대한한의학회지 제29권 제1호(2008년 3월)
J Korean Oriental Med 2008;29(1):39-46

Original Article

Acupuncture on ST36 Increases c-Fos Expression in vlPAG of Visceral Pain-induced Mice

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Background : Acupuncture has been used as a clinical treatment in Oriental medicine for various diseases including pain relief. The descending pain control system of periaqueductal gray (PAG) is a powerful pain control system in mammalians. Expression of c-Fos is used as a marker for stimuli-induced metabolic changes of neurons.

Objective : In the present study, the effects of acupuncture on analgesic effect in visceral pain were investigated through the writhing reflex and c-Fos expression in ventrolateral PAG (vlPAG) area using immunohistochemistry in mice.

Method : For the writhing test, mice were divided into five groups. Immediately after finishing the behavioral test, the animals were weighed and overdosed with Zoletil. After a complete lack of response was observed, the brains of the mice were dissected into serial coronal sections, and c-Fos immunohistochemistry was performed. Statistical analysis of all data was performed using one-way ANOVA.

Result : The present results showed that acupuncture affected the writhing reflex and that Choksamni (zusnali) acupoint and aspirin significantlysuppressed acetic acid treatment-induced increased writhing reflex, and the expression of c-Fos in vlPAG was significantly increased in the acupunctured group.

Conclusion : The present study suggests that acupuncture has an antinociceptive effect on acetic acid-induced visceral pain by increase of c-Fos expression in mice. Aspirin also showed analgesic effect, however the mechanism is different from the acupuncture.

Key Words : Acupuncture, visceral pain, antinociceptive, c-Fos; vlPAG

Introduction

Acupuncture has traditionally been used in Oriental medicine for the treatment and prevention of various diseases and for the control of pain[1-3]. It is known that acupuncture modulates neurotransmitter functions in the central nervous system (CNS)[2,3] and possesses such various positiveeffects as analgesia, promotion of homeostasis, induction of changes in the microcirculatory network, and improvement in brain circulation[3,4].

The Choksamni (zusnali) acupoint (ST36), near the kneejoint of the hind limb 1mm lateral to the anterior tubercle of the tibia, is one of the most effective acupuncture points for brain diseases with a wide range of analgesic, spasmolytic, and homeostatic effects[5-7]. Experimental studies have shown that acupuncture on ST36 stimulates cell proliferation in the dentate gyrus under pathologic conditions, including ischemia and diabetes[2,5]. Acupuncture on ST36 also decreased neuronal cell death following hemorrhage or ischemia in animals, thus demonstrating the neuroprotective effect of the ST36 against brain damage[8,9].

· received : 2007년 12월 13일
· accepted : 2008년 3월 5일
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Transmission of nociceptive information may be altered by many neuronal circuits within the CNS[10]. The descending pain control system consists of three major components: the periaqueductal gray (PAG) of the midbrain, the rostroventral medulla including the nucleus raphe magnus, and the spinal dorsal horn. Descending modulation of spinal nociceptive neurones from the periaqueductal gray matter (PAG) is one of the most extensively studied pain control systems[11]. PAG is rich in opioid receptors and opioid peptidesopiates are known to produce analgesia by activating descending pain control pathways, especially at the level of the PAG[12,13]. It is well documented that endogenous opioid peptides can activate PAG output neurons by inhibitory interneurons[14,15], and also reported that the analgesic effect induced by stimulation on the ventralateral PAG (vlPAG) is abolished by opioid antagonist naltrexon.

c-Fos is an immediate early gene, expression of which is used as a marker for stimuli-induced metabolic changes of neurons, and is also induced in the CNS under various conditions[16-18]. Mapping of the brain areas associated with the analgesic effects represented by c-Fos expression in either anesthetized or restrained animals has provided useful information for the understanding of its mechanisms of action.

In the present study, to gain insights into analgesic mechanism of acupuncture controlling acetic acid-induced visceral pain, we investigated the effect of acupuncture on c-Fos expression in the vlPAG region.

