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Neuroprotective effect of pyruvate and oxaloacetate during pilocarpine induced status epilepticus in rats

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ABSTRACT

Recent research data have shown that systemic administration of pyruvate and oxaloacetate causes an increased brain-to-blood glutamate efflux. Since increased release of glutamate during epileptic seizures can lead to excitotoxicity and neuronal cell death, we tested the hypothesis that glutamate scavenging mediated by pyruvate and oxaloacetate systemic administration could have a neuroprotective effect in rats subjected to status epilepticus (SE). SE was induced by a single dose of pilocarpine (350 mg/kg i.p.). Thirty minutes after SE onset, a single dose of pyruvate (250 mg/kg i.p.), oxaloacetate (1.4 mg/kg i.p.), or both substances was administrated. Acute neuronal loss in hippocampal regions CA1 and hilus was quantitatively determined five hours after SE onset, using the optical fractionator method for stereological cell counting. Apoptotic cascade in the hippocampus was also investigated seven days after SE using caspase-1 and -3 activity assays. SE-induced neuronal loss in CA1 was completely prevented in rats treated with pyruvate plus oxaloacetate. The SE-induced caspase-1 activation was significantly reduced when rats were treated with oxaloacetate or pyruvate plus oxaloacetate. The treatment with pyruvate and oxaloacetate caused a neuroprotective effect in rats subjected to pilocarpine-induced SE.

1. Introduction

Glutamate (Glu) is the major excitatory neurotransmitter in the nervous system. Glu regulates many brain functions and its synaptic concentration must be precisely controlled to avoid excessive excitation and toxicity. As a matter of fact, the brain has at least two mechanisms to control Glu extracellular concentration. The first is credited mainly to the presence, both on nerve terminals and on astrocytes, of members of a large family of Na^+-dependent Glu transporters which bind and take up Glu. This system ensures that the very high concentrations of Glu, transiently present after synaptic or astrocytic release, are soon decreased to concentrations at which Glu exerts neither overt excitatory nor excitotoxic activities (Danbolt, 2001; Sattler and Tymianski, 2001). The second mechanism accounts for the elimination of Glu from brain into blood in the face of an unfavorable concentration gradient between interstitial/cerebrospinal fluids (ISF/CSF) Glu and blood plasma (O'Kane et al., 1999). According to this mechanism, extracellular Glu is transported via Na^+-dependent transporters, located on the antiluminal membrane of brain capillaries being concentrated and accumulates into endothelial cells. When its concentration exceeds those found in plasma, Glu is facilitatively transported across the luminal membrane into blood. The brain-to-blood Glu efflux may also involve a glutamate–glutamine (Gln) cycle (yet to be demonstrated) between astroglial end feet and endothelial cells. In this pathway, excess Glu is transported into the astrocytic end feet where it is converted into Gln via the Gln synthetase. Gln exits from the end feet and is untaken by Gln transporters, present on the juxtaposed abluminal membrane of capillary endothelial cells (Lee et al., 1998). Once into the endothelial cell, Gln is converted back to Glu via the endothelial glutaminase, which now diffuses into the blood by facilitative transport. Such a mechanism could also sub-serve a neurometabolic coupling (Jakovcevic and Harder, 2007).

Under pathological conditions involving a brain insult such as ischemic stroke, traumatic brain injury or prolonged epileptic seizures, Glu is uncontrollably released from its neuronal and glial stores, via the reverse operation of the excitatory amino acid transporters (EAATs) (Vesce et al., 2007). In these circumstances, excess Glu is also regulated by the transporters associated with the ubiquitous and dense network of brain capillaries, leading to excitotoxic neuronal death in very large brain territories.

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One of the most severe acute neurological conditions, associated with excessive Glu release, is the *status epilepticus* (SE). SE is defined as an epileptic seizure lasting more than 30 min or as intermittent seizures, lasting for more than 30 min, during which the patient does not recover consciousness between repeated episodes (Leite et al., 2006). SE is one of the most common neurological emergencies and several prospective studies have reported an incidence of 10–20/100,000 amongst whites in Europe and the US (Hesdorffer et al., 1998; Coeytaux et al., 2000; Knake et al., 2001). Convulsive SE is the commonest form, representing 40–60% of all SE cases. Mortality is high, with one out of five dying in the first 30 days (Logroscino et al., 1997). The main neurological sequels of SE reported in the literature are cognitive impairment, brain damage-related deficits, and long-term development of recurrent seizures (Leite et al., 2006).

