, the intrathecal injection of exogenous nociceptin at T1–2 elicits an EA-like attenuation of the reflex increase in BP. The pretreatment of nociceptin with a nonselective opioid receptor antagonist naloxone does not alter the EA-like inhibitory influence of nociceptin on the gastric distension-induced pressor reflex, suggesting that at least part of the actions of nociceptin are independent of the opioid system. The microinjection of the NOP antagonist into either the dorsal horn or the IML at T1 significantly reversed the EA responses, supporting the observation that nociceptin may act as sympathetic interneurons in EA-related cardiovascular regulation, possibly through a dorsal horn–IML pathway. These results provide new information about the spinal mechanisms underlying the influence of EA on the autonomic and cardiovascular systems.

REFERENCES


