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ORIGINAL PAPER

A ketogenic diet did not prevent effects on the ectonucleotidases pathway promoted by lithium-pilocarpine-induced status epilepticus in rat hippocampus

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Abstract A Ketogenic Diet (KD) mimics the anticonvulsant effects of fasting, which are known to suppress seizures. The purinergic system has been investigated in the matter of epilepsy development, especially the nucleoside adenosine, which has been considered a natural brain anticonvulsant. During epileptic seizures, extracellular adenosine concentration rises rapidly to micromolar levels. Adenosine can exert its anticonvulsant functions, after its release by nucleoside bidirectional transport, or by production through the sequential catabolism of ATP by ecto-nucleotidases, such as E-NTPDases (ectonucleoside triphosphate diphosphohydrolases) and ecto-5′-nucleotidase. Here, we have investigated the effect of a ketogenic diet on the nucleotide hydrolysis and NTPDases expression in the lithium-pilocarpine (Li-Pilo) model of epilepsy. For the induction of Status Epileticus (SE), 21-day-old female Wistar rats received an i.p. injection of lithium chloride (127 mg/kg) and 18–19 h later an i.p. injection of pilocarpine hydrochloride (60 mg/kg). The control groups received an injection of saline. After induction of SE, the control and Li-Pilo groups received standard or ketogenic diets for 6 weeks. The lithium-pilocarpine exposure affected the ATP (a decrease of between 8 % and 16 %) and ADP (an increase of between 18 % and 22 %) hydrolysis in both groups whereas the diet did not impact the nucleotide hydrolysis. NTPDase2 and 3 mRNA expressions decreased in the Li-Pilo group (41 % and 42 %). This data highlights the participation of the purinergic system in the pathophysiology of this model of epilepsy, since nucleotide hydrolysis and NTPDase expressions were altered by Li-Pilo exposure, with no significant effects of the ketogenic diet. However, the interaction between purinergic signaling and a ketogenic diet on epilepsy still needs to be better elucidated.

Keywords Ketogenic diet · Ecto-nucleotidases · Status epilepticus · Rat hippocampus

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Introduction

The Ketogenic Diet (KD) was initially devised in 1921 to mimic the anticonvulsant effects of fasting, which were known to suppress seizures (Wilder 1921). Despite its long history of clinical use, it is still not entirely clear how a KD affects the brain and what mechanism(s) underlies its seizure-suppressive action. Because both a KD and fasting have beneficial effects on epilepsy, it has been assumed that they share a common mechanism in alleviating seizures (Schwartzkroin 1999; Janigro 1999). Besides, both a KD and fasting produce elevated blood levels of β-hydroxybutyrate (β-OHB) and acetoacetate,

it has been speculated that ketosis may have a beneficial effect upon brain seizure resistance. In addition to ketosis, other changes associated with a ketogenic diet might affect seizure activity. For example, changes in energy metabolism, in the lipid composition of cell membranes, in the level of brain water content, and in brain pH, have all been suggested as to playing a role in seizure suppression (Schwartzkroin 1999; Janigro 1999).

Temporal lobe epilepsy (TLE) is the most common form of human epilepsy. Patients often have a clinical history, including an initial precipitating injury (febrile convulsions, Status Epilepticus (SE) or trauma), followed by a latent period of several years, before the emergence of complex partial seizures (Jutila et al. 2001). The lithium-pilocarpine (Li-Pilo) model reproduces the most clinical, temporal and neuropathologic features of human TLE (Turski et al. 1989; Cavalheiro 1995; Dubé et al. 2001a, b). In adult rats, the systemic injection of pilocarpine associated with lithium leads to partial seizures and SE. This acute phase is followed by a latent seizure-free phase, preceding the expression of spontaneous recurrent seizures (SRS), which can be]observed until death (Cavalheiro 1995; Dubé et al. 2001a, b; Rigoulot et al. 2004).

Systemic administration of a large dose of pilocarpine leads to excessive cholinergic stimulation in the brain, which is manifested as an alteration in electro-encephalographic activity, cell damage and convulsions (Cavalheiro et al. 1987; Turski et al. 1989). Pre-treatment with lithium has been found to potentiate the seizure activity induced by pilocarpine (Honchar et al. 1983), which may be prevented by either cholinergic antagonists or anticonvulsive drugs (Morrisett et al. 1987a, b).

The involvement of the purinergic system in the physiopathology of epilepsy has been discussed at length (Abbracchio et al. 2009). The nucleoside adenosine has been considered to be a natural brain anticonvulsant (Boison 2005). In the healthy brain, the development and spread of seizures is thought to be prevented by a tonic anticonvulsant effect, mediated by endogenous adenosine, which is kept in the range of 25–250 nM (Dunwiddie and Masino 2001; Fredholm et al. 2001). However, during situations of metabolic stress, such as epileptic seizures or during periods of oxygen stress, extracellular adenosine concentration rises rapidly to micromolar levels, which is then able to activate all types of adenosine receptors (Berman et al. 2000; Pedata et al. 2001).

