The effect of aspartame metabolites on human erythrocyte membrane acetylcholinesterase activity

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Abstract

Studies have implicated aspartame (ASP) with neurological problems. The aim of this study was to evaluate acetylcholinesterase (AChE) activity in human erythrocyte membranes after incubation with the sum of ASP metabolites, phenylalanine (Phe), methanol (met) and aspartic acid (aspt), or with each one separately. Erythrocyte membranes were obtained from 12 healthy individuals and were incubated with ASP hydrolysis products for 1 h at 37 °C. AChE was measured spectrophotometrically. Incubation of membranes with ASP metabolites corresponding with 34 mg/kg, 150 mg/kg or 200 mg/kg of ASP consumption resulted in an enzyme activity reduction by −33%, −41%, and −57%, respectively. Met concentrations 0.14 mM, 0.60 mM, and 0.80 mM decreased the enzyme activity by −20%, −32% or −40%, respectively. Aspt concentrations 2.80 mM, 7.60 mM or 10.0 mM inhibited membrane AChE activity by −20%, −35%, and −47%, respectively. Phe concentrations 0.14 mM, 0.35 mM or 0.50 mM reduced the enzyme activity by −11%, −33%, and −35%, respectively. Aspt or Phe concentrations 0.82 mM or 0.07 mM, respectively, did not alter the membrane AChE activity. It is concluded that low concentrations of ASP metabolites had no effect on the membrane enzyme activity, whereas high or toxic concentrations partially or remarkably decreased the membrane AChE activity, respectively. Additionally, neurological symptoms, including learning and memory processes, may be related to the high or toxic concentrations of the sweetener metabolites.

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1. Introduction

Although aspartame (ASP) received Food and Drug Administration (FDA) approval in 1981 and has been judged safe by medical groups such as the AMA Council on Scientific Affairs [1], there has been persistent concern that the use of ASP, an O-methyl ester of the dipeptide l-a-aspartyl-l-phenylalanine, may result in adverse neurologic symptoms or other abnormalities [1,2]. Clinical studies have demonstrated that administration of ASP can alter some aspects of blood chemistry, in particular plasma phenylalanine (Phe) levels [3–5] and brain wave activity among epileptic children [6,7]. Moreover, since ASP’s approval numerous case reports have implicated ASP in the occurrence of such problems as seizures [7], memory loss [8], headache [9] and hypersensitivity reactions [7,9]. In addition, most of clinical studies investigating ASP have used as subjects healthy adults [9] and individuals likely to use large amount of ASP, like diabetics or in weight reduction [10,11]. Furthermore, other short-term studies have been conducted and none of these have suggested any relationship between ASP consumption and memory loss [3,11]. In contrast, various neurochemical effects due to ASP ingestion have been reported [8]. Certain brain amino acid levels, including aspt or Phe, have been shown to be increased after the consumption of the sweetener [12]. Taken collectively, these studies suggest that ASP might affect neurotransmitters and receptors and these effects may become more prominent with long-term consumption [3,12–14].
2. Subjects and methods

The study was approved by the Greek ethics committee and conducted according to the principles expressed in the declaration of Helsinki.

2.1. Subjects

The study population consisted of 12 healthy individuals, mean age 24 ± 5 years old.

2.2. Erythrocyte membrane preparation

Venous blood samples (7.0 ml) were collected into heparinized blood collection tubes from the individuals. Within 2 h of collection, the erythrocytes were sedimented by centrifugation at 2000 × g for 30 min at 4°C, three times, after three similar centrifugations, with a buffer solution (250 mM, tris (hydroxymethyl) aminomethane-HCl (Tris-HCl), pH 7.4, 140 mM NaCl, 1 mM MgCl2, 10 mM glucose. The erythrocytes were then resuspended in 1 ml of this buffer and stored at 4°C for up to 24 h before erythrocyte membrane preparation. The washed erythrocytes were lysed after freeze (−80°C) thawing (50°C) for five times, as described by Galbraith and Watts [18] and Kamber et al. [19]. The hemolysate was centrifuged at 35,000 g (×g) for 30 min at 4°C, as shown in Tables 1 and 2.

