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The effect of aspartame on acetylcholinesterase activity in hippocampal homogenates of suckling rats

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Abstract

Background: Neurological disturbances have been implicated with aspartame (ASP) consumption and the cholinergic system with acetylcholinesterase (AChE) seems actively involved.
Aim: To evaluate the effect of ASP and its metabolites on rat hippocampal AChE activity.
Methods: Hippocampal homogenate or pure enzyme AChE (eel *E. electricus*) was incubated with the sum or each of ASP components, phenylalanine (Phe), aspartic acid (asp) and methanol (MeOH) for 1 h at 37 °C. AChE activity was measured spectrophotometrically.
Results: Incubation of rat tissue or pure enzyme with the sum of ASP metabolites in concentrations in CSF (the concentrations were calculated according to the CSF/plasma concentration ratios) following 150 or 200 mg kg^{-1} of ASP consumption, resulted in significant enzyme activity reductions of 25 and 31% for hippocampal AChE and 11% ($p < 0.01$) and 19% for pure enzyme, respectively. Aspartic acid concentrations of 0.42 or 0.56 mM significantly reduced the enzyme activities by 13 and 20% for hippocampal AChE and 15 and 18% for pure enzyme, respectively. Phe concentrations of 0.042 or 0.083 mM decreased the enzyme activity by 12% ($p < 0.01$) and 20% ($p < 0.001$) for hippocampal AChE and 15 and 18% ($p < 0.001$) for pure enzyme, respectively. Methanol concentrations of 0.60 or 0.80 mM remarkably inhibited hippocampal AChE by about 18 and 22% and pure enzyme by about 14 and 20%, respectively.
Conclusions: Low concentrations of ASP components had no effect on hippocampal and pure AChE activity, whereas high or toxic concentrations remarkably decreased both enzyme activities. Muscarinic symptoms may be related to the latter concentrations of ASP metabolites.

Keywords: Acetylcholinesterase; Hippocampus; Aspartame; Phenylalanine; Methanol; Aspartate

1. Introduction

Aspartame (ASP) is one of the artificial sweeteners added to many soft beverages, cakes, etc. and its usage is interesting in health conscious societies. Upon ingestion, ASP is immediately absorbed from the intestinal lumen and metabolized to phenylalanine (Phe), aspartate (asp) and methanol (MeOH). Following ASP consumption, the concentrations of its metabolites are increased in the blood stream [1].

There are numerous reports on the toxic effects of ASP and various issues continue to be raised today, more than 20 years after ASP approval by the Food and Drug Administration (FDA) of USA. Concerns have been raised about possible adverse effects of the sweetener and its above-mentioned metabolic components. There are many accounts of situations in which ASP is believed to have caused negative effects on specific human functions. These include memory loss, seizures, headaches, confusion, dizziness, etc. [2]. Neurochemical changes following high ASP dose with dietary carbohydrates have also been reported [3]. All together, these studies show that the sweetener might affect brain neurotransmitters and receptors and that these effects may become more prominent following long-term consumption [4,5].

In a previous *in vitro* study, we reported a reduction in AChE activity in rat brain and diaphragm homogenates or pure enzyme of eel *E. electricus* incubated with Phe concentrations of 7–10 mg dl^{-1} or 0.4–1.8 mM (normal Phe values in the blood: 1–2 mg dl^{-1} or 0.06–0.12 mM) [6,7]. Furthermore,

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high Phe concentrations are commonly seen in the blood of phenylketonuric (PKU) children "off diet" [8]. In addition, we have reported that the above-mentioned high Phe concentrations caused a 30% inhibitory effect on erythrocyte-membrane AChE activity [8]. Although there are no reports relating AChE inhibition with Phe and clinical symptoms observed in PKU patients, we have found not only a remarkable inhibition (60%, $p < 0.001$) of the erythrocyte membrane AChE from PKU patients "off treatment", but also a significant reduction in their plasma neurotransmitter levels of dopamine, noradrenaline, adrenaline and serotonin [9].

Although red cell AChE activity is not always a direct reflection of the AChE activity in the brain and both enzymes are of different physiological relevance, we reported that high or toxic concentrations of ASP metabolites in the blood resulted in a partial or profound decrease in AChE activity in erythrocyte membranes from healthy subjects [10].

