Protective Effects of Bupivacaine against Kainic Acid-Induced Seizure and Neuronal Cell Death in the Rat Hippocampus

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The excessive release of glutamate is a critical element in the neuropathology of epilepsy, and bupivacaine, a local anesthetic agent, has been shown to inhibit the release of glutamate in rat cerebrocortical nerve terminals. This study investigated whether bupivacaine produces antiseizure and antixcitotoxic effects using a kainic acid (KA) rat model, an animal model used for temporal lobe epilepsy, and excitotoxic neurodegeneration experiments. The results showed that administering bupivacaine (0.4 mg/kg or 2 mg/kg) intraperitoneally to rats 30 min before intraperitoneal injection of KA (15 mg/kg) increased seizure latency and reduced the seizure score. In addition, bupivacaine attenuated KA-induced hippocampal neuronal cell death, and this protective effect was accompanied by the inhibition of microglial activation and production of proinflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor-α in the hippocampus. Moreover, bupivacaine shortened the latency of escaping onto the platform in the Morris water maze learning performance test. Collectively, these data suggest that bupivacaine has therapeutic potential for treating epilepsy.

Key words bupivacaine; anticonvulsion; neuroprotection; antiinflammation; kainic acid; hippocampus

Epilepsy is a common neurological disorder affecting people worldwide. Although the exact cause of seizures is unclear, a substantial amount of evidence supports the role of the excitatory neurotransmitter glutamate in epileptic seizures. For example, experimentally applying glutamate receptor agonists has induced seizures in rats. Conversely, glutamate receptor antagonists have exhibited antiepileptic-like properties and reduced seizure-induced brain damage in various animal models. Furthermore, a significant increase in glutamate levels has been observed in human epilepsy patients as well as in experimental models of epilepsy. Excessive glutamate release and activation of the glutamate receptors induces an increase in intracellular Ca2+ levels, subsequently triggering a cascade of cellular responses, including nitric oxide synthase (NOS) activation, enhanced oxygen free-radical production, and disturbed mitochondrial function, which ultimately causes inflammatory responses and neuronal cell death. Thus, influencing central glutamatergic neurotransmission may provide a potential target for epilepsy treatment. Consequently, several glutamatergic modulators are being developed to treat epilepsy, including N-methyl-D-aspartic acid (NMDA) receptor antagonists as well as metabotropic glutamate receptor agonists and antagonists. However, these drugs have been unsuccessful in clinical trials because of numerous side effects, such as ataxia and memory impairment. Therefore, seeking antiepileptic drugs that are safe and effective is necessary.

Bupivacaine is a Na+ channel blocker and is widely used in various clinical settings, including local anesthesia, antiarrhythmia, and pain control. Numerous studies have shown that bupivacaine can inhibit glutamatergic transmission by affecting glutamate receptors or by reducing the release of glutamate, and these effects are more potent than other local anesthetics. These results therefore suggest that bupivacaine has a glutamate-inhibiting property in addition to Na+ channel blocking. Considering that the excitotoxicity caused by excessive glutamate is a critical factor in the neuropathology of epilepsy, we hypothesized that bupivacaine plays a role in preventing excitotoxicity-induced seizure genesis. In this study, we used a kainic acid (KA)-injected rat seizure model to assess whether bupivacaine has an anticonvulsant effect and plays a neuroprotective role. Furthermore, we also evaluated the effect of bupivacaine on KA-induced cognitive impairment. KA is an analogue of glutamate, and the systemic administration of KA to animals induces epileptic seizures. These seizures cause neuronal cell death in specific brain regions, specifically in the hippocampus. Furthermore, KA-induced seizures and neuronal death have been proposed to be linked to the pathological release of glutamate. Based on behavioral and pathological similarities, KA has been widely used as a tool to experimentally mimic human temporal lobe epilepsy, the most common type of epilepsy.