Since ASP is immediately hydrolyzed in the intestinal lumen giving Phe, methanol (met) and aspartic acid (aspt) [3], it was important to evaluate AChE activity in the erythrocyte membranes of healthy individuals after incubation with various concentrations of each or the sum of ASP metabolites.

2.3. Measurement of erythrocyte membrane acetylcholinesterase (AChE) activity after incubation with ASP metabolites

Erythrocyte membranes were incubated with ASP metabolites at 37°C, as shown in Tables 1 and 2. ASP follows a hydrolysis process in the laboratory giving met, aspt and Phe 10:40:50 (w/w/w) [1]. Furthermore, ASP metabolites at 37°C were measured in blood after ingestion of various (10, 18, 34, 150 or 200 mg kg⁻¹) doses of the sweetener. Values represent mean ± S.D. a/b, a/f, g/i = P < 0.01, a/s, a/t, o/t, p/t = P < 0.001. The concentrations of ASP metabolites used in Mixes 1–5 are described in details in Section 2.

USA), and the value was subtracted from the total protein concentration.

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AChE activity (ΔAbs 405/Δt) mm⁻¹ mg⁻¹ protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.0 ± 0.12 x</td>
</tr>
<tr>
<td>ASP (10 mg kg⁻¹) Mix 1</td>
<td>3.0 ± 0.09 (NS)</td>
</tr>
<tr>
<td>ASP (18 mg kg⁻¹) Mix 2</td>
<td>2.80 ± 0.10 (f &lt; 0.01)</td>
</tr>
<tr>
<td>ASP (34 mg kg⁻¹) Mix 3</td>
<td>2.02 ± 0.10 (−33%)</td>
</tr>
<tr>
<td>ASP (50 mg kg⁻¹) Mix 4</td>
<td>1.78 ± 0.05 d (&lt;41%)</td>
</tr>
<tr>
<td>ASP (200 mg kg⁻¹) Mix 5</td>
<td>1.28 ± 0.07 f (&lt;52%)</td>
</tr>
</tbody>
</table>

The inhibition and its degree have been observed in each subject (N = 12). Values are expressed: means ± S.D. ab, af = NS; non statistically significant; a/d, a/f, b/d, b/c, c/d, c/e = P = 0.01; a/e, b/e, f/i = P < 0.001. The concentrations of ASP metabolites used in Mixes 1–5 are described in details in Section 2. Further, values represent mean ± S.D. a/b, a/f = NS; NS: non statistically significant; a/i, f/i, = P < 0.01, a/n, k/n = P < 0.001, a/s, a/t, o/t, p/t = P < 0.01. NS: non statistically significant.
the concentrations of ASP hydrolysis products measured in the human plasma, after its ingestion, are different than those found in the laboratory and reported by Stegink [3,4]. So, we decided to use the concentrations of ASP degradation parts (Phe, met, aspt), which are commonly measured in the blood of ASP consumers as follows [3]: ASP ingestion of 10 mg kg\(^{-1}\) Mix 1 (met = undetected + aspt 0.82 mM + Phe 0.07 mM) represents the additive and its components when drinking a beverage (12 oz). Mix 2 (met 0.07 mM + aspt 1.40 mM + Phe 0.06 mM) represents the sweetener and its components when drinking a beverage of 16 oz. In addition, ASP intake of 34 mg kg\(^{-1}\) Mix 3 (met 0.14 mM + aspt 2.80 mM + Phe 0.14 mM) represents the 99th percentile of projected daily ingestion, whereas ASP 150 mg kg\(^{-1}\) Mix 4 (met 0.60 mM + aspt 7.60 mM + Phe 0.35 mM) or 200 mg kg\(^{-1}\) Mix 5 (met 0.80 mM + aspt 10.00 mM + Phe 0.50 mM) may be taken by accident [3]. For the sake of comparison with the results of our previous reports [17,22], we decided to incubate the erythrocyte membranes with the mixtures (Mixes 1–5) or each one of ASP degradation products for 1 h, as we previously did. After 1-h incubation at 37°C, determination of AChE activity was performed spectrophotometrically.

