Anatomical evidence of regional specific effects of acupuncture on gastric motor function in rats

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Abstract

To obtain the anatomical evidences of possible neural pathways in mediating acupuncture-induced gastric motor responses, we studied c-Fos immunohistochemistry of the brain stem in response to acupuncture in rats. Acupuncture needles were inserted at the bilateral acupoints of ST-36 (lower limb) or ST-25 (abdomen) for 30 min. After acupuncture, the brainstem was removed for c-Fos immunohistochemistry. The total number of c-Fos immunopositive cells was counted in the dorsal motor nucleus of the vagus (DMV), the nucleus tractus solitarius (NTS) and the rostral ventrolateral medulla (RVLM). Acupuncture at ST-36, but not ST-25, significantly increased the number of c-Fos immunopositive cells at the DMV to 6.7 ± 0.4 cells/section, compared to that of controls (1.7 ± 0.2 cells/section) (n = 5, P < 0.05). Acupuncture at ST-25, but not ST-36, significantly increased the number of c-Fos immunopositive cells at the RVLM to 12.6 ± 0.8 cells/section, compared to that of controls (4.2 ± 0.7 cells/section) (n = 5, P < 0.05). Acupuncture at ST-36 also increased the number of c-Fos immunopositive cells at the medio-caudal and caudal NTS. On the other hand, acupuncture at ST-25 increased the number of c-Fos immunopositive cells at the medio-caudal and caudal NTS. It is suggested that somatic afferents activated by acupuncture at ST-36 is conveyed to the medio-caudal and caudal NTS and stimulates the DMV neurons. In contrast, somatic afferents activated by acupuncture at ST-25 is conveyed to the medio-caudal NTS and stimulates the RVLM neurons. The RVLM neurons are known as premotor sympahto-excitatory neurons that provide drive to the sympathetic preganglionic neurons in the intermediolateral nucleus of the spinal cord. Thus, acupuncture at ST-36 stimulates gastric motility via vagal efferents, while acupuncture at ST-25 inhibits gastric motility via sympathetic efferents in rats.

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1. Introduction

Acupuncture has been used for treating various gastrointestinal (GI) diseases, including irritable bowel syndrome (IBS) (Chan et al., 1997; Fireman et al., 2001), gastroparesis (Chang et al., 2001; Wang, 2004), functional dyspepsia (FD) (Diehl, 1999), constipation (Broidel et al., 2001; Zhu et al., 2003) and diarrhea (Anastasi and McMahon, 2003; Burgmann et al., 2004).

Acupuncture point (acupoint) of ST-36 is one of the most effective points, with a wide range of effects for analgesia (Chen et al., 1996; Han, 2003), immunity (Yu et al., 1998; Hahm et al., 2004), homeostasis and GI disorders. ST-36 is located near the knee joint approximately 10 mm lateral to the anterior tubercle of the tibia in humans.

Electroacupuncture (EA) at ST-36 and PC-6 (forearm) enhances gastric migrating myoelectrical complex in dogs (Qian et al., 1999). EA at ST-36 and PC-6 accelerates gastric emptying of liquid in dogs via a vagal dependent pathway.
(Ouyang et al., 2002). EA at ST-36 enhances restraint stress-induced gastric emptying in conscious rats (Tabosa et al., 2004). On the other hand, acupuncture point of ST-25 (abdomen) is used for treating chronic colitis and diarrhea (Lin et al., 1993; Yang and Yan, 1999). ST-25 is located 50 mm lateral to the umbilicus on the abdomen in humans.

The precise mechanism of acupuncture on GI motor function remains to be clarified. It has been showed that acupuncture at the lower limbs stimulates GI motility (Sato et al., 1993; Tatewaki et al., 2003), while acupuncture at the abdomen inhibits GI motility (Sato et al., 1993; Tada et al., 2003) in rats.

