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EFFECT OF TOPIRAMATE ON THE KAINATE-INDUCED STATUS EPILEPTICUS, LIPID PEROXIDATION AND IMMUNOREACTIVITY OF RATS

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Effect of topiramate on the kainate-induced status epilepticus, lipid peroxidation and immunoreactivity of rats. M. KUBERA, B. BUDZISZEWSKA, L. JAWORSKA-FEIL, A. BASTA-KAIM, M. LEŚKIEWICZ, M. TETICH, M. MAES, G. KENIS, A. MARCINIAK, S.J. CZUCZWAR, G. JAGŁA, W. NOWAK, W. LASOŃ. Pol. J. Pharmacol. 2004, 56, 553–561.

Topiramate, a new anticonvulsant, has been reported to possess neuroprotective effects in both *in vivo* and *in vitro* experiments. In the present study, the effect of topiramate (40 and 80 mg/kg *ip*) on the fully developed kainate-induced *status epilepticus* was evaluated in the rat. Injection of kainate (15 mg/kg *ip*) evoked recurrent limbic seizures which lasted several hours. Topiramate injected 1.5 h after kainate administration had no effect on the seizures and mortality of the animals. Biochemical study revealed that at 80 mg/kg *ip*, topiramate significantly attenuated the kainate-induced lipid peroxidation in the piriform cortex and showed similar tendency in the frontal cortex. Besides the central nervous system, the kainate-induced seizures evoked significant changes in immunoreactivity, such as reduction in thymus weight and the proliferative activity of splenocytes, and the splenocyte-increased production of interleukin-10, but not interferon-γ. Topiramate did not affect the kainate-induced reduction in thymus weight, but attenuated changes in the proliferative activity of splenocytes. It is concluded that topiramate, when given during the fully developed kainate-induced *status epilepticus* in rats, has no effect on seizures, but attenuates lipid peroxidation in piriform cortex and prevents certain changes in immunoactivity.

Key words: *topiramate, kainate, seizures, lipid peroxidation, immunoreactivity, cytokines*

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INTRODUCTION

Topiramate is a new antiepileptic drug which inhibits voltage-dependent sodium channels, blocks glutamate AMPA/kainate receptors and enhances the $GABA_A$ receptor-mediated chloride conductance [25, 30, 32]. An increasing body of evidence indicates that, due to its multiple mechanisms of action, topiramate possesses not only antiepileptic but also neuroprotective properties. The protective effects of topiramate against neuronal injury have been found in a model of global ischemia in gerbils, as well as in the focal cerebral ischemia and *status epilepticus* induced by hippocampal electric stimulation in rats [7, 17, 21, 29, 31]. Since *status epilepticus* is a life-threatening neurologic emergency which leads to neuronal degeneration of vulnerable brain regions, we decided to estimate the efficacy of topiramate in another, well-characterized model, i.e. the kainate-induced seizures.

Ip administration of kainate to rats results in long-lasting recurrent limbic seizures which are followed by neuronal loss, especially in the hippocampus, amygdala, piriform cortex and some thalamic nuclei [1, 16, 22]. In order to estimate the effects of topiramate on the fully developed *status epilepticus* associated with impaired GABAergic transmission and drug resistance, topiramate was administered 1.5 h after kainate injection. The effects of topiramate on the duration of seizures, mortality and lipid peroxidation as a marker of neurodegenerative processes were evaluated. Furthermore, since the kainate-induced seizures cause changes in the immune system, such as reduction of thymus weight and alterations in pro-inflammatory cytokine levels [4, 8, 14], we examined the effects of topiramate on the weight of primary and secondary immune organs and the proliferative activity of splenocytes.

MATERIAL and METHODS

Male Wistar rats (200–250 g), purchased from a licensed dealer, were used. The rats were housed in groups of six per cage, under a standard 12/12 h light-dark cycle (08.00/20.00), with food and water *ad libitum*. Experimental protocols were approved by the local Ethics Committee and complied with the guidelines of the responsible agency of the Institute of Pharmacology.

