Article

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Lipoic acid effects on glutamate and taurine concentrations in rat hippocampus after pilocarpine-induced seizures

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ABSTRACT

Pilocarpine-induced seizures can be mediated by increases in oxidative stress and by cerebral amino acid changes. The present research suggests that antioxidant compounds may afford some level of neuroprotection against the neurotoxicity of seizures in cellular level. The objective of the present study was to evaluate the lipoic acid (LA) effects in glutamate and taurine contents in rat hippocampus after pilocarpine-induced seizures. Wistar rats were treated intraperitoneally (i.p.) with 0.9% saline (Control), pilocarpine (400 mg/kg, Pilocarpine), LA (10 mg/kg, LA), and the association of LA (10 mg/kg) plus pilocarpine (400 mg/kg), that was injected 30 min before of administration of LA (LA plus pilocarpine). Animals were observed during 24 h. The amino acid concentrations were measured using high-performance liquid chromatograph (HPLC). In pilocarpine group, it was observed a significant increase in glutamate content (37%) and a decrease in taurine level (18%) in rat hippocampus, when compared to control group. Antioxidant pretreatment significantly reduced the glutamate level (28%) and augmented taurine content (32%) in rat hippocampus, when compared to pilocarpine group. Our findings strongly support amino acid changes in hippocampus during seizures induced by pilocarpine, and suggest that glutamate-induced brain damage plays a crucial role in pathogenic consequences of seizures, and imply that strong protective effect could be achieved using lipoic acid through the release or decrease in metabolization rate of taurine amino acid during seizures.

Key words: seizures, pilocarpine, amino acids, hippocampus, glutamate, taurine.

Efeitos do ácido lipóico nas concentrações de glutamato e taurina no hipocampo de ratos após convulsões induzidas por pilocarpina

RESUMO

As convulsões induzidas pela pilocarpina podem ser mediadas através do aumento do estresse oxidativo cerebral e das alterações na concentração dos aminoácidos. O presente estudo sugere que compostos antioxidantes podem produzir neuroproteção contra a neurotoxicidade em nível celular causada pelas convulsões. O objetivo deste estudo foi avaliar os efeitos do ácido lipóico (AL) no conteúdo de glutamato e taurina no hipocampo de ratos durante convulsões induzidas por pilocarpina. Ratos Wistar foram tratados por via intraperitoneal com solução salina 0,9% (controle), pilocarpina (400 mg/kg, pilocarpina), AL (10 mg/kg) e com a associação de AL (10 mg/kg); 30 min após com pilocarpina (400 mg/kg), que foi injetada 30 min após a administração de AL (AL + pilocarpina). Os animais foram observados durante 24 horas. As concentrações de aminoácidos foram

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determinadas por HPLC. No hipocampo dos ratos do grupo pilocarpina foi observado um aumento significativo de 37% na concentração de glutamato e uma diminuição de 18% no nível de taurina, quando comparado ao grupo controle. O pré-tratamento com o antioxidante reduziu significativamente o nível de glutamato em 28% e aumentou em 32% os níveis de taurina no hipocampo dos ratos, quando comparado ao grupo pilocarpina. Nossos resultados sugerem que ocorrem alterações na concentração dos aminoácidos no hipocampo de ratos durante as convulsões induzidas por pilocarpina, e que o glutamato pode desempenhar um papel crucial na fisiopatologia das convulsões, e que o efeito protetor poderia ser alcançado com pré-tratamento com ácido lipóico, provavelmente pelo aumento da liberação ou redução da taxa de metabolização dos aminoácidos durante as convulsões.

Palavras-chave: convulsões, pilocarpina, aminoácidos, hipocampo, glutamato, taurina.

Systemic injection of pilocarpine in rodents is an experimental model largely used to study the pathophysiology of seizures and to identify potential therapeutic agents for treatment of epilepsy. This seizures model demonstrates the potent pro-convulsant effect of pilocarpine and reproduces the behavioral, electroencephalographic and neurochemical alterations associated with seizures that are similar to those of temporal lobe epilepsy in humans[1-3]. Other researches suggest permanent changes in the concentration of the brain neurotransmitters during seizures and status epilepticus (SE) induced by pilocarpine[4-9]. An increase in dopamine level, a decrease in serotonin (5-HT) content, and also an excessive increase in concentration of 3,4-hydroxyphenylacetic acid (DOPAC) may occur in hippocampus and frontal cortex of adult rats during SE induced by pilocarpine[10].

Studies have demonstrated that the installation of seizures requires cholinergic stimulation, but some other neurotransmitter systems appear to be responsible for propagation and maintenance of seizures[11]. Although changes in concentrations of brain amino acid during seizures might be either a cause or a consequence of the ongoing epileptic activity, we have investigated the levels of glutamate and tyrosine during pilocarpine-induced seizures, as a first step in determining the possible role of these neurotransmitters during the propagation and maintenance of limbic seizures.