Materials and Methods

1. Experimental Animals

Experiments were performed on male ICR mice, weighing 32 - 36 g. Each animal was housed at a controlled temperature (20±2°C) and was maintained under 12 h light and 12 h darkness (lights on from 07:00 h to 19:00 h), with food and water made available *ad libitum*. The experimental procedures were performed in accordance with the animal care guidelines of the NIH and the Korean Academy of Medical Science.

2. Writhing test

Mice were divided into five groups:the normal saline-injection group (normal group), the acetic acid-injection group (control group), ST36-acupunctured and acetic acid-injection group, the non-acupoint-acupunctured and acetic acid-injection group, and aspirin-treated and acetic acid-injection group (n = 10 in each group). The mice were injected intraperitoneally with 0.2 ml of 1.5% acetic acid. Immediately after injection, the mice were placed in a large glass cylinder. The numbers of abdominal stretches were counted for 30 min after acetic acid injection.

3. Acupuncture and treatments

In the acupunctured groups, acupunctural treatment was given to the mice before acetic acid injection. For acupunctural stimulation at ST36, stainless acupuncture needles of 0.1 mm diameter were bilaterally inserted into ST36 and left in place for 20 min. In the non-acupuncture groups, in same condition to acupunctured group except both hips were used for the acupunctural stimuli(Fig. 1). In the aspirin-treated group, 100 mg/kg aspirin was injected intraperitoneally 1 h before acetic acid injection.

4. Tissue preparation

Immediately after finishing behavioral test, the animals were weighed and overdosed with

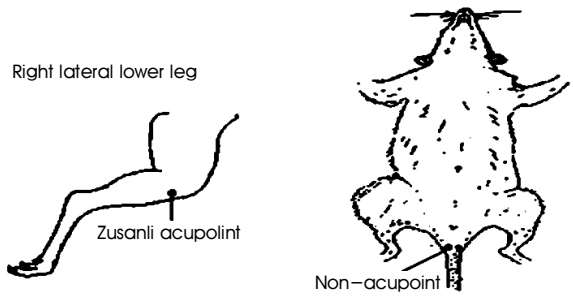


Fig. 1. Schematic illustration of acupoints.
Left : ST36 acupoint. Right: the non-acupoint.

Zoletil 50® (10 mg/kg, i.p.; Vibac Laboratories, Carros, France). After a complete lack of response was observed, the mice were transcardially perfused with 4% paraformaldehyde (PFA) in 100 mM phosphate buffer (PB, pH 7.4). The brains were dissected, postfixed in the same fixative overnight, and transferred into a 30% sucrose solution for cryoprotection. Serial coronal sections of 40 μm thickness were made using a freezing micritome (Leica, Nussloch, Germany).

5. c-Fos Immunohistochemistry

For analyzing c-Fos expression in vlPAG, 20 sections on average were selected from each brain region spanning from bregma -4.24 to -4.96 mm. For visualization of c-Fos expression, c-Fos immunohistochemistry was performed. In brief, the sections were drawn from each brain and incubated with rabbit anti c-Fos antibody (1:1,000, Santa Cruz Biotechnology, CA, USA) and the for another 1 h with biotinylated anti-rabbit antibody made in goat as a secondary antibody. Bound secondary antibody was then amplified with VECTASTAIN® Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA). The antibody-biotin-avidin-peroxidase complexes were visualized using 0.02% 3,3'-diaminobenzidine (DAB) with nickel and the sections were finally mounted on the gelatin-coated slides.

6. Data analysis.

The area of vlPAG region was measured and the number of c-Fos positive cellsin the vlPAG were counted and expressed as the number of cellsper mm^2 of vlPAG region. All measurements on immunohistochemistry were made using image with digital camera attached to light microscope (Olympus, Tokyo, Japan) and the Image-Pro Plus® software (Media Cybernetics Inc., Silver Spring, MD, USA).

