Acupuncture Inhibits Kainic Acid–Induced Hippocampal Cell Death in Mice

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Abstract: We examined whether acupuncture can reduce both the incidence of seizures and hippocampal cell death using a mouse model of kainic acid (KA)–induced epilepsy. ICR mice were given acupuncture once a day at acupoint HT8 (sobu) bilaterally during 2 days before KA injection. After an intracerebroventricular injection of 0.1 µg of KA, acupuncture treatment was subsequently administered once more (total 3 times), and the degree of seizure was observed for 20 min. Three hours after injection, the survival of neuronal cells and the expressions of c-Fos, c-Jun, and glutamate decarboxylase (GAD)-67 in the CA1 and CA3 were determined using immunohistochemistry and Western blotting techniques. Acupuncture reduced the severity of the KA-induced epileptic seizure and the rate of neural cell death, and it also decreased the expressions of c-Fos and c-Jun induced by KA in the hippocampus. Furthermore, acupuncture increased GAD-67 expressions in the same areas. These results demonstrated that it could inhibit the KA-induced epileptic seizure and hippocampal cell death by increasing GAD-67 expressions.

Key words: epilepsy, seizure, kainic acid, acupuncture, glutamate decarboxylase (GAD).

Epilepsy is among the most common serious brain disorders that affect people of all ages. Although various antiepileptic drugs are used in the treatment of epilepsy, they are effective in only 60%–70% of individuals. Moreover, they do not modify the disease process, but only suppress the symptoms of epilepsy, such as seizures [1, 2]. There is increasing interest in alternative therapies for the treatment of epilepsy, including acupuncture [3]. Acupuncture has been reported to have therapeutic effects on many neurological diseases, such as Parkinson’s disease [4] and intrastriatal hemorrhage [5] in rat models by protecting neuronal cell death. Some studies have suggested the effects of acupuncture on epilepsies in humans [6]; however, more-rigorous high-quality clinical trials are warranted to provide stronger evidence of the therapeutic effects of acupuncture on epilepsy [3]. Several studies on animal models attempted to show the antiepileptic actions of acupuncture [7, 8]; however, the biological basis underlying its anticonvulsive mechanism remains unclear.

The kainic acid (KA) model is widely used as an experimental model of human temporal lobe epilepsy because the intracerebroventricular injection of KA produces a loss of dentate hilar cells and hippocampal CA3 pyramidal cells similar to those observed in the human epileptic hippocampus [9]. Following KA-induced epilepsy, rat hippocampal neurons strongly express the immediate early gene (IEG) products, i.e., c-Fos and c-Jun, which encode transcription factors. A relationship between these up-regulated c-Fos and c-Jun proteins and hippocampal neuronal death or survival following KA-induced seizure has been suggested [10, 11]. However, it has been reported that c-Fos and c-Jun are not predictors of cell death or survival, but that they rather serve as markers of cells sensitive to KA excitotoxicity [12]. The increased expression of c-Fos and c-Jun induced by KA might be a marker of seizure activity or excitotoxicity or the activation of target genes [13, 14]. It is believed that the effects of KA are the result of alterations in glutamatergic and gamma-aminobutyric acid (GABA)-mediated neurotransmissions [15]. They may be mediated by the sustained activation of...
excitatory amino acid receptors, resulting in prolonged depolarization, neuronal edema, and cell death. Alterations in GABAergic neurotransmission are thought to play a role in the pathophysiology of the epilepsies [16, 17]. The regulation of GABA-mediated signaling involves several mechanisms. The modulation of GABA synthesis by the rate-limiting enzyme glutamate decarboxylase (GAD-67) is one such mechanism that plays a central role in the regulation of GABA-mediated signaling [18]. GAD-67 is an enzyme that catalyzes the decarboxylation of glutamate to GABA and CO₂, and the downregulation of GAD-67 in hippocampus was found in rat brains following status epilepticus [19]. Thus one of the principal mechanisms of current antiepileptic drugs is thought to be the enhancement of the inhibitory GABAergic system [1].

