Electroacupuncture Attenuates Inflammatory Pain by Activating AMPK/autophagy and Inhibiting iNOS and IL-1 β in Inflamed Tissues

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Research

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Abstract

Background

Electroacupuncture (EA) produces analgesic effects on inflammatory pain partially via activating adenosine monophosphate (AMP)-activated protein kinase (AMPK) pathway in the spinal cord. However, it is unclear whether EA activates AMPK in peripheral tissues in inflammatory pain. This study was aimed at determining whether EA promotes autophagy by activating AMPK to inhibit the expression of inflammatory mediators IL-1 β and iNOS in inflamed skin tissues.

Methods

In CFA-induced inflammatory pain in mice, mechanical allodynia and thermal hyperalgesia were tested 2 hours after EA treatment. The AMPK antagonist Compound C was injected intraperitoneally 30 minutes before EA treatment. The analgesic effects of AMPK activator 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) were determined and its effects on autophagy, IL-1 β and iNOS expression were detected. Also, the effects of the autophagy inhibitor 3-methyladenine (3-MA) on EA analgesia and iNOS/IL-1 β expression in inflamed skin tissues were examined. The phosphorylation of AMPK (Thr172) and total AMPK proteins, LC3BII/I, autophagy substrate protein p62, IL-1 β and iNOS were detected using Western blotting. Co-labeling of macrophages (CD68) with IL-1 β and iNOS was detected using immunofluorescence. In addition, after NR8383 macrophages were treated with CFA, the effects of AICAR and Compound C on autophagy were determined using stubRFP-sensGFP-LC3 Lentivirus..

Results

EA reduced CFA-induced inflammatory pain, activated AMPK and autophagy, and inhibited iNOS and IL-1 β expression in inflamed skin tissues. AICAR also attenuated CFA-induced hyperalgesia, promoted autophagy and inhibited iNOS and IL-1 β expression in vivo and in vitro. In addition, the AMPK inhibitor Compound C reversed the effect of EA on autophagy. Pretreatment with 3-MA, an inhibitor of autophagy, inhibited the effect of EA on inflammatory pain and expression of iNOS and IL-1 β in inflamed skin tissues.

Conclusions

EA treatment alleviated inflammatory pain by activation of AMPK, enhancing autophagy, and inhibiting iNOS and IL-1 β expression in the inflamed skin tissues.

Background

Inflammatory pain is an important clinical problem. Proinflammatory mediators released locally, such as prostaglandins, cytokines, chemokines, proteases, neuropeptides and growth factors result in nociceptor sensitization[1, 2]. Acupuncture can effectively alleviate arthritis pain, low back pain, and labor pain[3-5].

However, the mechanism of electroacupuncture (EA) analgesia needs further studies to better understand the effectiveness of EA analgesia and promote its clinical application.

AMPK (adenosine monophosphate-activated protein kinase) is an important analgesic target. Ozone can activate AMPK to relieve nerve injury-induced neuropathic pain in rats[6]. In a postoperative pain model, local administration of AMPK agonists can significantly alleviate pain[7]. Furthermore, EA may activate APN/AdipoR2-AMPK pathway in the spinal cord to alleviate inflammatory pain[8]. However, it remains unclear whether EA activates periphery AMPK in inflammatory skin tissues to play an analgesic effect.

AMPK activation can also enhance autophagy, which is closely related to analgesia[9, 10]. Previous study has uncovered that EA activates AMPK to promote autophagy, thus playing a protective role on myocardial infarction, and Compound C, an inhibitor of AMPK, reverses the effects of EA[11]. In the intracerebral hemorrhage rat model, acupuncture can improve neurological deficits by activating autophagy of perihemorrhagic penumbra[12]. Therefore, we hypothesized that EA may activate AMPK and promote autophagy associated with inflammatory pain.

iNOS (inducible nitric oxide synthase) in local inflammatory tissues and its production of a large amount of nitric oxide induce pain[13]. Inhibition of iNOS and nitric oxide production in activated macrophages alleviates inflammatory pain[14]. In the rat model of temporomandibular arthritis, EA inhibits inflammatory mediators including iNOS[15]. Moreover, inflammatory cytokines, such as IL-1 β , released by activated macrophages in peripheral inflammation play an important role in inflammatory pain[16]. In CFA-induced inflammatory pain, EA inhibits the release of IL-1 β and TNF- α [17]. Thus, EA may alleviate inflammatory pain by enhancing autophagy and then inhibiting the expression of iNOS and IL-1 β in the inflammatory skin tissues.