The enhancement of purinergic transmission, mediated through adenosine A_1 receptors agonist, has potentiated the protective activity of classical antiepileptic drugs (AEDs) (Borowicz et al. 2002). In this way, several approaches to reduce seizure damage are focused in an increase in adenosine levels, through adenosine administration and adenosine kinase inhibitors (Boison and Stewart 2009). Adenosine can exert its anticonvulsant functions, after its release by nucleoside bidirectional transport, or by production through sequential catabolism of ATP by ectonucleotidases, such as E-NTPDases (ectonucleoside triphosphate diphosphohydrolases) and ecto-5′-nucleotidase (Battastini et al. 1991; Bianchi and Spychala 2003; Robson et al. 2006; Zimmermann 2006). The augmentation of extracellular ATP in seizure-prone mice was suggested to be possible owing to decreased brain ATPase activity (Wieraszko and Seyfried 1989). In fact, animal models of epilepsy and convulsion, through exposure to pentylenetetrazole, pilocarpine and kainic acid, have been shown to alter the activity of ectonucleotidases (Bonan et al. 2000; Bruno et al. 2003). E-NTPDases are membrane-bounded enzymes with a catalytic site located in the extracellular medium that sequentially converts nucleosides triphosphates in monophosphates, especially the physiologically actives ATP and ADP (Zimmermann 2001). This family of enzymes includes eight members, identified as NTPDase1 to 8. While four of the NTPDases are cell-surface-located enzymes with an extracellularly facing catalytic site (NTPDase 1, 2, 3 and 8), the other four members have intracellular localization (Robson et al. 2006). These eight members of NTPDases enzymes have a considerable variation of hydrolysis rates and are the main source of AMP. Ecto-5′-nucleotidase hydrolyses monophosphate nucleosides, such as AMP, is directly involved in adenosine production in the extracellular medium (Bianchi and Spychala 2003). On other hand, the astrocyte-specific enzyme adenosine kinase (ADK) constitutes a major metabolic uptake system for adenosine, and therefore, largely regulates extracellular levels of adenosine (Boison 2008; Wetherington et al. 2008). Reduction of ADK expression has been seen after a KD treatment (Masino et al. 2011). In general, this myriad of enzymes contributes to control the level of nucleosides and nucleotides in the extracellular medium, promoting the regulation of nucleotide-mediated signaling of purinoceptors.

Considering the suggestion of purines as mediators of a ketogenic diet neuroprotective action and the important role of the ectonucleotidases pathway regarding the control of extracellular purine levels, the aim of this study was to evaluate the effect of a ketogenic diet on the nucleotide hydrolysis and NTPDases expressions in the pilocarpine model of epilepsy.

Experimental procedures

Materials

Nucleotides (ATP, ADP and AMP), Percoll, Trizma Base, Malachite Green Base, Comassie Brilliant Blue G, EDTA, HEPES and Pilocarpine Hydrocloride were purchased from

Sigma, St. Louis, Mo, U.S.A. All other reagents were of analytical grade.

Animals

21-day-old female Wistar rats came from the local breeding colony. They were maintained on a 12-h light/dark cycle in a ventilated room at 21 °C with free access to food and water. Rats were housed in groups of 4 animals per box. Each box had animals of the same experimental group. All animal procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Lithium-pilocarpine-induced SE

At 21 days old, 24 rats received an i.p. injection of lithium chloride (127 mg/kg); 18–19 h later, rats received an i.p. injection of pilocarpine hydrochloride (60 mg/kg), for the induction of SE. The control groups received an injection of the same amount of normal saline (n=11). After pilocarpine injection, all rats progressed to SE. The onset of SE was characterized by initial immobility and chewing, followed by repetitive clonic activity of the trunk and limbs. The rats then developed repeated rearing with forelimb clonus and falling, interspersed with immobility, chewing, and myoclonic jerks, occurring singularly, or in series (Zhao et al. 2004). Two (8.33 %) of the SE rats died. Twenty-four hours after the induction of SE, the rats received the diets as described below.

Diets

In the present study we chose a KD with 25 % protein in order to avoid undernutrition effects in the rats (Table 1). The animals were divided into four groups: The control group (submitted to an injection of saline and received standard diet (SD); n=5); The KD group (submitted to an injection of saline and received KD; n=6); The Li-Pilo group (submitted to lithium-pilocarpine–induced SE and received SD; n =11); and the Li-Pilo + KD group (submitted to a lithium-pilocarpine-induced SE model and received KD; n=11). All rats consumed food and water *ad libitum* for 6 weeks. At the end of the treatments they were with 9 weeks old.

Isolation of blood serum fraction and ketonemia determination

The rats were decapitated and drained from the cut surface. The blood samples were drawn and immediately centrifuged at 3,000 r.p.m. for 5 min at room temperature and the Ketonemia was determined by a semiquantitative kit (Keto-Diabur-Test®5000) from Roche Diagnostics (Mannheim, Germany).

