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INVOLVEMENT OF PERIPHERAL BETA-ENDORPHIN AND MU, DELTA, KAPPA OPIOID RECEPTORS IN ELECTROACUPUNCTURE ANALGESIA FOR PROLONGED INFLAMMATORY PAIN OF RATS

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Acupuncture is commonly used to relieve chronic pain worldwide. Accumulating evidence shows that peripheral opioid system plays an important role in inhibiting inflammatory pain. This study aimed to investigate the involvement of peripheral opioid system in electroacupuncture (EA) analgesia for prolonged inflammatory pain. Inflammatory pain was induced by an intraplantar injection of complete Freund's adjuvant (CFA) into the right hind paw. EA (2/100 Hz, 2 mA) was applied to the ipsilateral Zusanli (ST36) and Kunlun (BL 60) acupoints for 30 min once everyday. Block studies on EA analgesia were performed on day 18 after CFA injection by using α-helical corticotrophin-releasing factor (CRF), a CRF antagonist, and naloxone methiodide, a peripherally restricted opioid receptor antagonist. Paw withdrawal latency (PWL) to a noxious thermal stimulus was measured as the pain behavioral change. Radioimmunoassay for beta-endorphin (beta-END), Met-enkephalin (Met-ENK), and dynorphin A (DYN A) in paw inflammatory tissue and immunohistochemistry study for mu, delta, kappa opioid receptors in dorsal root ganglion (DRG) were carried out. A subsequent validation experiment by locally delivered exogenous beta-END was also performed. We found that EA significantly increased the PWL of rats injected with CFA from day 4 to day 18. Locally administered α-helical CRF or naloxone blocked EA-produced analgesia. EA increased beta-END level in the paw inflammatory tissues, while CFA raised the local levels of Met-ENK and DYN A. The increased beta-END level by EA was fully reversed by α-helical CRF. Intraplantar injection of exogenous beta-END alleviated prolonged inflammatory pain. EA also up-regulated the expressions of mu, delta, kappa opioid receptors in rat L5 DRG. In conclusion, peripheral local beta-END and three subtypes of opioid receptors may be involved in EA analgesia for prolonged inflammatory pain.

Acupuncture has a long history in disease treatment in China. Nowadays, acupuncture is used worldwide for pain relief. The American College of Physicians and the American Pain Society have issued joint clinical practice guidelines recommending acupuncture as one treatment option for chronic low back pain (1, 2), while the U.K. National Health Service now offers acupuncture comprising up to a maximum of 10 sessions over a period of up to 12 weeks for such patients (3). Electroacupuncture (EA), a commonly-used therapy in acupuncture which is referred to the application of an electrical stimulation to acupoints via acupuncture needles, has been widely used in pain control for decades (4-6).

Key words: inflammatory pain, electroacupuncture, analgesia, peripheral opioid

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Although the analgesic effect of acupuncture including EA has been well demonstrated, its biological basis is far from clear. The current primary mechanism involves the release of opioid peptides in the CNS in response to the selective activation of A beta and A delta nerve fibers by acupuncture, which inhibit nociceptive transmission in the superficial dorsal horn in a presynaptic way (6-9). However, the central opioid mechanism cannot fully explain why needling an acupoint adjacent to the locus of pain, a common clinical practice, is generally more effective in relieving pain than needling distant areas, and why the analgesic effect of acupuncture is mainly restricted to the ipsilateral side (10). These phenomena indicate the existence of a peripheral local mechanism underlying acupuncture analgesia.

Opioid receptors have been demonstrated on the peripheral sensory nerve fibers and their terminals (11, 12). Research showed that peripheral local application of morphine produced analgesia, while naloxone exacerbated pain under inflammatory conditions (13-16), indicating the involvement of peripheral opioid receptors in inflammatory pain relief. Besides opioid receptors, under the conditions of inflammation, local opioids can be released from immune cells infiltrating into inflammatory sites, which can be triggered by a stressful event, such as a cold water swim, and mediated by a variety of hormones and cytokines, such as corticotropin-releasing factor (CRF) and interleukin-1 beta. These local opioids inhibit inflammatory pain by interacting with peripheral opioid receptors (17-20). However, whether acupuncture can effectively stimulate the peripheral opioid system, and to what extent the peripheral opioid system is involved in acupuncture analgesia for chronic inflammatory pain have not been comprehensively investigated.

