The Effect of Galactose Metabolic Disorders on Rat Brain Acetylcholinesterase Activity

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To evaluate whether in classical galactosemia galactose (Gal), galactose-1-phosphate (Gal-1-P) and galactitol (Galtol) affect brain acetylcholinesterase (AChE) activity, various concentrations (1–16 mM) of these compounds were preincubated with brain homogenates of suckling rats as well as with pure eel *Electroforus electricus* AChE at 37 °C for 1 h. Initially, Galtol (up to 2.0 mM) increased (25%) AChE activity which decreased, thereafter, reaching the control value in high Galtol concentrations. Gal-1-P decreased gradually the enzyme activity reaching a plateau (38%), when incubated with 8–16 mM. However, when the usually found 2 mM of Galtol and 2 mM of Gal-1-P, concentrations in galactosemia were added in the incubation mixture simultaneously, brain AChE was stimulated (16%). Galtol or Gal-1-P modulated brain AChE as well as enzyme activity of *E. electricus* in the same way. Gal, Glucose (Glu) and glucose-1-phosphate (Glu-1-P) had no effect on AChE activity. It is suggested that Galtol as well as Gal-1-P can affect acetylcholine degradation acting directly on AChE molecule. Consequently the direct action of these substances on the enzyme might explain the brain cholinergic dysfunction in untreated galactosemia patients.

Introduction

Three enzyme deficiencies have been described in association with galactose disorders: galactose-1-phosphate uridyl transferase (EC 2.7.1.12) (classical galactosemia), galactokinase (EC 2.7.1.6) and galactose-4-epimerase (EC 5.1.3.2) (Segal, 1995). The first enzyme catalyses the formation of uridyldiphosphate (UDP)-galactose (Gal) and glucose-1-phosphate (Glu-1-P) from galactose-1-phosphate (Gal-1-P) and UDP-glucose. As a consequence of this block in the sugar nucleotide pathway, Gal-1-P accumulates in the lens, liver, kidney and brain (Waggoner *et al.*, 1990), whereas Gal accumulates in liver, brain and kidney. Galactitol (dulcitol) (Galtol), the sugar alcohol of Gal also accumulates in brain and lens because of the action of aldol reductase on Gal (Wells *et al.*, 1965). The cataracts, so common in this disorder, appear to be the result of intralenticular accumulation of Galtol (Schweitzer, 1995; Segal, 1995). Gal-1-P is believed to play a toxic role resulting in the generation of liver and cerebral dysfunction (Segal, 1995). The infant with deficiency of Gal-1-P uridyl transferase presents a severe clinical picture. The patients develop diarrhea, vomiting, dehydration, hyperbilirubinemia, hepatic dysfunction, prolongation of clotting times and they are at risk in developing severe mental retardation and seizures (Schulpis *et al.*, 1997). Renal aminoaciduria, proteinuria and renal tubular acidosis are also some of the symptoms.

One avenue of support of this view comes from patients with galactokinase deficiency galactosemia in whom no Gal-1-P accumulates, where Galtol buildup cataracts and increases of intracerebral osmolarity (Segal, 1995; Schulpis *et al.*, 1997). Feeding Gal to rats there was demonstrated a diminished responsiveness of synaptic endings to acetylcholine (ACh) (Berry *et al.*, 1981). Furthermore, acetylcholinesterase (AChE, EC 3.1.1.7) at nerve endings represents the molecular target of organophosphorus esters (OP) and carbamate toxicity. When the enzyme is blocked, it can no longer participate in the hydrolysis of ACh. Thus ACh action is enhanced and due to the widespread distribution of cholinergic functions, toxic effects involve the parasympathetic, motor and central nervous system (Sussman *et al.*, 1999).

The objective of this study was the evaluation of AChE activity in the rat brain homogenates in relation to various concentrations of Gal and its derivatives.

Materials and Methods

Animals

For the experiments conducted on homogenised rat brain, 21 day old Albino Wistar rats of both sexes (Saint Savvas Hospital, Athens, Greece) were used. The suckling rats with their mother were housed in a cage at constant room temperature (22± °C) under a 12hL:12hD (light 08.00–

20.00 h) cycle. Animals were cared for in accordance with the principles of the *"Guide to the Care and Use of Experimental Animals"* (Committee on Care and Use of Laboratory Animals, 1985).

Tissue preparation

Rats were sacrificed by decapitation. Whole brains from five rats were rapidly removed, weighed and thoroughly washed with isotonic saline. They 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 homogenizing vessel at 900 rpm (4–5 strokes). Then, the homogenate was centrifuged at 1,000×*g* for 10 min to remove nuclei and debris. In the resulting supernatant the protein content was determined according to Lowry *et al.* (1951) and then AChE activities were measured. The enzyme incubation mixture was kept at 37 °C.

Preincubation of GAL and its derivatives

Various concentrations (1–16 mM) of Gal (Sigma), Gal-1-P (Sigma), and Galtol (Sigma) were preincubated with 100 μg protein of whole brain homogenates or with 0.1 μg protein of eel *Electroforus electricus* pure AChE (Sigma) for 1h and then AChE activities were measured. The preincubation medium (about 1 ml) contained 50 mM Tris-HCl, pH 8.0 and 240 mM sucrose in the presence of 120 mM NaCl. For comparison, glucose (Glu) (Sigma) as well as glucose-1-phosphate (Glu-1-P) (Sigma) were also tested as above.