Neurobiological substrate of SE-related brain damage includes the excitotoxic effect of excitatory amino acids, particularly Glu (Ben-Ari and Schwarcz, 1986; Choi, 1988; Naffah-Mazzacoratti and Amado, 2002). Intense seizure activity causes massive Ca^{2+} influx, which results in increased intracellular and intra-mitochondrial membrane depolarization, superoxide production and activation of caspases (Gupta and Dettbarn, 2003; Persike et al., 2008; Henshall, 2007). The large increase in cytosolic Ca^{2+} evoked by activation of Glu receptors (NMDA and AMPA/kainate) seems to be a necessary step in the overall process of neuronal degeneration. This process triggers the acute neuronal cell death that occurs after SE (Maus et al., 1999; Fujikawa et al., 2000; Men et al., 2000).

Gottlieb et al. (2003) recently tested the hypothesis that a larger Glu concentration gradient between ISF/CSF and blood plasma could provide an increased driving force for the brain-to-blood Glu efflux. To achieve a decrease of blood Glu levels, they made use of the Glu scavenging properties of the blood resident enzymes glutamate-pyruvate transaminase (GPT) and glutamate-oxaloacetate transaminase (GOT), which transform Glu into 2-ketoglutarate in the presence of the respective Glu co-substrates, pyruvate (Pyr) and oxaloacetate (Oxa). They showed that the intravenous administration of Pyr and Oxa, which decreases blood Glu levels, accelerates the brain-to-blood Glu efflux. These results support the conclusion that the brain-to-blood Glu efflux can be modulated by changes in blood Glu levels and can be accelerated by blood Glu scavenging (Gottlieb et al., 2003). Accordingly, Zlotnik and colleagues recently tested the effects of blood Glu scavengers in a rat model of closed head injury (CHI) and observed a significant improvement of the neurological recovery in the Oxa-treated and Pyr-treated rats when compared with saline-treated controls (Zlotnik et al., 2007, 2008).

On these bases, we hypothesized that blood Glu scavenging induced by systemic Pyr and Oxa administration could be neuroprotective by increasing brain-to-blood Glu efflux and thus preventing excitotoxic neuronal cell damage caused by prolonged epileptic seizures. In order to test this hypothesis, in the present investigation we studied the effect of Pyr and Oxa administration in rats subjected to pilocarpine-induced SE (Cavalheiro, 1995). Pilocarpine-induced SE is a widely used model to study neurodegeneration in limbic structures after prolonged epileptic seizures, particularly the hippocampal formation (Cavalheiro et al., 1991).

2. Material and methods

2.1. Status epilepticus induction

Male Wistar rats (weight ~250 g) were housed in groups of five under a continuous 12 h/12 h light/dark cycle and had free access to food and water. Experimental rats were injected with 4% pilocarpine hydrochloride (350 mg/kg i.p., Merck). Scopolamine methyl nitrate (1 mg/kg s.c., Sigma) was injected 30 min before pilocarpine to reduce the peripheral cholinergic effects. Approximately 10 min after pilocarpine injection, animals developed partial limbic seizures with secondary generalization leading to self-sustained SE (Turski et al., 1983). After five hours, SE was blocked with diazepam (10 mg/kg i.p.). A control group received saline instead of pilocarpine (Group Saline).

2.2. Pyruvate and oxaloacetate administration

Based on previous experiments designed to evaluate the neuroprotective effect of pyruvate and oxaloacetate in vivo (Lee et al., 2001; Gottlieb et al., 2003; Gonzales-Falcon et al., 2003; Zlotnik et al., 2007), pyruvate solution (250 mg/kg, i.p., pH 7.4, Alfa Aesar) (Group Pilo + Pyr), oxaloacetate solution (1.4 mg/kg, i.p., pH 7.4, Calbiochem) (Group Pilo + Oxa) or both substances (Group Pilo + Pyr + Oxa) were administrated as single injection (1.5 ml) to rats thirty minutes after the development of SE. A control group received the same volume of saline instead of pyruvate and oxaloacetate (Group Pilo + Saline). Survival rates for each experimental group were calculated.

The effect of Pyr and Oxa administration on brain damage was studied five hours after SE onset (neuronal cell counting) and seven days later (caspase activation). These time points were chosen in order to estimate the impact of the treatment on acute/necrotic and late/apoptotic cell death (Fujikawa, 1996; Weise et al., 2005).