For this reason, in the present study we examined the effect of EA on prolonged inflammatory hyperalgesia induced by local injection of complete Freund's adjuvant (CFA) and the profile of peripheral opioid system. Block studies using α-helical CRF, a CRF antagonist, and naloxone methiodide, a peripherally acting opioid receptor antagonist, were carried out. In addition, validation experiment using exogenous beta-endorphin (beta-END) was performed to further investigate the involvement of peripheral opioid system in acupuncture analgesia for chronic inflammatory pain.

MATERIALS AND METHODS

Animal preparation and experimental design

Male Wistar rats (180-200 g body weight) were obtained from SLAC Laboratory Animal Co. Ltd., Shanghai, China. Rats were housed in temperature-controlled animal cages (25 ± 1°C) under a 12-h light, 12-h dark cycle, with free access to food and water. All animals were treated in accordance with the regulations of the State Science and Technology Commission for the care and use of laboratory animals (State Science and Technology Commission Order No.2, 1988).

Two experiments were conducted: (1) effect of EA on prolonged hyperalgesia and effects of α-helical CRF and naloxone methiodide on EA analgesia; (2) validation experiment using exogenous beta-END. In Experiment 1, rats were divided into the following groups (n = 8 per group): Normal, CFA, CFA + saline, CFA + EA, CFA + EA + saline, CFA + EA + α-helical CRF, CFA + EA + naloxone methiodide. In Experiment 2, rats were divided into CFA + saline and CFA + exogenous beta-END groups (n = 8 per group).

Induction of inflammatory pain

Inflammatory pain was induced by injecting 0.1 ml CFA (Sigma, USA) subcutaneously into the plantar surface of the right hind paw. The normal control rats were injected with the same volume of saline alone. Hyperalgesia was determined by a reduction of paw withdrawal latency (PWL) to a noxious thermal stimulus.

Measurement of thermal hyperalgesia

Thermal hyperalgesia was assessed by PWL to a noxious thermal stimulus using a plantar tester (Ugo Basile, Italy). Briefly, rats were placed in a clear plastic chamber and allowed to acclimatize for 30 minutes. A radiant heat stimulus (high-intensity projector lamp bulb) was positioned under the glass floor directly beneath the right hind paw. When the animal withdrew its hind paw, the heat stimulus was automatically stopped, and the time was recorded as thermal PWL. A 20-second cut-off was used to prevent tissue injury. PWL was established by averaging the latency of 3 tests with a 5-min interval between each test. PWL was measured pre-CFA/saline injection and 30 min after EA treatment or intraplantar administration of exogenous beta-END at the indicated time points post-CFA injection.

EA treatment

Ipsilateral Zusanli (ST36, 5 mm lateral to the anterior tubercule of the tibia) and Kunlun (BL60, at the ankle joint

level and between the tip of the external malleolus and tendo calcaneus) acupoints commonly used in acupuncture practice for pain relief were selected. We did not carry out sham acupuncture or acupuncture in other acupoints for control, considering that the analgesic effects of the two acupoints are well documented (10, 21), and that the study focused on the peripheral opioid mechanism but not the specificity of acupoints. Stainless-steel acupuncture needles of 0.25 mm in diameter were inserted 5 mm deep into the skin of the acupoints. A pair of electrodes from an electrical stimulator (LH-202H, Huawei Co. Ltd., Beijing, China) was attached to the ends of the needles. EA was applied using 2.0 mA, 2/100 Hz with pulse width 0.6ms at 2Hz and 0.2ms at 100Hz for 30 min once per day from D 1 to D 18 after CFA injection. No anesthesia was used during the EA treatment. Animals were calmed by placing the heads in black hoods with no physical restraint. No sign of stress, such as increased urination or defecation, was observed. All rats were killed on D 18, which is in the inflammation strengthening period in the chronic phase of CFA model (22-23), for paw and DRG samples.

Drug delivery

The following reagents were used: α-helical CRF (2 ng, Sigma); naloxone methiodide (50 μg, Sigma); rat beta-END (10 μg, Sigma). All reagents were dissolved in saline and intraplantarly (i.pl.) injected in 0.05 ml. The antagonists were delivered 10 min before EA treatment on D 18 in Experiment 1. Rat beta-END was administered from D1 to D 17 once every other day in Experiment 2. Rats in saline control groups received the identical procedure except for injecting the same volume of saline instead of drug.

