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Effects of caffeic acid on behavioral parameters and on the activity of acetylcholinesterase in different tissues from adult rats

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ABSTRACT

Acetylcholinesterase (AChE) is distributed throughout the body in both neuronal and non-neuronal tissues and plays an important role in the regulation of physiological events. Caffeic acid is a phenolic compound that has anti-inflammatory and neuroprotective properties. The aim of this study was to investigate in vitro and in vivo whether caffeic acid alters the AChE activity and behavioral parameters in rats. In the in vitro study, the concentrations of 0, 0.1, 0.5, 1.0, 1.5, and 2 mM of caffeic acid were used. For the in vivo study, five groups were evaluated: group I (control); group II (canola oil), group III (10 mg/kg of caffeic acid); group IV (50 mg/kg of caffeic acid) and group V (100 mg/kg of caffeic acid). Caffeic acid was diluted in canola oil and administered for 30 days. In vitro, the caffeic acid increased the AChE activity in the cerebral cortex, cerebellum, hypothalamus, whole blood, and lymphocytes at different concentrations. In muscle, this compound caused an inhibition in the AChE activity at concentrations of 0.5, 1.0, 1.5, and 2 mM when compared to the control ($P<0.05$). In vivo, 50 and 100 mg/kg of caffeic acid decreased the AChE activity in the cerebral cortex and striatum and increased the activity of this enzyme in the cerebellum, hippocampus, hypothalamus, pons, lymphocytes, and muscles when compared to the control group ($P<0.05$). The amount of 100 mg/kg of caffeic acid improved the step-down latencies in the inhibitory avoidance. Our results demonstrated that caffeic acid improved memory and interfered with the cholinergic signaling. As a natural and promising compound caffeic acid should be considered potentially therapeutic in disorders that involve the cholinergic system.

1. Introduction

Cholinesterases belong to a family of proteins that is widely distributed throughout the body in both neuronal and non-neuronal tissues and is classified as either acetylcholinesterase (AChE) or butyrylcholinesterase (BuChE) based on their substrate and inhibitor specificity (Anglister et al., 2008).

Acetylcholinesterase (E.C. 3.1.1.7, AChE) is a specific choline esterase that hydrolyzes predominantly choline esters. This enzyme is present in the brain, erythrocytes, lymphocytes and neuromuscular junction and plays an essential role in the regulation of physiological events involving the turnover of acetylcholine (Anglister et al., 2008; Szelenki et al., 1982; Gaspersic et al., 1999). AChE is one of the most efficient enzymes that rapidly hydrolyzes the neurotransmitter acetylcholine at cholinergic synapses as well as the neuromuscular junction (Anglister et al., 2008; Gaspersic et al., 1999). Besides its catalytic properties, AChE has potent effects in cellular adhesion, neurite extension and postsynaptic differentiation and has been accepted as the most important biochemical indicator of cholinergic signaling in the central nervous system (CNS) (Silman and Sussman, 2005). This enzyme is also widely accepted as having roles in non-neuronal tissues including hematopoietic differentiation (Deutsch et al., 2002) and regulation of the immune function (Kawashima and Fujii, 2003).

As a consequence of its key physiological role, AChE is the target of natural toxins (Senanayake and Román, 1992) and the therapeutic drugs designed to combat neuromuscular disorders and to alleviate the cholinergic deficiency associated with neurodegenerative diseases (Brenner et al., 2003). Furthermore, several studies have also demonstrated that natural substances and dietary components can affect the

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AChE activity in different tissues (Ahmed and Gilani, 2009; Schmatz et al., 2009).

Caffeic acid (3,4-dihydroxycinnamic acid) is a non-flavanoid catecholic compound present in many plants and occurs in the diet as part of fruits, tea and wine (Clifford, 1999). Even though caffeic acid is a phenolic compound especially abundant in coffee, it is chemically unrelated to caffeine (Higdon and Frei, 2006; Omar et al., 2012). It is known that caffeic acid has a broad spectrum of pharmacological activities including anti-inflammatory, antioxidant and immunomodulatory effects (Challis and Bartlett, 1995; Chan and Ho, 1997; Gulcin, 2006). Pharmacology studies have also shown that the caffeic acid exerts a protective effect against hydrogen peroxide-induced oxidative damage in the brain (Pereira et al., 2006), and cerebral ischemia (Zhou et al., 2006) and prevents brain damage as well as behavioral and biochemical changes caused by aluminum (Yang and Zho, 2008). Although many of these effects have been partially related to 5-lipoxigenase inhibition, the mechanisms involved in the protective activities of caffeic acid have not yet been fully understood. In addition, there are few studies about the effects promoted by caffeic acid on cholinergic signaling parameters in CNS as well in other tissues.

Lately, the effects of compounds present in the diet as an alternative source for the treatment of various diseases have been studied. In this context, considering the pharmacological properties of caffeic acid, the aim of this study was to investigate the effects of this compound on behavioral parameters as well as on the activity of the AChE enzyme in the brain, lymphocytes, whole blood and muscle of rats in order to investigate the potentially therapeutic use of this compound in disorders associated with cholinergic dysfunction.

2. Material and methods

2.1. Chemicals

Caffeic acid, acetylthiocholine iodide (ASCh), 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) and Triton X-100 were obtained from Sigma (Deisenhofen, Germany), while ethopropazine hydrochloride was obtained from Aldrich (Steinheim, Germany). Ficoll Hypaque (Lymphoprep™) was purchased from Nycomed Pharma (Oslo, Norway). All the other reagents used in the experiments were of analytical grade and of the highest purity.

