Neuroscience Letters 468 (2010) 277–281

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Myo-inositol treatment prevents biochemical changes triggered by kainate-induced status epilepticus

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ARTICLE INFO

Article history:
Received 16 September 2009
Received in revised form 3 November 2009
Accepted 4 November 2009

Keywords:
Epilepsy
Kainic acid
Myo-inositol
Glutamate receptors
Calcium/calmodulin-dependent protein kinase II

ABSTRACT

Identification of the compounds preventing the biochemical changes underlying the epileptogenesis process is of great importance. We have previously shown that myo-inositol (MI) administration reduces kainic acid (KA) induced seizure scores. MI treatment effects on biochemical changes triggered by KA induced status epilepticus (SE) were investigated in the present study. After SE one group of rats was treated with saline, whereas the second group with MI. Control groups received either saline or MI administration. Changes in the amounts of following proteins were studied in the hippocampus and neocortex of rats: GLUR1 subunit of glutamate receptors, calcium/calmodulin-dependent protein kinase II (CaMKII), and heat shock protein 90. No changes were found 28–30 h after experiments. However on 28th day of experiment the amounts of GLUR1 and CaMKII were strongly reduced in the hippocampus of KA treated animals but MI significantly halted this reduction. Obtained results indicate anti-epileptogenic features of MI on biochemical level.

Epilepsy is a heterogeneous group of disorders. It is the most common neurological disorder after the stroke, with a 2–3% lifetime risk of being given a diagnosis of epilepsy [3]. Despite the progress in the pharmacotherapy of epilepsy, about one-third of epileptic patients are resistant to current treatments [14,22]. Available therapy is only symptomatic and often ineffective [14,19,22]. At present there is no any anti-epileptic drug (AED) which could effectively prevent epileptogenesis, the process by which a normal brain becomes epileptic [14] and thus the search for a truly anti-epileptogenic drugs is a problem of outstanding importance for modern biomedical sciences.

Series of our previous experiments have revealed that water extract of plant *Aquilegia vulgaris* (medicinal herb for epilepsy and insomnia treatment) contains compounds altering binding of ligands to the benzodiazepine and γ-aminobuturic acid (GABA) binding sites of the GABA-$_A$ receptors [23]. We have identified two such compounds of this extract: (1) *myo*-inositol (MI) and (2) oleamide—sleep inducing lipid. MI inhibits ^{3}H-muscimol binding to GABA$_A$ receptors and oleamide stimulates binding of ^{3}H-flunitrezepam binding *in vitro* [23]. We have further shown that administration of MI significantly reduces the grade of seizures induced by pentylentetrazol or kainic acid (KA) [25].

The central nervous system is characterized with relatively high concentrations of MI as well as the means to synthesize it (reviewed in [10]). MI serves not only as a precursor molecule for inositol lipid synthesis, but also as a physiologically important osmolyte (reviewed in [10]). Alteration in MI deposition may play a role in a number of neuropathological conditions, either as a physiologically important osmolyte or as a precursor molecule for phosphoinositide synthesis (reviewed in [10]). MI abnormalities take place in temporal lobe epilepsy (TLE) [29] and Na^+/MI co-transporter is up-regulated after KA induced seizures [18]. Gene coding for MI monophosphatase 2 is likely to be febrile seizure susceptibility gene [17]. We have questioned if MI treatment could influence the biochemical changes following status epilepticus (SE)?

Local or systemic administration of KA in rodents leads to a pattern of repetitive limbic seizures and SE, which can last for several hours [6,13]. SE is followed by a latent period and precedes the chronic phase, which is characterized by the occurrence of spontaneous limbic seizures [4]. Kainate model replicates several phenomenological features of human TLE and is widely used as

Abbreviations: KA, kainic acid; SE, status epilepticus; αCaMKII, α-subunit of calcium-calmodulin-dependent protein kinase II; GLUR1, GLUR1 subunit of α-amino-3-hydroxy-5-methyl-4-isoxazole propionate type glutamate receptors; HSP90, heat shock protein 90; ANOVA, analysis of variance; *P*, probability; MI, myo-inositol; GABA, γ-aminobuturic acid.