Seizures were induced by *ip* injection of kainate (RBI, 15 mg/kg; in a volume of 2 ml/kg). Control rats received the solvent (physiological saline) *ip.* After injection, the rats were placed in individual cages and were continuously observed for the occurrence of convulsions. Ninety minutes after kainate administration, the rats which developed *status epilepticus* received *ip* injection of topiramate (Topamax; Cilag AG, Schaffhausen, Switzerland) or physiological saline, and were observed for another 5 h. The number of rats convulsing throughout 5 h, and the number of rats dead within a period of 72 h after kainate administration, compared to the total number of the tested animals, were recorded for each treatment group.

Effects of topiramate on the kainate-induced lipid peroxidation

Seventy two hours after kainate injection, the rats were killed by rapid decapitation and their brains were removed. The piriform and frontal cortices were dissected, rapidly frozen in liquid nitro-

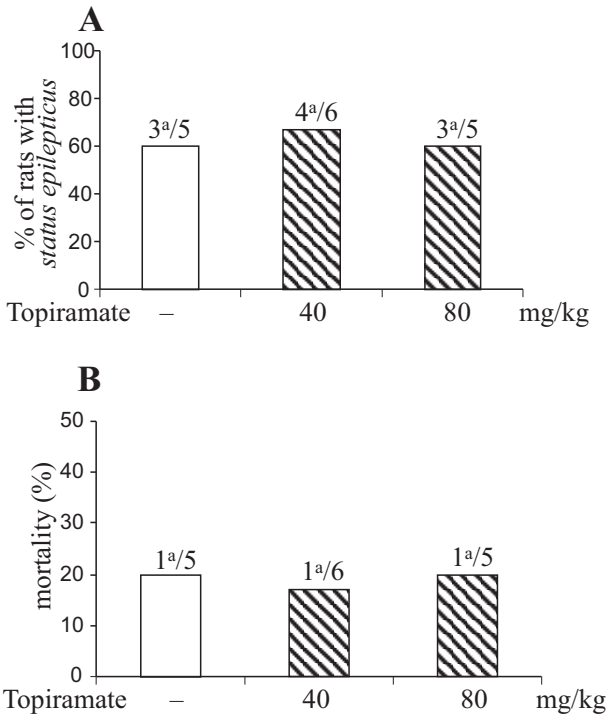

Fig. 1. The effect of topiramate (TOP) on kainate-induced *status epilepticus* (A) and mortality (B) in male rats. TOP was administered *ip* 1.5 h after kainate (KA) (15 mg/kg). The data represent the percentage of rats with *status epilepticus* within 5 h (A) or the percentage of rats dead throughout a period of 3 days (B) after KA administration. [a] number of animals with *status epilepticus* (A) or dead (B) *vs.* total number of animals

gen, and transported to Lublin for an assay of lipid peroxidation. The cortices were homogenized in 10 volumes (10 ml per gram of wet tissue) of an ice cold Tris buffer (pH 7.4) containing 5 mmol of butylated hydroxytoluene to prevent sample oxidation. After centrifugation at 3000 × g for 10 min at 4°C, 200 µl of the supernatant were assessed for the concentration of the lipid peroxidation end product, malondialdehyde (MDA), using a lipid peroxidation assay kit (Bioxytech LPO 586, Portland USA) according to the manufacturer's instructions. The reaction of MDA with the chromogenic reagent yielded a stable end product (with a maximum absorbance at 586 nm) after incubation at 45°C for 40 min [20]. The reaction was stopped on ice. The samples were centrifuged at 10 000 × g for 10 min, and the absorbance was measured at 586 nm. Using standard curves generated with samples of 1,1,3,3-tetrametoxypropane, the concentration of a chromophore in the solution was calculated and expressed as picomoles of MDA per milligram of protein. A protein content was determined by the method of Lowry et al. [18] using bovine albumin as a standard.

Estimation of topiramate effects on the kainate-induced immunological changes

Preparation of cell suspensions

Immediately after sacrifice, spleens were gently crushed in individual glass homogenizers. Cells were resuspended in RPMI-1640 medium (Sigma, USA), and were centrifuged at 500 × g for 5 min. Cell pellets were resuspended in the RPMI-1640 medium for an MTT assay, or in the medium supplemented with antibiotics and a 10% fetal bovine serum (FBS, Sigma, USA) for other studies.