MATERIALS AND METHODS

Chemicals Bupivacaine was obtained from AstraZeneca (Monts, France). KA, carbamazepine and neutral red was bought from Sigma-Aldrich (St. Louis, MO, U.S.A.). Fluoro-Jade B was obtained from Millipore (CA, U.S.A.). Mouse monoclonal anti-CDD1b antibody was bought from AbD Serotec (CA, U.S.A.). RNA Later Solution and miRNA Isolation Kit were purchased from Life Technologies (NY, U.S.A.). GoScript Reverse Transcription System kit was...
bought from Promega (WI, U.S.A.). SYBR Green PCR Master Mix was obtained from Roche Diagnostics (Mannheim, Germany).

Animals and Seizure Activity Adult male Sprague-Dawley rats (200–250 g) were randomly divided into 4 experimental groups: (1) a saline+saline (control) group; (2) a KA 15 mg/kg+saline group; (3) a KA+bupivacaine 0.4 mg/kg group; and (4) a KA+bupivacaine 2 mg/kg group. All animal procedures were conducted according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Far Eastern Memorial Hospital Animal Care and Utilization Committee (101-02-19). All efforts were made to minimize animal suffering and to reduce the number of animals used.

According to previous studies, the experimental design is shown in Fig. 1. Seizures were induced by intraperitoneally (i.p.) administering rats with KA (15 mg/kg) in phosphate-buffered saline (PBS). After KA administration, the animals were continuously monitored for seizure activity for 4 h. KA-induced behavioral seizure activity was rated using a scale devised by Racine. Wet dog shakes, facial clonus, and starling considered stage 1 seizure activity; head nodding is considered stage 2 activity; forelimb clonus is classified as stage 3 activity; forelimb clonus with rearing is regarded as stage 4 activity; and rearing, jumping, falling, and status epilepticus are considered stage 5 activity. Bupivacaine (0.4 and 2 mg/kg) was injected i.p 30 min before KA injection. The time of seizure initiation and seizure scores were analyzed.

After behavioral assessment, the rats were sacrificed 3 d after KA injection with an overdose of anesthetics (chloral hydrate, 650 mg/kg, i.p.). The animals were then perfused transcardially with saline (room temperature), followed by cold 4% paraformaldehyde in 0.1 M PBS. The brains were removed immediately and postfixed in the same fixative overnight at 4°C and then cryoprotected in 30% sucrose for 24–48 h. In addition, to investigate the effects of bupivacaine on proinflammatory cytokine (interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α) expression, a second group of rats was sacrificed at various time points (3 h, 1 d, and 3 d) after KA administration.

Neutral Red and Fluoro-Jade B Staining The frozen brains were sectioned coronally at a thickness of 30 μm in a cryostat at −20°C. The sections were mounted on silane-coated glass slides, air dried, and then stained with a neutral red solution. Fluoro-Jade B (Chemicon, Millipore Ltd., Billerica, MA, U.S.A.), a high affinity fluorescent marker for all degenerating neurons, was used to quantify cell loss. Staining for Fluoro-Jade B was performed as described previously. Neutral Red and Fluoro-Jade B-stained sections (10 μm) were mounted on gelatin-coated slides and dried at room temperature; subsequently, the slides were immersed for 5 min in a solution containing 1% sodium hydroxide in 80% ethanol. The slides were then immersed in 70% ethanol for 2 min and then in distilled water for 2 min. The sections were oxidized in 0.06% potassium permanganate for 15 min, washed with water, and then immersed in a 0.001% Fluoro-Jade B solution for 30 min in the dark. The slides were then washed in distilled water, air dried, cleared, and coverslipped. According to previous studies, the hippocampal CA3 is the most vulnerable area to excitotoxic lesions caused by KA. Therefore, the CA3 region was visualized under 100× magnification using an upright fluorescence microscope (Zeiss Axioskop 40, Goettingen, Germany), and digitized photomicrographs employed in analysis were captured using a digital camera (Nikon D80, Tokyo, Japan) between bregma −2.30 mm and −3.60 mm according to the rat brain atlas of Paxinos and Watson. To compare neuronal death among the experimental groups, an examiner blind to experimental conditions measured the number of Fluoro-Jade-B-positive cells in a 255×255 μm area of the hippocampal CA3 in 6 to 8 randomly chosen sections from each animal and then averaged.
this number for each animal by using a computer-assisted image analysis system (Image J; NIH Image, National Institutes of Health, Bethesda, MD, U.S.A.). Results are expressed as the mean±standard error of the mean (S.E.M.) of labeled cells per 0.1 mm².