Therefore, in the present study, we determined whether EA alleviates inflammatory pain and promotes autophagy by activating AMPK, which in turn inhibits the expression of IL-1 β and iNOS in inflammatory skin tissues.

Materials And Methods

1. CFA-induced inflammatory pain in mice

All experiments were approved by the ethics committee of Tongji Medical College, Huazhong University of science and technology. The surgical procedures of animal models were carried out in strict accordance with the ethical guidelines of the International Association for the study of pain. Male C57BL/6 mice (8-9 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. Mice were housed 4-6 per cage at 22-24 ° C, on a 12-h light/dark cycle with food and water *ad libitum*. As reported previously[18], 25 μ l of CFA (Sigma, F5881-10ml) was subcutaneously injected into the left posterior plantar side to induce inflammatory pain. The control group was injected with 25 μ l of normal saline (N.S.).

2. Electroacupuncture treatment

Han's acupoint stimulation electroacupuncture apparatus (LH202, China) was used for the treatment of inflammatory pain in mice. As reported previously, we selected the acupoints of Zusanli (ST36) and Shangjuxu (ST37)[19]. On the second day after the CFA injection, we started EA treatment. The control and CFA-injected mice were restrained with self-made clothes. Two filiform needles were inserted into the two acupoints on the left side of the mouse for 2-3 mm. The EA intensity was 1 mA, and the frequency was 2 Hz for 30 min. The treatment was administered once a day for 6 days. The sham EA group was inserted with the filiform needle at the same acupoint without stimulation for 30 min.

3. Nociceptive behaviors

All mice were conditioned in the test environment for 30 min before the behavior test. The baseline withdrawal threshold was measured for three consecutive days before CFA injection, and the threshold on the third day was taken as the baseline. EA was performed on the second day after CFA injection, and the nociceptive behavior was detected 2 hours after EA.

The mechanical withdrawal threshold of mice was measured using von Frey filaments[20]. After a 30-min adaptation period, we used von Frey filaments (Wood Dale, Stoelting, Italy) to vertically stimulate the plantar surface of the left hind paw for 5 s. The mechanical pain withdrawal threshold test was repeated twice in each mouse, and the average value was obtained finally.

The thermal pain threshold was measured using a hot plate method. The surface temperature of the hot plate was maintained at 53 ° C. We recorded the withdrawal latency when the hind paw of mice swung quickly after placing the mice on the hot plate. We set 20 seconds as the cutoff time to prevent skin damage[21]. The hot plate experiment was repeated every 5 minutes three times and the average value was counted.

4. Western blotting

Mice were deeply anesthetized with 10% chloral hydrate. The inflamed skin tissues were removed quickly and put into the Eppendorf tube on the ice. Then, the skin tissues were minced with scissors, and NR8383 cells of macrophage cell line after treatment were collected by centrifuging at 1000 *g* for 5 min at room temperature. The skin tissues or NR8383 cells were then lysed by RIPA Lysis Buffer, containing protease inhibitor (100 μ l RIPA with 1 μ l PMSF or 1 μ l phosphatase inhibitor Cocktail). The homogenate was then transferred to an Eppendorf tube and centrifuged at 4°C for 15 minutes. After centrifugation, the supernatant was transferred to a new EP tube and placed on ice. The concentration was measured with BCA kit (Beyotime, #P0012). We took the same quality protein from each sample and added SDS-PAGE loading buffer to denature at 98°C. Then, we used SDS-PAGE gel to separate proteins using electrophoresis. The protein was transferred to a PVDF membrane (MERCK MILLIPORE, Germany), blocked with 5% skimmed milk or 5% BSA prepared with 0.01 M TBST solution (for phosphorylated protein) for 1 hour. The PVDF membrane was incubated with primary and secondary antibodies. The

