Electroacupuncture Stimulation Alleviates Inflammatory Pain in Male Rats by Suppressing Oxidative Stress

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Summary

In the present study, we focused on whether the analgesic effect of Electroacupuncture (EA) is related to the regulation of oxidative stress. We established a chronic inflammatory pain model in male rats by a single injection of complete Freund's adjuvant (CFA) and then treated the animals with daily EA stimulation at the site of "zusanli". The analgesic effect of EA was evaluated by measuring the paw withdrawal threshold (PWT) when rats received mechanical and thermal pain stimulation. The levels of inflammation-related molecules and oxidative stress-related markers in the spinal cord were measured by western blotting or ELISA kits. EA stimulation and antioxidants effectively increased the PWT in CFA rats. Co-treatment of CFA rats with the ROS donor t-butyl hydroperoxide (t-BOOH) further decreased the PWT and weakened the analgesic effect of EA. EA treatment inhibited inflammation and oxidative stress, as shown by decreased levels of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, and MDA and increased activity of SOD and catalase. Moreover, EA reduced the expression of p-p38, p-ERK, and p-p65 and simultaneously downregulated the expression of TRPV1 and TRPV4 in CFA rats. In an *in vitro* study, direct stimulation with t-BOOH to the C6 cells increased the production of TNF-α, IL-1β, IL-6, activated p38, ERK, and p65 and up-regulated the expression of TRPV1 and TRPV4, and these effects could be prevented by the ROS scavenger PBN. Taken together, our data indicate that the inhibition of oxidative stress and the generation of ROS contribute to the analgesic effect of EA in male CFA rats.

Key words

Electroacupuncture • Inflammatory pain • Oxidative stress • Reactive oxygen species • TRPV1

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Introduction

Chronic pain (CP) is a common symptom associated with various diseases. CP is characterized by persistent nociceptive hypersensitivity, in which patients experience a marked reduction in the thresholds required to induce pain [1-2]. CP has a high prevalence worldwide and causes great stress to human society. The morbidity of chronic pain is 20-45 % and it varies by country, such as England and Canada 11 %, Sweden 40 %, USA 2-45 %, and New Zealand 14-24 %, which accounts for 30-40 % of patients attending pain clinics in that country. In the USA, the number of adults with chronic pain is estimated to be 100 million, with an annual cost of up to US $635 billion [2-3]. Chronic inflammatory pain (CIP) is a subtype of CP usually caused by trauma, bacterial and viral infection, chemical stimulation and surgery [4]. At present, pain management interventions for chronic pain are insufficient, providing inadequate pain relief and having multiple health and societal adverse effects. Therapeutic drugs, such as opioid receptor agonists and nonsteroidal anti-inflammatory drugs, are available to treat CIP. However, these drugs usually cause side effects, including respiratory depression, drug addiction and gastrointestinal adverse reactions [5]. Acupuncture is a well-known analgesic therapy with remarkable effects and a long history in Asia. Many clinical studies have confirmed that acupuncture and electroacupuncture (EA) exhibit the

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superiority of rare side effects compared with drug analgesia [6-7]. However, the exact analgesia mechanism of acupuncture is still unclear.

Oxidative stress is an important characteristic of inflammatory and damaged tissues. In previous investigations [8-9], oxidative stress and its related factors have been reported to be involved in CIP. Inflammatory and oxidative stress biomarkers are associated with pain intensity, pressure pain threshold and quality of life in myofascial pain syndrome. Moreover, reactive oxygen species (ROS) generated during oxidative stress are increased in the ipsilateral psoas major muscle after complete Freund's adjuvant (CFA) injection, and pharmacological inhibition of ROS by phenyl-N-tert-butylnitrone (PBN) reduced CFA-evoked CIP. Interestingly, recently published reports [10-11] have disclosed that EA has a definite effect on oxidative stress in several animal models. In brief, EA has been shown to reduce the astrocyte number and oxidative stress in aged rats with surgery-induced cognitive dysfunction and inhibit oxidative stress during the acute phase of cerebral ischemia-reperfusion in rats. However, whether EA therapy has an effect on oxidative stress remains unclear. We hypothesized that the analgesic effect of EA might be achieved by reducing oxidative stress. Microglia regulates neuronal and synaptic activities to change pain behavior within minutes to tens of minutes following treatment with microglial activators and inhibitors. Insights into the important role of microglia in pain (specific in male than female) were revealed [12-13]. Therefore, in the present study, we established CFA-induced inflammatory pain model *in vivo*, and used C6 glioma cells investigate the role of oxidative stress in the analgesic effects of EA.

Materials and Methods

Animals

Male Sprague-Dawley rats (180-200 g) were obtained from the experimental animal center of Anhui Medical University. The rats were housed six per cage with free access to food and water, and they were kept in a temperature-controlled (22-24 °C) room under a 12 h/12 h light/dark cycle. All procedures were approved by the Animal Care and Use Committee of Anhui Medical University (Hefei, China, LLSC20183042), and conducted in accordance with the Guide for the Care and Use of Laboratory Animals (1985), NIH, Bethesda.

