Pharmacology, Biochemistry and Behavior 95 (2010) 88–91

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

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

Lipoic acid blocks seizures induced by pilocarpine via increases in δ-aminolevulinic dehydratase and Na^+, K^+-ATPase activity in rat brain

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ARTICLE INFO

Article history:
Received 30 September 2009
Received in revised form 1 December 2009
Accepted 14 December 2009
Available online 21 December 2009

Keywords:
Lipoic acid
Seizures
δ-aminolevulinic dehydratase
Na^+, K^+-ATPase
Pilocarpine

ABSTRACT

In the present study we investigated the effects of lipoic acid (LA) on δ-aminolevulinic dehydratase (δ-ALA-D) and Na^+, K^+-ATPase activities in rat brain after seizures induction by pilocarpine. Wistar rats were treated with 0.9% saline (i.p., control group), lipoic acid (10 mg/kg, i.p., LA group), pilocarpine (400 mg/kg, i.p., pilocarpine group), or the combination of LA (10 mg/kg, i.p.) with pilocarpine (400 mg/kg, i.p.), 30 min before administration of LA (LA plus pilocarpine group). After the treatments all groups were observed for 1 h. The enzyme activities (δ-ALA-D and Na^+, K^+-ATPase) were measured using spectrophotometric methods, and the results were compared with that obtained from saline and pilocarpine-treated animals. Neuroprotective effects of LA against seizures were evaluated based on those enzyme activities. The pilocarpine group showed a reduction in δ-ALA-D and Na^+, K^+-ATPase activities after seizures. In turn, LA plus pilocarpine abolished the appearance of seizures and reversed the decreased in δ-ALA-D and Na^+, K^+-ATPase activities produced by seizures, when compared to the pilocarpine seizing group. The results from the present study demonstrate that preadministration of LA abolished seizure episodes induced by pilocarpine in rat, probably by increasing δ-ALA-D and Na^+, K^+-ATPase activities in rat brain during seizures.

1. Introduction

The cholinergic system has been implicated in a variety of behavioral functions, including learning, control of cognition and memory, circadian cycle's synchronization and control of the body temperature. It is also involved in some electroencephalographic wave generation and regulation of the vigilance states (sleep–waking control) (Crouzier et al., 2006). Muscarinic cholinergic agonists, such as pilocarpine, have effects on slow wave sleep and rapid eye movement induction (Gamundí et al., 2003). Moreover, a high dose of pilocarpine is used to induce a pattern of repetitive limbic seizures and a status epilepticus state in rodents, which can last for several hours (Turski et al., 1989). The epileptic model induced by pilocarpine is a useful animal model to study the development and understanding of the neuropathology of temporal lobe epilepsy. This status epilepticus model is interesting because it reproduces behavioral and electroencephalographic alterations which are similar to those of human temporal lobe epilepsy (Turski et al., 1989).

Reactive oxygen species (ROS) have been implicated in the development of seizures and status epilepticus induced by pilocarpine (Freitas et al., 2004; Shin et al., 2008). For example, there is a temporal correlation between ROS formation and seizure development (Kim et al., 2002). In previous studies, we have demonstrated that pilocarpine induced seizure episodes in adult rats. Importantly, the seizure episodes induced by pilocarpine are related to oxidative stress, with increases on lipid peroxidation level and nitrite formation (Erakovié et al., 2000; Freitas et al., 2005). However, the effects of seizures related oxidative stress on δ-ALA-D and Na^+, K^+-ATPase activities have not been studied yet.