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doi:10.1016/j.neulet.2009.11.012

animal preparation to understand the basic mechanisms of epileptogenesis [4,15].

A number of molecular changes take place at the early and latent phase after SE, which may contribute to the process of epileptogenesis and therefore the drugs or molecules that can block damage-induced plastic changes may also suppress the development of epilepsy [13–15].

The present work was aimed to study the effects of MI treatment on biochemical changes following KA induced SE before the onset of chronic seizures. The quantitative changes in the following proteins were investigated; (i) α-subunit of calcium-calmodulin-dependent protein kinase II (αCaMKII); (ii) GLUR1 subunit of α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) type glutamate receptors (GLUR1) and (iii) heat shock protein 90 (HSP90). The reasons for choosing these proteins for the study of MI treatment effects were the following: (i) there is a strong evidence that decreased expression of αCaMKII is sufficient to induce limbic epilepsy ([5,8] reviewed in [16]); (ii) the amount of GLUR1 immunopositive neurons is known to be drastically reduced after SE [27]; (iii) whereas the amount of HSP90 is not changed [11]. Here we have shown that daily MI treatment significantly attenuates the decrease in the amounts of αCaMKII and GLUR1 in the hippocampus of rats on 28th days following KA induced SE.

Male Wistar rats received intraperitoneal injection of kainic acid (10 mg/kg, Sigma) dissolved in phosphate-buffered saline. Each animal was placed into an individual plastic cage for observation lasting 4 h. Seizures and tonic–clonic convulsions were recorded. Seizures were scored according to a modified Racine scoring system from 0 to 6: (0) no motor seizures; (1) freezing, staring, mouth or facial movements; (2) head nodding or isolated twitches, rigid posture; (3) tail extension, unilateral–bilateral forelimb clonus; (4) rearing, in which the mice sit in an immobile state on their rear haunches with one or both forepaws extended; (5) clonic seizures, loss of posture, jumping and forepaws extended; (6) tonic seizures with hindlimb extension [9,20]. 28 animals with seizures grade 4–6 were selected. The same amount of control rats received saline injections.

Experiments were carried out on four groups of Wistar rats and each group was divided in two subgroups according to the time of decapitation (the first or the 28th day after the start of experiment). Animals of the first and second groups were comprised of 28 animals with seizures grade 4–6 (see above). Six hours following kainic acid treatment first group received saline injection (KA + Sal group), whereas the second group with the same time interval received MI (30 mg/kg) injection (KA + MI group). Each of these two groups was divided further in two subgroups according to the time of decapitation. Half of KA + Sal group received saline injection on the next morning and half of the KA + MI group received MI injections and animals from the both subgroups were killed 10 h afterward. Thus this time corresponded to the 28–30 h following KA treatment (1st day). Remaining rats were treated twice daily either by saline (KA + Sal group) or MI (KA + MI group) during the following 27 days and decapitated. From the 25th day of experiment rats were monitored for the existence of seizures for 11–12 h during the day. No seizures were observed during this indicated period. One animal died from each group and thus further biochemical experiments were carried out on six animals per each subgroup.

The third and fourth groups of animals were not treated by chemical convulsant. Rats of the one group received saline injection twice a day (Con + Sal group) and another group twice a day MI injection (30 mg/kg, Con + MI group). They were killed according to the same time schedule as groups treated with KA. During the whole experiment rats were housed in cages with free access to water and food. All experiments on animals were carried out at the I. Beritashvili Institute of Physiology and were performed in compliance with approved Institutional Animal Care Guidelines.

After decapitation two regions were removed from each brain—hippocampus and neocortex and frozen immediately on dry ice. Tissue from each region and single rat formed a single sample. We have carried out pilot experiments for the estimation of sample size by power analysis for 1-way ANOVA. The number of animals used was estimated to be the minimum required for adequate statistical analysis.

