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NEUROSCIENCE

Electroacupuncture induces antihyperalgesic effect through endothelin-B receptor in the chronic phase of a mouse model of complex regional pain syndrome type I

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Abstract

Complex regional pain syndrome (CRPS) is a disorder that involves abnormal inflammation and nerve dysfunction frequently resistant to a broad range of treatments. Peripheral nerve stimulation with electroacupuncture (EA) has been widely used in different clinical conditions to control pain and inflammation; however, the use of EA in the treatment of CRPS is under investigation. In this study, we explore the effects of EA on hyperalgesia and edema induced in an animal model of chronic post-ischemia pain (CPIP model) and the possible involvement of endothelin receptor type B (ET_B) in this effect. Female Swiss mice were subjected to 3 h hind paw ischemia/reperfusion CPIP model. EA treatment produced time-dependent inhibition of mechanical and cold hyperalgesia, as well as edema in CPIP mice. Peripheral administration (i.pl.) of BQ-788 (10 nmol), an ET_B antagonist, prevented EA-induced antihyperalgesia while intrathecal administration prolonged EA's effect. Additionally, peripheral pre-treatment with sarafotoxin (SRTX S6c, 30 pmol, ET_B agonist) increased EA anti-hyperalgesic effect. Furthermore, the expression of peripheral ET_B receptors was increased after EA treatments, as measured by western blot. These results may suggest that EA's analgesic effect is synergic with ET_B receptor activation in the periphery, as well as central (spinal cord) ET_B receptor blockade. These data support the use of EA as a nonpharmacological approach for the management of CRPS-I, in an adjuvant manner to ET_B receptor targeting drugs.

Keywords Chronic pain · Chronic post-ischemia pain · Neuropathic pain · Sanyinjiao (SP6) · Zusanli (ST36)

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Introduction

Complex regional pain syndrome of type-I (CRPS-I) is a multifactorial nervous dysfunction originated from an ischemic lesion [10]. Clinical manifestations include regional pain, allodynia, hyperalgesia, edema, changes of skin temperature, and vasomotor dysfunctions [43]. As CRPS-I responds in a refractory way to several treatments [30]. there is continuous search for a better understanding of the pathophysiology underlying its clinical manifestations [4, 39].

As researchers seek to establish more effective approaches to reduce the signs and symptoms of CRPS-I [34], an animal model has been developed and is being widely used: the so-called chronic post-ischemic pain (CPIP) model which mimics CRPS-I by inducing ischemia followed by a rapid reperfusion period [24].

Involvement of the endothelinergic system in the pathophysiology of CRPS-I has been previously suggested, as microvascular lesions in endothelial cells [31] are often implicated in ischemia and reperfusion (IR). Additionally, preclinical and clinical studies have highlighted the participation of the endothelinergic system in the signaling of pain [9, 18].

It is well established that endothelins (ETs) exert their effects through two subtype receptors: ET_A and ET_B and that ET-1 binds equally to either receptor [20]. Peripheral ET_B receptors are expressed in endothelial cells [11], smooth muscle cells [38], in macrophages [37], and in keratinocytes [19]. In the nervous system, ET_B receptors are expressed in structures such as the sciatic nerve, the dorsal root ganglion (DRG), and the hypothalamus, among others [3, 13]. Activation of ET_B receptors at the periphery induces analgesia [31], while the spinal blockage of this receptor by administration of Bq-788 (ET_B receptor antagonist) reduces nociception [22].

In the spinal cord, the concentration of ET-1 increases, following peripheral nerve injury in rats, as is the case of the spinal nerve ligation [22]. In addition, spinal cord administration of ET-1 induces antihyperalgesia in animal models of neuropathic and inflammatory pain [16, 17]. Moreover, an increase in spinal ET-1 has been shown to induce mechanical hyperalgesia in an animal model of CRPS-I [22].

Neuronal stimulation is emerging in modern medicine as an interesting approach to help control organ dysfunction and re-establish physiological homeostasis [42]. While manual acupuncture uses the mechanical stimulation of needles inserted at specific reactive sites in the body (the so-called acupoints) to control pain among other conditions [47], electroacupuncture (EA) consists of the stimulation of the same acupoints with low amperage electric current [47]. EA in acupoints ST36 (stomach 36) and SP6 (spleen 6) has been shown to reduce mechanical hyperalgesia in animal models of neuropathy [6, 12, 25] and inflammation induced by complete Freund's adjuvant (CFA) [15]. However, the effect of EA on the signs and symptoms of CRPS-1 has not yet been investigated, nor has the mechanism of action adjacent to this effect been yet fully elucidated.

In both patients and animal models, CRPS induces an acute (inflammatory) and posteriorly a chronic (neuropathic) phase [6]. Despite extensive literature on ischemic lesions, the chronic phase of the disease is not well studied, since the majority of the research has focused on the nociceptive response during the acute phase of CRPS-I.

In this context, the present study was carried out to determine the involvement of peripheral and central (spinal cord) ET_B receptors in the antihyperalgesic effect of EA in an animal model of CPIP/CRPS-I, in the chronic phase of the disease.

Material and methods

Animals

All experiments were conducted using female Swiss mice (25–35 g), housed at 22 ± 2 °C under a 12-h light/dark cycle (lights on at 6:00 am) and with free access to food and water. The animals were acclimated to the laboratory for at least 1 h before the tests that were carried out between 8:00 and 12:00 am. All animal care and experimental procedures were carried out in accordance with the National Institutes of Health Animal Care Guidelines (NIH publications number 80-23) and were previously approved by the Ethics Committee of the Federal University of Santa Catarina (protocol number 15.045.2.07. IV).

Chemical reagents

The following substances were used: acetone (Audaz Brazil, SP, Brazil), anti-endothelin B receptor antibody (Abcam, Cambridge, MA, USA); ET_B receptor antagonist Bq-788 (Sigma-Aldrich, St Louis, MO, USA), Anti-beta Actin antibody (HRP) (Sigma-Aldrich, St. Louis, MO, USA), isoflurane (Biochimico, Itatiaia, RJ, Brazil); pentobarbital sodic (Intervet, SP, Brazil); ET_B receptor agonist sarafotoxin 6c (SRTX S6c)—sarafotoxins are a family of peptides identified from the venom of a snake Atractaspis engaddensis that exhibits high selectivity for the ET_B receptor subtype over the ET_A subtype [20] (Sigma-Aldrich Co., St. Louis, MO, USA).

Chronic post-ischemic pain (CPIP)/complex regional pain syndrome-type I (CRPS-I)

CPIP induction was performed as previously described [31]. Mice were anesthetized with sodium pentobarbital (55 mg/kg, i.p.) and supplemented with up to 27.5 mg/kg (i.p.), when necessary. After induction of anesthesia, an elastic O-ring for braces (Elástico Ligadura 000–1237, Uniden) with 1.2 mm internal diameter was placed around the animals' right hind limb, proximal to the ankle joint. The O-rings were selected to provide a tight-fit that produced ischemia and were left on the limb for 3 h as initially described. The O-ring was always positioned at a point on the limb, just proximal to the medial malleolus by sliding it off the outside of a 100-mL pipette tip after the right hind paw was inserted into the pipette as far as possible. Naive animals were anesthetized but not subjected to the IR procedure.