Proliferative response of splenocytes to mitogen stimulation in vitro

The proliferative response of spleen cells was described earlier by Kubera et al. [13]. Briefly, rat spleen cells were obtained from kainate- and/or topiramate-treated animals; 4×10^5 splenocytes per ml were stimulated with 2.5 µg/ml of concanavalin A (Con A). The cells were incubated in 96-well plates at 37°C for 72 h, at a final volume of 0.2 ml. Cell proliferation was determined by adding 0.5 µCi of [^{3}H]-thymidine per well (ICN, USA, SpA 6.7 Ci/mmol) 16 h before the end of the incubation.

Colorimetric MTT assay

The MTT assay is based on the conversion of a yellow tetrazolium salt to a colored formazan product by enzymes in viable cells. This assay is widely used for cell proliferation and cytotoxicity studies. The cellular bioreduction of MTT is associated with mitochondrial dehydrogenases and with enzymes of the endoplasmatic reticulum; it involves the reduced pyridine nucleotides NADH and (to a lesser extent) NADPH. The concentration of the colored product can be measured spectrophotometrically and is proportional to the number of viable cells. This method was described by us previously [15]. In short, MTT (3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma, USA) was dissolved in PBS at 5 mg/ml. A stock MTT solution (10 µl per 100 µl of splenocytes; a suspension of 4×10^6 rat splenocytes per ml) was added to all the wells and the plates were incubated at 37°C for 1.5 h. Thereafter 100 µl of a lysine buffer (acid-isopropanol; POCh, Poland), were added to each well, and the plates were read 30 min later with a Uniscan II reader (Labsystem, Finland) at a wave length of 570 nm.

Determination of cytokines

Rat splenocytes were tested for their ability to produce interleukin-10 (IL-10) and interferon-γ (IFN-γ) after mitogen stimulation. Splenocyte suspensions were seeded at a concentration of 4×10^6 cells/ml in 24-well Corning tissue culture plates, and then were stimulated with a Con A solution (2.5 µg/ml). Cell-free supernatants were collected 72 h later and stored at –20°C.

For an enzyme-linked immunosorbent assay (ELISA), 150 µl of the appropriate anti-lymphokine "capture" antibody (for IL-10: 0.5 µg/ml MoAb from BD-Pharmingen, Dendermonde, Belgium, catalogue number 555083, clone A5-7; for IFN- γ: 2 µg/ml MoAb from U-CyTech, Utrecht, The Netherlands, clone DB-1) were added to a 96-well flat-bottom high-binding plate and incubated overnight at 4°C. The wells were then washed twice with a washing solution (PBS, containing 0.1% Tween 80). Two hundred µl of the "blocking solution", PBS with 3% BSA, were added to each well, and the plates were incubated at 37°C for 1 h. The wells were washed twice. Aliquots of the dilution of the tested supernatants (125 µl for IL-10 studies and 100 µl for IFN-γ studies) were added to the experi-

mental wells. For both cytokines, the plates were incubated at 37°C for 2 h. The plates were washed five times with PBS containing 0.01% Tween-80 and detection antibodies were added for 1 h at 37°C (125 µl of 0.5 µg/ml MoAb-biotin: BD-Pharmingen 555084, clone A5-7 for IL-10 studies, and 100 µl of 1 µg/ml Poab-biotin from U-CyTech, Utrecht, The Netherlands for IFN-γ). The plates were washed five times, and afterwards streptavidin conjugated horse-radish peroxidase (DiaMed-Eurogen, Tessenderlo, Belgium) was added for a 20 min incubation at 37°C. After a final fivefold wash, 100 µl of the substrate solution (DiaMed-Eurogen, Tessenderlo, Belgium) were added to each well. A blue color developed within 20 min. The reaction was stopped by the addition of 50 µl of 1M H_2SO_4 to each well. The blue color then turns to yellow, and the color intensity in each well is determined by reading the plate at 450 nm in an ELISA-plate reader.

The amount of lymphokines in each well was determined on the basis of a standard curve which was generated using lymphokine standards.

The intra-assay CV values for both those analyses did not exceed 10%. In our laboratory detection limits were 5 pg/ml for IFN-γ, and 10 pg/ml for IL-10. The viability of cells was checked with trypan blue.