Brain tissue samples were rapidly homogenized in a buffer: 20 mM Tris–HCl (pH 7.4), 0.32 M sucrose, 1 mM ethylenediaminetetraacetic acid, 0.5 mM ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid containing cocktail of protease inhibitors and centrifuged at 1000 × *g* for 10 min. The supernatant (nuclear-free homogenate) was used in subsequent experiments. A concentrated solution of sodium dodecyl sulphate (SDS) was added to the homogenate fraction to give a final concentration of 5%. In all fractions, protein concentration was determined in quadruplicate using a micro bicinchoninic acid protein assay kit (Pierce). Aliquots containing 30 μg of protein and of equal volume were applied to the gels. SDS gel electrophoresis and Western blotting were carried out as described previously [24]. After protein had been transferred onto nitrocellulose membranes, the membranes were stained with Ponceau S solution to confirm transfer and the uniform loading of the gels. The membranes were then washed with phosphate-buffered saline + 0.05% Tween 20 and cut according to the molecular weight standards in 3 parts to carry out separate immunostaining with used antibodies. The upper part of the filters were stained with antibodies against GLUR1 (Chemicon International), the second part with monoclonal antibody against HSP90 (Sigma), the third part was stained with monoclonal antibody (Chemicon International) against αCaMKII. This part of the filter was stripped and equal protein loading was verified by total actin staining (antibodies from Santa Crus Biotechnology Inc.) of the membrane.

Standard immunochemical procedures were carried out using peroxidase-labelled secondary antibodies and SuperSignal West Pico Chemiluminescent substrate (Pierce). The blots were then exposed with intensifying screens to X-ray films pre-flashed with Sensitize (Amersham). The optical densities of bands corresponding to the GLUR1, HSP90, αCaMKII and actin measured using LabWorks 4.0 (UVP). The autoradiographs were calibrated by including in each gel four standards comprising the homogenate fraction from the brain of untreated rats. Each standard contained 15–60 μg total protein. Optical density was proportional to the amount of GLUR1, HSP90 and αCaMKII (see Fig. 1). To obtain the data given in Fig. 2 the optical density of each band from an experimental sample (e.g. hippocampus of KA + MI rat, 1 day) was divided by the optical density which, from the calibration of the same autoradiograph, corresponded to 30 μg of total protein in the standard [24]. The data expressed in this way will be referred to as "relative amount" of GLUR1, HSP90 or αCaMKII.

Because of the functional differences between the hippocampus and neocortex, data from these two regions were analyzed separately. Time points after the starting of experiment were also analyzed separately. Within the region data for the amounts of GLUR1, HSP90 and α-CaMKII were analyzed by the one-way analysis of variances (one-way ANOVAs). Planned comparisons between were made using *t*-tests. All statistical tests were two-tailed.

Antibodies against GLUR1 subunit of AMPA glutamate receptor bound to a band of molecular weight 103 kDa, corresponding to rat GLUR1 subunit; antibodies against HSP90 bound to a band of molecular weight 86 kDa corresponding to a rat HSP90 protein; and antibodies against αCaMKII bound to a band of 52 kDa corresponding to rat αCaMKII (Fig. 1a–c):

Brain regions, time after starting of experiments and protein levels.

