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Non-opioid-dependent anti-inflammatory effects of low frequency electroacupuncture

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Abstract

Low frequency electroacupuncture, which is commonly used in pain relief, is known to induce opioid-mediated analgesia. This study examined the contribution of the opioid system in mediating the anti-inflammatory effects of low frequency EA in a standard model of acute inflammation, the carrageenan-induced edema model. Carrageenan was injected in the hind paw of anesthetized rats and low frequency electroacupuncture was applied to acupoints equivalent to Zusanli (St 36) and Sanyinjiao (Sp 6) in humans just prior to the induction of inflammation in the ipsilateral leg. Induction of Fos protein, reflecting neuronal activation, was investigated in the spinal cord with immunohistochemistry. It was found that electroacupuncture strongly inhibited the carrageenan-induced edema by over 60%, and suppressed the associated Fos expression in the superficial laminae (I–II) of the ipsilateral dorsal horn by 50%. Neither the anti-edematous effect nor the suppression of Fos expression in the superficial spinal laminae was affected by intraperitoneal injection of the opioid antagonist naloxone. These results demonstrate that low frequency electroacupuncture is capable of inhibiting peripheral inflammation and the associated central neuronal activity via a non-opioid-dependent mechanism.

Keywords: Acupuncture; Naloxone; Carrageenan inflammation; c-*fos*; Dorsal horn; Pain

1. Introduction

A major application of acupuncture is for pain relief [12]. However, the mechanism underlying the pain-relieving effect is far from clear. Activation of the opioid system is a well documented phenomenon during acupuncture [17]. Thus, the analgesic effects of acupuncture can be attenuated either by systemic treatment of the opioid antagonist naloxone [34,41], or by intracerebral microinjection of naloxone into opioid receptor-containing brain sites [55]. Moreover, opioid release in the cerebrospinal fluid has been found in human following acupuncture [9,18]. The colocalization of Fos-containing neurons and beta-endorphin-positive neurons in the anterior lobe of the pituitary gland further suggests that the pituitary gland is activated by acupuncture to release opioids [37]. However, despite the ample evidence supporting the concept that acupuncture activates the endogenous opioid system to produce analgesia [17,47,49], the role of opioids in mediating acupuncture-induced analgesia in human subjects is controversial [7,34,38,43]. Most notably, the pain-relieving effects of acupuncture in chronic pain patients are naloxone-resistant [21,27,29]. Apparently, discrepancies exist between the results of experimental studies and clinical findings with regards to the role of opioids. It seems that opioids only contribute to the transient antinociceptive effect of acupuncture [34,41,49], whereas acupuncture relief from the more complex clinical pain is non-opioid-dependent [11,21,23,27,29]. It is therefore necessary to study the mechanism of acupuncture and the contribution of opioids using different animal models.

Many pain conditions that have been treated by acupuncture, such as tendonitis [22], oesteoarthritis [1,19,48], and rheumatoid arthritis [32,33], involve inflammation. Evidence is accumulating that acupuncture has anti-inflammatory effects [28], in addition to analgesia. However, the role of opioid activation in mediating the anti-inflammatory effect of acupuncture is uncertain, although opioids are known to have anti-inflammatory properties both within the central

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nervous system and in the periphery [20,46]. It has been reported that naloxone partially blocks the anti-inflammatory effect of acupuncture in a capsaicin-induced edema model [5], but similar findings have not yet been shown in other models of inflammation.

The carrageenan (CA)-induced edema is a widely used model for screening anti-inflammatory drugs [50]. The first objective of the current study was to use this model to examine the anti-edematous effect of low frequency electroacupuncture (EA) and the contribution of the opioid system to this phenomenon. Low frequency EA is known to produce opioid-dependent analgesia and to inhibit Fos expression evoked by noxious stimulation in the dorsal horn of the spinal cord [26,49]. Fos is the protein product of the c-*fos* gene, which is considered one of the candidate immediate early genes for coupling excitation of neurons to long-term adaptive modifications of transcription. The expression of Fos protein has been used previously as a marker for increased neuronal activity in response to inflammation as well as to noxious stimulation [3,4,26,53]. However, little is known about the effect of acupuncture on c-*fos* expression evoked by acute local inflammation without association with pain. The second objective was therefore to determine the effect of acupuncture on c-*fos* expression in the spinal cord associated with inflammation.

