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The Effect of Topiramate on Hippocampal Neuronal Death and Expression of Glutamate Receptor in Kainate-induced Status Epilepticus Model

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Excitotoxicity and epileptogenesis have often been associated with glutamate receptor activation. Accumulating evidences indicates that topiramate (TPM), an antiepileptic drug with multiple mechanisms of action has neuroprotective activity. We explored the neuroprotective effect of TPM on the status epilepticus (SE)-induced hippocampal neuronal death. After development of SE by kainite injection (15 mg/Kg), rats were treated with TPM (10 mg/kg) for 1 week. The neuronal death was detected by Apop tag *in situ* detection kit, and the expression levels of glutamate receptors were semi-quantitatively analyzed by immunoblot. Kainate-induced SE caused a significant neuronal death and cell loss in CA1 and CA3 regions of hippocampus at 1 week. However, treatment of TPM for 1 week after SE markedly reduced hippocampal neuronal death. The expression of N-methyl-D-aspartate (NMDA) receptor subunit 1, was increased by SE, but was not affected by 1 week treatment of TPM. The expressions of NMDA receptor subunit 2a and 2b were not changed by either SE or TPM. As for α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) glutamate receptors (GluR), kainate-induced SE markedly up-regulated GluR1 expression but down-regulated GluR2 expression, leading to increased formation of Ca^{2+} permeable GluR2- lacking AMPA receptors. TPM administration for 1 week attenuated SE-induced expression of both the up-regulation of GluR1 and down-regulation of GluR2, reversing the ratio of GluR1/GluR2 to the control value. In conclusion, TPM protects neuronal cell death against glutamate induced excitotoxicity in kainate-induced SE model, supporting the potential of TPM as a neuroprotective agent.

Key words – kainate, status epilepticus, glutamate receptor, neuroprotection, topiramate

Kainic acid (KA) and pilocarpine are widely used tools for the induction of experimental epilepsy[3]. KA and pilocarpine models replicate several phenomenological features of human temporal lobe epilepsy and can be used as animal preparations to understand the basic mechanisms of epileptogenesis[55,14].

Local or systemic administration of KA or pilocarpine in rodents leads to a pattern of repetitive limbic seizures and status epilepticus (SE), which can last several hours[55]. A somewhat variable latent period follows SE and precedes the chronic phase, which is characterized by the occurrence of spontaneous limbic seizures. The brain damage induced by SE in such preparations may be considered as an equivalent of the initial precipitating injury event, usually a prolonged febrile convulsion, which is commonly found in patients with mesial temporal lobe epilepsy[33]. Neuropathological changes such as neuronal loss in several hippocampal subfields and reorganization of mossy fibers into the molecular layer of the fascia dentata are observed in both models and are similar to hippocampi from patients with hippocampal sclerosis[54]. These abnormal changes have been suggested to be anatomical substrates for epileptogenesis[54,2].

Glutamate is the principal fast excitatory neurotransmitter in the brain. Excitotoxicity and epileptogenesis have often been associated with glutamate receptor activation[35,4,43, 29]. Glutamate-operated ion channels form the major excitatory neurotransmitter system in the central nervous system and are involved in synaptic plasticity[5,31] and in pathophysiological conditions including epilepsy[11]. The ionotropic glutamate receptors (GluRs) have been categorized on the basis of their preferred agonists: α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA), kainate (KA) and N-methyl-d-aspartate (NMDA) receptors. Activation of the NMDA receptor has been shown to play a key role in neuronal signaling, long-term potentiation(LTP)[5,13], altered neuronal excitability in the kindling model[20], and delayed neuronal cell death[12,34]

Potential neuroprotective effects of the antiepileptic drug, topiramate (TPM) have been evaluated using primary neu-