The amino acids are involved in several physiological events, including brain development and ageing, in physiological integration among brain structures, and in the processes of learning and memory[12]. Neurotransmitter systems alterations can be implicated in seizures due an increase in their oxidative metabolism or by a decrease in their syntheses and/or release[7,9]. Epileptic activity can occur through a wide range of local neurochemical changes that affects several neurotransmitters, such as adenosine, norepinephrine, dopamine, 5-HT, glutamate, γ-amino butyric (GABA), aspartate, tyrosine, taurine and glutamine[11,13-15].

Studies have demonstrated changes in mobilization rate of amino acid in hippocampus of rats during seizures[13]. In addition, little is known about the effects of antioxidant compounds in amino acid concentration in hippocampus of adult rats after seizures pilocarpine-induced. Despite the fact that several studies clearly indicate the importance of amino acids in epileptic phenomenon[16,17], it is important to verify the effects of antioxidant drugs in cerebral amino acid levels during seizures. Based on these facts, there is an increasing evidence of the involvement of augmented glutamatergic transmission during seizures pilocarpine-induced. We have decided to investigate the effects of lipoic acid in glutamate and taurine concentrations in the hippocampus of adult rats after seizures pilocarpine-induced.

METHOD

Male Wistar rats (250-280g; 2-month-old) were used. Animals were housed in cages with free access to food and water and were kept with standard light-dark cycle (lights on at 07:00h a.m.). The experimental protocols were approved by the Faculty Ethics Committee. The experiments were performed according to the Guide for the care and use of laboratory of the US Department of Health and Human Services, Washington, DC (1985). All doses are expressed in milligrams per kilogram and were administered in a volume of 10 ml/kg injected intraperitoneally (i.p.).

In a set of experiments, the animals were divided in four groups and treated with lipoic acid (10 mg/kg, i.p., n=20) or 0.9% saline (i.p., n=20) and 30 min later, they received pilocarpine hydrochloride (400 mg/kg, i.p.). The treatments previously described represent the LA plus pilocarpine and pilocarpine groups, respectively. The third and fourth groups received alone 0.9% saline (i.p., n=20, control group) and lipoic acid (10 mg/kg, i.p., n=20, LA group), respectively. After the treatments the animals were observed during 24 h to determinations

of behavioral changes, such as appearance of peripheral cholinergic reactions, such as miosis, piloerection, chromodacryorrhea, diarrhea, masticatory and stereotyped movements, seizures, status epilepticus and mortality rate. The survivors were killed by decapitation and their brains dissected on ice to remove hippocampus to amino acid determinations. The pilocarpine group was constituted by those rats that presented seizures; SE for a period longer than 30 min and that did not died during 24 h.

Immediately after rats were decapitated, the hippocampus, striatum and frontal cortex were removed on an ice-chilled plate, weighed, and stored at –120°C. Tissues were ultrasonically homogenized in a 0.1 M solution of $HClO_4$ containing 0.02% HSER (10 µg/ml) (as internal standard for amino acids) in a proportion of 15 µg solution for each milligram wet tissue. The samples were then centrifuged at 11.000×g at 4°C for 40 min. the supernatant as filtered and injected into high-performance liquid chromatograph (HPLC) system. HPLC system was used simultaneously to analyze amino acids[18].

Amino acid determination was performed after a pre-column derivatization procedure. The reagent was prepared by dissolving 27 mg OPA in 1 ml methanol followed by 5 µl mercaptoethanol (BME) and 9 ml 0.1M sodium tetraborate (pH 9.3). This OPA/BME stock solution was diluted threefold in tetraborate just before the use. Precolumn amino acid derivatization was accomplished by mixing 50 µl samples with 100 µl working OPA/BME reagent at exactly 2 min before its injection into the analytical column.

Gradient HPLC system, used to analyze amino acids, was composed of two pumps linked to a programmable gradient controller (Milton Roy), coupled with an electrochemical detector (potential +0.9V; Spark Holland). A rheodyne injector with a 20 µl loop was connected to a Lichorospher 100 RP-18, 5 µm (125×4mm) columns, with a flow rate of 1ml/ min. Amino acids were eluted with a gradient solvent system as described by Joseph and Davies[19].

Mobile phase A contained sodium phosphate 0.05M (pH 5.5) buffer plus 0.03% NaCl/methanol 20%. Mobile phase B consisted of sodium phosphate 0.05M (pH 5.5) buffer plus 0.03% NaCl/methanol 80%. Standard mixtures of amino acids were injected at the beginning and of each set of six analyses to control the performance of system. Amino acid recoveries were made after addition of 200 µg of taurine and glutamate to 10 ml of 0.1 M of $HClO_4$ containing 0.02% of $Na_2S_2O_5$ and 100 µg of HSER. Tissues were then homogenized in this solution, and amino acids were quantified. The recovery of amounts added was determined by subtracting the amount originally in the tissue. The values obtained were expressed as mmol/g tissue wet weight.

