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Effects of phenylalanine and phenylpyruvate on ATP-ADP hydrolysis by rat blood serum

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Summary. The nucleotide (ATP-ADP)/nucleoside (adenosine) ratio in the circulation can modulate the processes of vasoconstriction, vasodilatation and platelet aggregation. The main objective of the present study with rat blood serum was to evaluate the possibility of changes in nucleotide hydrolysis by phenylalanine (Phe) and phenylpyruvate (PP), the levels of which could increase in the circulation of individuals with phenylketonuria. Results demonstrated that Phe in the range 1.0–5.0 mM inhibited the ADP hydrolysis by rat serum. The effect of inhibition by Phe on ATP hydrolysis appeared only at a concentration of 5.0 mM. PP had no significant effect upon nucleotide hydrolysis. Kinetic analysis indicated that the inhibition of ADP and ATP hydrolysis by Phe in rat blood serum is uncompetitive. Conversely, Phe and PP did not affect the hydrolysis of p-nitrophenyl-5′-TMP by rat serum.

Keywords: ATP Diphosphohydrolase – Apyrase – Phenylalanine – Phenylpyruvate – Phenylketonuria – Circulatory problems

Introduction

Phenylketonuria (PKU) is the most frequent inborn error of amino acid metabolism. The primary biochemical defect, an inability to convert phenylalanine to tyrosine, is due to a deficiency of hepatic phenylalanine hydroxylase (PAH) (Scriver et al., 1989). The deficiency of phenylalanine hydroxylase leads to increased serum and brain levels of phenylalanine (Phe) and its deaminated metabolites. Neurological disfunction is commonly found in human PKU (Tourian and Sibury, 1983) and has been mainly attributed to increased circulating Phe levels, but the mechanisms by which this occurs are poorly understood (Hommes, 1991). It is important to evaluate the idea that phenylalanine metabolites can contribute to the pathophysiology of PKU in humans (Kaufman, 1989). Of the phenylalanine metabolites, phenylpyruvate (PP) is exceptional because, in the milimolar range in vitro, it can inhibit a number of enzyme activities (Kaufman, 1977).

ATP diphosphohydrolase (apyrase, EC 3.6.1.5) is a general designation for enzymes that hydrolyze ATP, ADP and other triphospho- and diphosphonucleosides to their equivalent monophosphonucleosides and inorganic phosphate (Meyerhof, 1945). This enzyme is a widespread enzyme and has been demonstrated in: a) plant tissue; b) insects; c) parasites; d) synaptosomes from peripheral nervous system and e) in a number of mammalian sources (Battastini et al., 1991; Battastini et al., 1998; Frassetto et al., 1993; Handa et al., 1996; Ketlun et al., 1992; Oliveira et al., 1997; Pilla et al., 1996; Ribeiro et al., 1990; Sarkis et al., 1986). The ecto-nucleotidase activity of ATP diphosphohydrolase (CD39) has been proposed to regulate a variety of physiological conditions including cardiac function, hormone secretion, immune responses, neurotransmission and platelet aggregation, by modulating circulating levels of nucleotides in the blood (Chadwick and Frischauf, 1998; Todorov et al., 1997; Marcus et al., 1997; Gayle et al., 1998). The 5′-nucleotide phosphodiesterase (PDEase, NPPase, PC-1, EC 3.1.4.1) is a microsomal enzyme that releases mononucleoside-5′-monophosphate from the 3′-OH terminal of the nucleotides. Then both enzymes can

hydrolyse ATP and ADP and in association with a 5′-nucleotidase can modulate the levels of nucleotide/nucleoside in the bloodstream. Serum PDEase was characterized in fetal serum and is known to increase in hepatoma and it is used as a marker for liver disease (Sakura et al., 1998).

The importance of adenine nucleotides ATP, ADP and AMP, and their nucleoside derivative, adenosine, as coronary artery vasodilators is well established. Previous studies have shown that at equimolar doses ATP and ADP are considerably more potent as vasodilators than either AMP or adenosine (Oliveira et al., 1997). Adenosine diphosphate (ADP) is a nucleotide known to induce changes in platelet shape and aggregation, to promote the exposure of fibrinogen binding sites and to inhibit stimulated adenylate cyclase (Colman, 1990), while adenosine triphosphate (ATP) competitively inhibits ADP-induced platelet aggregation (Coade and Person, 1989).