Electroacupuncture treatment

EA treatment was performed according to the procedures described in previous studies [23, 32]. Mice were slightly sedated with 1–2% isoflurane delivered via a nose cone to

minimize restraint-induced stress, then stainless acupuncture needles (Dong Bang, 0.18 mm/diameter and 8 mm/length) with electrodes soldered to their handles were inserted with 0.3 mm of depth into the acupoints SP6 and ST36 ipsilateral to the injured paw [44]. The needles were positioned with approximately 3 mm of depth in each acupoint and the intensity of the electrostimulation was increased until a mild twitch was observed at the tibialis anterior muscle (ST36) and flexor digitorum muscle (SP6), usually obtain with the intensity of 2 to 3 mA. In total, this needling procedure typically lasted than 20 s. The acupoints used in this study were selected because they stimulate the tibial nerve (SP6) and the fibular nerve (ST36) which are related to the segmental innervation the hind paw of the animal [41], moreover, several studies have demonstrated the effects of these acupoints to control pain and inflammation in different animal models [28, 45]. The parameters for electrostimulation included a dense-disperse asymmetric balanced wave (F1 = 2 Hz, 0.7 ms pulse with, 5 s of stimulation; F2 = 10 Hz, 0.2 ms pulse with, 5 s of stimulation) with alternating polarities using the NKL EL-608 electrostimulator (NKL produtos eletronicos, Brusque, SC). These selected EA parameters were used based on previous studies, which revealed that 2 to 10 Hz frequencies are effective to inhibit inflammatory and neuropathic pain [28, 45]. To investigate the most effective treatment duration, 5, 10, and 20 min of electrical stimulation were tested in different groups of animals.

To determine if the electrostimulation and the needle position were relevant to the treatment, two different needling control groups were included. In control group 1, the same procedures of the active EA group were performed but no electrical current was delivered to the ST36 and SP6 acupoints. In control group 2, needles were inserted bilaterally with 3 mm of depth into a nonacupoint located at the gluteal region, at the height of the iliac crest 5 mm from the midline and electrical current was delivered with the same parameters of the active EA group [29]. The rationale for selecting this nonacupoint was to observe the effect of the electrical stimulation into a region of the hind limb (gluteus inferior nerve) that was not directly related to the innervation of the paw.

Study outline

One day prior to the IR procedure, baseline hyperalgesia, edema, and temperature were recorded. Mechanical hyperalgesia was assessed by the von Frey test after daily EA treatments for seven consecutive days (7 to 14 or 14 to 21 post-IR). Additionally, on days 3, 7, 14, and 21, the time course of the effect of EA was assessed. Different EA durations (5, 10, and 20 min long continuous stimulation) were performed only on the third and seventh day and the time course of the effect was evaluated. After determining the treatment duration that produced the best antihyperalgesic effect (20 min), cold hyperalgesia was also evaluated, and an analysis of the time course of the antihyperalgesic effect of EA on the third and seventh day was performed. The time course of the effect was evaluated and then the daily effect of EA for seven consecutive days (from 7 to 14) was recorded. Analysis of the effect of EA on paw edema and temperature was performed from the first to the third day after IR. After characterizing the antihyperalgesic effect of EA, the next step was to analyze the involvement of peripheral and central (spinal cord) ET_B receptors in EA effect during the chronic phase of CPIP. To this end, on the 14th day after IR, the mice were pre-administered intraplantarly (i.pl.) or intrathecally (i.t.) with saline or ET_B receptor antagonist (Bq-788) and treated with EA, as well as with the peripheral ET_B receptor agonist, SRTX S6c and treated with EA, mechanical hyperalgesia was then evaluated. Groups for the respective controls were also used. Finally, on fourteenth day after IR (chronic phase of CPIP), we analyzed the expression of peripheral and central (spinal cord) ET_B receptors in the paw and spinal cord tissues of EA-treated animals (Fig. 1).

Behavioral measurement: mechanical and thermal hyperalgesia

Mechanical hyperalgesia was assessed with a von Frey monofilament (0.6 g, VFH, Stoelting, Chicago, IL, USA). The animals were individually placed in a 9 × 7 × 11 cm, bottomless observation acrylic chamber positioned on a 6 mm (70 × 6 mm) wire mesh platform 40 cm. Paw withdrawal frequency obtained in 10 right hind paw stimulations with the von Frey monofilament was recorded as indicative of mechanical hyperalgesia.

To characterize baseline response, the animals were evaluated on day-1 (1 day previous to IR). Only the animals that responded 20% or less to the von Frey stimuli were selected for the study. The filament test was applied perpendicularly to the plantar surface with sufficient pressure to provide the curvature of the filament, thereby obtaining total pressure. In addition, the animals were evaluated when all four paws were accommodated on the mesh, and the withdrawal response was recorded only when the animal completely removed the paw from the support mesh [27].

Mechanical hyperalgesia was evaluated 0.5, 1, and 2 h after the treatment to verify the time course of its anti-hyperalgesic effect. To investigate the effects of repeated treatments, EA was performed once a day. Mechanical hyperalgesia was evaluated 0.5 h after each treatment (time with maximal inhibition observed in the acute treatment) for seven consecutive days (7 to 14 and 14 to 21 post-IR).

To assess hyperalgesia to cold stimulus, the acetone drop method [4] with minor modifications was used. The amount of time the animals spent flicking/stamping or licking the plantar aspect of the hind paw during a 20-min observation

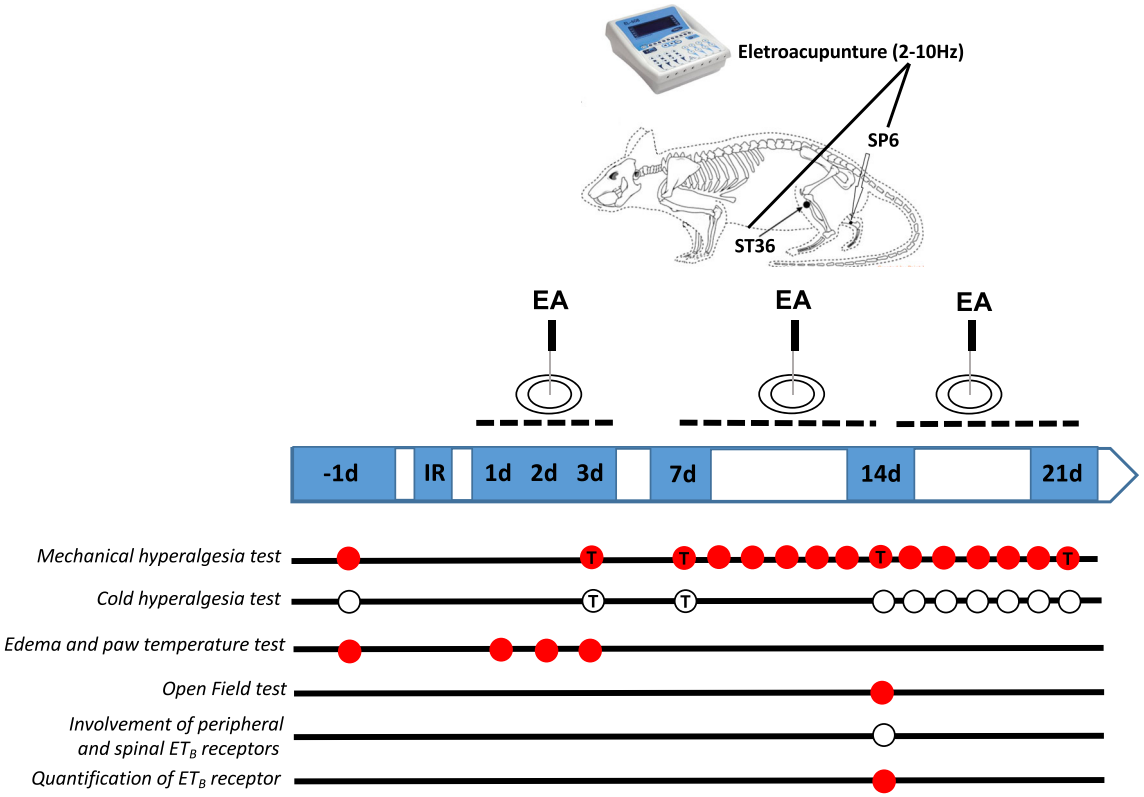

Fig. 1 Schematic representation of the experimental design. EA electroacupuncture; IR ischemia and reperfusion; d days; T time course analysis

period was recorded with a chronometer and considered to be indicative of cold hyperalgesia. The evaluations were performed before (0) IR and on days 3, 7, 14, and 21 post-IR.