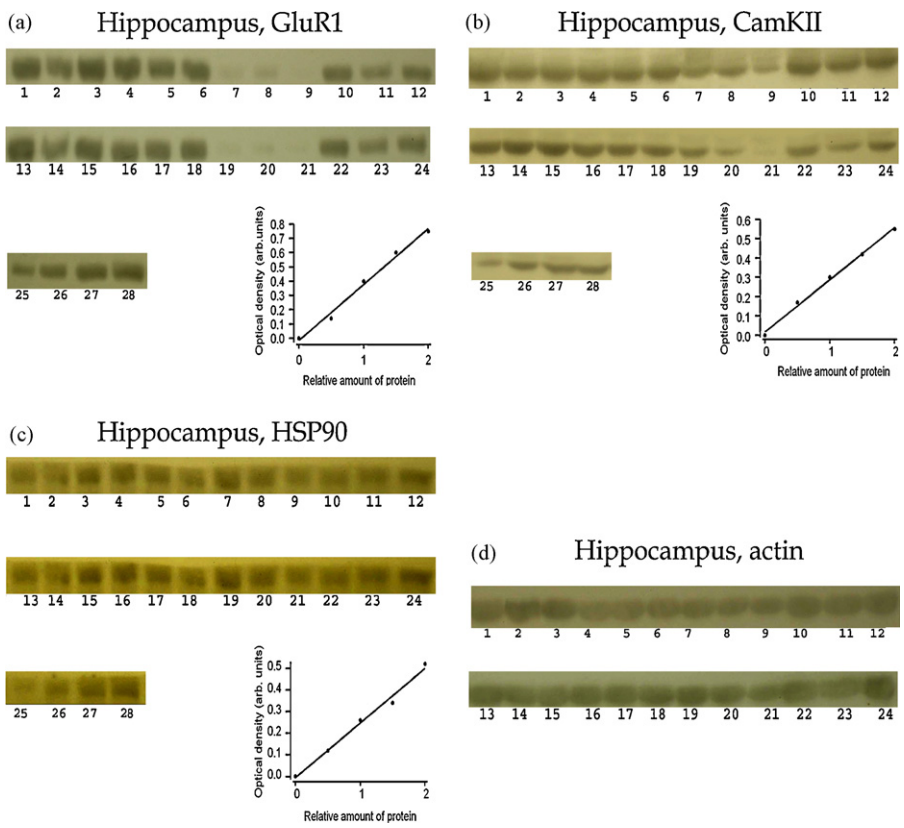

Fig. 1. Representative Western blot autoradiographs of homogenate fractions of rat brain hippocampus from four groups of rats on 28th day of experiment. Blots were stained with antibodies against GLUR1 (a), αCaMKII (b), HSP90 (c) and total actin (d). The optical densities of the bands were linearly related to the amounts of proteins in the bands (see calibration plots for GLUR1, αCaMKII and HSP90). Each lane was derived form a single sample. Lanes 1–3 and 13–15 are samples from Con + Sal group (a–d); lanes 4–6 and 16–18 from Con + MI group (a–d); lanes 7–9 and 19–21 from KA + Sal group (a–d); lanes 10–12 and 22–24 from KA + MI group (a–d).

Hippocampus and neocortex, 1 day: the amount of neither of the protein was changed significantly by experimental conditions in hippocampus or neocortex (see Table 1).

Hippocampus 30 days—GLUR1: the amount of GLUR1 subunit of AMPA glutamate receptors was significantly influenced by experimental conditions (see Table 1). The mean amount of GLUR1 subunit was drastically reduced (more than for 95%) in the hippocampus of KA + Sal group of rats and was significantly lower as compared to two groups of control rats (KA + Sal vs Con +Sal, $t = 7.19$; $p = 0.001$; KA + SAL vs Con +MI, $t = 14.6$; $p = 0.0001$) (Figs. 1a and 2a). The mean amount of GLUR1 AMPA receptors in the KA + MI group was in the middle between the KA and control groups: significantly lower as compared to two control groups (KA + MI vs Cont + SAL, $t = 3.06$; $p = 0.022$; KA + MI vs Con + MI, $t = 4.32$; $p = 0.002$), but significantly higher as compared to KA group (KA + Sal vs KA + MI, $t = 9.53$; $p = 0.0001$). Thus MI treatment significantly halts the drastic reduction of GLUR1 AMPA receptors induced by KA evoked status epilepticus.

CaMKII: the amount of CaMKII was significantly influenced by experimental conditions (see Table 1). The changes in general were similar to those observed for GLUR1 AMPA receptor. The mean amount of CaMKII in KA + Sal group was significantly decreased (approximately by 60%) as compared to two control groups (KA + Sal vs Con + Sal, $t = 3.81$; $p = 0.004$; KA + Sal vs Con + MI, $t = 5.6$; $p = 0.001$) (Figs. 1b and 2b). The mean amount of CaMKII in the KA + MI group was significantly more as compared to KA + Sal group ($t = 2.46$; $p = 0.04$) and was more near and not different from two control groups (KA + MI vs Con + Sal, $t = 0.62$; $p = 0.554$; KA + MI vs Con + MI, $t = 1.15$; $p = 0.292$). The mean amounts in the two control groups were not different from each other ($t = 0.58$; $p = 0.578$). Thus, the changes in general were similar to those observed for GLUR1 AMPA receptor.