The brain is more susceptible to ROS damage due to the large lipid content of myelin sheaths and the high rate of brain oxidative metabolism (Rauca et al., 1999). In this context, the thiol redox state is an essential parameter associated with major biologic processes such as oxidative stress, intracellular redox homeostasis and gene expression (Sies, 1999). Lipoic acid (LA) which plays an essential role in mitochondrial dehydrogenase reactions, has recently gained considerable attention as an antioxidant. Lipoate, or its reduced form, dihydrolipoate, reacts with ROS such as superoxide radicals, hydroxyl radicals, hypochlorous acid, peroxyl radicals, and singlet oxygen. It also protects membranes by interacting with vitamin C and glutathione reduced, which may in turn

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doi:10.1016/j.pbb.2009.12.011

recycle vitamin E (Packer et al., 1995). Recent studies showed that lipoic acid exerts anticonvulsant effects in an epilepsy model induced by pilocarpine (Freitas, 2009; Freitas et al., 2010).

In addition to its antioxidant activities, dihydrolipoate may exert prooxidant actions through reduction of iron. LA administration has been shown to be beneficial in a number of oxidative stress models such as ischemia, diabetes, and neurodegenerative diseases (Packer et al., 1997). Furthermore, LA can function as a redox regulator of proteins in neurodegenerative diseases (Genestra, 1997). Brain regions were chosen according to the neuropathology associated with status epilepticus that has been described in the hippocampus, striatum and frontal cortex. The aim of this study was to evaluate the effects of lipoic acid on δ-aminolevulinic dehydratase (δ-ALA-D) and Na^+, K^+-ATPase activities in rat brain after administration of high dose of pilocarpine.

2. Material and methods

The protocols for the animals experiments described in this study were performed in accordance with international (EEC Directive of 1986, 86/609/EEC) and national rules and institutional guidelines as prescribed by the ethical committee for animal experiments of the Federal University of Piaui (UFPI).

2.1. Animals and experimental procedures

Adult male Wistar rats (250–280 g) were maintained in a temperature controlled room (26 ± 1 °C), with a 12 h light/dark cycle and food and water provided *ad libitum*. All 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).

The following substances were used: pilocarpine hydrochloride and alpha-lipoic acid (Sigma, Chemical USA). All doses are expressed in milligrams per kilogram and were administered in a volume of 10 ml/kg injected intraperitoneally (i.p.). A total of 96 rats were treated with either 10 mg/kg lipoic acid (i.p.) or 0.9% saline (i.p.). 30 min after the treatments 48 rats from each above group were randomized to pilocarpine hybrochloride administration. Thus there are 4 groups of rats in this set of experiments: group 1, lipoic acid and pilocarpine co-administration ($n=24$); group 2, pilocarpine plus saline treatment ($n=24$); group 3, lipoic acid alone administration ($n=24$); and group 4, saline treatment serves as control ($n=24$). After the treatments, the animals were recorded in 30 cm × 30 cm chambers with: latency to first seizure (any one of the behavioral indices typically observed after pilocarpine administration: wild running, clonus, tonus, clonic–tonic seizures), number of animals that died after pilocarpine administration. Previous work has shown that convulsions and deaths occurred within 1 and 24 h respectively post pilocarpine injection, so we decided to record the phenotypes of the animals for 1 h after pilocarpine administration. At the end of observations, the survivors were killed by decapitation and their brains were dissected on ice to remove hippocampus, striatum, frontal cortex and cerebellum for determinations of LA effects on δ-ALA-D and Na^+, K^+-ATPase activities. The pilocarpine administration rat group was constituted by those presented seizures, SE for over 30 min and non-phenotype survisors.

The drug dosages of pilocarpine (400 mg/kg) and lipoic acid (10 mg/kg) were determined by previous study in our lab (Freitas, 2009) and in the present study (data not shown). The drug doses used in this present study are not equivalent to those used by humans because rats have different metabolic rates.

