Kinetics of Glutamate and γ-Aminobutyric Acid in Cerebrospinal Fluid in a Canine Model of Complex Partial Status Epilepticus induced by Kainic Acid

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ABSTRACT. An imbalance of excitatory and inhibitory transmitters in the brain has been suggested to cause epileptic seizures. In this study, we investigated the kinetics of glutamate (GLU) and γ-aminobutyric acid (GABA) in cerebrospinal fluid (CSF-GLU and CSF-GABA, respectively) using a high performance liquid chromatography (HPLC) in a canine model of complex partial status epilepticus (CPSE) induced by the microinjection of kainic acid (KA) into the unilateral amygdala. During the acute phase (3, 6, 12 and 48 hr after the onset of CPSE), CSF-GLU was significantly increased, while CSF-GABA was decreased, although not significantly. In the chronic phase, both CSF-GLU and CSF-GABA were significantly lower than normal at 72 hr after the onset of CPSE, and their levels returned to normal at 2 months. Results of the present study demonstrate that CSF-GLU is gradually increased in relation with seizure severity, and suggested the possibility that CSF-GABA was consistently decreased during CPSE induced by KA in dogs.

KEY WORDS: cerebrospinal fluid, GABA, glutamate, kainic acid, status epilepticus.

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MATERIALS AND METHODS

This study was performed in conformity with the Animal Care and Use Committee of Nippon Veterinary and Animal Science University.

Normal animals: Thirteen clinically healthy Beagle dogs (1–3 years old, mixed gender, 8–12 kg) were used as the normal group.

CPSE model animals: Details of the canine KA-induced CPSE model were described previously [5, 6]. Briefly, a total of eleven healthy beagle dogs (8–10 kg, mixed gender, 1–3 years old) were used. A cannula for KA injection was stereotactically inserted into the left amygdala of each animal under general anesthesia induced by intravenous injection of sodium thiopental and maintained by inhalation of isoflurane. After 7–14 days of the surgery, nine dogs were injected slowly with 1.5 μg of KA dissolved in phosphate buffered saline (PBS) under freely-moving conditions without anesthesia. The dogs were divided into 3 groups as follows.

For the acute phase group, one each of five animals were examined at 3, 6, 12, 24 and 48 hr after the onset of CPSE [6]. For the chronic phase group, four animals were examined at approximately 72 hr (the end of CPSE) and 2 months after the onset of CPSE [5]. Two dogs were treated with PBS instead of KA, and were used as controls for the chronic group (PBS group).

CSF sampling: The CSF samples used in this study were identical to those used in the previous studies [5,6]. CSF was collected by cisternal puncture under light general anesthesia using thiopental. At least 1 ml of CSF was collected from each animal and stored at −76°C until amino acid analysis.

In the acute phase group, CSF was collected from five animals before KA injection and from one animal at 3, 6, 12,
In the chronic phase and PBS groups, CSF was collected from 4 animals before KA injection, just after the end of CPSE (approximately 72 hr after the onset of CPSE), and at 2 months after KA injection.