Determination of AChE activity

AChE activity was determined according to the method of Ellman *et al.* (1961). The reaction mixture (1 ml) contained 50 mM Tris-HCl, pH 8.0 and 240 mM sucrose in the presence of 120 mM NaCl. Protein concentration was 80–100 μg/ml incubation mixture for the homogenised brain and 0.1 μg/ml for the eel *E.electricus* pure AChE. Then, 0.030 ml of 5.5'-dithionitrobenzoic acid (DTNB) and 0.050 ml of acetylthiocholine iodide, used as a substrate, were added and the reaction was started. The final concentrations of DTNB and substrate were 0.125 and 0.5 mM, respectively. The

reaction was followed spectrophotometrically by absorbance ($\Delta\overline{OD}$) at 412 nm.

Statistical analysis

The data were analyzed by using two-tailed Student's *t*-test. A p value of < 0.05 was considered statistically significant.

Results and Discussion

The effect of various concentrations (1–16 mM) Galtol as well as Gal-1-P on brain AChE activity are presented in Figure 1. AChE activity was markedly increased (about 25%, p<0.001), when the brain homogenate was incubated with the usually found concentrations (up to 2 mM) of the Galtol in the untreated galactosemic patients. Thereafter, the enzyme activity was decreased reaching the control value, when incubated with high concentrations (4–16 mM). Galtol modulated *E.electricus* pure AChE activity (non-membrane bound enzyme) in the same way.

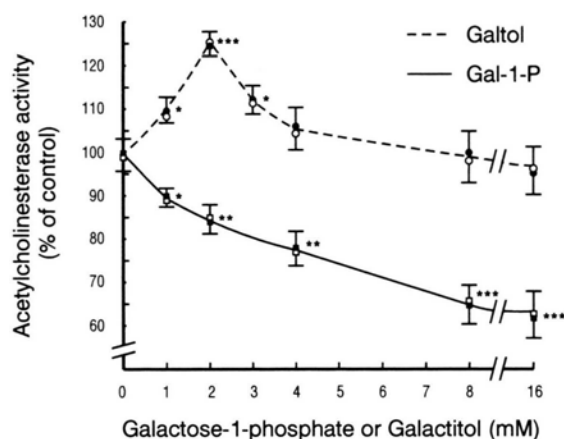Furthermore, incubation of brain homogenate with 1 mM of Gal-1-P resulted in 10% (p<0.05) in-

Fig. 1. Effects of different concentrations of Galactitol (Galtol – – –) or galactose-1-phosphate (Gal-1-P——) on AChE activity. Galtol was preincubated for 1h with rat brain homogenates (●) or *E.electricus* pure AChE (o). Gal-1-P was preincubated for 1h with rat brain homogenates (■) or *E.electricus* pure AChE (□). Control value of brain AChE activity was 0.960±0.040 $\Delta\overline{OD}$/min × mg protein and that of the pure enzyme 1.23±0.04 $\Delta\overline{OD}$/min × μg protein. Values represent means ±SD of five experiments. The average values of each experiment arise from three independent determinations.
*: p<0.05; **: p<0.01; ***: p<0.001; as compared to control.

hibition of AChE. Concentrations of the ester (2 mM or 4 mM) usually found in patients with classical galactosemia (Segal, 1995) resulted in reduced enzyme activity 15% ($p < 0.01$) and 22% ($p < 0.01$) respectively, reaching a plateau of inhibition 38% ($p < 0.001$) when 8–16 mM of Gal-1-P was added in the incubation mixture. Additionally, pure *E.electricus* AChE was inhibited in the same way when incubated with the same concentrations.

Interestingly, when 2 mM of Galtol and Gal-1-P were added in the incubation medium simultaneously, brain AChE activity increased 16% ($p < 0.01$). However, high concentrations (16 mM) of the substances in the incubation mixture resulted in a 35% ($p < 0.001$) inhibition of the enzyme. Neither Gal, Glu nor Glu-1-P all tested over the range of 1–16 mM, had any statistically significant effect on brain AChE activity ($p > 0.05$).

Gal-1-P inhibitor effect (figure) seems to represent a different type of interaction than a simple phosphorylation of AChE, since Glu-1-P did not cause any measurable action. Thus, the observed statistically significant influence of AChE by Gal-1-P vs Glu-1-P could be the result of a different configuration induced by Gal and Glu. Gal as an epimer of Glu at C-4 differs in configuration only at one asymmetric center (Segal, 1995). In contrast, Galtol stimulated brain AChE as well as *E.electricus* pure AChE. This Gal derivative might produce an enzyme stimulation by changing the configurating the AChE molecule. Galtol, as a polyol might loose its protons, acquiring negative charges. AChE stimulation might also be due to the interaction of these negative charges with NH_4^+ terminals of AChE. Interestingly, as it was found, the simultaneous addition of each substance (Galtol, Gal-1-P) in the brain homogenate caused an AChE stimulation which can affect ACh degradation in patients with untreated galactosemia. Since the two molecules are derivatives of the same biochemical pathway, their simultaneous existence in the synaptic microenvironment is likely. The fact that these two molecules exert opposite actions on the activity of AChE may indicate that this pair of molecules has a regulatory effect on the activity of the enzyme when present at normal concentrations and at the correct molar ratio. If these conditions are not met, these molecules alter the enzyme activity, either positively or negatively, possibly depending on the molecular ratios. Therefore, in the case of the simultaneous brain accumulation of these compounds, as it is presented in the untreated patients with classical galactosemia, cholinergic dysfunction of their central nervous system, especially in infants with developing brain, might be observed (Schulpis *et al.*, 1997).

Galtol as well as Gal-1-P modulated brain AChE activity in the same way as pure *E.electricus* AChE (Fig. 1). AChE is an ectoenzyme of brain synaptic membranes. Consequently, these compounds do not influence the enzyme indirectly but they act on AChE molecule directly. This direct action of Galtol and Gal-1-P on AChE molecule might explain the mechanism of impairment of cholinergic functions of the brain of patients with galactose metabolic disorders.

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