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ronal astroglial cultures or astroglial-enriched cultures from newborn rats exposed to excitotoxic concentrations of glutamate or kainite[17,38]. TPM [2,3:4,5-bis-O-(1-methylethylidene)β-D-fructo-pyranose sulfamate] is a broad-spectrum antiepileptic drug with multiple mechanisms of action[58]. These include a negative modulatory effect on the AMPA and kainate subtypes of glutamate receptors (GluR)[19,50], a positive modulatory effect on $GABA_A$ receptors[57], attenuation of voltage-gated Na^+ channel activity[15], a negative modulatory effect on a neuronal L-type Ca^{2+} channels [60], and enhancement of some types of and K^+ channel currents[21]. TPM also inhibits some isozymes of carbonic anhydrase, particularly CA-II and CA-IV[16]. Based on these findings, it was hypothesized that TPM might have neuroprotective capabilities by affecting the subtype composition of AMPA GluRs[8,1,24], which determines Ca^{2+} permeability of receptor channels formed.

The aim of this study was to investigate whether TPM has protective effect on SE-induced hippocampal neuronal death via modulating the expression of subtypes of GluRs.

Materials and Methods

Chemicals and reagents

Apoptag *in situ* apoptosis detection kit was purchased from Oncor (Gaithersburg, MD, U.S.A.). The antibody to NMDA receptor subunit type 1 (NMDAR1) were purchased from BD Transduction Laboratories (San Diego, CA, USA). The antibodies to GluR1, GluR2, NMDAR2a and NMDAR2b were obtained from Chemicon International Inc (Temecula, CA, U.S.A.). All the other drugs were purchased from Sigma (St. Louis, MO, U.S.A.) except where indicated.

Animals and experimental design

Twenty male Sprague Dawley rats weighing 150~200 g participated in the study. Seizures were induced by intraperitoneal injection of kainate (15 mg/kg in saline; Tocris, Bristol, UK). Animals were monitored behaviorally for seizures for 6 h after injection. Seizure intensity was evaluated using the scale described by Racine[41]. SE was defined as prolonged seizures for at least 1 h, which was terminated by intraperitoneal administration of diazepam (30 mg/kg) if persisted longer. All the kainate-treated rats received subcutaneous lactated Ringer (3~5 ml) at the end of the period of SE to replenish fluids. Five rats out of seventeen died during or after status epilepticus (survival rate; 70.6%) and five rats which developed only very mild seizures were excluded from the study.

Entire investigation was conducted on 3 experimental groups. Group I rats (controls) received intraperitoneal and subsequently subcutaneous injection of normal saline with the equivalent volume to the kainate and topiramate, respectively (n=3). Group II rats (SE) received intraperitoneal injection of kainate and underwent SE (n=4). Group III rats received treatment with TPM (10 mg/Kg) daily for a week after development of kainate-induced SE (n=3). Animals were sacrificed at 1 week after seizure induction.

Assessment of neuronal damage with TUNEL and propidium iodide (PI) staining

Neuronal damage was assessed by direct fluorescence detection of digoxigenin labeled genomic DNA in the brain sections with simultaneous nuclear staining using Apop Tag *in situ* apoptosis detection kit. Double-stranded DNA breaks identifying apoptotic cells were detected *in situ* by the TUNEL technique on tissue sections which had been fixed in formalin and embedded in paraffin. After deparaffinization with xylene, hydration in graded ethanols and washing in PBS, the sections were treated with 20 mg/ml proteinase K (Boehringer Mannheim Gmbh, Mannheim, Germany) for 30 min at room temperature. After washing, endogenous peroxidase activity was quenched with 2% hydrogen peroxide (Sigma, St. Louis, MO, U.S.A.). The tissue was then reacted with terminal deoxynucleotidyl transferase (TdT) and digoxigenin-labeled UTP, followed by fluorescein-labeled anti-digoxigenin antibody. PI stain was used for counter stain and mounted under a glass coverslip. The slides mounted with Vectashield and examined under the confocal laser scanning microscope (LSM 510, Carl Zeiss, Germany).

