Endogenous Anandamide and Cannabinoid Receptor-2 Contribute to Electroacupuncture Analgesia in Rats

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Abstract: Acupuncture is widely used clinically to treat acute and chronic pain conditions, but the mechanisms underlying its effect are not fully understood. Although endocannabinoids are involved in modulation of nociception in animal models and in humans, their role in acupuncture analgesia has not been assessed. In this report, we determined the effect of electroacupuncture (EA) on the level of anandamide in the skin tissue and the role of cannabinoid CB1 and CB2 receptors in the analgesic effect of EA in an animal model of inflammatory pain. Inflammatory pain was induced by local injection of complete Freund's adjuvant (CFA) into the hind paw of rats. Thermal hyperalgesia was tested with a radiant heat stimulus, and mechanical allodynia was quantified with von Frey filaments. The anandamide concentration in the skin tissue was measured by using high-performance liquid chromatography. EA, applied to GB30 and GB34, at 2 and 100Hz significantly reduced thermal hyperalgesia and mechanical allodynia induced by CFA injection. Compared with the sham group, EA significantly increased the anandamide level in the inflamed skin tissue. Local pretreatment with a specific CB2 receptor antagonist, AM630, significantly attenuated the antinociceptive effect of EA. However, the effect of EA was not significantly altered by AM251, a selective CB1 receptor antagonist. These findings suggest that EA potentiates the local release of endogenous anandamide from inflamed tissues. Activation of peripheral CB2 receptors contributes to the analgesic effect of EA on inflammatory pain.

Perspective: This study shows that electroacupuncture increases the anandamide level in inflammatory skin tissues, and CB2 receptors contribute to the analgesic effect of electroacupuncture in a rat model of inflammatory pain. This information improves our understanding of the mechanisms involved in the analgesic effect of acupuncture.

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Key words: Anandamide, hyperalgesia, inflammatory pain, acupuncture, analgesia, cannabinoid receptor-2 (CB2).

Acupuncture is an ancient therapeutic technique and is an important part of traditional Chinese medicine. Acupuncture is rapidly gaining interest and recognition by the West, and its analgesic effect has been validated in several clinical studies.22,23 Electroacupuncture (EA), a modified therapeutic method based on the theory of traditional manual acupuncture, is also effective to treat pain.45,46 It is generally accepted that the endogenous opioid system plays a key role in acupuncture analgesia.17,21 However, the involvement of other mechanisms, such as the endocannabinoid system, in EA analgesia is still uncertain.

Anandamide is the first endocannabinoid isolated from the porcine brain9 and can activate both cannabinoid receptor-1 (CB1) and cannabinoid receptor-2 (CB2) receptors.38 Local application of cannabinoids and endocannabinoids produces antinociceptive effects in rodent models of acute inflammatory pain. For example, when injected into the hind paw of the rat, anandamide reduces hyperalgesia induced by carrageenan38 or pain...
responses triggered by formalin injection. Other studies also suggest that peripheral cannabinoids are involved in inhibition of persistent inflammatory pain. The effect of anandamide and endocannabinoids on hyperalgesia is reduced by a CB1 receptor antagonist. Thus, CB1 receptors appear to be involved in the analgesic actions of cannabinoids.

Using newly developed selective CB2 receptor agonists, recent studies suggest that activation of CB2 receptors inhibits inflammatory pain responses. For instance, the selective CB2 receptor agonist GW405833 decreases carrageenan-induced mechanical allodynia by as much as 50%. Local injection of another selective CB2 receptor agonist, AM1241, into the inflamed paw also reverses carrageenan-induced thermal hyperalgesia. Moreover, coadministration of the CB2, but not the CB1, receptor antagonist blocks the inhibitory effects of intraplantar injection of anandamide on innocuous and noxious stimuli-evoked responses of spinal dorsal horn neurons in rats with hind paw inflammation. Intraplantar injection of the CB2 receptor agonist JWH-133 also suppresses mechanically evoked responses of spinal dorsal horn neurons in carrageenan-treated rats, and this effect is blocked by local administration of the CB2 antagonist SR144528 but not by the CB1 antagonist SR141716A. These studies strongly suggest that activation of peripheral CB2 receptors can inhibit inflammatory pain. We have shown previously that EA increases the number of CB2 receptor-positive cells in the skin tissue of rats with complete Freund’s adjuvant (CFA)-induced inflammation in the hind paw. Therefore, we reasoned that EA could mobilize the endocannabinoid system by increasing the inhibitory endocannabinoid tone under conditions of inflammation and produce its antinociceptive effect through activation of peripheral CB2 receptors.

