Nitric Oxide in the Gracile Nucleus Mediates Depressor Response to Acupuncture (ST36)

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Chen, Shuang and Sheng-Xing Ma. Nitric oxide in the gracile nucleus mediates depressor response to acupuncture (ST36). J Neurophysiol 90: 780–785, 2003. First published April 2, 2003; 10.1152/jn.00170.2003. The purpose of these studies was to determine the role of the gracile nucleus and the effects of l-arginine-derived nitric oxide (NO) synthesis in the nucleus on the cardiovascular responses to electroacupuncture (EA) stimulation of “Zusanli” (ST36). Arterial blood pressure and heart rate were monitored during EA stimulation of ST36 following microinjections of agents into gracile nucleus. EA ST36 produced depressor and bradycardiac responses in anesthetized Sprague-Dawley rats. The cardiovascular responses to EA ST36 were blocked by bilateral microinjection of neuronal NO synthase (nNOS) antisense oligos into gracile nucleus and blocking the hypotensive and bradycardiac responses to EA ST36. The cardiovascular responses to EA ST36 were attenuated by bilateral microinjection of neuronal NO synthase (nNOS) antisense oligos into gracile nucleus. Microinjection of l-arginine into gracile nucleus facilitated the hypotensive and bradycardiac responses to EA ST36. The results demonstrate that blockade of neuronal conduction in the gracile nucleus inhibited the cardiovascular responses to EA ST36. The hypotensive and bradycardiac responses to EA ST36 are modified by influences of l-arginine-derived NO synthesis in the gracile nucleus. We conclude that NO plays an important role in mediating the cardiovascular responses to EA ST36 through gracile nucleus.

INTRODUCTION

Acupuncture and electroacupuncture (EA) have been used for centuries in treatment and prevention of diseases including cardiovascular diseases and diseases with hypertensive syndromes (Ernst and Lee 1986; Qian 1986). Sympathoinhibition and widespread vasodilation are induced by low-frequency transcutaneous nerve stimulation (Kaada 1982; Kaada and Eieben 1983) and EA stimulation of Zusanli (ST36) in human (Ernst and Lee 1986). Depressor effects are also produced by EA applied to acupuncture points (acupoints) in spontaneous hypertensive rats (Utsunomiya et al. 1987) and in norepinephrine (NE)-induced hypertensive rats (Lin and Li 1981). Han et al. (1986) have demonstrated that analgesic effect of EA is mediated by opioid peptides in the periaqueductal gray. The sympathoinhibition induced by low-frequency EA-like stimulation is not antagonized by noloxone, an opioid-receptor-blocking drug (Kaada 1982; Kaada and Eieben 1983). It has been suggested that activation of somatic afferents and acupuncture stimulation might modulate intestinal motility and cardiovascular responses via somatosympathetic reflex (Sato et al. 1992, 1993). However, the neural pathways and neurotransmitters responsible for the cardiovascular effects of EA ST36 are unclear. It has been demonstrated that nitric oxide (NO) plays an important role in the central regulation of sympathetic tone and mediates tonic inhibition of renal sympathetic nerve activity and arterial blood pressure (Ma and Long 1991; Ma et al. 1995; Sakuma et al. 1992). NO in the brain stem plays an inhibitory role in the central modulation of somato-cardiac sympathetic C-reflex (Li et al. 1995).

The somatotopic organization of the gracile nucleus receiving peripheral somatosensory afferents from the hindlimb has been demonstrated with electrophysiological mapping studies and anterograde axons tracing techniques in various mammals (Cliffer et al. 1992; Leem et al. 1994; Ueyama et al. 1994). Neurons in the gracile nucleus are activated by peripheral nociceptive stimulation of the sciatic nerve (Leem et al. 1994; Ueyama et al. 1994). Previous studies have shown that neurons in the gracile nucleus which receive somatosensory afferent inputs originating in nociceptors project to the thalamus (Cliffer et al. 1992; Leem et al. 1994). A number of recent studies have suggested that gracile nucleus is an integration center for cutaneous and visceral information flowing into the thalamus, which plays an important role in somatic and visceral pain processing (Al-Chaer et al. 1996a,b, 1997).

