

# Effects of L-Phenylalanine on Acetylcholinesterase, (Na<sup>+</sup>,K<sup>+</sup>)-ATPase and Mg<sup>2+</sup>-ATPase Activities in Adult Rat Whole Brain and Frontal Cortex

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Acetylcholinesterase, (Na<sup>+</sup>,K<sup>+</sup>)-, Mg<sup>2+</sup>-ATPase, L-Phenylalanine

The effect of different L-phenylalanine (Phe) concentrations (0.12–12.1 mM) on acetylcholinesterase (AChE), (Na<sup>+</sup>,K<sup>+</sup>)-ATPase and Mg<sup>2+</sup>-ATPase activities was investigated in homogenates of adult rat whole brain and frontal cortex at 37 °C. AChE, (Na<sup>+</sup>,K<sup>+</sup>)-ATPase and Mg<sup>2+</sup>-ATPase activities were determined after preincubation with Phe. AChE activity in both tissues showed a decrease up to 18% ( $p < 0.01$ ) with Phe. Whole brain Na<sup>+</sup>,K<sup>+</sup>-ATPase was stimulated by 30–35% ( $p < 0.01$ ) with high Phe concentrations, while frontal cortex Na<sup>+</sup>,K<sup>+</sup>-ATPase was stimulated by 50–55% ( $p < 0.001$ ). Mg<sup>2+</sup>-ATPase activity was increased only in frontal cortex with high Phe concentrations. It is suggested that: a) The inhibitory effect of Phe on brain AChE is not influenced by developmental factors, while the stimulation of Phe on brain Na<sup>+</sup>,K<sup>+</sup>-ATPase is indeed affected; b) The stimulatory effect of Phe on rat whole brain Na<sup>+</sup>,K<sup>+</sup>-ATPase is decreased with age; c) Na<sup>+</sup>,K<sup>+</sup>-ATPase is selectively more stimulated by high Phe concentrations in frontal cortex than in whole brain homogenate; d) High (toxic) Phe concentrations can affect Mg<sup>2+</sup>-ATPase activity in frontal cortex, but not in whole brain, thus modulating the amount of intracellular Mg<sup>2+</sup>.

## Introduction

Phenylketonuria (PKU) is a group of recessively inherited metabolic disorders, in which the conversion of phenylalanine (Phe) to tyrosine is impaired. The metabolic disorder is due to an autosomal recessive gene that codes for a type of phenylalanine hydroxylase that has reduced enzymatic activity, resulting in abnormally high levels of Phe in body fluids (Kupfermann, 1991). An excessive increase of blood Phe results in mental retardation (IQ < 60) (Missiou-Tsagaraki *et al.*, 1988), seizures and other neurophysiological and psychological dysfunctions (Behbenhani and Langenbeck, 1982). High Phe concentrations in plasma (0.3–5.4 mM) may occur in sick humans, and are harmful especially during the first year of life, while this “damage” can be prevented with an appropriate dietary control (Missiou-Tsagaraki *et al.*, 1988).

The rapid and precise communication between neurons, necessary for the performance of nervous system functions, is made possible by two signaling mechanisms: excitability and synaptic transmission.

Neural excitability was found to be influenced by Phe in rat brain (Iarosh *et al.*, 1987) while experimental hyperphenylalaninemia in 3–17 day-old rats leads to reduced myelinogenesis (Burri *et al.*, 1990), which could result in a decrease of the axonal conduction velocity. Reduced myelinogenesis is consistent with the observation which showed that high Phe concentrations in the body fluids inhibit brain protein synthesis by inhibition of the transport of amino acids across the blood-brain barrier (Oldendorf, 1973; Antonas and Coulson, 1975) or by direct interference with the protein synthesis apparatus (Taub and Johnson, 1975; Huges and Johnson, 1977).

Alterations in synaptic transmission are also implicated in brain dysfunctions in PKU and several experimental data suggest that the principal cause for the brain dysfunction is the impairment in the neurotransmitter amine synthesis (Blau, 1979). Increased Phe concentrations, by decreasing the availability of the precursors tryptophan and tyrosine (Aragon *et al.*, 1982) might be the primary cause of serotonin and catecholamine depletion in PKU (Herrero *et al.*, 1983). Since the aromatic amino acids (tryptophan, tyrosine, Phe) are the



biosynthetic precursors for the neurotransmitters serotonin, dopamine and norepinephrine (NA), the aromatic amino acids uptake into the brain can modify their conversion to neurotransmitters and thereby can modify their release from neurons and influence brain functions (Fernstrom, 1994). Moreover high Phe concentrations induce changes of brain electrical function, which may be mediated in part through inhibition of catecholamine synthesis (Krause *et al.*, 1986).