Table 1 Composition of control and ketogenic diets

Control	g/100 g	Ketogenic	g/100 g
Lard	4,5	Lard	65,5
Soy oil	0,5	Soy oil	2,0
Soy protein[a]	25	Soy Protein[a]	25
Fiber	1,0	Fiber	1,0
Salt Mix[b]	4,0	Salt Mix[b]	4,0
Vitamin Mix[c]	1,0	Vitamin Mix[c]	1,0
DL-methionine[d]	0,3	DL-methionine[d]	0,3
Carbohydrates	63,7	Carbohydrates	1,2

[a] Soy protein purity 92 % (BUNGE)

[b] Mineral mixture (Roche, São Paulo, Brazil), mg/100 g of ration: NaCl, 557; Kl, 3.2; KH2PO4, 1,556; MgSO4, 229; CaCO3, 1,526; FeSO4 _7H2O, 108; MnSO4 _ H2O, 16; ZnSO4 _ 7H2O, 2.2; CuSO4 _ 5H2O, 1.9; and CoCl2 _ 6H2O, 0.09

[c] Vitamin mixture (Roche, São Paulo, Brazil), mg/100 g of ration: vitamin A, 4; vitamin D, 0.5; vitamin E, 10; menadione, 0.5; choline, 200; PABA 10; inositol 10 mg; niacin, 4; pantothenic acid, 4; riboflavin, 0.8; thiamin, 0.5; pyridoxine, 0.5; folic acid, 0.2; biotin, 0.04; and vitamin B-12, 0.003

[d] DL-methionine (Delaware, Porto Alegre, Brazil)

Subcellular fraction preparation

The animals were killed by decapitation and their brains were removed and placed into an ice-cold isolation medium (320 mM sucrose, 5 mM HEPES, and 0.1 mM EDTA, pH 7.5) and were immediately dissected on ice. The total hippocampi were gently homogenized in 5 volumes of ice-cold isolation medium with a motor-driven Teflon-glass homogenizer. The synaptosomes were isolated as described previously (Nagy and Delgado-Escueta 1984). Briefly, 0.5 ml of crude mitochondrial fraction was mixed with 4.0 ml of an 8.5 % Percoll solution and layered onto an isosmotic Percoll/sucrose discontinuous gradient (10 %/16 %). The synaptosomes that banded at 10 %/16 % Percoll interface were collected with wide tip disposable plastic transfer pipettes. The synaptosomal fractions were washed twice at 15,000 × g for 20 min with the same ice-cold medium to remove the contaminating Percoll and the synaptosome pellet was re-suspended to a final protein concentration of approximately 0.5 mg/ml. The material was prepared fresh daily and maintained at 0–4 °C throughout preparation.

Enzyme assays

The reaction medium used to assay the ATP and ADP hydrolysis from hippocampal synaptosomes was essentially as described previously (Battastini et al. 1991) and

contained 5.0 mM KCl, 1.5 mM $CaCl_2$, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose and 45 mM Tris–HCl buffer, pH 8.0, in a final volume of 200 μl. The reaction medium used to assay the 5′-nucleotidase activity (AMP hydrolysis) contained 10 mM $MgCl_2$, 0.1 M Tris–HCl, pH 7.0, and 0.15 M sucrose in a final volume of 200 μl (Heymann et al. 1984). The synaptosomal preparation (10–20 μg protein) was added to the reaction mixture and pre-incubated for 10 min at 37 °C. The reaction was initiated by the addition of ATP, ADP or AMP to a final concentration of 1.0 mM and stopped by the addition of 200 μl 10 % trichloroacetic acid. The released inorganic phosphate (Pi) was measured as previously described by Chan et al. (1986) and expressed as nmol of Pi released by minute by milligram of protein. In both enzyme assays, incubation times and protein concentrations were chosen in order to ensure the linearity of the reactions. Controls with the addition of the enzyme preparation after the addition of trichloroacetic acid were used to correct non-enzymatic hydrolysis of the substrates. All samples were run in triplicate. The percentage of alteration of enzymatic activity was calculated assuming the respective control animal activity as 100 %.

Protein determination

Protein was measured by the Coomassie Blue Method (Bradford 1976), using bovine serum albumin as standard.