Paw temperature

The temperature of the ventral and dorsal surface of the right and left hind paws was evaluated using a Thermal Image Camera (Testo 880®) with an accuracy of ± 0.1 °C and an infrared spectrum range of 7.5 to 13 m [33]. The cold/hot color pallet was used with temperature variation between 20 and 40 °C. The result was obtained through the difference of the right paw in relation to the left. Evaluations were conducted on days 1, 2, and 3 after IR, 30 min after the treatments.

Paw edema

Edema evaluation was performed by measuring the thickness of the right hind paw with a digital micrometer (Insize®, SP, Brazil) [8]. The results were expressed as the difference between the measure obtained and the baseline (before IR) evaluation of the same paw. Evaluations were conducted on days 1, 2, and 3 after IR, 30 min, 1, 2, and 24 h after the treatments.

Open field test

In this test, a wooden apparatus (box) measuring 40 × 60 × 50 cm was used, and the floor of the box was divided into 12 equal squares. Locomotor activity was evaluated by counting the number of squares the animals crossed with all paws over a 6-min evaluation period [36].

Involvement of peripheral ET_B receptors in the anti-hyperalgesic effect of EA

To test the hypothesis that peripheral ET_B receptors are involved in the anti-hyperalgesic effect of EA, different groups of mice subjected to IR were also treated with an ET_B receptor agonist, SRTX S6c alone (30 pmol/i.pl.) [35] or in combination with EA (20 min). Mechanical hyperalgesia was evaluated 15, 30, 60, 120, and 150 min after the treatments. IR + Control group 1 was also evaluated in parallel.

Since it has been shown that activation of ET_B peripheral receptors produces analgesia [31], we evaluated the involvement of these receptors in the anti-hyperalgesic effect of EA, analyzing whether the effect of SRTX S6c (30 pmol/i.pl.), EA or the combination of the two could be prevented by peripheral administration of Bq-788 (10 nmol/i.pl.). To this end, mice were subjected to IR, pre-treated with Bq-788 (i.e., fixed volume of 10 μl), and after 15 min, were treated with SRTX S6c (30 pmol/i.pl.), with EA or the combination of the two.

Mechanical hyperalgesia was evaluated 30 min after the treatments and control groups were evaluated in parallel.

Involvement of central (spinal cord) ET_B receptors in the anti-hyperalgesic effect of EA

Initially, we evaluated the effect of the i.t. administration of the ET_B receptor antagonist upon mechanical hyperalgesia induced by IR. Different groups of animals previously subjected to IR were treated with Bq-788 (3–30 nmol/i.t.). Mechanical hyperalgesia was evaluated 15, 30, 60, 120, 180, and 210 min after the treatments. IR + control group 1 was also evaluated in parallel.

Since it has been shown that blocking ET_B receptors produces analgesia [16, 17, 22], we evaluated the involvement of these receptors in the anti-hyperalgesic effect of EA directly by blocking ET_B receptors and by combining EA with Bq-788 administration (ET_B receptor antagonist). To this end, different groups of mice subjected to IR were treated with Bq-788 (3 nmol, i.t.) or with 20-min EA alone and in association. Mechanical hyperalgesia was evaluated 15, 30, 60, 120, 180, and 210 min after the treatments. The IR + control group 1 was also evaluated in parallel.

Western blotting

Quantification of ET_B receptor in muscle and spinal cord

The samples were collected on the 14th day after IR, 30 min after the daily treatment (the animals had been treated once a day for seven consecutive days). Samples of plantar flexor tissue of the right hind paw and spinal cord of the lumbar region (segment L4-L6) were collected and stored in the freezer (− 80 °C).

Spinal cord tissue samples were manually homogenized with micropistils in ice-cold RIPA buffer containing 1% protease inhibitor (Sigma-Aldrich, St Louis, MO), while the paw muscles were immersed in liquid nitrogen, pulverized and immediately placed in tubes containing RIPA buffer, and then incubated on ice for 30 min. The tubes containing the lysates were centrifuged at 10.000 rpm for 20 min at 4 °C, and the supernatants were collected. Protein concentration was determined using the Bradford method.

The electrophoretic separation was conducted using 30 μg of protein per well in 10% polyacrylamide gel electrophoresis (SDS-PAGE), running in a Mini-PROTEAN® Tetra cell apparatus under a PowerPac TM HC power supply (both from Bio-Rad, CA, USA). The proteins were transferred onto a PVDF membrane (Bio-Rad Laboratories Inc., Hercules, CA, USA), blocked in 5% BSA (prepared in TBS-T buffer, pH 7.4; concentration in mmol/L: 20 Tris-HCl, 137 NaCl, 0.1% Tween 20) and incubated overnight at 4 °C with primary antibodies to ET_B receptor (dilution 1:1000; Abcam, Cambridge, MA, USA). Peroxidase-conjugated monoclonal antibody against β-actin (dilution 1:45000) was used as a loading control for all samples tested. After incubation with primary antibodies, the membranes were washed three times (10 min each) with TBS-T solution and incubated with the specific secondary antibody conjugated to horseradish peroxidase (HRP) at room temperature for 1 h. The membranes were washed another three times (10 min each) with TBS-T solution and exposed to HRP substrate (Pierce Biotechnology, Rockford, IL, USA), and immune complexes were visualized by chemiluminescence using Chemidoc MP System (Bio-Rad Laboratories). Bands were quantified by densitometry using the software from the manufacturer (Image Lab; version 4.1; Bio-Rad Laboratories, Hercules, CA, USA). Values were normalized using the data obtained for β-actin and expressed as arbitrary units. In these experiments, the following groups ($n = 8$) were analyzed: Naive, IR + control 1, and IR + EA 20 min.

Statistical analysis

The results were analyzed with the Graph Pad Prism program (version 6.0 - La Jolla, California, USA). Initially, the Shapiro-Wilk normality test was applied to evaluate the normality of the data. The results are expressed as means ± standard deviation (SD) for continuous variables. The data was analyzed using both one-way analysis of variance (ANOVA) with the Student Newman-Keuls test and two-way ANOVA with repeated measures. Differences with a value of $P < 0.05$ were considered significant.

Results

EA reduces mechanical hyperalgesia in mice subjected to IR

The results illustrated in Fig. 2a–d demonstrate that the experimental model of CPIP induced mechanical hyperalgesia, when compared to the baseline results, i.e., before the animals were submitted to IR (− 1 day). Figure 2 shows the time course effect of EA treatment on the 3rd (panel a), 7th (panel a), and 14th day (panel d) after IR; as well as he results of EA daily treatments from the 7th to 14th day (panel c). In the evaluations performed on the 3rd, 7th, and 14th day after IR (Fig. 2a–d), no statistically significant difference was observed between the IR + control 1 group in relation to the IR + control 2 group, for this reason, in the subsequent evaluations, only IR + control 1 group was used.