2.3. Perfusion, histology and neuronal cell counting

Rats were anaesthetized with chloral hydrate and transcardially perfused with a solution of paraformaldehyde (PF 4%) in phosphate buffer (PB 0.1 M). The brains were removed immediately after perfusion and post-fixed with a solution of PF 4% and sucrose (30%) in PB 0.1 M. Fifty-micron coronal sections through the entire extension of the hippocampus were obtained using a cryostat (−18 °C), mounted on glass slides, and stained with cresyl violet (Nissl).

The estimative of total number of neurons in the CA1 hippocampal sub-region and the hilus of the dentate gyrus was obtained in five animals per group using the stereological method optical fractionator (West et al., 1991). Briefly, every fifth section was selected, resulting in a section sampling fraction of 0.2 (*ssf* = 0.2). In each section, the hippocampal subfield CA1 and the hilus of the dentate gyrus were identified according to a brain atlas (Paxinos and Watson, 1982). Disector counting probes (25 μm × 25 μm) were uniform and randomly distributed through the hippocampus (right and left). Each disector correspond to an area (*a*) of 625 μm^2 and the distance between counting frames (*x,y* step) was 250 μm, resulting in an area sampling fraction of 0.01 (*asf* = 0.01). Neuronal cell bodies (tops) were counted through the entire thickness of each section, resulting in a thickness sampling fraction of 1 (*tsf* = 1). The estimative of total neuronal cell number (*N*) for each region was calculated using the formula (West et al., 1991):

$$N = \sum Q^{-} \cdot \frac{1}{ssf} \cdot \frac{1}{asf} \cdot \frac{1}{tsf}$$

where ΣQ^{-} is the number of counted neurons, *tsf* is the thickness sampling fraction, *asf* is the area sampling fraction, and *ssf* is the section sampling fraction. A pilot study showed that this sampling scheme produced acceptable coefficients of error (*CE*) and variance (*CV*) (West et al., 1991; Keuker et al., 2001).

2.4. Caspase assays

Caspase-1 and -3 activities were studied in five animals per group using the method described by Thornberry et al. (1997) and modified by Belizario et al. (2001). Rats were killed, hippocampi were dissected at 4 °C and added to 20 mM HEPES buffer (pH 7.4) that contained 2 mM EDTA, 0.1% CHAPS, 10% sucrose, 0.1% PMSF, 0.1% benzamidin, 0.1% antipain, 0.1% TLCK, 0.1% chemostatin and 0.1% pepstatin (5 μl homogenization buffer/mg tissue). Homogenates were obtained by mechanically disrupting the tissue three times on dry-ice, with thawing in an ice bath, interpolated by 1 min of moderate vortex shaking. Samples were centrifuged at 12,000 × *g* for 40 min at 4 °C to remove cellular debris. Total proteins were determined in the supernatants using the Bio-Rad Protein Assay (Bio-Rad Labs, Germany). Homogenates (100 μg/protein) were incubated at 37 °C with the selective tetrapeptide substrates: Trp-Glu-His-Asp (Ac-WEHD-AMC, 4 μM) or Asp-Glu-Val-Asp (Ac-DEVD-AMC, 4 μM) for caspase-1 and -3 respectively, in a final volume of 150 μl. For a negative control, homogenates were pre-incubated for 10 min at 37 °C with commercial inhibitors to caspase-1 (Ac-WEHD-CHO, 1 μM) or caspase-3 (Ac-DEVD-CHO, 1 μM), followed by the addition of the respective substrate. Activity was measured continuously over 90 min on a GENius Tecan Austria G.M.B.H. Spectrofluorimeter, using λ_{ex} = 360 nm and λ_{em} = 465 nm. The peptide hydrolysis reaction velocities were expressed as units of fluorescence per min (RFU/min).

2.5. Statistics

Variance analysis (Two-way ANOVA) and Bonferroni post hoc were used to compare the estimative of neuronal cell numbers, including the right and left hemispheres. Data are presented as mean ± S.E. and differences were considered significant when $p \leq 0.05$. One-way ANOVA followed by Tukey's test was used to compare the activity of the different caspases. Data are presented as mean ± S.D. and

Table 1
Neuronal cell counting and caspases activities in the hippocampus of experimental and control groups. Values for cell counts (absolute numbers) and caspases activities (RFU/min/μg of protein) are expressed as means ± standard deviation.

