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Proline induces alterations in nucleotide hydrolysis in rat blood serum

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Abstract

The main objective of the present study was to evaluate the *in vivo* (acute and chronic) and *in vitro* effects of proline on serum nucleotide hydrolysis. For acute administration, 29-day-old rats received one subcutaneous injection of proline (18.2 μmol/g body weight) or an equivalent volume of 0.9% saline solution (control) and were sacrificed 1 h, 3 h or 12 h later. Results showed that acute proline administration provoked a decrease in ATP (42%) and ADP (49%) hydrolysis when rats were sacrificed 1 h after the injection. Furthermore, in rats killed 3 h and 12 h after acute injection, no change in nucleotide hydrolysis were observed. For chronic treatment, buffered proline was injected subcutaneously twice a day at 10 h intervals from the 6th to the 28th day of age. Rats were sacrificed 3 h or 12 h after the last injection. Chronic administration of proline did not alter the nucleotide hydrolysis when the rats were killed 12 h after the last injection, but decreased ATP (15%) and ADP (32%) hydrolysis when rats were sacrificed 3 h after the last injection. The *in vitro* effect of proline (3.0 μM − 1.0 mM) on serum nucleotide hydrolysis was also investigated; results showed that 1.0 mM proline significantly increased ATP (45%), ADP (55%) and AMP (49%) hydrolysis. The data indicate that proline *in vivo* and *in vitro* alters nucleotide hydrolysis, which may be involved in the pathogeny of hyperprolinemic patients. (Mol Cell Biochem **292:** 139–144, 2006)

Key words: hyperprolinemia, proline, NTPDase, nucleotidases, 5′-nucleotidase, experimental model

Introduction

NTPDase (ATP diphosphohydrolase, CD39, EC 3.6.1.5) is a general designation for enzymes that hydrolyze ATP, ADP and other triphospho- and diphospho-nucleosides to their equivalent monophosphonucleosides and inorganic phosphate in the presence of Ca^{2+} or Mg^{2+} [1]. These enzymes are associated, as ecto-enzymes, with the synaptic plasma membrane [2]. The ecto activities of NTPDases have been proposed to regulate a variety of physiological conditions including cardiac function, hormone secretion, immune responses, neurotransmission and platelet aggregation, by modulating the nucleotide levels in the blood [3–6]. The AMP produced is subsequently hydrolyzed to adenosine by the action of an ecto-5′-nucleotidase (EC 3.1.3.5), a key enzyme in this pathway, which is a membrane protein anchored to the cell surface by a phosphatidylinositol glycan [7, 8].

The enzyme, ATP diphosphohydrolase, belongs to the group of nucleotidases and, recently, we described [9] this enzyme in rat serum in a soluble form that can be classified as a NTPDase 1 (an enzyme that hydrolyzes ATP and ADP equally well) [10]. The roles of adenine nucleotides (ATP, ADP and AMP) and their nucleoside derivative, adenosine, as compounds with opposite effects are well established. In this context, studies have shown that

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ATP is a vasoconstrictor and may be cytotoxic, while ADP causes platelet aggregation [11–12]. In contrast, adenosine produced by nucleotide degradation is a vasodilator, inhibits platelet aggregation and presents neuromodulator effects [11, 12].

Studies from our laboratory have shown that ATP diphosphohydrolase activity is reduced in synaptosomes of rat cerebral cortex by 2.0 mM phenylalanine [13] and also that this amino acid significantly inhibits the *in vitro* ATP and ADP hydrolysis activity of rat blood serum [14]. In addition, acute administration of arginine is able to decrease the activities of the enzymes involved in nucleotide hydrolysis in rat blood serum [15]. Furthermore, homocysteine administration alters the activities of ATPase, ADPase and 5′-nucleotidase, both in the central nervous system (CNS) and in the serum of adult rats [16].

Type II Hyperprolinemia (HPII) is an autosomal recessive disorder of amino acid metabolism, caused by the hepatic deficiency of Δ-1-pyrroline-5-carboxilic acid dehydrogenase activity, which results in proline (Pro) accumulation in plasma and tissues of the affected individuals [17]. Although neurological and other dysfunctions are found in a considerable number of patients, the exact mechanism(s) involved remain poorly understood. Since the strategy of using animal models is useful to better understand the pathophysiology of diseases, we have developed a chemical experimental model of hyperprolinemia [18], in which the Pro levels are similar to those found in human hyperprolinermia type II [17]. Rats subjected to this experimental model of hyperprolinemia present reduction in the activities of brain acetylcholinesterase and serum butyrylcholinesterase [19]. In addition, studies show that Pro provokes alteration in the cardiovascular system via actions in the brainstem [20].

Considering that the ratio nucleotides/nucleoside in the circulation could present some changes that could evoke responses in both CNS and circulatory system, in the present study we investigate the *in vivo* (acute and chronic) and *in vitro* effects of Pro on serum nucleotide hydrolysis.