It may be postulated that the enzymes ATP diphosphohydrolase, phosphodiesterase and 5′-nucleotidase present at minimum in the blood wall, platelets and lymphocytes are involved in modulating the ATP-ADP/adenosine ratio in the circulation and thus can modulate the processes of vasoconstriction, platelet aggregation and vasodilatation.

In a previous work carried out in our laboratory we demonstrated that ATP diphosphohydrolase activity is reduced in synaptosomes in rat cerebral cortex by 2.0 mM Phe and some of its metabolites (Wyse et al., 1994).

The main objective of the present study was to evaluate the effects of phenylalanine (Phe) and phenylpyruvate (PP), metabolites that can accumulate in the circulation in inborn metabolic errors, on serum nucleotide hydrolysis.

Materials and methods

Chemicals

Nucleotides, phenylalanine and phenylpyruvate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of analytical grade.

Isolation of blood serum fraction

Blood was drawn after decapitation of male Wistar rats (approximately 60 days old). Blood samples were centrifuged in plastic tubes for 5 minutes at 5,000 g, 20°C, and the obtained serum was kept on ice (Yegutkin, 1997). Serum was used immediately in experiments.

Measurement of p-nitrophenyl-5′-timidine monophosphate

p-Nitrophenyl-5′-timidine monophosphate (p-Nph-5′-TMP) hydrolysis was determined essentially as described by Sakura et al., (1996). The reaction mixture containing 0.5 mM p-Nph-5′-TMP as a substrate in 100 mM TRIS-HCL, pH 8.9, was incubated with 0.5 mg to 2.0 mg protein serum at 37°C for 8 minutes in a final volume of 0.2 mL. The reaction was stopped by the addition of 0.2 mL 0.2 N NaOH. Incubation times and protein concentrations were chosen to ensure the linearity of the reaction (results not shown). The amount of p-nitrophenol was measured at 400 nm. Controls to correct for non-enzymatic hydrolysis were performed by adding serum after the reaction was stopped with NaOH. All samples were assayed in duplicate. Enzyme activities were generally expressed as nanomoles (nmol) of p-nitrophenol released per minute per milligram of protein.

Measurement of ATP and ADP hydrolysis

ATP and ADP hydrolysis was determined using a modification of the method described by Yegutkin (1997). The reaction mixture containing 3.0 mM ADP or ATP as substrate, 112.5 mM TRIS-HCl, pH 8.0, was incubated with 0.5 mg to 2.0 mg protein serum at 37°C in a final volume of 0.2 mL. The reaction was stopped by the addition of 0.2 mL 10% TCA. The amount of Pi liberated was measured by the method of Chan et al. (1986). Incubation times and protein concentrations were chosen to ensure the linearity of the reaction (results not shown) and absorbance was measured at 630 nm. Controls to correct for non-enzymatic hydrolysis were performed by adding serum after the reaction was stopped with TCA. All samples were assayed in duplicate. Enzyme activities were generally expressed as nanomoles of Pi released per minute per milligram of protein. The inhibitors, phenylalanine and phenylpyruvate were added from aqueous solution.

Protein determination

Protein was measured by the Coomassie Blue method using bovine serum albumin as standard (Bradford, 1976).

Characterization of enzyme inhibition

The inhibition type was characterized by the Lineweaver-Burk plot.

Statistical analysis

Data were analyzed by one-way ANOVA, followed by the Duncan multiple range test. $P < 0.05$ was considered to represent a significant difference in statistical analysis used. All analyses were performed with an IBM compatible computer using the SPSSPC software.