AChE is a key enzyme of the muscarinic cholinergic system, involved in learning and memory [5]. Moreover, muscarinic receptor binding has been shown to be altered in forebrain and midbrain regions of chickens during passive avoidance learning [11].

ASP is immediately hydrolysed in the intestinal lumen giving Phe, MeOH and asp [1]. The aim of the present study was to evaluate AChE activity in hippocampal homogenate of suckling rats, after incubation with various concentrations of each or the sum of ASP metabolites. In addition, in order to elucidate whether ASP metabolites directly and/or indirectly affect hippocampal AChE, similar concentrations of ASP components were incubated with pure enzyme from eel *E. electricus*.

2. Materials and methods

2.1. Animals

Suckling (21 days) Albino Wistar rats of both sexes (Saint Savas Hospital, Athens, Greece) were used in all experiments. Under our *in vitro* experimental conditions, there were no differences in ASP action on hippocampal AChE activity between male and female rats. Body weight was 50 ± 12 g (mean $\pm$ S.D.). The animals were housed in separate cages with their mothers at a constant room temperature (22 ± 1 °C) under a 12 h L:12 h D (light 08:00–20:00 h) cycle. For the mothers, food and water were provided *ad lib*. Animals were cared for in accordance with the principles of the "Guide to the Care and Use of Experimental Animals" [12].

2.2. Tissue preparation

Animals were euthanised by decapitation. The hippocampus (50.2 ± 9.0 mg) of each of the 60 suckling rats (10 rats per group) was rapidly removed, weighed and thoroughly washed with isotonic saline. All tissues were homogenized in 10 vol. ice-cold (0–4 °C) medium containing 50 mM Tris(hydroxymethyl)-aminomethane–HCL (Tris–HCL), pH 7.4 and 300 mM sucrose using an ice-chilled glass homogenising vessel at 900 rpm (4–5 strokes). Then the homogenate was centrifuged at $1000 \times g$ for 10 min to remove nuclei and debris (the hippocampal homogenates contain the whole cell machinery). In the resulting supernatant, the protein content was determined according to the method of Lowry et al. [13] and then the enzymatic activities were measured.

Moreover, the enzymatic activity was measured after incubation of ASP metabolites with pure AChE from eel *E. electricus* (Sigma, C-3389). The enzyme incubation mixtures of all experiments were kept for 60 min at 37 °C.

2.3. Measurement of hippocampal homogenate AChE and pure enzyme activities after incubation with ASP metabolites

Hippocampal homogenates or pure enzyme were incubated with each or the sum of ASP metabolites at 37 °C.

ASP follows a hydrolysis process in the laboratory giving MeOH, asp and Phe at a ratio of 10:40:50 (w:w:w) [1].

Concentrations of ASP hydrolysis products are immediately measured in the human plasma after ASP ingestion are different than those found in the laboratory and reported by Stegink et al. [1,14]. Moreover, MeOH concentrations in the cerebrospinal fluid (CSF) are similar to those in plasma. On the contrary, CSF/blood concentration ratios for Phe and asp are 1/6 and 1/18, respectively [15]. ASP metabolite concentrations may be related to the symptoms from the central nervous system. Thus, we decided to use the possible cerebrospinal fluid (CSF) concentrations of ASP degradation products (MeOH, asp, Phe) of ASP consumers (the concentrations were calculated according to CSF/plasma ratios), as follows:

ASP ingestion of 10 mg kg^{-1} of Mix 1 (corresponding to CSF concentrations of MeOH = undetected, asp = 0.05 mM, Phe = 0.012 mM) represents the additive and its components when drinking a beverage (12 oz).
ASP intake of 34 mg kg^{-1} of Mix 2 (corresponding to CSF concentrations of MeOH = 0.14 mM, asp = 0.16 mM, Phe = 0.023 mM) represents the 99th percentile of projected daily ingestion.
ASP ingestion of 150 mg kg^{-1} of Mix 3 (corresponding to CSF concentrations of MeOH = 0.60 mM, asp = 0.42 mM, Phe = 0.042 mM) and of 200 mg kg^{-1} of Mix 4 (corresponding to CSF concentrations of MeOH = 0.80 mM, asp = 0.56 mM, Phe = 0.083 mM) represent sweetener doses that may be taken by accident [1]. For the sake of comparison with the results of our previous studies [6,7,16], we decided to incubate tissue homogenates or pure AChE with the mixtures (Mixes 1–4) or each one of ASP degradation products for 1 h at 37 °C, as we previously did.