primary antibodies used include phosphorylation of AMPK (*p*-AMPK) (Thr172) (1:1000; Cell Signaling Technology, #2535), AMPK (1:1000; Cell Signaling Technology, #2532), iNOS (1:1000; Abcam, #178945), IL-1 β (1:1000; R&D Systems, #MAB5011), LC3B (1:1000; Cell Signaling Technology), autophagy substrate protein p62 (1:1000; Cell Signaling Technology), and β -actin (1:10,000; Santa Cruz Biotechnology, sc-47778). Secondary antibodies used were anti-rabbit HRP and anti-mouse HRP (1:20000). We used Image J software (NIH, USA) for blot analysis.

5. Immunofluorescence

Mice were deeply anesthetized with 10% chloral hydrate and were perfused with 100 ml normal saline preheated at 37°C and then infused with 100 ml of 4% paraformaldehyde dissolved in 0.1 M PBS at 4 °C. The skin tissues were taken out and put into 4% paraformaldehyde, fixed for 8 hours, and washed with 0.1M PBS three times, and then dehydrated with 30% sucrose. The skin tissues were cut into 15 μ m-thick sections on a freezing microtome.

The primary antibody was incubated overnight at 4 °C. Then the corresponding secondary antibody was incubated at room temperature, and DAPI solution was added to the skin tissues. After sealing with cover glass, slides were photographed with a fluorescence microscope (Olympus, BX51, Japan). The primary antibodies used were rabbit anti-*p*-AMPK (1:250; Abcam, #ab23875), rabbit anti iNOS (1:300; Abcam, #178945) , rabbit anti-IL-1 β (1:300; Santa Cruz biotechnology, #sc-7884), and mouse anti-CD68 (1:200; Abcam, #ab955)[22]. Secondary antibodies were purchased from Jackson ImmunoResearch and included donkey anti-rabbit IgG with dylight 594 (1:600) and donkey anti-mouse IgG with Dylight 488 or Dylight 594 (1:600).

6.NR8383 Cell Culture

A rat [macrophage cell line](#) (NR8383) was purchased from Procell (Procell, Wuhan, China) and cultured in DMEM high glucose medium (Gibco, Thermo Fisher Scientific, MA, USA) supplemented with penicillin/streptomycin (100 U/10 mg/mL) (Gibco, Thermo Fisher Scientific, MA, USA) and 20 % (v/v) [fetal calf serum](#) (Biological Industries, Kibbutz Beit, Israel). NR8383 cells were cultured in 50 ml flasks (Corning, New York, USA) under standard cell culture conditions (37 °C, 5% CO₂).

NR8383 cells were treated with DMSO (Vehicle of Compound C, v/v 1%), CFA, or CFA plus AICAR plus vehicle (CFA + AICAR+vehicle), or CFA plus AICAR plus Compound C (CFA + AICAR+ Compound C) overnight. The AMPK [agonist](#) AICAR (0.5 mM, Abcam) was used to activate AMPK 30 min before CFA administration. To inhibit AMPK, cells were pre-treated with Compound C (20 μ M, Abcam) 1 h before AICAR.

7. LC3 dual fluorescence Lentivirus

LC3 dual fluorescence Lentivirus autophagy flow detection system (Cat.GPL2001, Shanghai Genechem Co.,Ltd.) was used to detect autophagy flow changes. The product was Part#GPL2001A, stubRFP-

sensGFP-LC3 Lentivirus, 5×10^7 TU. NR8383 cells transfected with stubRFP-sensGFP-LC3 lentivirus were cultured using 24-well cell culture plates coated with glass slides coating polylysine (sterile water, W/V concentration 0.01%). NR8383 cells were treated with DMSO (Vehicle of Compound C), CFA, or CFA plus AICAR plus vehicle (CFA + AICAR + vehicle), or CFA plus AICAR plus Compound C (CFA + AICAR + Compound C) overnight. The AMPK agonist AICAR (0.5 mM, Abcam) was used to activate AMPK 30 min before CFA administration, and Compound C was used 1 h before AICAR. After treatment, the cells were fixed with 4% paraformaldehyde, stained with DAPI and washed with PBS. The round glasses were placed on glass slides with an anti-fluorescence quenching coating. Finally, fluorescence microscope (Olympus, BX51, Japan) was used for image capturing.