2.2. Animals

Male Wistar rats (70 days, 280–300 g) from the Central Animal House of the University Federal of Santa Maria (UFSM) were used. Animals were maintained at a constant temperature (23 ± 1 °C) on a 12 h light/dark cycle with free access to food and water. The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, (COBEA) which are in accordance with international guidelines. After the acclimatization period, the rats were submitted to the experimental protocols.

2.3. In vitro experiments with caffeic acid

Ten adult male Wistar rats were used for the in vitro experiments. After the acclimatization period, animals were submitted to euthanasia after being previously anesthetized with halothane and the brain structures, blood and muscle were removed. The caffeic acid was dissolved in the methanol and the different concentrations (0, 0.1, 0.5, 1.0, 1.5 and 2 mM) were used in the assay of the AChE activity.

2.4. In vivo experiments with caffeic acid

Animals were divided into five groups (10 rats per group): group I (control); group II (canola oil), group III (treated with 10 mg/kg of caffeic acid); group IV (treated with 50 mg/kg of caffeic acid) and group V (treated with 100 mg/kg of caffeic acid). Caffeic acid was freshly prepared in canola oil and was administered by gavage once a day for 30 days. One day after the end of the treatment with caffeic acid, animals were subjected to behavioral tests. After this procedure, animals were submitted to euthanasia after being previously anesthetized with halothane and the brain, blood and muscle were removed for the AChE assay.

2.5. Behavioral procedure

2.5.1. Inhibitory avoidance

For the evaluation of memory we used the inhibitory avoidance task, which is commonly used to assess memory in rats and mice and it is considered a very simple test. Training was done only once and had an important biological value (Izquierdo et al., 2000a,b, 2002). One day after the end of the treatment, animals were subjected to training and tested in a step-down inhibitory avoidance apparatus according to Guerra et al. (2006). Animals were trained in a one-trial step-down inhibitory avoidance paradigm. The inhibitory avoidance apparatus consisted of a $30 \times 25 \times 25$-cm box with a 2.5-cm high, 7-cm wide, and 25-cm-long platform covering the left side of the grid floor. During training, animals were gently placed on the platform facing the left rear corner of the training box. Immediately after stepping down with their four paws on the grid, they received a 4-s 0.4 mA scrambled foot shock. Immediately after training, they were returned to their home cage. Memory retention was evaluated in a test session carried out 24 h after training, in which trained animals were placed on the training box platform, and the step-down latency (s) was measured. A cutoff time of 300 s was imposed on step-down latency during testing session. The apparatus was cleaned with 30% ethyl alcohol before and after each rat occupied it.

2.5.2. Open field

Immediately after the inhibitory avoidance test session, all animals were transferred to an open field in order to assess exploratory activity. Animals were placed on the center quadrant of a round open field (56 cm diameter) with the floor divided into ten equal areas. An observer, who was not aware of the pharmacological treatments, manually recorded the number of crossing and rearing responses over 5 min. Crossing was defined as the total number of areas crossed with the four paws and rearing was defined as the total number of stand up responses on two paws. This test was carried out to identify possible motor disabilities that might have influenced inhibitory avoidance performance at testing.

2.6. AChE assay in brain structures

In both in vitro and in vivo experiments, the brain was separated into the striatum (ST), hippocampus (HP), cerebral cortex (CC), cerebellum (CB), hypothalamus (HY) and pons (PN) and placed in a solution of 10 mM Tris–HCl, pH 7.4, on ice. Brain structures were homogenized in a glass potter in Tris–HCl solution. Aliquots of resulting brain structure homogenates were stored at −30 °C until utilization. Protein varied for each structure: ST (0.4 mg/ml), HP (0.8 mg/ml), CC (0.7 mg/ml), CB (0.6 mg/ml) PN (0.7 mg/ml) and HY (0.6 mg/ml); these concentrations were determined by the Coomassie blue method according to Bradford (1976) using bovine serum albumin as standard solution.

The AChE enzymatic assay was determined by a modification of the spectrophotometric method of Ellman et al. (1961) as previously described by Rocha et al. (1993). The reaction mixture (2 ml final volume) was composed of 50 mM K^+-phosphate buffer, pH 7.5 and 1 mM of DTNB. The method is based on the formation of the yellow anion, 4,4-dithio-bis-acid-nitrobenzoic measured by absorbance at 412 nm during 2 min of incubation at 25 °C. The reaction was initiated by adding 0.8 mM acetylcholine iodide. All samples were run in duplicate

or triplicate, and the enzyme activity was expressed in micromoles ASCh/h/mg of protein.

2.7. AChE assay in whole blood

Whole blood AChE activity was determined by the method of Ellman et al. (1961) modified by Worek et al. (1999). The blood was collected in vaccutainer tubes using EDTA as anticoagulant. Samples were hemolyzed with phosphate buffer, pH 7.4 containing Triton X-100 and stored at −30 °C for 1 week. The specific activity of whole blood AChE was calculated from the quotient between the AChE activity and hemoglobin content and the results are expressed as mU/μmol Hb.

2.8. AChE assay in lymphocytes

The blood was collected in vaccutainer tubes using EDTA as anticoagulant. The peripheral lymphocytes were isolated using Ficoll Hypaque density gradient as described by Böyum (1968). Lymphocyte viability and integrity were confirmed by determining the percentage of cells, excluding 0.1% trypan blue and measuring lactate dehydrogenase (LDH) activity (Bergmeyer, 1983).