Previous studies have demonstrated that stimulation of the somatic sensory nerve results in changes in sympathetic nerve activity and arterial blood pressure (Cliffer et al. 1992; Samso et al. 1994). Our recent studies have found that NO in the gracile nucleus plays an inhibitory role in cardiovascular responses to stimulus-evoked somatosympathetic reflexes (SSR) in rats (Chen and Ma 2002). Neuronal NO synthase (nNOS) immunoreactivity is enhanced in the gracile nucleus by EA applied to the hindlimb acupoints (Ma and Li 2002). The purpose of the present study was to determine the role of the gracile nucleus in the cardiovascular responses evoked by electrical stimulation of hindlimb acupoint, ST36. The effects of l-arginine-derived NO synthesis in the gracile nucleus on the cardiovascular responses to EA stimulation of ST36 were...
examined by microinjections of l-arginine and nNOS antisense oligodeoxynucleotides (olidos) into the area.

METHODS

Animal preparation

All experiments were performed using adult (5–8 mo) male Sprague-Dawley rats. The protocol was approved by the Harbor–UCLA Animal Care and Use Review Committee and was in accord with AAALAC and National Institutes of Health guidelines. The animals were maintained on a 12-h light/dark cycle in temperature- and humidity-controlled rooms. Food and water were available ad libitum. The rats were anesthetized by an intraperitoneal injection of urethan (1.3 g/kg), which maintained a stable anesthesia throughout surgery, microinjections, and EA stimulations as previously described (Ma and Long 1991; Ma et al. 1995; Chen and Ma 2002). Femoral venous cannulae were implanted for systemic delivery of drugs. The femoral arterial catheter was connected for recording arterial blood pressure and heart rate. Polyethylene catheters (PE 50) filled with heparinized saline were inserted into the right femoral artery for recording systemic arterial blood pressure. Heart rate was monitored by a tachograph (Grass 7P4H) triggered by the arterial blood pressure wave. After tracheal cannulation, the animals breathed spontaneously throughout the experiment. A heating pad was used to maintain body temperature at 37°C.

Electroacupuncture stimulation

EA was applied at the pairs of acupuncture points Zusanli (ST36) in rats anesthetized by urethan as described above. The needle electrodes (27-gauge sharpened stainless-steel insect pins) were inserted percutaneously into a depth of 2–4 mm (cutaneous and muscle) at the points of ST36, at the depression below the knee from the anterior crest of the tibia (Ernst and Lee 1986; Lee and Beitz 1993). EA stimulation was performed using a Grass S48 stimulator connected to each pair of needle electrodes. Biphasic pulse electrical stimuli with three frequencies 3, 10, and 30 pulses/s were applied to the acupoints. As previously described, the parameters of stimulation were high-intensity stimuli (6 V, duration 1.0 ms) to activate the Ao/Aö and C fibers (Han et al. 1986; Ku et al. 1993; Lee and Beitz 1993; Wang et al. 1994). As a control for the specific acupoint effects, stimulation using the same parameters were applied to the “nonacupoints” located nearby ST36 in the hamstring muscles as described (Lee and Beitz 1993).

Microinjection in the gracile nucleus

Rats were placed in a stereotaxic apparatus with the head flexed at 45° to facilitate exposure of the obex. The dorsal surfaces of the medulla oblongata were exposed through removal of a small section of the posterior cranium and the area postrema was visualized under a microscope. Bilateral microinjections were made into the gracile nucleus at approximately 1.0 mm posterior to the calamus scriptorius, 0.5–1.0 mm lateral from midline and at a depth of 0.5 mm to the surface of the medulla (Leem et al. 1994; Ma and Long 1991). Single-barrel glass cannula (1.14 mm OD and 0.5 mm ID, World Precision) were pulled; the outer tip diameter was approximately 50 μm. The cannula was connected to an electronically controlled nanoliter injector (A203XVZ, World Precision). Agents were dissolved in artificial cerebral spinal fluid (pH adjusted to 7.4) and were given in a volume of 50 nl over a period of 5 s.

To identify the site of microinjection, 2% pontamine sky blue was injected into the gracile nucleus when the experiment was completed. The animals were deeply anesthetized; the brains were removed and stored in 10% paraformaldehyde solution. The frozen brain tissue was sectioned in the coronal plane (40 μm). Histological verification was carried out with reference to the rat brain in stereotaxic coordinates (Paxinos and Watson 1997). The stained area in the brain stem containing the gracile nucleus was examined under a light microscope. The results from the animals with injections diffusing out of the gracile nucleus were excluded from statistical data.

Protocol

The rats were randomly divided into five groups. Arterial blood pressure and heart rate were monitored and allowed to stabilize for ≥20 min. Cardiovascular responses were induced by EA stimulation of ST36 using 6 V with a duration of 1.0 ms at 3, 10, 30 pulse/s for 10 s in random order in the same rat. Frequency-response curves were obtained for the changes in arterial blood pressure and heart rate induced by each stimulation at intervals of 5–7 min, to ensure that arterial blood pressure was completely maintained to the baseline level. Lidocaine (10 μmol), l-arginine (3 nmol) versus vehicle (same volume), were microinjected into the gracile nucleus. Before and after administration of each compound into the gracile nucleus, arterial blood pressure and heart rate were observed following EA stimulation of ST36.