8. Statistical analysis

The results were expressed by mean $\pm$ SEM. Two-way analysis of variance (ANOVA) and Bonferroni post hoc test were used to analyze the thermal and mechanical withdrawal thresholds between different groups. One-way ANOVA and Newman Keuls post hoc test (SPSS, version 11.0) was used to analyze the expression levels of proteins. $P < 0.05$ was considered as statistically significant.

Results

1. EA reduces CFA-induced inflammatory pain and activates AMPK and autophagy in inflamed skin tissues

Compared with the control group, EA treatment significantly reduced mechanical and thermal hyperalgesia in CFA-treated mice on the 2nd - 7th day ($p < 0.05$, Fig. 1A, C). In contrast, sham EA did not affect the mechanical and thermal hyperalgesia in CFA-treated mice ($p > 0.05$, Fig. 1A, C). EA treatment had no significant effect on the withdrawal thresholds on the contralateral plantar in CFA-treated mice ($p > 0.05$, Fig. 1B). These results suggest that EA reduces inflammatory pain induced by CFA in mice.

The level of *p*-AMPK (phosphorylation of Thr172 site) in skin tissues was significantly higher in the CFA group than in the control group ($p < 0.05$). EA treatment, but not sham EA, significantly increased the level of *p*-AMPK in skin tissues of CFA-treated mice (Fig. 1D and F). There was no significant difference in the total protein level of AMPK in skin tissues among groups (Fig. 1D and E). These data suggest that EA activates AMPK in inflamed skin tissues.

The level of autophagy markers LC3II/I in skin tissues was significantly lower in the CFA group than in the control group ($p < 0.05$). EA treatment, but not sham EA, significantly increased the level of LC3II/I in skin tissues from CFA-treated mice (Fig. 1D and G). Also, the level of autophagy substrate protein p62 in skin tissues in the CFA group was significantly higher than that in the control group ($p < 0.05$). EA treatment, but not sham EA, significantly reduced the level of p62 in skin tissues from CFA-treated mice ($p < 0.05$; Fig. 1D and H). These results suggest that EA activates autophagy in inflamed skin tissues.

2. EA inhibits CFA-induced expression of IL-1 β and iNOS in inflamed skin tissues

iNOS and IL-1 β are markers of activated macrophages[23]. The levels of iNOS and IL-1 β in skin tissues were significantly higher in the CFA group than in the control group ($p < 0.05$). EA treatment, but not sham EA, significantly reduced the levels of iNOS and IL-1 β in skin tissues from CFA-treated mice ($p < 0.05$; Fig. 2A, B). Furthermore, immunofluorescence images showed co-labeling of macrophages (CD68) with iNOS and IL-1 β , respectively (Figure 2 D and E). These results suggest that CFA increases the levels of iNOS and IL-1 β in local tissues and that EA inhibits the expression of iNOS and IL-1 β in inflamed skin tissues.

3. AMPK activator alleviates inflammatory pain, and promotes autophagy, and inhibits iNOS and IL-1 β in inflamed skin tissues.

A single subcutaneous administration of AICAR (20 μ g), an AMPK activator, significantly reduced mechanical and thermal hyperalgesia. The analgesic effects of AICAR lasted for more than 4 hours on day 7 after CFA injection ($p < 0.05$; Figure 3 A and B).

After AICAR treatment for 2 hours, the skin tissues were obtained for immunoblotting and immunofluorescence. The level of LC3II/I in inflamed skin tissues was significantly lower in the CFA group than in the control group ($p < 0.05$). AICAR significantly increased the level of LC3II/I in inflamed skin tissues from CFA-treated mice ($p < 0.05$; Figure3 C and E). Furthermore, the level of p62, iNOS and IL-1 β in inflamed skin tissues were significantly higher in the CFA group than in the control group ($p < 0.05$). AICAR significantly reduced the level of p62, iNOS and IL-1 β in inflamed skin tissues from CFA-treated mice ($p < 0.05$) (Figure3 C, F-H). Electron microscopy images show that autophagosomes in macrophages of inflammatory skin tissues from the CFA group and CFA plus AICAR group(Figure3 D). These data suggest that AMPK activation produces an analgesic effect, activates autophagy, and inhibits the level of iNOS and IL-1 β levels in inflamed skin tissues.