Analysis of gene expression by semi-quantitative RT-PCR

The analysis of the E-NTPDases expressions was carried out by a semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) assay. The hippocampi had its total RNA extracted with a TRIzol reagent according to the manufacturer's instructions (Invitrogen, USA). Afterwards, the cDNA species were synthesized with SuperScript First-Strand Synthesis System for RT-PCR from 3 μg of total RNA and Oligo DT in accordance with the suppliers (Invitrogen, USA). RT reactions were performed for 50 min at 42 °C. cDNA (0.1 μl) was used as a template for the RT-PCR, with specific primers for all of the enzymes analyzed (primers sequences described below). β-actin-PCR was performed as a control for the cDNA synthesis. PCR reactions were performed (total volume of 25 μl) using a concentration of 0.4 μM of each primer, 2 mM (β-actin, E-NTPDase1 and 3) or 4 mM (E-NTPDase2) of $MgCl_2$ and 1 U Taq polymerase in the supplied reaction buffer. To improve the conditions of E-NTPDase 1, 5 % PCR glycerol was added to the reaction medium. Conditions for E-NTPDases PCR were as follows: an initial 1 min denaturation step at 94 °C, 1 min at 94 °C, a 1 min annealing step at 65 °C (E-NTPDase1 and E-NTPDase3) or 66 °C (NTPDase2), a 1 min extension step at 72 °C for 35 cycles and a final 10 min extension at 72 °C (Oses et al. 2007). Conditions for β-actin PCR were as follows: an initial 1 min denaturation step at 94 °C, 1 min at 94 °C, a 1 min annealing step at 58.5 °C, a 1 min extension step at 72 °C for 35 cycles and a final 10 min extension at 72 °C. The amplification products were: E-NTPDase 1: 543 bp, E-NTPDase 2: 331 bp, E-NTPDase 3: 267 bp and β-actin: 210 bp. PCR products were submitted to electrophoresis using a 1 % agarose gel. Band intensities were analyzed in a semi-quantitative manner using the software ImageJ 1.37 for Windows. The following set of primers were used: E-NTPDase 1: Primer 1 - 5′-GAT CAT CAC TGG GCA GGA GGA AGG-3′, Primer 2 - 5′- AAG ACA CCG TTG AAG GCA CAC TGG -3′, E-NTPDase 2: Primer 1 - 5′-GCT GGG TGG GCC GGT GGA TAC G -3′, Primer 2- 5′-ATT GAA GGC CCG GGG ACG CTG AC -3′, E-NTPDase 3: Primer 1- 5′- CGG GAT CCT TGC TGT GCG TGG CAT TTC TT-3′, Primer 2 - 5′- TCT AGA GGT GCT CTG GCA GGA ATC AGT -3′, β-actin: Primer 1- 5′- TAT GCC AAC ACA GTG CTG TCT GG -3′, Primer 2- 5′- TAC TCC TGC TTC CTG ATC CAC AT -3′.

Statistical analysis

The weights of the rats receiving SD or KD and submitted to saline or lithium-pilocarpine injection were analyzed along the treatment using a Two Way analysis of variance, considering time and treatment as factors. The effect of diet and lithium-pilocarpine treatment on ATP, ADP and AMP hydrolysis from hippocampal synaptosomes of 21-day-old rats submitted to KD was expressed as the mean ± SEM of at least five animals and analyzed using the Two Way analysis of variance considering diet and drug as factors. Multiple comparisons of means were performed by Bonferroni method by using diet and drug as factors. Results of lithium-pilocarpine on gene expression were shown as the mean ± SEM of optical density and analyzed by Student's T test. The significance level was established at $p<0.05$.

Results

In the present study, we chose a KD with 25 % protein, in order to avoid undernutrition effects in the rats. With this treatment, the animals remained apparently healthy and a semi-quantitative test showed that the blood levels of ketone bodies in the serum of control rats were lower than 0.5 mmol/L, while animals fed on the ketogenic diet developed an increased level of ketosis (1–5 mmol/L) (data not shown).

The weight of each animal was registered from the first day of injection (saline or lithium/pilocarpine) up to the sixth week later. Rats receiving KD and saline, or lithium-

pilocarpine, lost weight during the third and fourth week after injection, in comparison to the control group ($p<0.05$) (Fig. 1).

Hydrolysis of nucleotides from hippocampus of young rats treated with SD or KD and exposed to a lithium-pilocarpine model of epilepsy was analyzed (Fig. 2). The factor lithium-pilocarpine exposure affected the ATP and ADP hydrolysis in both groups whereas the diet did not impact the nucleotide hydrolysis (Fig. 2a and b). ATP hydrolysis was reduced in the SD and KD group after lithium-pilocarpine exposure (8 % and 16 %, respectively, $F(1;29)=6.06$, $p=0.02$). ADP hydrolysis was increased in the SD and KD group after lithium-pilocarpine exposure (18 % and 22 %, respectively, $F(1;27)=7.38$, $p=0.0114$). AMP hydrolysis was not affected ($F(1;28)=0.21$, $p=0.6470$).

As lithium-pilocarpine induced alteration in the ATP and ADP hydrolysis in an independent way of diet supplied, we performed the evaluation of NTPDase1, 2 and 3 gene expressions in saline and lithium-pilocarpine groups. Statistical analysis revealed a decrease in NTPDase2 and 3 mRNA expression (41 % and 42 %, respectively, $p<0.05$) (Fig. 3).

Discussion

Here, we evaluated the relation between a ketogenic diet and the enzymatic cascade responsible for nucleotide availability after the development of *status epileticus* induced by lithium-pilocarpine. The first consideration will focus on