Groups	CA1 (number of cells × 10^3)	Hilus (number of cells × 10^3)	Caspase-1 (WEHDase activity)	Caspase-3 (DEVDase activity)
Control	219.8 ± 24.9	60.0 ± 7.8	23 ± 18	5 ± 4
Pilo + Saline	167.5 ± 31.0[a]	39.2 ± 3.8[a]	53 ± 11[c]	11 ± 2[c]
Pilo + Pyr	184.6 ± 10.2	52.8 ± 9.5	41 ± 15[c]	11 ± 3[c]
Pilo + Oxa	183.3 ± 12.7	39.6 ± 6.2	32 ± 3[c,d]	9 ± 2[c]
Pilo + Pyr + Oxa	233.6 ± 13.0[b]	47.9 ± 8.6	35 ± 6[c,e]	10 ± 3[c]

[a] $p < 0.05$ compared with control group (saline).
[b] $p < 0.05$ compared with groups pilo + saline, pilo + pyr and pilo + oxa.
[c] $p < 0.001$ compared with control group (saline).
[d] $p < 0.01$ compared with group pilo + saline.
[e] $p < 0.05$ compared with group pilo + saline.

differences were considered significant when $p \leq 0.05$. Freeman-Halton extension for Fisher's exact test (table 2X4) was used to compare the survival rates in different experimental groups.

2.6. Ethics

All procedures were approved by the Local Ethics Committee (CEP. 1913/06) and are in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Every effort was taken to minimize the number of animal used and distress of the animals.

3. Results

3.1. Neuronal cell counting

A significant reduction of hippocampal neurons (CA1 and hilus) was observed in the group Pilo + Saline, when compared to the control group Saline + Saline (Table 1, Fig. 1). SE-induced neuronal loss in CA1 was completely prevented in rats treated with pyruvate plus oxaloacetate (Group Pilo + Pyr + Oxa). Treatment with pyruvate or oxaloacetate alone did not prevent neuronal loss in CA1. On the other hand, SE-induced neuronal loss in the hilus was prevented only in rats that received pyruvate alone (Group Pilo + Pyr).

3.2. Caspases activity

Seven days after pilocarpine-induced SE, a significant increase in the caspase-1 and caspase-3 activity was observed in all experimental groups when compared to controls ($p < 0.001$) (Table 1). Treatment with Oxa and Pyr + Oxa to rats presenting SE, reduced significantly the caspase-1 activation in the hippocampus whereas have no effect on caspase-3.

3.3. Effects on SE severity

The administration of pyruvate or oxaloacetate did not change seizure semiology and severity during SE in experimental rats. Mortality during SE was 34% in the group Pilo + Saline, 29% in the group Pilo + Pyruvate, 7% in the group Pilo + Oxa and 25% in the group Pilo + Pyr + Oxa. Fisher's exact test did not show significant differences amongst groups ($P = 0.38$).

4. Discussion

In humans, several brain insults are characterized by excessive Glu brain levels. These include acute disorders such as stroke, traumatic brain injury, bacterial meningitis and prolonged seizures (Castillo et al., 1996; Spranger et al., 1996; Zauner et al., 1996; Men et al., 2000; Ma et al., 2003) or chronic diseases such as glaucoma, amyotrophic lateral sclerosis or human immunodeficiency virus dementia (Shaw et al., 1995; Ferrarese et al., 2001; Spreux-Varoquaux et al., 2002). We focused our study on epileptic seizures, particularly SE, since it is not only accompanied by a large increase of Glu in brain fluids but there is also a tight correlation between SE-related brain damage and the development of chronic epilepsy (Olney, 1985; Leite et al., 1990; Cavalheiro et al., 1991; Lemos and Cavalheiro, 1995; Fujikawa, 2005).