After the isolation of the lymphocytes, the AChE activity was determined according to the method described by Ellman et al. (1961) modified by Fitzgerald and Costa (1993). Briefly, proteins of all samples were adjusted to 0.1–0.2 mg/ml. The amount of 0.2 ml of intact cells was added to a solution containing 1.0 mM acetylthiocholine, 0.1 mM of DTNB, and 0.1 M phosphate buffer (pH 8.0). Immediately before and after incubation for 30 min at 27 °C, the absorbance was read on a spectrophotometer at 412 nm. AChE was calculated from the quotient between lymphocyte AChE activity and protein content and the results are expressed as μmol/h/mg of protein.

2.9. AChE assay in muscle

The gastrocnemius muscles was homogenized in a glass potter in Tris–HCl (pH 7.2) solution and centrifuged for 1000 *g* for 15 min. Aliquots of the resulting centrifugation were used to determine the AChE activity. The AChE enzymatic assay in muscle was determined by a modification of the spectrophotometric method of Ellman et al. (1961). The reaction mixture (2 ml final volume) was composed of 24 mM K^+-phosphate buffer, pH 7.2 and 10 mM of DTNB. The reaction was initiated by adding 0.8 mM acetylcholine iodide. All samples were run in duplicate or triplicate and enzyme activity was expressed in micromoles ASCh/h/mg of protein.

2.10. Protein determination

Protein was measured by the method of Bradford (1976) using bovine serum albumin as standard.

2.11. Statistical analysis

Because the distributions of data of inhibitory avoidance test latencies were not normally distributed, the statistical analysis of test step-down latencies was carried out by Kruskal–Wallis followed by post hoc analyses (nonparametric Dunn's test) and the results were expressed as the median and interquartile range. Crossing, rearing, latency to training and biochemical analysis were normally distributed and were analyzed by one-way ANOVA followed by post hoc Duncan's multiple range tests and the results were expressed as mean ± SEM. $P<0.05$ was considered to represent a significant difference between groups.

3. Results

3.1. In vitro experiments with caffeic acid

Results demonstrated that in vitro caffeic acid altered the AChE activity in different brain structures. It can be observed in Fig. 1 that caffeic acid significantly increased the AChE activity in the cerebral cortex at concentrations of 1.0 mM (18%), 1.5 mM (27%), and 2.0 mM (37%); in the cerebellum at concentrations of 1.5 mM (20%) and 2.0 mM (22%); and in the hypothalamus at concentrations of 1.5 mM (42%) and 2.0 mM (42%) when compared to the control group ($P<0.05$). In the striatum, hippocampus and pons caffeic acid did not affect in vitro the AChE activity at any concentration evaluated.

Fig. 2 shows the effects of caffeic acid on the AChE activity in whole blood. It can be observed that caffeic acid significantly increased the AChE activity in whole blood only at a high concentration (2 mM (−32%)); whereas in the lymphocytes, caffeic acid significantly increased the enzyme activity at concentrations of 0.5 mM (26%), 1.0 mM (56%), 1.5 mM (68%), and 2.0 mM (68%) when compared with the control group ($P<0.05$) (Fig. 3). On the other hand, this compound in vitro caused a significant inhibition in the AChE activity in muscle at concentrations of 0.5 mM (28%), 1.0 mM (33%), 1.5 mM (37%), and 2.0 mM (42%) when compared to the control group ($P<0.05$) (Fig. 4).

3.2. In vivo experiments with caffeic acid

3.2.1. AChE activity

When rats were treated with caffeic acid, the AChE activity was altered in different brain structures. Fig. 5 shows that in the cerebral cortex ,the AChE activity was significantly decreased in the groups treated with 10 mg/kg (23%), 50 mg/kg (25%) and 100 mg/kg (28%) of caffeic acid when compared to the control group ($P<0.05$). Similarly, in the striatum the AChE activity was also significantly inhibited in the groups treated with 10 mg/kg (36%), 50 mg/kg (36%), and 100 mg/kg (37%) of caffeic acid in relation to the control group ($P<0.05$). On the other hand, in the hippocampus the AChE activity was significantly increased in the animals treated with 10 mg/kg (29%), 50 mg/kg (40%) and 100 mg/kg (40%) of caffeic acid as well as in the pons at dosages of 10 mg/kg (22%), 50 mg/kg (24%), and 100 mg/kg (22%) of caffeic acid in comparison with the control group ($P<0.05$) (Fig. 5). In addition, a significant increase was also observed in the AChE activity in the hypothalamus of animals treated with 50 mg/kg (53%) and 100 mg/kg (40%) of caffeic acid, while in the cerebellum this increase was observed only in rats treated with 10 mg/kg (27%) and 50 mg/kg (33%) of caffeic acid ($P<0.05$) (Fig. 5).

Fig. 6 shows the effect of caffeic acid on the AChE activity in lymphocytes. This compound significantly increased the AChE activity in lymphocytes only in animals treated with 100 mg/kg (73%) ($P<0.05$). In relation to the AChE from muscle, it can be observed that caffeic acid significantly increased the enzyme activity in all animals treated with this compound (10 mg/kg (59%), 50 mg/kg (65%) and 100 mg/kg (37%)) when compared to the control group ($P<0.05$).

In addition, it is also important to note that controls were performed in vitro and in vivo to correct the vehicle (methanol or canola oil) interference and no difference between vehicle and control enzyme was observed (Figs. 1–7, groups methanol and oil).

3.2.2. Behavioral tests

Fig. 8 shows the effect of oral administration of caffeic acid (10, 50, 100 mg/kg) once a day during 30 days on test step-down latencies of rats subjected to the inhibitory avoidance task. Statistical analysis (Kruskal–Wallis) revealed a significant effect of treatment [$F_{(4,35)}=11.54$; $P<0.05$, Fig. 8]. Post hoc analysis showed that caffeic acid (100 mg/kg) significantly increased step-down latencies compared to the respective control group, suggesting that oral administration of caffeic acid improves memory in the inhibitory avoidance task.