After 1 h incubation at 37 °C, determination of AChE activity was performed spectrophotometrically.

2.4. Determination of AChE activity

AChE activity was determined according to the method of Ellman et al. [17], as modified by Tsakiris et al. [7]. The reaction

mixture (1 ml) contained 50 mM Tris–HCl, pH 8 and 240 mM sucrose in the presence of 120 mM NaCl and 80–100 μg of protein of hippocampal homogenate or 0.1 μg of protein of the eel *E. electricus* pure AChE as well as in the presence or absence of ASP (Fluca, 47135), MeOH (Merck), asp (Sigma, A-7219) or Phe (Sigma, P-2126). Finally, 0.050 ml 5,5′-dinitrobenzoic acid (DTNB) and 0.050 ml acetylthiocholine iodide (substrate) were added, and the reaction was started (reaction time the first 3 min). The final concentrations of DTNB and substrate were 0.125 and 0.5 mM, respectively. The reaction was measured spectrophotometrically by the increase in absorbance ($\Delta\overline{OD}$) at 412 nm.

3. Statistical analysis

Data were analysed by one-way ANOVA. All analyses were performed with an IBM PC using the SPSS 10.0 statistical package. The p-values <0.05 were considered statistically significant.

4. Results

As shown in Table 1, abuse or toxic doses (150 or 200 mg kg^{-1}) of ASP consumption that may result in high concentrations of its components in the CSF significantly decreased hippocampal and pure AChE activities (by 31 and 11%, respectively, for abuse doses and by 31 and 19%, respectively, for toxic doses). Lower concentrations (e.g. 34 mg kg^{-1} or less) had no effect on the enzyme activities.

As illustrated in Table 2, concentrations of each of ASP components (MeOH, asp, Phe), possibly found in the CSF following consumption of abuse (150 mg kg^{-1}) and/or toxic (200 mg kg^{-1}) doses of the sweetener, resulted in a remarkable reduction of the enzyme activities in rat hippocampus as well as pure AChE, as follows:

150 mg kg^{-1} of ASP consumption corresponding to CSF concentrations of 0.60 mM MeOH, 0.42 mM asp and 0.042 mM Phe resulted in reductions of hippocampal enzyme activity by 18, 13 and 12%, respectively. Moreover, the previously mentioned concentrations of ASP metabolites also caused reductions of pure enzyme activity by 14, 15 and 15%, respectively.

In addition, toxic doses of ASP (200 mg kg^{-1}) corresponding to CSF concentrations of 0.80 mM MeOH, 0.56 mM asp and 0.083 mM Phe resulted in reductions of hippocampal AChE activity by 22, 20 and 20%, respectively. Similar reductions of 20, 18 and 18%, respectively, were observed in pure enzyme activity.

Table 1
Effect of the incubated doses of ASP on AChE activity in rat hippocampal homogenate and on pure enzyme

Treatment		AChE activity	
Blood	CSF[a]	($\Delta\overline{OD}$ min^{-1} mg protein^{-1}) Hippocampus	($\Delta\overline{OD}$ min^{-1} mg protein^{-1}) Pure enzyme
Control		0.397 ± 0.016	1.23 ± 0.04
Mix 1			
ASP (10 mg kg^{-1})		0.400 ± 0.012 (NS)	1.26 ± 0.05 (NS)
MeOH none	None		
asp 0.82 mM	0.05 mM		
Phe 0.07 mM	0.012 mM		
Mix 2			
ASP (34 mg kg^{-1})		0.389 ± 0.019 (NS)	1.17 ± 0.06 (NS)
MeOH 0.14 mM	0.14 mM		
asp 2.80 mM	0.16 mM		
Phe 0.14 mM	0.023 mM		
Mix 3			
ASP (150 mg kg^{-1})		0.298 ± 0.012*** (−25%)	1.09 ± 0.02** (−11%)
MeOH 0.60 mM	0.60 mM		
asp 7.60 mM	0.42 mM		
Phe 0.35 mM	0.042 mM		
Mix 4			
ASP (200 mg kg^{-1})		0.274 ± 0.014*** (−31%)	0.99 ± 0.03*** (−19%)
MeOH 0.80 mM	0.80 mM		
asp 10.00 mM	0.56 mM		
Phe 0.50 mM	0.083 mM		

Mix 1, Mix 2, Mix 3 and Mix 4 are the incubated concentrations of the sum of ASP metabolites corresponding to CSF levels. The mixture (Mixes 1–4) was derived from the quick degradation of the used ASP in the solutions (for details see Section 2).