2. Materials and methods

2.1. Experimental animals and model of acute inflammation

The protocol of the experiments was approved by the Animal Ethics Committee of the Hong Kong Baptist University. Sprague–Dawley rats weighing 200–300 g were used. Light anesthesia was maintained with chloral hydrate (400 mg/kg i.p., with supplement doses of 100 mg/kg when required), which was chosen because the duration and depth of anesthesia are linearly related to the dose [14]. Inflammation was induced by injection of 1 mg of carrageenan λ (Sigma, St. Louis, MO, USA) in 0.1 ml of pyrogen-free water into the plantar surface of the left hind paw subcutaneously [50]. Paw volume was measured with the use of a plethysmometer (Ugo Basile, Comerio, Italy) prior to the CA injection and at 1 h intervals for 4 h after the injection. For edema studies, animals were divided into three groups: an EA group, which received EA for 45 min and then intraplantar CA injection (EA + CA, $n = 12$), a control group with CA injection only (CA, $n = 12$), and a naloxone-blocking group (Nal + EA + CA, $n = 12$). Pilot experiments were also carried out to examine the effect of EA applied 1 h after CA injection. In these experiments, animals of both the treatment (CApt + EA, $n = 9$) and the control (CApt, $n = 9$) groups received intraplantar CA injection immediately after anesthesia. For experiments involving the study of Fos immunoreactivity, a vehicle control group (H_2O, $n = 6$), which received intraplantar injection of 0.1 ml pyrogen-free water, was also introduced. Animals of the CA or H_2O group received CA or H_2O injection 45 min after anesthesia, at the same time as other groups receiving CA injection following EA.

2.2. Electroacupuncture treatment

Low frequency EA (4 Hz), which had been found to activate the endogenous opioid system [26,49], was used as the mode of stimulation. Two stainless steel acupuncture needles were inserted perpendicularly into points corresponding to acupoints Zusanli (St 36) and Sanyinjiao (Sp 6) in humans, which were located according to anatomical landmarks. St 36 was at the juncture of proximal 1/5 and distal 4/5 between the lateral condyle of the tibia and the lateral malleolus, 2 mm lateral to the anterior border of the tibia; Sp 6 was at the juncture of the proximal 4/5 and distal 1/5 of the distance between the medial condyle of the tibia and the medial malleolus, between the tibia and the fibula. Needle insertion was ipsilateral to the CA injection site, approximately 6 mm deep for St 36 and 3 mm for Sp 6. EA treatment was given just prior to CA injection, as pilot experiments found that EA given after CA injection was ineffective in reducing edema (Table 1). The EA stimulation, consisting of 0.45 ms square wave pulses at 4 Hz lasting for 45 min, was delivered by a constant current EA machine (CEFAR Acus II, Lund, Sweden) to produce a moderate muscle twitch. The intensity of stimulation was typically 0.6–0.8 mA.

2.3. Naloxone blocking experiments

Naloxone hydrochloride powder (Sigma) was freshly prepared in 0.9% NaCl solution just prior to use. An initial dose of naloxone (5 mg/kg i.p.) was injected 15 min prior to EA stimulation. A supplement dose (2.5 mg/kg i.p.) was administered every 30 min for 2 h [26].

As the focus of this study was to determine the contribution of opioid activation during EA, it was important to determine whether the dose of naloxone was adequate in blocking opioid receptors. To this end, a separate series of

Table 1
Baselines and maximal changes of paw volume expressed as median/range

Experimental group	Baseline volume (ml)	Maximum Δ edema (ml)
EA given 1 h after CA injection		
CApt ($n = 9$)	1.69/0.32	0.49/0.50
CApt + EA ($n = 9$)	1.68/0.27	0.54/0.31
EA given prior to CA injection		
CA ($n = 12$)	1.65/0.58	0.53/0.51
EA + CA ($n = 12$)	1.66/0.53	0.19/0.26*
Nal + EA + CA ($n = 12$)	1.66/0.59	0.16/0.26*
Vehicle control		
H_2O ($n = 6$)	1.66/0.28	0.12/0.05

* $P < 0.01$, compared with CA (control).