4. Compound C reverses the analgesic effect of EA, and the effects of EA on AMPK activation, autophagy enhancement, and inhibition of iNOS and IL-1 β expression in inflamed skin tissues

In CFA-induced inflammatory pain, the mice were treated with EA every day from the 2nd day to the 7th day. Mice were injected(i.p.) with an AMPK inhibitor, Compound C, 30 min before EA, and the mechanical and thermal hyperalgesia were tested 2 hours after EA treatment. EA treatment significantly reduced mechanical and thermal hyperalgesia in CFA-injected mice ($p < 0.05$, Fig. 4A and B). However, co-treatment with Compound C blocked the EA effect on mechanical and thermal hyperalgesia in CFA-treated mice ($p > 0.05$, Fig. 4A and B).

The level of p-AMPK in inflamed skin tissues was significantly higher in the CFA group than in the control group ($p < 0.05$). The level of p-AMPK in inflamed skin tissues in CFA plus Compound C plus EA group was significantly lower than that in CFA plus vehicle plus EA group (Fig. 4C and E). There was no significant difference in the total protein level of AMPK in inflamed skin tissues among groups ($p > 0.05$, Fig. 4C and D). These results suggest that the analgesic effect of EA is mediated by activating AMPK.

The level of LC3II/I in inflamed skin tissues was significantly lower in the CFA group than in the control group ($p < 0.05$). EA treatment significantly increased the level of LC3II/I in inflamed skin tissues from CFA-injected mice ($p < 0.05$). The level of LC3II/I in inflamed skin tissues in CFA plus Compound C plus EA group was significantly lower than that in CFA plus vehicle plus EA group (Fig. 4C and E). Also, the levels of p62, iNOS and IL-1 β in inflamed skin tissues in the CFA group were significantly higher than those in the control group ($p < 0.05$). EA treatment significantly reduced the level of p62, iNOS and IL-1 β in inflamed skin tissues from CFA-injected mice ($p < 0.05$). The level of p62, iNOS and IL-1 β in inflamed skin tissues in CFA plus Compound C plus EA group was significantly higher than that in CFA plus vehicle plus EA group (Fig. 4C and E). These results suggest that EA activates AMPK to promote autophagy and inhibit iNOS and IL-1 β in inflammatory skin tissues.

5. AMPK activation promotes autophagy, and inhibits the expression of iNOS and IL-1 β in the NR8383 macrophage cell line.

IL-1 β and iNOS are mainly released by activated macrophages and are the markers of inflammatory macrophages[24]. NR8383 cells, a rat alveolar macrophage cell line, were used to determine whether AMPK activation regulates autophagy and expression of iNOS and IL-1 β . Treatment with CFA overnight significantly decreased the level of LC3II/I and the increased p62, iNOS and IL-1 β in NR8383 cells (Fig 5 A-E, $p < 0.05$). NR8383 cells were preincubated with a specific AMPK agonist, AICAR, with or without the specific AMPK inhibitor Compound C. CFA-induced changes in the levels of LC3II/I and the activated form of p62, iNOS and IL-1 β were all blocked by AICAR pretreatment. Also, the effect of AICAR was abolished by Compound C cotreatment (Fig. 5A-E, $p < 0.05$). To further confirm the autophagy induced by AICAR, NR8383 cells were transfected with RFP-GFP-LC3 Lentivirus to detect autophagy flux. As shown in Fig 5F, the number of yellow (overlay of RFP and GFP) and red (RFP) puncta both increased in the merged images after CFA treatment, and AICAR decreased the number of green and yellow puncta. Compound C blocked the effects of AICAR (Fig. 5 F). Thus, AMPK activation plays an anti-inflammatory role by promoting autophagy.