2.2. δ-Aminolevulinic dehydratase (δ-ALA-D) activities determinations

δ-ALA-D activies in the rat brains of 4 groups above mentioned were assayed by measuring the rate of product porphobilinogen (PBG) formation according to the method described by Sassa (1982). The rat number of each drug treated group was 6, and saline control was 9. An aliquot of 200 µl of S1 from each sample was incubated for 3 h at 37 °C. Enzymatic reaction was initiated by adding the substrate (δ-ALA) to a final concentration of 2.2 mM in a medium containing 45 mM phosphate buffer, pH 6.8. The reaction was stopped by adding 250 µl 10% TCA with 10 mM $HgCl_2$, and reaction product was determined using modified Erlich's reagent at 555 nm. The reaction was typically linear in relation to protein concentration and time of incubation. The enzymatic activity was presented as nmol PBG/mg protein/h.

2.3. Na^+, K^+-ATPase activities determinations

Rat brain homogenates from the 4 groups above mentioned were centrifuged at 4.000 × *g* at 4 °C for 10 min and the S1 was used for assay of protein Na^+, K^+-ATPase. The homogenates of rat brains from each drug treated group ($n=6$) and saline control group ($n=9$) were centrifuged at 4.000 × *g* at 4 °C for 10 min and the S1 was used for the assay of Na^+, K^+-ATPase. The assay reaction was set up in a 500 µl reaction buffer (3 mM MgCl, 125 mM NaCl, 20 mM KCl and 50 mM Tris–HCl, pH 7.4), and initiated with the addition of ATP to a final concentration of 3.0 mM. Control samples were carried out under the same conditions with the addition of 0.1 mM ouabain. The reaction was incubated at 37 °C for 30 min, and stopped by adding 250 µl 10% TCA with 10 mM $HgCl_2$. Released inorganic phosphate (Pi) was measured by the method of Fiske and Subbarow (1925). Enzyme activity was presented as nmol Pi/mg protein/min.

2.4. Statistical analysis

Results of latency to first seizure and neurochemical alterations between different groups were compared using ANOVA and the Student–Newman–Keuls test as post hoc test, because these results show a parametric distribution. The numbers of animals that seizured and that survived were calculated as percentages respectively, and compared with a nonparametric test (χ^2). In all situations statistical significance was reached at p less-than-or-equals, slant 0.05. The statistical analyses were performed with the software GraphPad Prism, Version 3.00 for Windows, GraphPad Software (San Diego, CA, USA).

3. Results

3.1. Effects of lipoic acid on pilocarpine-induced seizures

All the animals studied showed generalized tonic–clonic convulsions with status epilepticus, and 61% survived the seizures. Pilocarpine induced the first seizure at 34.93 ± 0.65 min. All animals pretreated with the lipoic acid selected for this study were observed for 1 h before pilocarpine injection for manifested alterations in behavior, such as peripheral cholinergic signs (100%), tremors (50%), staring spells, facial automatisms, wet dog shakes, rearing and motor seizures (25%), which develop progressively within 1–2 h into a long-lasting status epilepticus (25%). Table 1 shows that when administered at the dose (10 mg/kg) before pilocarpine, lipoic acid reduced by 75% the percentage of animals that seized, increased (126%) latency to the first seizure (126.13 ± 1.05 min) and increased (39%) the survival percentage as compared with the pilocarpine-treated group (Table 1). None of the animals that received injections of isotonic saline (control) or lipoic acid alone showed seizure activity (Table 1).

3.2. Effects of lipoic acid on δ-ALA-D activity prior to pilocarpine-induced seizures

We found no apparent change in hippocampal δ-ALA-D activity in group 3 rats which were treated with LA alone. However, hippocampal δ-ALA-D acitivity in group 2 rat brains with pilocarpine was significantly decreased ($p<0.0001$) when compared to the saline control group 4. In the LA and pilocarpine co-treated group 1 the δ-ALA-D

Table 1
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	34.93 ± 0.65	100	61	24
LA plus pilocarpine	126.13 ± 1.05[b]	25[a]	100[a]	24
LA	00	00	100[a]	24

Animals were pretreated acutely, intraperitoneally, with lipoic acid (10 mg/kg, i.p.) and 30 min afterwards received 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 pilocarpine group (ANOVA and Student–Newman–Keuls test).

activity was similar to that in the saline control group 4. In other words, the decreased δ-ALA-D activity in the pilocarpine only treated group was brought back to the saline control group activity level by lipoic acid ($p<0.0001$) (Fig.1). LA alone did not alter δ-ALA-D activity, which was inhibited in pilocarpine group ($p=0.3234$).