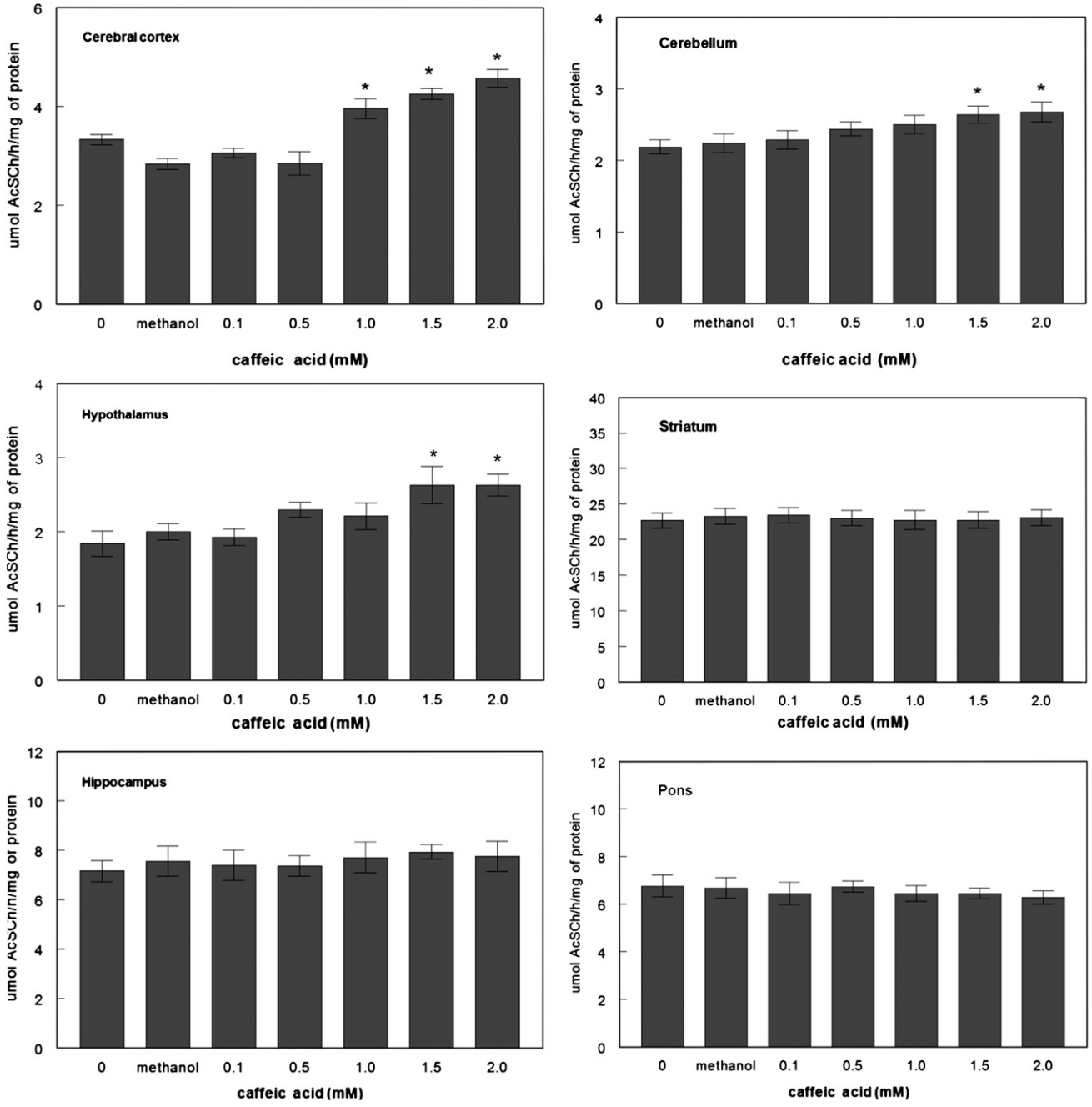

Fig. 1. In vitro effects of caffeic acid on the AChE activity in the cerebral cortex, cerebellum, striatum, hippocampus, hypothalamus, and pons of rats. Each bar represents mean ± SEM. AChE activity is expressed as μmol of acetylthiocholine/h/mg of protein. *Different from control ($P<0.05$ n = 5). ANOVA–Duncan's test.

Table 1 shows the effect of oral administration of caffeic acid on training step-down latencies in the inhibitory avoidance task and exploratory behavior in the open field immediately after the inhibitory avoidance testing session. Statistical analysis (one-way ANOVA) revealed that oral administration of caffeic acid did not alter the training step-down latencies and the number of crossing or rearing responses in a subsequent open-field testing session (F values shown in Table 1), suggesting that caffeic acid once a day during 30 days did not cause gross motor disabilities during testing.

4. Discussion

Caffeic acid, due to its therapeutic properties, represents a promising candidate for the treatment of many diseases. Although in literature it is established that caffeic acid has many effects on human heath, the mechanisms involved in these beneficial properties have not yet been fully understood. In this study we evaluated in vitro and in vivo the effects the caffeic acid on the AChE activity in the brain, whole blood, lymphocytes and muscle of rats as well as the effect of this compound on memory.

Our results in the brain showed that caffeic acid altered in vitro and in vivo the AChE activity, but these alterations were not homogeneous in all evaluated brain structures. In the cerebral cortex, cerebellum and hypothalamus, caffeic acid significantly increased the AChE activity at concentrations varying from 1.0 to 2.0 mM, while in the striatum, hippocampus and pons this compound did not alter the enzyme activity (Fig. 1). On the other hand, treatment for 30 days with caffeic acid at the dosage of 10 mg/kg, 50 mg/kg and 100 mg/kg inhibited significantly the AChE activity in cerebral cortex and striatum and increased the enzyme activity in the hippocampus, hypothalamus and pons (Fig. 5).