In the present study, we examined (1) whether acupuncture could reduce KA-induced seizure and hippocampal cell death. We then observed (2) whether it blocks the cell overexcitation induced by KA by observing the expressions of c-Fos and c-Jun and (3) whether it could increases in the inhibitory GABAergic system by observing the expression of GAD-67 in order to evaluate the potency of acupuncture as an antiepileptic strategy.

METHODS

Animals and grouping. Male ICR mice (8 weeks old, Orientbio Inc., Korea) weighing 20–25 g were housed at room temperature (22 ± 3°C) under a standard 12-h light/dark cycle (lights on at 07:00 h) and were given unlimited access to food and water. The animals were handled in accordance with current guidelines for animal research, the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, 1985), and all efforts were made to minimize animal suffering and to reduce the number of animals used. The mice were randomly assigned to one of three groups: the saline group (n = 12), which would be injected with normal saline and would not receive acupuncture treatment; the KA group (n = 12), which would be injected with KA and would not receive acupuncture treatment; and the Acu group (n = 12), which would be injected with KA and would receive acupuncture treatment bilaterally at HT8 acupoints.

Acupuncture treatment and kainic acid injection. From 10:00 to 10:30 a.m., the mice in the Acu group were lightly immobilized, and acupuncture needles (0.18 × 8 mm, Dongbang Acupuncture Inc., Korea) were inserted at the bilateral HT8 acupoints. HT8 was located on the palmar surface of the forelimbs, between the fourth and fifth metacarpal bones [20]. The position of the acupoints in mice corresponded anatomically to the location in humans. Acupoints in the Heart Meridian (HT) have been used to treat psychopathic or neurological disorders such as epilepsy. Among these acupoints, HT8 (Sobu) is traditionally known as the strongest for balancing homeostasis by regulating the excitatory or inhibitory functions in the body [20]. The depth of inserted needles was 1 mm, and we turned the needles at a rate of two spins per second for 15 s, removing them immediately afterward. The entire treatment lasted for 30 s, and the animals of the other groups were also immobilized in a similar fashion for 30 s. The treatment was repeated once a day from 2 days before KA injection and directly after it (total 3 times). We did not include a nonacupoint control group with KA injection in this study because our preliminary study showed that this group exhibited no improvement in seizure activity compared to the KA group. KA (Sigma, St. Louis, MO) was injected intracerebroventricularly at bregma with a 50 µl Hamilton microsyringe fitted with a 26-gauge needle, which was inserted to a depth of 2.4 mm according to Laursen and Belknap’s method [21]. The injection volume was 5 µl (0.02 µg/µl) in the KA and Acu groups. The mice in the saline group underwent the same procedure, but normal saline was injected intracerebroventricularly instead of KA.

Behavioral test. Acupuncture treatment or immobilized holding was performed for 30 s after the saline or KA injections, and we directly observed the behavioral changes in the mice for 20 min to determine the severity of seizure activity. The standard of the score was used with Morrison’s method on a scale from 0 to 7, with 0 representing normal behavior and 7 representing death. Typical activities such as walking, exploring, sniffing, and grooming ceased after the KA injection, and the mouse became motionless (stage 1). This immobility was frequently followed by a period of forelimb and/or tail extension, giving the appearance of a rigid posture (stage 2). Repetitive scratching, circling, or head bobbing (stage 3) was followed in most animals by seizure behaviors comprised of forelimb clonus, as well as by rearing and falling (stage 4). This pattern repeated continuously in some cases (stage 5). The surviving mice exhibited more severe tonic-clonic seizures characterized by barrel rolling and the inability to rise (stage 6) [22].