Preparation of hippocampal protein

After decapitation, rat brain was transferred immediately to ice-cold Hepes buffered saline (HBS; 142 mM NaCl, 2.4 mM KCl, 1 mM $MgCl_2$, 5 mM D-glucose, 0.1 mM EGTA and 10 mM Hepes [pH 7.5]) and left in -20℃ for 10 min. Brain was homogenized with Ultra-Turrax T25 homogenizer (Jandel & Kunkel, Germany) in the homogenization medium (320 mM Sucrose, 1mM EGTA, 0.1 mM EDTA and 10 mM Hepes [pH 7.5]) containing the protease inhibitor mixture (0.3 mM phenylmethylsufonyl fluoride, 2 μg/ml leupeptin, 4 μg/ml aprotinin and 0.8 μg/ml pepstatin A).

Nuclei and debris were removed after low speed centrifugation (2,500 g for 10 min) at 4℃ and the postnuclear supernatant was incubated with 2% Tween for 1 h in ice to extract membranous proteins. The protein concentration of the final suspension was measured using Bradford's method.

Western blotting

The rat brain membrane proteins were lysed in boiling 5'sodium dodecyl sulfate (SDS) sample buffer. The lysates were boiled for 5 min, and loaded in each lane (20 μg per lane) to be separated by SDS-polyacrylamide gel electrophoresis (PAGE). After transferring the proteins onto a nitrocellulose membrane, the membrane was blocked with Tris-buffered saline-Tween (TBST; 20 mM Tris [pH 7.5], 145 mM NaCl, 0.05% Tween-20) containing 5% skim milk overnight at 4℃, then incubated with primary antibodies (anti-GluR1 & 2 and NMDA receptor1, 2a & 2b) for 1 h at room temperature. After washing three times for 10 min each with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (3000:1, Amersham Life Science Ltd., Buckingamshire, England) for 1 h at room temperature. After washing three times with TBST, the membranes were developed and quantified with an enhanced chemiluminescence detection system (LAS-1000plus, Fuji, Japan).

Results

Behavioral manifestation of SE

Administration of kainate (15 mg/kg, i.p.) caused typical sequential behavioral changes. Shortly after receiving kainate, rats demonstrated strong immobility and staring (stage 1). Within 40 min, this behavior was replaced by automatism such as repetitive head nodding, chewing and blinking, which were often interrupted by increased locomotor activity or 'wet dog shakes' (stage 2). After 90 min of kainate injection, low-intensity tonic-clonic seizures, mostly of the forelimbs (stage 3), usually began and generally progressed into full generalized seizures including rearing, jumping, loss of posture and falling (stage 4-5). Seizures were accompanied by copious salivation and foaming at the mouth. Seizures of stage 4 or 5 were maintained for 2~4 h if not terminated with diazepam. These stages of seizures lasting at least 1 h were regarded as SE and demonstrated in about 41% of kainate-injected rats (7 out of 17 rats).

Behavioral and physical changes after topiramate injection

TPM injection for 1 week induced discernible changes in both the behavior and body weight. TPM-injected rats looked restless, anxious, and fierce compared to those of SE group. There was significant weight loss in TPM treatment group compared to the others (Table 1).

Effect of TPM on hippocampal neuronal death after kainate-induced SE

To investigate whether SE induces hippocampal neuronal death and whether TPM has protective effects on it, neuronal damage was assessed by either structural integrity of hippocampus or the presence of end tail DNA streak in apoptotic cells. In control group of saline-injected mice without SE development, apoptotic cells were not detected and structures of hippocampal area were well demarcated (Fig. 1). However, significant numbers of apoptotic cells in hippocampal area were observed in mice that had experienced kainate-induced SE. In spite of increased apoptosis, the structural integrity of hippocampus was fairly well maintained, presumably due to the short time interval after initiation of apoptosis. In TPM treated group, the numbers of apoptotic cells were significantly reduced compared to SE group, implying a neuroprotective effect of TPM.