Striatal δ-ALA-D activity was not modified in rats which had seizure episodes induced by 400 mg/kg pilocarpine ($p=0.0931$). LA alone did not alter δ-ALA-D activity, which was inhibited in the pilocarpine group ($p=0.6547$). δ-ALA-D activity was not altered in the LA plus pilocarpine group ($p=0.2659$), when compared to pilocarpine (Fig. 1).

In the frontal cortex, δ-ALA-D activity was inhibited in rats, which had seizure episodes induced by 400 mg/kg pilocarpine ($p<0.0001$). LA alone did not alter δ-ALA-D activity, which was inhibited in the pilocarpine group ($p=0.2664$). δ-ALA-D activity was increased in the LA plus pilocarpine group ($p<0.0009$), when compared to the pilocarpine only group (Fig. 1). However, δ-ALA-D activity remained unaltered in the cerebellum from the groups treated with LA plus pilocarpine ($p=0.5316$), pilocarpine ($p=0.3249$) and LA ($p=0.2001$) (Fig. 1).

3.3. Effects of lipoic acid on Na^+, K^+-ATPase activity prior to pilocarpine-induced seizures

Hippocampal Na^+, K^+-ATPase activity was decreased in the pilocarpine group ($p<0.0178$). Na^+, K^+-ATPase activity data from the LA and pilocarpine co-administrated groups showed that preadministration with LA significantly stimulated this enzymatic activity in the hippocampus of seizured rats ($p<0.0113$). Na^+, K^+-ATPase activity was not altered in the LA group ($p=0.9761$) (Fig. 2).

Striatal Na^+, K^+-ATPase activity was inhibited in the pilocarpine group ($p<0.0082$). Na^+, K^+-ATPase activity data revealed that preadministration with LA significantly stimulated this enzyme in striatum of seized rats ($p<0.0031$). Na^+, K^+-ATPase activity was not altered in LA group ($p=0.9851$) (Fig. 2).

In the frontal cortex, Na^+, K^+-ATPase activity was not modified in the pilocarpine group ($p=0.2025$). Na^+, K^+-ATPase activity data revealed that preadministration with LA failed to stimulate this enzyme in frontal cortex of seized rats ($p=0.9463$). Na^+, K^+-ATPase activity was not altered in LA group ($p=0.9749$) (Fig. 2). Similarly, the Na^+, K^+-ATPase activity remained unaltered in rat cerebellum of groups treated with LA plus pilocarpine ($p=0.9509$), pilocarpine ($p=0.9463$) and LA ($p=0.9876$) (Fig. 2).

4. Discussion

Previous studies have demonstrated that δ-ALA-D and Na^+, K^+-ATPase activities were targets of free radical attack during oxidative stress, and these activities were decreased in oxidative stress environment (Lima et al., 2008). LA is an antioxidant that is both fat and water soluble. This important characteristic enables LA to easily cross the blood brain barrier and protect neurons in the brain (Freitas, 2009). In the present study, we investigated the effects of LA on brain oxidative stress and susceptibility to seizures induced by pilocarpine in adult rats. Our results demonstrated that preadministration of LA reduced seizures by 75% of animals treated with pilocarpine only, and caused an elevation in the latency for the first seizure episode. These data support the idea that the decrease of seizure susceptibility by LA treatment is due to a decrease in ROS formation, suggesting that ROS are involved in the establishment of seizures induced by pilocarpine. Recently, our research group has reported that oxidative stress is, at least in part, involved in installation or propagation of seizures induced by pilocarpine (Freitas, 2009; Freitas et al., 2010).