**HPLC Analysis of CSF-GLU and CSF-GABA:** CSF-GLU and CSF-GABA were analyzed by high-performance liquid chromatography (HPLC). The HPLC system (JASCO, Tokyo, Japan) consisted of 2 pumps (PU-980), a column oven (CO-965), an autosampler (AS-950), a fluorescence detector (UV-970) and an integrator (LCSS-905). Two ODS columns (Pegasil ODS, 4.6 mm × 30 mm and 4.6 mm × 250 mm, Senshu, Tokyo, Japan) were used as a guard column and analyzing column, respectively, at 40°C. The mobile phase consisted of 100 mM sodium acetate (pH 5.55: solvent A) and methanol (solvent B), and was eluted at a flow rate of 1.0 ml/min. A gradient protocol was carried out as follows: a linear gradient from 85% to 50% of solvent A for the first 32 min, a linear gradient from 50% to 10% of solvent A for the next 8 min, and an isotropic elution of 10% solvent A for the last 5 min. Fluorescence of the eluant was monitored for 46 min with excitation and emission wavelengths set at 340 nm and 455 nm, respectively. To equilibrate the column between each run, the 85% solvent A was isocratically eluted for 9 min. CSF samples were thawed immediately before use. An amino acid standard solution (Amino acid standard solution, Wako pure chemical, Osaka, Japan) or CSF samples were mixed with an aliquot of OPA solution (10 mg of o-phtalalddehyde and 10 µl of 2-mercaptoethanol in 10 ml of 0.5 M of sodium bicarbonate) at a ratio of 1 to 2 (30 µl of sample into 60 µl of OPA solution) at 2 min before injection, then 20 µl of the mixture was loaded. GLU and GABA were identified by superimposition onto standards. Values of CSF-GLU and CSF-GABA were calculated by analyzing standards with known concentrations, and presented as nmol/ml and pmol/ml, respectively.

**Statistical analysis:** For the chronic-phase and the normal groups, the averages and standard deviations (SD) of CSF-GLU and CSF-GABA levels were calculated and expressed as average ± SD. The levels of CSF-GLU and CSF-GABA at each time point (before KA injection, at 72 hr and 2 months after the onset of CPSE) in the chronic phase group were compared with the normal group using the Student’s t-test. A significant difference was assumed when p<0.01. For the acute phase and PBS groups, a difference of more than the mean ± 2SD of the normal group was assumed as significant due to their small sample numbers.

**RESULTS**

**Summary of KA-induced CPSE:** The detail of symptoms, electroencephalograms (EEGs), and MRI changes after KA injection were described previously [5, 6]. All animals injected with KA showed initial limbic seizures that consisted of salivation, mastication, mydriasis and facial twitching within 30 min after injection, which were synchronized seizure discharges of the injected unilateral amygdala. The frequency of the seizures increased gradually and developed into CPSE at 1–3 hr after KA injection. At 3–6 hr after the onset of CPSE, diffusion-weighted imaging (DWI) and fluid-attenuated inversion recovery imaging (FLAIR) of MRI showed hyperintensity in the amygdala and hippocampus of the KA-injected side. Seizures were generalized frequently, and also started from the contralateral limbic systems (the opposite to the injected side) at approximately 5–10 hr after KA injection. MRI of 12, 24, and 48 hr after the onset of CPSE showed increasing hyperintensity on T2-weighted imaging, FLAIR, and DWI resulted from progressive cytotoxic and/or vasogenic edema in the amygdala and hippocampus bilaterally. This CPSE lasted for 1–3 days, and the animals recovered gradually. After two-months, the brain histopathology of the KA-dogs revealed extended necrosis of the bilateral amygdala and sclerosis of the bilateral hippocampi.

**CSF-GLU and CSF-GABA:** Representative chromatograms of a normal dog and a KA-treated dog are shown in Fig. 1.

![Fig. 1. Chromatograms of amino acids in the CSF from a normal dog (A) and from a KA treated dog (B) at 48 hr after the onset of CPSE.](image-url)
Acute phase group: Time-course changes in CSF-GLU and CSF-GABA after the onset of CPSE are shown in Figs 2A and 2B, respectively. The level of CSF-GLU showed a gradual increase after the onset of CPSE, although the severity of the seizures varied in each animal. The levels of CSF-GLU from 6 hr to 48 hr after the onset of CPSE were higher than the mean $\pm$ 2SD of the levels of the normal group ($1.26 \pm 0.47$ nmol/ml). On the other hand, the levels of CSF-GABA after the onset of CPSE were lower than those of the normal group ($131 \pm 63$ pmol/ml) at all time points, although the values were within the mean $\pm$ 2SD of the normal group.