Table 1. The changes in body weight after topiramate injection in rats experienced kainate- induced status epilepticus.

Group	*N*	Day 0	Day 7
control	3	152±3.0 g	196±11.4 g
Status epilepticus	4	158±4.7 g	189±14.5 g
Topiramate	3	156±5.2 g	125±7.0 g*

* $p<0.05$ compared to control

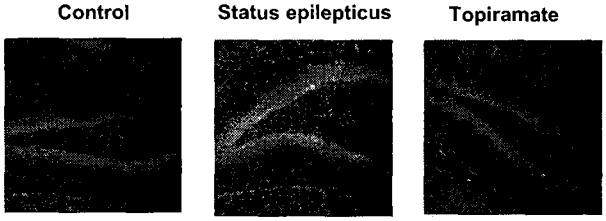

Fig. 1. Topiramate prevents hippocampal neuronal death induced by status epilepticus. Topiramate was administered for 1 week to rats which experienced kainate-induced status epilepticus. Frozen sections at the level of dorsal hippocampus were fixed and DNA fragmentation was detected using TUNEL stain. Note that topiramate decreased neuronal death induced by status epilepticus (X100).

Effect of TPM on expression of glutamate receptors after kainate-induced SE

Subunit combination of AMPA glutamate receptors is an important determinant of neuroexcitability. To investigate whether neuronal death after kainate-induced SE is mediated by neuroexcitability due to increased formation of Ca^{2+}-permeable AMPA receptors and whether TPM affects on this, the expression of ionotropic glutamate receptors were semi-quantitatively assessed with immunoblotting. The expression of NMDA receptor subunit 1 was increased by kainate-induced SE (Fig. 2). However, TPM did not affect the SE-induced expression of NMDA receptor subunit 1. The expression levels of NMDA receptor subunit 2a and 2b were not changed significantly by either SE or TPM treatment. Among the AMPA glutamate receptors we tested, GluR1 expression was markedly up-regulated, but GluR2 expression became significantly down-regulated after 1 week of SE, resulting in the relatively high ratio of GluR1/GluR2 (Fig. 3). Interestingly, TPM administration for 1 week attenuated SE-induced expression of both the up-regulation of GluR1 and down-regulation of GluR2, reversing the ratio of GluR1/GluR2 to the control value.

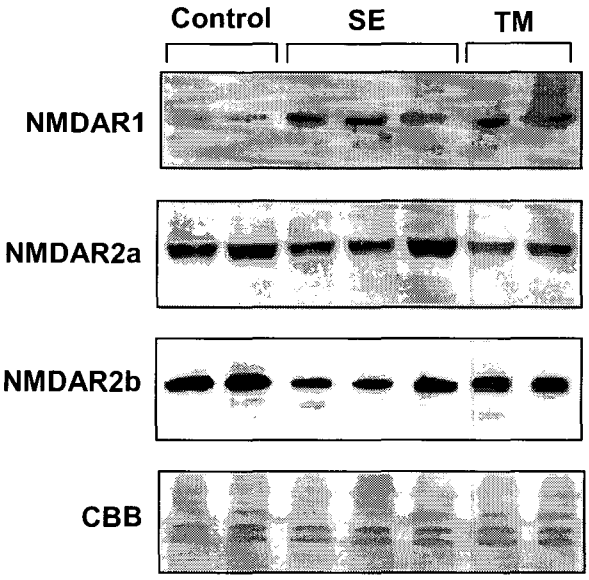

Fig. 2. Expression of NMDA receptor proteins after kainate-induced status epilepticus. After status epilepticus was induced by kainate injection, Topiramate was administered for 1 week. Hippocampal brain proteins were subjected to western blots using antibodies against the NMDA receptor 1, 2a and 2b (NMDAR1, 2a, & 2b). Topiramate did not significantly affect the expression levels of NMDAR induced by status epilepticus. SE, status epilepticus; TM, topiramate; CBB, Coomassie stain.