Regarding cholinergic brain systems, experimental results showed their possible involvement during Phe action. The 6R-L-erythro-5, 6, 7, 8-tetrahydrobiopterin (6R-BH4), a natural cofactor for Phe hydroxylases, has direct acetylcholine (ACh) releasing action *in vivo* in the rat hippocampus (Ohue *et al.*, 1991), while hyperphenylalaninemia leads to a decrease in density of muscarinic receptors of the hippocampus and other brain areas (Hommes, 1993 and 1994). Since it has been shown that the hippocampus is involved in the acquisition and long-term storage of information, and frontal cortex is involved in associative learning, a decrease in neurotransmitter receptor density may contribute to the basis for mental retardation in PKU.

Comparison of the effects of frontal cortex lesions between rats, monkeys and humans has shown that the behavioral symptoms are remarkably similar (Kolb, 1984). These behavioral symptoms are induced by perturbation of functions such as: motor, response inhibition, temporal ordering, spatial orientation, social or affective behavior, behavioral spontaneity, olfaction, habituation, associative learning, homeostasis, response to sensory stimuli etc.

The aim of this work was to investigate the effect of different Phe concentrations on the activity of three brain enzymes: a) Acetylcholinesterase (AChE, EC 3.1.1.7), the role of which is very important in the ACh cycle, including the release of ACh (Kouniniotou-Krontiri and Tsakiris, 1989), b) Na<sup>+</sup>,K<sup>+</sup>-ATPase (EC 3.6.1.3), an enzyme implicated in neural excitability (Sastri and Phillis, 1977), metabolic energy production (Mata *et al.*, 1980) and Na<sup>+</sup>-dependent tryptophan uptake system (Herrero *et al.*, 1983) and c) Mg<sup>2+</sup>-ATPase, the role of which is to maintain high brain intracellular Mg<sup>2+</sup>, changes of which can control rates of protein synthesis and growth of the cell (Sanui and

Rubin, 1982). The above enzymes were estimated in homogenates of whole brain and frontal cortex of adult rats. Adult rat experiments were necessary in relation to PKU since phenylketonuric patients usually discontinue their therapeutic special diet when they reach adulthood (Schuelt *et al.*, 1980). Diet discontinuation results in the pathological increase of Phe concentration in plasma and consequently in brain. The effects of Phe on adult rat whole brain homogenates are discussed in relation to those in suckling rats which have been previously reported (Tsakiris *et al.*, 1998).

## Materials and Methods

### Animals

Albino adult (4 mo) Wistar rats of both sexes (Saint Savvas Hospital, Athens, Greece) were used in all experiments. Body weight was 225 ± 10 g (mean ± SD). The rats were housed four in a cage, at a constant room temperature (22 ± 1 °C) under a 12hL:12hD (light 08.00–20.00 h) cycle and acclimated 1 week before use. 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" (Committee on Care and Use of Laboratory Animals, 1985).

### Tissue preparation

Animals were sacrificed by decapitation. Whole brains from five rats or frontal cortexes from twenty rats were rapidly removed, weighed and thoroughly washed with isotonic saline. 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 homogenizing vessel at 900 rpm (4–5 strokes). Then, the homogenate was centrifuged at 1000×g for 10 min to remove nuclei and debris. In the resulting supernatant, the protein content was determined according to the method of Lowry *et al.* (1951) and then the enzyme activities were measured. The enzyme incubation mixture was kept at 37 °C.

### Phe preincubation

The enzymatic activity measurements were carried out on homogenised rat whole brain or fron-

tal cortex. The activity was determined after 1 h preincubation of the homogenate with 0.12, 0.24, 0.48, 0.9, 1.8 or 12.1 mM of Phe at 37 °C. AChE activity was also investigated as a function of time of Phe action on the enzyme. These mentioned intracellular concentrations of Phe (0.12–1.80 mM) correspond to 6 times the concentration in plasma (Scriver and Rosenberg, 1973) (0.72–10.8 mM). Phe concentrations of 0.3–5.4 mM are usually found in the plasma of phenylketonuric patients (Missiou-Tsagaraki *et al.*, 1988).