Immunohistochemistry. Three hours after KA injection, the animals were perfused with 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (PB). The brains were removed from the cranium, postfixed for a day, washed in 0.1 M PB, and immersed in a 30% sucrose solution for storage at 4°C prior to sectioning. Frozen sections (40 µm) were cut using a cryostat. Hippocampal sections (~1.82 to ~2.06 mm from the bregma) from each animal were stained. To identify degenerating neurons in the hippocampus, cresyl violet staining was performed. Sections were mounted on silane-coated slides, air-dried, and incubated for 1 min in a 1% solution of cresyl violet, then washed thoroughly in cold tap water, rinsed briefly in 1% acetic acid solution for 10 s, dehydrated by immersion in ascending grades of alcohol, cleared with xylene, and cov-
erslipped using mounting fluid. Sections were immunohistochemically processed to detect c-Fos, c-Jun, and GAD-67 in the mouse hippocampus. Briefly, the sections were incubated in a primary antibody to a dilution of 1:1,000 for 24 h at 4°C. C-Fos (Santa Cruz Biotechnology Inc., Santa Cruz, CA), c-Jun (Santa Cruz Biotechnology Inc.), and GAD-67 (Sigma) were used separately as primary antibodies. After washing in 0.05 M phosphate-buffered saline (PBS), the sections were incubated with biotinylated antimouse or rabbit IgG (Vector Laboratories Inc., Burlingame, CA) for 1 h at room temperature. The sections were then incubated with ABC reagent (Vector Laboratories Inc.) for 1 h at room temperature, washed in PBS, and incubated for 5 min in 0.02% diaminobenzidine (DAB) and 0.003% hydrogen peroxide in 0.1 M tris-HCl–buffered saline (pH 7.5). After the DAB reaction, the tissues were rinsed with PBS, mounted on gelatin-coated slides, air-dried, dehydrated, and c overslipped. The histo logical pictures were taken using a bright-field BX51 microscope (Olympus, Japan) and DP70 camera (Olympus).

The levels of cresyl violet and GAD-67 expressions were measured quantitatively in terms of optical density (OD) on the pyramidal layers of CA1 or CA3 using Image-pro plus 5.1 (Media Cybernetics, Silver Spring, MD). For normalizing the differences of OD among each picture, we subtracted the OD of the primary somatosensory cortex, where there were no changes after 3 hours of KA injection [24], from that of each target region (CA1 and CA3). And the number of c-Fos and c-Jun positive cell bodies in the CA1 and CA3 of the hippocampus was counted from each section and analyzed using an image analyzer (Optimas version 6.5, Media Cybernetics). Cell counts were expressed as the mean number of cells per unit area (mm²).

Western blotting. To confirm the immunohistochemical data, we performed Western blot analysis. The animals were sacrificed at 3 h after receiving the KA injection, and the hippocampus was extracted from the skull and put on ice as soon as possible. The hippocampus samples were homogenized and lysed in ice-cold homogenization buffer containing 50 mM Tris-base (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% glycerol, 10 mM NaF, 10 mM Na-pyrophosphate, and protease inhibitors (0.1 mM phenylmethanesulfonylfluoride, 5 µg/ml apro tin, and 5 µg/ml leupeptin). Equal amounts of the extracted protein (50 µg) were resolved using an 8%–12.5% SDS-PAGE and transferred to nitrocellulose membranes. After being blocked using 5% nonfat dry milk in Tris-buffered saline (TBS, pH 7.6), the membranes were incubated overnight at 4°C with anti–c-Fos, c-Jun (rabbit polyclonal; Santa Cruz Biotechnology Inc.), GAD-67 (mouse monoclonal; Sigma), and actin (mouse monoclonal; Sigma) antibodies that were diluted 1:1,000 in blocking solution. The membranes were further incubated with an antirabbit or antimouse horseradish peroxidase-conjugated antibody (Pierce, Rockford, IL). The membranes were treated with an enhanced chemiluminescence solution (ECL kit; Pierce). The gel bands were analyzed using gel-scanning integrated optical density software (Molecular Dynamics, Sunnyvale, CA).