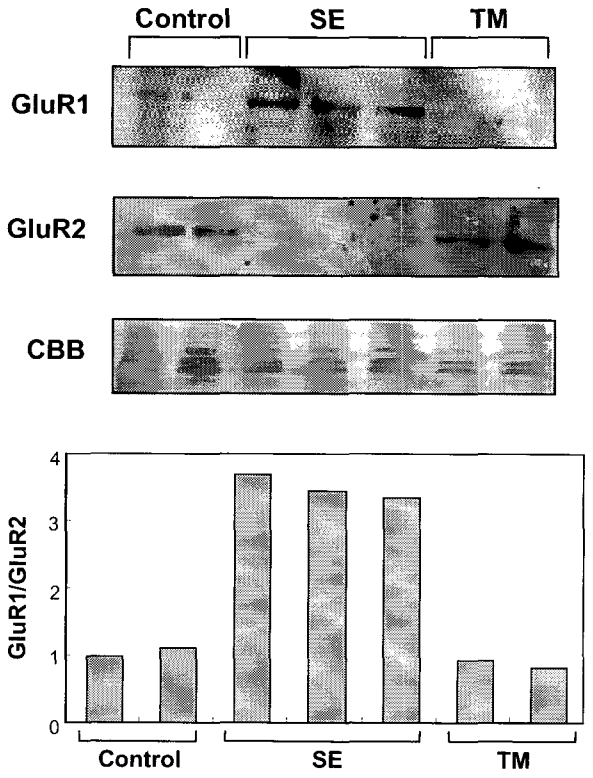

Fig. 3. Expression of AMPA glutamate receptor proteins after kainate-induced status epilepticus. After status epilepticus was induced by kainate injection, Topiramate was administered for 1 week. Hippocampal brain proteins were subjected to western blots using antibodies against the AMPA receptor subunit glutamate receptor 1, 2 (GluR1, 2). Note that the expression of GluR1 increased and GluR2 decreased after status epilepticus, which was reversed by Topiramate treatment. SE, status epilepticus; TM, topiramate; CBB, Coomassie stain.

Discussion

Seizures have many causes and can be precipitated by many types of brain injury such as SE, stroke, head trauma and anoxia[52]. These types of brain injuries can produce a single seizure or lead to the development of epilepsy. The specific molecular signals produced by a brain injury that can induce the development of epilepsy in a previously normal brain are unknown. Several well-established epilepsy models involve the use of SE to produce epileptic animals[7,30]. The pilocarpine or kainate model of epilepsy in rodents is a well characterized model that employs SE to induce epileptogenesis and has many features similar to limbic epilepsy in man[37]. Many of the pathophysiological changes observed in epileptic human brain tissue have been shown to be present in SE-induced epileptic

rats, including hippocampal sclerosis[37], mossy fiber sprouting[37], neuronal hyperexcitability[23], altered receptor function and interictal spike pattern on EEG[27]. Thus, SE-induced epilepsy provides a powerful tool to study the molecular mechanisms underlying epileptogenesis.

Many molecular signals are triggered by SE through activating receptors in neuronal membranes. Neuronal cell loss in the CA1 subfield of the hippocampus in kainate-induced epileptic models has been described previously[26]. Mossy fiber sprouting is a common sequelae following SE[6,56]. Although the present study didn't demonstrate spontaneous recurrent seizure, we observed significant cell loss and neuronal death in the CA1 and CA3 subfield of the hippocampus 1 week after SE. In addition, it was demonstrated that TPM apparently reduced SE-induced neuronal death, one of the changes associated with epileptogenesis.