6. Pretreatment with 3-MA, an inhibitor of autophagy, inhibits the effects of EA on inflammatory pain and expression of iNOS and IL-1 β

EA treatment markedly reduced mechanical and thermal hyperalgesia induced by CFA injection ($p < 0.05$, Fig. 4A and B). However, pretreatment with 3-MA (15mg/kg, i.p. injection) reversed the EA effect on hyperalgesia in CFA-injected mice ($p > 0.05$, Fig. 6A and B). The results suggest that the analgesic effect of EA is mediated by autophagy.

The level of iNOS and IL-1 β in inflamed skin tissues was significantly higher in the CFA group than in the control group ($p < 0.05$). EA significantly reduced the levels of iNOS and IL-1 β in inflamed skin tissues from CFA-injected mice ($p < 0.05$). The levels of iNOS and IL-1 β in inflamed skin tissues in the CFA plus 3-MA plus EA (CFA+3-MA+EA) group were significantly higher than that in CFA plus vehicle plus EA group (Fig. 4C and E). These data suggest that in inflammatory skin tissues, autophagy mediates the anti-inflammatory effect of EA.

Discussion

Many studies have shown that AMPK activation reduces inflammatory pain[25, 26]. Further research found that EA also plays analgesic effects on inflammatory pain partially via activating the AMPK pathway in the spinal cord[8]. In the present study, we provide a new information that EA treatment reduces inflammatory pain by activation of AMPK, enhancing autophagy and inhibiting the expression of iNOS and IL-1 β in inflamed skin tissues.

AMPK is an energy sensor regulating energy homeostasis and metabolic stress[27]. AMPK is sensitive to cellular ATP depletion and is activated by upstream kinases such as LKB1 (liver kinase B1) and Ca²⁺/calmodulin-dependent protein kinase β in mammalian cells[28]. In addition, the activation of AMPK in macrophages reduces the production of TGF- β 1 (Tumor growth factor β 1), thus reducing the chronic inflammation and fibrosis in Duchenne muscular dystrophy[29]. Activating AMPK in dorsal root ganglion (DRG) neurons inhibits the activity of TRPA1 (Transient receptor potential ankyrin 1) and participates in analgesia of AICAR in diabetic neuropathic pain[30]. Activation of AMPK inhibits AMPA receptors in the spinal cord and produces an analgesic effect in the neuropathic pain model[31]. In our study, EA significantly activated the AMPK and relieved inflammatory pain, which was reversed by the AMPK inhibitor Compound C. The key role of AMPK in analgesia effect of EA is consistent with previous studies[8, 32].

Autophagy is a conserved process in which substrates in the cytoplasm are transported to lysosomes via double-membrane-bound vesicles (autophagosomes). Substrates for autophagy include a large amount of cytoplasm, protein aggregates, macromolecular complexes and organelles[33]. Autophagy plays an important role in regulating inflammation[34]. Koumine inhibits astrocyte activation and pro-inflammatory cytokines releasing by promoting autophagy, thus alleviating neuropathic pain[35]. Also, P2X7R antagonist Brilliant Blue G promotes autophagy to alleviate microglia activation of trigeminal nucleus caudalis and relieves chronic migraine[36]. We showed for the first time that autophagy was reduced in inflamed skin tissues, which was promoted by EA. In addition, blocking AMPK with Compound C reversed the effects of EA on autophagy activation. EA-activated AMPK probably promotes autophagy, which is involved in EA analgesic effect on inflammatory pain.

Down-regulation of inflammatory mediators is an important mechanism of EA analgesia[37-39]. iNOS is a zinc-bridged homodimer quadruple structure, which enables inducible nitric oxide synthase to convert L-Arg (arginine) to L-citrulline and produces nitric oxide[40]. Macrophages stimulated by inflammation produce a large amount of iNOS, which is a marker of inflammatory macrophages[41]. In the early phase of acute inflammatory pain following oral surgery, the high expression of iNOS increased the production of nitric oxide and pain[42]. Our data showed that iNOS was significantly increased in inflammatory skin tissues, which was inhibited by EA. Pretreatment with an autophagy inhibitor, 3-MA, inhibited the analgesic effect of EA and the down-regulation of iNOS by EA. Activation of autophagy reverses LPS-induced iNOS and IL-6 expression in BV2 microglia[43]. Therefore, our results suggest that EA inhibits the iNOS expression in inflamed tissues by activating autophagy.