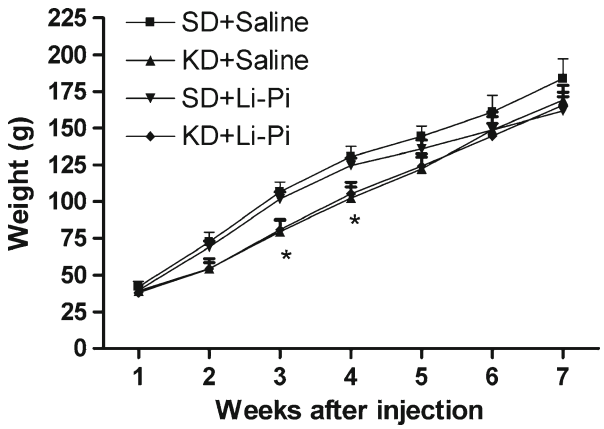

Fig. 1 Weight of rats receiving standard or ketogenic diet submitted to saline or lithium-pilocarpine injection. Weekly evaluation started on the first day of injection and stopped 6 weeks later. SD + Saline: standard diet plus saline injection, ($n=5$); KD + saline: ketogenic diet plus saline injection, ($n=6$); SD + Li-Pilo: Standard diet plus lithium-pilocarpine injection, ($n=11$); KD + Li-Pilo: ketogenic diet plus lithium-pilocarpine injection ($n=11$). Points represent mean ± S.E.M. * represents significant difference from respective controls considering $p<0.05$. Statistical analysis was performed using Two Way analysis of variance

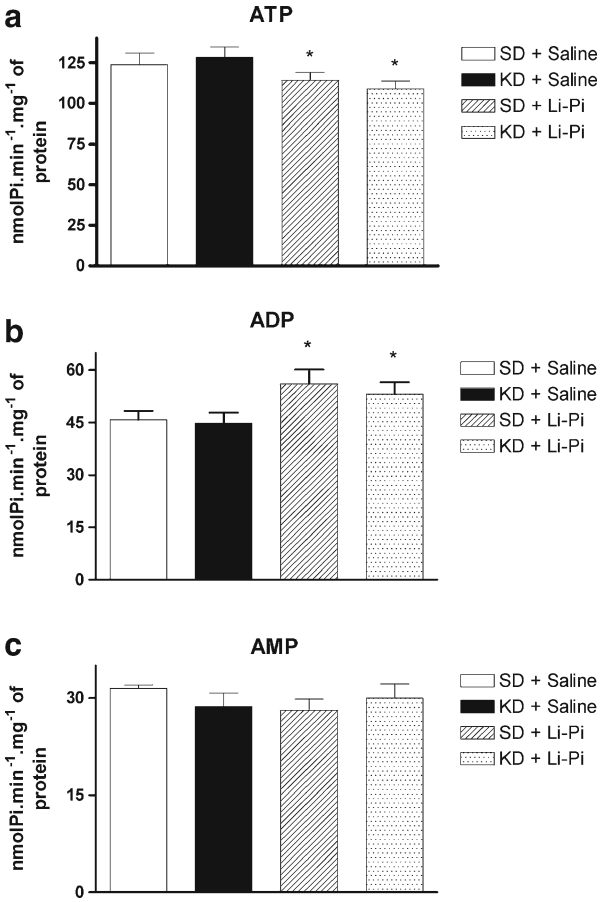

Fig. 2 Effect of lithium-pilocarpine treatment on ATP, ADP and AMP hydrolysis from hippocampal synaptosomes of rats submitted to a ketogenic diet for 6 weeks. *Bars* represent mean ± S.E.M. Statistical analysis was performed using Two Way analysis of variance. Multiple comparisons of means was performed by Bonferroni method by using diet and drug as factors. * indicates significant difference between the respective control (SD-saline or KD-saline) and Lithium-pilo- treated animals considering $p<0.05$

the weight of the rats during diet treatment. A KD treatment has been shown to reduce animal weight (Langston and Myers 2011), whereas no alteration was registered in humans receiving a KD chronic treatment (Tagliabue et al. 2012). In fact, in our study, animals lost weight only during the middle of the treatment, recovering the standard weight up to the end of it. Despite the classical effect of weight loss during a treatment with a KD and ketonemia, KD per se did not alter the nucleotide hydrolysis.

As nucleotides play an important role in neurotransmission and neuromodulation, especially during epileptic episodes, we have checked the effect of lithium-pilocarpine-induced SE on nucleotide hydrolysis by hippocampal synaptosomes. Our results have shown that ATP hydrolysis was reduced whereas ADP hydrolysis was increased after lithium-pilocarpine-induced SE. Previous findings have shown that nucleotide hydrolysis were altered after pilocarpine and kainate models of epilepsy, indicating that the