Statistical analysis. All the data were expressed as the mean ± SEM, and the histological data were analyzed by one-way ANOVA with a Scheffe post hoc test.

RESULTS

Acupuncture suppresses KA-induced seizure behavior

After injecting KA, we evaluated the severity of seizure behaviors in the KA group (KA injection without acupuncture) and the Acu group (KA injections plus acupuncture) according to Morrison’s method [22]. We observed forelimb clonus as well as rearing and falling (stages 4 and 5) in the KA group (3 [2.75, 3], median [25%, 75%] at 20 min). However, head bobbing (stage 3) was the most severe seizure behavior observed in the Acu group, and the degrees of seizure in most animals were stage 1 or 2, such as motionless and tail extension (0.5 [0, 1], median [25%, 75%] at 20 min). In the saline group, the seizure was not observed.

Acupuncture prevents KA-induced cell death in the hippocampus

Besides seizure behavior, we observed the KA-induced cell death in the CA1 and CA3 using cresyl violet. The optical densities of the KA group (CA1: 4.50 ± 3.14 and CA3: 3.18 ± 2.46) were significantly lower than those of the saline group in CA1 (23.70 ± 1.15, p < 0.001) and CA3 (15.03 ± 1.64, p < 0.01). This result showed neuronal death in the hippocampus of the KA group. However, the Acu group showed a substantial protective effect against the hippocampal cell death induced by KA in the CA1 (19.10 ± 2.37, p < 0.001 vs. KA group) and CA3 (13.16 ± 2.50, p < 0.05 vs. KA group). These results showed that acupuncture protects the hippocampal neuronal cell death induced by KA (Fig. 1).

Acupuncture inhibits KA-induced expressions of c-Fos and c-Jun

It was previously reported that the administration of KA at a convulsant dose induced the activation of protooncogenes, such as c-Fos and c-Jun, and that they are markers of excitotoxicity, especially during the acute period [12]. Therefore we postulated that acupuncture at acupoint HT8 reduces the induction of Jun and Fos by KA. In our study, the expression of c-Fos was increased in the CA1 and CA3 of the KA group. In contrast, those of the Acu group blocked the KA-induced c-Fos expression in those areas (p < 0.001 and 0.01 in the CA1 and CA3 vs. the KA group, Fig. 2, A and B). The Western blot analyses revealed that the upregulated c-Fos expressions were
completely blocked by the acupuncture treatment (Fig. 2C). The changes in c-Jun–positive neurons in the hippocampus are presented in Fig. 3A. The neurons in the CA1 and CA3 were significantly increased after KA injection compared to the saline-injected group. However, those of the Acu group were significantly decreased in these areas (each \( p < 0.001 \) and 0.05, Fig. 3B) compared to those of the KA group. We observed a very similar pattern in the Western blot analysis with the results of the immunohistochromies; we found that acupuncture completely blocked the expression of c-Jun induced by KA (Fig. 3C).

**Acupuncture modulates the alterations of GAD-67 induced by KA**

KA alters GABA-mediated neurotransmission and results in neuronal cell death [15]. GAD-67, which plays an important role in the modulation of GABA synthesis, is regarded as a marker for cell bodies of GABAergic neurons and is known to be down-regulated after KA injection [24]. Therefore we examined whether acupuncture treatment could regulate the expression of GAD-67. These positive neurons were observed in the CA1 and CA3 of the saline group. The density of GAD-67 neurons was reduced in those areas of the KA group in comparison to the saline group (each \( p < 0.05 \), Fig. 4A). However, it was significantly increased in the CA1 (\( p < 0.05 \)) and CA3 (\( p < 0.01 \)) of the Acu group in comparison to the KA group (Fig. 4B). The results of the Western blot showed that acupuncture increased the decreased levels of the GAD-67 protein that occurred after the KA injection (Fig. 4C).