In the present study, we used an established rat model of inflammatory pain to determine the analgesic effect of EA and its effect on the level of endogenously released anandamide in inflamed skin tissues. We also examined the role of peripheral CB1 receptors and CB2 receptors in the antinociceptive effect of EA.

Materials and Methods

Animal Models

Experiments were carried out on male adult Sprague-Dawley rats (180–200 g) purchased from Experimental Animal Center of Tongji Medical College of Huazhong University of Science and Technology. All procedures were approved by the Animal Care Committee at Huazhong University of Science and Technology and conformed to the ethical guidelines of the International Association for the Study of Pain. The rats were individually housed in cages with a 12-hour light/dark cycle and had free access to food and water.

Inflammation was induced by injecting 50 μL of CFA (Sigma; St Louis, MO) subcutaneously into the dorsal surface of left hind paw of rats using a 25-gauge hypodermic needle. CFA injection into the dorsal surface of the hind paw was used in the previous study by Cook et al. The injections were carried out under light anesthesia by means of ether inhalation. We selected the dorsal surface of the hind paw as the injection site to produce an inflammatory pain focus in the gallbladder channel of foot Shaoyang, where GB 30 and GB 34 are located, according to the traditional Chinese medicine meridian theory.

Electroacupuncture Treatment

In the EA treatment group, the rats received EA administration on the ipsilateral Huantiao (GB30) and Yanlingquan (GB34) once every other day, starting at the second day after CFA injection. EA (1 mA and 0.1 ms) was administered at 2 or 100 Hz for 30 minutes. Current was delivered with a modified current-constant Han’s Acupoint Nerve Stimulator (LH202; Huawei Co Ltd, Beijing, China). GB30 and GB34 were chosen based on their effective use in reducing inflammatory pain in rats.

Two acupuncture needles were inserted into 2 acupoints that correspond to GB30 and GB34 in humans: GB30 is located at the junction of the lateral one third and medial two thirds of the distance between the greater trochanter and the hiatus of the sacrum; and GB34 lies on the lateral aspect of the leg, in the depression anterior and inferior to the head of the fibula in rats. During EA treatment, each rat was placed under an inverted clear plastic chamber (approximately 4 × 4 × 11 cm) but was neither restrained nor given any anesthetic. The animals remained awake and still during EA treatment and showed no evident signs of distress. For sham control, acupuncture needles were inserted ipsilaterally into GB30 and GB 34 without electrical stimulation or manual needle manipulation.

Drug Administration

AM630 is a selective CB2 receptor antagonist with a 70- to 165-fold selectivity for the CB2 receptor in vitro. AM251 is a CB1 receptor antagonist and has a 300-fold selectivity for CB1 receptors. AM 251 (30 μg) and AM 630 (30 μg) (Alexis Biochemicals; Lausen, Switzerland) were dissolved in the vehicle solution containing 5% Tween 80 and 5% DMSO in normal saline. AM251, AM630, or the vehicle (50 μL) was injected subcutaneously into the dorsal surface of the left hind paw 5 minutes before each EA treatment. The dose of AM 251 and AM 630 was selected on the basis of a previous study by Malan et al and in our preliminary dose-response study to select the lowest dose that produces a consistent effect.

Behavioral Testing

The behavioral tests were performed 3 times before CFA injection and once every other day, starting from the first day to 7 days (for EA analgesic effect) or 15 days (for EA plus CB1 or CB2 antagonist) after CFA injection. The animals were habituated to the testing environment for 30 minutes. Thermal hyperalgesia was assessed by exposing the mid-plantar surface of the hind paw to a beam of radiant heat through a transparent glass surface using a plantar analgesia meter for paw
stimulation (Ugo Basile, Italy), as previously described. The withdrawal latency was recorded for both left and right hind paws as the time taken from the onset of radiant heat stimulation to withdrawal of the hind paw. The left or the right hind paw was tested 4 times with a 4- to 5-minute interval.