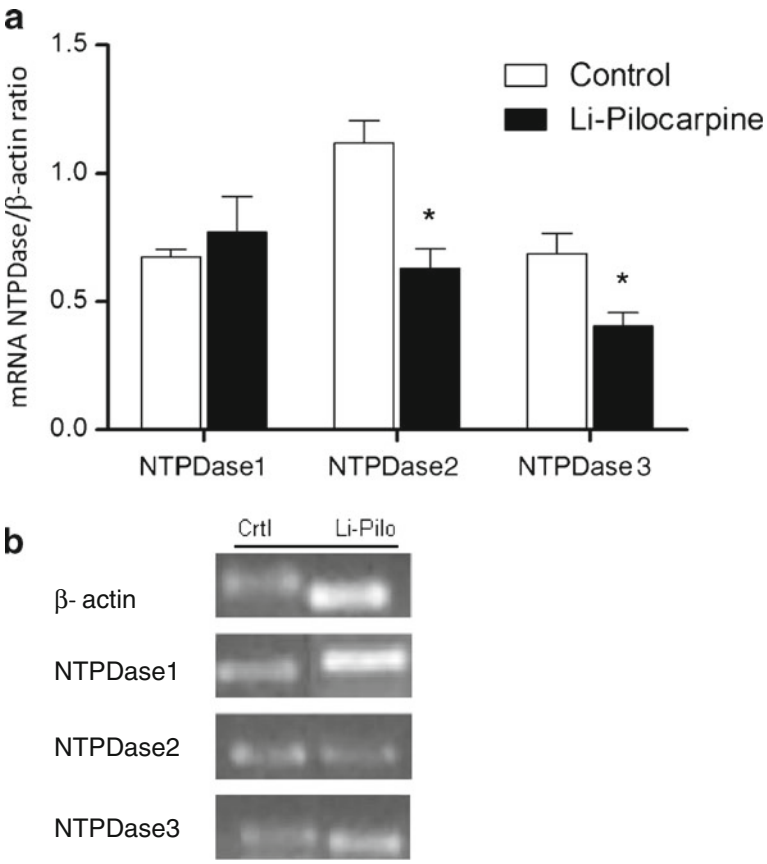

Fig. 3 Effect of lithium-pilocarpine treatment on NTPDase 1, 2 and 3 mRNA transcript levels from hippocampi of rats submitted to a ketogenic diet for 6 weeks. *Bars* represent mean ± S.E.M of optical density. * Represents a significant difference at $p<0.05$ in relation to control rats. The data is from at least three different experiments. Statistical analysis was performed using Student's *T* test

regulation of ectonucleotidase pathway may play a modulatory role during the evolution of behavioral and pathophysiological changes related to temporal lobe epilepsy (Bonan et al. 2000). We have previously shown that chronic treatment with lithium increases the ecto-nucleotidase activities in rat hippocampal synatosomes (Wilot et al. 2004). Although we do not know what effect would happen after an acute injection of lithium, we can assume that this is not affecting the presented results, since in the present study, we observed a decrease in ATPase and an increase in ADPase activities, while no effect was observed on AMPase activity. Then, it is improbable that a single injection would alter the results obtained with chronic treatment of 1 month, to the point of reversing the effect on the expression and activity of the NTPDases.

Considering the changes observed in ATP and ADP hydrolysis, we evaluated the expression of main NTPDases that could contribute to nucleotide hydrolysis in SNC, the NTPDases1, 2 and 3. NTPDases2 and 3 had their expression decreased in lithium-pilocarpine exposed animals. The reduction of NTPDases expressions can be related to the decrease of ATP hydrolysis, especially when we consider that NTPDase2, hydrolyses ATP, 30 times more than ADP (Zimmermann 2006). Meanwhile, NTPDase3 hydrolyzes ATP 3 times more than ADP (Zimmermann 2006). As we evaluated the expression of the whole hippocampal tissue, we could not discharge glial and endothelial contributions. In hippocampus, immunohistochemistry showed an increased ATP hydrolysis in the neuropil and in the subgranular layer of the dentate gyrus; the latter had previously been assigned to the activity of NTPDase 2 (Shukla et al. 2005; Langer et al. 2008) contributing to the idea that the reduction of NTPDase 2 can reflect, even partially, in the ATP hydrolysis reduction. Since NTPDase 1 and 3 activities contribute strongly to ADP hydrolysis, and expression of NTPDase3 is decreased, we could assume that the activity of NTPDase 1 is crucial for the increased ADP hydrolysis observed in this study. However, NTPDase1 has not only been strongly associated with microglia and blood vessels, but also with the neuropil of defined brain regions (Langer et al. 2008). In this way, specific alterations on the synaptic cleft could be masked by the observation of the general cellular content of hippocampus.

The participation of the purinergic system on the anticonvulsive effect of a KD has been based on the ability of increased levels of ATP to promote an adenosine burst on the synaptic environment (Masino and Geiger 2008). This is understandable when we assume that adenosine is able to reduce neuronal activity by K^+ channel - mediated hyperpolarization through adenosine A_1 receptor activation (Abbracchio et al. 2009). The increase of ATP levels in the extracellular medium after a KD treatment was shown in mice (Nakazawa et al. 1983) and the source of this

nucleotide has been attributed mainly to astrocytes (Guzman and Blazquez 2001). Additionally, some observations support the hypothesis of reduced excitatory transmission, such as a reduction of glutamate content, playing a role in the anti-convulsive effect of a KD (Patel et al. 1988). Studies of our group, focusing the effect of a KD on rat blood serum hydrolysis of nucleotides, reported an increase of nucleotides hydrolysis by a KD per se and in association with lithium-pilocarpine induced SE (Da Silveira et al. 2010). In the present study, the lack of effect on nucleotide breakdown, after a KD treatment, raises the question that, in basal conditions, ketones could have no ability to alter the energy balance able to affect nucleotide hydrolysis in a synaptic cleft. Additionally, during lithium-pilocarpine exposure, a KD was also unable to affect nucleotide hydrolysis, despite the fact that nucleotide release increased during the SE. Considering that in the present study we have evaluated the nucleotide hydrolysis in synaptosomal fractions, we have no information about the contribution of nucleotide breakdown from glial cells, which certainly could contribute to a better approach of biological events occurring in the synaptic environment.