Results

In the blood serum of adults rats, the ADP hydrolysis was 78%, at a concentration of 1.0 mM Phe, in relation to control enzyme activity (no Phe added). This inhibition of 22% was statistically significant ($P < 0.05$) for ADP hydrolysis. The inhibition was concentration dependent, with the enzyme activity being markedly reduced in the presence of 2.5 mM and 5.0 mM Phe (41% and 64% inhibition, respectively,

Fig. 1. Effect of phenylalanine upon ADP hydrolysis by rat blood serum. Bars represent mean ± SD for 4 independent experiments. The values for ADP hydrolysis are in nmoles of Pi/min/mg of protein. *p < 0.05; **p < 0.01
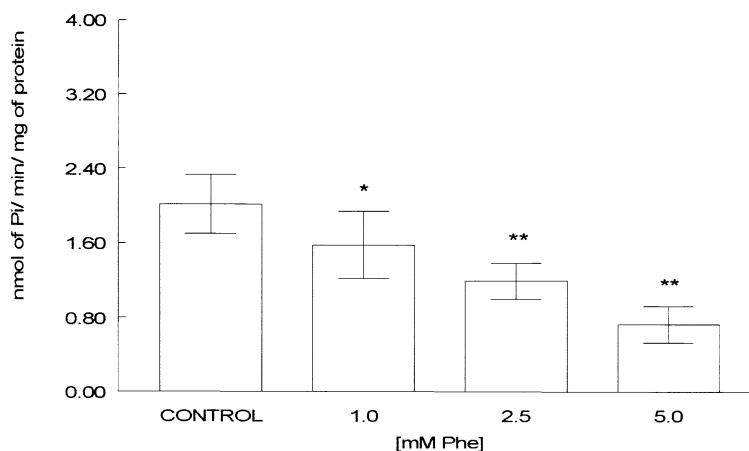
Fig. 2. Effect of phenylalanine upon ATP hydrolysis by rat blood serum. Bars represent mean ± SD for 3 independent experiments. The values for ATP hydrolysis are in nmoles of Pi/min/mg of protein. **p < 0.01
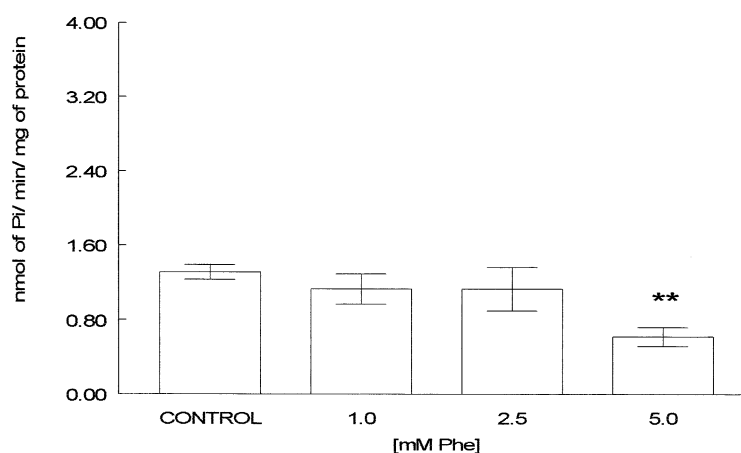
in relation to control activity for ADP hydrolysis) (P < 0.01) (Fig. 1).

Phe at a concentration of 1.0 mM and 2.5 mM did not significantly change the ATP hydrolysis by rat blood serum. The hydrolysis of this nucleotide was significantly inhibited only at 5.0 mM Phe (53%). This inhibition was statistically significant (P < 0.01) (Fig. 2).

The kinetic of the interaction of Phe with the enzyme activity able to hydrolyse ADP and ATP in adult rat blood serum was determined. The Lineweaver-Burk double-reciprocal plot was analyzed over a range of ADP (Fig. 3A) or ATP (Fig. 3B) concentrations (0.2–0.4 mM) in the absence and presence of Phe (4.0 mM and 6.0 mM). The data indicated that the type of inhibition is uncompetitive by Phe and the same for ADP and ATP hydrolysis in rat blood serum. It is important to note that the same pattern of inhibition for both nucleotides suggest the presence of only one active site for hydrolysis of ATP and ADP.