pilot experiments was carried out using the hot water tail flick reflex paradigm, as previously described [36]. Briefly, conscious rats were tested for latency of tail flick reflex by immersing the tail into 53 °C water. The baseline latency was found to be 5.34 ± 0.31 s (mean ± S.E.M., $n = 7$). All the animals were then given morphine (5 mg/kg, i.p.; Antigen, Liberty, USA), and the tail flick latency was again assessed. Immediately after morphine injection, no tail flick reflex was observable up to 150 s, when the test was discontinued. Then four of the seven animals were given naloxone (5 mg/kg, i.p.), and the tail flick reflex was measured again 10 min later. The tail flick latency of animals which had been injected with naloxone ($n = 4$) was 5.1 ± 1.0 s, being reverted back to the baseline level. In contrast, the remaining three animals which did not receive naloxone injection had a tail flick latency of 33.5 ± 2.8 s at the same time point, much longer than that of the naloxone-injected group. These results demonstrated that naloxone at 5 mg/kg dosage used for the experiments was effective in blocking opioid receptors.

2.4. Detection of Fos immunoreactivity

At the end of each experiment, 4 h after CA or vehicle control injection, the animals were deeply anaesthetized with sodium pentobarbitone (60 mg/kg i.p.), and perfused transcardially with 200 ml of 0.01 M phosphate-buffered saline (PBS, pH 7.4) followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The spinal cord was removed from L3-L5, postfixed for 4 h in the same fixative and cryoprotected overnight in 30% sucrose in PB at 4 °C. Frontal frozen 40 μm-thick sections were cut with a cryostat. Every fifth section was collected in PBS and prepared for immunostaining. The sections were washed in 0.01 M PBS, incubated in methanol with 0.3% H_2O_2 for 30 min, then rinsed again in 0.01 M PBS. The sections were then incubated in a solution containing 0.01 M PBS, 0.4% Triton X-100, 0.1% bovine serum albumin and 1% normal goat serum for 30 min in room temperature. The rabbit Fos polyclonal antibody (Cambridge Research Biochemicals, Valley Stream, NY, USA) was added to the same solution (1:2000 dilution) and the sections were incubated at 4 °C for 48 h. After the incubation in primary antibody, the sections were washed in 0.01 M PBS (pH 7.4) and incubated with biotinylated goat anti-rabbit IgG for 3 h, followed by incubation in avidin-biotin-peroxidase complex (Vectastain, Vector Laboratories, Burlingame, CA, USA) for 2 h at room temperature. Between each incubation step, the sections were rinsed in three 10-min washes of PBS. Spinal cord sections were placed on an agitator during each incubation and rinse step. The horseradish peroxidase reaction product was visualized by using the 3,3′-diaminobenzidine (DAB) kits from Vector Laboratories, with nickel chloride solution and a standardized development time of 5 min. The reaction was terminated by two consecutive 0.01 M PBS washes. Finally, the sections were mounted on gelatin-coated slides, rinsed in a series of 95% ethanol, 100% ethanol, and xylene, and then coverslipped. Typically, tissues from three different experimental groups were processed together, so as to minimize the variability associated with different reactions. Fos-immunoreactive (Fos-IR) cells were first identified by the appearance of black DAB reaction product distinguishable from background staining at 4× magnification, and then verified by visualizing the DAB product within the cell nucleus at 20×. To determine the specificity of the immunostaining for Fos protein, controls were carried out in which the Fos primary antibody was omitted from the staining protocol. No Fos-positive cells were stained in the controls.

2.5. Quantification and statistical analysis

Spinal sections were examined under a light microscope by an investigator blind as to the experimental group and treatment. The number of Fos-IR neurons was counted in the superficial (I–II) and deep (III–V) laminae of the dorsal horn in 10 sections per animal, and averaged to generate a regional mean per animal. StatView for Windows (SAS Institute Inc. Cary, NC, USA) was used for data management and statistical analysis. Data were presented as mean ± S.E.M. for comparison between groups, or as median and range to indicate the distribution of the data. The differences in mean values between different experimental groups, and between different time points, were evaluated by ANOVA followed by the Fisher's PLSD test. Statistical significance was inferred when $P < 0.05$.

3. Results

3.1. Effects on paw edema

The baselines and maximal changes of paw volume in different experimental groups are summarized in Table 1. In the control group, the CA-induced paw edema reached a maximum at about 3 h, which represented a 32.9 ± 3.3% (mean ± S.E.M., $n = 12$) increase from the pre-CA injection volume (Fig. 1). A similar pattern and severity of edema were seen in control animals receiving CA immediately after anesthesia (CApt), or 45 min after anesthesia (CA). Pilot experiments showed that EA delivered 1 h after CA injection did not alter the development of edema (Table 1). In contrast, EA pretreatment prior to intraplantar injection of CA markedly reduced the subsequent development of paw edema. In comparison with the control group (CA), paw edema of the EA + CA group was inhibited by 56, 62, 64 and 65% at 1, 2, 3 and 4 h post CA injection, respectively ($P < 0.01$ for all the time points; see Fig. 1).