Radioimmunoassay for beta-END, Met-ankephalin (Met-ENK), and dynorphin A (DYN A) in ipsilateral paw inflammatory tissue

Ipsilateral paw inflammatory tissue beta-END, Met-ENK, and DYN A were measured using a Rat ^{125}I beta-END RIA Kit, Rat ^{125}I Met-ENK RIA Kit, and Rat ^{125}I DYN A RIA Kit (the Department of Neurobiology of the Second Military Medical University of Chinese PLA, Shanghai, China) according to the manufacturer's instructions respectively.

Immunohistochemistry study of ipsilateral L5 dorsal root ganglion (DRG) mu, delta and kappa opioid receptors (MOR, DOR, KOR)

On D 18, rats were deeply anesthetized with 10% (w/v) chloral hydrate (3.5 ml/Kg, i.p.) and transcardially perfused with 150 ml cold sterilized saline followed by 500 ml cold, fresh 4% (w/v) paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). The ipsilateral L5 DRGs were removed, postfixed in the same fixatives for 2 h, and then placed successively in 15% (w/v) and 30% (w/v) sucrose solution overnight at 4°C. Tissues were embedded in OCT (Bayer Corp., Elkhart, IN), frozen, and then cut into 14-μm sections. Sections were mounted onto gelatin-coated slides, incubated in PBS with 0.3 % H_2O_2 for 30 min at 37°C to block endogenous peroxidase, and in PBS (PH 7.4) containing 5% goat serum for 60 min at 37°C to prevent nonspecific binding. Sections were respectively incubated with a rabbit polyclonal anti-rat KOR (1:1000; Abcam, USA), MOR (1:2500; Abcam, USA), and DOR (1:100; Alomone, Israel) overnight at 4°C, followed by incubation with the appropriate biotinylated goat anti-rabbit secondary antibody (1:750; ZSGB-BIO, China), and HRP-conjugated avidin (1:400; ZSGB-BIO, China). Diaminobenzidine tetrahydrochloride substrate (ZSGB-BIO, China) was then added for 60 s at 37°C. After that, sections were washed, dehydrated in alcohol, cleared in xylene, and finally mounted in dibutylpthalate polystyrene xylene (Merck, Germany).

The method of quantification for DRG staining has been described previously (24). Briefly, the total number of MOR, DOR, and KOR-immunoreactive (IR) cells was counted by an observer blinded to the experimental protocol. This number was divided by the total number of neurons in each DRG section, and the percentage of MOR, DOR, and KOR-IR neurons was calculated. Five rats per group were used for analysis.

Statistical analysis

All data were expressed as mean ± SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) *post-hoc* test for multiple comparisons in Experiment 1, and using the non-paired Student's *t*-test in Experiment 2. The criterion for statistical significance was $P < 0.05$.

RESULTS

EA inhibited CFA-induced local prolonged hyperalgesia

Mean PWL in all experimental groups at each time point is shown in Fig. 1. Before CFA injection, no significant difference in the basic PWL of the right hind paw of rats was found in all groups ($p>0.05$). Intraplantar injection of CFA into the right hind paw significantly decreased rats' PWL from D 1 to D 18 compared to that in the normal group ($p<0.001$). EA, administered after CFA injection once per day, significantly increased CFA-injected rats' PWL on D 4 ($p<0.05$). And till D 18, PWL in the EA group was