Inflammatory pain model and treatment

Intrathecal catheter surgery was performed. In brief, after the rats were anesthetized with 5 % sodium pentobarbital (0.2 ml/100 g, intraperitoneal injection), a PE-10 intrathecal catheter was implanted into the intrathecal space of the spinal cord at L4-L6 level. After filling the catheter with sterile endotoxin-free PBS, rats were individually housed to protect the catheter from gnawing. Three days after surgery, 20 µl of 2 % lidocaine was injected intrathecally, a paralysis of lower limbs occurred within 30 s and recovered within 30 min indicated successful implantation of the catheter. These rats without signs of spinal cord damage were applied for experimentation. A total of thirty-six rats were used in the present study, and each group contained six rats. CIP was induced in the rats as we reported previously [14]. Briefly, the rats received an intraplantar injection of 100 µl of CFA (Sigma-Aldrich Corp., St. Louis, MO, USA) on the plantar surface of the left hind paw. Each milliliter of the injection contained 1 mg heat-killed and dried *Mycobacterium tuberculosis*, 0.85 ml paraffin oil and 0.15 ml mannide monooleate. Control animals were injected with the same volume of 0.9 % saline. After injection, all CFA animals were randomly divided into several groups: (1) CFA group, received 0.1 ml CFA injection; (2) CFA+t-BOOH (t-butyl hydroperoxide, ROS donor, Sigma-Aldrich Corp., St. Louis, MO, USA) group, received 0.1 ml CFA injection and daily intrathecal injection of 0.10 mg t-BOOH dissolved in 5 µl saline; (3) CFA+EA group, received 0.1 ml CFA injection together with daily EA stimulation for 7 days (7 d); (4) CFA+EA+t-BOOH, received 0.1 ml CFA injection, daily EA stimulation and intrathecal injection of 0.10 mg t-BOOH; (5) CFA+PBN (phenyl-N-tert-butylnitrone, ROS scavenger, Sigma-Aldrich Corp., St. Louis, MO, USA) group received a 0.1 ml CFA injection together with a daily intraperitoneal injection of PBN (150 mg/kg). Moreover, the normal group included six healthy rats, which received a 0.1 ml saline injection.

EA treatment was performed based on a previously published method [11,14]. In brief, animals were anaesthetized during the EA stimulation sessions. To minimize the potential impact of the anesthetic on the EA, we used 2 % sevoflurane as an anesthetic, which has little effect on the automatic nervous system [15-16]. The rats received EA treatment with these stimulator parameters: 2/100 Hz, 0.5 to 1.5 mA (initial strength of 0.5 mA, increased by 0.5 mA every 10 min) for a total of

30 min. We applied a total of seven bilateral sessions of EA therapy (30 min per session, one session per day) over seven days. Electrostimulation was performed with a pulse width of 0.6 ms at 2 Hz and 0.2 ms at 100 Hz. The rats were loosely immobilized. Four stainless steel acupuncture needles of 0.25 mm in diameter were inserted at a depth of 5 mm into bilateral "zusanli" (ST36, 5 mm lateral to the anterior tubercle of the tibia) and "Kunlun" (BL60, at the ankle joint level and between the tip of the external malleolus and tendon calcaneus) acupoints. Electrical stimulation was produced by a Trio 300 electrical stimulator (Trio 300, ITO Corporation, Germany).

Cell culture and treatment

C6 glioma cells were obtained from American Type Culture Collection (RRID:CVCL_0194, Manassas, VA, USA), seeded in culture dishes with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin, and routinely cultured in a humidified incubator at 37 °C supplemented with 5 % CO_2. When the cells reached 70 % to 80 % confluency, they were treated with 50 μM t-BOOH and 100 μM PBN for 24 h. After 24 h of incubation, the whole-cell extracts were collected for biochemical analyses.

ROS detection

Intracellular ROS levels were measured using a ROS assay kit (Beyotime, Shanghai, China). Briefly, the cells were seeded in 24-well plates and treated with 50 μM t-BOOH and 100 μM PBN for 24 h. After being washed with PBS, the cells were loaded with 10 μM DCFH-DA and incubated for 40 min at 37 °C. The cells were then washed with PBS and maintained in culture medium. Tissue samples from the spinal cord were made into single-cell suspensions, and ROS levels were detected in the same manner.

Paw withdrawal threshold

Behavioral tests were performed 2 h after EA stimulation. The paw withdrawal threshold (PWT) was used to assess inflammatory pain. PWT was determined by the von Frey behavioral test and Hargraves' test. Mechanical sensitivity to the pain was measured by testing the force of responses to stimulation with three applications of electronic von Frey filaments (North Coast Medical, Gilroy, CA, USA), while thermal pain was assessed with three applications using a Hargraves' test IITC analgesiometer (IITC Life Sciences., Model 390 G, CA, USA).

Assessment of inflammatory cytokines

Seven days after CFA/0.9 % saline injection, all animals were sacrificed. The spinal cords were collected and homogenized. The levels of TNF-α, IL-1β, and IL-6 in the tissues were assessed by using ELISA kits (R&D Systems, Minneapolis, MN, USA). Moreover, the C6 culture supernatant was collected, and the levels of TNF-α, IL-1β, and IL-6 were measured in the same manner.

Assessment of oxidative stress

Seven days after CFA/0.9 % saline injection, all animals were sacrificed. To measure oxidative stress levels, biomarkers in C6 cells and the spinal cord of rats were detected. The malondialdehyde (MDA) content and catalase (Cat) and superoxide dismutase (SOD) activity in tissue homogenates were measured with reagent kits provided by Beyotime Biotechnology Institute (Shanghai, China).