Statistics

Fisher's exact probability test was used for a statistical analysis of the effects of topiramate on seizures and mortality. Immunological and biochemical data were subjected to a two-way analysis of variance (ANOVA), followed by Duncan's or LSD test.

RESULTS

Ip administration of kainate evoked recurrent limbic seizures in rats, as described previously [1]. Briefly, the first behavioral changes, observed 30 min after kainate injection, were wet dog shakes and intermittent periods of immobility. The frequency of the shakes gradually decreased as convulsions (facial muscle myoclonus, forelimb clonus, bizzare position accompanied with hypersalivation) occurred. 90 min after kainate injection, ca. 50 % of the animals developed a *status epilepticus* associated with rearing, head nodding, salivation and occasional falling down. The seizures lasted approximately 7 h.

Effects of topiramate on the kainate-induced status epilepticus and mortality

Topiramate at doses of 40 or 80 mg/kg had no remarkable effect on the fully developed *status epilepticus* in kainate-injected rats (Fig. 1A). No significant effect of either dose of topiramate on the kainate-induced mortality was observed (Fig. 1B).

Effect of topiramate on the kainate-induced lipid peroxidation

A biochemical assay showed that the kainate-induced seizures increased lipid peroxidation in the frontal and piriform cortices by 70 and 171%, respectively. The rats that received kainate injection but did not develop seizures showed no changes in lipid peroxidation (data not shown). Topiramate at a dose of 80 mg/kg significantly reduced the kainate-induced lipid peroxidation in piriform cortex, but had no effect on that parameter when it was used at the lower dose (40 mg/kg) (Fig. 2). Similarly, topiramate at a dose of 80 mg/kg, but not at a dose of 40 mg/kg, decreased kainate-induced lipid peroxidation in the frontal cortex, however, this effect was not statistically significant (data not shown). No effect of topiramate alone on the MDA concentration was observed.

Effect of topiramate on the kainate-induced changes in the immunoreactivity of rats

Seventy two hours after kainate injection, the rats showed a marked decrease (by ca. 50%) in the

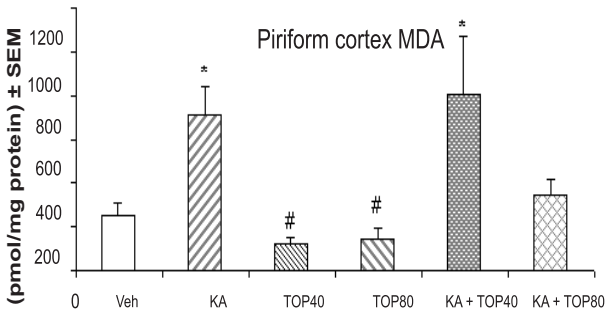

Fig. 2. The effect of topiramate (TOP) on kainate (KA)-induced lipid peroxidation in piriform cortex. TOP was administered *ip* 1.5 h after KA (15 mg/kg). Lipid peroxidation was assessed 72 h after KA administration. The results are shown as the means ± SEM obtained from 3–5 rats. * $p < 0.05$ *vs.* vehicle group; # $p < 0.05$ *vs.* KA-treated rats (ANOVA following Duncan's test)

