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Ascorbic acid prevents cognitive deficits caused by chronic administration of propionic acid to rats in the water maze

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Abstract

Propionic acidemia is an inherited neurometabolic disorder characterized by progressive neurological deterioration with psychomotor delay/mental retardation, convulsions and coma, and whose pathophysiology is poorly unknown. In the present study, we investigated the effect of chronic administration (from the 5th to the 28th days of life) of propionic acid (PA), the major metabolite accumulating in tissues of patients affected by propionic acidemia, on the cognitive performance of adult rats in the Morris water maze task. PA doses ranged from 1.44 to 1.92 μmol/g body weight as a function of animal age. Control rats were treated with saline in the same volumes. Chronic postnatal days (5–28) PA treatment had no effect on body weight. However, it impaired spatial performance in the water maze. We also determined the effect of ascorbic acid (AA) administered, alone or combined with PA, on the same behavioral parameters in order to test whether free radicals could be responsible for the behavioral alterations observed in PA-treated animals. AA was able to prevent the behavioral alterations provoked by PA, implying that oxidative stress may be involved in these effects. Furthermore, we also investigated the total radical-trapping antioxidant potential (TRAP) in the hippocampus of the animals. We observed that TRAP was significantly reduced in the brain of propionic acidemic rats and that co-administration of AA prevented this effect. The results provide evidence that early PA treatment induces long-lasting behavioral deficits, which are possibly caused by oxygen reactive species generation, and suggest that oxidative stress may be involved in the neuropathology of propionic acidemia.

Keywords: Propionic acid; Propionic acidemia; Water maze; Ascorbic acid; Antioxidants

1. Introduction

Propionic acid (PA) is found in large amounts in tissues of patients with propionic acidemia, one of the most frequent genetic neurodegenerative disorders of organic acid metabolism caused by severe deficiency of propionyl CoA carboxylase deficiency. The blockage of the metabolic pathway gives rise to increased amounts of propionyl CoA, which is spontaneously converted to PA (Fenton et al., 2001). The levels of the acid in the blood and cerebrospinal fluid (CSF) usually are as high as 2.5–5 mM during crises, but can be even higher in the brain (Hoffmann et al., 1993). Encephalopathy is the clinical hallmark of propionic acidemia. Among the neurologic symptoms often present, psychomotor delay/mental retardation, focal and generalized convulsions, cerebral atrophy and EEG abnormalities are the most frequent. However, the mechanisms underlying the pathophysiology of the neurologic dysfunction of propionic acidemia are poorly known.

Oxidative stress has been demonstrated to be related to the pathophysiologic mechanisms involved in brain injury in various common neurodegenerative disorders, including Parkinson's disease, Alzheimer's disease and Huntington's disease (Matés et al., 1999; Alexi et al., 2000; Halliwell and Gutteridge, 1999). Moreover, recent studies demonstrated that chronic antioxidant treatment improves cognitive performance in animal models of various neurodegenerative diseases, such as Parkinson's disease (Dunnett and Björklund, 1999) and Alzheimer's disease (Praticò and Delanty, 2000), and also in aged rats (Socci et al., 1995). As regard to propionic acidemia, we recently provided some evidence

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that PA induces free radical generation in the cerebral cortex of rats in vitro (Fontella et al., 2000). In vitro stimulation of superoxide by PA was also observed in blood cells (Nakao et al., 1998). Finally, a further study demonstrated that oxidative stress may be involved in the pathology of propionic acidemic patients, since plasma α-tocopherol levels in these patients are significantly reduced as compared to age-matched controls (Moyano et al., 1997).

A chemically induced model of propionic acidemia was recently developed in our laboratory, in which the plasma and brain PA levels are of the order of 1–5 mM and 1 μmol/g, respectively (Brusque et al., 1999). By using this model, we have shown that high sustained levels of PA provoked long-term learning deficit in rats submitted to the open field and to the shuttle avoidance tasks (Brusque et al., 1999).