Western blot analysis

Seven days after CFA/0.9 % saline injection, all animals were sacrificed. The spinal cord (L4-L6 segments) was removed immediately and rinsed in ice-cold PBS and subjected to protein extraction. The protein concentration of the tissue lysates and the whole-cell extracts was detected with a bicinchoninic acid protein assay kit (Thermo Scientific, Grand Island, NY, USA). Samples were denatured and then separated by 10 % SDS-PAGE and then transferred to a PVDF membrane (Millipore, CA). The membranes were blocked with 5 % low-fat milk in TBST for 1 h and then incubated with antibodies against phosphorylated (p)-p38 (ab4822, Abcam, Cambridge, MA, USA), p-ERK (ab47310, Abcam, Cambridge, MA, USA), p-p65 (ab76302, Abcam, Cambridge, MA, USA), substance P (SP, df6541, Affinity Biosciences, Cincinnati, OH, USA), TRPV1 (ab203103, Abcam, Cambridge, MA, USA), TRPV4 (ab39260, Abcam, Cambridge, MA, USA) and NADPH oxidase-4 (ab154244, NOX4, Abcam, Cambridge, MA, USA). Afterwards, the blots were washed with Tris-buffered saline and Tween 20, incubated with a horseradish peroxidase-conjugated anti-rabbit secondary antibody and finally visualized in ECL solution (Thermo Scientific, Grand Island, NY, USA). GAPDH was used as an internal control.

Statistical analysis

Statistical analyses were performed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA). All experimental data are expressed as the SD (standard deviation). The data differences between groups were analysed using one-way ANOVA followed by individual *post hoc* multiple comparisons. $P<0.05$ was considered to represent statistical significance.

Results

ROS mediate EA effects in the CFA-induced pain model

As shown in Figure 1A-1B, both mechanical and thermal hyperalgesia were measured and used to evaluate the analgesic activity of EA treatment. Intraplantar injection of CFA resulted in a reduction in mechanical and thermal pain thresholds on d3, d5, and d7 after injection. EA treatment induced significant and long-lasting antihyperalgesic effects on CFA rats ($P<0.01$). To investigate the role of oxidative stress involved in EA stimulation therapy, we injected t-BOOH (ROS donor) *via* an intrathecal catheter or antioxidant treatment (PBN) in CFA rats. Compared with the CFA group, treatment with t-BOOH further reduced the values of mechanical hyperalgesia and thermal hyperalgesia, while treatment with the antioxidant PBN caused obvious improvements in the values of mechanical and thermal hyperalgesia, respectively ($P<0.01$). However, it was interesting to see that the antihyperalgesic effect of EA was abolished in rats that received t-BOOH, and was further enhanced in rats that received PBN ($P<0.01$, Fig. 1, Fig. S1 in Supporting materials).

EA treatment inhibits oxidative stress and reduces ROS generation in CFA-treated rats

At the end of the study, we detected MDA, SOD, and Cat levels in the tissues of the spinal cord. As shown in Figure 2A-2C, CFA injection significantly increased MDA and decreased SOD and Cat levels in the tissues ($P<0.01$), suggesting the occurrence of oxidative stress in the CFA rats. The CFA rats given t-BOOH showed increased MDA and decreased SOD and Cat, while the rats that received the antioxidant PBN exhibited decreased MDA and increased SOD and Cat levels compared with those of CFA rats ($P<0.05$). In addition, the inhibitory effect of EA