Several authors have reported that oxidative stress is associated with seizures, and that antioxidant compounds are important defenses in the removal of ROS (Skaper et al., 1998; Bustamante et al., 1998; Kutluhan et al., 2009). According to previous report, in the present study, LA pretreatment, the most important non-enzymatic antioxidant, was able to abolish seizure episodes induced by pilocarpine, as well as preventing the development of oxidative stress. The protective effect of LA was demonstrated by the decreases in lipid peroxidation

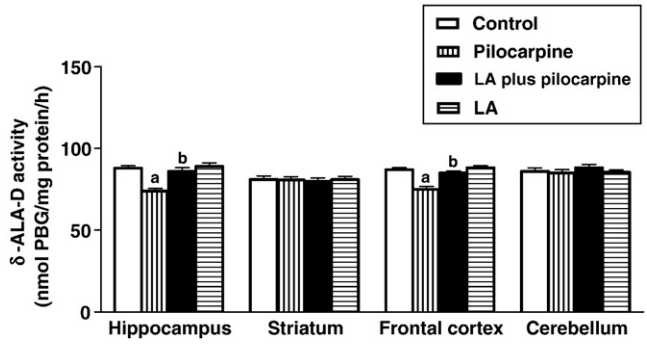

Fig. 1. Effects of pretreatment with lipoic acid on δ-aminolevulinic dehydratase (δ-ALA-D) activities in rat brain prior to pilocarpine-induced seizures. Male rats (250–280 g, 2 months old) were treated with a single dose of pilocarpine (400 mg/kg, i.p., pilocarpine group, $n=5$), LA group with lipoic acid (10 mg/kg, i.p., LA group, $n=5$) and the control animals with 0.9% saline (i.p., control group, $n=7$). The LA plus pilocarpine group was treated with LA (10 mg/kg, i.p.) and 30 min afterwards received pilocarpine (400 mg/kg, i.p., LA plus pilocarpine group, $n=5$). Animals were observed for 1 h and then subsequently sacrificed. Results are expressed as mean ± S.E.M for the number of animals shown inside in parentheses. The differences between experimental groups were determined by Analysis of Variance. [a]$p<0.05$ as compared to control animals (*t*–Student–Neuman–Keuls test); [b]$p<0.05$ as compared to P400 group (*t*–Student–Neuman–Keuls test).

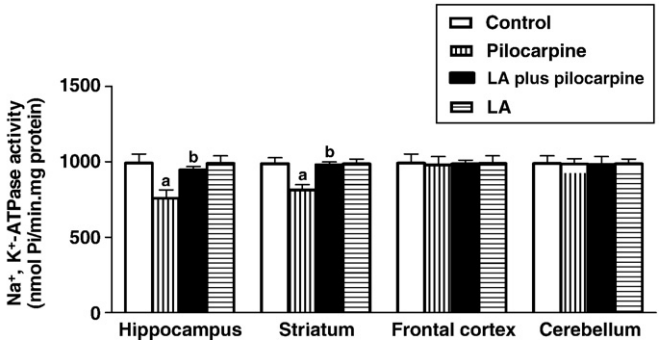

Fig. 2. Effects of pretreatment with lipoic acid on Na^+, K^+-ATPase activities in rat brain prior to pilocarpine-induced seizures. Male rats (250–280 g, 2 months old) were treated with a single dose of pilocarpine (400 mg/kg, i.p., pilocarpine group, $n=5$), LA group with lipoic acid (10 mg/kg, i.p., LA group, $n=5$) and the control animals with 0.9% saline (i.p., control group, $n=7$). The LA plus pilocarpine group was treated with LA (10 mg/kg, i.p.) and 30 min afterwards received pilocarpine (400 mg/kg, i.p., LA plus pilocarpine group, $n=5$). Animals were observed for 1 h and then subsequently sacrificed. Results are mean ± S.E.M for the number of animals shown inside in parentheses. The differences between experimental groups were determined by Analysis of Variance. [a]$p<0.05$ as compared to control animals (*t*–Student–Neuman–Keuls test); [b]$p<0.05$ as compared to P400 group (*t*–Student–Neuman–Keuls test).