Mechanical allodynia was assessed by placing an animal on an elevated mesh floor, and the tactile threshold was measured by using an electronic von Frey anesthesiometer (Ugo Basile, Italy) applied to the plantar surface of the left and right hind paws. The force (g) needed to produce a paw withdrawal response was tested 4 times for each paw separated by 2- to 3-minute intervals. A mean value of 4 consecutive measurements was taken for each paw.

High-Performance Liquid Chromatography Quantification of Anandamide

Chemical and Sample Preparation

Anandamide, R (+)-methanandamide, DBD-COCl, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma (St Louis, MO). Acetonitrile (gradient grade) was from Kermel (Tianjin, China). Stock solutions of anandamide, and R (+)-methanandamide and DBD-COCl were prepared in anhydrous acetonitrile.

Extraction and derivatization procedures were based on the method described by others with some modifications. The skin tissue was excised from rats based on the method described by others with some modifications. The skin tissue was excised from rats based on the method described by others with some modifications. The skin tissue was excised from rats based on the method described by others with some modifications.

The removal of endogenous anandamide from the samples was necessary to obtain accurate quantification results. Ananda- mide is a naturally occurring endogenous cannabinoid that is present in the skin and other tissues. It is a potent agonist of cannabinoid receptors and has been implicated in various physiological processes, including pain modulation.

For quantification of the samples, a standard curve for anandamide over the validated range was generated for each analytical run. The contribution of endogenous anandamide in control samples used for calibration was eliminated by subtracting the peak area ratio of anandamide to its internal standard for the first point (ie, blank samples) in the standard curve from all subsequent points. Furthermore, quality control samples of 5 anandamide concentrations (70, 140, 290, 580, and 1150 pmol/mL) were incorporated in duplicates into each run. The results of the quality control samples provided the basis of accepting or rejecting the run.

Statistical Analysis

All data are presented as mean ± SEM and analyzed either with Student’s t test or with a 1-way analysis of variance (ANOVA) followed by a Newman-Keuls post hoc multiple comparison test. A P value of less than .05 was considered significant.

Results

Effect of EA on Thermal Hyperalgesia and Mechanical Alldynia

Three groups of animals (2 Hz EA, 100 Hz EA, and sham EA) were used for this series of experiments. CFA injection elicited typical inflammatory responses, including redness, edema, and hypersensitivities to noxious stimuli in the injected paw in all 3 groups of rats. These symptoms lasted for about 2 weeks as described before. The baselines of the thermal latency and mechanical withdrawal threshold were similar in the 3 groups. A large and similar decrease in the baseline was observed 1 day after CFA injection in all 3 groups (Fig 1, A and B).
EA was applied to GB30 and GB34 for 30 minutes 2, 4, and 6 days after CFA injection. Sham EA had no effect on the thermal hyperalgesia and tactile allodynia elicited by CFA injection. At 100 Hz, EA significantly increased the thermal withdrawal latency 5 days after CFA injection, compared with that in the sham EA group \( (P < .01, \text{Fig } 1A) \). At both 2 and 100 Hz, EA significantly increased the thermal withdrawal latency and tactile threshold 7 days after CFA injection, compared with that in the sham EA group \( (P < .05, \text{Fig } 1, \text{A and B}) \).

**Effect of EA on the Concentration of Anandamide in Skin Tissue**

For this series of experiments, 5 groups of rats were used: naïve control, CFA alone, CFA plus sham EA, CFA plus 2 Hz EA, and CFA plus 100 Hz EA. Endogenous anandamide in the skin tissue was detected in all the groups 7 days after CFA injection (ie, 1 day after the third EA treatment). The retention time of anandamide and the internal standard \( R \) \(-\text{methanandamide} \) was 24 and 25.8 minutes, respectively (Fig 2A). The concentration of anandamide in the skin tissue ranged from 410 pmol to 930 pmol/g (Fig 2B).

The anandamide level between the naïve control and CFA alone groups was not significantly different (Fig 2B). Also, the anandamide concentration in the sham EA group was not significantly different from that in the control and CFA alone groups (Fig 2B). However, the anandamide level in the 2 Hz and 100 Hz EA groups was significantly higher than that of the sham EA group \( (P < .05, \text{Fig } 2B) \).