IL-1 β produced can act on the IL-1RI (IL-1 receptor type I) of dorsal root ganglion neurons in inflammatory pain of a knee joint[44]. Autophagy specifically targets pro-IL-1 β for lysosomal degradation, thereby indirectly reducing the production of mature IL-1 β [45]. In the arthritis model, low-intensity pulsed ultrasound upregulated the autophagy level of macrophages, inhibited the production of mature IL-1 β through autophagic degradation, and improved the pain gait patterns[46]. In our study, EA inhibited the expression of mature IL-1 β (p17) in inflammatory skin tissues, which was reversed by pretreatment with the autophagy inhibitor 3-MA. This finding suggests that EA may reduce inflammatory pain by promoting autophagy to decrease IL-1 β in inflamed skin tissues.

AMPK is an upstream signaling of autophagy, which may explain why pretreatment with Compound C, an inhibitor of AMPK, inhibited the effect of EA on the expression of iNOS and IL-1 β expression as well as autophagy. Meanwhile, AICAR, an AMPK agonist, attenuated CFA-induced hyperalgesia, promoted autophagy, and inhibited iNOS and IL-1 β expression *in vivo* and *in vitro*.

Conclusions

In summary, our study provides new information that EA can inhibit the expression of iNOS and IL-1 β in macrophages by activating AMPK and subsequently promoting autophagy in inflamed skin tissues. This peripheral action of EA extends our understanding of the mechanism of EA analgesia and supports the clinical application of EA for treating inflammatory pain.

Abbreviations

AMPK Adenosine monophosphate (AMP)-activated protein kinase; EA Electroacupuncture; STAT3 Signal Transducers and Activators of Transcription 3; iNOS inducible nitric oxide synthetase; IL-1 β Interleukin-1 β ; IL-1ra Interleukin-1 receptor antagonist; IL-1RI Interleukin-1 receptor; NF- κ B nuclear factor kappa B; CFA Complete Freund's Adjuvant; ROS reactive oxygen species; AICAR 5-aminoimidazole-4-carboxamide ribonucleoside; GFP green fluorescent protein; WB Western blot; IF Immunofluorescence.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

ML, HX, WL, CL, GC conceived and designed the experiments. HX performed most of the experiments and drafted the first manuscript. YL helped with the Western blotting experiment. YZ and TW helped with the rat model and behavior test experiments. YL, XH, HZ helped with immunofluorescence experiments. LL helped with the data collection. XY and HP participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed of this study are included in this published article.

Ethics approval and consent to participate

The animal experiments were approved by the ethics committee of Tongji Medical College, Huazhong University of Science and Technology.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

EA reduces CFA-induced inflammatory pain and activates AMPK and autophagy in inflamed skin tissues. A-C, time course of effects of EA on mechanical withdrawal threshold on the ipsilateral (A) and contralateral (B) hindpaw and heat withdrawal latency (C) in mice treated with vehicle or CFA. Data are expressed as mean $\pm$ SEM ($n = 10$ mice per group). * $p < 0.05$, compared with respective baseline (time 0); # $p < 0.05$ compared with the value in the CFA group at the same time points. D-H, Representative gel images (D) and mean changes (E-H) show the effects of EA treatment on protein levels of p-AMPK, AMPK, LC3 and p62 in the skin tissues of mice injected with CFA (D). Summary data show the relative protein level of AMPK, p-AMPK, LC3II/I and p62 in skin tissues of mice ($n = 3$). * $p < 0.05$, compared with the vehicle control group; # $p < 0.05$, compared with the CFA group, & $p < 0.05$, compared with the CFA+EA group.

Figure 2

EA inhibits the expression of iNOS and IL-1 β of macrophages in inflammatory skin tissues. Panel A is representative gel images of iNOS and IL-1 β . (B) Summary data show the relative protein level of iNOS and IL-1 β in skin tissues of mice (n=3). Compared with the control group (Control) * $p < 0.05$, compared with the CFA model group (CFA) # $p < 0.05$, and compared with the CFA + EA group & $p < 0.05$. C and D, CD68(green) and iNOS(red), CD68 and IL-1 β (red) co-labeling represent images.