Values are expressed as means ± S.D. of four independent experiments; the average value of each experiment was derived from three determinations of the enzyme activity.

[a] Calculated concentrations of the ASP metabolites in the CSF after consumption of several amounts of the sweetener.

** $p < 0.01$ (vs. control).

*** $p < 0.001$ (vs. control).

Table 2
Effect of each ASP metabolite CSF concentrations, separately, corresponding to the consumption of 10, 34, 150 or 200 mg ASP kg^{-1} on rat hippocampal AChE and pure enzyme activity

Treatment	AChE activity	
	($\Delta\overline{OD}$ min^{-1} mg $protein^{-1}$) Hippocampus	($\Delta\overline{OD}$ min^{-1} mg $protein^{-1}$) Pure enzyme
Control	0.397 ± 0.016	1.23 ± 0.04
Methanol (MeOH) (mM)		
None	0.402 ± 0.012 (NS)	1.21 ± 0.05 (NS)
0.14	0.377 ± 0.021 (NS)	1.18 ± 0.06 (NS)
0.60	0.325 ± 0.010*** (−18%)	1.05 ± 0.04** (−14%)
0.80	0.309 ± 0.012*** (−22%)	0.98 ± 0.05*** (−20%)
asp (mM)		
0.05	0.396 ± 0.011 (NS)	1.25 ± 0.03 (NS)
0.16	0.389 ± 0.012 (NS)	1.11 ± 0.04** (−10%)
0.42	0.345 ± 0.007** (−13%)	1.05 ± 0.05** (−15%)
0.56	0.317 ± 0.015*** (−20%)	1.00 ± 0.03*** (−18%)
Phe (mM)		
0.012	0.395 ± 0.020 (NS)	1.20 ± 0.06 (NS)
0.023	0.377 ± 0.018 (NS)	1.16 ± 0.06 (NS)
0.042	0.349 ± 0.008** (−12%)	1.05 ± 0.04***(−15%)
0.083	0.317 ± 0.012*** (−20%)	1.00 ± 0.03*** (−18%)

Values are expressed as means ± SD of four independent experiments. The average value of each experiment was derived from three determinations of the enzyme activity.
NS: not statistically significant.
** $p < 0.01$ (vs. control).
*** $p < 0.001$ (vs. control).

On the contrary, low concentrations of the above metabolites, corresponding to the consumption of lower doses of ASP (10 or 34 mg kg^{-1}), had no effect on both hippocampal and pure AChE activities.

5. Discussion

The *per os* loading with large amounts of ASP is claimed to be the cause of some ailments. Human studies have confirmed the safety of ASP, although they suggest that ASP consumption may constitute a hazard to humans [18]. An important question is whether the ingestion of ASP at various doses is still safe for humans. The effect of ASP in humans probably depends on its metabolite concentrations.

Orally ingested ASP is metabolized and absorbed as mentioned above. The above-mentioned metabolites are absorbed from the intestinal lumen and reach the portal blood in a manner similar to that of amino acids and MeOH arising from dietary proteins and polysaccharides [1].

In speculating parallel changes in brain and blood, both asp and Phe are transported from plasma to CSF in competition with other amino acids. Moreover, movement of large neutral amino acids, such as Phe across the blood–brain barrier is mediated by a common high affinity, low capacity transport system. A large excess of Phe, as found in a previous study in ASP consumers [1], will saturate this carrier system excluding other amino acids, such as tyrosine and tryptophane from entry into the brain. Thus, high levels of Phe interfere with the conversion of tyrosine to the biogenic amines dopamine, adrenaline and serotonin possibly affecting AChE activity, as has been previously reported [1,10,15].