On the 3rd day after IR (Fig. 2a), 20-min EA treatment significantly ($P < 0.001$) reduced mechanical hyperalgesia up to 1 h after the treatment. 10-min EA was effective for only 0.5 h ($P = 0.007$) after treatment, and 5-min EA had no

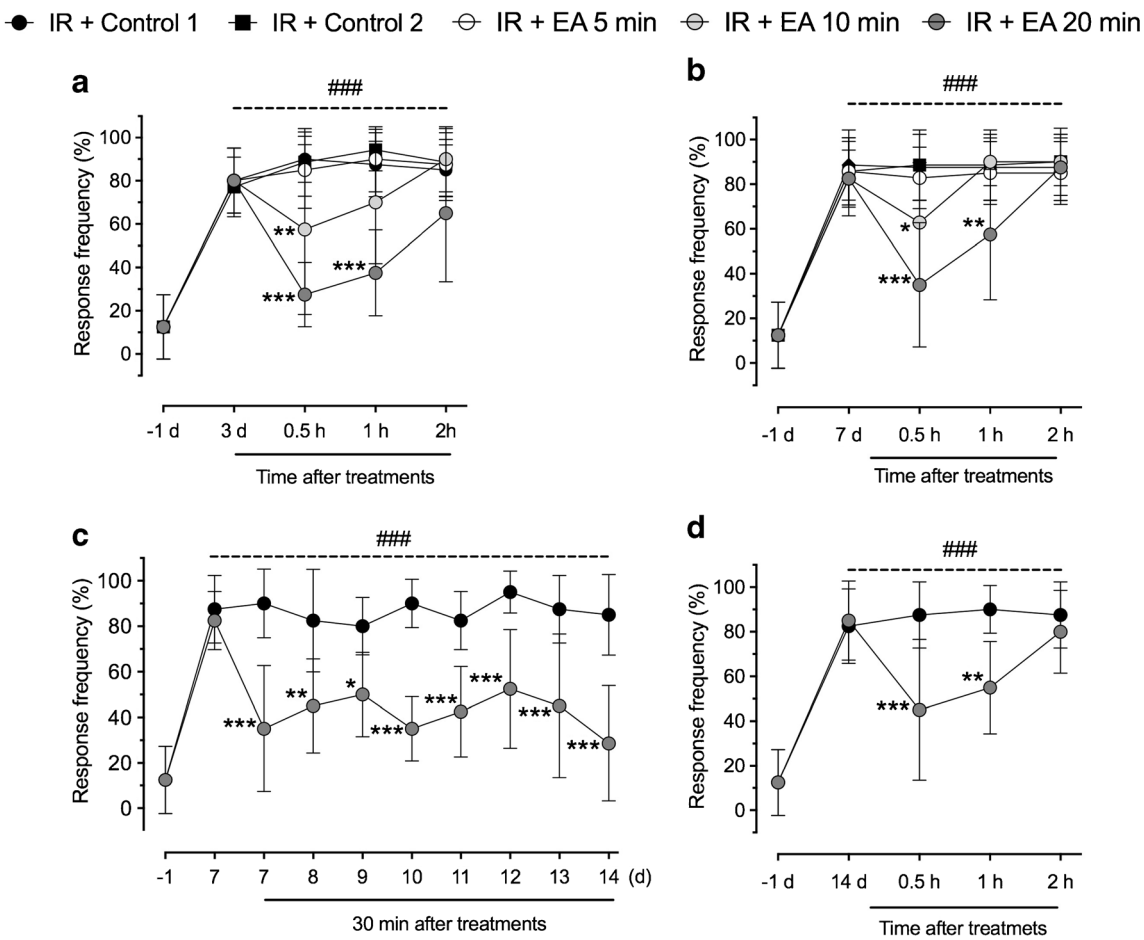

Fig. 2 Antihyperalgesic effect of EA after IR. Time course evaluation of the treatment with EA on the 3rd (**a**), 7th (**b**), and 14th (**d**) day after IR. Evaluation of hyperalgesia after 30 min of daily EA for 7 consecutive days (**c**). Data were expressed as mean ± standard deviation (SD) ($n = 8$ animals). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ when compared with the IR + control group. ### $P < 0.001$ when compared to baseline before IR (− 1 day). Two-way ANOVA followed by the Bonferroni test. EA electroacupuncture; IR ischemia and reperfusion; d days; h hours

analgesic effect. Figure 2b shows that on the 7th day after IR, 20-min EA reduced mechanical hyperalgesia for up to 1 h [0.5 h ($P < 0.001$) and 1 h ($P = 0.005$)]; 10-min EA was effective for up to 30 min [0.5 h ($P = 0.046$)] and 5-min EA did not affect paw withdrawal threshold. Confirming our previous observations, 20-min EA was more effective than 10 and 5-min EA, with the highest efficacy obtained 0.5 h after treatment.

Given the results, daily 20-min EA was conducted from day 7 to 14 post-IR and the evaluations were conducted 0.5 h after treatment. In these parameters, EA reduced mechanical hyperalgesia from the 7th to the 14th day ($P < 0.001$) post-IR when compared to the IR + control 1 group (panel c). On the 14th day after IR (panel d), daily 20-min EA continued to present the same profile, reducing mechanical hyperalgesia 0.5 h ($P < 0.001$) and 1 h ($P = 0.002$) after treatment, when compared to the IR + control group 1. Thus, no cumulative antihyperalgesic effect was observed with the daily EA treatment.

The results shown in Fig. 3a–c demonstrate that IR increased paw withdrawal frequency when compared to baseline evaluations, i.e., before IR (− 1 day). The results of EA performed at different times after IR on day 14 are shown on panel a, daily treatment from day 14 to day 21 on panel b, and on day 21 on panel c. The results presented on the 14th day after IR (panel a) demonstrate that 20-min EA reduced mechanical hyperalgesia 0.5 h ($P < 0.001$) and 1 h ($P < 0.001$) after the treatment, when compared to the group IR + control 1. On the 21st day after IR (panel B) after daily 20-min EA sessions, for 7 consecutive days, mechanical hyperalgesia reduction was only observed 0.5 h ($P < 0.001$) after treatment, when compared to the IR + control 1 group, which indicates there is no cumulative effect. With daily 20-min EA, from the 14th to the 21st day, it was possible to observe that EA reduced mechanical hyperalgesia on a daily basis 0.5 h after treatment, until the 21st ($P = 0.002$) day post-IR.

EA reduces cold hyperalgesia in CPIP mice

The results shown in Fig. 4a–c demonstrate the development of cold allodynia after IR when compared to baseline

