Effect of Electroacupuncture on ERK Activation in Carrageenan-induced Inflammatory Pain Model

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The present study found that EA pre-treatment effectively attenuated both spinal ERK activation and hyperalgesia against carrageenan-induced inflammation, whereas EA co-treatment with carrageenan injection unexpectedly elevated ERK activation in a synergistic manner and virtually had no analgesic effect. Therefore, we have concluded that the molecular mechanism of EA anagesia may be related to the inhibition of spinal ERK activation. Further experiments are required to find the intermediate candidates which transmits the pain-alleviating signals on the way of inhibiting ERK activation by EA.

Key words : electroacupuncture, ERK, carrageenan

Introduction

Various noxious stimuli such as carrageenan, formalin, capsaicin, complete Freund's adjuvant cause tissue inflammation[1-3]. These stimuli cause peripheral pain sensitization at the tissue damage site and are transferred through Aδ or C nerve fibers to spinal cord where central sensitization is formed. In the spinal cord dorsal horn (SCDH) neurons, a series of signal transduction cascades including ionotropic and metabotropic receptors, protein kinases-PKA, PKC or MAP kinase family, and so on transmits noxious signals inside nerve cells[4,5]. Especially, extracellular signal-regulated kinase (ERK) among MAPK kinase family is known to play an important role in pain signaling mechanism[2,6,7].

The phosphorylation of spinal ERK leads to hyperalgesia and eventually correlates with the development of pain hypersensitivity[8-10].

Acupuncture has been used to treat a variety of illness including inflammatory pain in traditional oriental medicine[11] and electroacupuncture (EA) which is one of acupuncture techniques has widely used to relieve acute and chronic inflammatory pain in patients. Studies on EA using experimental pain models also show that EA can effectively attenuate hyperalgesia associated with inflammatory pain[12,13]. Although underlying mechanism of EA analgesia is still not fully understood, it is suggested that the analgesic effect of EA may be connected with various neural mechanisms including PGE2, GABA, opioid analgesia, NMDA-mediated pain signaling, and MAPK signaling[10,13-15].

Increasing evidences show that ERK activation in the SCDH neurons has a pivotal role in leading to central sensitization[2,6-10]. Therefore, in the present study, we investigated whether molecular mechanism of EA analgesia is related to attenuation of spinal ERK activation in the carrageenan-induced inflammatory pain model. In addition, we studied the difference of analgesic effect to carrageenan-induced pain between EA co- and pre-treatment.

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· Received : 2010/08/31 · Revised : 2010/09/13 · Accepted : 2010/09/15

Materials and Methods

1. Animals and carrageenan treatment

Male Sprague-Dawley rats (8 weeks) were purchased from Dooyeol Biolab (Seoul, Korea) and adapted at 12:12h light-dark cycle for 1 week before the study. Food and tap water were provided ad libitum. The experimental procedures were conducted under the Animal Care Guidelines of the Animal Experimental Committee of Pusan National University. 100 ㎕ of 1.5% λ-carrageenan (20049, Fluka, USA) was injected into subcutaneous tissue of the right hind paw to induce inflammatory response.

2. EA stimulation

In all experiment, two pairs of stainless-steel acupuncture needles (Dong-Bang, South Korea) of 0.25 mm diameter were bilaterally inserted into rat's hind legs on the same acupoint as Zusanli (ST36) and Sanyinjiao (SP6) in human. The needles at Zusanli and Sanyinjiao acupoint were connected to the cathode and anode of Electro-pulse generator (A300 Pulsemaster, World precision instruments, USA), respectively. Electrical pulses were given to rats in the following constant conditions: 2 Hz frequency, 499 ms pulse train, 1 ms width, and 500 ms event interval. The pulse intensity was gradually increased from 1 mA through 2 mA to 3 mA at 10 minute interval. All EA manipulation was carried out under 2% isoflurane (Choongwae pharm corporation, South Korea) anesthesia.