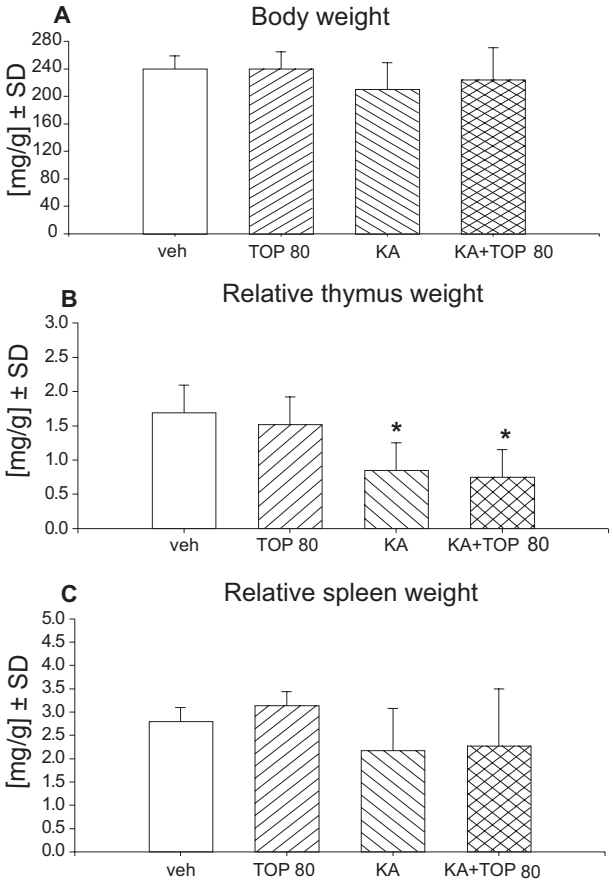

Fig. 3. The effect of topiramate (TOP) and kainate (KA) on the body weight (A), relative thymus weight (B) and relative spleen weight (C) 72 h after KA injection. The relative spleen and thymus weight was estimated as a ratio of thymus weight in mg to body weight in g. All the results are shown as the mean ± SD. The significance of differences between the means was evaluated by the LSD test following a two-way analysis of variance. * $p < 0.05$ *vs.* vehicle (veh) group, n = 4

thymus weight, whereas the weight of their spleens did not differ from that of the control animals. Topiramate (80 mg/kg) had no effect on either the thymus or the spleen weight in either saline- or kainate-injected animals (Fig. 3). The splenocytes of kainate-injected animals exhibited a weaker proliferative response to Con A, whereas topiramate elicited an opposite effect. The proliferative response of splenocytes in rats pretreated with kainate and topiramate was not different from that in the control, but was statistically significantly higher than in kainate-treated animals (Fig. 4.). Topiramate moderately increased the metabolic activity of splenocytes in control, but not in kainate-treated rats (Fig. 5). Furthermore, a statistically significant increase in IL-10 production was observed

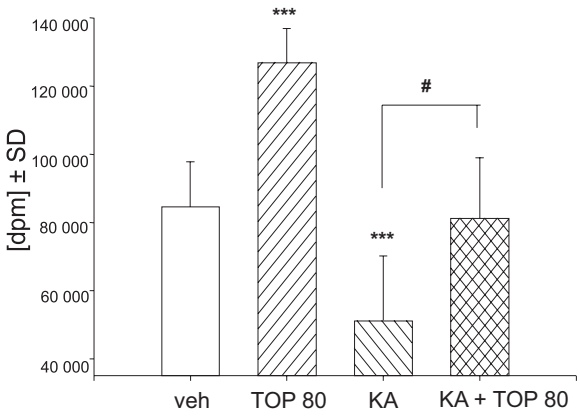

Fig. 4. The effect of topiramate (TOP) and kainate (KA) on the proliferative activity of splenocytes after concanavalin (Con A) (2.5 μg/ml) stimulation, assessed 72 h after KA injection. All the results are shown as the means ± SD. The significance of differences between the means was evaluated by the LSD test following a two-way analysis of variance. *** $p < 0.001$ *vs.* vehicle (veh)-treated control rats; # $p < 0.001$ *vs*. KA-treated group, n = 4

in non-stimulated splenocytes obtained from topiramate- or kainate-treated rats with and without the *status epilepticus*. Moreover, the IL-10 production was significantly higher in non-convulsing rats in comparison with rats with the *status epilepticus* (Tab. 1). Con A had also a strong stimulatory effect on IFN-γ production but this response was affected neither by kainate nor topiramate administration (Tab. 1).

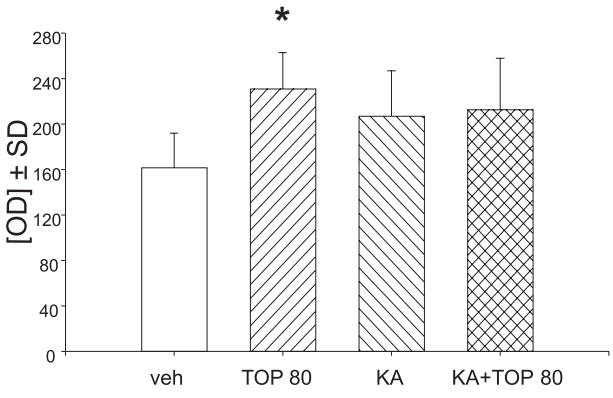

Fig. 5. The effect of topiramate (TOP) and kainate (KA) on the metabolic activity of splenocytes (an MTT test), examined 72 h after KA injection. The metabolic activity was expressed in optical density units. All the results are shown as the means ± SD. The significance of differences between the means was evaluated by the LSD test following a two-way analysis of variance. * $p < 0.05$ *vs.* vehicle (veh)-treated control rats, n = 4