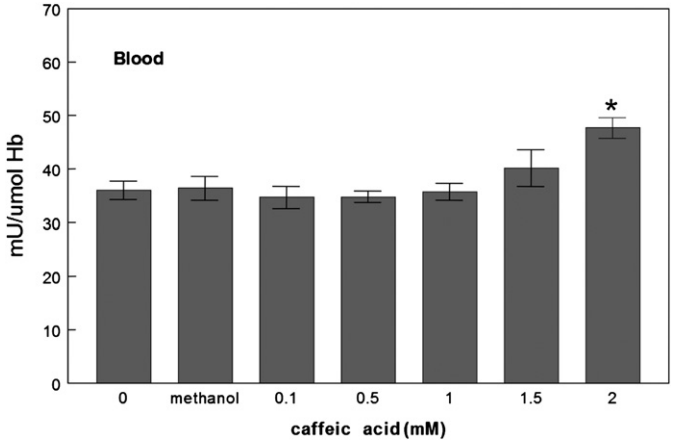

Fig. 2. In vitro effect of caffeic acid on the AChE activity in the whole blood of rats. Each bar represents mean ± SEM. AChE activity is expressed as mU/μmol Hb. *Different from control ($P<0.05$ n = 5). ANOVA–Duncan's test.

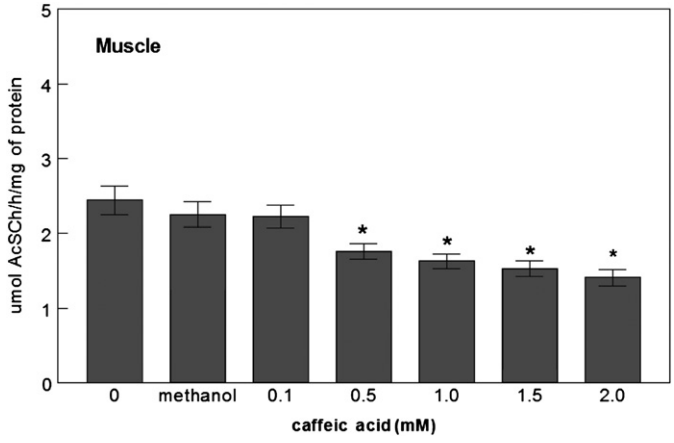

Fig. 4. In vitro effect of caffeic acid on the AChE activity in the muscle of rats. AChE activity is expressed as μmol of acetylthiocholine/h/mg of protein. Each bar represents mean ± SEM. *Different from control $P<0.05$, ANOVA–Duncan's test.

A possible explanation for the differences in relation to the AChE activity in brain regions is the fact that AChE exists in a variety of molecular forms that differ in solubility and type of membrane attachment rather than in catalytic activity. In the brain, AChE occurs mainly as tetrameric G4 forms (membrane bound) together with the monomeric G1 form (cytosolic) (Das et al., 2001). Literature shows that many drugs, including those of therapeutic use, did not affect any form of AChE equally well, most importantly, sometimes these drugs behaved differently, with the same isoform from different brain areas (Zhao and Tang, 2002). Based on this, we can suggest that caffeic acid also may have the form specific selectivity in relation to the AChE from brain regions. Although this hypothesis must be confirmed with further studies, these findings open doors to the discovery of mechanisms about more specific targets of the caffeic acid in the CNS.

The AChE activity measured in CNS has been extensively studied not only because it is involved in the cholinergic neurotransmission (Silman and Sussman, 2005; Soreq and Seidman, 2001) but also because the deleterious consequences of its inhibition (Lotti, 1995) and its action as therapeutic target in neurodegenerative diseases (Rakonczay, 2003). AChE is one of the most efficient enzymes known, and its presence leads to rapid deactivation of acetylcholine at the cholinergic synapse (Silman and Sussman, 2005). Previous studies have showed that natural substances and dietary components such as resveratrol (Schmatz et al., 2009), quercetin (Tota et al., 2010), curcuminoids (Ahmed and Gilani, 2009) and chlorogenic acid (Know et al., 2010) altered the AChE activity

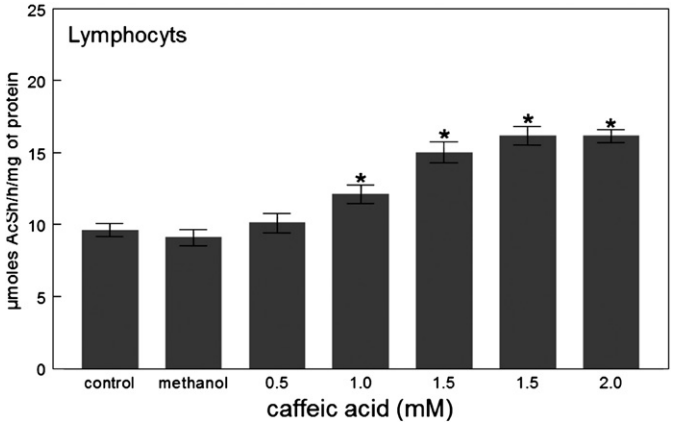

Fig. 3. In vitro effect of caffeic acid on the AChE activity in lymphocytes of rats. AChE activity is expressed as μmol of acetylthiocholine/h/mg of protein. Each bar represents mean ± SEM. *Different from control $P<0.05$. ANOVA–Duncan's test.

in CNS. Although it has been demonstrated that caffeic acid has neuroprotective effects in many experimental conditions (Yang et al., 2007; Kalonia et al., 2009; Kart et al., 2009; Sul et al., 2009) our findings led us to propose that alterations in the AChE activity induced by caffeic acid could alter the acetylcholine level interfering in the cholinergic neurotransmission.