It has been demonstrated that acupuncture-evoked afferent input converge in several nuclei in the brain stem, such as the rostral ventrolateral medulla (RVLM) (Li et al., 2001; Lu et al., 2004), the nucleus tractus solitarius (NTS) (Toney and Mifflin, 2000; Lee et al., 2001; Liu et al., 2004) and the Barrington’s nucleus (Iwa et al., 2006a). It is conceivable that several nuclei in the brain stem play an important role in mediating acupuncture-induced gastric motor responses.

c-Fos is expressed by various stimuli and expression of c-Fos can be used as a marker for stimulus generated neuronal activation. Numerous studies of acupuncture-induced analgesia demonstrated c-Fos is expressed by acupuncture within the brain and the spinal cord (Dai et al., 1992; Lee and Beitz, 1992, 1993; de Medeiros et al., 2003). To obtain the anatomical evidences of possible neural pathway in acupuncture-induced gastric motor responses, we studied c-Fos immunohistochemistry of the NTS, the RVLM and dorsal motor nucleus of vagi (DMV) in response to acupuncture.

2. Materials and methods

2.1. Animals

Male Wistar rats weighting 250–300 g were maintained on a 12:12-h light-dark cycle (8:00–20:00) with free access to food and water. All animals were kept in individual cages in a controlled environment with constant temperature (21–24 °C).
All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by ethical committee of Meiji University of Oriental Medicine.

2.2. Acupuncture procedure in conscious rats

It is highly possible that acupuncture affects GI functions via peripheral and central nervous system (CNS). The anesthetic agents may interfere with the possible neural pathway, which mediates acupuncture-induced GI functions. A previous study indicates that various anesthetic agents (pentobarbital, ketamine, chloral hydrate, urethane, and metofane) eliminate evoked firing and suppressed spontaneous firing in the CNS (West, 1998). The neuron at the CNS may stop its discharging in response to natural stimulation during anesthesia. It is likely that anesthetic agents may interfere the neural activation at the CNS induced by acupuncture. To avoid any possible influence of anesthesia on GI motility in response to acupuncture, we performed acupuncture stimulation in an awake condition.

The body of rats was wrapped by a jacket and the rats were hanged by a fixed stand in order to restrain the body movement. The rats were divided into three groups as follows: non-treated, treated restraint only, treated with restraint and acupuncture. Non-treated rats did not receive any restraint or acupuncture.

Acupuncture needle used in this experiment was 0.25 mm in diameter and 40 mm in length (Seirin; Shizuoka, Japan). Needles were inserted to a depth of 5 mm into the skin and underlying muscles at either ST-36 (lower limb) or ST-25 (abdomen) bilaterally. ST-36 is located at 5 mm lateral and lower from the anterior tubercle of the tibia in rats (Tang et al., 1997). ST-25 is located 20 mm above the symphysis pubis and 5 mm lateral from the midline in rats.

Acupuncture needles inserted into bilateral acupoints were stimulated by electrical square pulse width (0.1 ms, 1 mA and 2 Hz) for 30 min using an electrical stimulator (SEN-3201, Nihonkoden; Tokyo, Japan), as previously described (Iwa et al., 2006a,b).

Another group of rats received an insertion of acupuncture needles for 30 min without electrical stimulation (leaving...
The rats received restraint only served as controls. After finishing EA or LN, the needles were removed and rats were returned to their cages.

In the fasting state, acupuncture at ST-36 induces dual effects, either stimulatory or inhibitory, on gastric motility in conscious rats (Tatewaki et al., 2003). In contrast, our preliminary study showed that EA at ST-36 causes augmentation of postprandial gastric contractions, while EA at ST-25 causes inhibition of postprandial gastric contractions in conscious rats (unpublished observations). To avoid the dual effects of acupuncture on the fasting gastric motor activity, we utilized non-fasted rats in the current study.

2.3. c-Fos immunohistochemistry

To investigate the possible neural pathways in mediating acupuncture-induced gastric motor responses, we performed c-Fos immunohistochemistry of the brain stem. Ninety minutes after finishing EA or LN, the rats were anesthetized with pentobarbital sodium (50 mg/kg IP). As previously reported (Mazda et al., 2004), rats were transcardially perfused for 10 min with 0.01 M PBS to wash out the blood and then perfused with a fixative (4% paraformaldehyde, 0.5% glutaraldehyde, and 0.2% picric acid in 0.1 M phosphate buffer) at 4°C for 10 min. The brain was removed from the skull and immersed for 24 h in the post fixative (4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer) at 4°C and washed for 4 days with several changes of 0.1 M phosphate buffer containing 15% sucrose. The brain was cut into 20 μm coronal sections in a cryostat and collected in 0.1 M PBS containing 0.3% Triton X-100 (PBST).