Immunohistochemical Staining Immunohistochemical staining of CD11b positive cells was performed to analyze microglial activation after KA treatment. The frozen brain was cut into 30-μm-thick coronal sections in a cryostat at −20°C, and free-floating staining was then performed using an immunohistochemical avidin biotin complex (ABC) method. After the sections were rinsed 3 times with PBS, the sections were blocked with 1% normal horse serum containing 0.3% Triton X-100 for 1 h at room temperature. The sections were incubated with a mouse monoclonal anti-CD11b antibody (1:500 diluted in blocking solution) overnight at 4°C. The sections were then incubated with a horse biotinylated antimouse secondary antibody (Vector, CA, U.S.A.) for 2 h and subsequently incubated with ExtraAvidin peroxidase (1:1000, Sigma-Aldrich) for 1 h at room temperature. After the sections were rinsed in 0.1 M PBS for 20 min, the sections were reacted with a 0.025% 3,3′-diaminobenzidine tetrahydrochloride (DAB) solution in PBS containing 0.0025% hydrogen peroxide for 6 min. The sections were then mounted on gelatin-coated glass slides, air dried, dehydrated, cleared with xylene, and coverslipped with the Entellan mounting medium (Merck, Darmstadt, Germany).

Real-Time Quantitative Polymerase Chain Reaction (PCR) Rats were killed through decapitation. The hippocampi were rapidly dissected and immediately placed into the RNAlater Solution (Life Technologies) and stored at 4°C overnight. The hippocampi were homogenized with TissueRuptor (Qiagen, CA, U.S.A.), and total RNA extraction was conducted using an mirVanaTM miRNA Isolation Kit (Life Technologies). Total RNA was reverse transcribed into cDNA by using the GoScript™ Reverse Transcription System kit (Promega).

Quantitative real-time PCR was performed using the SYBR Green PCR Master Mix (Roche Diagnostics). Amplification was conducted using the LightCycler® 480 System (Roche Diagnostics). The following LightCycler® experimental run protocol was used: 95°C for 5 min for one cycle, followed by 10 s of denaturation at 95°C, 10 s of annealing at 60°C, and 10 s of primer extension at 72°C for 55 cycles. All cytokine primers were designed using the LightCycler®® Probe Design Software 2.0 (Roche Diagnostics). The primers and product size of each gene are listed as follows: IL-1β (151 bp); 5′-TGG CAA GGA GAG ACA AAC-3′ (forward) and 5′-TTT CCA TCT TCT TCT TGG ATT-3′ (reverse), IL-6 (154 bp); 5′-AAT GGA GAA GTT AGA GTA GCA GA-3′ (forward) and 5′-GAG GAA TGT CCA CAA GAT A-3′ (reverse), TNF-α (154 bp); 5′-CTT CCC TCC CTC AGA GC-3′ (forward) and 5′-CCG GCC TCC CAA ATA AAT ACA-3′ (reverse). The primer for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, is listed as follows: 5′-TCC CAT TCT TCC ACC TTT GAT-3′ (forward) and 5′-TGG CCA AGG GTT TCT TAC TC-3′ (reverse). The product size of this gene was 155 bp. Amplification specificity was examined using a melting curve according to the manufacturer's instructions. The comparative threshold method (ΔΔCT method) was used to examine the relative quantification of the samples (LightCycler® 480 Software, Version 1.5, Roche Diagnostics). Fold induction was calculated using the 2−ΔΔCT method as previously described.36