In the present study, we also determined the effect of PP on ADP-ATP hydrolysis by rat blood serum, considering that PP is a metabolic product of Phe. We evaluated the effect of PP on nucleotide hydrolysis by rat blood serum in the range of 1.0–5.0 mM. PP had no significantly effect on the nucleotide hydrolysis activity at the concentrations studied when compared to the control enzyme activity (no PP added, results not shown). The effect of PP or Phe addition was the same for both nucleotides.

Since there are at least two enzymes in rat serum able to hydrolyse ATP and ADP, called ATP diphosphohydrolase and phosphodiesterase, we evaluated the effect of Phe and PP on the hydrolysis of p-Nph-5′-TMP, which is a substrate marker for the latter enzyme. We observed that Phe did not change the phosphodiesterase activity when tested at 5.0 mM (11.83 ± 0.96 and 13.55 ± 2.05 nanomoles of p-nitrophenol liberated/min/mg of protein, mean ± SD for six experiments, respectively for control and test). This result indicates that the phosphodiesterase from
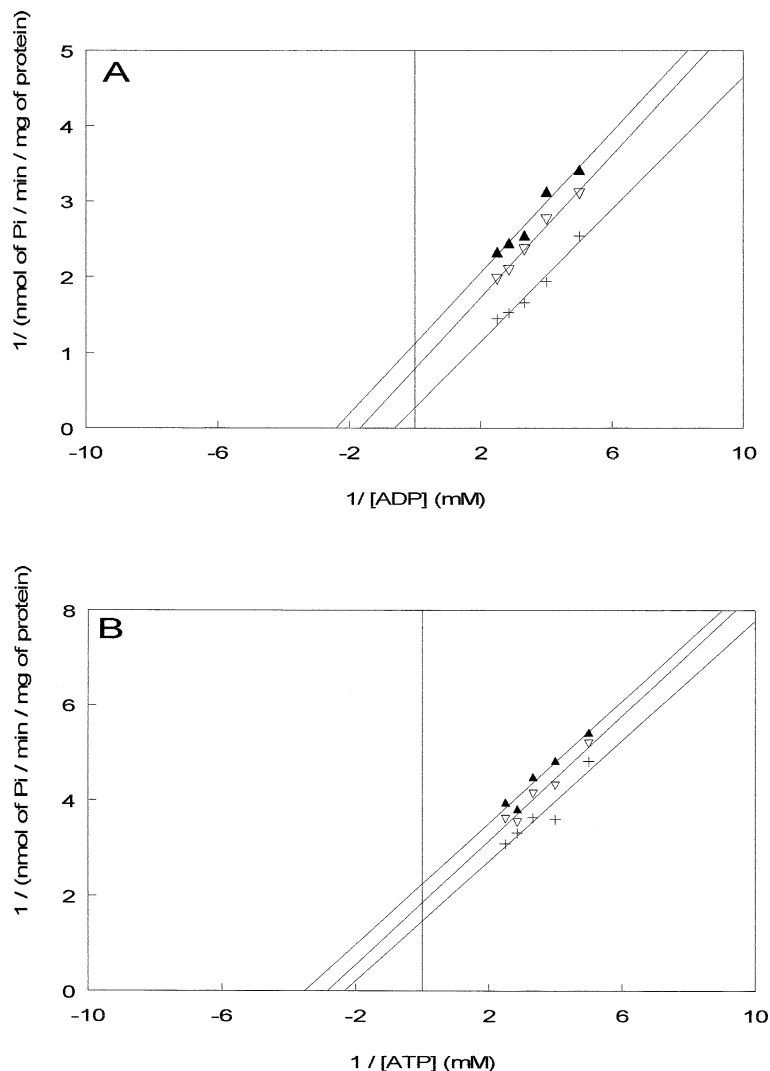
Fig. 3. Kinetic analysis of the inhibition of ATP diphosphohydrolase by Phe in rat blood serum. The graphs show double-reciprocal plots of ATP diphosphohydrolase for ADP (**A**) and ATP (**B**) concentrations (0.2–0.4 mM) in the absence of Phe (+) and in the presence of 4.0 mM (∇), 6.0 mM (▲) Phe. All experiments were repeated at least three times and similar results were obtained. Data presented are from individual experiments

rat blood serum is not affected by the metabolite Phe. The significance of the difference in behavior for ATP-ADP and p-Nph-5′-TMP hydrolysis will be discussed later on.