Therefore, in the present investigation, we initially determined the influence of early high sustained levels of PA on the spatial performance of adult rats on learning and memory tasks in the Morris water maze. Since some data in the literature suggest that oxidative stress may be involved in the pathophysiology of the neurological dysfunction of propionic acidemia (Moyano et al., 1997; Fontella et al., 2000), we also evaluated the influence of concomitant administration of the naturally occurring free radical scavenger, ascorbic acid (AA), on the behavioral alterations provoked by PA treatment in order to evaluate whether reactive oxygen species (ROS) could be implicated in these alterations and whether in vivo administration of antioxidants would be able to protect rat performance on this task. Finally, total radical-trapping antioxidant potential (TRAP) was measured in the hippocampus of the treated animals to better evaluate the role of oxidative stress on the behavioral effects detected.

2. Materials and methods

2.1. Chemicals

Unless otherwise stated, reagents were purchased from Sigma (St. Louis, MO, USA).

2.2. Subjects

A total of 72 male Wistar rats from our breeding stock were used. Pregnant rats were housed in individual cages and left undisturbed during gestation. Forty-eight hours after delivery, litters were culled to eight male pups; rats were weaned at 21 days. The animals were divided so that in each cage there was the same number of rats for each treatment (saline, PA, AA, PA+AA). Forty-four animals were used in the behavioral tests, whereas 28 in the biochemical tests. All animals had free access to commercial chow and water, and were kept on a 12-h light/dark cycle at 24±1 °C. The experimental protocol was approved by the Ethical Committee of Federal University of Rio Grande do Sul and complied with the National Institute of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

2.3. Treatment

Saline-buffered PA, pH 7.4, was administered subcutaneously, twice a day, from the 5th to the 28th days of life to produce chemically induced propionic acidemia. PA doses were calculated to achieve 1.0–5.0 mM plasma PA concentrations and were as follows: 5–12 days of life, 0.72 μmol/g body weight; 13–19 days of life, 1.68 μmol/g body weight; 20–28 days of life, 1.92 μmol/g body weight (Brusque et al., 1999). The brain concentrations of PA were around 1 μmol/g brain. Control animals received saline subcutaneously in the same volumes and frequency. AA was administered at the dose of 100 μg/g body weight alone (AA) or combined with PA (AA+PA). All solutions were prepared so that each animal received 10 μl solution/g body weight.

2.4. Cognitive tasks

2.4.1. Morris water maze

The animals were left to recover for approximately 1 month. On the 60th day of life, spatial learning/memory was tested in the Morris water maze (Morris et al., 1982; Netto et al., 1993), which consisted of a black circular pool (200 cm in diameter, 100 cm high), theoretically divided into four equal quadrants for the purpose of analysis. The pool was filled to a depth of 50 cm with water (23±1 °C). The experimenter remained at the same location, approximately 50 cm from the outside edge of the tank, on each trial. A video camera was mounted above the center of the tank and each trial was recorded.

2.4.2. Acquisition phase

Rats had daily sessions of four trials per day for 5 days to find the submerged platform that was located in the center of a quadrant of the tank and remained there throughout training. We observed that all animals of each group were able to swim in a normal way during all trials. On each trial, the rat was placed in the water, facing the edge of the tank, in one of the four standard start locations (N, S, W and E). The order of the start locations was varied in a quasirandom sequence, so that for each block of four trials, any given sequence was not repeated on consecutive days. The rat was then allowed 60 s to search for the platform. Latency to find the platform and swimming speed were measured in each trial. Once the rat located the platform, it was permitted to remain on it for 10 s. If it did not find the platform within this time (60 s), it was guided to it and allowed to remain on it for 10 s. After each trial, the rats were removed, dried in a towel and put back in their home cages. The interval between trials was 15–20 min (Warren and Juraska, 2000).