Cardiovascular responses to ST36 stimulation in two additional groups of rats were examined by the presence of nNOS antisense oligos to block local NO generation in the gracile nucleus. nNOS antisense and sense oligos were designed and synthesized by Bio-gnostik (Chemi-Con, Temecula, CA) based on rat nNOS gene sequence. nNOS sense or antisense oligos were dissolved in artificial cerebral spinal fluid (pH adjusted to 7.4) and given in a volume of 50 nl over a period of 30 s. After cardiovascular effects were obtained by EA stimulation of ST36, nNOS sense or antisense oligos (20 pmol) were microinjected bilaterally into gracile nucleus. Arterial blood pressure and heart rate were monitored for 2 h. The cardiovascular responses to EA ST36 were measured at 10-min intervals before and 15, 30, 45, 60, and 90 min after injection of sense and antisense oligos. Time-response curves of nNOS sense or antisense oligos in the gracile nucleus were obtained for the changes in arterial blood pressure and heart rate induced by EA stimulation of ST36.

Data analysis

Results are expressed as mean ± SE. Mean arterial pressure (MAP) and heart rate are presented using mmHg and beats per minute, respectively. Heart rate and changes in MAP were obtained by measurement of a peak response to each EA stimulation from baseline values. The effects of central pretreatments on EA responses are expressed as changes in MAP and heart rate was induced by EA stimulation as compared with control values. Two-way ANOVA and Fisher’s least significant differences were used to analyze significant difference. P values <0.05 were considered significant.

RESULTS

Effects of lidocaine in the gracile nucleus on cardiovascular responses to EA ST36

In rats anesthetized with urethan, EA stimulation of ST36 produced decreases in MAP and heart rate but the same stimulation on the nonacupoints caused slight cardiovascular responses (Fig. 1). The cardiovascular responses to EA ST36 were initiated within 15–30 s and lasted 2–3 min after a stimulation of 10 s. Figure 2 shows cardiovascular responses to EA stimulation of ST36 with three different frequencies. Frequency-dependent depressor and bradycardia were elicited by EA stimulation of ST36. Immediately following EA stimulation of ST36 with the highest frequency (30 pulse/s), decreases in MAP and heart rate were 16 ± 1 mmHg and 49 ± 4
beats/min. For the same stimulation on the nonacupoints, decreases in MAP and heart rate were 2 ± 1 mmHg and 5 ± 1 beats/min, much lower than the responses induced by EA ST36 (n = 7, P < 0.05). MAP and heart rate were not significantly altered by stimulation of nonacupoints compared with baseline values.

To determine the roles of gracile nucleus in mediating the effect of ST36 stimulation, lidocaine (10 μmol) was injected bilaterally into the nucleus. The baseline of the MAP is 109.1 ± 1.9 mmHg and the mean heart rate is 343 ± 6 beats/min. Lidocaine in the gracile nucleus did not alter the baseline of MAP and heart rate. Following microinjection of lidocaine into gracile nucleus, hypotensive and bradycardiac responses to EA stimulation of ST36 were significantly blocked at all of the three different frequencies (n = 7, Fig. 2).

\( l \)-arginine microinjected into the gracile nucleus

To determine the effects of \( l \)-arginine in the gracile nucleus on the cardiovascular responses to EA stimulation of ST36, \( l \)-arginine (3 nmol) and vehicle were injected into the gracile nucleus (n = 5). The basal MAP and heart rate in rats were 108.1 ± 1.8 mmHg and 323 ± 5 beats/min, respectively. After microinjection of \( l \)-arginine into the gracile nucleus, the MAP was 105.3 ± 1.2 mmHg, and the mean heart rate was 331 ± 12 beats/min. The baselines of MAP and heart rate were not altered by microinjection of \( l \)-arginine or vehicle into the gracile nucleus. The frequency-dependent hypotensive and bradycardiac responses to ST36 stimulation were significantly enhanced by the presence of \( l \)-arginine in the gracile nucleus, as shown in Fig. 3. The responses to \( l \)-arginine on ST36 stimulation occurred 2–5 min after drug administration and lasted for 10–15 min, and the responses reversed at 20 min after the injection. Administration of vehicle has no effect on the cardiovascular responses to EA stimulation of ST36.