3. Western blot analysis

For Western blot analysis, the dorsal part of ipsilateral L4-5 segments of the spinal cords were obtained after each experiment. Tissue samples were homogenized in lysis buffer and centrifuged. Supernatants were collected and the amount of total protein was measured by protein assay kit (BioRad, USA). Equal amounts of protein were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Whatman protranTM, Dassel, Germany). Membranes were blocked with 5% skim milk in PBST buffer (0.1% (v/v) Tween-20 in PBS buffer). After washing with PBST buffer, the membranes were incubated overnight with the following primary antibodies: rabbit anti-rat ERK1 (sc94, Santacruz biotechnology, USA), rabbit anti-rat p-p44/42 MAPK (#9101s, Cell-signaling, USA). The membranes were then washed with PBST buffer for 30 min and incubated for 1 h with the following secondary antibodies: goat anti-mouse IgG-HRP (sc-2005, Santacruz, USA), goat anti-rabbit IgG-HRP(sc-2004, Santacruz, USA). After washing with PBST buffer, antibody-specific proteins were detected by chemiluminiscence methods (Fluorchem Q, Alpha Innotech, USA). Each immunoblot area of ERK and pERK at each time point was captured and analyzed using Image J program (version 1.42q, NIH, USA).

4. Paw withdrawal latency (PWL) test

PWL was recorded in the response to the thermal hyperalgesia using Plantar Test System (Cat. No. 37370, Ugo Basile, USA). Infrared source was positioned under the rat's right hind paw, and the time to detach hind paw from the bottom was automatically measured. The Infrared intensity was adjusted to 80 according to the withdrawal reflex time (approximately 10.5 sec) of normal rats and the cut-off time to 15 sec to prevent plantar tissue damage.

5. Data Analysis

Data was expressed as mean±SEM. Calculations of means, standard errors, one or two way ANOVA and Holm-Sidak method for the pairwise multiple comparison were performed using SigmaPlot version 11.2 software (SPSS, Chicago, IL, USA). $P<0.05$ was considered to indicate statistically significant differences.

Results

1. Effect of EA Co-treatment on ERK Activation

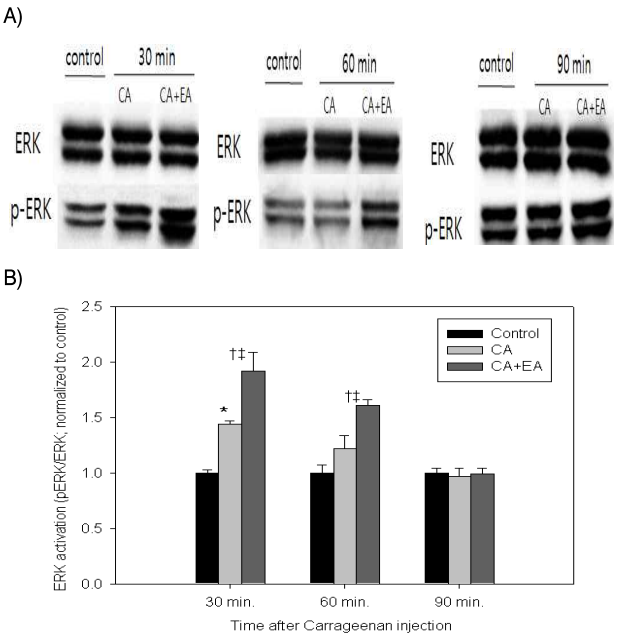

Fig. 1. Effect of EA co-treatment on spinal ERK activation in carrageenan-induced inflammation. (A) Representative immunoblots of dorsal spinal cord proteins at time points indicated. (B) Graph showing densitometric result on ERK activation. Data are shown normalized to levels detected in control sample which ran on the same gel. The results are presented as mean±SEM. $^{*}P<0.05$ or $^{\dagger}P<0.01$ as compared to the control, and $^{\ddagger}P<0.05$ as compared to the CA group.