Morris Water Maze Test Spatial learning and memory performance of rats was measured using the Morris water maze task at three days after KA treatment. The Morris water maze consisted of a round plastic pool (200 cm in diameter, 50 cm height) filled with water (30 cm depth) at 26±1°C and virtually divided into four equivalent quadrants: northeast, northwest, southeast, and southwest. Rats were trained to escape from the water by swimming to a hidden platform (10 cm diameter, 20 cm height) placed in the northwest quadrant of the pool and submerged 2 cm below the water surface. Rats were trained for four consecutive days before testing. Each rat was given four trials per day and each trial was separated by 1 h. For each trial, rats were randomly placed into one of four quadrants facing the wall and then allowed to swim freely until they found and climb onto the hidden platform. Each rat was given 120 s to find and mount the platform. When successfully reaching the hidden platform, the rat was allowed to rest on the platform for 30 s. The inter-trial interval of the Morris water maze test was 1 min. The latency to reach the platform and total distance traveled for each trial were recorded using a camera connected to a video recorder and a compute-rized tracking system (Video Tracking Record System Version 1.17, SINGA Technology Corporation, Taipei, Taiwan).

Statistical Analysis Data are expressed as the mean±S.E.M. The data reported were analyzed using one-way ANOVA accompanied by post hoc least significant difference (LSD) comparison tests for multiple comparisons. Analysis was conducted using SPSS software (17.0; SPSS Inc., Chicago, IL, U.S.A.). p<0.05 was considered to represent a significant difference.

RESULTS

Bupivacaine Pretreatment Attenuated KA-Induced Seizures KA administered i.p. at a dose of 15 mg/kg induced epileptic seizures in 88% of the rats. The latency and score of the seizures were 32.8±3 and 4.6±0.3 min, respectively (Figs. 2A, B). Administering bupivacaine (0.4 or 2 mg/kg, i.p.) 30 min before KA administration increased seizure latency [F(4, 32)=7.812, p=0.001; Fig. 2A] and reduced the seizure score [F(4, 32)=5.587, p=0.002; Fig. 2B]. Similar observations were made using the clinical antiepileptic drug carbamazepine (40 or 80 mg/kg, i.p.) (Fig. 2).

Bupivacaine Pretreatment Reduced KA-Induced Neuronal Cell Death in the CA3 Area of the Hippocampus The neuronal death that occurred after KA administration (15 mg/kg, i.p., 3 d) was verified using neutral red and Fluoro-Jade B staining. As shown in Figs. 3A and B, neutral red staining indicated a significant neuronal loss in the hippocampal CA3 of KA-injected rats compared with that of the saline-treated rats (control). Administering bupivacaine (0.4 or 2 mg/kg, i.p.) 30 min before KA administration substantially reduced KA-induced neuronal death in the CA3 region (Figs. 3C, D). A similar protective effect of bupivacaine against neuronal death was observed by using Fluoro-Jade B staining. In Fig. 3E, no staining was observed in the saline-injected rats (control). KA treatment caused a substantial increase in the number of Fluoro-Jade-B-positive neurons in the CA3 region of the hippocampus (p=0.001; Fig. 3F). In rats pretreated with bupiva-
caine (0.4 or 2 mg/kg), the number of Fluoro-Jade-B-positive neurons in CA3 was decreased compared with that in KA-treated rats \( F(2, 22) = 4.909, p = 0.017; \) Figs. 3E–I].