#### 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. Then, 0.030 ml 5,5'-dithionitrobenzoic acid (DTNB) and 0.050 ml 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 the increase in absorbance ( $\Delta OD$ ) at 412 nm.

#### Determination of Na<sup>+</sup>,K<sup>+</sup>-ATPase and Mg<sup>2+</sup>-ATPase activities

Na<sup>+</sup>,K<sup>+</sup>-ATPase activity was calculated as the difference between total ATPase activity (Na<sup>+</sup>,K<sup>+</sup>,Mg<sup>2+</sup>-dependent) and Mg<sup>2+</sup>-dependent ATPase activity. Total ATPase activity was assayed in an incubation medium consisting of 50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 20 mM KCl, 4 mM MgCl<sub>2</sub>, 240 mM sucrose, 1 mM ethylenediaminetetraacetic acid K<sub>2</sub>-salt (K<sup>+</sup>-EDTA), 3 mM disodium ATP, and 80–100 µg protein of the homogenate, in a final volume of 1 ml. Ouabain (1 mM) was added to determine the activity of the Mg<sup>2+</sup>-ATPase. The values of Mg<sup>2+</sup>-dependent ATPase were similar in the presence of ouabain in the reaction mixture as also in ouabain absence and without NaCl and KCl. The reaction was started by adding ATP and was stopped after a 20 min incubation period by the addition of 2 ml of a mixture of 1% lubrol and 1% ammonium molybdate in 0.9 M H<sub>2</sub>SO<sub>4</sub> (Atkinson *et al.*, 1971;

Bowler and Tirri; 1974). The yellow colour which developed was read at 390 nm.

#### Statistical analysis

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

#### Results

The time-course of AChE activity during incubation of the whole brain homogenate with 0 (control), 0.48, 0.9 and 1.8 mM of Phe is shown in Fig. 1. In the absence of Phe the enzyme activity remained at a steady level during the 120 min duration of the experiment. In the presence of Phe, AChE activity declined progressively (decrease 18%). The above decrease (*p*<0.01), attained a steady level after 30 min of Phe action when Phe concentration was 0.48 mM and after 20 min when Phe concentration was 0.9 or 1.8 mM. A similar time-course of AChE activity was observed during incubation of frontal cortex homogenates with 0, 0.48, 0.9 and 1.8 mM of Phe. In the presence of Phe, there was also a 18% decrease of the enzyme activity, which reached a steady level after 30 min of Phe action when Phe concentration was 0.48 mM and after 20 min when Phe concentration was 0.9 or 1.8 mM.

Moreover, the effects of different concentrations of Phe on AChE and Na<sup>+</sup>,K<sup>+</sup>-ATPase activi-

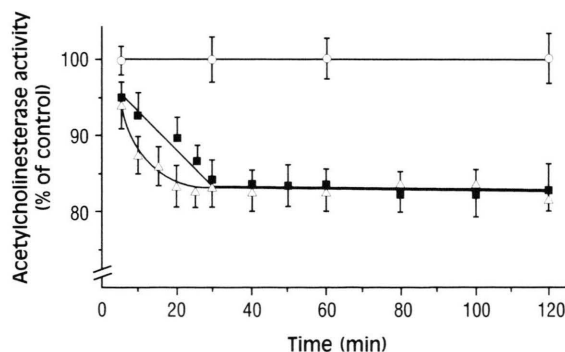


Fig. 1. Time-dependent effect of Phe on AChE activity determined in adult rat homogenised whole brain. Points and vertical bars represent mean values  $\pm$  SD for Phe concentrations 0 mM (○), 0.48 mM (■) as well as 0.9 and 1.8 mM (△). Values represent means  $\pm$  SD of four experiments. The average value of each experiment arises from three determinations. Control value of AChE activity was  $0.800 \pm 0.032 \Delta OD/\text{min} \times \text{mg protein}$ .

ties were investigated in whole brain homogenates. The results of this study, illustrated in Fig. 2, showed that 1 hour of Phe action on AChE resulted in a statistically significant decline of AChE activity by about 10% ( $p<0.05$ ) for a Phe concentration of 0.1 mM, 13% ( $p<0.01$ ) for 0.24 mM of Phe and 18% ( $p<0.01$ ) for Phe concentrations ranging from 0.48 to 12.1 mM. As shown in this Figure, one hour of Phe action induced a 20% ( $p<0.05$ ) stimulation of Na<sup>+</sup>,K<sup>+</sup>-ATPase for a Phe concentration of 0.24 mM, a 28% ( $p<0.01$ ) stimulation for 0.48 mM of Phe and a 30–35% ( $p<0.01$ ) stimulation for Phe concentrations ranging from 0.9 to 12.1 mM.