Statistical analysis of all data was performed using one-way ANOVA followed by Tukey's HSD post-hoc test. Results are presented as the mean ± standard error of the mean (SEM). Differences were considered significant for $P < 0.01$

Results

1. Effect of acupuncture on writhing reflex in mice

The number of writhing reflexes in the normal group was 0±0. The number of writhing reflexes increased to 35.33±3.57 in the acetic acid-

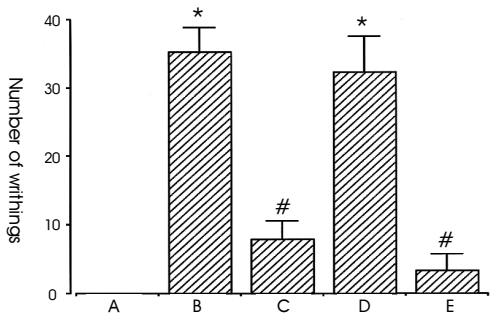


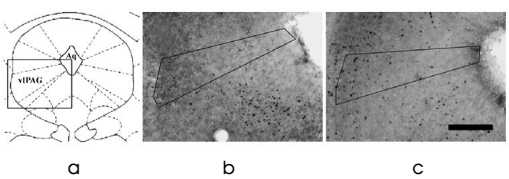
Fig. 2. Effect of acupuncture on writhing reflex in mice.
Number of writhing in each group. A, Normal group; B, Control group; C, ST36-acupunctured and acetic acid-injection group; D, non-acupoint-acupunctured and acetic acid-injection group; E, aspirin-treated and acetic acid-injection group. Results are presented as mean ± SEM. * represents $P < 0.01$ compared to the normal group. # represents $P < 0.05$ compared to the control group.

injection group, but this number significantly decreased to 8.00±2.70 in the ST36-acupunctured and acetic acid-injected group. The number was 32.33±5.20 in the non-acupoint-acupunctured and acetic acid-injection group, while the aspirin-treated and acetic acid-injection group showed significant decrease at 3.4±5.41.

The number of writhing reflexes was increased by acetic acid injection, while acupuncture on ST36 and aspirin treatment with acetic acid significantly reduced the number of writhing reflexes. The present results showed that acupuncture on ST36 alleviated visceral pain induced by acetic acid (Fig. 2).

2. Effect of acupuncture on c-Fos expression in mice vlPAG

The number of c-Fos positive cells in the



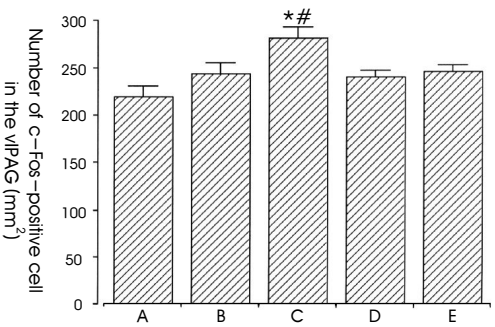


Fig. 3. Modulation of acupuncture on c-Fos expression in the ventrolater periaqueductal gray (vlPAG).
Upper: Photomicrographs of c-Fos expression in vlPAG. a, location of vlPAG; b, acetic acid-injection group; c, ST36-acupunctured and acetic acid-injection group. The scale bar represents 500 μm. Lower: Number of c-Fos-positive cells in the vlPAG. A, Normal group; B, Control group; C, ST36-acupunctured and acetic acid-injection group; D, non-acupoint-acupunctured and acetic acid-injection group; E, aspirin-treated and acetic acid-injection group. Results are presented as mean ±SEM. * represents $P < 0.01$ compared to the normal group. # represents $P < 0.05$ compared to the control group.

vlPAG was 219.79±11.13 in the normal group. The number of c-Fos positive cells in the vlPAG was 245.20±10.61in the acetic acid injection group. This number increased to 282.39±11.13 in the ST36-acupunctured and acetic acid-injected group. The number was 241.85±6.52 in the non-acupoint-acupunctured and acetic acid-injection group and 246.81± 6.60 in the aspirin-treated and acetic acid-injection group.