We also observed in this study that animals treated with caffeic acid only at the dose of 100 mg/kg increased the step-down latencies in relation to the control group, suggesting that caffeic acid improved the learning and memory of rats in the inhibitory avoidance task (Fig. 8), although the exact mechanism involved in this effect is still unknown. The inhibitory avoidance task is commonly used as a parameter to evaluate the learning and memory in rats and mice (Izquierdo et al., 2000a,b, 2002). This task involves the formation of memories through an aversive stimulus (electric shock). Although this aversive stimulus can induce emotional components of fear and anxiety in the animal, this task was performed in animals treated with caffeic acid in order to evaluate only the process of learning and memory formation under these conditions. Some other behavioral parameters to evaluate other types of memory, such as object recognition, could be used to investigate if the caffeic acid could improve the memory in other conditions (non-aversive). These results are important to complement our findings related to the caffeic acid effect with the improvement of learning and memory.

Research regarding caffeic acid on behavioral parameters including memory tests is scarce in the literature, although some studies have shown that the acute administration of caffeic acid (1 mg/kg) increased the number of entries and the time spent in the open arms of a plus maze suggested an anxiolytic-like effect. The treatment for 15 days (10 and 30 mg/kg) reverted the alterations in memory and learning induced by aluminum (Yang et al., 2008) but it remains unknown if caffeic acid could be interfering in such behavior. Another important aspect to be discussed is that studies have demonstrated that caffeic acid phenethyl ester (CAPE), a derivate of caffeic acid, protects blood brain barrier integrity (Aladag et al., 2006; Zhao et al., 2012) and has relaxing properties on arteries (Cicala et al., 2003; Long et al., 2009). Moreover, it has also been reported that caffeic acid and CAPE are able to ameliorate glucose metabolism and uptake (Huang et al., 2009; Lee et al., 2007; Tsuda et al., 2012). Taken together, the vasodilator effect and the ability to improve the cellular energetic metabolism in the brain can explain, in part, the alterations in memory observed in the animals treated with caffeic acid with 100 mg/kg.

On the other hand, it is known that AChE inhibitors are used by improving the memory and other cognitive deficits by increasing the acetylcholine concentration in the extracellular medium. In this