L-glutamate is the major excitatory transmitter in the vertebrate CNS[45, 36]. In addition to its action as a synaptic neurotransmitter, it produces long-lasting changes in neuronal excitability, synaptic organization, neuronal migration during development, and neuronal viability. The excitatory responses of this endogenous excitatory amino acid are mediated by a number of pharmacologically and functionally distinct membrane receptors, i.e., the ionotropic NMDA, kainate and AMPA receptors as well as metabotropic receptors. Excitotoxicity is believed to be mediated by an excessive synaptic release of L-glutamate and the consequent overstimulation of glutamate receptors, as attenuation of synaptic transmission and application of glutamate receptor antagonist were shown to be neuroprotective[44,49].

Although the molecular basis of glutamate-mediated toxicity is still uncertain, studies of neurotoxicity in cultured neurons have established important pathological roles for intracellular ionic changes caused by glutamate, especially the influx of Ca^{2+} and Na^{+}, or efflux of K^{+}[10,46]. Neuronal activity can lead to a marked increase in the concentration of cytosolic Ca^{2+}, which then functions as a second messenger that mediates a wide range of cellular responses, or can lead to metabolic derangements such as the formation of free radicals and cell death[46,11]. Although it is generally accepted that the NMDA subtypes play a major role, mainly owing to their high Ca^{2+} permeability[42,9]. AMPA receptors also have Ca^{2+} permeability depending on their subtype composition. A wide variety of functionally distinct receptor isoforms are present in neuronal tissue due to alternatively spliced exonic sequences in the respective mRNA as well as to RNA editing of the different subunits. The GluR2 subunit is unique in that it can undergo RNA editing to encode a positively charged arginine(R) residue in the membrane-associated segment of the subunit, while unedited subunits contain a neutral glutamine(Q) residue at this position[22,51]. This editing, which occurs with a very high efficiency, determines the Ca^{2+} permeability of AMPA receptor complexes to make GluR2(R) containing AMPA receptors impermeable to Ca^{2+} or other divalent cations[25]. AMPA receptor subunits also show divergence in the sequence and length of their C-terminal tail, a region that is now known to be responsible for interaction with different intracellular proteins to regulate receptor function, targeting, and trafficking[47]. While a few studies have demonstrated that Ca^{2+} permeability alone may not determine neuronal vulnerability to AMPA receptor-mediated excitotoxicity, Ca^{2+} elevation still concern to excitotoxicity.

In this study, we demonstrated that the expression of GluR1 increased and GluR2 decreased after SE and that this change was reversed by TPM administration. While the GluR1 subunit has attracted interest in studies of synaptic plasticity and organization[31], little is known about its role in excitotoxic neuronal cell death. Lissin and colleagues reported that application of glutamate or AMPA to cultured neurons evoked a rapid and selective redistribution of GluR1 subunits[28], which is unlikely to be as direct consequence of excitotoxicity[45]. Zukin and colleagues provided evidence for the importance of the GluR2 subunit in excitotoxic cell death[53]. They showed that brief forebrain or global brain ischemia triggers a specific decrease in the expression of the GluR2 subunit in hippocampal neurons of the CA1 region[53,40], which was associated with an increased Ca^{2+} permeability of AMPA receptors in these cells. They also reported that SE caused down-regulation of GluR2 mRNA, but not of GluR1 mRNA, in hippocampal CA1 and CA3, as indicated by *in situ* hybridization, about 8 h later, but still before cell death[59], Ying et al. proposed that the increase in GluR4 could contribute to lowering the relative abundance of GluR2, a change that might increase formation of AMPA receptors, which lack a GluR2 subunit and have enhanced Ca^{2+} permeability[59]. Unfortunately, these *in vivo* studies do not allow for a clear distinction as to whether the enhanced neurotoxicity is solely due to increased entry through AMPA receptor or to other GluR2-linked mechanisms[39,18]. In our study, the expression of GluR2 subunit

significantly decreased after SE with that of GluR1 subunit unchanged or slightly increased, which is consistent of the results of other previous studies. After SE, increased ratio of GluR1 to GluR2 is known to be associated with an increased Ca^{2+} permeability and contribute to subsequent neuronal cell death.