In summary, we have observed that the epilepsy model of lithium-pilocarpine is able to alter nucleotide hydrolysis and NTPDase expressions, with no significant effect of a ketogenic diet on these observations. This data highlights the participation of the purinergic system in the pathophysiology of this model of epilepsy. However, the interaction between purinergic signaling and a ketogenic diet on epilepsy still needs to be better elucidated.

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References

Abbracchio MP, Burnstock G, Verkhratsky A, Zimmermann H (2009) Purinergic signaling in the nervous system: an overview. Trends Neurosci 32(1):19–29

Battastini AM, Da Rocha JB, Barcellos CK (1991) Characterization of an ATP diphosphohydrolase (EC 3.6.1.5) in synaptosomes from cerebral cortex of adult rats. Neurochem Res 16:1303–1310

Berman RF, Fredholm BB, Aden U, O'Connor WT (2000) Evidence for increased dorsal hippocampal adenosine release and metabolism during pharmacologically induced seizures in rats. Brain Res 872(1–2):44–53

Bianchi V, Spychala J (2003) Mammalian 5′-nucleotidases. J Biol Chem 278(47):46195–46198

Boison D (2005) Adenosine and epilepsy: from therapeutic rationale to new therapeutic strategies. Neuroscientist 11:25–36

Boison D (2008) The adenosine kinase hypothesis of epileptogenesis. Prog Neurobiol 84:249–262

Boison D, Stewart K-A (2009) Therapeutic epilepsy research: from pharmacological rationale to focal adenosine augmentation. Biochem Pharmacol 78(12):1428–1437

Bonan CD, Walz R, Pereira GS, Worm PV, Battastini AM, Cavalheiro EA, Izquierdo I, Sarkis JJ (2000) Changes in synaptosomal ectonucleotidase activities in two rat models of temporal lobe epilepsy. Epilepsy Res 39(3):229–238

Borowicz KK, Luszczki J, Czuczwar SJ (2002) 2-Chloroadenosine, a preferential agonist of adenosine A1 receptors, enhances the anticonvulsant activity of carbamazepine and clonazepam in mice. Eur Neuropsychopharmacol 12:173–179

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Bruno AN, Oses JP, Amaral O, Coitinho A, Bonan CD, Battastini AM, Sarkis JJ (2003) Changes in nucleotide hydrolysis in rat blood serum induced by pentylenetetrazol-kindling. Brain Res Mol Brain Res 114(2):140–145

Cavalheiro EA (1995) The pilocarpine model of epilepsy. Ital J Neurol Sci 16:33–37

Cavalheiro EA, Silva DF, Turski WA, Calderazzo-Fihlo LS, Bortolotto A, Turski L (1987) The susceptibility of rats to pilocarpine-induced seizures is age-dependent. Dev Brain Res 37:43–58

Chan K, Delfert D, Junges KD (1986) A direct colorimetric assay for Ca2+-ATPase activity. Anal Biochem 157:375–380

Da Silveira VG, Cognato GP, Müller AP, Figueiró F, Bonan DC, Perry MLS, Battastini AMO (2010) Effect of ketogenic diet on nucleotide hydrolysis and hepatic enzymes in blood serum of rats in a lithium-pilocarpine-induced status epilepticus. Metab Brain Dis 25:211–217

Dubé C, Da Silva Fernandes MJ, Nehlig A (2001a) Age-dependent consequences of seizures and the development of temporal lobe epilepsy in the rat. Dev Neurosci 23:219–223

Dubé C, Boyet S, Marescaux C, Nehlig A (2001b) Relationship between neuronal loss and interictal glucose metabolism during the chronic phase of the lithium-pilocarpine model of epilepsy in the immature and adult rat. Exp Neurol 167:227–241

Dunwiddie TV, Masino SA (2001) The role and regulation of adenosine in the central nervous system. Annu Rev Neurosci 24:31–55

Fredholm BB, Ijzerman AP, Jacobson KA, Klotz KN, Linden J (2001) International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. Pharmacol Rev 53(4):527–552

Guzman M, Blazquez C (2001) Is there an astrocyte-neuron ketone body shuttle? Trends Endocrinol Metab 12:169–173

Heymann D, Reddington M, Kreutzberg GW (1984) Subcellular localization of 5′-nucleotidase in rat brain. J Neurochem 43:971–978

Honchar MP, Olney JW, Sherman WR (1983) Systemic cholinergic agents induce seizures and brain damage in lithium-treated rats. Science 2388:323–325

Janigro D (1999) Blood-brain barrier, ion homeostatis and epilepsy: possible implications towards the understanding of ketogenic diet mechanisms. Epilepsy Res 37(3):223–232

Jutila L, Ylinen A, Partanen K, Alafuzoff I, Mervaala E, Partanen J, Vapalahti M, Vainio P, Pitkänen A (2001) MR volumetry of the entorhinal, perirhinal, and temporopolar cortices in drug-refractory temporal lobe epilepsy. AJNR Am J Neuroradiol 22 (8):1490–1501