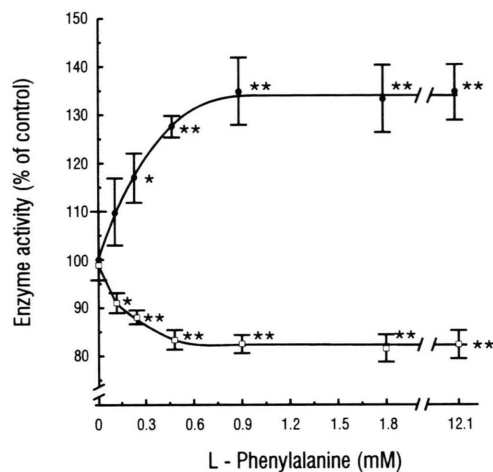


Fig. 2. Effect of different Phe concentrations on AChE (□) and Na<sup>+</sup>,K<sup>+</sup>-ATPase (●) activities determined in homogenised whole brain. The activity control values were  $0.800 \pm 0.032 \Delta OD/min \times mg$  protein for AChE and  $2.11 \pm 0.22 \mu mol Pi/h \times mg$  protein for Na<sup>+</sup>,K<sup>+</sup>-ATPase. Values represent means  $\pm$  SD of four experiments. The average value of each experiment arises from three determinations. In all cases a 1h preincubation of the homogenate with different Phe concentrations preceded the substrate addition. \* $p<0.05$ ; \*\* $p<0.01$ ; compared to control.

Mg<sup>2+</sup>-ATPase activity was found to be  $8.21 \pm 0.38 \mu mol Pi/h \times mg$  protein in homogenised whole brain of adult rats, while Phe, in the concentrations used in this study, appeared unable to affect the enzyme activity ( $p>0.05$ ).

The effects of different Phe concentrations on AChE and Na<sup>+</sup>,K<sup>+</sup>-ATPase activities in homogenates of frontal cortex are illustrated in Fig. 3. One hour of Phe action induced a 13% ( $p<0.05$ ) inhibition of AChE for a Phe concentration of 0.24 mM and a 18% ( $p<0.01$ ) inhibition for Phe concentra-

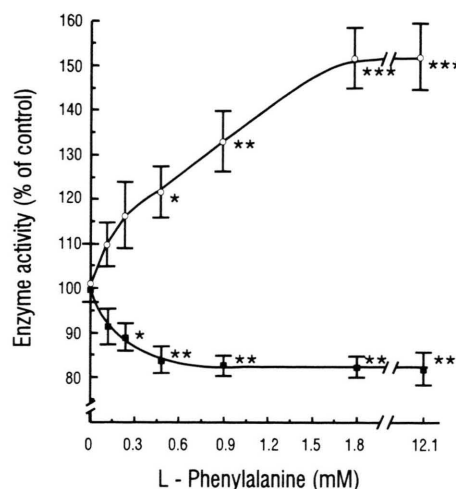


Fig. 3. Effect of different Phe concentrations on AChE (■) and Na<sup>+</sup>,K<sup>+</sup>-ATPase (○) activities determined in homogenised frontal cortex. The activity control values were  $0.196 \pm 0.006 \Delta OD/min \times mg$  protein for AChE and  $6.95 \pm 0.69 \mu mol Pi/h \times mg$  protein for Na<sup>+</sup>,K<sup>+</sup>-ATPase. Values represent means  $\pm$  SD of four experiments. The average value of each experiment arises from three determinations. In all cases a 1h preincubation of the homogenate with different Phe concentrations preceded the substrate addition. \* $p<0.05$ ; \*\* $p<0.01$ ; \*\*\* $p<0.001$ ; compared to control.

tions ranging from 0.48 to 12.1 mM. As shown in this figure, one hour of Phe preincubation resulted in a stimulation of Na<sup>+</sup>,K<sup>+</sup>-ATPase by about 20% ( $p<0.05$ ) for a Phe concentration of 0.48 mM, 35% ( $p<0.01$ ) for 0.9 mM of Phe and 50–55% ( $p<0.001$ ) for Phe concentrations ranging from 1.8 to 12.1 mM.