HSP90: The amount of HSP90 was not significantly changed by experimental conditions (see Table 1). The mean amounts of protein did not differ significantly between the experimental groups (Figs. 1c and 2c).

Neocortex 30 days: the amount of neither of the protein was changed by experimental conditions (see Table 1).

We have investigated changes in the amounts of the three following proteins: HSP90, GLUR1 and CaMKII after KA induced SE. Two of them—GLUR1 and CaMKII revealed time-dependent, region-specific changes, namely strong quantitative decrease in the hippocampus on 28th day after KA treatment. Similar trends were also described by others [5,8,27], but the main aim of our experiments was to investigate if the MI treatment could interfere with these changes! Indeed our findings show that MI daily treat-

Table 1
Summary of one-way ANOVAs results. *F*, variance ratio from one-way ANOVA; *P*, probability.

	Hippocampus		Neocortex	
	1st day	28th day	1st day	28th day
GLUR1				
$F_{3,20}$	0.40	31.32	1.98	1.46
P	0.753	0.0001	0.151	0.256
CaMKII				
$F_{3,20}$	0.34	6.72	1.94	0.25
P	0.795	0.003	0.157	0.858
HSP90				
$F_{3,20}$	0.10	0.98	1.71	0.19
P	0.957	0.423	0.197	0.902