In order to determine the contribution of the endogenous opioid system in mediating the anti-inflammatory effects of EA, the opioid receptor antagonist naloxone was adminis-

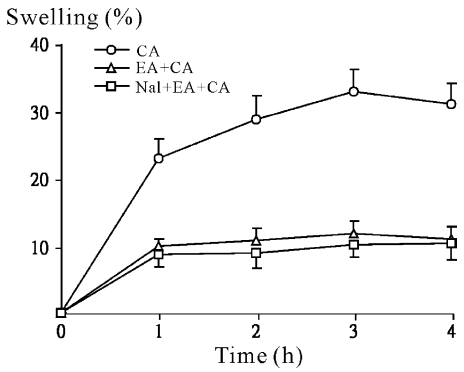

Fig. 1. Line graph showing the inhibitory effect of low frequency electroacupuncture (EA) on carrageenan (CA)-induced inflammation in anaesthetized rats. EA was applied for 45 min prior to subplantar injection of 1% carrageenan at the ipsilateral hind paw. Each value represents the mean ± S.E.M. in 12 animals. Compared with the CA injection only group, animals pretreated with EA showed a significant reduction in edema ($P < 0.01$). Intraperitoneal injection of naloxone (Nal) did not alter the anti-edematous effect of EA ($P > 0.5$ for all time points measured). CA: CA injection alone; EA + CA: EA pretreatment prior to CA injection; Nal + EA + CA: naloxone treatment of animals with CA-induced inflammation and pretreated with EA.

tered prior to, during and after EA (see Methods). As seen in Fig. 1, there was no significant difference in edema between the naloxone treatment group (Nal + EA + CA) and the EA + CA group ($P > 0.5$ for all time points, $n = 12$ for both groups), indicating that the anti-edematous effect of EA was not dependent on opioid receptors.

3.2. Fos expression in the spinal cord

Fos immunoreactivity was used to monitor the pattern of neuronal activation in the spinal cord following CA-induced inflammation, and the effect of EA or EA with naloxone. As mentioned above, for comparison a vehicle control group (H_2O), which received H_2O injection at the hind paw and displayed small degree of edema (Table 1), was also included. In the CA control group, Fos-IR neurons were mainly found in the medial part of the superficial laminae (I–II) ipsilateral to the side of the injection (Figs. 2A, and 3). The mean number of Fos-IR neurons in the ipsilateral side was 22.2 ± 0.91 in the superficial laminae and 6.2 ± 0.48 in the deep laminae (Fig. 4). Compared with the CA control group, EA pretreatment reduced the number of Fos-IR neurons by 50% in the ipsilateral superficial laminae to 11 ± 0.37 ($P < 0.001$), but had no effect in the deep laminae (mean = 5.17 ± 0.48, $P > 0.05$). In the H_2O group, the number of Fos-IR neurons in the superficial laminae (7 ± 0.91, $n = 6$) was significantly lower than in the other groups ($P < 0.01$). No difference was found in the number of Fos-IR neurons in the deep laminae (III–V) between all groups (Fig. 4).

Naloxone treatment of animals with CA-induced inflammation and EA pretreatment did not change the pattern of Fos expression compared with the EA + CA group (Figs. 2–4). The number of Fos-IR neurons in the ipsilateral side of the Nal + EA + CA group was 11.67 ± 0.99 in the superficial laminae and 5 ± 0.58 in the deep laminae,