**Bupivacaine Pretreatment Inhibited the KA-Induced Microglial Activation in the CA3 Area of the Hippocampus**

The microglial activation that occurred after KA administration (15 mg/kg, i.p., 3 d) was verified by detecting the expression of CD11b, a surface marker used for microglia. In the saline-treated rats (control), microglial cells in the CA3 region exhibited a resting morphology with small cell bodies and thin processes (Fig. 4A). Conversely, the number of microglial cells in KA-injected rats increased substantially in the CA3 region. These cells exhibited enlarged cell bodies with considerably shorter and thicker processes (indicating the activation state; Fig. 4B). CD11b assessment showed no obvious microglial activation in the saline control group. Microglial cells in the CA3 region exhibited a resting morphology with small cell bodies and thin processes (Fig. 4A). KA-induced microglial activation was substantially suppressed in rats pretreated with bupivacaine (0.4 or 2 mg/kg); most of the microglial cells were in a ramified or resting state (Figs. 4C, D).

**Bupivacaine Pretreatment Decreased the KA-Induced Proinflammatory Cytokine Production in the Hippocampus**

We analyzed the effect of bupivacaine on the mRNA expression of proinflammatory cytokines IL-1β, IL-6, and TNF-α in the hippocampus 3, 24, and 72 h after KA administration. These proinflammatory cytokines are implicated in KA-induced microglia activation and neuronal death.\(^{12,37}\) In Fig. 5, expression of IL-1β, IL-6, and TNF-α mRNA in the hippocampus increased at all times after KA administration \( (p<0.05) \). When bupivacaine (0.2 or 4 mg/kg) was administered 30 min before KA administration, KA-induced expression of these proinflammatory genes decreased by 43–53% depending on the target gene (Figs. 5A–C).

**Bupivacaine Pretreatment Prevented the KA-Induced Cognitive Impairment**

In Fig. 6, we evaluated the effect of bupivacaine on KA-induced cognitive impairment by water maze test at 3 d after KA administration. The mean latencies and distances swam to reach the platform was significantly increased in KA-injected rats compared with that of the saline-treated rats (control) \( (p<0.001) \). When bupivacaine (0.2 or 4 mg/kg) was administered 30 min before KA administration, the mean latencies \( F(3, 17)=130.12, p=0.0001; \) Fig. 6A] and distances swam to reach the platform \( F(3, 17)=19.437, p=0.0001; \) [Fig. 6B] were significantly decreased in comparison to KA-treated rats.

**DISCUSSION**

Excessive release of glutamate is a critical factor in the neuropathology of epilepsy,\(^{6,7,21}\) and regulating its release may be a crucial mechanism of antiepileptic drugs. Bupivacaine is used clinically as a local anesthetic. Because we previously determined that bupivacaine depresses glutamate release from nerve terminals,\(^{20}\) we hypothesized that it has an anticonvulsant effect. This hypothesis was confirmed in this study by applying the KA model to adult rats. We used this model because KA-induced seizure activity and brain damage are similar to human temporal lobe epilepsy.\(^{23,25,26,38}\) Furthermore, these KA-induced alterations were associated with the excessive release of glutamate.

In this study, administering KA (15 mg/kg, i.p.) induced epileptic seizures. This result is consistent with the results obtained when other research group applied the same dose of...
Administering bupivacaine (0.4 or 2 mg/kg, i.p.) or carbamazepine (40 or 80 mg/kg, i.p.) before a KA injection resulted in an anticonvulsive effect, increasing the latencies to onset of seizure and reducing the seizure score. The present data indicate that bupivacaine is more potent than carbamazepine in the inhibiting seizure activity, since the dose of carbamazepine is higher than bupivacaine. However, our finding is inconsistent with previous studies, which showed that bupivacaine at a dose of 10–20 mg/kg produces seizures, suggesting that different mechanisms of action might be involved. Therefore, the role of bupivacaine on epilepsy is apparently dependent on the administered dose; a low dose results in...
anticonvulsant activity, whereas a high dose causes convulsion. Further clinical testing is needed to determine whether a lower dosage of bupivacaine can be safely administered to patients with epilepsy. On the other hand, it has been shown that bupivacaine, at high concentration range (1–10 mM), can cause neurotoxicity. In a clinical setting, bupivacaine is well-known to produce central nervous system (CNS) toxicity in patients who exceed the recommended dosage (2 mg/kg).