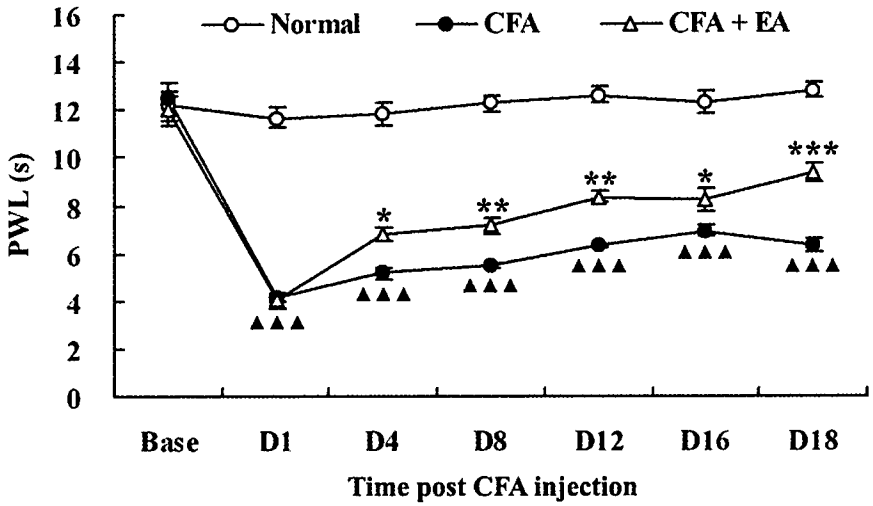

Fig. 1. *Effect of EA on paw withdrawal latency (PWL) of rats to a noxious thermal stimulus. PWL of the ipsilateral hind paws was measured at the indicated time points. Data are presented as mean ± SEM, n = 8 per group.* ▲*P < 0.001, CFA group vs Normal group; *P < 0.05, **P < 0.01, ***P < 0.001, CFA + EA group vs CFA group.*

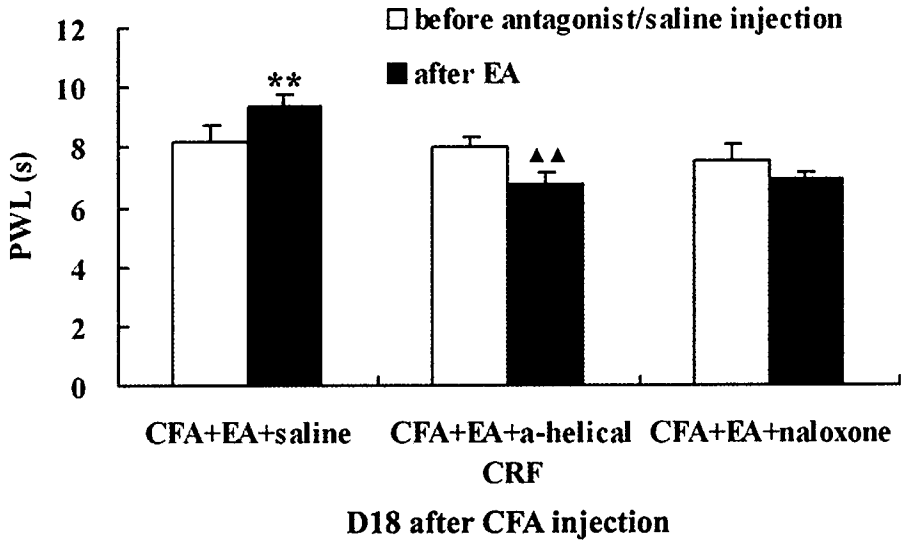

Fig. 2. *Effects of α-helical CRF and naloxone methiodide on EA inhibition of hyperalgesia. Rats were intraplantarly injected with saline, α-helical CRF and naloxone methiodide followed by EA treatment on D 18. Data are presented as mean ± SEM, n = 8 per group.* ***P < 0.01, PWL after EA treatment vs PWL before local saline injection in CFA + EA + saline group;* ▲▲*P < 0.01, PWL after EA treatment vs PWL before local α-helical CRF injection in CFA + EA + α-helical CRF group.*

still much longer than that of the CFA control group ($p<0.001$).

Effects of locally administered α-helical CRF and naloxone on EA analgesia

As shown in Fig. 2, EA significantly increased rats' PWL on D 18 after CFA injection ($p<0.01$, PWL after EA treatment *vs* PWL before local saline injection). The increase in PWL caused by EA treatment was reversed by intraplantar injection of α-helical CRF, and local administration of naloxone methiodide.