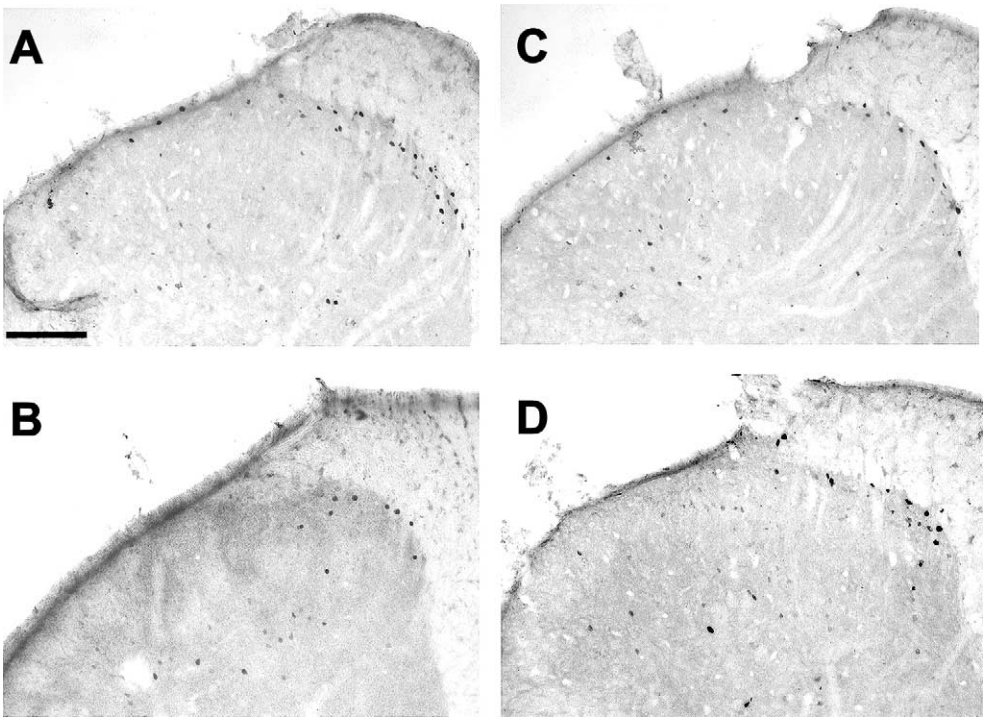

Fig. 2. Photo showing examples of Fos-IR cells in the dorsal horn of the spinal cord after subplantar injection of 1% carrageenan (A), vehicle control injection (B), electroacupuncture pretreatment and carrageenan injection (C), and naloxone blocking of the pretreatment effect of EA (D). Calibration: 200 μm.

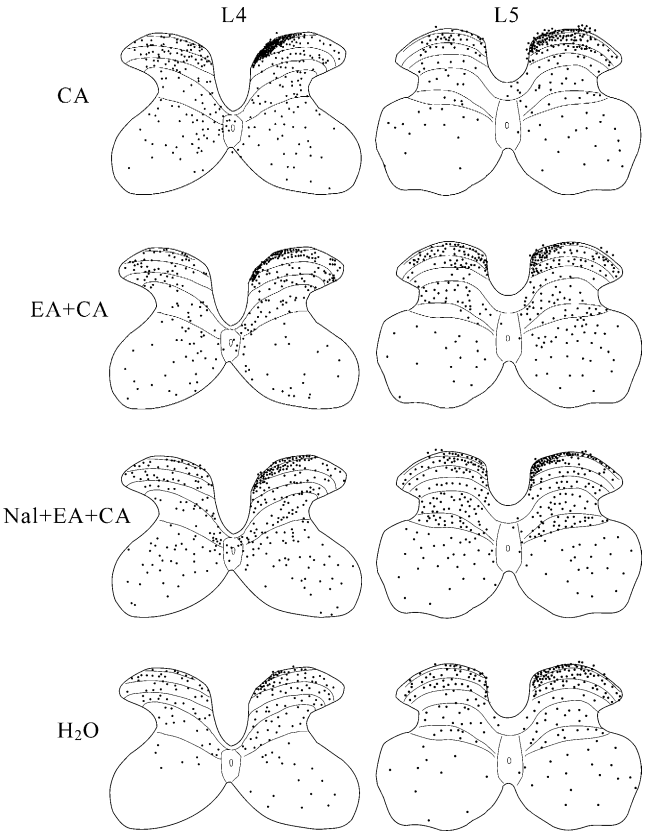

Fig. 3. Schematic drawing of frontal sections of the spinal gray matter showing the distribution of Fos-IR cells at L4–5 of representative animals from each experimental group. Each dot represents one Fos-IR cell. Fos-IR cells from the five most heavily labeled sections of an experiment have been combined onto a section. Note that Fos expression in the superficial laminae (I–II) evoked by carrageenan injection (CA) is inhibited by EA pretreatment (EA + CA), and the inhibitory effect is not affected by naloxone (Nal + EA + CA). H_2O: vehicle control group. See Fig. 1 legend for other keys.