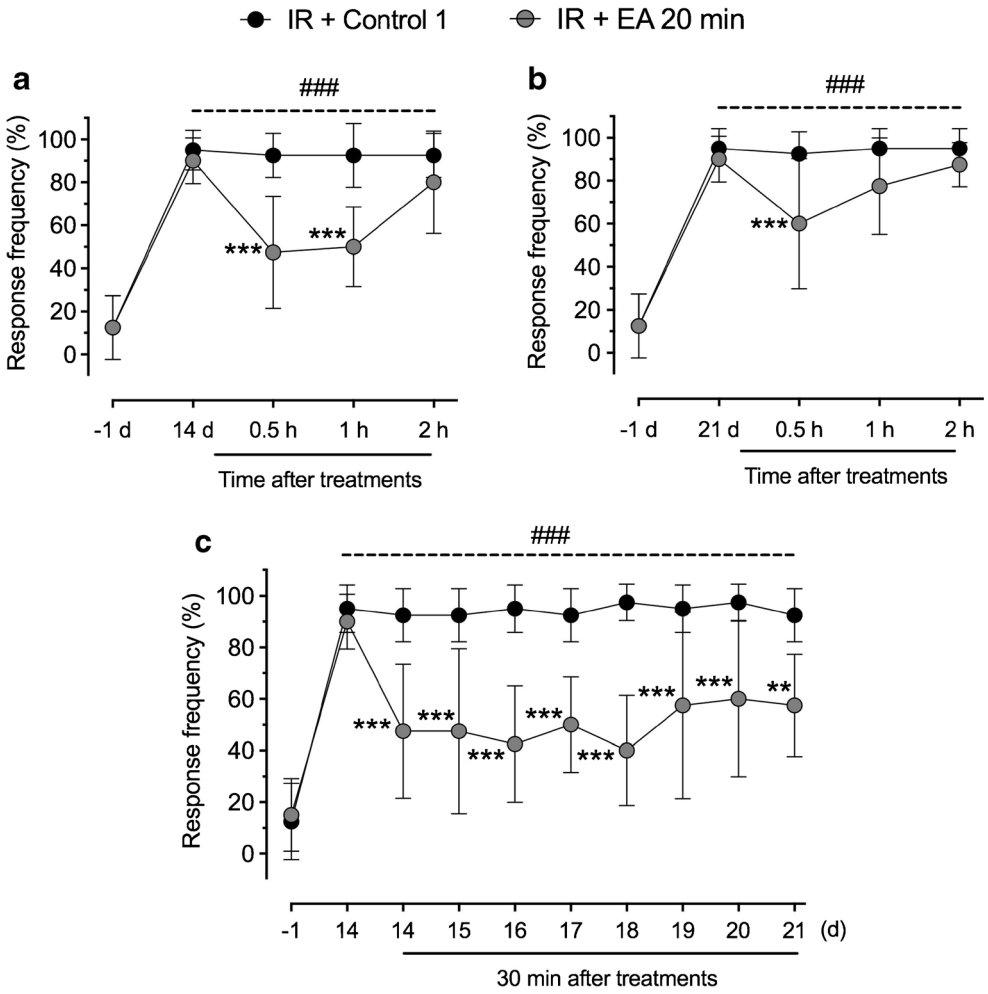

Fig. 3 Antihyperalgesic effect of EA from 14th to 21st day after IR. Time course evaluation of the treatment with EA on the 14th (**a**) and 21st (**b**) day after IR. Evaluation of hyperalgesia after 30 min of daily EA treatment for 7 consecutive days (**c**). Data were expressed as mean ± standard deviation (SD) ($n = 8$ animals). * $P < 0.05$, ** $P < 0.01$ when compared to the IR + control group. ### $P < 0.001$ when compared to baseline before IR (− 1 day). Two-way ANOVA followed by the Bonferroni test. EA electroacupuncture; IR ischemia and reperfusion; d days; h hours

evaluations, i.e., before IR (− 1 day). Figure 4 shows the effect of 20-min EA on day 3 (panel a), 7 (panel b), and after daily treatments from day 14 to day 21 (panel c). Panel a of Fig. 4 represents the acute phase of the CPIP model. 20-min EA reduced cold allodynia 0.5 h after treatment ($P < 0.001$) when compared to the IR group + control 1 (Fig. 4a), on the 3rd day after IR. However, in the chronic phase of the CPIP model, i.e., on the 7th day after IR (panel b), 20-min EA reduced allodynia to cold 0.5 h ($P < 0.001$), 1 h ($P = 0.007$), and 2 h ($P = 0.015$) after treatment, whereas 10-min EA effect lasted for only 0.5 h ($P < 0.001$), demonstrating thus that 20-min EA is more effective. Twenty-minute EA reduced cold allodynia on all treatment days until the 19th day ($P = 0.003$), when compared to the IR + control group 1 (Fig. 4c). Cold hyperalgesia thresholds were also drastically reduced in IR + control group 1 on the 20th and 21st days post-IR.

EA decreases edema, but does not affect paw temperature

Figure 5, panels a and b, demonstrates data regarding the surface temperature of the paw after IR. Neither the IR procedure nor the daily treatment for 3 days with EA altered paw temperature (neither on the ventral surface nor on the dorsal surface of the paw). In panels c and d, it was observed that on the 1st, 2nd, and 3rd days after IR, there was an increase ($P < 0.001$) in paw edema IR + control 1 group, when compared to the naive group. There was a significant reduction of paw edema ($P = 0.03$) induced by 20-min EA on the 1st day, 24 h after the treatment, when compared to the IR + control 1 group.

EA does not affect locomotive activity

In the open-field test conducted after 7 days of daily treatment with EA (14th day after IR), it was verified that 30 min after EA, the animals' locomotor activity was not affected, when compared to the group IR + control 1. The mean number of crosses over 6 min for the groups was 120.6 crossings for the naive, 118.3 crossings for the IR + control 1 group, and 118.3 crossings for the IR + EA 20 min group (Supplementary Fig. S1).

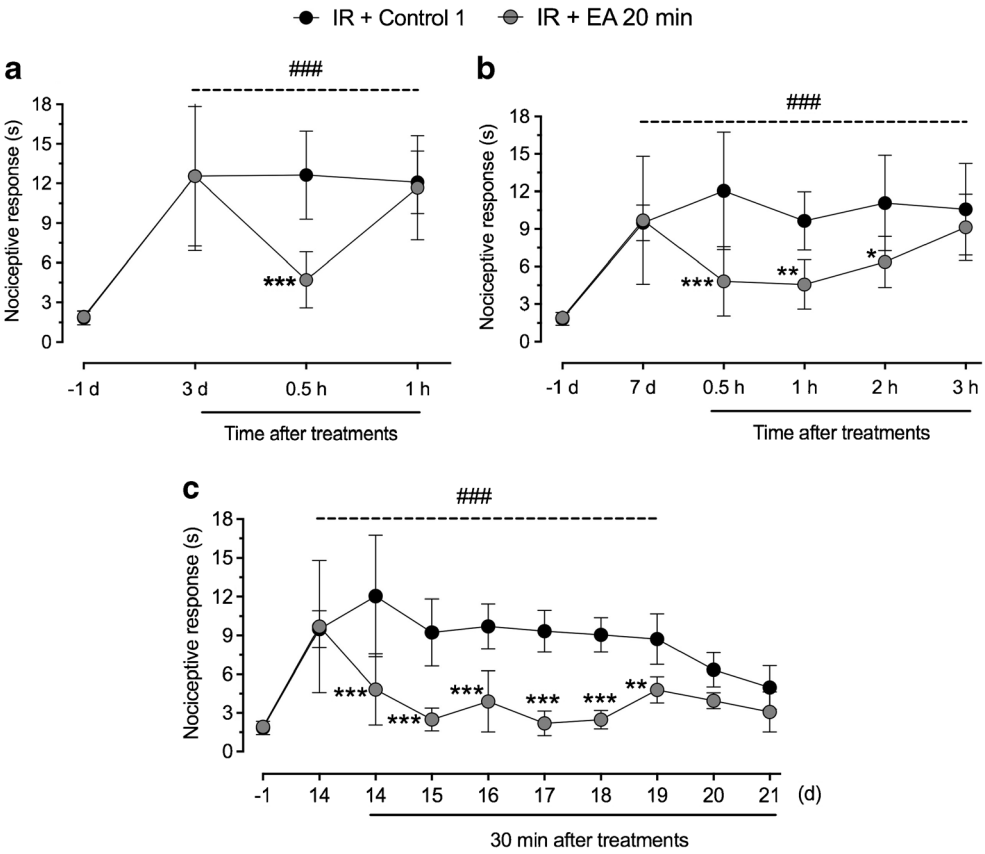

Fig. 4 EA reduces cold hyperalgesia in mice subjected to IR. Time course evaluation of the treatment with EA on the 3rd day (**a**) and 7th (**b**) day after IR. Evaluation of cold hyperalgesia after 30 min of daily EA treatment for 7 consecutive days (**c**). Data were expressed as mean ± standard deviation (SD) ($n = 8$ animals). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ when compared with the IR + control group. ### $P < 0.001$ when compared to baseline before IR (− 1 day). Two-way ANOVA followed by the Bonferroni test. EA electroacupuncture; IR ischemia and reperfusion; d days; h hours

Peripheral ET_B receptors are involved in the anti-hyperalgesic effect of EA in the chronic phase of CPIP/CRPS-I

In Fig. 6a, the administration of SRTX S6c (30 pmol/i.pl.) was shown to reduce mechanical hyperalgesia 15 and 30 min after the treatment ($P < 0.001$), as well as after 20-min EA, in the later, for up to 1 h ($P < 0.001$). When both treatments were associated, i.e., SRTX S6c (30 pmol/i.pl.) and 20-min EA, the effect lasted for up to 2 h, suggesting an added effect ($P < 0.001$).

Figure 6b shows that i.pl. administration of Bq-788 (10 nmol/i.pl.) on the 14th day after IR prevented the antihyperalgesic effect induced by SRTX S6c ($P < 0.001$), EA ($P < 0.001$), or the association of the treatments ($P < 0.001$). These data suggest the involvement of the peripheral ET_B receptor in EA induced antihyperalgesia.