Table 1
Effect of postnatal chronic PA, AA and PA+AA administration on body weight of rats

Group	Age of animals (days)				
	5	10	15	20	28
Control	11.44±0.412	19.77±0.572	27.56±0.503	35.44±1.79	67.33±3.05
PA	11.33±0.408	19.77±0.434	27.78±0.682	36.22±1.78	67.78±2.69
AA	11.36±0.337	19.81±0.536	27.55±0.474	36.27±1.13	70.36±1.77
PA+AA	11.85±0.404	20.57±0.685	28.57±0.922	38.14±1.24	72.86±3.40

Data are expressed as mean±S.E.M. for 11 rats in each group. There was no significant difference between groups (one-way ANOVA).

2.4.3. Probe trial

One day after the last training trial, each rat was subjected to a probe trial (60 s) in which there was no platform present. The time spent in the quadrant of the former platform position was taken as a measure for spatial memory.

2.4.4. Reversal learning (reversal phase)

Two weeks after the probe trial, reversal training, similar to the acquisition, was started. Animals were trained to find the hidden platform, now located in the quadrant diagonally opposite (reversed) to the initial location, for 4 days (four trails per day). Latency to find the platform was determined in each trial. On Day 5, all animals were submitted to a second probe trial. Each rat was placed in the water maze without the platform and was allowed to swim for 60 s. The period of time spent in the quadrant where the platform was initially located was registered (Block and Schwarz, 1998).

2.4.5. Open field task

Fifteen days after the reversal learning probe trial, rats were submitted to the open field task. The apparatus consisted of a wooden box measuring 60×40×50 cm with a glass front wall, whose floor was divided by black lines into 12 equal squares. The animals were gently placed facing the rear left corner of the arena and the number of squares crossed with the four paws recorded for 2 min to evaluate motor activity (Walsh and Cummins, 1976).

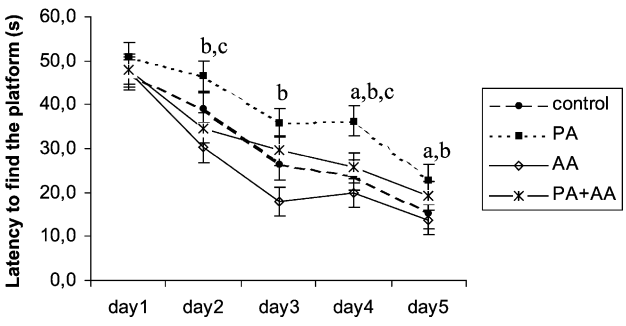

Fig. 1. Effect of propionate on acquisition learning. Data represent mean±S.E.M. latency to find the platform across blocks of four trials on each day (n=11 rats/group). PA=propionic acid; AA=ascorbic acid; PA+AA=propionic acid+ascorbic acid. P<.05, significantly different from controls (a), AA group (b), PA+AA group (c). Two-way ANOVA is shown in the text.

2.4.6. Total TRAP

TRAP represents the total antioxidant capacity of the tissue and was determined by measuring the luminol chemiluminescence intensity induced by 2,2′-azo-bis (2-amidinopropane) (ABAP) (Lissi et al., 1992) at room temperature. For these experiments, the animals were sacrificed 18 h after the last injection when they had 29 days of age. Brain was rapidly removed, the hippocampus was separated and homogenized 1:10 (wt/vol) in 0.1 M glycine buffer, pH 8.6, which was also used to prepare the other solutions. Four milliliters of 10 mM ABAP was added to the vial and the background chemiluminescence was measured. Ten microliters of 4 mM luminol were then added and the chemiluminescence was measured. This was considered to be the initial value. Ten microliters of 80 μM Trolox or homogenates was added and chemiluminescence was measured until it reached the initial levels. The addition of Trolox or tissue homogenate to the incubation medium reduces the chemiluminescence. The time necessary to return to the levels present before the addition was considered to be the induction time. The induction time is directly proportional to the antioxidant capacity of the tissue and was compared to the induction time of Trolox. The results are reported as nanomoles of Trolox per milligram of protein.