The sections were processed for c-Fos immunohistochemistry and adjacent sections were stained with cresyl violet to identify the boundary of the brain nucleus.

The sections were incubated with c-Fos antibody (Ab5; Oncogene, San Diego, CA) diluted 1:10,000 in PBST at 4°C for 2 days. After washing for 30 min with PBST, the sections were incubated in biotinylated anti-rabbit IgG (Vector...
Laboratories, Burlingame, CA) diluted 1:1000 in PBST at room temperature for 2 h. The sections were then washed and placed in avidin–biotin peroxidase complex diluted 1:2000 in PBST for 1.5 h at room temperature. Immunoreactivity was visualized by incubating with 0.05 M Tris HCl buffer (pH 7.6) containing 0.01% diaminobenzidine, 1% ammonium nickel sulfate, and 0.0003% H2O2 for 30 min at room temperature. The stained sections were mounted on gelatin-coated glass slides, dehydrated with graded ethanol, and cover slipped with Entellan (Merck, Darmstadt, Germany). The sections were observed under light microscope (Olympus BX51, Olympus, Tokyo, Japan) and images were transported by CCD camera (DP12, Olympus, Tokyo, Japan).

Coronal frozen sections (20 μm) of the brain were cut at the interaural levels of −4.68 to −4.8 mm, −2.8 to −3.72 mm and −4.68 to −5.6 mm, respectively, according to the atlas of Paxinos and Watson (Paxinos and Watson, 1998). In the NTS, divided into 3 sections as follows: the medial NTS (interaural levels of −4.68 to −4.8 mm), the caudal NTS (interaural levels of −5.3 to −5.6 mm) and the medio-caudal NTS (section between the medial and the caudal NTS) (Fig. 1). Under the light microscopy at ×100 magnification, the total number of c-Fos immunopositive cells was counted in each brain nucleus. A mean value of each brain nucleus was determined by sampling from three randomly selected sections cut through the specific brain nuclei.

2.4. Statistical analysis

A mean value of each brain nucleus was calculated by sampling from three randomly selected sections. Data are expressed as mean±SE. Statistical comparisons among multiple groups were first made using a one-way analysis of variance (ANOVA), and if differences were detected, Post hoc analysis was then performed using Stat View 5.0 (Abacus Concept; Berkeley, CA). Scheffe test was used to test the significance. P<0.05 was considered to be significant.

Fig. 4. c-Fos immunohistochemistry at the caudal NTS and DMV in rats treated with restraint only (A), restraint plus EA at ST-36 (B) and restraint plus EA at ST-25 (C). In rats treated with EA at ST-36, the number of c-Fos immunopositive cells was increased in the caudal NTS (B). CC; central canal.
3. Results

3.1. Effects of EA or LN on c-Fos expression in the NTS

In the medial area of NTS, the number of c-Fos immunopositive cells in both ST-36 (lower limb) and ST-25 (abdomen) groups was not significantly increased (Figs. 2 and 6A).

In the medio-caudal area of NTS, the number of c-Fos immunopositive cells significantly increased in response to both EA and LN at ST-25 to 13.7±0.5, 12.5±0.3 cells/section respectively, compared to that of restrained rats (3.5±0.5 cells/section) (n=5, P<0.05) (Figs. 3 and 6B). Both EA and LN at ST-36 also significantly increased c-Fos expression in the medio-caudal NTS (Figs. 3 and 6B).

In the caudal area of NTS, both EA and LN at ST-36, but not ST-25, significantly increased the number of c-Fos immunopositive cells to 11.6±0.9, 10.2±0.6 cells/section respectively, compared to that of restrained rats (2.7±0.6 cells/section) (n=5, P<0.05) (Figs. 4 and 6C).