The pilocarpine model is one of the most commonly studied chemical-inductive models for epilepsy (Turski et al., 1983, 1986; Leite et al., 1990; Cavalheiro et al., 1991; Cavalheiro, 1995; Arida et al., 2006; Curia et al., 2008). Morphological analysis of the brain after pilocarpine-induced SE demonstrates that the hippocampal subfield CA1 and the hilus of dentate gyrus are particularly susceptible to neuronal cell loss (Turski et al., 1983, 1986). Neuronal death occurs mainly by excitoxic injury caused by the activation of glutamatergic pathways in the course of SE (Cavalheiro et al., 1994; Costa et al., 2004). In the present investigation, SE-induced neuronal loss in CA1 was completely prevented in rats treated with Pyr plus Oxa. Moreover, neuronal damage in the hilus was prevented in rats that received Pyr alone. These results confirm previous studies showing the neuroprotective effect of Pyr (Izumi et al., 1994; Maus et al., 1999; Monaghan et al., 1989; Lee et al., 2001; Gonzales-Falcon et al., 2003). This neuroprotective effect is related to the potential of Pyr and Oxa to activate the blood resident enzymes GTP and GOT which increases the brain-to-blood Glu efflux (O'Kane et al., 1999; Gottlieb et al., 2003). Other hypothesis for the neuroprotective effect of Pyr and Oxa is related with the capacity of these subtracts to cross hematoencephalic barrier and normalize ATP and NAD+ (Sheline et al., 2000; Lee et al., 2001). For instance, Oxa can contribute to an improvement of NAD-linked mitochondrial energetics, via an enhancement of malate-aspartate shuttle, which increases hydrogen peroxide scavenging (Desagher et al., 1997; Zlotnik et al., 2007). In our experiments, we did not observe significant neuroprotective effects of Oxa (alone) during pilocarpine-induced SE. In fact our results suggest a neuroprotective effect of Oxa only when it is associated with Pyr. Further experiments must be done in order to test the efficacy of different protocols for Oxa administration in preventing neuronal damage induced by SE.

It is noteworthy that the quantitative techniques used here were sufficiently sensitive to detect even small changes in neuron number. Coefficients of error provide a standardized statistic for evaluating the precision of neuron number estimates derived by modern stereological techniques (Slomianka and West, 2005). Averaged across animals in each experimental group, this parameter ranged from less than 5% in the CA1 pyramidal cell field to less than 3% for the hilus of the dentate gyrus. The total number of hilar neurons per hippocampus computed in the present study (39.200 ± 3.882) compares closely to the number reported by Jiao and Nadler (2007) (37.580 ± 1.594), Buckmaster and Dudek (1997) (41.093 ± 1.284), who used essentially the same optical disector approach, and by Miki et al., 2005 (35.200 ± 1.600), who used a physical disector approach. The similarity of our results with

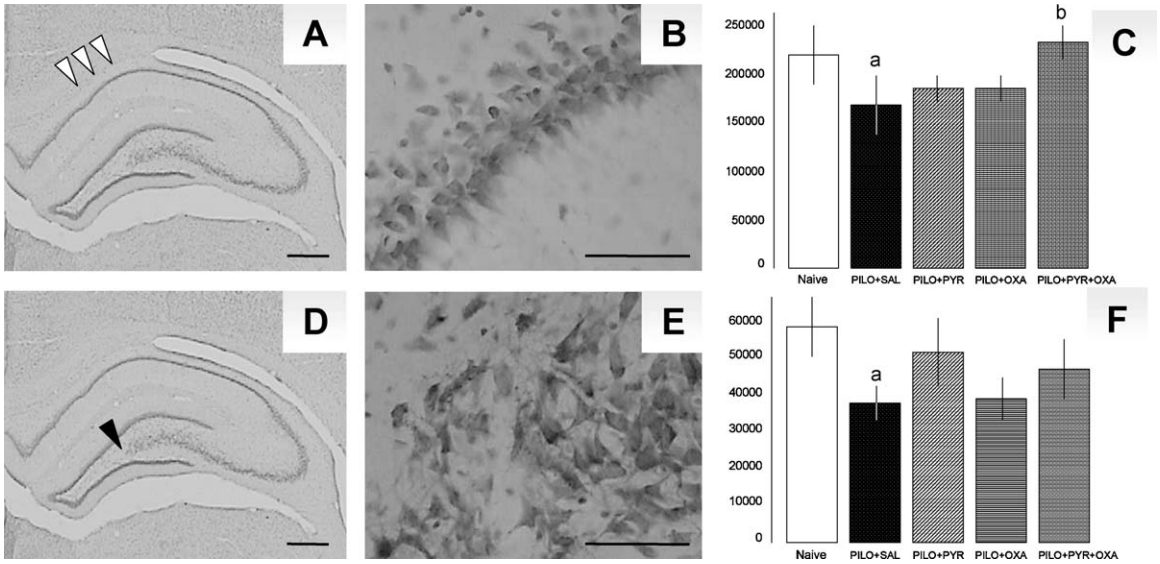

Fig. 1. Representative pictures of panoramic (A, D) and high (B, E) magnification views of the hippocampal subfields and respective neuronal cell counts (C, F). Panels A, B and C represent the CA1 subfield (white arrowheads). Panels D, E and F represent the hilus of the dentate gyrus (black arrowhead). Calibration bars correspond to 100 microns. (a) $p < 0.05$ compared with control group (naive). (b) $p < 0.05$ compared with groups pilo + saline, pilo + pyr and pilo + oxa.

previously reported values demonstrates high precision in the stereological estimates of neuronal number.