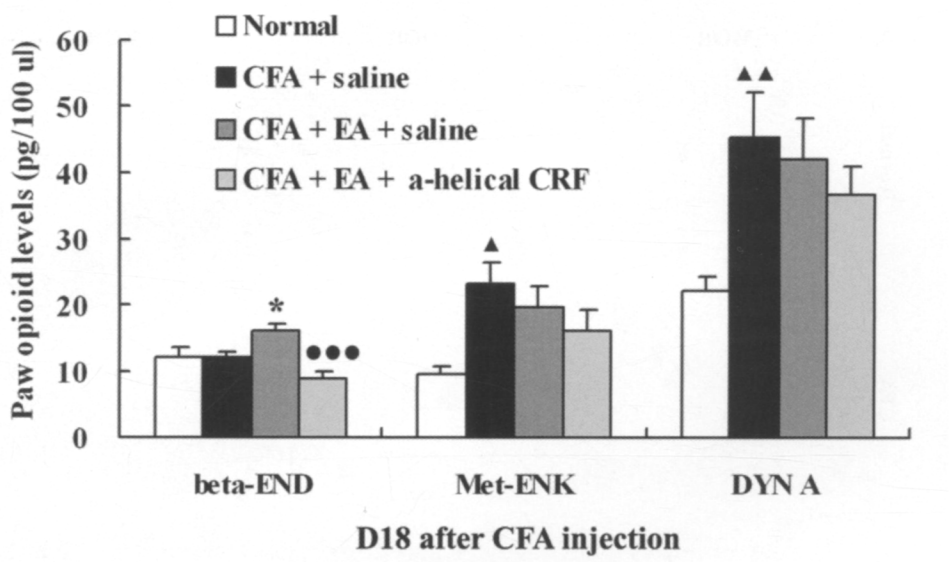

Fig. 3. *Effects of EA and locally administered α-helical CRF on the ipsilateral paw inflammatory tissue opioid levels on D 18. Supernatants from paw inflammatory tissue homogenates were used immediately for the radioimmunoassay of beta-END, Met-ENK and DYN A. Data are presented as mean ± SEM, n = 8 per group. ▲P < 0.05, ▲▲P < 0.01, CFA + saline group vs Normal group; *P < 0.05, CFA + EA + saline group vs CFA + saline group; ***P < 0.001, CFA + EA + α-helical CRF group vs CFA + EA + saline group.*

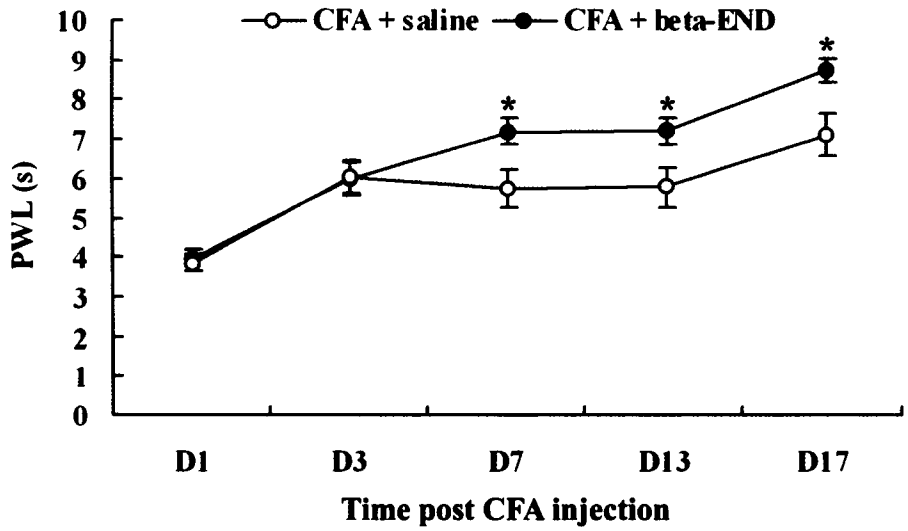

Fig. 4. *Effect of exogenous beta-END intraplantarly applied on rats' PWL. PWL of the ipsilateral hind paw was measured at the indicated time points. Data are presented as mean ± SEM, n = 8 per group. *P < 0.05, CFA + beta-END group vs CFA + saline group.*

Effects of EA and α-helical CRF on opioid levels in local inflammatory tissues

As shown in Fig. 3, Met-ENK and DYN A levels in the ipsilateral hind paw were remarkably increased by prolonged inflammatory pain, compared with those in the normal group (P<0.05, P<0.01). EA significantly increased local beta-END (P<0.05), but without significantly affecting Met-ENK and DYN A levels of CFA-injected rats compared to those of the CFA group. Locally administered α-helical CRF fully reversed the increase in beta-END level caused by EA.