This experiment was designed to test the effect of EA co-treatment toward the spinal ERK activation in carrageenan-induced pain model. EA was applied just after intraplantar injection of carrageenan. Rats were divided into the following 3 groups (n=5 per group): control (normal rats), CA (rats injected intraplantarly with carrageenan), and CA+EA (EA-treated rats immediately after carrageenan injection), respectively. As shown in Fig. 1, In the CA group, the spinal ERK phosphorylation increased about 1.5-fold over the control group at 30 min after carrageenan injection ($P<0.05$), and then returned to the basal level at 90 min. In the CA+EA group, the

spinal ERK phosphorylation increased about 1.9-fold over the control group at 30 min after carrageenan injection (P<0.01), and then returned to the basal level at 90 min. Unexpectedly, the level of phosphorylated ERK in the CA+EA group was even greater than that in the CA group at 30 min and 60 min after carrageenan injection.

2. Effect of EA Pre-treatment on ERK Activation

This study was designed to determine the effect of EA pre-treatment before carrageenan injection in the spinal ERK activation. EA was applied for 30 minutes before carrageenan injection. Rats were divided into the following 3 groups (n=5 per group): control, CA, EA (EA-treated rats), and PreEA+CA (EA-treated rats for 30 minutes before CA injection). As shown in Fig. 2, in the CA group, the spinal ERK phosphorylation increased about 1.4-fold over the control group at 30 minutes after carrageenan injection (p<0.001). The preEA+CA group showed a significant decrease of the ERK phosphorylation compared to the CA group (p<0.01). The EA group did not show significant difference compared to the control group.

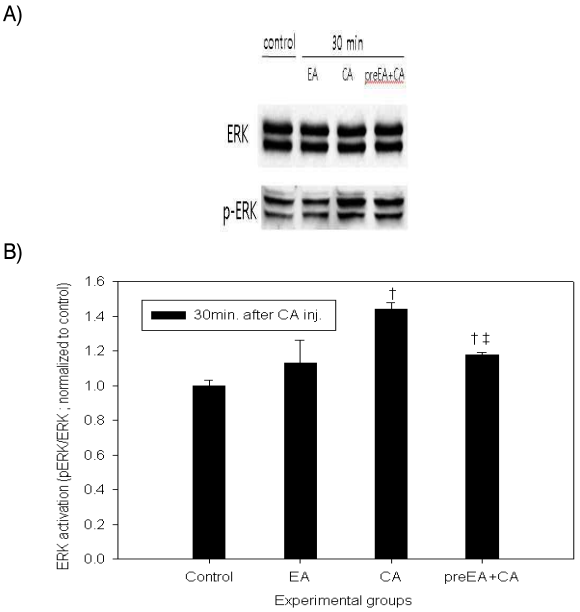

Fig. 2. Effect of EA pre-treatment on spinal ERK activation in carrageenan-induced inflammation. (A) Representative immunoblot of spinal cord protein at 30 minutes after carrageenan injection. (B) Graph showing densitometric result on ERK activation. Data are shown normalized to levels detected in control sample which ran on the same gel. The results are presented as mean±SEM. † P < 0.01, as compared to the control ,and ‡ P < 0.05 as compared to CA group.

3. Effect of EA pre- or co-treatment on Hyperalgesic Behavior

This study was designed to evaluate the analgesic effect of EA pre- or co-treatment on carrageenan-induced hyperalgesia by PWL test. PWL was measured at each 1 hour interval until three hours in the following 4 groups (n=6 per group): control, CA, CA+EA, and PreEA+CA, respectively. One hour after CA injection, PWL of all treated groups increased compared to the control group. Especially, the elevation of PWL in preEA+CA group was most significant (P<0.001). Two hours after carrageenan injection, the PWL of both CA and CA+EA group (6.0±0.4 and 7.2±0.5 sec, respectively) was significantly shorter than that of control group (10.7±0.7 sec) (P<0.001). However, the PWL of preEA+CA group (10.1±1.6 sec) showed significant increase compared to CA group (P<0.001) and approached to the level of control group. This tendency continued next one hour and there was no significant difference of PWL between control and preEA+CA group. This difference of PWL between EA co-treatment and pre-treatment group was consistent with the result of spinal ERK activation between two groups.