In the present study, we found TPM administration reversed SE-induced up-regulation of expression of GluR1/GluR2. We do not know the underlying molecular mechanisms of TPM-induced change of GluR1/2 expression. Further studies are needed to differentiate the sites of modification by TPM to be transcriptional or posttranslational, including mRNA stability and ubiquitin-proteasome-mediated degradation of receptor proteins. By contrast with the effect of TPM on AMPA receptors, it did not affect significantly the expression of NMDA receptors. It was reported that TPM reduced neuronal excitability and could contribute to the anticonvulsant efficacy by specific blockade of the kainite-induced excitatory conductance[57,48]. Their studies revealed that the effect of TPM on kainate-evoked currents was temporally biphasic. TPM partially blocked kainate-evoked currents with an early-onset reversible phase (phase I) and a late-onset phase (phase II) that occurred after at 10 to 20 min delay and did not reverse during a 2 h washout period. John et al. examined whether the blocking effect of TPM was specific for excitatory amino acid receptors activated by kainite (mediated by activation of AMPA and kainate receptors), the sensitivity of NMDA-mediated currents to TPM. TPM had no effect on NMDA-evoked currents at a concentration (100 μM) and exposure time in which kainate-evoked currents were blocked by > 90%[57].

Although this study demonstrated the effect of TPM on the AMPA receptors, we could not exclude it from other properties of TPM such as the activity of voltage activated Na^+ and Ca^{2+} channels, $GABA_A$ receptor, and some isozymes of carbonic anhydrase (CA). Because we did not demonstrate spontaneous recurrent seizure activity, it is difficult to imply these findings directly to the epileptogenesis. However, we observed significant cell loss and neuronal death in the CA1, CA3 subfield of the hippocampus after status epilepticus and then TPM apparently reduced the neuronal death. These observations provide that the neuronal death is associated with status epilepticus and TPM has protective effect against neuronal cell death after status epilepticus.

In summary, the present study demonstrated that TPM protects neuronal cell death against glutamate induced excitotoxicity in kainate-induced SE model, supporting the potential of TPM as s neuroprotective agent. Though further studies including more cases are required, these results may contribute to antiepileptic potential drug development in epilepsy.

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초록 : Kainate 유발 간질중첩증 모델에서 topiramate가 해마 신경세포사와 glutamate 수용체 발현에 미치는 영향

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신경흥분독성과 간질발작발현은 glutamate 수용체활성과 연관이 있다고 알려져 있다. α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA), kainate 수용체에 대한 glutamate 활성을 포함하는 다양한 기전을 가진 항전간제인 Topiramate는 신경보호작용을 가진다는 증거가 제시되어 Topiramate가 간질발작 후 해마의 glutamate 수용체 발현에 미치는 효과를 관찰하였다. 흰쥐에 kainate를 복강 내 주사하여 간질중첩증을 유발시킨 후 Topiramate를 1주일 주사하였다. Apop tag in situ detection kit를 이용하여 세포손상을 관찰한 결과 kainate 유발 간질중첩증 1주일 후 해마의 CA1, CA3에서 심각한 세포사를 보였으나, Topiramte 처리 군에서는 세포사가 현저히 감소하였다. 간질중첩증 이후 NMDA 수용체 아형 1,2a, 2b 발현이 현저히 증가했으나 Topiramate 처치에 의해 NMDA 수용체의 발현에는 뚜렷한 변화가 없었다. AMPA 수용체에서는 GluR1이 간질중첩증 이후 현저히 상향조정 되었고 GluR2는 현저히 하향조정 되었다. Topiramate 1주일 처리 시 간질중첩증으로 인해 변화된 GluR1과 GluR2의 발현이 역전되었다. 결론적으로 Topiramate는 간질중첩증에 의한 GluR1/GluR2 발현비의 증가로 유발되는 흥분성 신경세포사를 억제시킴으로써 신경보호작용이 있는 것으로 보인다.