Table 1. The effect of kainate (KA) and topiramate (TOP 80) on cytokine production

Rat treatment	Con A	IFN-γ (IU/ml)	IL-10 (pg/ml)
Vehicle	–	10 ± 9	314 ± 147
	+	843 ± 295	2442 ± 737
KA	–	19 ± 12	764 ± 160*
	+	1116 ± 955	2998 ± 1463
TOP 80	–	58 ± 27	660 ± 280*
	+	1424 ± 474	2933 ± 729
KA + TOP 80	–	46 ± 31	557 ± 191
	+	1391 ± 663	2661 ± 292
KA without *st. epilepticus*	–	34 ± 15	1236 ± 294**#
	+	1079 ± 685	3644 ± 3436

The data are expressed as the means ± SD; n = 4 for each group. The ability of splenocytes to produce cytokines was determined in duplicate for each animal. * $p < 0.05$, ** $p < 0.001$ *vs.* vehicle-treated control rats; # $p < 0.01$ *vs.* KA-treated rats with *status epilepticus* (LSD test); for more information see the Materials and Methods

DISCUSSION

The present study has shown that when topiramate is administered during the fully developed kainate-induced *status epilepticus* to rats, it has no effect on seizures and mortality. The lack of effect of topiramate on the *status epilepticus* contrasts with the efficacy of this drug in preventing seizures, when it is given prior to a convulsant [26, 27]. The *status epilepticus* is characterized by the failure of some inhibitory neuronal mechanisms, e.g. $GABA_A$ receptors become less sensitive to the inhibitory action of benzodiazepine, while their responsiveness to barbiturates is maintained [9, 10]. These findings suggest that the *status epilepticus* differentially affects the functional activity of various modulatory sites of the $GABA_A$ receptor complex and, possibly, that the interaction between topiramate and the $GABA_A$ receptor is also disturbed.

It is well known that the *status epilepticus* triggers an excitotoxic cascade, in the final phase of which free radicals are generated and lipid peroxidation takes place. Although it is commonly agreed that the kainate-induced *status epilepticus* increases lipid peroxidation in some limbic regions, there are some differences regarding the time-course of these changes. Dal-Pizzol et al. [5] showed that the lipid peroxidation occurring in the hippocampus in the acute phase of *status epilepticus* can be extended to at least 12 h after a spontaneous recovery from the *status epilepticus* in both pilocarpine or kainate models. Other studies reported a significant increase in MDA concentration in the rat hippocampus after 4 and 24 h, which remained unchanged 48 h after kainate administration [12]. On the other hand, Bruce and Baudry [3] demonstrated that kainate increased MDA content in the rat hippocampus and piriform cortex after 8 and 16 h, which was followed by its significant decrease after 48 h and 5 days.

We found that lipid peroxidation in the frontal and piriform cortices was significantly enhanced at 72 h following kainate administration. The increase in the level of MDA was considerably higher in the piriform cortex, which correlates well with higher vulnerability of this structure to seizure-induced damage**.** Topiramate, used at the higher dose, significantly reduced the kainate-induced increase in MDA content in piriform cortex and to a lesser degree that in frontal cortex. These findings suggest that topiramate may interfere with excitotoxic processes even at their later stage. The lack of effect of the lower dose suggests that in order to induce neuroprotective effects, high doses of this drug should rather be used. This observation is in agreement with the findings of other studies that doses as high as 100 and 200 mg were effective against the ischemia-induced neuronal damage [17]. Thus, topiramate attenuated one of the important biochemical stages of the excitotoxic cascade, i.e. lipid peroxidation in the rat piriform cortex.