Mg<sup>2+</sup>-ATPase activity was found to be  $6.34 \pm 0.67 \mu mol Pi/h \times mg$  protein in homogenised frontal cortex. It was increased by 18% ( $p<0.05$ ) and 36% ( $p<0.01$ ) by Phe concentrations of 0.9 mM and 1.8 or 12.1 mM, respectively. Lower Phe concentrations were not able to affect this enzyme activity ( $p>0.05$ ).

## Discussion

In our previous work (Tsakiris *et al.*, 1998), where AChE activity was investigated vs Phe concentrations incubated in whole brain homogenates of suckling rats and in pure eel *E. electricus* AChE, the enzyme activity declined in the presence of Phe in a similar way and reached a 18% decrease, as it was found in whole brain homogenates of



adult rats (Fig. 2). Therefore, the direct inhibitory effect of Phe on brain AChE is not influenced by developmental factors.

It has been reported that whole brain homogenate Na<sup>+</sup>,K<sup>+</sup>-ATPase activity in suckling rats was increased 60–65% with 0.9–12.1 mM of Phe (Tsakiris *et al.*, 1998), while in adult rats it was increased by only 30–35% with similar concentrations of Phe (Fig. 2). This Na<sup>+</sup>,K<sup>+</sup>-ATPase stimulatory effect induced by Phe demonstrates an indirect effect of Phe on the enzyme (Tsakiris *et al.*, 1998), possibly relating to the Phe transformation to noradrenaline (NA) (Doulgeraki *et al.*, 1999), which binds to NA receptors affecting thereafter the function of Na<sup>+</sup>,K<sup>+</sup>-ATPase (Swann, 1983 and 1984). Therefore, NA may be formed from Phe in reduced amounts, because the enzymes involved in this transformation mechanism may have a decreased activity dependent on developmental factors. Some authors have suggested that the density of alpha- and beta-adrenergic receptors is decreased during aging in some brain areas (Greenberg, 1986; Slesinger *et al.*, 1988; Pascual *et al.*, 1991). Therefore, NA formed from Phe may bind to a lower number of receptors in adult brain, while it may bind to many more receptors in suckling brain.

Na<sup>+</sup>,K<sup>+</sup>-ATPase was differentially stimulated by high Phe concentrations in whole brain and frontal cortex (Figs 2 and 3). This may be due to the higher concentration of adrenergic receptors found in frontal cortex, to which NA is bound affecting Na<sup>+</sup>,K<sup>+</sup>-ATPase activity to a higher degree (50–55%). The fact that Na<sup>+</sup>,K<sup>+</sup>-ATPase is differentially stimulated by high Phe concentrations in whole brain and frontal cortex, suggests that Phe levels in whole brain or in a distinct brain area may induce different changes in the neural excit-

ability (Sasthy and Phillis, 1977) and metabolic energy production (Mata *et al.*, 1980).

In this study, it is reported that high (toxic) Phe concentrations can affect Mg<sup>2+</sup>-ATPase activity in frontal cortex but not in whole brain. It seems that the intracellular Mg<sup>2+</sup> in frontal cortex is affected by high Phe levels, a fact which may influence the protein synthesis of the brain area and growth of the cells (Sanui and Rubin, 1982).

Since it has been shown that hippocampus is involved in long-term storage of information and frontal cortex is involved in associative learning, damage to these structures by hyperphenylalaninemia may provide a clue to the global mental retardation observed in untreated PKU (Hommes, 1993 and 1994). Furthermore, high Phe concentrations in these structures were found to cause a loss of muscarinic receptors (Matsuo and Hommes, 1988; Hommes, 1993 and 1994), while 6R-BH<sub>4</sub> has a direct ACh releasing action *in vivo* (Ohue *et al.*, 1991). An AChE activity decrease, induced by Phe, such as the decrease observed in this study, in addition with an increased ACh release, result in enhancement of synaptic ACh and could lead to a decrease of muscarinic receptors density, while the AChE activity decrease has been found to be related to a decline of spontaneous quantal ACh release in diaphragm (Kouniniotou-Krontiri and Tsakiris, 1989). The decline of AChE activity induced by Phe, which was found in this study, could not be excluded from being involved in the mental retardation observed in PKU.

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