3.2. Effects of EA or LN on c-Fos expression in the DMV

In the DMV, the number of c-Fos immunopositive cells was remarkably increased in response to both EA and LN at ST-36 to 6.7±0.4, 5.3±0.6 cells/section respectively, compared to those of restrained rats (1.7±0.2 cells/section) (n=5, P<0.05) (Figs. 2 and 7A). However, both EA and LN at ST-25 did not affect the expression of c-Fos in the DMV.

3.3. Effects of EA or LN on c-Fos expression in the RVLM

In EA at ST-25 treated rats (12.6±0.8 cells/section), the number of c-Fos immunopositive cells in the RVLM was significantly higher, compared to those of restrained rats (4.2±0.7 cells/section) (n=5, P<0.05) (Figs. 5 and 7B). LN at ST-25 also significantly increased c-Fos expression in the RVLM. On the other hand, both EA and LN at ST-36 did not affect the number of c-Fos immunopositive cells in the RVLM.

Fig. 5. c-Fos immunohistochemistry in the RVLM in rats treated with restraint only (A), restraint plus EA at ST-36 (B) and restraint plus EA at ST-25 (C). EA at ST-25, but not ST-36, significantly increased the number of c-Fos immunopositive cells in the RVLM (C).
The number of c-Fos immunopositive cells at the NTS, RVLM and DMV was not significantly different between non-restraint group and restraint group (Figs. 6 and 7).

4. Discussion

It has been demonstrated that somatic afferents from the skin and muscle are involved in the control of GI motor functions. In 1913, Lehman showed that electrical stimulation of the sciatic nerve inhibited small intestinal motility in dogs (Lehman, 1913). In 1927, Ruhmann showed that mechanical and thermal stimulation of the abdominal skin affects gastric motility in humans (Ruhmann, 1927). Sensory stimulation of the abdominal skin by pinching inhibits gastric motility, while the pinching of a hind paw enhances gastric motility in rats (Kametani et al., 1979).

Acupuncture treatment involves the insertion of thin needles into the skin and underlying muscle layer. Thus, this procedure may stimulate the somatic afferent nerves of the skin and muscles.

NTS is the primary brainstem relay for visceral information from cardiovascular, respiratory and GI systems. NTS is
adjacent to the dorsal motor nucleus of the vagus (DMV) and composes the dorsal vagal complex (DVC). The DVC integrates vago–vagal reflex which play a major role in the regulation of GI function (Travagli et al., 1992; Washabau et al., 1995). NTS also receives somatic afferent inputs (Gamboa-Esteves et al., 2001). NTS neurons are activated by cutaneous mechanical stimulus to the hind limb in rats (Toney and Mifflin, 2000). The neurons of the NTS and the spinal cord were labeled by injection of neuroanatomical tracers to ST-36 in rats (Lee et al., 2001). Acupuncture at the facial acupoints increased the number of c-Fos immunopositive cells in the NTS in rats (Liu et al., 2004). These suggest that somatic stimulation induced by acupuncture is conveyed to the NTS through the spinal cord.

It is well known that acupuncture has regional specific effects. Acupuncture at the lower limbs stimulates gastric motility (Sato et al., 1993; Tatewaki et al., 2003), while acupuncture at the abdomen inhibits gastric motility (Sato et al., 1993; Tada et al., 2003) in rats. The excitatory gastric responses to hind limb stimulation are abolished by severance of the bilateral vagi (Sato et al., 1993). Others also showed that the stimulatory effects of acupuncture at ST-36 are abolished by muscarinic receptor antagonists, nicotinic receptor antagonists and vagotomy in conscious rats (Tatewaki et al., 2003). In contrast, the inhibitory gastric responses to abdominal stimulation are abolished by severance of the sympathetic nerve branches to the stomach in anesthetized rats. We have previously showed that gastric relaxations induced by acupuncture on the abdomen are abolished by spinal cord transection and splanchnic ganglionectomy, but not by vagotomy (Tada et al., 2003). Acupuncture-induced gastric relaxations were inhibited by guanethidine, propranolol and hexamethonium.