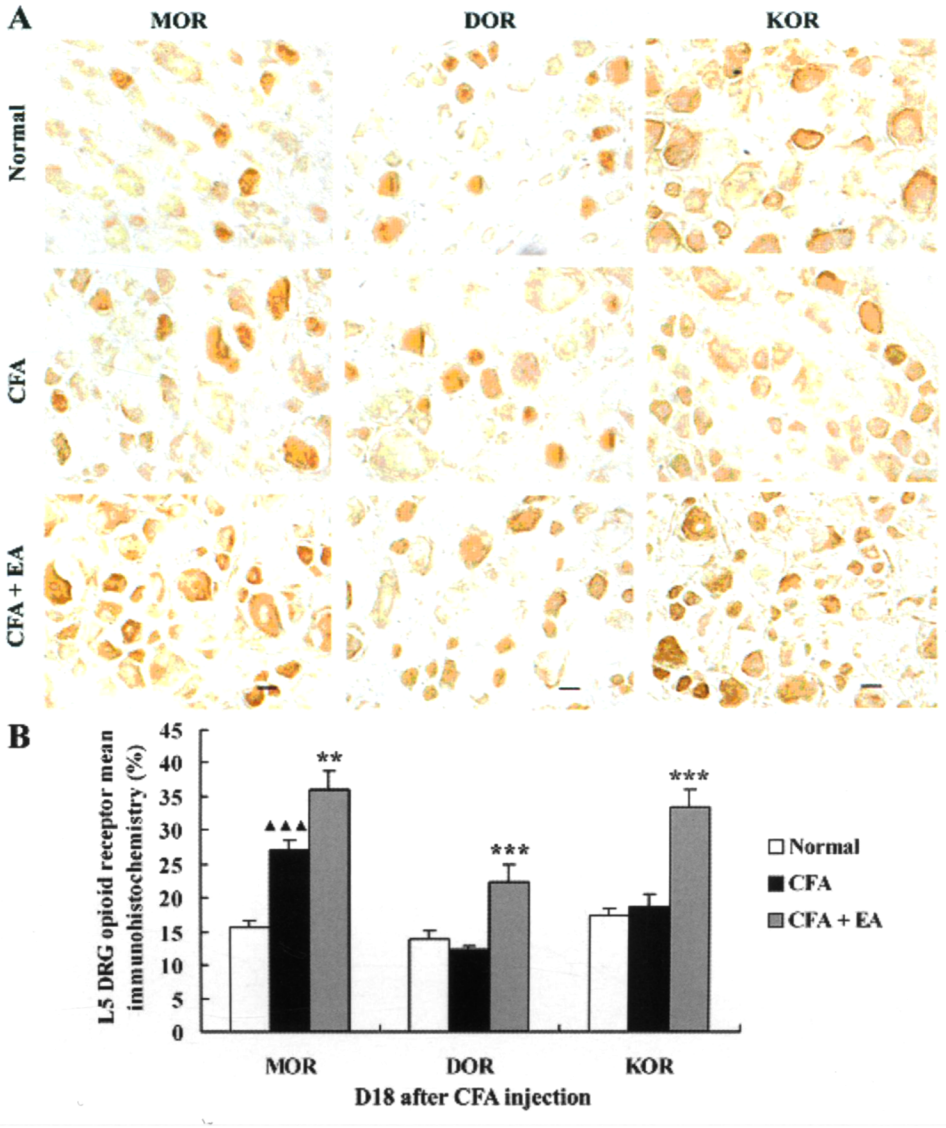

Fig. 5. *A) Representative bright-field micrographs showing MOR, DOR, KOR positive neurons in L5 DRGs of rats in the Normal, CFA, CFA + EA groups. MOR-, DOR-, and KOR-IR were mainly seen in small DRG neurons.* ***B)*** *Statistic analysis of L5 DRG opioid receptor immunohistochemistry. Scale bar, 20 μm. Data are presented as mean ± SEM, n = 5 per group.* ▲▲▲*P < 0.001, CFA group vs Normal group; **P < 0.01, ***P < 0.001, CFA + EA group vs CFA group.*

Exogenous beta-END alleviated CFA-induced prolonged hyperalgesia

To further testify whether local beta-END is involved in EA analgesia, the analgesic effect of exogenous beta-END administered locally was examined. Intraplantarly delivered exogenous beta-END significantly increased rats' PWL from D 7 to D 17 after CFA injection, compared to those of the saline control group ($p<0.05$) (Fig. 4). We also found that, the intraplantar injection itself, indeed an intervention, had an obvious effect on PWL of inflammatory pain rats on D 17 by comparing that of the CFA control group as presented in Fig. 1.