Figure 3

AICAR attenuates CFA-induced pain behavior, and promotes autophagy and inhibits iNOS and IL-1 β expression. A and B, time course of effects of AICAR (20 μ g/20 μ l, plantar injection) on mechanical withdrawal threshold on the ipsilateral (A) and heat withdrawal latency (B) in mice treated with vehicle or CFA. Data are expressed as mean $\pm$ SEM (n = 7 mice per group). Two-way ANOVA revealed a significant difference at *p < 0.05 vs. Control group and #p < 0.05 vs. CFA+VEH group. C, Representative gel images of LC3II/I, iNOS and IL-1 β in inflammatory skin tissues. D, Electron microscopic images of inflammatory skin tissues in CFA group and CFA plus AICAR group. The images showed macrophages and autophagosomes in macrophages of inflammatory tissues. E-H, Summary data show the relative protein level of LC3II/I, iNOS and IL-1 β in skin tissues of mice. *p < 0.05 vs. Control group, and #p < 0.05 vs. CFA+VEH group. VEH is vehicle, solvent (Normal saline) of AICAR.

Figure 4

Compound C inhibits the effects of EA on pain, AMPK activation, autophagy, iNOS and IL-1 β . Compound C (20mg/kg) was injected intraperitoneally 30 minutes before EA treatment from day 2 to day 7, and pain behavior was measured after EA treatment. A and B, time course of effects of EA and Compound C on mechanical withdrawal threshold (A) and heat withdrawal latency (B) in mice treated with vehicle or Compound C in CFA-induced inflammatory pain mice. Data are expressed as mean $\pm$ SEM (n = 8 mice per group). * p < 0.05, compared with control; # p < 0.05 compared with the value in the CFA group at the same time points. Representative gel images (C) and mean changes (D-I) show the effects of EA and Compound C treatment on protein levels of p-AMPK, AMPK, LC3 and p62 in the skin tissues of mice injected with CFA. Summary data show the relative protein level of AMPK, p-AMPK, LC3II/I, p62, iNOS and IL-1 β in skin tissues of mice (n = 3). *p < 0.05, compared with the vehicle control group; #p < 0.05, compared with the CFA group, &p < 0.05, compared with the CFA+VEH+EA group. CC is the abbreviation of Compound C. VEH is the Vehicle (5%DMSO in normal saline), the solvent of Compound C.

Figure 5

Activation of AMPK promotes autophagy and inhibits iNOS and IL-1 β expression in CFA-induced NR8383 cells. A Representative gel images show the protein level of LC3II/I, p62, iNOS and IL-1 β in NR8383 cells treated with DMSO, CFA(100 μ g/ml), or CFA plus VEH plus AICAR(0.5 mM) (CFA +VEH+ AICAR), or CFA plus Compound C(20 μ M)plus AICAR(CFA + Compound C+AICAR). B-E Summary data show the relative protein level of LC3II/I, p62, iNOS and IL-1 β in NR8383 cells(n=3 /group). * $p < 0.05$, compared with the CFA model group (CFA) # $p < 0.05$. CC is the abbreviation of Compound C, VEH is the Vehicle (1% DMSO), the solvent of Compound C.

Figure 6

Pretreatment with 3-MA inhibits the effect of EA on pain behavior, iNOS and IL-1 β expression. From the 2nd to 7th day, mice were injected intraperitoneally 3-MA(15mg/kg) 30 minutes before EA treatment. After 2 hours of the last EA treatment, the local inflammatory skin tissues were obtained for WB experiments. A and B, time course of effects of EA and 3-MA on mechanical withdrawal threshold (A) and heat withdrawal latency (B) in mice treated with vehicle or 3-MA in CFA-induced inflammatory pain mice. (n = 6 mice per group) . Panel C is the representative gel of iNOS and IL-1 β . Panel D and E are the statistical diagrams of iNOS and IL-1 β (n = 3/group). Compared with the control group (Control) * p< 0.05, compared with the CFA model group (CFA) # p< 0.05, compared with the CFA + VEH + EA group &p< 0.05. VEH is the Vehicle (normal saline), the solvent of 3-MA.