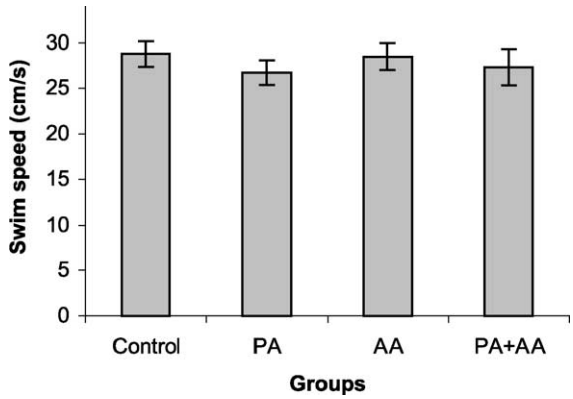

Fig. 2. Effect of propionate on swim speed. Data represent mean±S.E.M., expressed in centimeters per second (n=11 rats/group). PA=propionic acid; AA=ascorbic acid; PA+AA=propionic acid+ascorbic acid. There were no significant differences between groups. One-way ANOVA is shown in the text.

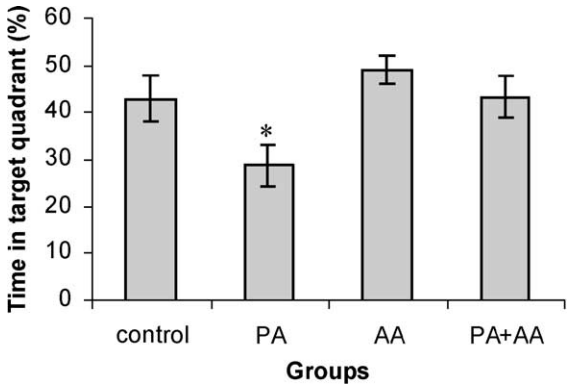

Fig. 3. Time spent in the quadrant of the former platform position (as percentage of total time) during the probe trial for controls, PA-, AA- and PA+AA-treated rats (n=11 rats/group). PA=propionic acid; AA=ascorbic acid; PA+AA=propionic acid+ascorbic acid. Values are means±S.E.M. *P<.05, significantly different from the other groups (Duncan's multiple range test). One-way ANOVA is shown in the text.

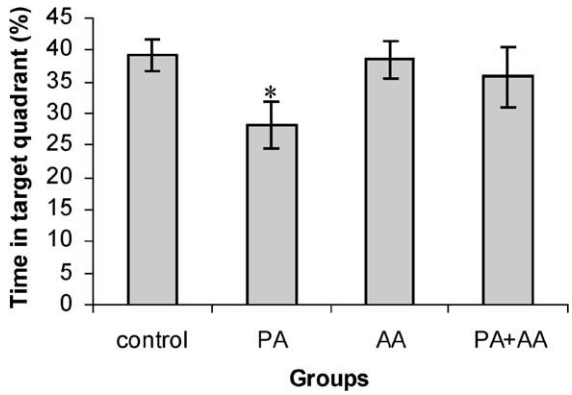

Fig. 5. Time spent in the quadrant of the former platform position (as percentage of total time) during the second probe trial for controls, PA-, AA- and PA+AA-treated rats (n=11 rats/group). Values are mean±S.E.M. *P<.05, significantly different from the other groups (Duncan's multiple range test). One-way ANOVA is shown in the text.

2.5. Statistical analysis

Data from rat's behavior were analyzed by one- or two-way analysis of variance (ANOVA) (Treatment×Days). When appropriate, post hoc comparisons were assessed using the Duncan's multiple range test. In some experiments, the nonparametric Kruskal–Wallis test was used because the parameters did not fit a normal distribution. A value of P<.05 was considered to be significant. F values are presented only if P<.05.

3. Results

Body weight of rats submitted to chronic PA treatment did not differ from those of control rats nor from rats receiving ascorbate (Table 1), indicating that chronic administration of PA does not provoke malnutrition.