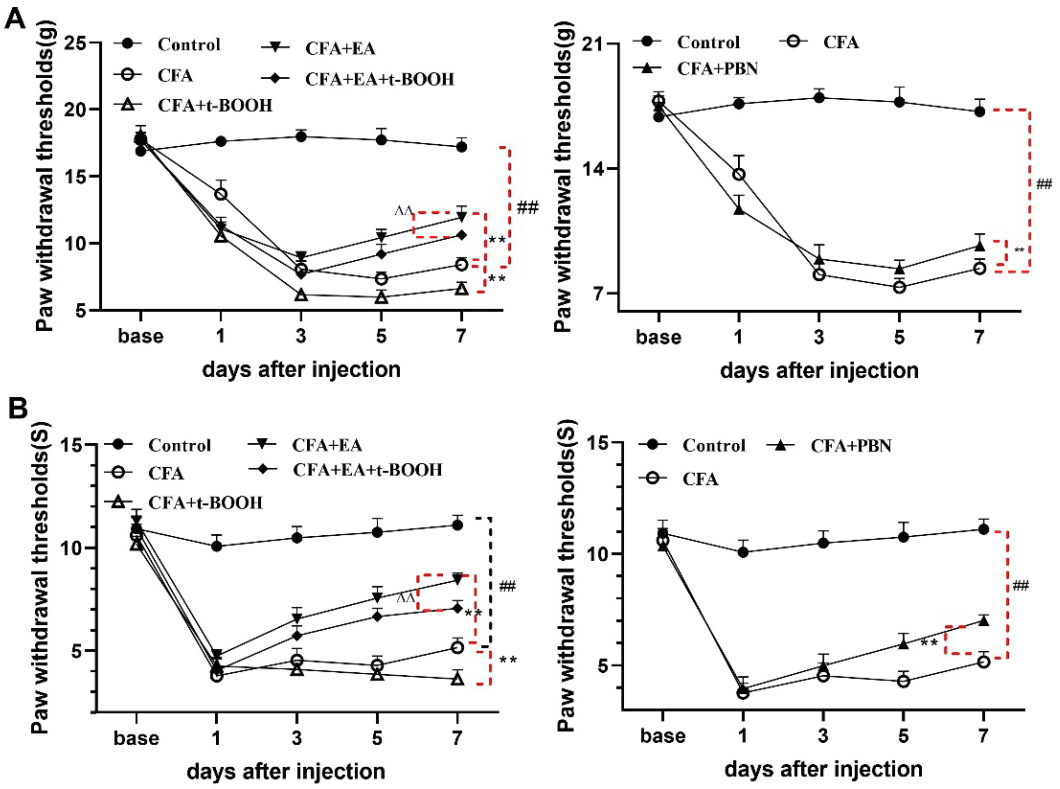

Fig. 1. ROS mediate the withdrawal threshold after mechanical and thermal stimulation in EA-treated CFA rats. (**A**) Mechanical sensitivity of rats was measured by assessing the force of responses to stimulation with electronic von Frey filaments. (**B**) Thermal sensitivity of rats was measured using a Hargraves' test IITC analgesiometer. EA: electroacupuncture; CFA: complete Freund's adjuvant; t-BOOH: tert-butyl hydroperoxide; PBN: phenyl-N-tert-butylnitrone; values represent the mean ± standard deviation; n=6 per group for each time point; ## $P<0.01$ vs. the control group; ** $P<0.01$ vs. the CFA group; ^^ $P<0.01$ vs. the CFA+EA group.

on oxidative stress was attenuated in rats that received t-BOOH (P<0.01). As shown in Figure 2D-2E, we detected tissue ROS generation and the oxidative enzyme NOX4. The results were similar to those of the oxidative stress parameters. In brief, CFA injection caused increased levels of NOX4 and ROS generation. Both EA and antioxidant treatment significantly reduced NOX4 expression and ROS generation in the spinal cord of CFA rats (P<0.05). However, local injection of t-BOOH into CFA rats not only increased ROS production and NOX4 expression in CFA rats but also alleviated the inhibitory effects of EA on ROS and NOX4 (P<0.01).

ROS mediate the anti-inflammatory effect of EA in CFA-treated rats

To evaluate the effect of ROS on the EA-mediated inflammatory response in CFA-treated rats, we detected the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 in the spinal cord using ELISA kits. The results shown in Figure 3 indicate that CFA injection increased TNF-α, IL-1β and IL-6 expression in CFA rats (P<0.01). However, EA stimulation significantly downregulated the expression of TNF-α, IL-1β, and IL-6 compared with that in the CFA group (P<0.01). Moreover, we found that CFA rats given t-BOOH showed increased levels of TNF-α, IL-1β and IL-6, while the rats that received antioxidants exhibited decreased levels of TNF-α, IL-1β and IL-6 (P<0.05). In addition, the anti-inflammatory effect of EA was inhibited in rats that received t-BOOH (P<0.01).

ROS are involved in EA-mediated pain-related substances in CFA-treated rats

p38, ERK and p65 are well-known downstream functional molecules of the ROS-mediated pathway [17], and it has been shown that p-p38, p-ERK and p-p65 activate chronic inflammatory response-related gene transcription. ROS have been shown to promote the expression of inflammatory pain-related proteins, such as SP, TRPV1 and TRPV4 [18-19]. As shown in Figure 4A-4B, CFA injection increased the expression of p-p38, p-ERK, and p-p65 and upregulated the expression of SP, TRPV1 and TRPV4 in CFA-treated rats (P<0.01). EA or antioxidant treatment significantly decreased the expression of p-p38, p-ERK, and p-p65 and simultaneously reduced the levels of SP, TRPV1 and TRPV4 (P<0.05). In contrast, treatment with t-BOOH increased the levels of p-p38, p-ERK, p-p65, SP and TRPV1 in both CFA and EA-treated CFA rats (P<0.01).

ROS mediate proinflammatory cytokine- and pain-related molecule levels in C6 cells

Spinal microglia dysfunction in microglia has persisted in the spotlight as a major pathomechanism of male chronic pain [12-13]. Therefore, to investigate the direct role of ROS in CIP, we evaluated the effect of t-BOOH on proinflammatory cytokine expression, pain-related substances and receptors in C6 glioma cells. As shown in Figure 5A, incubation of C6 glioma cells with t-BOOH for 24 h increased ROS production. However, stimulation of C6 by PBN inhibited t-BOOH-induced ROS generation. As shown in Figure 5B-5E, t-BOOH incubation caused an increase in proinflammatory cytokine (TNF-α, IL-1β, IL-6) production and upregulation of p-p38, p-ERK, p-p65, SP, TRPV1 and TRPV4 in the C6 glioma cells (P<0.01), and all of these biological effects were inhibited by treatment with PBN, suggesting a direct role of ROS in CIP.

Discussion

As a complementary and alternative medicine for the treatment of chronic pain, EA has been accepted in Asian countries for many years. A number of studies have proven its good analgesic effect [6-7]. In the present study, EA stimulation improved the manifestation of mechanical and thermal hyperalgesia in CFA rats, which is consistent with our previous report [14]. During repeated daily stimulation, we observed a significant difference in the PWTs between the CFA+EA group and the CFA group, beginning on Day 3 and lasting until the end of the study. However, interestingly, there was no significant improvement in the inflammatory response on Day 3, as we did not observe any relief of paw swelling in the rats (data not shown). Therefore, the appearance of an anti-inflammatory effect of EA may occur later than its analgesic effect. On the other hand, it could be presumed that EA induced an analgesic effect in an anti-inflammatory independent manner. In a previous study, the analgesic mechanism of EA was well documented to be based on its anti-inflammatory effect. Previous investigations reported that EA reduces CIP by inhibiting the activation of the NLRP3 inflammasome [20] or by suppressing P2X3 expression [21]. Moreover, we have previously confirmed that EA therapy alleviates CIP by regulating anti-inflammatory mediators and mediating SP expression [12]. However, an analgesic mechanism of EA unrelated to anti-inflammation remains unclear.