The kainate-induced seizures lead to severe changes not only in the central nervous system, but also in the immune one. In agreement with our previous studies on rats and mice, the kainate-induced seizures produced a dramatic decrease in the thymus weight [4, 14]. Such a marked reduction in the thymus weight may be due to a direct toxic effect of kainate on thymocytes (despite the fact that kainate receptors have not yet been found in the thymus), or to an elevated corticosterone plasma level during the kainate-induced seizures [8]. A corticosteroid-induced thymus atrophy was also observed

in some kinds of stress, or after a pathogenic infection [11]. Alternatively, the effect of kainate on immununoactivity may be connected with lesions of the brain structures involved in immunoregulation. It has been shown that brain lesioning affects the humoral and cell-mediated immunity, as well as the thymus weight. The kainate-evoked seizures led to both a neuronal loss of hippocampal pyramidal cells and a reduction of the relative thymus weight in mice [4]. A significant thymus weight reduction was also observed following electric lesion of the hypothalamus in mice [19]. The massive reduction of the thymus weight observed in kainate-injected animals is probably responsible for the decrease in splenocyte proliferative response to Con A stimulation. In our previous study, a significant diminution of the ability of splenocytes to produce IL-2 after Con A stimulation was also described [14].

In the present study, the decrease in the thymus weight after kainate injection was not affected by topiramate. On the other hand, topiramate given to kainate-pretreated animals significantly enhanced the proliferative activity of their splenocytes. Moreover, topiramate alone also significantly potentiated the proliferative activity of splenocytes, which is difficult to explain. Topiramate antagonizes glutamate AMPA/kainate receptors, enhances $GABA_A$ receptor activity, and blocks voltage-dependent sodium channels, but the possible participation of these mechanisms in the effect of topiramate on immunoactivity remains unclear. The effect of GABA treatment on the immune cells was the focus of a few studies only. Those studies showed that GABA increased the cytotoxic activity of T cells [2], but potently decreased T cell proliferation *in vitro* in response to anti-CD3 and antigen-specific stimulation, and attenuated the T cell-dependent DTH response *in vivo* [28].

Although the expression of AMPA receptors on the immune cells has not been examined so far, functional studies [23] suggest their presence there. To the best of our knowledge, the effect of topiramate on immune cell activity has not been studied to date, but some investigations have been conducted with NBQX – another AMPA/kainate antagonist. NBQX has been found not to alter the proliferative activity of antigen-primed T cells *in vitro*, which indicates its lack of effect on this immune parameter [23].

According to the above-cited literature data on the role of $GABA_A$ and AMPA receptors in lymphocyte proliferation, topiramate should suppress rather than stimulate the proliferative activity of splenocytes. Also the ability of topiramate to block voltage-dependent sodium channels is unlikely to be responsible for the stimulation of immunocyte proliferation, since other blockers of this channel (carbamazepine, valproate) decrease the proliferation of lymphocytes (our unpublished data).

Another interesting finding of this study is the enhanced ability of splenocytes to produce IL-10 in kainate-injected rats which did not develop a *status epilepticus*. Since IL-10 is an anti-inflammatory cytokine, it may be speculated that the increase in IL-10 concentration is a part of an endogenous anticonvulsant mechanism responsible for seizure inhibition in some kainate-injected rats. Indeed, it has been reported that endogenous and exogenous pro-inflammatory cytokines promote seizure generation in various models of experimental epilepsy and in humans, whereas antibodies against these cytokines produce an opposite effect [6, 24].

Surprisingly, both topiramate and kainate enhanced the basal production of anti-inflammatory cytokine – IL-10. Despite unidirectional changes in the cytokine level, the mechanism and functional significance of kainate and topiramate action on this parameter may be different. Topiramate-induced IL-10 production may be a part of its hitherto unknown anticonvulsant mechanism, whereas the increase in the cytokine level after kainate injection may be an adaptive (protective) response to a harmful agent administration. Modulatory effect of kainate and topiramate on IL-10 production cannot be regarded as an unspecific reaction, since the level of the other cytokine (INF-γ) was not affected by these substances.

Summing up, this study revealed that topiramate had no effect on termination of kainate-induced *status epilepticus* or mortality, but attenuated lipid peroxidation in the piriform cortex and reversed the kainate-induced decrease in proliferative activity of splenocytes. This indicates that topiramate may attenuate some deleterious effects of kainate on the central nervous system and immune parameters.

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