In vitro Influence of Phenylalanine on Acetylcholinesterase Activity and DNA

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Acetylcholinesterase (AChE) is a significant component of the membrane contributing to the permeability changes during synaptic transmission and conduction. Phenylketonuria is a group of metabolic disorders in which phenylalanine (Phe) is highly elevated in blood (up to 0.1M) resulting in mental retardation etc. AChE activity was measured spectrophotometrically after incubation with various Phe concentrations. Phe interaction with DNA was evaluated with an established HPLC method. Phe was found to inhibit AChE almost 40%, at a concentration of 5 mM, whereas a 62.5% DNA peak exclusion (molecular interaction) was observed when Phe was incubated with DNA at a concentration of 3 mM. In addition the ratio of DNA: Phe determined the potency of the observed molecular effect.

Introduction

Acetylcholinesterase (AChE) is a biological significant component of the membrane, contributing to its integrity and to the permeability changes occurring during synaptic transmission and conduction. It is a membrane-bound enzyme with its active side exposed at the external leaflet of the bilayer (ectoenzyme). The effect of small amphipathic compounds (eg. cholesterol, steroid hormones, negatively charged phospholipids etc.) on the activity of some plasma membrane-bound enzymes was suggested that alter the membrane fluidity, causing functional changes in the allosteric properties of integral enzymes (Lotti, 1995). When the enzyme is blocked, it can no longer participate in the hydrolysis of acetylcholine (ACh). Thus, ACh action is enhanced and due to the widespread distribution of cholinergic functions, toxic effects involve the parasympathetic, sympathetic, motor and central nervous systems (Deliconstantinos and Tsakiris, 1985; Sussman *et al.,* 1991). On the other hand, phenylketonuria (McKusick 261600) is a group of metabolic disorders in which the amino acid phenylalanine (Phe) is highly elevated in blood (greater than 20μg/μl, 0.1 M) (Hsia *et al.,* 1968) resulting in mental retardation, which is the main clinical manifestation in the untreated patients (Chambers and Levi 1992; Bickel 1985). Additionally, Burri et al showed that experimental hyperphenylalaninemia in 3–17 d-old rats leads to reduce myelinogenesis and unexplained decreased DNA levels at postnatal day 14. Moreover, nerve conduction studies showed the presence of a minor sensory neuropathy which in rare cases may also affect peripheral motor nerves. This neuropathy did not have features of a central-peripheral distal axonopathy which argues against a toxic-nutritional causation (Ludolph *et al.,* 1996).

Therefore, it was of interest to examine, *in vitro,* the effect of Phe on the AChE enzymatic activity in relation to the observed direct molecular interaction of the amino acid with DNA.

Materials and Methods

Human cholinesterase acetyl Type XIII in a lyophylized powder form, (0.25–1U/mg) was purchased from Sigma, L-phenylalanine (Serva) and Physostigmine (Famar) were tested at final concentrations 1, 2, 5, 10, 20 mM in triplicate. AChE activity was measured on the basis of the yellow 5-thio-2-nitrobenzoate on reaction to 5 mM butyrylthiocholine (BTC) with 0.25 mM, 5.5'-dithiobis-2 nitrobenzoic acid (DTNB) (Ellman *et al.,* 1961). Incubation time 30 min at final assay volume 1 ml. Maximum absorbance at 405 nm directly propotional to AChE activity was measured with UV-Vis spectrophotometer (Hitachi 2000).

AChE activity was expressed in μmol/mg protein/min. Complete inhibitory effect (100%) was performed by physostigmine at concentration 7 mM.

Calf thymus DNA type XV prepared by a modification of Aposhian and Kornberg (1962) method was purchased from Sigma Chemical Co. and doxorubicin (Doxo) from Pharmacia. HPLC grade

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solvents were obtained from Lab. Scan. A Hewlett-Packard HPLC series 1050 and a Lichrospher RP-18 column (250x4 mm, 5μm) were used.

DNA solutions were prepared in water (0.10 g/l) and kept at 4 °C. Doxo (0.5 g/l) was used as a typical intercalating agent with major binding (100%) capability. Phe-Doxo was tested at six final concentrations ranged 0.1–3 mM vs DNA at 0.05 g/l concentration.

Column was equilibrated with a H_2O:CH_3OH (80:20) mixture. Test samples and DNA solutions were introduced in a ratio (1:1, v/v) with zero time incubation into the sample loop (20μl). All solvents and solutions were filtered through 0.22 μm Millipore membrane filters.

All samples were tested in triplicate. DNA binding is expressed as a percentage of DNA peak exclusion in the chromatogram.

Obtained results were analysed by Student's t-test. p-values < 0.05 were considered statistically significant.

Results and Discussion

As shown in Table I, AChE activities were significantly decreased when Phe incubated with concentrations higher than 5 mM reaching an inhibitory effect up to 65% at concentration 20 mM. The effect of Phe-Doxo on DNA is presented in Table II. A 62.5% DNA peak exclusion was observed at concentration 3 mM of the amino acid vs DNA at concentration 0.05 g/l. Chromatograms of DNA and DNA plus Phe and Doxo at concentrations 1.5 mM and 0.2 mM respectively indicating the DNA interaction are shown in Figure 1.