Discussion

Circulating nucleotides are known to be important signaling molecules, potentiating a variety of physiological responses. It is well established that ATP and ADP can promote contraction of smooth muscle cells (Kennedy et al., 1985) and platelet activation and it is worth noting that platelet activation is the first event in the thrombotic process (Vermeylen, 1986). Many authors have provided evidence that platelet aggregation and thrombus formation are controlled at least in part by ADP, since its removal inhibits thrombus formation.

The ratio of nucleotides (ATP, ADP and AMP)/ nucleoside (adenosine) in the circulation is controlled by ecto-nucleotidases of the ATP diphosphohydrolase family. This enzyme is present on the endothelial cell surface of bovine aorta and in smooth muscle cells (Lieberman et al., 1977; Yagi et al., 1991) and may represent a critical mechanism of control of vascular homeostasis, contributing to inhibit the thrombogenic event. A phosphodiesterase (PDEase) activity was described in serum fraction obtained from human umbilical cord blood by Sakura et al. (1998) and this enzyme could be involved, amongst others, in ATP-ADP hydrolysis. The other enzyme in blood serum that could be involved in ATP-ADP hydrolysis is an

ATP diphosphohydrolase (apyrase). Some evidence exists (results not shown) to demonstrate the possible co-existence of these two enzymes in rat blood serum. It is exciting to speculate that the nucleotide hydrolysis observed under our assay conditions occurs by the action of an ATP diphosphohydrolase (apyrase), since our investigation demonstrates an inhibition of ATP-ADP hydrolysis by Phe and we observed that this metabolite does not inhibit the hydrolysis of p-Nph-5′-TMP, a substrate marker for the phosphodiesterase (PDEase) activity. Although the enzyme involved in nucleotide hydrolysis is as yet unknown, we present results demonstrating that Phe is able to inhibit this activity in rat blood serum. Respect of the different concentrations to inhibit ATP and ADP hydrolysis this fact could be explained because both substrates are substrates for many enzymes in the rat serum.

The present study showed that Phe significantly inhibited *in vitro* ATP and ADP hydrolysis activity of rat blood serum. On the other hand, PP did not alter significantly the ATP and ADP hydrolysis by the same fraction. We have tested other amino-acids (methionine and alanine) on ATP and ADP hydrolysis but both did not change the nucleotide hydrolysis (results not shown). The enzyme that are involved in nucleotide hydrolysis in blood serum obtained from adult rats certainly is not analkaline phosphatase taken in account that 1.0mM of leva-misole did not change the ATP/ADP hydrolysis (data not shown).

The kinetic analysis of the effects of Phe on ATP-ADP hydrolysis indicated an uncompetitive inhi-bition. It is important to note that the *in vitro* actions of Phe occur at concentrations of Phe equivalent to the levels of this metabolite circulating in patients with inborn errors of metabolism (IEM). Further studies are required to verify if the patients with inborn errors of metabolism could present increases in the levels of ATP-ADP nucleotides in the circulation and, in turn, demonstrate related deleterious effects.

The inhibition of ATP-ADP hydrolysis can result in a significant increase in ATP and ADP levels in the vascular system. Thus, if the role of ATP in the vascular system as a vasodilator is well established and the ADP nucleotide is demonstrated to induce changes in platelet shape and aggregation, individuals with untreated phenylketonuria may present vascular-circulatory problems.

The results presented in this study should be considered to be important, since the knowledge of the exact mechanism of a possible deleterious enzyme inhibition is the first approach for the design of drugs able to avoid this inhibition. Further experiments will be necessary to obtain more detailed information about a possible relationship between three variables: the increase in the levels of Phe, the increase in the levels of nucleotides and the appearance of vascular-circulatory disturbs.

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