The present results showed that c-Fos expression in the vlPAG was increased by acupuncture on ST36, suggesting acupuncture on ST36 activates the descending pain control system in the vlPAG region (Fig. 3).

Discussion

Acupuncture has been used for controlling various kinds of pain including lower back pain, chronic elbow pain, and toothache. Acupuncture-induced analgesia has been studied in patients with diabetic neuropathy and neuropathic animal models[19,20].

Choksamni(zusnali;ST[36]) is the converging point of the stomach meridians (ST) and has a character of regulating Ki(Qi) and blood, and strengthening spleen and stomach. Therefore, they are widely used for hemiplegia, hypertension, diseases of the digestive system, and enervation[21].

In the present results, intraperitoneal injection of acetic acid increased the number of writhing reflexes, showing that acetic acid induced visceral pain in mice. Acupuncture on ST36 suppressed the number of writhing reflexes, demonstrating that acupuncture on ST36 exerted analgesic effect on acetic acid-induced visceral pain. However, acupuncture on non-acupoints did not show analgesic effect.

c-Fos has been used to identify activated neurons within the CNS expressed rapidly by various stimuli. c-Fos protein appears 30 min after stimulation and its expression lasts a few hours depending on the type and strength of the stimuli. c-Fos has been considered as a marker for activated neurons[16,22,23]. c-Fos expression represents activated neurons induced by various kinds of stimuli including noxious stimulation[24-27]. c-Fos expression has been used to investigate the antinociceptive effect of acupuncture[28,29].

The midbrain PAG is believed to be an important component in the endogenous pain control system[14]. Neurons in the PAG of the midbrain are connected to the rostroventral medulla, and some neurons of the rostroventral medulla have inhibitory connections with neurons in the lamina of the spinal cord[30]. Opioid peptides and opiates produce analgesia by activating the descending pain control system, especially at the level of the PAG[11,12,31]. vlPAG is a crucial region for the descending control of the transmission of pain from the spinal cord dorsal horn. Several reports have indicated that electrical stimulation on the vlPAG selectively inhibited responses to noxious stimuli in a variety of pain test conditions[32-36]. This antinociceptive effect of vlPAG has been reported in tail-flick tests and in long lasting tonic pain tests induced by subcutaneous injections of formalin[37-41]. Consistent evidences on the antinoceptive regulation of vlPAG have been provided[10,42]. Acupuncture is known to activate PAG neurons[43].

In this study, c-Fos expression in the vlPAG was increased only by acupuncture on ST36. The results suggested that acupuncture on ST36 should activate the descending pain

control system in the vlPAG region, exerting antinociceptive effect.

Aspirin is one of the widely usednon-steroidal anti-inflammatory drugs (NSAIDs), and it reduces synthesis of prostaglandins which are derived from arachidonic acid. Prostaglandins play key roles in inflammation in peripheral pain. Aspirin produces analgesic and anti-inflammatory effects through inhibiting cyclooxygenase enzyme activity.

According to these results, aspirin significantly inhibited the number of acetic acid-induced writhing reflexes, demonstrating that aspirin exerted analgesic effect on acetic acid-induced visceral pain. However, c-Fos expression in the vlPAG was not significantly changed by aspirin, suggesting that the analgesic effect of aspirin is not associated with descending pain control system in the vlPAG[44,45)].

It suffices to say that acupuncture on ST36 exerted analgesic effect on acetic acid-induced visceral pain, while acupuncture on non-acupointsdid not show analgesic effect. This analgesic effect of acupuncture on ST36 may occur through activation of the descending pain control system in the vlPAG. Further study is required to determine which mechanisms make aspirin and the acupuncture on ST38 analgesic act differently.

Conclusion

The present study suggests that acupuncture has an antinociceptive effect on acetic acid-induced visceral pain by increase ofc-Fos expression in mice. Aspirin also showed analgesic effect, however the mechanism is different from the acupuncture.

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