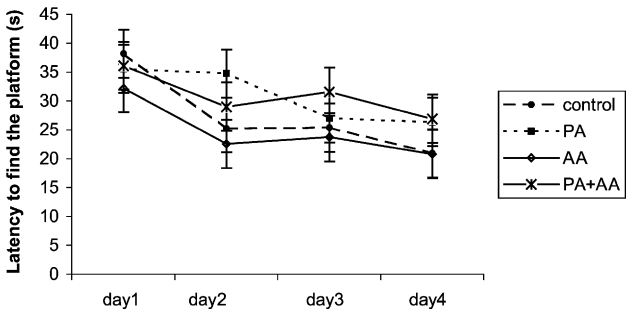

Fig. 4. Effect of propionate on reversal learning. Data represent mean± S.E.M. latency to find the platform across blocks of four trials on each day (n=11 rats/group). PA=propionic acid; AA=ascorbic acid; PA+AA=propionic acid+ascorbic acid. There was no difference between groups. Two-way ANOVA is shown in the text.

3.1. Acquisition phase

Fig. 1 shows that all groups of animals improved water maze acquisition performance, i.e., decreased the latency to find the platform along time from the first to the last day of training (Day 5) [F(3,43)=788, P<.0001]. However, group comparisons revealed that PA-treated animals presented a higher latency to find the platform than saline- and AA-treated rats along time [F(3,43)=4.65, P<.01], i.e., the rate of acquisition over time was lower for the PA-treated group. Furthermore, ANOVA showed no significant difference among the swim speeds of all four groups [F(3,43)=0.452, P>.05] (Fig. 2).

3.2. Probe trial

On the probe trial, with the platform removed, PA-treated rats failed to remember the precise location of the platform, spending significantly less time in the training quadrant than control and PA+AA groups [F(3,43)=4.319, P<.05] (Fig. 3).

3.3. Reversal learning session

Fig. 4 shows that all groups of rats, including the PA-injected animals, had the same rate of acquisition in the

Table 2
Effect of postnatal chronic PA, AA and PA+AA administration on performance (number of crossings) in the open field task

Group	Mean	S.E.M.
Control	33.6	1.71
PA	37.7	2.35
AA	33.7	2.08
PA+AA	36.4	2.45

Data are presented as mean±S.E.M. for 11 rats in each group. There was no significant difference between groups (Kruskal–Wallis test).

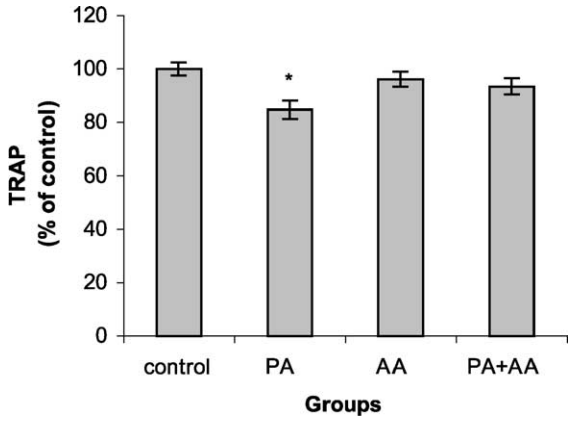

Fig. 6. Effect of chronic administration of PA on TRAP in hippocampus. Results are mean±S.E.M. for seven animals per group, expressed as percentage of controls (mean=15.7 as nmol Trolox/mg protein). *P<.05 compared to control (Duncan's multiple range test). One-way ANOVA is shown in the text.

second phase (reversal learning) [F(3,43)=0.442, P>.05] and learned the task strategy [F(3,43)=267, P<.0001] (Fig. 4). However, analysis of the swimming performance during the second probe trial (Fig. 5) revealed that animals submitted to PA administration spent significantly [F(3,43)=4.09, P<.05] less time in the quadrant of the former platform position than all other groups. These results suggest that they could not remember the exact position of the platform. Moreover, the observation that rats receiving chronic co-administration of PA and AA showed good retention indicates that ascorbate could prevent the memory deficit of PA-treated rats.