Results are expressed as means ±S.E.M. for the number of experiments, with all measurements performed in duplicate. The Student-Newman-Keuls test was used for multiple comparisons of means of two groups of data. Differences were considered significant at $p<0.05$. Differences in experimental groups were determined by two-tailed analysis of variance.

RESULTS

According to our previous studies[3,20], immediately after pilocarpine administration, animals persistently had behavioral changes, including initial akinesia, ataxic lurching, peripheral cholinergic signs (miosis, piloerection, chomodacryorrhea, diarrhea and masticatory automatisms), stereotyped movements (continuous sniffing, paw licking, rearing and wet dog shakes that persisted for 10-15 min), clonic movements of forelimbs, head bobbing and tremors. These behavioral changes progressed to motor limbic seizures as previously described by Turski et al.[3]. Pilocarpine induced the first seizure at 35.00±0.70 min. Limbic seizures persisted for 30-50 min evolving to SE in all rats. In the latter experiments, 60% of animals died during the 24 h observation period. The animals pre-treated with lipoic acid 30 min before with pilocarpine (LA plus pilocarpine) developed cholinergic reactions, stereotyped movements and tremors; 10% (02/20) had seizures, 10% (02/20) built up to status epilepticus and no one animal died. Table shows that lipoic acid (10 mg/kg) administration before pilocarpine treatment reduced by 50% the percentage of animals that seized ($p<0.0001$), increased latency (154%) to the first seizure (89.00±1.95 min) ($p<0.0001$) and increased (60%) the survival ($p<0.0001$) when compared to the pilocarpine only group. None of the control animals (saline or lipoic acid) showed seizures.

Effects of lipoic acid in glutamate and taurine concentrations in hippocampus of adult rats during seizures induced by pilocarpine are presented in Figures 1 and 2. Glutamate level was markedly increased in pilocarpine group, when compared with corresponding values for the control group [$T(14)=3.860$; $p<0.0017$]. During acute phase of seizures induced by pilocarpine, it was observed a significant (18%) decrease in taurine content [$T(14)=2.416$; $p<0.0300$], when compared with corresponding values for the control group.

Post hoc comparison of means indicated a significant decreases of 28% in hippocampal glutamate level [$T(12)=3.277$; $p<0.0066$] of rats pretreated with lipoic acid, when compared with the pilocarpine group. Post hoc comparison of means indicated a significant (32%) increase in hippocampal taurine content of rats pretreated with lipoic acid [$T(12)=4.876$; $p<0.0004$], when compared to pilocarpine group.

Table. Effect of pretreatment with lipoic acid on pilocarpine-induced seizures and lethality in adult rats.

Groups	Latency to first seizures (min)	Percentage seizures	Percentage survival	Number of animals/group
Pilocarpine	35.00±0.70	60	40	24
LA plus pilocarpine	89.00±1.95[c]	10a	100[a]	24
LA	0	0[a,b]	100[a]	24

Animals were pretreated acutely, intraperitoneally, with lipoic acid and 30 min after receiving pilocarpine 400 mg/kg, i.p. Results for latency to first seizure are expressed as mean ±S.E.M of the number of experiments shown in the table. Result for percentage seizures and percentage survival are expressed as percentages of the number of animals from each experimental group. [a]p<0.0001 as compared with pilocarpine group (χ^2-test); [b]p<0.0001 as compared with LA plus pilocarpine group (χ^2-test); [c]p<0.0001 as compared with pilocarpine group (ANOVA and Student-Newman-Keuls test).

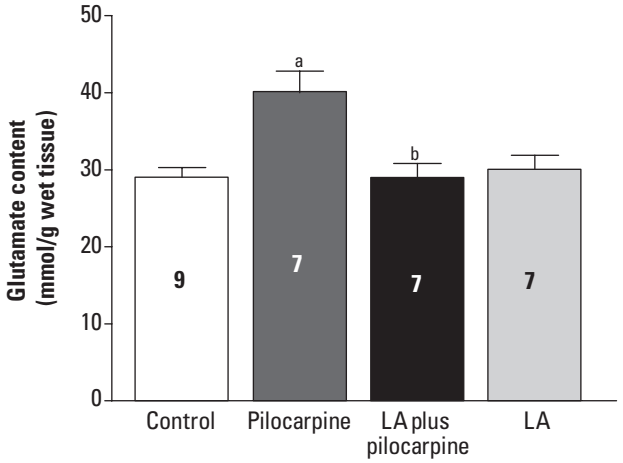

Fig 1. Effects of lipoic acid on glutamate concentration in rat hippocampus after pilocarpine-induced seizures. [a]p<0.0001 as compared with control group (ANOVA and Student-Newman-Keuls test); [b]p<0.0001 as compared with pilocarpine group (ANOVA and Student-Newman-Keuls test).