The dose of bupivacaine used to inhibit KA-induced seizures in our study, therefore, may be considered safe.

Systemically administering KA to animals causes neuronal cell death or neurodegeneration in specific brain regions, such as the hippocampus, piriform cortex, thalamus, and amygdala. In the hippocampus, CA3 pyramidal cells are the most severely damaged after KA administration. Such a phenomenon occurs because the density of KA receptors is the highest in the CA3 region. In this study, administering KA (15 mg/kg, i.p.) caused considerable neuronal death in the hippocampal CA3; this result is consistent with those of previous studies. However, bupivacaine pretreatment (0.4 or 2 mg/kg) reduced this KA-induced neuronal loss, suggesting that bupivacaine acts as a potent neuroprotective agent as well as an anticonvulsant agent.

Neuroinflammation is included in the pathogenesis of numerous acute and chronic neurological disorders. Inflammatory processes, including microglial activation and the consequent production of various neurotoxic factors (including free radicals and proinflammatory cytokines), are believed to contribute to KA-induced neuronal death. In our study, we observed that the amount of activated microglia immunostained by CD11b antibodies and the production of proinflammatory cytokines (IL-1β, IL-6, and TNF-α) in the hippocampus was higher in KA-treated rats than in saline-treated rats. Bupivacaine pretreatment reduced the amount of activated microglia and production of these proinflammatory cytokines, suggesting that bupivacaine performs an antiinflammatory function that underlies, at least in part, its protective action against the excitotoxicity induced through KA.

From the previous report, it was clear that KA-induced seizures and neurodegeneration is associated with cognitive impairment. In this study, we also observed that administering KA (15 mg/kg, i.p.) caused considerable cognitive impairment as shown by increasing the latencies and distances swam to reach the platform in water maze tests. This phenomena was shortened by bupivacaine pretreatment (0.4 or 2 mg/kg), indicating that bupivacaine has a protective effect against KA-induced cognitive deficit. This observation together with the fact that antiepileptic drugs have been reported to cause cognitive side effects, we suggested that bupivacaine acts as a useful adjuvant to the conventional antiepileptic therapy.

In conclusion, our data show that bupivacaine produces significant anticonvulsant, antiinflammatory, and neuroprotective effects in KA-treated rats, which constitute a well-characterized model of temporal lobe epilepsy. Moreover, bupivacaine exerts a protective effect on KA-induced cognitive impairment. Although the relevance of our findings to in vivo clinical situations has yet to be determined, our investigation enhances the understanding of bupivacaine action in the brain and demonstrates the therapeutic potential of this local anesthetic in treating epilepsy and neurological diseases associated
Fig. 5. Bupivacaine Reduces KA-Induced Proinflammatory Cytokines Expression in the Hippocampus

Relative quantification of (A) IL-1β, (B) IL-6 and (C) TNF-α mRNA expression 3, 24 and 72 h after KA-injection in rat hippocampus measured with real-time PCR. Data are expressed as mean±S.E.M. (n=6). *p<0.05, **p<0.001, ***p<0.0001.

Fig. 6. Bupivacaine Protects against KA-Induced Cognitive Impairment

The rats were pretreated with saline or bupivacaine (0.4 or 2 mg/kg, i.p.) 30 min before KA (15 mg/kg, i.p.) injection. The Morris water maze test including latency (A) and total distance traveled (B) were performed at 3 d after KA administration, according to the methods. Data are expressed as mean±S.E.M. (n=6). *p<0.001, as compared with the KA-treated group.
with excitotoxicity.

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Conflict of Interest The authors declare no conflict of interest.

REFERENCES

21) Meldrum BS. The role of glutamate in epilepsy and other CNS disorders. Neurology, 44 (Suppl. 8), S14–S23 (1994).