AChE activity was determined according to the method of Ellman et al. [21] as modified by Tsakiris et al. [17]. The reaction mixture (1 ml) contained 50 mM Tris–HCl, pH 8.0, 240 mM sucrose and 120 mM NaCl in the presence of 80–100 μg protein of erythrocyte membranes as well as in the presence or absence of ASP, met, or Phe. Quinidine sulfate (2.10^{-2} M) was also added to the mixture, in order to inhibit pseudocholinesterase activity. Finally, 0.030 ml 5.5'-dithionitro-benzoic acid (DTNB) and 0.050 ml acetylthiocholine iodide, which was used as substrate, and the reaction was started. 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 (ΔOD) at 412 nm.

2.4. Statistical analyses

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

3. Results

As illustrated in Table 1, the amount of ASP ingestion, which correlates with the consumption of a 12 oz beverage (Mix 1) or 16 oz beverage (Mix 2), when incubated with erythrocyte membranes, did not alter AChE activity. On the contrary, ASP metabolite concentrations which correspond to the 99th percentile of the daily intake of the sweetener, which corresponds to the consumption of four to five beverages (12 oz) (Mix 3) as well as toxic doses (150 mg kg\(^{-1}\) and/or 200 mg kg\(^{-1}\)) (Mixes 4 and 5, respectively), resulted in a remarkable decrease of the human erythrocyte membrane enzyme activity.

As shown in Table 2, the metabolite met, significantly statistically inhibited AChE activity after incubation of the erythrocyte membranes with met concentrations usually determined in blood and related to ASP consumption of 34 mg kg\(^{-1}\) 24 h\(^{-1}\) or with those measured in blood when taken toxic doses of ASP (150 or 200 mg ASP kg\(^{-1}\)). In addition, incubation of the erythrocyte membranes with concentrations of the dicarboxylic acid aspt, which correlated with ASP ingestion 34, 150, or 200 mg kg\(^{-1}\), resulted in a reduction of AChE activity by –20%, –35%, and –47%, respectively.

Also, significant inhibition of the membrane enzyme activity was observed after 1-h incubation with high concentrations of the aromatic acid Phe, corresponding to Phe blood levels, which were found after ingestion of abuse doses of the sweetener. Lower concentrations of Phe had no or minor effect on AChE activity (Table 2).

4. Discussion

ASP is one of the artificial sweeteners added to many soft beverages, cakes etc., and its usage is increasing in health-conscious societies. Upon ingestion, this artificial sweetener is immediately absorbed from the lumen and metabolized to Phe, aspt and met. Their concentrations are found increased in the blood stream [3,4].

In speculating about parallel changes in brain and blood, both aspt and Phe are transported into CNS in competition with other amino acids. 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 our previous study [22] and probably in ASP consumers [3], will saturate this carrier system excluding other amino acids, such as tyrosine (Tyr) from entry into the brain. Thus, high levels of Phe interfere with the conversion of Tyr to the biogenic amines dopamine and noradrenaline affecting AChE activity as found previously [22].

Aspt, the dicarboxylic amino acid may exert toxic effects when administered at very high doses, although species susceptibility varies considerably. Neonatal rodents administered large doses of aspt may develop hypothalamic neuronal necrosis. However, there is disagreement over the ability of aspt to produce neuronal necrosis in infant primates [3,4,23]. It is not surprising that the per oz loading with large amounts of ASP is claimed to be the cause of some ailments [9]. Human studies have affirmed the safety of ASP [23], although suggest that ASP consumption might constitute a hazard to humans [24,25]. An important question is whether the ingestion of ASP at abuse doses is still safe to humans, because consumers, including children, might unintentionally take a large amount of ASP commonly used in many products. The effect
of ASP on humans is probably dependent on its metabolite concentrations.

Of the ASP metabolites, met is a toxicant that causes systemic toxicity [26]. The primary metabolic fate of met is the direct oxidation to formaldehyde and then into formate. The severity of clinical findings in met intoxication correlated better with formate levels [27]. Mean peak blood methanol concentrations exceeded 2 mg dl⁻¹ in subjects administered abuse doses of ASP and were still lower than those reported in met intoxication [4]. However, another study [28] revealed that the cellular glutathione content and activity of glutathione-related enzymes were decreased in met intoxication. In this study, the observed inhibition of the erythrocyte membrane AChE activity after incubation with high and/or toxic met concentrations may be due to the production of oxidative stress as a consequence of the cellular glutathione reduction [29].