\( n \)NOS antisense oligos microinjected into the gracile nucleus

The basal MAP and heart rate were 100.3 ± 2.6 mmHg and 328 ± 8 beats/min. Bilateral microinjection of antisense oligo into gracile nucleus did not alter the baseline of MAP and heart rate.

FIG. 2. Frequency-dependent changes in MAP (top) and heart rate (bottom) induced by EA ST36 in anesthetized Sprague-Dawley rats. Parameters of stimulation: 6 V, 1 ms pulse duration, 3, 10, and 30 Hz for 10 s. ANOVA revealed significant differences of hypotensive and bradycardiac responses to EA stimulation of ST36 following microinjection of lidocaine into gracile nucleus compared with control (n = 7/group). *P < 0.05, compared with control; **P < 0.05, compared with 3 Hz stimulation; ***P < 0.05, compared with 10 Hz treatment.

FIG. 3. Frequency-response curves for changes in MAP in responses to EA stimulation of ST36 before and after microinjection of \( l \)-arginine into the gracile nucleus in anesthetized rats. ANOVA showed that depressor responses to EA ST36 were significantly enhanced by microinjection of \( l \)-arginine into the gracile nucleus (\( P < 0.05, n = 5 \) group, compared with control). Other details are shown in legend to Fig. 2.
rate. After microinjection of antisense oligo into the gracile nucleus, the MAP is 97.1 ± 1.8 mmHg and heart rate is 339 ± 15 beats/min. Figure 4 shows the time-course reduction in cardiovascular responses to EA stimulation of ST36 by microinjection of nNOS antisense oligos in the gracile nucleus (n = 5). The hypotensive and bradycardiac responses to EA ST36 were significantly inhibited at 30, 45, and 60 min after the injection (P < 0.05). The maximum inhibitory responses occurred at 45 min and the responses reversed at 90 min after the injection. The EA ST36-evoked responses were not altered by injection of nNOS sense oligos into the gracile nucleus (n = 5, Fig. 4). Figure 5 presents a medullary coronal section summarizing the locations of gracile nucleus sites for microinjection during cardiovascular studies.

**DISCUSSION**

We examined the effects of L-arginine-derived NO synthesis in the gracile nucleus on the cardiovascular responses to EA stimulation of ST36. The major new findings of these studies are as follows: 1) lidocaine in the gracile nucleus blocks the cardiovascular responses to EA ST36; 2) L-arginine in the gracile nucleus enhances depressor and bradycardia induced by EA ST36; and 3) cardiovascular responses to EA ST36 are inhibited by suppression of the nNOS gene in the gracile nucleus. This is the first evidence showing that hypotensive and bradycardiac responses to EA stimulation of ST36 are enhanced by L-arginine in the gracile nucleus, but attenuated by either lidocaine or suppression of the nNOS gene in the gracile nucleus. The results suggest that the gracile nucleus is an important central site in the neural pathways of the cardiovascular responses to EA ST36. L-arginine-derived NO in the gracile nucleus contributes to cardiovascular effects induced by EA ST36.

Acupuncture has long been used for the treatment of a wide spectrum of cardiovascular diseases and diseases with hypertensive syndromes (Ernst and Lee 1986; Lin and Li 1981; Qian 1986). The therapeutic effects of EA have been studied in various animal experiments. Ohsawa et al. (1995) demonstrated that arterial blood pressure and sympathetic nerve activity were decreased by acupuncture-like stimulation with frequency of about 1 Hz to hindlimb in anesthetized rats. In nonanesthetized spontaneously hypertensive rats, stimulation of somatic afferents in the sciatic nerve to mimic EA caused a decrease in arterial blood pressure (Yao et al. 1982). Our results are consistent with these reports and demonstrate that a somatosympathetic pathway is involved in the mechanism of EA ST36 elicited cardiovascular responses.

The gracile nucleus receives peripheral somatosensory nociceptive inputs that trigger the SSR (Cliffer et al. 1992; Samso et al. 1994). Recently, a number of reports have suggested that the gracile nucleus is an integration center for cutaneous and visceral information flowing into the thalamus, which plays an important role in somatic and visceral pain processing (Al-Chaer et al. 1996a,b, 1997). Our recent studies have demonstrated that the L-arginine-derived NO synthesis in the gracile

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**FIG. 4.** Time-course histogram of antisense oligos to neuronal NO synthase (nNOS) in the gracile nucleus on the cardiovascular responses caused by EA ST36 in rats. Microinjection of nNOS antisense oligos into the gracile nucleus revealed significant differences in depressor (top) and bradycardiac responses (bottom) to EA ST36 (*P < 0.05, **P < 0.01, ANOVA, compared with sense oligos treatment, n = 5/group). Microinjection of nNOS sense oligos into the gracile nucleus did not alter the responses to stimulation of ST36. Other details are shown in legend to Fig. 2.