Previous studies on pilocarpine model showed that cell death occurs by necrosis or apoptosis (Fujikawa, 1996, 2005; Fujikawa et al., 2000, 2002, 2007; Henshall, 2007). In contrast to acute cell death, which occurs in the first 24–48 h and is predominantly necrotic, secondary or delayed neuronal cell death occurring at later stages has been identified to be predominantly apoptotic (Kermer and Klocker, 1999; Snider et al., 1999; Weise et al., 2005). Caspases are considered the common apoptosis execution pathway, and its activation raises structural alterations that characterize apoptosis (Henkart and Gristein, 1996). In the present investigation, we evaluated two types of caspases: caspase-1, related with inflammatory process, and caspase-3, which executes the apoptosis (Earnshaw et al., 1999; Henkart and Gristein, 1996). As previously demonstrated in the pilocarpine model (Persike et al., 2008) we also observed an increased activity of caspases-1 and -3 seven days after SE. Treatment with Pyr and/or Oxa did not prevent the increase of caspases activation, but it was significantly less pronounced (only for caspase-1) when rats were treated with Oxa or Pyr + Oxa. This result suggests that early Glu scavenging did not prevent late apoptotic neuronal cell death. In fact, Weise et al. (2005) observed that significant neuronal cell loss occurred in brain regions that showed activated caspase-3 expression. Areas with the highest levels of activated caspase-3 expression displayed the most extensive neuronal cell loss (Weise et al., 2005). In the present work, the increase of caspase-3 activity was not modified by Pyr and/or Oxa administration 30 min after SE. Nevertheless, it remains to be determined if late or prolonged Glu scavenging prevents SE-induced caspase activation and late neuronal cell loss.

Blood glutamate scavenging has been demonstrated to be neuroprotective in terms of neurological outcome. Zlotnik and colleagues tested the hypothesis that Pyr- or Oxa-mediated blood Glu scavenging causes neuroprotection in a rat model of closed head injury (CHI), in which there is a well established deleterious increase of Glu in brain fluids. They observed highly significant improvements of the neurological status of rats submitted to CHI following an intravenous treatment with Pyr or Oxa (Zlotnik et al., 2007, 2008). The neuroprotective effects of Pyr contrast with those observed following Oxa treatment since the neurological recovery of rats treated with Oxa after CHI was more complete and in markedly stronger correlation with the decrease of blood Glu levels. Thus, unlike Oxa that was suggested to exert its neuroprotective effects mainly via its blood Glu scavenging activity, Pyr is likely to use additional neuroprotective mechanisms particularly when administered at high doses (Zlotnik et al., 2008). Although these conclusions were taken from a rat model of CHI, some may be applied to our model of acute SE since both models involve Glu-mediated brain injury. Future investigations focused on long term behavioral outcome after SE may also include the monitoring for the occurrence of spontaneous recurrent seizures which are the hallmark the chronic phase of the pilocarpine model of epilepsy (Arida et al., 2006; Leite et al., 2006).

As stated above, previous studies have demonstrated that systemic administration of Pyr and Oxa in rats produces blood Glu scavenging and increased brain-to-blood Glu efflux (Gottlieb et al., 2003; Zlotnik et al., 2007, 2008). In this context, an important issue to be addressed is the impact of Glu drop off on brain tissue, particularly neuronal cells. Preliminary results of our group indicate that naive animals (not subjected to SE) that received Pyr or Pyr + Oxa show neuronal damage in the hippocampus (unpublished data). Moreover, Gonzalez et al. (2005) showed that rapid injection of large doses of Pyr (1–2 g/kg, i.v.) in naive rats produced a proconvulsive effect. These findings suggest that further experiments must be conducted in order to evaluate the possible deleterious effects of abnormal brain-to-blood Glu efflux on brain tissue.

5. Conclusion

The acute neuronal cell loss in the hippocampus (CA1 subfield) induced by SE was completely prevented in rats treated with pyruvate plus oxaloacetate. Moreover, the late caspase-1 activation was significantly reduced when rats were treated with oxaloacetate or pyruvate plus oxaloacetate. These data support the idea that the treatment with pyruvate and oxaloacetate causes a neuroprotective effect in rats subjected to pilocarpine-induced SE.

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