Additionally, we observed a slight decrease of AChE activity when the membranes were incubated with Phe concentrations, usually measured in humans after ingestion a dose of ASP corresponding to the 99th percentile of ASP daily dose (Table 2), whereas no effect was observed on the enzyme activity when incubated with lower Phe concentrations (Table 2). On the contrary, a remarkable inhibition by the erythrocyte membrane enzyme activity was measured when the membranes were incubated with Phe concentrations related to abuse doses of the sweetener (Table 2). Almost the same degree of the enzyme inhibition was observed in the erythrocyte membrane AChE activity of phenylketonuric (PKU) patients “off diet”, in whom their plasma neurotransmitter levels were found very low, whereas Phe levels were determined very high in their blood [17,30]. The high degree of AChE inhibition in erythrocyte membranes from healthy individuals after incubation with high Phe [17] concentrations may be caused by the indirect influence of the high concentrations of the amino acid on AChE in the membrane bilayer through lipid(s)–protein interactions as shown in our previous report [17]. Furthermore, we cannot exclude that the observed decrease of the membrane AChE activity may be due to an oxidative stress as observed in phenylketonuric patients “off diet” [17,30].

Aspartic acid (aspt), another ASP metabolite, is a dicarboxylic amino acid which may exert toxic effects when administered at very high doses although species susceptibility varies considerably [4]. In this study, high or toxic doses of aspt, when incubated with the erythrocyte membranes resulted in a significant (−20%) or very significant reduction (−50%) of AChE activity, respectively (Table 2). The observed enzyme activity inhibition may be due to an increase of reactive oxygen species (ROS) production and/or increases of intracellular Ca²⁺ by the amino acid as reported by Sureda et al. [31].

Orally ingested ASP components are immediately absorbed from the lumen and reach the portal blood in a manner similar to that of amino acids and met arising from dietary protein or polysaccharides [4,25]. The inhibitory action of ASP metabolites (Table 1) may simultaneously work on the erythrocyte membrane AChE activity either by producing an oxidative stress and/or through the indirect influence of the metabolites on AChE in the membrane bilayer through lipid(s) peroxidation, as suggested in our previous in vivo findings: It was reported that the erythrocyte membrane AChE activity was evaluated significantly decreased in PKU patients “off diet” with low antioxidant status [17,30].

Numerous studies have implicated muscarinic cholinergic receptors and memory [32,33]. In the rabbit, elevated muscarinic binding has been shown in the anterodorsal nucleus early in the learning process. It has been suggested that nicotinic transmission may be important in delayed response tasks, while the muscarinic system may be involved, in general working memory processes [34–36]. These studies lead us to hypothesize that it might exist an alteration in brain muscarinic system after high or toxic doses of ASP consumption and that the most available human tissue for AChE study was the human erythrocyte membranes. Also, it might be suggested that elevations of ASP hydrolysis products in the blood, especially aspt and Phe concentrations, may take place, in some degree, in the brain inducing modulations not only on the enzyme activity but also in the neurotransmitter levels, including acetylcholine and biogenic amines, as found in our previous work [17], resulting in learning difficulties and memory loss [4,8].

5. Conclusions

ASP metabolites may partially inhibit the erythrocyte membrane AChE activity, when incubated with their concentrations corresponding to the 99th percentile of the daily intake of the sweetener, whereas a remarkable decrease of the enzyme activity was observed after incubation with ASP hydrolysis products related to abuse doses of the additive. ASP metabolite concentrations commonly found in beverages had no effect on AChE activity. Oxidative stress and/or an indirect effect of ASP metabolites on the membrane bilayer through lipid(s) peroxidation may contribute to the reduction of the human erythrocyte membrane AChE activity.

Neurological symptoms, memory loss and/or learning process, headaches, confusion, visual difficulties and dizziness, observed after high or abuse doses of ASP consumption, may be related to the partial or remarkable decrease of the AChE activity.

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