EA enhances expression of peripheral ET_B receptors in the chronic phase of CPIP/CRPS-I

Figure 7 demonstrates that naive animals constitutively express the ET_B receptor in the flexor leg muscle. Fourteen days after paw IR, there was no significant increase in the expression of this receptor. However, in the animals subjected to IR and treated with 20-min EA, there was an increase ($P = 0.03$) in ET_B receptors expression in the flexor muscle of the paw, when compared to the IR + control group 1. Therefore, 20-min EA increases the expression of ET_B receptors in the periphery on the 14th day after IR, after 7-day consecutive treatments.

Spinal ET_B receptor contributes to the anti-hyperalgesic effect of EA in the chronic phase of CPIP/CRPS-I

The data presented in Fig. 8 demonstrate that the experimental model induced ($P < 0.001$) mechanical hyperalgesia, when compared to the baseline evaluation, i.e., before the animals were submitted to IR (− 1 day). In panel A, it can be seen that i.t. administration of Bq-788 (3 nmol) reduced mechanical hyperalgesia 15 ($P < 0.001$), 30 ($P < 0.001$) and 60 min ($P = 0.002$) after treatment. However, the dose of 10 nmol, Bq-788 (i.t.) induced a significantly statistical effect only 15 min after treatment ($P = 0.014$). Treatment with Bq-788 (30 nmol/i.t.) did not reduce mechanical hyperalgesia.

In Fig. 8b, the antihyperalgesic effect of the most effective dose of Bq-788 (3 nmol/i.t) was demonstrated on the 14th day after IR. Bq-788 and 20-min EA induced antihyperalgesia for up to 1 h after the treatments. However, when Bq-788 was

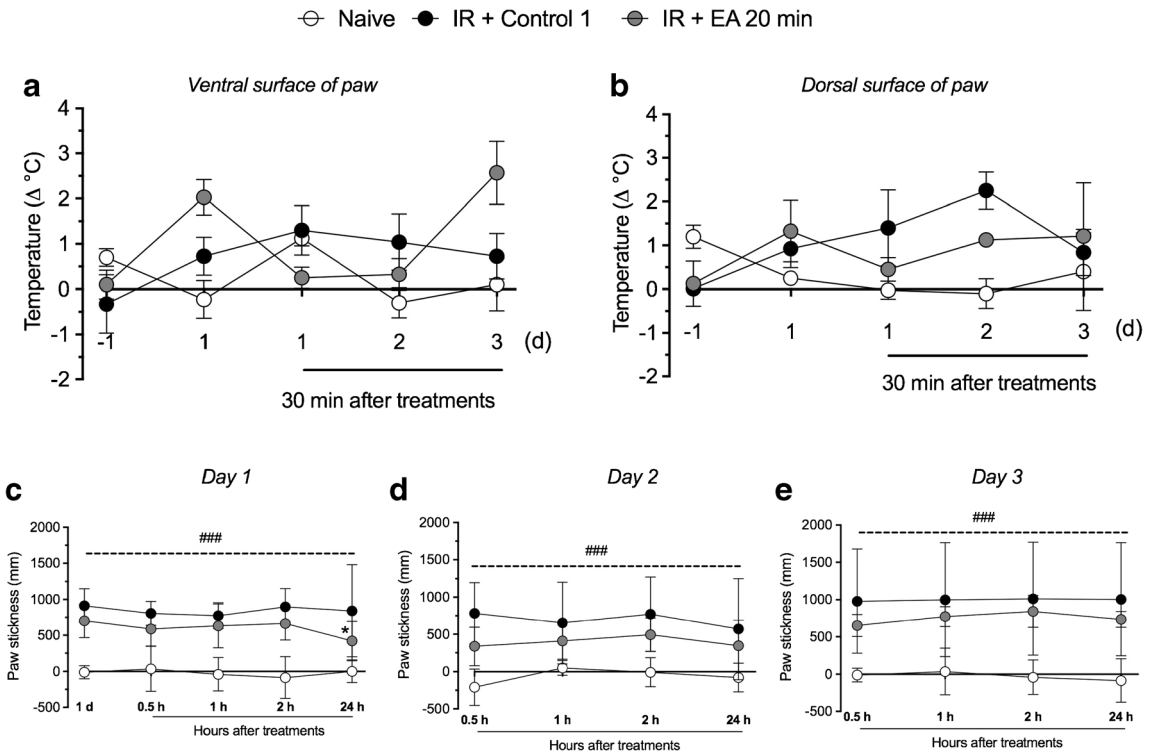

Fig. 5 Effect of EA on paw temperature and edema in mice subjected to IR. Quantification of the ventral surface temperature (**a**), dorsal surface (**b**) and paw edema (**c**–**e**). Data were expressed as mean ± standard deviation (SD) ($n = 8$ animals). * $P < 0.05$, when compared with the IR + control 1 group, # $P < 0.05$ and ### $P < 0.001$ when compared to naive. Two-way ANOVA followed by the Bonferroni test. EA electroacupuncture; IR ischemia and reperfusion; d days; Δ delta; °C degrees centigrade; mm millimeters

associated with EA, a reduction ($P < 0.001$) in mechanical hyperalgesia was achieved for up to 3 h after treatment. The results suggest the central (spinal cord) involvement of the ET_B receptors in the antihyperalgesic effect of EA.

EA did not affect the expression of spinal ET_B receptors in the chronic phase of CPIP/CRPS-I

In Fig. 9, it is possible to observe that naive animals constitutively express the ET_B receptor in the spinal cord; however, 14 days after IR, there was no significant increase in the expression of this receptor. Additionally, no changes in ET_B receptor expression were evidenced in the animals subjected to IR and treated with 20-min EA. Therefore, 20-min EA did not influence the expression of ET_B receptors in the spinal cord after 7 consecutive daily treatments.

Discussion

Endothelins are peptides that have their effects mediated by ET_A and ET_B receptors, which induce vasoconstriction and vasodilation, respectively [31, 48]. The expression of these receptors on pain pathways suggests their participation in pain modulation. ET_B receptors expressed in paw muscles during CPIP, could be an interesting target for the treatment of CRPS-I [31]. Furthermore, central (spinal cord) ET_B receptors are known to reduce mechanical hyperalgesia in the CRPS-I animal model [22].

Despite extensive literature on ischemic lesions, the chronic phase of CRPS-I is not well studied, as most of the research has focused on its acute phase. Therefore, this study sought to determine the involvement of peripheral and central (spinal cord) ET_B receptors in the antihyperalgesic effect of EA in the chronic phase of an animal model of CPIP/CRPS-I (14 days post-IR).

Our results demonstrate for the first time that IR induces hyperalgesia (to mechanical and cold stimuli) in both the acute (inflammatory) and chronic (neuropathic) phases of the disease, and that even a single treatment with EA effectively reduces it. These results agree with the literature that demonstrated that ST36 and SP6 EA reduces mechanical hyperalgesia in an animal model of neuropathy [6, 12, 25] as well as in the model of persistent inflammatory pain [15].

In regard to cold hyperalgesia, this has been shown that paw IR induces hyperalgesia to cold stimuli which is prevented by peripheral administration of Bq-788, 2 days post-IR [31]. Thus, the results of the present study confirm and extend literature data by demonstrating that EA reduces cold hyperalgesia in the neuropathic phase of CPIP/CRPS-I.