Chronic phase group: The levels of CSF-GLU before the KA injection, at the end of CPSE and at 2 months after the KA injection in the chronic phase group were $1.83 \pm 0.10$ nmol/ml, $0.58 \pm 0.23$ nmol/ml and $1.73 \pm 0.77$ nmol/ml, respectively (Fig. 3A). The levels of CSF-GLU at the end of CPSE were significantly lower than the levels of the normal group ($p<0.01$). The levels of CSF-GABA were at the same time points were $218 \pm 41, 42.0 \pm 9.3, 151 \pm 43$ pmol/ml, respectively (Fig. 3B). The CSF-GABA was also significantly lower ($p<0.01$) than that of the normal group at the end of CPSE. Neither CSF-GLU nor CSF-GABA of the PBS group showed significant changes at any time point.

DISCUSSION

In the present study, 1) CSF-GLU showed a significant increase with time at the early stage of KA-induced CPSE, while CSF-GABA remained at levels lower than those of normal dogs although these differences were not significant, 2) both CSF-GLU and CSF-GABA at the end stage of CPSE showed the lowest concentrations, which were significantly lower than those of normal dogs, and 3) both CSF-GLU and CSF-GABA returned to the normal levels at 2 months after CPSE.

Using a feline epilepsy model induced by KA injection into the hippocampus, Griffith et al. reported that the levels of CSF-GLU and CSF-GABA showed no significant change during CPSE [4]. They concluded that CSF-GLU and CSF-GABA were not altered during the acute phase of CPSE because the seizure type continually and variously changed from arrest to generalized tonic-clonic seizures. Nakase et al. [12] reported that CSF-GLU increased, but GLU in the brain tissue decreased during the seizure in a feline kindling model. In addition, they reported that the release of CSF-GLU was more prominent in serious generalized seizures than in partial seizures. Their data suggested that the severity of the seizures might relate to the release of GLU into the CSF. In our KA-induced CPSE model in
dogs, generalized seizures were also frequently observed, even in the acute phase, and the seizures and resultant brain damage were more serious than those observed in the feline models [4, 5, 21]. The difference in seizure severities between canine- and feline-CPSE models might be a reason for the increased CSF-GLU during acute phase of canine CPSE. Furthermore, this increase in CSF-GLU indicates the possibility of severe brain damage in the present model resulting from the excitotoxicity by excessive extracellular GLU concentration [5], whereas, in feline KA model, there were no remarkable histological changes in the amygdala [4, 21]. In addition, the canine model did not develop into chronic recurrent spontaneous seizures because of severe amygdala damage. This pattern is much different from those of KA-induced epilepsy models in other animal species [5, 21].

In the present canine model, the severity of the seizure and the signal intensity observed in the MRI gradually increased up to 48 hr after the onset of CPSE [5, 6]. The continuous increase in CSF-GLU seemed to correlate with this development of seizure severity and MRI changes. To examine this relationship, although it has not been established in the present model [5], an objective stage classification of the symptoms of seizure, such as a kindling model, may be required. Previously, the changes in GLU and GABA were examined by a microdialysis method in the rat hippocampus based on the stage classification for an amygdala-kindling model [22], as well as in a rat model systematically injected with KA [20]. In a kindling model, GLU was 3 times higher than the standard level at stage 3 (i.e., amygdaloid seizures: arrest, salivation, facial twitching) and 5 times greater at stage 5 (i.e., kindling completion: generalized tonic-clonic seizures). In the rat KA model, GLU was higher than the normal control at any stage of seizures. In the kindling model, GABA concentration increased at stage 5. On the other hand, in a KA model, GABA slightly increased during partial seizure, then significantly decreased during the transient stage from partial seizure to the secondary generalized seizures, and finally increased during the secondary generalized seizure. In the present study, CSF-GABA was lower than normal at any time point though not significant in the acute phase. This potential decrease in CSF-GABA might be caused by either an insufficient level of GLU available for synthesis of GABA due to an increase of GLU consumption, or a dysfunction of glutamate transporters induced by the excess release of GLU.