3.4. Open field

The analysis of motor activity (number of crossings) of rats submitted to the open field task showed no significant effect [F(3,43)=0.470, P>.05] of the treatments used, suggesting that spatial deficits found in the PA-treated group in the water maze task cannot be credited to motor impairment (Table 2).

3.5. Total TRAP

We observed that chronic PA administration significantly diminished the antioxidant capacity (TRAP) and that co-administration of AA prevented this effect in the hippocampus of 29-day-old rats [F(3,27)=3.558, P<.05] (Fig. 6).

4. Discussion

Propionic acidemic patients, even under restricted treatment protocols, present a variable degree of psychomotor delay/mental retardation, suggesting that the presently available therapeutic regimens may not be fully appropriated and that new treatment strategies should be designed. Considering that the mechanisms of neurological dysfunction in this disease are poorly known, it is therefore expected that clarification of the pathophysiology of the brain damage in propionic acidemia would contribute to better therapeutic approaches. In this context, it has been recently suggested that increased free radical production caused by propionate may be related to the neurologic damage characteristic of propionic acidemia (Nakao et al., 1998; Fontella et al., 2000). Alternatively, reduction of energy production caused by propionate may also be related to the neurological damage characteristic of these patients (Brusque et al., 1997; Wyse et al., 1998; Matsuishi et al., 1991).

In the present study, we produced high sustained levels of PA in the blood (1–5 mM) and brain (1 μmol/g brain) of developing rats (5th to the 28th days of life), similar to those found in human propionic acidemia by using a previously described chemically induced experimental model for this human condition (Brusque et al., 1999; Fenton et al., 2001). This animal model does not exactly mimic human propionic acidemia, in which, besides PA, other metabolites accumulate in lesser amounts. However, it reproduces the main feature of the disorder, which is high sustained levels of PA. PA was administered during a period of great cellular proliferation and synaptogenesis in various cerebral structures involved in learning/memory in rats (Dutra et al., 1993; Winick and Noble, 1965; Roisen et al., 1981; Dreyfus et al., 1984). The animals were allowed to recover for 30–45 days, and, thereafter, their cognition was tested in the Morris water maze and their motor activity measured in the Morris water maze (swim speed) and in the open field (number of crossings) tasks. We first observed that chronic PA administration had no effect on body weight, implying that chronic PA injection does not cause malnutrition in the animals. This observation is important since malnourished animals may behave differently in neurobehavioral tests. Therefore, this undesirable variable in behavioral studies can be ruled out as a cause of the behavioral alterations observed in PA chronically treated rats.

PA-treated rats presented a delay in the acquisition on the water maze task, i.e., they reached the platform with higher latency to find the platform than saline- and ascorbate-treated animals along time, indicating that chronic administration of PA causes a deficit in spatial learning (Block et al., 1995). We also verified that rats receiving chronic PA administration stayed for a significantly shorter time (28% of total session time) in the quadrant where the platform was formerly located, as compared to all other groups (43–49%)—a fact that reflects an impairment of reference memory. More important, such learning/memory deficits could be prevented by co-administration of AA, a free radical scavenger. Therefore, the ability of antioxidant treatment to prevent cognitive deficit in this study suggests an association between free radicals/oxidative damage and cognitive impairment in propionic acidemia.

We also found that PA-treated rats, as well as the other experimental groups, learned the task strategy, as revealed by control-like latencies in the reversal learning phase. However, rats receiving PA spent less time in the second probe trial, as compared to all others groups, strongly indicating a memory deficit. Once more, the simultaneous administration of ascorbate prevented this behavior deficit.

Deficits in spatial navigation shown in PA-treated rats cannot be attributed to a decreased motor activity since the number of crossing responses (open field) and the swim speed (water maze) were evaluated and revealed that all groups had the same motor activity.