Aspartic acid, the dicarboxyl amino acid, may exert toxic effects when administered at very high doses, although species susceptibility varies considerably [1]. Neonatal rodents administered large doses of asp may develop hypothalamic neuronal necrosis [18]. However, there is a disagreement over the ability of *N*-methyl-D-aspartate (NMDA) to produce neuronal necrosis in infant primates [19]. In this study, high or toxic doses of asp incubated with hippocampal or pure AChE caused significant reductions of the enzyme activity. If these results come into whole cell machinery, the observed inhibition may be due to an increase in reactive oxygen species production and in intracellular Ca^{2+} concentration caused by the metabolite, as reported previously. In addition, free radicals attack on unsaturated bonds of membrane fatty acids results in an autocatalytic process called membrane lipid(s) peroxidation, which can indirectly impair the function of membrane AChE [19].

MeOH, another ASP metabolite, is a toxicant that causes systematic toxicity [20–22]. The primary metabolic fate of MeOH is the direct oxidation to formaldehyde and then into formate. Mean peak blood MeOH concentrations exceeded 2 mg dl^{-1} in subjects administered abuse doses of ASP, but were still lower than those reported in MeOH toxication [20]. In this study, the observed inhibition of the rat tissue and pure enzyme AChE activity after incubation with high MeOH concentrations indicates an indirect effect of the metabolite through induction of oxidative stress (as a consequence of cellular glutathione reduction [21]) and an interaction with the lipid bilayer of the hippocampal cell membrane, as well as a direct effect of the ASP component on the enzyme protein [22]. Recently, Rico et al. [23] reported that methanol exposure remarkably decreased zebrafish brain AChE activity, suggesting a neurodegenerative event promoted by MeOH.

Additionally, we observed a significant decrease in the activities of rat hippocampal as well as pure AChE when incubated with Phe concentrations corresponding to the ingestion of high and/or toxic ASP doses. A similar degree of enzyme inhibition was observed in the erythrocyte membrane AChE activity of phenylketonuric (PKU) patients "off diet" whose plasma neurotransmitter levels were found to be very low, and Phe levels were determined very high [9]. The observed high degree of AChE inhibition of rat hippocampal AChE activity may be due to an indirect effect of high Phe concentrations on the membrane bilayer, through lipid(s)–protein interactions as shown in our previous studies [6,19]. Similarly, the inhibitory effect of high Phe concentrations on pure AChE indicates a possible direct action of the metabolite on the positively charged site of the enzyme [6]. According to the above experimental results (Table 1), it may be suggested that the effect of ASP and its metabolites on AChE activity appeared to represent a more complex procedure. Firstly, ASP and its metabolites probably work by changing the membrane lipid bilayer microenvironment causing functional modulation of the AChE leading to a reduction of the enzyme activity (indirect effect). Secondly, an extra effect (direct) could be the result of the charge of the

above ASP metabolites on the anionic subsite of the enzyme. Above suggestions may explain, in this preliminary stage, the observed differences of the relative inhibitory effect between AChE in homogenate preparations and pure enzyme activities. In the hippocampus homogenate case (more affected), where other cellular components may be present, the overall inhibitory effect could reflect the sum of indirect plus direct effect, while in the pure enzyme form (less affected) the effect seems to represent mostly a direct ionic interaction only between the inhibitory ASP agents on the enzyme.

Orally ingested ASP is immediately absorbed. Obviously, the inhibitory effects of the above-mentioned ASP metabolites (which enter the CSF through the blood–brain barrier) may be due to a simultaneous action on hippocampal cell membrane, affecting AChE indirectly. In addition, we cannot exclude the possibility of a direct action of ASP components on the positively charged sites of the enzyme.

In general, cholinergic system is closely implicated with CNS functions [11], without excluding the involvement of other neurotransmitter systems in chronic ASP-induced neurological symptoms.

6. Conclusions

ASP metabolites inhibit the activity of hippocampal AChE as well as pure enzyme when they were incubated with concentrations related to abuse or toxic doses of the sweetener. ASP metabolite concentrations corresponding to the consumption of a beverage had no effect on AChE activity.

Neurological symptoms, such as seizures, headaches, confusion, dizziness, observed after the consumption of high or abuse doses of ASP [18,24] may be related to a decrease in hippocampal AChE activity.

People on weight reduction programs and especially pregnant women may take care to avoid ASP consumption, since all ASP components, especially Phe and asp, cross the placenta in a concentration-dependent manner, and then the blood–brain barrier entering the CSF of the fetus [15].

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