A novel finding in this study is that both CSF-GLU and CSF-GABA significantly decreased at the end of CPSE. In our model CPSE terminated at approximately 72 hr after the onset. Therefore CSF-GLU gradually increased during CPSE and then fell below the standard level at the end of CPSE. Ito [7] reported that GLU in the brain tissue decreased during seizures (while CSF-GLU increased due to the GLU in the brain tissue released into the CSF) and then increased at 30 min after the end of the seizures. This change of GLU in the brain tissue suggested a transient enhancement of GLU synthesis after the excess GLU release. The precursor of GLU as a neurotransmitter is thought to be glutamine, and the released GLU may be recovered by astrocytes via the glutamate transporter and changed to glutamine. The expression of glutamate transporters has been reported to increase in the acute phase of KA- and Fe (3+) -induced seizure models [13, 19, 24]. Thus it may be possible that the decrease of CSF-GLU at the end of CPSE in the present study resulted from the enhancement of GLU-uptake into astrocytes. In fact astrocytic proliferation began to appear from 48 hr after the onset of CPSE [5], and severe astrocytosis was found in the bilateral limbic system at 2 months [6].

In our model the CSF-GABA level tended to be lower than normal control during CPSE and was significantly decreased at the end of CPSE. Generally, excitation in the brain (excitatory postsynaptic potential) induces the release of GABA into the synaptic cleft, which, in turn, induces an inhibitory postsynaptic potential that inhibits excess excitation [8, 10]. Excess GABA is taken up into synaptic terminals or astrocytes via GABA transporters (GAT). However, when the excitation is more excessive and GABA in the synaptic cleft is insufficient, GABA may be released through GAT (reversed GABA release) rather than taken up through GAT [10, 14]. In fact, the increase of GABA in the hippocampal neuronal clefts has been reported in a clinical case of human intractable epilepsy and a kindling model, and has been considered a compensatory inhibitory mechanism responsive to seizures [3, 22, 23]. Paradoxically, the level of GABA release in the brain has been reported to decrease during the epileptic seizures in relation with the decrease of GAT in a kindling model [10]. Thus, the kinetics of GABA during seizures remains controversial, due to differences in the types of seizures in clinical cases and the types and species of the models. It is unlikely that the GAT level decreases in the present model because the present model is of an acute phase of CPSE and not of spontaneous epilepsy or a chronic phase of the model. Another possible mechanism of the decrease of GABA may be a decrease in the synthesis of GABA due to insufficient GLU resulting from excessive release of GLU or a dysfunction of glutamate transporters as mentioned above.

At the end of CPSE (approximately 72 hr after the onset of CPSE), the behavior and EEGs of the animals returned to normal and were free from any seizures [5], in spite of a significant decrease in CSF-GLU and -GABA. Further question remains as to whether the end of CPSE was induced by the depletion of GLU or by the neuronal death of the epileptic focus (bilateral amygdala and hippocampi in this model). It is thought that the depletion of GLU resulted from the dysfunction of synthesis of GLU within neurons of epileptic focus. Perhaps neuronal death had begun from 6–12 hr after the onset of CPSE, as shown by MRI and histology, and further neuronal death was suggested by significant increase in neuron-specific enolase (NSE) in the CSF at the end of CPSE [5]. Therefore, the neuronal death of the entire epileptogenic area may be completed at 72 hr after the onset of CPSE.
In the present study, the kinetics of CSF-GLU and CSF-GABA in a canine KA-induced CPSE model were characterized. In human medical and/or experimental study of epilepsy, it has been suggested that the expression and the function of transporters and receptors might play an important role in changing of the levels of GLU and GABA. To elucidate the pathophysiological mechanisms in canine epilepsy, further comprehensive and/or exquisite studies using biochemical and molecular biological approaches for channels, receptors, and transporters are necessary.

REFERENCES