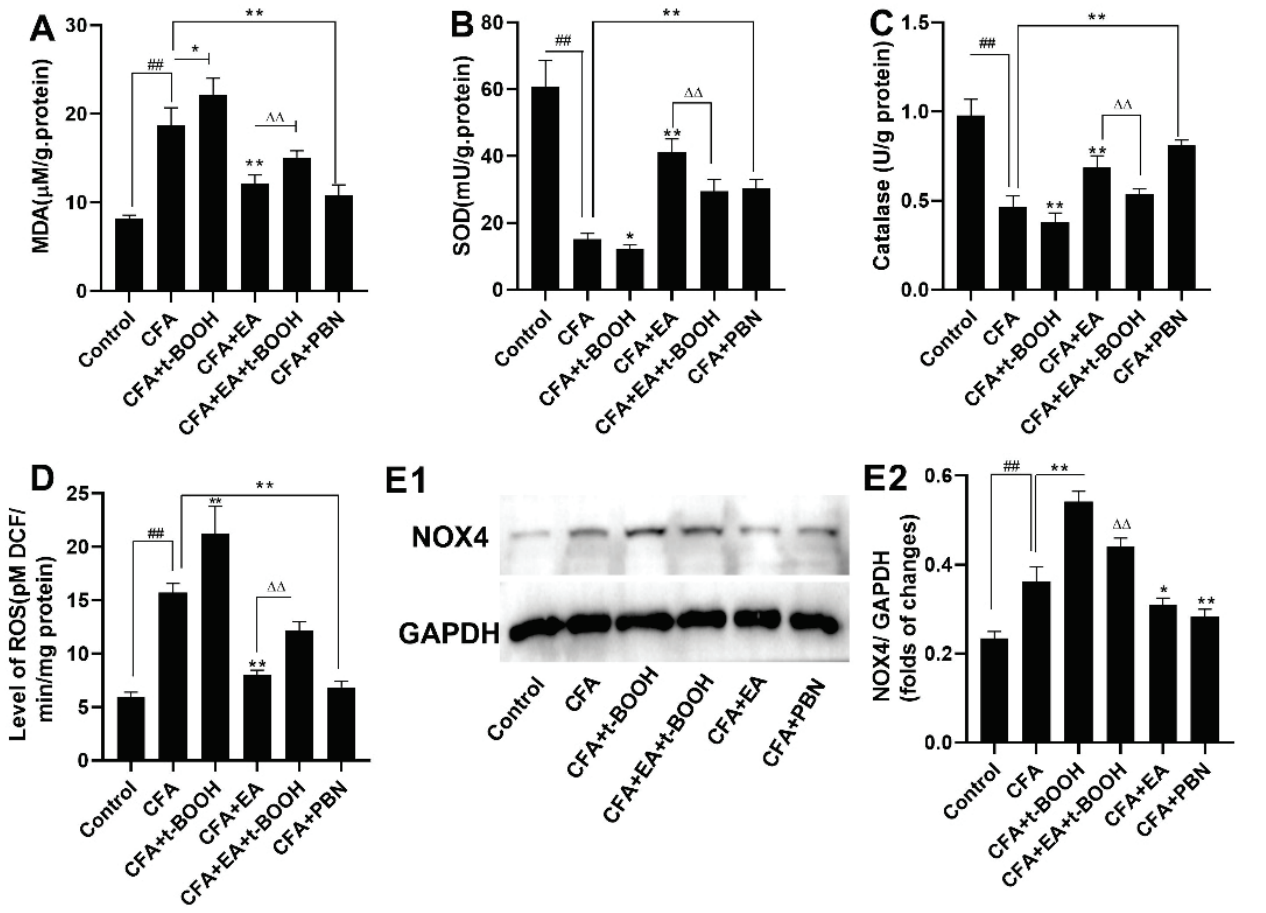

Fig. 2. EA treatment inhibits oxidative stress in CFA rats. MDA, SOD and Cat were measured by commercial kits. ROS in tissues was measured by DCFH-DA staining, and NOX4 levels were measured by western blotting. (**A**) MDA; (**B**) SOD; (**C**) Cat; (**D**) ROS; (**E**) NOX4; EA: electroacupuncture; CFA: complete Freund's adjuvant; t-BOOH: tert-butyl hydroperoxide; PBN: phenyl-N-tert-butylnitrone; the data are represented as the mean ± standard deviation; n=6 per group; $^{\#\#}$ P<0.01 vs. the control group; * P<0.05; ** P<0.01 vs. the CFA group; $^{\Delta\Delta}$ P<0.01 vs. the CFA+EA group.