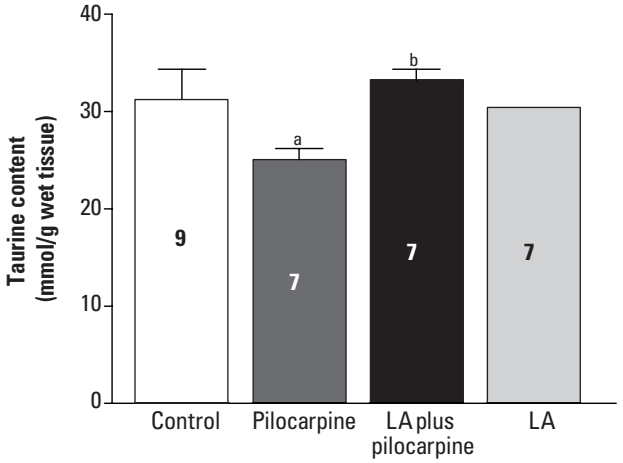

Fig 2. Effects of lipoic acid on taurine concentration in rat hippocampus after pilocarpine-induced seizures. [a]p<0.0001 as compared with control group (ANOVA and Student-Newman-Keuls test); [b]p<0.0001 as compared with pilocarpine group (ANOVA and Student-Newman-Keuls test).

DISCUSSION

Changes in amino acid brain concentration are, of course, crude approximations of changes which may be occurring at the synaptic level. Furthermore, some of these amino acids have metabolic roles in brain, in addition to their as neurotransmitter functions[6]. The changes we have found do appear to be secondary to any general derangement produced by seizures in amino acid metabolism, for several reasons. We observed an increase in glutamate and a decrease in taurine in hippocampus of rats during seizures.

Several amino acids have been associated with the mechanism of pilocarpine-induced seizures[18]. Significant differences in tyrosine and glutamate contents were evident in hippocampus during the installation of seizures induced by pilocarpine. Other studies have shown that during the chronic phase of pilocarpine-induced seizures, the GABA, dopamine and glutamine levels in hippocampus were increased, suggesting that a neuronal release may occur and under the same conditions, decreased aspartate and glutamate levels[18] were verified.

Our work showed that hippocampal glutamate and taurine levels, increased and decreased during acute phase of seizures, respectively, suggesting that these amino acids can have a important function during installation of seizures. Therefore, it is likely that the amino acids studied can be interconnected in epileptic activity. In support to this finding, an increase in hippocampal glutamate content was observed by Cavalheiro et al.[17], suggesting an idea of decreases in oxidative metabolism and cellular death observed in hippocampal neurons during the chronic phase of seizures in rats. On the other hand, the taurine level in hippocampus of rats was not altered during chronic phase of seizures induced by pilocarpine. In contrast of these findings, Yablonsky-Alter et al.[21] reported an increase in the release of neuroprotective amino acid taurine in the striatum rats treated with cocaine.

In the present study we have examined whether the pretreatment with lipoic acid can reverse the alterations observed in glutamate and taurine in hippocampus of adult rats after seizures induced by pilocarpine. Generation of reactive oxygen species is currently viewed as one of the process through which epileptic activity exert their

deleterious effects on brain[22]. These reactive oxygen species in the absence of an efficient cellular defence mechanism cause peroxidation of membrane poly unsaturated fatty acids. Brain is particularly susceptible to peroxidation due to simultaneous presence of high levels of poly unsaturated fatty acids and iron[23], which is the target of free radical damage. It has been demonstrated that seizures induced by pilocarpine produces changes in oxidative metabolism and interacts with glutamatergic receptors to produced part of its stimulatory action on the central nervous system[24-27]. The fall in glutamate content, after pretreatment with lipoic acid, is most readily explained as a consequence of inhibiting their release or increasing their metabolization[28-30]. Moreover, the lipoic acid can reduce the metabolization rate and/or augment the release and syntheses of neuroprotective taurine, suggesting a protective action of this amino acid against the excitotoxicity mediated by glutamate during seizures.

Our results clearly show that the cholinergic receptors are stimulated by pilocarpine administration during installation of seizures; moreover, several neurotransmitter systems can be involved also in initiation and in propagation and maintenance processes during establishment of seizures. Therefore, lipoic acid represents a possible neuroprotective agent against the risks of acute phase of seizures induced by pilocarpine. Results suggest the involvement in lipoic acid action mechanism of the neuroprotective amino acid (taurine) during seizures induced by pilocarpine in rats. Lipoic acid could provide further insights for neuroprotection and may lead to the development of effective therapeutic strategies against epilepsy in humans.

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