**Role of CB1 and CB2 Receptors in the Effect of EA on Thermal Hyperalgesia**

The purpose of this protocol was to assess the role of CB1 and CB2 receptors in EA-produced analgesic effect on thermal hyperalgesia caused by CFA injection. A specific CB1 receptor antagonist, AM251,14 and a highly selective CB2 receptor antagonist, AM630,39 were used. It has been shown that neither AM251 nor AM630 injected into the paw alone affects the thermal and mechanical sensitivity in rats.8 For this reason, we did not include AM251 or AM630 alone groups in this study. EA was applied to GB30 and GB34 at 2 Hz for 30 minutes on 2, 4, 6, 8, 10, 12, and 14 days after CFA injection. AM251 (30 \( \mu \text{g} \) in 50 \( \mu \text{L} \)), AM 630 (30 \( \mu \text{g} \) in 50 \( \mu \text{L} \)), or an equal volume of vehicle was injected into the dorsal surface of the hind paw 5 minutes before EA treatment.

One day after CFA injection, evident thermal hyperalgesia developed in all 3 groups \( (P < .01, \text{Fig } 3A) \). Coadministration of AM630 in the same hind paw significantly reduced the effect of EA on thermal hyperalgesia 3 to 15 days after CFA injection \( (P < .05, \text{Fig } 3A) \). EA treatment with AM630 but not AM251 produced a significantly smaller increase in the thermal withdrawal latency, compared with that in the EA plus vehicle group \( (P < .05, \text{Fig } 3A) \).

**Role of CB1 and CB2 Receptors in the Effect of EA on Tactile Allodynia**

We next determined the role of CB1 and CB2 receptors in EA-produced analgesic effect on tactile allodynia elicited by CFA injection. One day after CFA injection, there was a large and similar reduction in the tactile withdrawal threshold in all 3 groups \( (P < .01, \text{Fig } 3B) \). Pretreatment with AM630 in the same hind paw significantly reduced the effect of EA on tactile allodynia 3 to 15 days after CFA injection \( (P < .05, \text{Fig } 3B) \). EA treatment with AM630 but not AM251 produced a significantly smaller increase in the mechanical withdrawal threshold, compared with that in the EA plus vehicle group \( (P < .05, \text{Fig } 3B) \).

**Discussion**

Our study provides new information about the mechanisms underlying the antinociceptive effect of EA on
inflammatory pain. We found that, in the case of inflammatory pain, EA at 2 and 100 Hz had similar effects on thermal hyperalgesia and tactile allodynia 7 days after CFA injection. At 100 Hz, EA appeared to be more effective in reducing paw withdrawal latency to thermal stimulation than that produced by 2 Hz EA 5 days after CFA injection. It has been shown that 2 Hz stimulation can mobilize enkephalin, which acts on \( \mu \) - and \( \delta \) -opioid receptors; whereas 100 Hz stimulation releases dynorphin, which binds to \( \kappa \) -opioid receptors to attenuate pain in both animals and humans. However, our current study indicates that both 2 and 100 Hz EA can elevate the tissue level of anandamide to the same degree.

The endocannabinoid system comprises 2 cannabinoid receptor subtypes, CB1 and CB2, a number of endogenous ligands [including anandamide and 2-arachidonoyl glycerol (2-AG)], a high-affinity reuptake transport system, and endocannabinoid synthesizing and metabolizing enzymes. We observed that CFA injection alone did not increase the anandamide level. Similarly, Beaulieu et al. also reported no increases in the tissue endocannabinoid levels after formalin injection. Interestingly, we found that EA at both 2 and 100 Hz significantly increased the anandamide concentration in inflamed tissues 7 days after CFA injection, which was the time EA produced optimal effects on thermal latency and mechanical threshold. Thus, the antinociceptive effect of EA is correlated to the increase in endogenous anandamide levels in the tissue. Previous studies suggest that the inhibitory effects of endogenous anandamide in the skin are likely mediated by peripheral actions. The amounts of anandamide and palmitylthanolamide in the rat paw skin are 5- to 10-fold higher than those measured by the same method in the rat brain and plasma. Thus, the basal levels of anandamide and PEA in the skin are sufficient to cause tonic activation of local cannabinoid receptors. Our findings highlight the important role of local endogenous anandamide in EA-produced antinociceptive effect in the rat model of inflammatory pain.