To evaluate the clinical potential of EA, the mice were treated in the two phases. EA was effective both in the

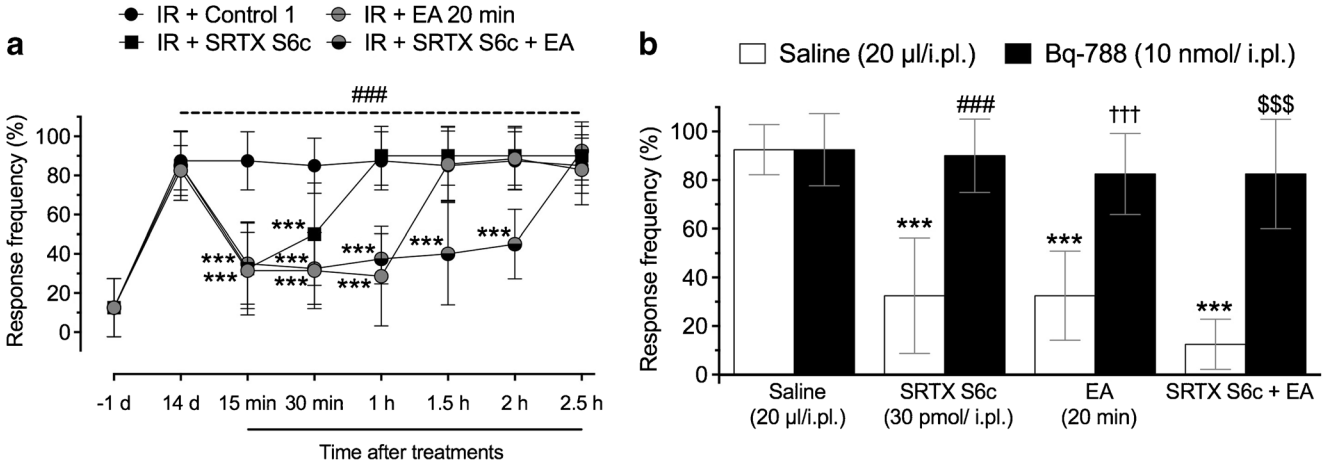

Fig. 6 Peripheral ET_B receptors participate in the antihyperalgesic effect of EA. Effect of peripheral ET_B receptor on mechanical hyperalgesia with administration of SRTX S6c (i.pl.) and EA on the 14th day after IR (**a**) and Bq-788 (i.pl.), EA and SRTX S6c (i.pl.) on the 14th day after IR (**b**). Data were expressed as mean ± standard deviation (SD) ($n = 8$ animals). *** $P < 0.001$ when compared to the IR + control group 1. ### $P < 0.001$, when compared to the saline group (20 µl/i.pl.) + SRTX S6c (30 pmol/ i.pl.) (**b**) or when compared to baseline before IR (− 1 day, **a**). ††† $P < 0.001$, when compared to the saline group (20 µl/i.pl.) + IR EA 20 min (**b**). $P = 0.001$ when compared to the saline group (20 µl/i.pl.) + (SRTX S6c 30 pmol/i.pl.) + IR EA 20 min) (**b**). Data were expressed as mean ± standard deviation (SD) ($n = 8$ animals). One-way or two-way ANOVA followed by Bonferroni tests (**a**) or Student Newman-Keuls test (**b**). EA electroacupuncture; min minutes; h hours; d days; SRTX S6c sarafotoxin S6c (ET_B receptor agonist); Bq-788 ET_B receptor antagonist; i.pl. intraplantar

inflammatory and in the neuropathic phase without affecting locomotor activity. Additionally, EA did not produce a cumulative effect. On the contrary, on the 14th day, EA had a shorter-lived effect when compared to other evaluation days.

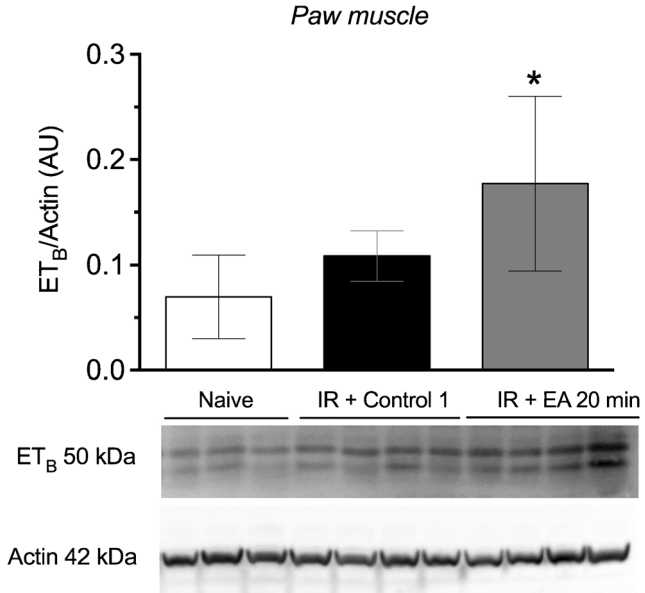

Fig. 7 EA induces increased expression of peripheral ET_B receptors in mice subjected to IR. Effect of cumulative treatment with EA from day 7 to 14 after IR on the expression of the ET_B receptor in the paw muscle. Data were expressed as mean ± standard deviation (SD) ($n = 8$ animals). * $P < 0.005$ when compared to the IR + group control 1. One-way ANOVA followed by Student Newman-Keuls test. EA electroacupuncture; IR ischemia and reperfusion; ET_B receptor for endothelin B; UA arbitrary units

Tolerance to repeated treatment with EA has been associated with the nociceptin/orphanin fq system. It has been shown that tolerance observed with EA is prevented by intracerebroventricular administration of the antibody against orphanin fq in rats [15, 40]. The authors concluded that EA may enhance the endogenous release of orphanin fq in the central nervous system (CNS) which acts as an antagonist to the antihyperalgesic effect of EA in the brain.

In addition, it is known that electrical stimulation itself can induce tolerance, as with TENS. It has been previously demonstrated that cholecystokinin B receptor blockade (CCK-B) prevented the tolerance induced by repeated transcutaneous electrical nerve stimulation (TENS) in rats with kaolin/carrageenan-induced knee inflammation [7]. Since octapeptide-cholecystokinin (CCK-B receptor agonist) plays an important role in tolerance induced by both morphine and EA, the blockade of this receptor potentiated the effect of EA and prevented tolerance. Thus, these peptidergic mediators may mediate tolerance observed in repeated treatment with EA [14].

Another finding of the present study was the demonstration that EA reduced edema but not the increase in paw temperature in the inflammatory phase of CPIP/CRPS-I. These findings corroborate previous observations that EA reduces inflammation and edema in the CFA [26] and carrageenan models [21], with potential to treat chronic inflammatory conditions [45].

Studies have highlighted the implication of the endothelinergic system in pain processing [20], by activating its receptors with distinct functions at both peripheral and central (spinal cord) sites, for example, activation of the ET_B