The three-dimensional atomic structure of AChE has only recently been determined and probably our knowledge on molecular interactions between AChE and inhibitors will be revised. These findings reconsider the anionic subsite of AChE attraction to the quarternary nitrogen of ACh in relation to the major role of aromatic residues that line the walls and floor of the active-site gorge (Sussman *et al.*, 1991). Table I demonstrates the changes of AChE activity at different Phe concentrations. These results obtained from the *in vitro* effect of Phe on the enzyme at concentrations 5–20 mM of the amino acid. These values of Phe

Table I. Effect of Phe concentrations on the AChE activity.

		AChE activity (μmol/mg protein/min)	P
Control		3.10±0.14[a] (100%)[b]	
Phe(mM)	1	3.25±0.26 (+ 5%)	0.436 (NS)
	2	3.24±0.21 (+ 4%)	0.403 (NS)
	5	1.91±0.23 (−39%)	0.002
	10	1.82±0.13 (−42%)	0.0001
	20	1.05±0.16 (−65%)	0.0001

[a] Values are expressed as mean ± SD.
[b] Numbers in parenthesis is shown% inhibition of the enzyme.
NS, non significant.

Table II. Effect on DNA caused by Doxorubicin (Doxo) and L-phenylalanine (Phe).

Compounds in (g/l) and (mM)	% DNA peak size exclusion
DNA (0.05) ± Doxo (0.1) (0.1)	45.8±5.4[a]
DNA (0.05) ± Doxo (0.25) (0.2)	100
DNA (0.05) ± Doxo (0.5) (0.5)	100
DNA (0.05) ± Phe (0.1) (0.7)	9.3±3.4[a]
DNA (0.05) ± Phe (0.25) (1.5)	31.6±8.5[a]
DNA (0.05) ± Phe (0.5) (3.0)	62.5±9.1[a]

[a] Mean ± SD (n = 3).

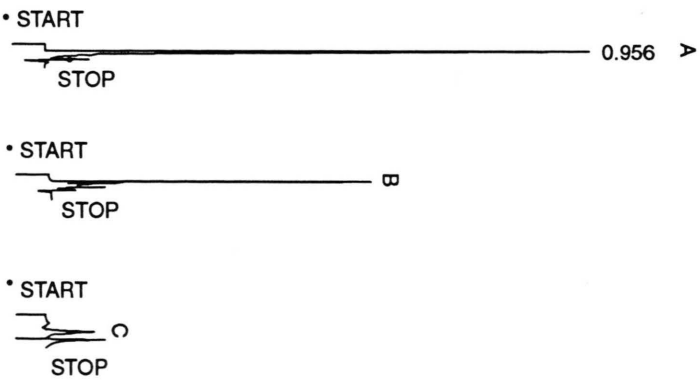

Fig. 1. Chromatograms of DNA (0.05 g/l) (A), DNA (0.05 g/l) + Phe (1.5 mM) (B), DNA (0.05 g/l) + Doxo (0.2 mM) (C). Chromatographic conditions: column octadecylsilane Lichrospher RP-18: eluent, water: methanol 80:20(v/v); Flow rate, 1.0 ml/min; injection volume, 20μl; detector UV set at 254 nm; temperature 25 °C; retention time 0.97 min.

are usually observed among untreated phenylketonuric patients (Hsia *et al.*, 1968). It could be suggested that Phe action is not direct but probably works by changing the membrane lipid bilayer microenvironment, causing functional modulation of the membrane of AChE. Moreover, positively charged drugs can selectively perturb lipid-bilayer membranes (Lotti, 1995) on effect that could cause the observed progressive inhibition of AChE. Activity kinetic studies are under way.

On the other hand, the association of cationic molecules such as polyamines and the antitumor agent Doxo with the negatively charged DNA induces important molecular changes in DNA. These effects have been recently observed chromatographically (Karikas *et al.*, 1997). Hence strong effects on DNA (100% DNA peak exclusion) caused by Doxo is due to the intercalating effect of polyaromatic cations plus hydrogen bonding and strand breakage (Silverman, 1992). Consequently, the observed moderate molecular interaction of Phe with DNA (62.5% peak size exclusion) should involve its ionized amino group (ionic interaction) as well as its aromatic moiety through partial intercalation. According to a recent study (Schulpis *et al.*, 1997) where Phe analogues were tested the potency of the phenomenon attributed to ionic interactions which is increased when the number of amino groups increases, whereas the ratio of DNA: Phe determined the potency of the observed DNA peak exclusion (Table II). Burley *et al.* (1996) having shown that two Phe residues of TATA box binding protein interacted with the T-A base pair by Van der Waals forces could reinforce the hypothesis of the partial intercalation of the amino acid with DNA.

In conclucion, these preliminary *in vitro* experimental observations cannot, in this stage, be easily extrapolated into *in vivo* reality. The expected brain-neurons damage in PKU patients could be demonstrated through future *in vivo* studies of Phe on both AChE and DNA.

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