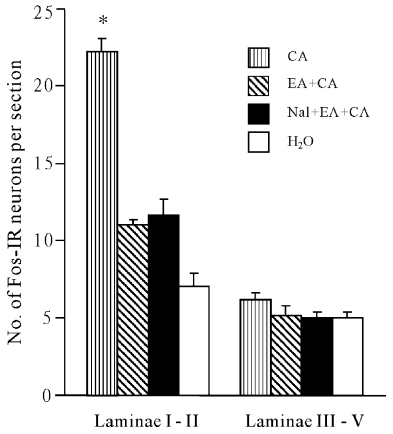

Fig. 4. Histogram showing the mean ± S.E.M. of Fos-IR cells in the dorsal horn of the spinal cord from different experimental groups. The number of Fos-IR neurons were counted for the superficial (I–II) and deep (III–V) laminae of the dorsal horn in 10 sections per animal, and averaged to generate a regional mean. In the superficial laminae, both the EA + CA and the Nal + EA + CA groups show about 50% less Fos-IR neurons compared with the CA group, and the H_2O group had the least Fos-IR neurons ($^*P < 0.01$; ANOVA). There is no difference between the EA + CA group and the Nal + EA + CA group for both superficial and deep laminae. See Figs. 1 and 3 for keys.

similar to the EA + CA group ($P > 0.05$, $n = 6$; Fig. 4). This result indicated that the inhibitory effect of EA on Fos expression evoked by CA inflammation was not mediated by opioid receptors either.

4. Discussion

The results of our study demonstrate unequivocally that low frequency EA exerts non-opioid-dependent inhibitory effects on CA-induced edema and Fos expression in the superficial laminae of the ipsilateral spinal cord. The anti-edematous effect of EA is consistent with recent studies, which reported that acupuncture is effective in inhibiting inflammation in other experimental models, including capsaicin-induced edema [5], collagen and adjuvant arthritis [13,25], as well as CA-induced inflammation [53]. The CA-induced acute inflammation consists of a series of complex reactions that involve a number of mediators including histamine, serotonin, arachidonic acid metabolites, cytokines and neuropeptides. According to Vinegar et al. [50], CA inflammation begins with a nonphagocytic response followed by a phagocytic response. The nonphagocytic response, occurring in the first 60 min after CA injection, consists of cytoplasmic injury of dermal mast cells and mast cell degranulation, damage to cytoplasmic organelles of endothelial cells of blood vessels, as well as expression of interleukin-1 (IL-1) protein from the cytoplasm of injured endothelial cells. Mast cell injury causes histamine and serotonin release, and IL-1 attracts phagocytes to the site of irritation. Release of substance P is also a key feature in the early stage of CA inflammation [15].

The findings that EA pretreatment inhibited CA-induced edema from the onset suggested that EA was at least effective in inhibiting some of the reactions seen in the nonphagocytic phase, such as mast cell degranulation, IL-1 expression, or release of substance P. Of interest is also the report that EA suppresses IL-1β expression in spleen cells and synovial tissue of the arthritic mice [13]. Furthermore, the release of substance P in the periphery, the spinal cord and the superficial laminae of the trigeminal nucleus is inhibited by EA [52,54,56]. It remains to be clarified, however, whether EA is capable of directly inhibiting the phagocytic response, or the reduction in phagocytic response is merely secondary to the inhibition of the nonphagocytic processes. In this regard, it is worth to note that in our pilot study, in which EA had been applied 60 min after CA injection, EA was ineffective in inhibiting edema development. Of interest is the report that transcutaneous electrical nerve stimulation (TENS), which is similar to EA, is ineffective in reducing joint swelling when administered several hours after inflammation induced by a mixture of 3% kaolin and 3%

carrageenan, although it is effective in reducing hyperalgesia [44].

Questions may be raised as to the clinical relevance of the present study, in which EA has been found to have a "preventive" effect, rather an antagonizing effect on inflammation. The preventive nature of EA may reflect the limitation of this therapeutic technique, which only inhibits certain reactions to an inflammatory process. In this regard, it is interesting to note that acupuncture has been given preoperatively, rather than postoperatively, to reduce postoperative pain, nausea and vomiting [24]. Further studies are under way in our laboratory to determine which reactions to the CA-induced inflammation are inhibited by EA.