**FIG. 5.** Coronal section of the dorsal medulla illustrating the histologically verified sites of microinjection in the gracile nucleus during EA ST36. Cu: cuneate nu; cu: cuneate fasciculus; AP: area postrema; CC: central canal; pyx: pyramidal decussation; Gr: gracile nucleus; so: solitary TR; Sol: nu of the solitary TR; 10: dorsal motor nu of vagus; 12: hypoglossal nu; SolI: nu sol tr, intermed. Closed circle: microinjection tips in the gracile nucleus.
nucleus facilitates the cardiovascular responses to stimulus-evoked inhibitory SSR and attenuates the responses to excitatory SSR (Chen and Ma 2002). Lidocaine (lignocaine) is a local anesthetic that reduces the conductance of the Na\(^{+}\) channel, and the excitatory transmitter release (Kaneda et al. 1989). It has been reported that intrathecally administered lidocaine can eliminate the A-δ and C fiber SSR evoked by the electrical stimulation of the tibial and radial nerves (Wang et al. 1994). In our study, the inhibitory cardiovascular responses to EA stimulation of ST36 are blocked by microinjection of lidocaine into the gracile nucleus. These results support the previous concept that the cardiovascular responses to SSR is inhibited by NO in the gracile nucleus (Chen and Ma 2002) and further suggest that the gracile nucleus plays an important role in the SSR neural pathway mediating cardiovascular activities elicited by EA ST36. The results also agree with the investigators who have reported that the gracile nucleus is an important site in autonomic regulation through interaction of peripheral somatosensory information with central pathways.

NO is an important diffusible neurotransmitter, which produces many biological functions in the brain, and NO in the brain plays an important role in the central regulation of cardiovascular responses. Recent studies have demonstrated that the depressor effect of EA ST36 on hypertensive rats can be reduced by microinjection of NO blocker into ventral peri-aqueductal gray matter (Li et al. 2001). NOS-positive neurons are distributed from the Zusanli point projecting to the ganglia of L4 to S1 (Xiong et al. 1998). Jang et al. (2003) showed that nNOS expression is increased in peri-aqueductal gray area of rats with streptozotocin-induced diabetes and acupuncture stimulation of ST36 suppressed the diabetic enhancement in the nNOS expression. Our recent studies revealed that EA stimulation of the cutaneous hindlimb acupoints induces nNOS expression in the gracile nucleus of SD rats, which may contribute to therapeutic effects of acupuncture (Ma and Li 2002). It seems that EA-induced nNOS expressions may vary in different brain areas and may be different from diabetic rats compared with normal rats. The results of the present study showed that microinjection of L-arginine into the gracile nucleus enhanced the depressor and bradycardiac responses to EA stimulation of ST36. It is well known that NO is synthesized from L-arginine catalyzed by nNOS in neurons. The data suggest that L-arginine is transferred into NO, and the newly formed NO produces inhibitory regulation of EA ST36 functions in the gracile nucleus.

Recent studies demonstrated that neuronal gene expression in the brain can be selectively blocked in vivo using antisense oligonucleotides complementary to strategically chosen sequences within the target mRNA (Maeda et al. 1999; Neckers and Whitesell 1993). It was reported that microinjection of nNOS antisense oligos into NTS produces decreases in MAP and heart rate (Maeda et al. 1999). The present results show that microinjection of nNOS antisense oligos into the gracile nucleus attenuates the cardiovascular responses to EA ST36. The changes started 30 min after the administration of nNOS antisense oligos. The largest responses during the observation occurred at 45 min after the injection and lasted 60 min. The time responses of our studies are similar to the effects of microinjection of the NOS antisense oligos into the brain stem nuclei (Chen and Ma 2002; Maeda et al. 1999). The results suggest the suppression of nNOS gene in the gracile nucleus modify the cardiovascular response to EA ST36.

In summary, EA ST36 produces depressor and bradycardia and the effects are blocked by the presence of lidocaine in the gracile nucleus. L-arginine and suppression of nNOS gene in the gracile nucleus affect the inhibitory cardiovascular responses to EA ST36. We conclude that L-arginine-derived NO in the gracile nucleus contributes to central cardiovascular responses to EA ST36.

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