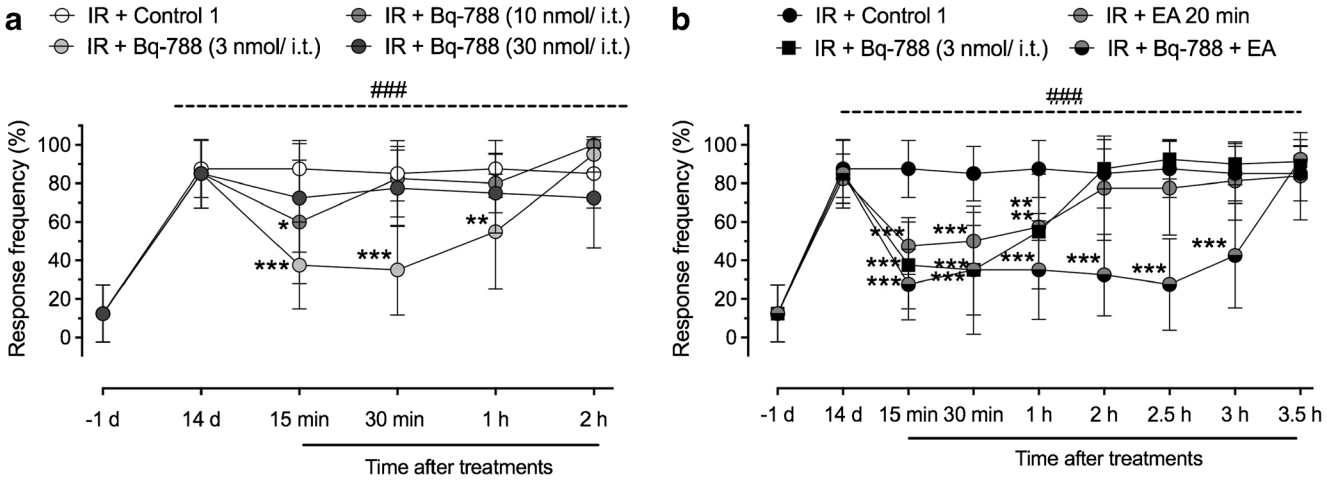

Fig. 8 The antihyperalgesic effect of EA is mediated by central ET_B receptors. Dose response curve of the effect of the ET_B receptor antagonist (Bq-788, i.t., **a**). Effect of the association of EA with the administration of the ET_B receptor Bq-788 i.t. antagonist upon mechanical hyperalgesia (**b**). * $P < 0.05$ (**a**), ** $P < 0.01$ (**a**) and *** $P < 0.001$ when compared to the IR + control group 1. It is considered ### $P < 0.001$ when compared to baseline before RI. Data were expressed as mean ± standard deviation (SD) ($n = 8$ animals). Two-way ANOVA followed by the Bonferroni test. EA electroacupuncture; min minutes; h hours; d days; Bq-788 ET_B receptor antagonist; i.t. intrathecal

receptor in the periphery produces analgesia [31, 35], whereas centrally (spinal cord), produces nociception [22].

As expected, peripheral administration of SRTX S6c or EA reduced mechanical hyperalgesia in the chronic phase of CPIP/CRPS-I, although a more significant effect was evidenced with the association of the two therapies. The findings suggest that these therapies could share the same mechanism of action, i.e., activation of peripheral ET_B receptors.

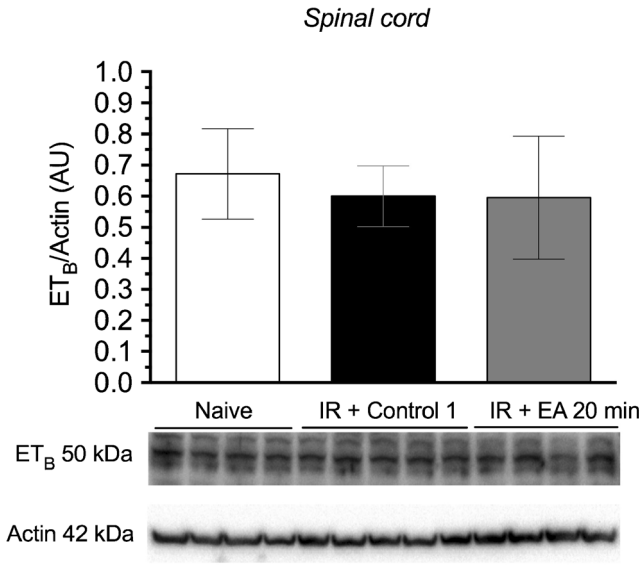

Fig. 9 EA does not alter the expression of central ET_B receptors in mice subjected to IR. Effect of cumulative treatment with EA from day 7 to 14 after IR upon ET_B receptor expression in the spinal cord. Data were expressed as mean ± standard deviation (SD) ($n = 8$ animals). * $P < 0.005$ when compared to the IR + group control 1. One-way ANOVA followed by Student Newman-Keuls test. EA electroacupuncture; IR ischemia and reperfusion; ET_B receptor for endothelin B; UA arbitrary units

Therefore, we suggest that the observed effects are related to the activity of SRTX S6c on the ET_B receptor expressed (1) in the vessel, which induces vasodilatation that is in opposition to the state of chronic hypoperfusion present in the CPIP model; and (2) in the primary nociceptive neuron, which induces the release of opioids causing analgesia. The same has been observed in other studies [31] who administered compound IRL-1620, another ET_B receptor agonist, in the CPIP model in mice, but on the second day after IR. A study has shown that the manual stimulation of ST36 and SP6 acupoints may also be effective in increasing blood flow in the lower limb [46], pointing to a possible involvement of the ET_B receptor in this effect, as similar results are obtained with the activation of the ET_B receptor by SRTX 6c.

The results show that the pharmacological blockade of peripheral ET_B receptors in the neuropathic phase of CRPS-I prevented both the effect of SRTX 6c and EA. It also prevented the antihyperalgesic effect induced by the combination of the two therapies. Furthermore, the expression of peripheral ET_B receptors on the 14th day post-IR was increased when compared to naive animals, although the results were not statistically significant. However, daily treatment with EA induced a significant increase in ET_B receptor expression in the paw muscle of these animals. Taken together, the results suggest the involvement of peripheral ET_B receptors in the antihyperalgesic effect of EA.

ET-1 produces pain by the activation of ET_B receptors in the spinal cord. It has been demonstrated in an animal model of neuropathic pain induced by spinal root ligation that the administration of Bq-788 reduces mechanical hyperalgesia [22]. Data from the present study showed that i.t. administration of Bq-788 on the fourteenth day after IR also reduced mechanical hyperalgesia, in a dose-dependent manner, in

mice subjected to CPIP/CRPS-I. However, IR did not affect ET_B receptor expression in the spinal cord. These findings corroborate previous studies that have also shown that blocking the ET_B receptor in the spinal cord by Bq-788 produces a dose-dependent antihyperalgesic effect, however in rats, and over a period of 21 days [22].

After determining the antihyperalgesic effect of the ET_B receptor antagonist administered in the spinal cord, the next step of the present study was to analyze the involvement of ET_B receptors on EA's effect. The findings of the present study also demonstrated that although daily EA did not affect ET_B receptor expression in the spinal cord, i.t. treatment with an ET_B receptor antagonist potentiated the antihyperalgesic effect of EA, suggesting the involvement of the central (spinal cord) ET_B receptor in the effect of EA.

Since ET-1 is the main endogenous activator of the central (spinal cord) ET_B receptor and it has been shown that plasma concentrations of this peptide are increased in the CPIP/CRPS-I model, it is very likely that mechanical hyperalgesia could also be sustained by the action of ET-1 upon central (spinal cord) ET_B receptors. In line with this claim, it has been described that the synthesis of ET-1 is modulated by physiological and pathophysiological factors. In addition, factors such as nitric oxide (NO), prostacyclin, and atrial natriuretic hormone have been shown to inhibit the production of ET-1 [5]. A number of studies have shown the effect of EA on NO modulation [1]. Specifically, EA in acupoint ST36 reduced orofacial thermal hyperalgesia in rats, an effect that was prevented by specific inhibitors of neuronal nitric oxide synthase and the induced nitric oxide synthase. In addition, the authors observed that concentrations in spinal brain fluid and plasma were four and three times higher, respectively, after EA [2]. Thus, a plausible explanation for the effect observed here is related to the increase of NO induced by EA that could in turn induce the decrease in the concentrations of ET-1 in the spinal cord and thus reducing ET_B receptor activation and hyperalgesia. However, future studies analyzing plasma and tissue concentrations of ET-1 in the CPIP/CRPS-I model on the fourteenth day post-IR and after seven consecutive days of EA are required to confirm this hypothesis.

It can be concluded from these results that daily EA is effective in reducing the signs and symptoms of CPIP/CRPS-I and that peripheral and central ETB receptors participate in the antihyperalgesic effect of EA in the chronic phase of the syndrome, in an opposite manner, in the periphery and the spinal cord (centrally). These results demonstrate EA analgesic effect in yet another chronic painful condition that is rather difficult to treat. In sum, this study deepens our knowledge about EA mechanism of action by demonstrating the involvement of ETB receptors, and adds to the understanding of the pathophysiological events induced by CRPS-I.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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