Effect of EA on opioid receptor expressions in DRG

Representative L5 DRG sections of animals in Normal, CFA, CFA + EA groups are shown in Fig. 5. Immunohistochemical visualization showed that

opioid receptor-IR neurons were mainly small-to-medium DRG cells (20–50μm). The expression of MOR-IR neurons in the CFA group (27.0±1.5%) was significantly increased compared to that in the normal group (15.7±1.0%). The increased MOR expression was further significantly up-regulated to 36.1±2.8% by EA. EA also significantly enhanced DOR and KOR expressions to 22.3±2.7% and 33.5±2.6%, compared to 12.5±0.4% and 18.8±1.8 % in the CFA group, respectively.

DISCUSSION

In the present study, we systematically investigated the involvement of peripheral opioid system in EA analgesia for treating CFA-induced peripheral inflammatory pain in rats. We found that EA could effectively inhibit prolonged inflammatory pain. Our findings suggest that local beta-END and three subtypes of opioid receptors may be implicated in EA analgesia for chronic inflammatory pain.

The rat model of unilateral inflammatory pain induced by CFA has been extensively used in the study of peripheral opioid system (19, 25). Most of these studies employed CFA model with a duration of no more than 4 days. Considering that a model of prolonged inflammatory pain may be in more accordance with acupuncture practice and has a greater clinical significance for chronic pain control, we focused on the second chronic inflammation stage of CFA model (26), and thus examined the effect of EA on hyperalgesia until D 18 after CFA administration, and performed the subsequent block studies.

Since CRF can induce opioid peptide release from immunocytes in local inflamed tissue (18-19), the finding that EA analgesia was blocked by locally delivered α-helical CRF, a CRF antagonist, suggests a role of CRF-mediated peripheral opioids in EA analgesia. To testify this, we measured local opioid levels and the effects of CRF antagonist and EA on them. The prolonged inflammatory pain significantly increased Met-ENK and DYN A levels, supporting the existence of intrinsic pain inhibition in inflammation. EA significantly inhibited prolonged inflammatory pain, and increased local beta-END level. Corresponding to its blockage of EA analgesia, α-helical CRF totally reserved the increase in local beta-END level caused by EA. Furthermore, the validation experiment also showed a suppression of locally applied exogenous beta-END on prolonged inflammatory pain. Taken together, these findings support that EA can promote beta-END release at the inflammatory site, which is mediated by CRF, and participates in EA analgesia.

We also demonstrated that intraplantar administration of naloxone methiodide effectively blocked EA analgesia. Considering that naloxone methiodide does not pass the brain-blood barrier (27), and thus no central effect was expected, the above finding indicates a role of peripheral oipioid receptors in EA analgesia. Previous studies from other researchers have demonstrated that spinal MOR and DOR but not KOR are involved in EA analgesia in CFA and capsaicin-induced inflammatory pain (28-29). However, few data can be obtained on the regulatory effect of acupuncture on peripheral opioid receptors. In the present study, EA not only enhanced MOR and DOR expressions in DRG, but also significantly up-regulated KOR expression. In addition, these opioid receptor-IR cells were mainly small-to-medium DRG neurons responsible for nociceptive transmission. Considering that Met-ENK displays preferential binding to DOR, and DYN A has high affinity for KOR (30), the up-regulated DOR and KOR by EA may further strengthen the intrinsic analgesia mediated by the increased Met-ENK and DYN induced by prolonged inflammatory pain, which may in turn contribute to EA analgesia. In all, these results suggest that three subtypes of opioid receptors in DOR neurons may play a role in EA analgesia.

In summary, our experiments demonstrate that EA can inhibit prolonged inflammatory pain, and the peripheral beta-END, CRF being a mediator, and three subtypes of opioid receptors may be involved in the process. Our study provides a deep insight into the peripheral opioid mechanism underlying acupuncture analgesia, and supports that EA is an effective treatment for patients with chronic inflammatory pain.

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