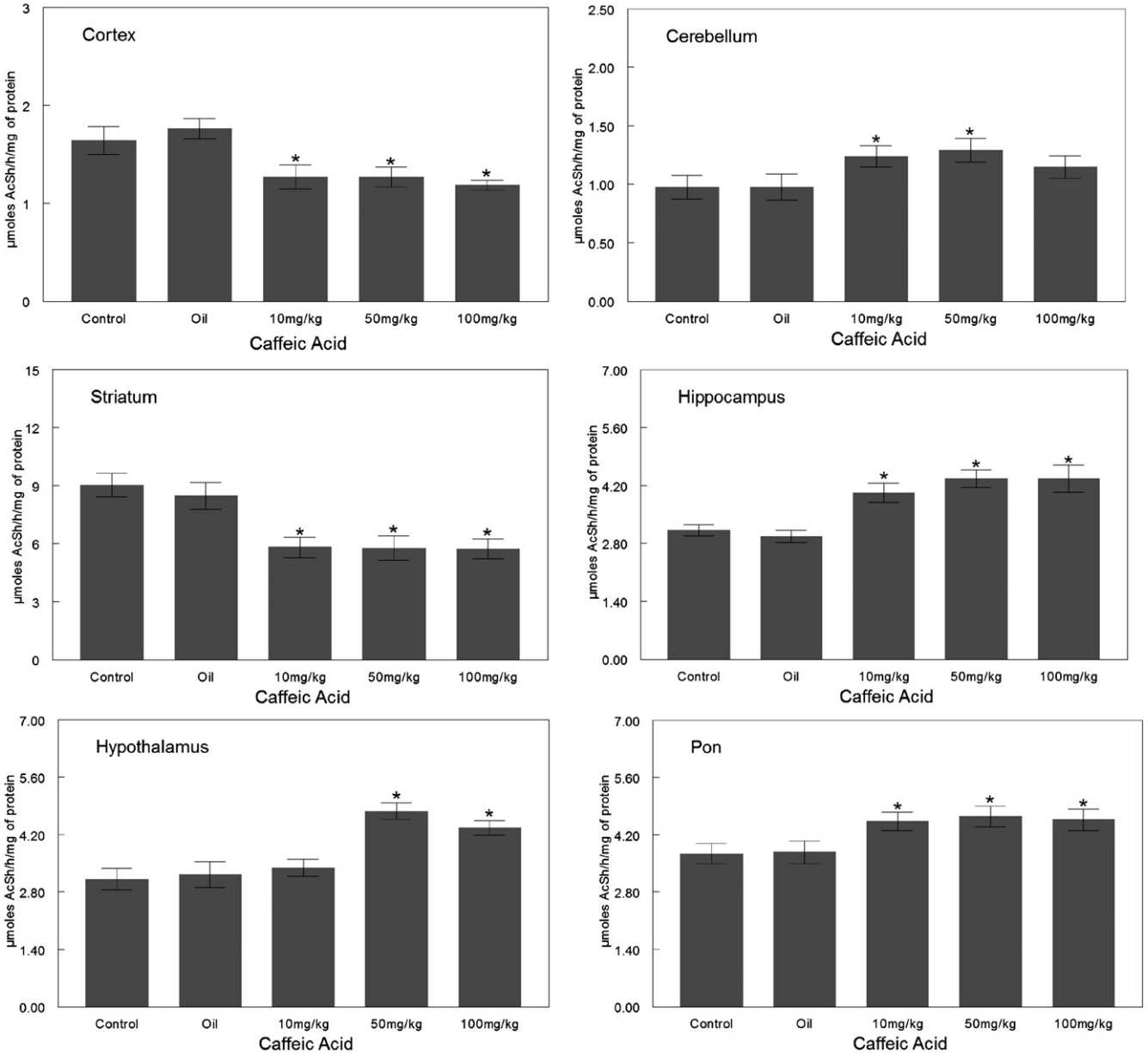

Fig. 5. In vivo effects of caffeic acid on the AChE activity in the cerebral cortex, cerebellum, hippocampus, hypothalamus, and pons of rats. Each bar represents mean ± SEM. AChE activity is expressed as µmol of acetylthiocholine/h/mg of protein. *Different from control ($P<0.05$). ANOVA–Duncan's test.

study, in vivo treatment with caffeic acid decreased the AChE activity in the cortex and striatum. This effect can be important and may be associated with the improvement of the memory observed. Furthermore, the cholinergic system has synaptic and extra-synaptic functions (Soreq and Seidman, 2001). Studies have demonstrated that the cholinergic system is involved in brain circulation (Wahl and Schilling, 1993; Bertrand et al., 1996; Moser et al., 2003). Based on these findings, caffeic acid can modulate AChE activity, and depending on the tissue involved it contributes to the cerebral circulation and improvement of cognitive performance.

In the present investigation, we also demonstrated that caffeic acid in vitro and in vivo increased the AChE activity in whole blood and lymphocytes from rats, demonstrating that this compound also affected the cholinergic signaling in non neural cells. In blood cells, AChE showed a similar structure to the enzyme that occurs in neurons, and the catalytic subunits resemble which was found in synaptic AChE (Thiermann et al., 2005). AChE is expressed in several types of hematopoietic lineages and can contribute to cell regulation (Wessler and Kirkpatrick, 2001). Studies showed that AChE is related to progenitor blood cells mainly for cell expansion of megakaryocytics and erythrocyte lineages (Soreq and Seidman, 2001; Soreq et al., 1994; Grisaru et al., 1999). Moreover, the activity of this enzyme has been used as an indicator of human cell aging with lowered levels commonly associated with older human red blood cells (Prall et al., 1998).

In addition, it has been known that lymphocytes possess a complete cholinergic system including acetylcholine, choline acetyltransferase, muscarinic and nicotinic receptors and AChE enzyme (Kawashima and Fujii, 2000, 2003). Acetylcholine synthesized and released from lymphocytes has been considered an immunomodulator acting via both muscarinic and nicotinic receptors present in these cells (Wessler and Kirkpatrick, 2001). In this context, AChE emerges as a potential contributor to the pathway controlling inflammatory and immune response in the blood. In fact, it has been demonstrated that inhibitors of the AChE reduce lymphocyte proliferation and the secretion of pro-inflammatory

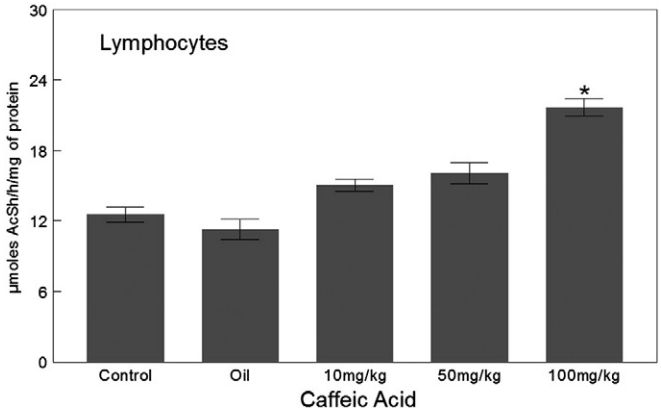

Fig. 6. In vivo effect of caffeic acid on the AChE activity in lymphocytes of rats. AChE activity is expressed as µmol of acetylthiocholine/h/mg of protein. Each bar represents mean ± SEM. *Different from control $P<0.05$. ANOVA–Duncan's test.

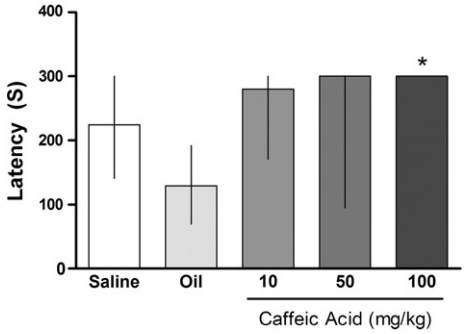

Fig. 8. Oral administration of caffeic acid (10, 50 or 100 mg/kg) once a day for 30 days in the training and test of inhibitory avoidance task. Caffeic acid, 100 mg/kg, improves memory in inhibitory avoidance test in adult rats. Data are the median ± interquartile range for 8 animals in each group. *$P<0.05$ compared with the oil group.

cytokines and may attenuate inflammation by increasing the acetylcholine concentration in the extracellular space (Nizri and Hamra-Amitay, 2006).