Langer D, Hammer K, Koszalka P, Schrader J, Robson S, Zimmermann H (2008) Distribution of ectonucleotidases in the rodent brain revisited. Cell Tissue Res 334:199–217

Langston JL, Myers TM (2011) Diet composition modifies the toxicity of repeated soman exposure in rats. Neurotoxicology 32(6):907–915

Masino SA, Geiger JD (2008) Are purines mediators of the anticonvulsivant/neuroprotective effects of ketogenic diet? Trends in Neuroscience 31(6):273–278

Masino SA, Li T, Theofilas P, Sandau US, Ruskin DN, Fredholm BB, Geiger J, Aronica E, Boison D (2011) A ketogenic diet suppresses

seizures in mice through adenosine A1 receptors. J Clin Invest 121(7):2679–2683

Morrisett RA, Jope RS, Snead OC 3rd (1987a) Effects of drugs on the initiation and maintenance of status epilepticus induced by administration of pilocarpine to lithium-pretreated rats. Exp Neurol 97(1):193–200

Morrisett RA, Jope RS, Snead OC 3rd (1987b) Status epilepticus is produced by administration of cholinergic agonists to lithium-treated rats: comparison with kainic acid. Exp Neurol 98 (3):594–605

Nagy A, Delgado-Escueta AV (1984) Rapid preparation of synaptosomes from mammalian brain using nontoxic isoosmotic gradient material (Percoll). J Neurochem 43(4):1114–1123

Nakazawa M, Kodama S, Matsuo T (1983) Effects of ketogenic diet on electroconvulsive threshold and brain contents of adenosine nucleotides. Brain Dev 5(4):375–380

Oses JP, Viola GG, de Paula Cognato G, Júnior VH, Hansel G, Böhmer AE, Leke R, Bruno AN, Bonan CD, Bogo MR, Portela LV, Souza DO, Sarkis JJ (2007) Pentylenetetrazol kindling alters adenine and guanine nucleotide catabolism in rat hippocampal slices and cerebrospinal fluid. Epilep Res 75(2–3):104–111

Patel S, Chapman AG, Millan MH, Meldrum BS (1988) Epilepsy and excitatory amino acid antagonists. In: Lodge D (ed) Excitatory amino acids in health and disease. Wiley, London, pp 353–378

Pedata F, Corsi C, Melani A, Bordoni F, Latini S (2001) Adenosine extracellular brain concentrations and role of A2A receptors in ischemia. Ann N Y Acad Sci 939:74–84, Review

Rigoulot MA, Koning E, Ferrandon A, Nehlig A (2004) Neuroprotective properties of topiramate in the lithium-pilocarpine model of epilepsy. J Pharmacol Exp Ther 308:787–795

Robson SC, Sévigny J, Zimmermann H (2006) The E-NTPDase family of ectonucleotidases: structure function relationships and pathophysiological significance. Purinergic Signal 2(2):409–430

Schwartzkroin PA (1999) Mechanisms underlying the anti-epileptic efficacy of the ketogenic diet. Epilepsy Res 37(3):171–180

Shukla V, Zimmermann H, Wang LP, Kettenmann H, Raab S, Hammer K, Sévigny J, Robson SC, Braun N (2005) Functional expression of the ecto-ATPase NTPDase2 and of nucleotide receptors by neuronal progenitor cells in the adult murine hippocampus. J Neurosci Res 80:600–610

Tagliabue A, Bertoli S, Trentani C, Borrelli P, Veggiotti P (2012) Effects of the ketogenic diet on nutritional status, resting energy expenditure, and substrate oxidation in patients with medically refractory epilepsy: A 6-month prospective observational study. Clin Nutr 31(2):246–249

Turski L, Ikonomidou C, Turski WA, Bortolotto ZA, Cavalheiro EA (1989) Review: cholinergic mechanisms and epileptogenesis. The seizures induced by pilocarpine: a novel experimental model of intractable epilepsy. Synapse 3:154–171

Wetherington J, Serrano G, Dingledine R (2008) Astrocytes in the epileptic brain. Neuron 58:16

Wieraszko A, Seyfried TN (1989) Increased amount of extracellular ATP in stimulated hippocampal slices of seizure prone mice. Neurosci Lett 106:287–293

Wilder RM (1921) The effect of ketonemia on the course of epilepsy. Mayo Clin Bull 2:307–308

Wilot LC, Da Silva RS, Ferreira OJ, Bonan CD, Sarkis JJF, Rocha E, Battastini AMO (2004) Chronic treatment with lithium increases the ecto-nucleotidase activities in rat hippocampal synatosomes. Neurosc Letters 368:167–170

Zhao Q, Stafstrom CE, Fu DD, Hu Y, Holmes GL (2004) Detrimental effects of the ketogenic diet on cognitive function in rats. Pediatric Res 55(3):498–506

Zimmermann H (2001) Ecto-nucleotidases: some recent developments and a note on nomenclature. Drug Dev Res 52:44–56

Zimmermann H (2006) Ectonucleotidases in the nervous system. Novartis Found Symp 276:113–128