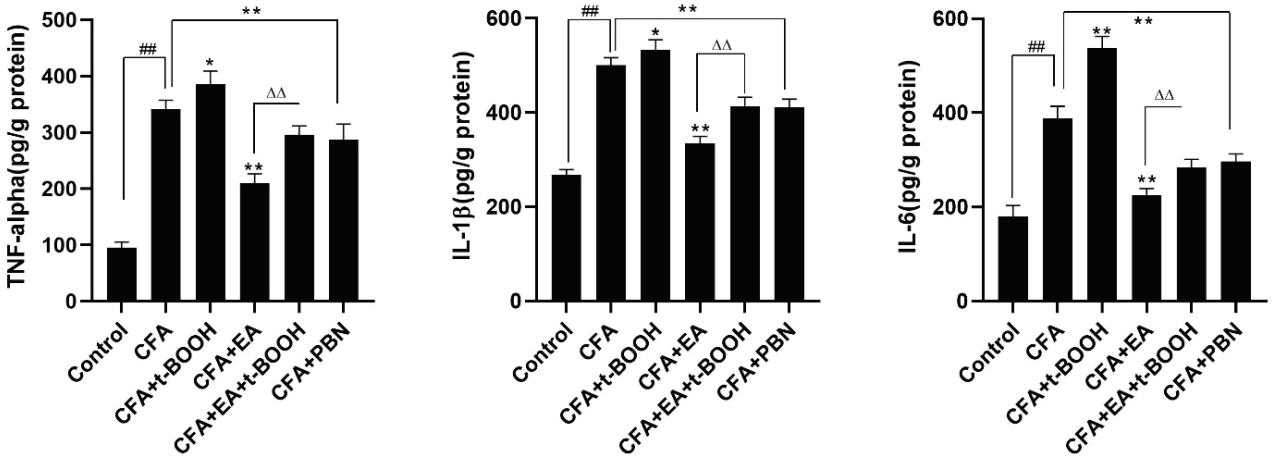

Fig. 3. ROS regulate the anti-inflammatory effects of EA treatment in CFA rats. The cytokines TNF-α, IL-1β and IL-6 in the spinal cord were detected by ELISA kits. EA: electroacupuncture; CFA: complete Freund's adjuvant; t-BOOH: tert-butyl hydroperoxide; PBN: phenyl-N-tert-butylnitrone; data are represented as the mean ± standard deviation; n=6 per group; $^{\#\#}$ P<0.01 vs. the control group; * P<0.05; ** P<0.01 vs. the CFA group; $^{\Delta\Delta}$ P<0.01 vs. the CFA+EA group.