The results of the present study clearly indicate that early chronic administration of PA to rats provokes long-lasting behavioral changes related to learning and memory in the water maze, in agreement with previous findings of our laboratory showing that high sustained levels of PA applied during the spurt phase of brain development provoke learning/memory deficit in the open field and the shuttle avoidance tasks (Brusque et al., 1999). However, we must emphasize that the water maze task, by measuring spatial learning and memory, can detect more subtle behavioral/learning impairments than the open field and active shuttle avoidance tasks (Block, 1999). These observations are particularly important in view of the significant mental retardation characteristic of propionic acidemic children. Therefore, it is possible that the brain PA concentrations achieved in our chemical model of propionic acidemia (1 μmol/g) may be high enough to induce biochemical alterations in pathways involved in learning, memory and interaction with the environment. However, further studies are necessary to clarify these points.

We also found that AA administration per se did not significantly affect any of the neurobehavioral parameters analyzed, and this is in agreement with the literature showing no improvement in cognitive performance in animals or humans under antioxidant treatment (Perrig et al., 1997). However, antioxidant treatment improves cognitive function evaluated in the water maze testing in aged rats, which were demonstrated to have a better acquisition and better memory retention compared to vehicle-treated control aged rats after 2 months of treatment (Socci et al., 1995). A study performed in humans demonstrated similar results of improved cognitive function secondary to long-term co-administration of combined antioxidants, such as ascorbate, vitamin A, vitamin B6, α-tocopherol, zinc and selenium, which were achieved in aged geriatric patients with dementia-related psychological scores (Clausen et al., 1989). Therefore, it appears that long-term antioxidant treatment can improve cognitive performance in certain tasks in aged animals and humans, where cognitive impairment associated with neural death occurs. This is interesting in view of recent studies demonstrating that increased generation of ROS is responsible for some of the detrimental effects of aging (Halliwell, 1992; Harman, 1992; Matés et al., 1999). Furthermore, an increased body of evidence points to increased oxidative stress in various neurodegenerative disorders such as Parkinson's disease, Huntington's disease and Alzheimer's disease (Halliwell, 1992). Therefore, the present results demonstrating that cognitive deficit caused by PA administration can be prevented by co-administration of AA, a recognized free radical scavenger, suggest that the long-lasting impairment of cognition provoked by PA may be caused by oxidative brain damage. In this context, we also found in the present study that chronically PA-treated rats presented a significant diminution of total TRAP measurement in the hippocampus, reflecting a reduction of the antioxidant defenses in the brain of the animals, which might be attributed to an excess of free radicals. It should be noted that the hippocampus is important for spatial learning, and therefore necessary for a good performance in the water maze task.

At this point, it should be stressed that propionic acidemia is a genetic neurodegenerative disorder whose neuropathology (cortical atrophy and basal ganglia damage) is strongly indicative of neural loss, similar to what is found in aging and in the common neurological disorders above mentioned (Fenton et al., 2001).

In conclusion, the present study confirms that chronic administration of PA provokes long-lasting learning/memory deficits and decreases the antioxidant defenses in the hippocampus. It also demonstrates that AA treatment prevents the deficient spatial cognition of PA chronically treated animals in the water maze task, as well as the TRAP reduction in the hippocampus. Our data suggest a causal association between free radicals/oxidative damage and decreased cognitive function provoked by PA administration, and consequently provide further support for the free radical hypothesis causing brain damage in propionic acidemia. Whether oxidative stress is the main cause of the neurological manifestations of propionic acidemic patients remains to be elucidated. On the other hand, although the plasma concentrations of PA achieved in the animals were similar to those of propionic acidemia, it is difficult to extrapolate our findings to the human condition. However, the results of the present study and others indicating the involvement of oxidative stress in the pathophysiology of propionic acidemic patients (Moyano et al., 1997; Nakao et al., 1998; Fontella et al., 2000) suggest that antioxidants could represent an adjuvant therapeutic approach in propionic acidemia in order to prevent the neurological damage and learning disabilities in these patients.

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