Although studies revealed that caffeic acid exhibits radioprotective effects in human lymphocytes (Devipriya et al., 2008) and that CAPE has anti-inflammatory properties by inhibiting (IL-2) gene transcription and IL-2 synthesis in stimulated T cells (Márquez et al., 2004), our results suggest that the increase in the AChE activity in lymphocytes by caffeic acid will lead to a decrease in the acetylcholine levels that could contribute to the onset of low-grade inflammation. These findings suggest that caffeic acid may exert negative effects that demand further investigations. In this line, other studies described that this compound has pro-oxidant activity in the presence of transition metal ions such as iron and copper causing oxidative DNA damage in lymphocytes (Ahmad et al., 1992; Yamanaka et al., 1997; Galati and Brien, 2004; Bhat et al., 2007). In addition, Wu et al. (2001) also observed that caffeic acid exhibits proglycative effects causing elevation of oxidative stress and inflammation in monocytes, macrophages and vascular endothelial cells.

AChE is also one of the key functional proteins in neuromuscular transmission by rapidly hydrolyzing acetylcholine molecules after its binding to receptors (Gaspersic et al., 1999). It has been demonstrated that the absence of AChE leads to marked alterations in muscle function including the contractile properties and the lack of resistance to fatigue (Mouisel et al., 2006; Vignaud et al., 2008). In addition, many diseases such as myasthenia gravis have been associated with disturbances in the acetylcholine homeostasis in the neuromuscular junction and in some cases inhibitors of AChE are used for the treatment of the symptoms (Kokontis and Gutmann, 2000; Shleton, 2002).

In the present study we demonstrated that caffeic acid was capable of inhibiting in vitro the AChE activity from the muscle at concentrations varying from 0.5 to 2 mM (Fig. 4), while this enzyme activity was increased when rats were treated with 10, 50 and 100 mg/kg of caffeic acid for 30 days (Fig. 7). Considering the results obtained in vivo the increase in the AChE activity caused by the ingestion of caffeic acid may increase the hydrolysis of acetylcholine at the neuromuscular junction, decreasing the levels of acetylcholine molecules for interaction with receptors. This activation of AChE by caffeic acid may modify acetylcholine homeostasis that may trigger cholinergic deficits in neuromuscular junction.

Another important aspect is the discrepancy in relation to the results obtained in vivo and in vitro, mainly in the brain and muscle (Figs. 1, 4, 5 and 7). The lack of uniformity in the profile of AChE may be a reflection of the route of administration, dose and time of exposure, absorption and metabolism of caffeic acid in the organism. Taken together, these associations can contribute to the differences observed between AChE activity in vivo and in vitro induced by caffeic acid.

Our laboratory has demonstrated that AChE activity is altered in CNS and lymphocytes in many pathological and experimental conditions (Battisti et al., 2009; Schmatz et al., 2009; Mazzanti et al., 2009; Kaizer et al., 2009). These findings reinforce the hypothesis that AChE may be considered an important therapeutic target in different tissues. In this line, compounds that may interfere with the activity of this enzyme may be important research targets regarding the treatment of inflammatory, cognitive and neurochemical dysfunctions. In the present study, based on the compiled results we demonstrated that caffeic acid exerts an effect in the cholinergic system by altering the AChE activity in different tissues.

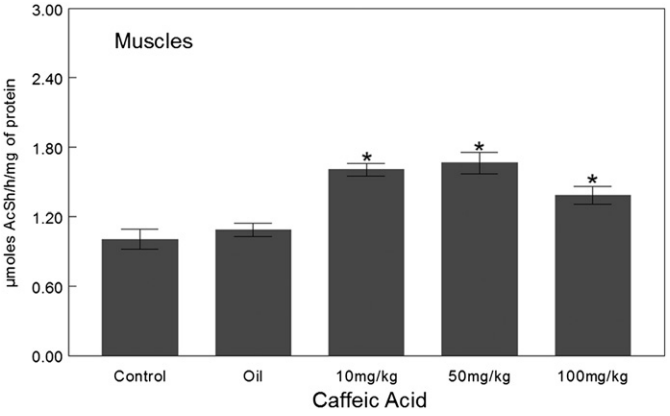

Fig. 7. In vivo effect of caffeic acid on the AChE activity in the muscle of rats. AChE activity is expressed as µmol of acetylthiocholine/h/mg of protein. Each bar represents mean ± SEM. *Different from control $P<0.05$, ANOVA–Duncan's test.

Table 1
Oral administration of caffeic acid (10,50 or 100 mg/Kg) once a day for 30 days on the latency of training (inhibitory avoidance task) and behavior of rats (number of crossing and rearing responses) in the open field immediately after the inhibitory avoidance testing session. Data are means ±SEM for 8 animals in each group.

Group	Training	Crossing	Rearing
Saline	6.37 ± 1.54	23.38 ± 4.32	11.13 ± 2.41
Oil	7.127 ± 0.97	17.38 ± 1.11	7.00 ± 0.96
10 mg/kg	5.37 ± 1.11	18.88 ± 3.30	8.75 ± 1.88
50 mg/kg	15.25 ± 7.06	17.50 ± 3.02	8.87 ± 1.69
100 mg/kg	10.75 ± 2.27	17.88 ± 2.78	11.63 ± 2.87
Statistical analysis	$F_{(4,35)} = 1.377$; $P > 0.05$	$F_{(4,35)} = 0.662$; $P > 0.05$	$F_{(4,35)} = 0.835$; $P > 0.05$

Data are means ± SEM for 8 animals in each group.

5. Conclusion

In conclusion, we have shown in this study that caffeic acid in vitro and in vivo altered the AChE activity in different structures and cells and improved memory. Further studies are necessary to elucidate the exact mechanism by which this compound affects the AChE activity and thus provide important information regarding the use of caffeic acid as therapeutic option in disorders associated with the cholinergic system.

Conflict of interest

The authors declare that there is no conflict of interest.

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