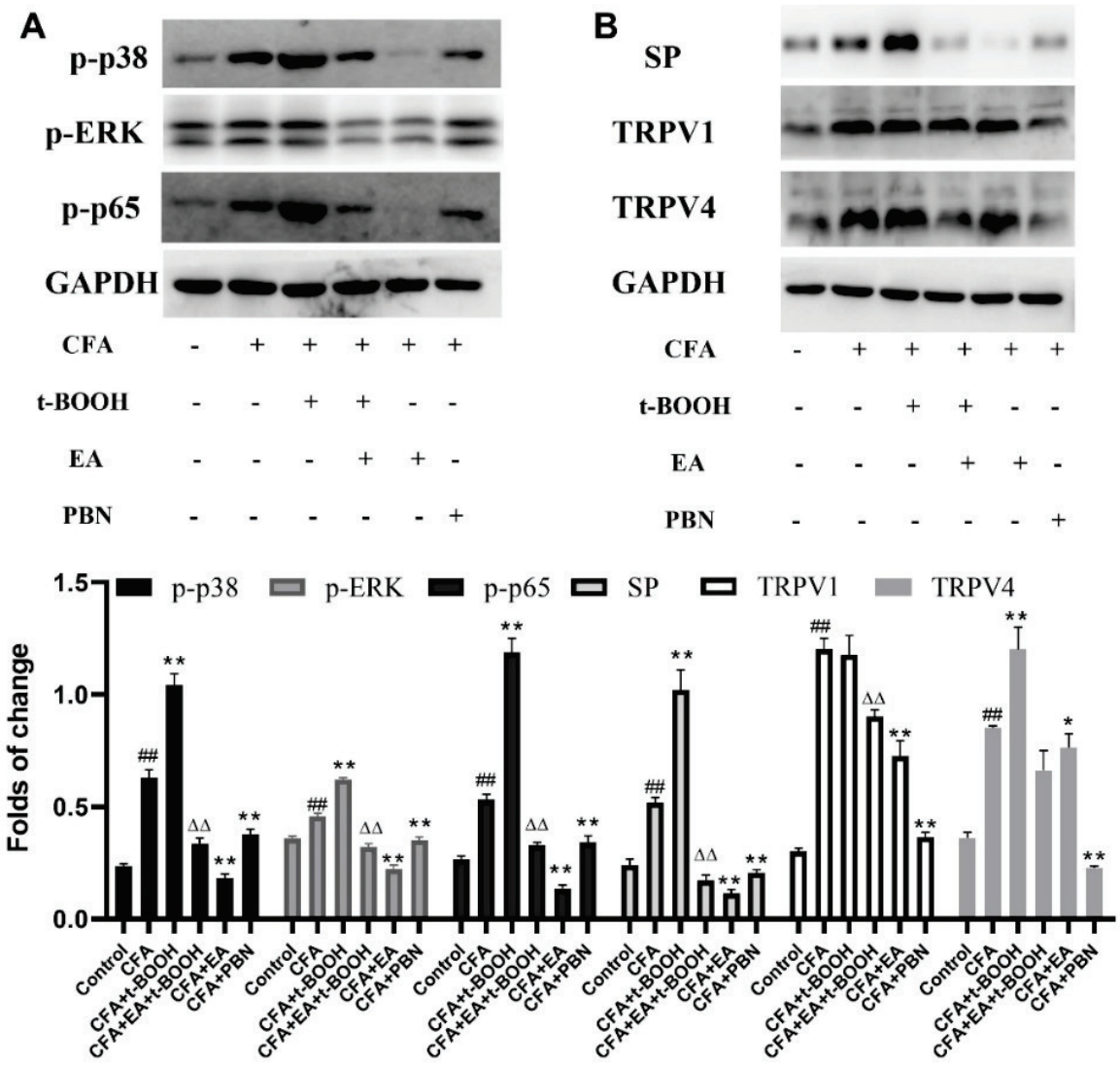

Fig. 4. ROS are involved in EA-mediated pain-related substances in CFA-treated rats. Inflammatory pain-related substances in the spinal cord (L4-L6) of hyperalgesic rats were evaluated and analysed by western blotting. (**A**): Downstream molecules of ROS; (**B**): Inflammatory pain-related substances; EA: electroacupuncture; CFA: complete Freund's adjuvant; t-BOOH: tert-butyl hydroperoxide; PBN: phenyl-N-tert-butylnitrone (ROS scavenger); data are represented as the mean ± standard deviation; n=6 per group; $^{\#\#}$ P<0.01 vs. the control group; * P<0.05; ** P<0.01 vs. the CFA group; $^{\Delta\Delta}$ P<0.01 vs. the CFA+EA group.

The positive role of oxidative stress-generated ROS in the progression of CIP has been well demonstrated recently. Elevated ROS, especially OH, in spinal cord-sensitized dorsal horn neurons produces hyperalgesia in normal rats, and systemic injection of the ROS scavenger PBN relieves mechanical allodynia in a dose-dependent manner [22-23]. Moreover, ROS and lipid peroxidation inhibitors reduce mechanical sensitivity in a rat model of chronic neuropathic pain [24]. Nevertheless, it is still unclear whether oxidative stress/ROS are an important target of EA. In the present study, local injection of the ROS donor t-BOOH exacerbated CIP, and antioxidant therapy by treatment with PBN effectively inhibited pain manifestation in CFA rats, although they both showed limited effects. These results are similar to the data reported previously, indicating that, similar to chronic inflammation, oxidative stress is an important factor involved in the development of CIP. In the current report, EA significantly reduced MDA and ROS but increased SOD and Cat levels in CFA rats, suggesting a regulatory effect on oxidative stress. In particular, injection of t-BOOH weakened the analgesic effect of EA, while co-treatment of PBN and EA showed synergistically analgesic effect. Thus, we concluded that EA improves CIP performance by inhibiting oxidative stress, although we do not know whether the effect occurs in a direct or indirect manner. To the best of our knowledge, the present study is the first to confirm the ROS-mediated analgesic effects of EA treatment.