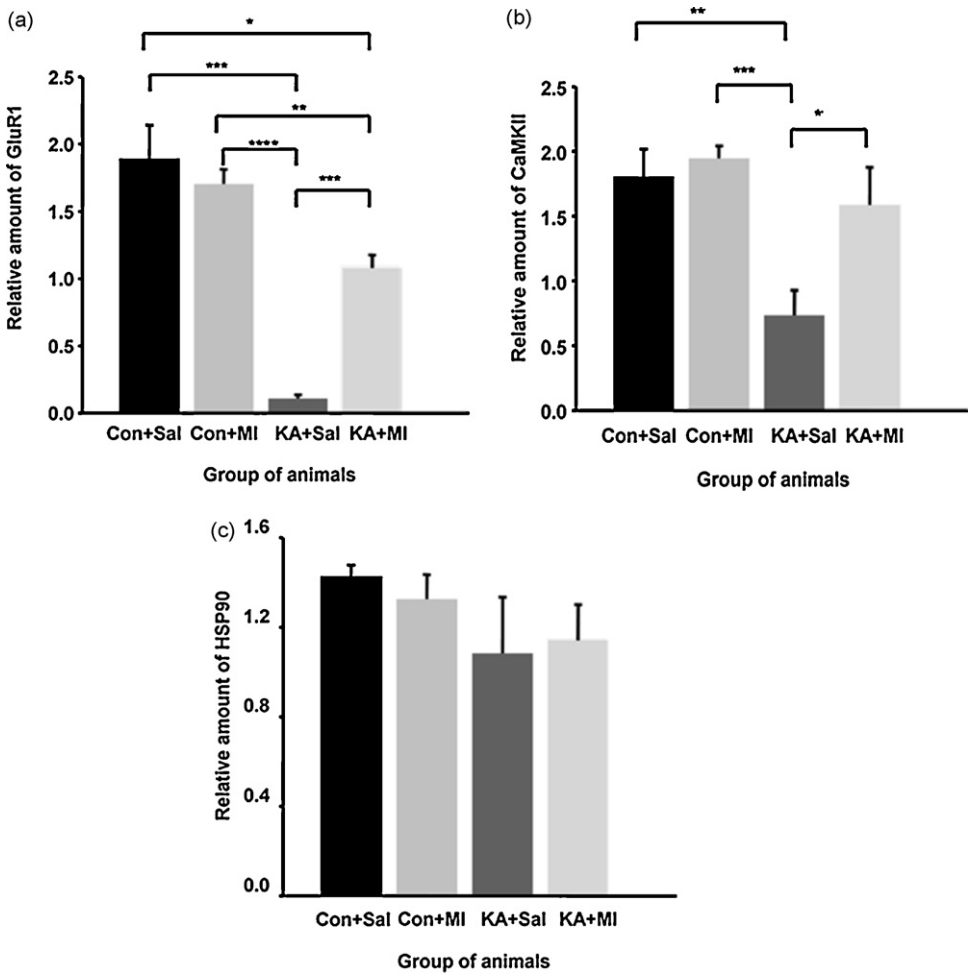

Fig. 2. Mean relative amounts of GLUR1 (a), CaMKII (b) and HSP90 (c) of rat brain hippocampus from four groups of rats on 28th day of experiment. Error bars represent standard errors of the means. (a) The difference between KA + Sal and Con + Sal groups is significant (***$p = 0.001$). The difference is also significant between KA + Sal and Con + MI groups (**** $p = 0.0001$). The mean amount of GLUR1 in the KA + MI group is significantly lower as compared to Con + Sal group (* $p = 0.022$) and Con + MI group (** $p = 0.002$), but significantly higher as compared to KA + Sal group (***$p = 0.001$). (b) The mean amount of CaMKII in KA + Sal group is significantly lower as compared to Con + Sal group (**$p = 0.004$), to Con + MI group (***$p = 0.001$) and to KA + MI group (*$p = 0.04$). (c) The mean amounts of HSP90 are not significantly different from each other.

ment significantly halts some biochemical changes initiated by KA induced SE. The same intensity MI administration to the group of rats not treated by KA does not produce any significant changes in the amounts of studied proteins, indicating that MI exerts specific influence on molecular machinery of epileptogenesis induced by KA. According to our knowledge this is the first report about the molecular effects of MI long-term treatment.

Data demonstrating anti-convulsant potential of MI were earlier obtained from experiments focused on lithium-pilocarpine induced seizures [1,12]. Lithium could act through decrease of MI level (inositol depletion hypothesis [2]) and this assumption was the main reason of studying of MI influence on lithium-pilocarpine model of seizures. However some data indicated, that mechanism other than phosphoinositide repletion contributed to the MI response [30].

Another series of experiments have shown that changes in MI levels are not restricted only to lithium-pilocarpine induced seizures and take place in another models of epilepsy and also in humans with TLE. Experiments on KA induced seizures have shown that Na^+/MI co-transporter is up-regulated in various parts of hippocampus shortly after seizures [18]. TLE patients are characterized with MI abnormalities; seizure focus has an increase level of MI, whereas areas of seizure spread have a lowered level of MI [29]. Gene coding for myo-inositol monophosphatase 2 is likely to be febrile seizure susceptibility gene [17]. Thus, changes in MI metabolism could be involved in different types of epilepsy.

What could be the mechanisms of MI action under these experimental conditions? During intense neuronal excitation massive influx of Na^+, Ca^{2+} and Cl^- takes place, which leads to water inward flow and cellular swelling [7,21]. Under such conditions various enzymes cannot function further in normal way [31]. For compensation of this disturbed condition neuronal and other cells accumulate high concentration of small organic osmolytes, including MI, which do not perturb the functioning of the enzymes ([26,28], reviewed in [18]). Up-regulation of Na^+/MI co-transporter after KA induced seizures also indicates for beneficial effects of MI accumulation for the normalization of cellular functions [18]. Our experiments revealed that *in vitro* MI changes the binding of 3^H muscimol to rat brain membranes [23] and therefore modulation of GABA-A receptors after KA induced seizures could be also involved in protective features of MI.

We have shown earlier that MI significantly decreases the seizure severity induced either by PTZ or KA [25]. These results along with another sets of data [1,12] indicate that, MI as an anticonvulsant could be effective for many types of induced seizures. In the present study, we have shown that daily treatment by MI could halt biochemical events initiated by KA induced SE. These data point to the anti-epileptogenetic properties of MI treatment on a molecular level.

Acknowledgements

Supported by the Ilia Chavchavadze Tbilisi State University and I. Beritashvili Institute of Physiology.

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