Various analytical methods have been developed to determine tissue levels of anandamide from a variety of sources. For instance, mass spectrometry coupled with liquid or gas chromatography has been used by many groups to assess tissue concentrations of anandamide. However, because this expensive and complex instrumentation is not always available, a simple HPLC method with fluorometric detection for the determination of anandamide in the plasma has been developed. We modified and validated HPLC-fluorometric detection method to assess concentrations of anandamide in

![Figure 2](image-url)
inflamed skin tissues. Our study is the first to detect endogenous anandamide in the skin tissue with this method. The basal concentration of anandamide in the skin tissue may be derived from epidermal keratinocytes from skin tissues. Other investigators have also detected endocannabinoids in the rat skin tissue. The exact mechanisms underlying the potentiating effect of EA on the tissue anandamide level are not clear. The endocannabinoids are produced by various cells, including cells in the nervous system and immune system. Both anandamide and 2-arachidonoxy glycerol undergo depolarization-induced synthesis and release from neurons and astrocytes. In humans, endocannabinoids are preferentially generated from stimulated lymphocytes and macrophages. It has been shown that human epidermal keratinocytes express an anandamide synthesizing phospholipase D and constitute a part of the peripheral endocannabinoid system. Moreover, our previous work has shown that EA increases the number of CB2 receptor-positive cells, including keratinocytes and recruited immune cells, which may synthesize and release anandamide in the rat skin tissue during inflammation. Electrical stimulation in the acupoint of rat hind leg may influence synthesis, releasing, reuptake, and degradation of anandamide from epidermal keratinocytes and recruit immune cells in inflammatory skin tissues. After EA treatment, the synthesized and released anandamide may exceed its reuptake and degradation, resulting in an increased concentration in the skin tissues. These possibilities need to be investigated in future studies.

Cannabinoid CB1 receptors are localized primarily in the central nervous system and the primary afferent neurons. On the other hand, cannabinoid CB2 receptors are mainly expressed in immune tissues. Such a difference in the distribution between CB1 and CB2 receptors suggests a different role of CB1 and CB2 receptors in pain and EA analgesia. In our present study, we found that the antinociceptive effect of EA in rats with hind paw inflammation was attenuated by the CB2 receptor antagonist AM630. However, the CB1 receptor antagonist AM251 did not significantly alter the effect of EA on thermal hyperalgesia and mechanical allodynia induced by CFA injection. Our results suggest that, under the inflammatory pain condition, peripheral CB2 receptors contribute to the analgesic effect of EA. This finding is consistent with previous reports showing that peripheral CB2 receptors can modulate inflammatory hypersensitivity. Indeed, previous behavioral, electrophysiological, and neurochemical studies support a role of CB2 receptors in modulating inflammatory pain.

Because the analgesic effect of EA was significantly attenuated by the CB2, but not CB1, receptor antagonist, our data suggest that EA can increase the level of anandamide in the inflamed tissue and inhibit inflammatory pain through activation of CB2 receptors. Notably, anandamide has the ability to stimulate transient receptor potential vanilloid 1 (TRPV1) receptors, which may contribute to the development of hyperalgesia. However, many studies have shown that an increased anandamide level in the tissue produces antinociception. For example, a low concentration of anandamide (10 nM) inhibits neurotransmitter release from nociceptive primary sensory neurons, whereas high concentrations of anandamide (0.1 to 10 μM) have opposite effects. Because the concentration of anandamide in the skin increased by EA was in the pM range, such a low concentration of anandamide may preferentially inhibit primary afferent nerves to produce an analgesic effect. Additionally, EA may reduce inflammatory pain responses by inhibition of the release and production of proinflammatory cytokines or neurotrophins.

In summary, we found that EA at 2 and 100 Hz increases the anandamide level in inflamed skin tissues, and CB2 receptors contribute to the antinociceptive
effect of EA in the rat model of inflammatory pain. Therefore, EA treatment could potentiate the endo-

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