These suggest that the inhibitory gastric responses to abdominal stimulation are mediated via sympathetic nerves, while the excitatory gastric responses to hind limb stimulation are mediated via vagus nerves. However, the mechanism of regional differences of acupuncture points has not been fully studied.

In our current study, the number of c-Fos immunopositive cells in the medio-caudal and caudal NTS was increased by EA at ST-36, while the number of c-Fos immunopositive cells in the medio-caudal NTS was increased by EA at ST-25. This raises the possibility that acupuncture stimulation at the hind limb and abdomen may activate different neurons of the NTS. The number of c-Fos immunopositive cells in the DMV was significantly increased by EA at ST-36, but not by ST-25. This suggests that acupuncture to ST-36 stimulates the DMV and vagal efferents, results in stimulating gastric contractions. This pathway may also include the activation of the NTS neurons at the medio-caudal and caudal sites.

The VLM plays an important role in integrating sympathetic outflow in the brain stem. The RVLM especially regulates cardiovascular system and vasomotor reflex. The NTS neurons project to the RVLM (Agarwal and Calaresu, 1991; Suzuki et al., 1997; Kantzides et al., 2005). Neurons double-labeled with c-Fos and enkephalin in the RVLM are significantly increased in cats treated by EA at PC-5 and PC-6 acupoints (forearm) (Guo et al., 2004). EA at PC-5 and PC-6 significantly decreases gastric distension-induced pressor response. This response is reversed by microinjection of naloxone into the RVLM, suggesting that depressor effect of EA is mediating via opioids in the RVLM (Li et al., 2002).

Our current study showed that EA at ST-25, but not ST-36, significantly increased the number of c-Fos immunopositive cells in the RVLM. This suggests that acupuncture to the abdomen stimulates the RVLM through the medio-caudal NTS, results in stimulation of gastric relaxations.

The inhibitory effects of restraint stress are mediated via stimulating sympathetic efferents (Nakade et al., 2005) and/or inhibiting vagal efferents (Tache et al., 1987). However, the number of c-Fos immunopositive cells at the NTS, RVLM and DMV was not significantly different between non-restraint group and restraint group. This suggests that our current method of restraint used during the acupuncture may be much milder than that usually used for loading restraint stress (Nakade et al., 2005, 2006).

In our current study, we compared the efficacy of acupuncture procedure with and without EA on c-Fos expression. Leaving needle (LN), which is one of the procedure frequently used in the clinical practice, leaves inserted needles without any stimulation. On the other hand, EA is a combined procedure with LN and electrical stimulation on the inserted needles. EA is more frequently used than LN in clinical and research settings in recent years.

Our current study showed that the number of c-Fos immunopositive cells in the DMV was significantly increased by LN at ST-36 as well as EA (2 Hz) at ST-36. Similarly, the number of c-Fos immunopositive cells in the RVLM was significantly increased by LN at ST-25 as well as EA (2 Hz) at ST-25. This suggests that a simple technique of LN may have a similar efficacy with EA (2 Hz) on stimulating the neurons at the brain stem in rats.

In summary, EA at ST-36 increased the number of c-Fos immunopositive cells in the medio-caudal NTS, caudal NTS and DMV, whereas EA at ST-25 increased the number of c-Fos immunopositive cells in the medio-caudal NTS and RVLM. This suggests that acupuncture at ST-36 elicits gastric contractions via the medio-caudal NTS, caudal NTS and DMV, while acupuncture at ST-25 elicits gastric relaxations via the medio-caudal NTS and RVLM.

The series of somato-autonomic reflex investigations has provided good evidence of cutaneous input in autonomic control of GI motility (Lehman, 1913; Ruhmann, 1927; Kametani et al., 1979). In the NTS, sensory input from either acupuncture at ST-36 or ST-25 is conveyed to either the DMV or the RVLM, respectively. The verification of neural pathway may contribute to the better understanding of the mechanisms of the regional difference of acupuncture in regulating GI motor function.
References