It was also found that EA was capable of inhibiting Fos expression evoked by acute somatic inflammation in the superficial laminae of the spinal cord. A dense collection of Fos-IR neurons was observed in the superficial laminae (I–II) of L3–5 spinal segments 4 h following ipsilateral CA injection. This finding is consistent with previous studies in conscious animals [3]. The concentration of Fos expression at the superficial laminae is also congruent with the fact that a high proportion of neurons in the superficial laminae are responsive to noxious inputs [51]. Inhibition of Fos expression is evident following EA (Figs. 2–4), and this is indicative of a reduction in neuronal activity. Increase in neuronal activity following injury, or central sensitization [42], is responsible for hyperalgesia and neurogenic inflammation. Buritova and Besson [3] observed a positive correlation between the size of the edema and the number of Fos-IR neurons in the dorsal horn. The present study also found that the decrease in peripheral edema and the number of Fos-IR neurons in the superficial laminae were similar in magnitude following EA. Our findings thus support the concept that there is a close association between the severity of peripheral inflammation and the extent of neuronal activation in the superficial dorsal horn. An important question related to this is whether the inhibition of Fos expression by EA is secondary to edema reduction or vice versa. Previously, EA has been shown to inhibit Fos expression evoked by somatic and visceral pain [6,26,53], and by neuropathic and arthritic pain [10,25]. Based on electrophysiological findings that the responsiveness of dorsal horn neurons is inhibited by EA or TENS [31,40], it has been proposed that the suppression of Fos expression is due to a direct central inhibition of nociceptive transmission in dorsal horn neurons. However, it should be noted that the central inhibition of nociception is susceptible to naloxone blockade [26,40,45], whereas the present inhibitory effect on Fos expression was not. Therefore, the possibility that the present inhibitory effect on Fos expression was, at least in part, secondary to the inhibition of inflammation in the periphery should be considered.

Our results stand in clear contrast to previous studies, which showed that administration of naloxone, at a dose similar to that used in the current experiment, reduces the inhibitory effects of EA on Fos expression evoked by noxious stimulation and on capsaicin-induced edema [5,26]. The reason for the discrepancy still has to be determined. It should be noted, however, that previous studies used different experimental models, in which a different inflammatory agent [5] or a nociceptive stimulation [26] had been employed. We have chosen the CA-induced edema model because this has been used extensively in the study of inflammation and evaluation of anti-inflammatory therapeutics in both conscious and anaesthetized animal preparations [4,31,50]. In contrast to a conscious animal preparation, the anaesthetized preparation has certain advantages. In particular, anesthesia avoids the stress caused by application of experimental procedures, including the prolonged restraining during EA stimulation and repeated handling for paw volume measurements. Stress is known to induce a number of immediate physiological changes, including activation of the endogenous opioid system and the hypothalamo–pituitary–adrenal axis, both of which may have significant effects on inflammation [2,8]. Furthermore, the anesthetized preparation prevented additional mechanical stimulation of the inflamed paw due to movement activity and weight bearing. This may irritate the sensitized peripheral receptors of the inflamed paw and aggravate local inflammation as well as Fos expression in the spinal cord [30]. As such, the inflammatory response observed was caused primarily by the irritant CA. However, one of the disadvantages of the current preparation was the inability to measure nociceptive reflexes. Nevertheless, the present anesthetized preparation provided a model that allows a focused examination of the anti-inflammatory effect rather than of the analgesic effect of acupuncture.

Previous studies have shown that a number of neurohormonal systems can be activated by somatic stimulation of nociceptive nature to produce anti-inflammatory effects [16,35,39]. However, the EA simulation employed in the present study is unlikely to activate a significant number of C-fibers, as the intensity of stimulation has been deliberately kept within the range comparable with its clinical use, and the stimulation is not painful when applied to human subjects (personal observation). Further experiments are required to determine the contribution of specific systems. At present, we can only conclude that there is a non-opioid-dependent mechanism mediating the anti-inflammatory effect of low frequency EA. Our results are consistent with the clinical findings that the pain-relieving effects of acupuncture are naloxone-resistant [21,27,29]. It is suggested that at least two distinct mechanisms of action are involved in pain relief by low frequency EA: an opioid-dependent analgesic effect and a non-opioid-dependent anti-inflammatory effect. Recognition of the latter mechanism provides an explanation for the naloxone-resistant effects of acupuncture observed in previous clinical studies.

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