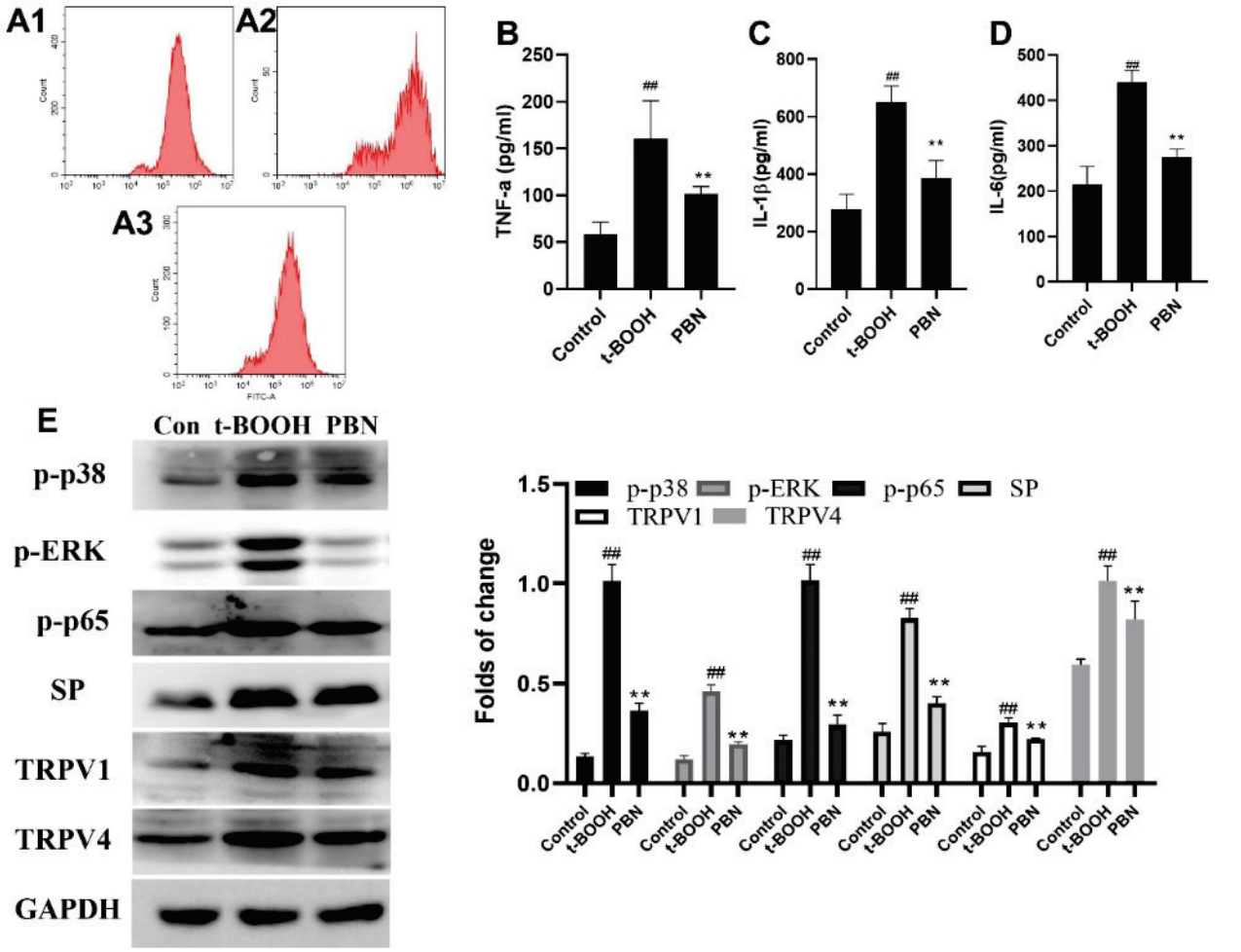

Fig. 5. ROS induced proinflammatory cytokine and CIP-related substance production in C6 glioma cells. C6 cells were treated with t-BOOH (50 μM) and PBN (100 μM) for 24 h. (**A**): ROS were assayed by a DCFHA probe (A1: CON; C2: t-BOOH; C3: PBN); (**B-D**): cytokines were measured by ELISA kits; (**E**): expression of p-p38, p-ERK, p-p65, SP, TRPV1 and TRPV4 was determined by western blotting. GAPDH was used as a loading control. t-BOOH: tert-butyl hydroperoxide; PBN: phenyl-N-tert-butylnitrone (ROS scavenger); data are represented as the mean ± standard deviation; n=4 per group; $^{\#\#}$ P<0.01 vs. the control group; ** P<0.01 vs. the t-BOOH group.

Although the precise mechanism of cytokine-based signaling relevant to the development of CIP is yet to be fully defined, the experimental evidence indicates that inflammatory cytokines mediate CIP responses. Intramuscular injection of TNF-α induces muscle hyperalgesia in rats [25]. Neutralizing antibodies against the IL-1 receptor reduce pain-associated behavior in mice with experimental neuropathy [26]. As microglia play key role in the regulating pain behavior, therefore, we selected C6 cells to investigate proinflammatory cytokine and CIP-related substance production in response to ROS [12]. In the present study, we found that EA stimulation significantly reduced the production of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 both *in vivo* and *in vitro*, and these data are consistent with our previous report. Moreover, although injection of t-BOOH weakened the regulatory effect of EA on the TNF-α, IL-1β, and IL-6 levels, the effect was limited and not fully abolished. Therefore, it is speculated that EA could indirectly affect CIP-related inflammatory mediators by targeting ROS and that EA exerts a direct regulatory effect on TNF-α, IL-1β, and IL-6.

We further tested downstream molecules (kinases or transcription factors) of ROS [27-28], mitogen-activated protein kinases (MAPKs) and NF-κB, all of which exhibit a close relationship with the development of CIP. MAPKs play a critical role in regulating inflammatory gene expression and include three major members: ERK, JNK and p38 MAPK [29-30]. It has been reported that the expression of p-p38 in the dorsal root ganglion (DRG) is enhanced and that p38 contributes to inflammatory neuropathic pain by increasing the Nav1.8 current density in DRG neurons [31]. In addition, it has been shown that upregulation of p-ERK in DRG neurons contributes to

chronic neuropathic pain [32]. Moreover, inflammation is associated with activation of the NF-κB signaling pathway. Activation of the NF-κB subunit p65 enhances gene translocation that drives proinflammatory cytokine (TNF-α, IL-6, etc.) expression [33]. In the present investigation, as we expected, direct stimulation with t-BOOH activated p38, ERK, and p65 both *in vivo* and *in vitro*, and these changes could be inhibited by EA treatment. Our results clearly show that the EA-induced inhibitory effect on oxidative stress and ROS generation contributes to its mediation of the inflammatory response and downregulation of pain-related functional molecules.

Further analysis was performed on downstream channels of ROS. TRP channels are polymodal signal detectors that respond to different physical and chemical stimuli and are involved in the perception of temperature, osmolarity, pressure, pH, and chemicals. The TRP channel superfamily contains numerous members, and the role of TRPV1 and TRPV4 in CIP have been extensively studied.

In previous reports, the CIP model amplifies TRPV1 receptor responses in the adult rat spinal dorsal horn, and transgenic mice injected with short interfering RNAs that silence TRPV1 expression show impaired pain behavior and sensitivity [34-35]. Moreover, genetic deletion of TRPV4 reduced the spiking frequency in response to von Frey filaments, and TRPV4 knockout mice showed smaller electromyographic responses to colonic distension [36]. Interestingly, TRPV channels are regulated not only by inflammatory mediators but also by oxidative stress. It has been recently reported that oxidation sensitizes TRPV2 to chemical and heat stimuli [37]. EA stimulation suppresses the enhanced TRPV1 channel activity in DRG neurons of paclitaxel-treated rats, and pharmacological blockade of TRPV1 mimics the analgesic effects of EA on pain hypersensitivities [38]. In agreement with these observations, we found similar results in the CIP model of rats. We also noticed that t-BOOH stimulation not only increased the expression of TRPV1 and TRPV4 in CIP rats but also impaired the inhibitory effect of EA treatment on TRPV1 and TRPV4.

In summary, we found that EA improved chronic pain performance in rats by reducing inflammatory cytokine secretion, inhibiting the activation of inflammation-related kinases and transcription factors, and blocking the activation of channels, effects all based on the regulation of oxidative stress. Our data suggest that inhibition of oxidative stress and ROS generation is necessary for the clinical benefit of EA. ROS have not been previously implicated in the antinociceptive actions of EA. Therefore, our present result further broadens our understanding of the analgesic mechanism of EA.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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