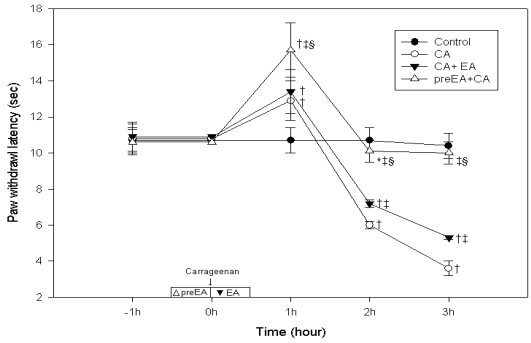

Fig. 3. Effect of EA pre- or co-treatment on hyperalgesic behavior elicited by carrageenan-induced inflammation. Note the significant PWL increase of the preEA+CA group in comparison with CA group. The results are presented as mean±SEM. * P < 0.01, † P < 0.001 as compared to the control, ‡ P < 0.001 as compared to CA group, and § P < 0.001 as compared to CA+EA group.

Discussion

EA has shown to effectively alleviate the pain induced by inflammation in various types of rat models[16,17]. Meanwhile, ERK, as well as c-fos, has been chosen as a marker to prove the mechanism of central sensitization caused by peripheral inflammation[18]. Nevertheless, little is known about the involvement between EA and ERK activation, yet. In this study, we induced inflammation on rat's hind paw by injecting carrageenan and then, examined whether there is a close association between the inhibition of ERK activation and the analgesic effect of EA by using immunoblots and PWL test, respectively.

An important finding in our study is that EA pre-treatment significantly suppressed the spinal ERK phosphorylation induced by carrageenan, whereas EA co-treatment with carrageenan injection unexpectedly led to more increased ERK activation in a synergistic manner than

carrageenan injection alone. EA pre-treatment also showed more analgesic effect than EA co-treatment against carrageenan-induced hyperalgesia in PWL test. Some researchers have reported that EA could alleviate hyperalgesic pain when it was treated before carrageenan injection. Barbara B. Garrido-Suarez et al[19]. reported that PWL which was significantly reduced by carrageenan-induced inflammation returned to the basal level after EA pre-treatment, and this tendency continued by 4 hours after carrageenan injection. Yu-Qiu Zhang et al[20]. also reported that prophylactic EA treatment made the expression of c-fos, another inflammatory marker, effectively reduced in a carrageenan-induced pain model. From the study that ERK activation was involved in pain sensitization[8], it could be postulated that the suppression of ERK phosphorylation may lead to alleviate pain. That idea was consistent with our result that EA pre-treatment attenuated both ERK activation and hyperalgesia induced by carrageenan.

As the mechanism of suppressing the spinal ERK activation, EA may activate the descending inhibitory pathway. Consequently, endogenous opioid peptides can suppress the release of neurotransmitters such as glutamate, substance P and BDNF from the primary afferent C fibers, resulting in downregulation of ERK signalling pathway in the spinal cord dorsal horn postsynaptic neuron. Yu-Qiu Zhang et al[20]. also mentioned about the suppression of neurotransmitters release as EA anagelsic mechanism by showing that co-treatment of both EA and excitatory amino acid receptor antagonists synergistically attenuated hyperalgesia in a carrageenan-induced pain model.

Interestingly, one hour after carrageenan injection in our experimental condition, PWL of all treated groups was significantly increased compared to the control group. Especially, PWL of preEA+CA group was most increased among that of treated groups, which was also statistically significant. There are no other reports concerning this phenomenon. It is likely that peripheral edema might temporarily suppress the transmission of thermal pain in the hind paw to the spinal cord.

Our result indicated that EA alone did not act as a noxious stimulant unlike carrageenan, in that there was not statistically significant ERK activation in case of treating EA alone. However, our result also showed that EA co-treatment with carrageenan injection synergistically increased ERK activation in the present experimental condition. Probably, EA co-treatment seems to act as a kind of noxious stimulant because EA was additively delivered with the gradually increasing intensity (1-2-3mA at 10 minutes intervals) during receiving the carrageenan-induced pain stimuli. We suggest that this tendency might be more extinguishing because both EA and carrageenan-induced pain signals were transmitted through the same ipsilateral side in our experimental condition. In addition, since we used the ipsilateral dorsal side of spinal cords for immunoblot, the ERK phosphorylation of CA+EA group might be significantly increased.

Acknowledgements

This work was supported for two years by Pusan National University Research Grant.

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