levels and nitrite formation (Freitas, 2009). These results suggest that the enhanced oxidative stress in the pilocarpine-treated group may be attributable to its failure to respond to glutathione peroxidase, combined with the perturbed GSH status (data not show). In fact, a number of studies have previously reported low GSH levels in epilepsy models (Mueller et al., 2001; Freitas et al., 2005; Sleven et al., 2006). Furthermore, in vivo studies support that GSH depletion in the brain can cause mitochondrial dysfunction and contribute to excitotoxic neuronal damage (Heales et al., 1995).

Na^+, K^+-ATPase and δ-ALA-D activities are regulated by the sulfhydryl redox state (Lima et al., 2008; Ahamed et al., 2008). The alterations of these activities increase cellular excitability and facilitate the appearance or propagation of seizures induced by pilocarpine. However, how those enzymes act in different brain regions in pilocarpine-induced seizures model, and what is the influence of LA administration on these two enzyme activities have not been studied yet. The results presented in this report showed that the appearance of seizure episodes induced by pilocarpine occurred in association with the inhibition of δ-ALA-D activity, suggesting that seizures, oxidative stress and δ-ALA-D activity are related events. Accordingly, previous studies have demonstrated that δ-ALA-D activity was inhibited during seizures (Prigol et al., 2007). This finding suggests that decrease in hippocampal δ-ALA-D activity is likely to be produced during the installation of seizures induced by pilocarpine in adult rats, whereas the same decrease observed in the striatum and frontal cortex can exert an important role during the propagation and/or maintenance of seizures.

Additionally, Na^+, K^+-ATPase activity was elevated only in the LA pretreated group, which indicated the decreases on TBARS and nitrite levels (Freitas, 2009), as well as on the appearance of seizure episodes in 75% of animals. In this context, considering that Na^+, K^+-ATPase enzyme plays a pivotal role in cellular ionic gradient maintenance and it is particularly sensitive to ROS (Ullrich et al., 2007), failure of Na^+, K^+-ATPase activity may increase cellular excitability and facilitate the appearance and/or propagation of convulsions. Moreover, our studies demonstrated that δ-ALA-D activity was more sensitive to oxidative damage than Na^+, K^+-ATPase activity. It is important to consider that the activities of enzymes in cerebellum did not suffer any change during the acute phase of seizure and no change was seen in such activity after pretreatment with LA. These results suggest that the cerebellum is not involved in the establishment of seizures induced by pilocarpine. These data are consistent with previously reported results on the enzymatic actitiy of catalase in the cerebellum of adult rats after pilocarpine-induced seizures (Freitas et al., 2004).

We can infer that LA pretreatment displayed antioxidant properties and protected the brain against the seizure episodes and induced alterations in oxidative stress generation in the brains from adult rats, since the group pretreated with LA presented unaltered Na^+, K^+-ATPase and δ-ALA-D activities in cerebellum during seizures. However, these activities of sulfhydryl containing enzymes are sensitive to oxidizing agents (Ahamed et al., 2008), and the reduction of the activities in seizures induction are reversible by pretreatment with LA in the LA plus pilocarpine group. Our results suggest that the effects of LA as an anticonvulsant are related to the maintenance of the oxidant homeostasis and lipid peroxides (Cruz-Aguado et al., 1999; Keenoy et al., 2001).

Collectively, the results from the present study demonstrate that preadministration of LA was able to protect the brain against seizure episodes induced by pilocarpine in adult rats by reducing oxidative stress, principally in the hippocampus.

Acknowledgements

We would like to thank Stenio Gardel Maia for her technical assistance. This work was supported in part by grants from CNPq, Brazil. The I.M.S.S., C.M. F. and R.M.F. are fellows from CNPq.

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