**DISCUSSION**

The present results demonstrated that acupuncture treatment at acupoint HT8 reduces KA-induced epileptic seizure and hippocampal cell death in mice. Moreover, it increases GAD-67 expression and inhibits c-Fos and c-Jun induction in the hippocampus after the KA treatment.

GABA is the principal inhibitory neurotransmitter in...
the mammalian brain, and the impairment of GABAergic transmission by genetic mutations or the application of GABA receptor antagonists induces epileptic seizures [25]. Therefore drugs augmenting GABAergic transmission are used for antiepileptic therapy. A loss in the subsets of hippocampal GABA neurons is observed in the tissues of animal epilepsy models and patients with temporal lobe epilepsy. Electrophysiological and neurochemical studies indicate a compensatory increase in GABAergic transmission at certain synapses [26, 27]. These findings suggest that hippocampal GABA neurons play an important role in epilepsy.

Fig. 2. The effect of acupuncture on kainic acid (KA)–induced c-Fos immunoreactivity in the mouse hippocampus. (A) The expressions of c-Fos–positive cells in the hippocampus. Sections were stained for c-Fos–positive cells (reddish brown). The expressions of c-Fos were present at very low levels in the hippocampi of the saline-treated group. The expressions were increased in the CA1 and CA3 after the KA injection. However, acupuncture treatment blocked the expression of c-Fos in the same regions. The scale bar represents 100 µm. (B) The number of c-Fos–positive cells in the CA1 and CA3. Acupuncture significantly blocked the increase in c-Fos positive cells by KA. All data are presented as the mean ± SEM (**p < 0.01 and ***p < 0.001, KA vs. Acu). (C) Western blot analysis for c-Fos expression. Acupuncture treatment suppressed the protein levels of c-Fos.
Acupuncture and electroacupuncture (EA) exhibit a variety of neuromodulatory functions in the central nervous system, such as the triggering of the release of neuropeptides, the regulation of neuronal gene expression, and the enhancement of neurogenesis [28, 29]. The effects of acupuncture on seizures and their mechanisms of action have been reported. For example, EA partially inhibits kainic acid-induced epilepsy by increased taurine transporter [8]. EA also inhibits the seizures in penicillin-induced epilepsy and the induction of nNOS and iNOS, which are increased during epilepsy [30]. Furthermore, the effect of EA on high oxygen pressure-induced convul-
The effect of acupuncture for the epileptic seizure has been studied along with its relationship to the GABA concentration in murine brains [31], and EA produced the neuroprotective actions as a result of GABA increase in a cerebral ischemia rat model [32]. So we supposed that acupuncture may reduce KA-induced epileptic activity and hippocampal cell death by increasing the levels of GABA neurons.

In the present study, the KA-induced c-Fos and c-Jun expression levels were inhibited by acupuncture treatment. It has been reported that c-Fos and c-Jun are markers of cells that are sensitive to KA excitotoxicity [12]. Thus the suppression of KA-induced c-Fos and c-Jun induction by acupuncture could be explained by the inhibition of KA excitotoxicity via increased levels of GABA neurons. The principal mechanisms for current antiepileptic drugs are thought to be the enhancement of the inhibi-
tory GABAergic system or the inhibition of neuronal excitability [1]. Therefore our results imply that acupuncture may be a good alternative therapeutic tool for the treatment of epilepsy, especially for patients who do not respond well to the current antiepileptic drugs.

**CONCLUSION**

In the present study, we showed that acupuncture inhibits KA-induced epileptic seizure activity and hippocampal cell death in mice. Acupuncture also reduced the KA-induced c-Fos and c-Jun expressions in the hippocampus. Moreover, it improved the decreased expressions of GAD-67 by KA treatment. These results indicate that acupuncture protects against KA excitotoxicity in the hippocampus through the up-regulation of inhibitory GABA neurons.

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**REFERENCES**