Materials and methods

Animals and reagents

Male Wistar rats were obtained from the Central Animal House of the Department of Biochemistry of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. The animals from our own breeding stock were maintained on a 12 h light/12 h dark cycle at a constant temperature (22 ± 1°C), with free access to water and protein commercial chow. Nucleotides and Pro were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of analytical grade.

Proline administration

Pro was dissolved in 0.9% NaCl and the pH adjusted to 7.2–7.4 with 0.1 N NaOH. For acute treatment, 29-day-old rats received one single subcutaneous injection of Pro correspondent to 18.2 μmol/g of body weight and control rats received an equivalent volume of saline. The animals were killed 1 h, 3 h or 12 h after injection by decapitation without anaesthesia.

For chronic treatment, the solution was administered subcutaneously twice a day at 10 h intervals from the 6th to the 28th day of age, as described by Pontes *et al.* [18]. During the first 8 days of treatment, the rats received 12.8 μmol of Pro/g body weight, from day 14 to 17 the rats received 14.6 μmol of Pro/g body weight, from 18 to 21 days of life the rats received 16.4 μmol of Pro/g body weight and from 22 to 28 days the rats received 18.2 μmol of Pro/g body weight. Rats subjected to this treatment achieved plasma Pro levels of between 1.0 and 2.0 mM, similar to those found in hyperprolinemic type II patients [17]. Control animals received saline injections in the same volumes as those applied to Pro-treated rats. The animals were killed 3 h or 12 h after the last injection by decapitation without anaesthesia.

For *in vitro* studies, the serum of untreated 29-day-old rats was used to evaluate the effect of Pro on serum nucleotide hydrolysis. The experiments were performed using different concentrations of Pro (in the range of 3.0 μM–1.0 mM) in the presence of ATP, ADP and AMP as a substrate in the incubation medium described below. The control samples were performed without Pro addition.

Isolation of blood serum fraction

Blood serum was drawn after decapitation of male Wistar rats (29 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 [21]. Serum was used immediately for experiments.

Ethics

The study was performed in accordance with the University Ethics Committee guidelines for experiments with animals.

Measurement of ATP, ADP and AMP hydrolysis

ATP, ADP and AMP hydrolysis were determined using a modification of the method described by Yegutkin [21]. Briefly, as described by Oses *et al.* [9] the reaction mixture containing 3.0 mM ATP, ADP or AMP as substrate, 112.5 mM Tris-HCl, pH 8.0, was incubated with 0.5 mg to 0.8 mg of protein serum at 37°C for 40 minutes 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.* [22]. Incubation times and protein concentrations were chosen to ensure the linearity of the reaction (results not shown) and absorbance was measured at 630 nm. In order to correct non-enzymatic hydrolysis, controls were performed by adding the serum after the reaction was stopped with TCA. All samples were assayed in triplicate. Enzyme activities were expressed as nanomoles of Pi released per minute per milligram of protein.

Protein determination

Protein was measured by the Coomassie Blue method, according to Bradford [23] using bovine serum albumin as standard.

Statistical analysis

Data were analyzed by Student's *t*-test or by one way ANOVA followed by the Duncan multiple range test when the *F*-test was significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC compatible computer. Values of $P < 0.05$ were considered to be significant.

Results

The effect of acute administration of Pro on ATP, ADP and AMP hydrolysis in the blood serum of rats was studied. Figure 1 shows that rats subjected to acute administration of Pro, killed 1 h after injection present a significant decrease in ATP (42%) [$t(8) = 6.38$; $p < 0.01$] and ADP (49%) [$t(8) = 5.75$; $p < 0.01$] hydrolysis, whereas this treatment did not alter AMP hydrolysis [$t(8) = 1.13$; $p > 0.05$] when compared to control groups (saline-treated rats). Table 1 demonstrates that rats sacrificed 3 h and 12 h after acute injection did not present any alterations in nucleotide hydrolysis, respectively.

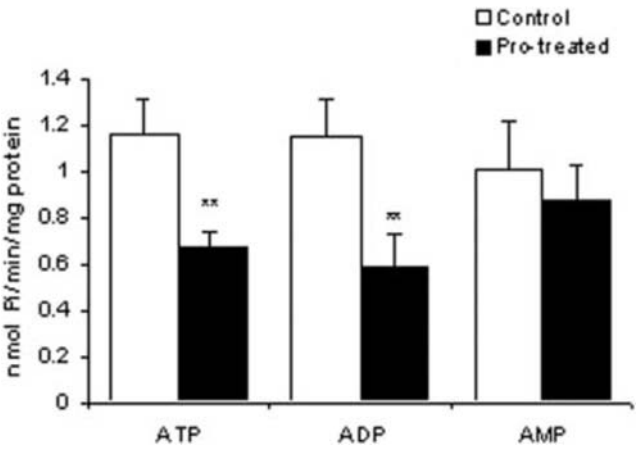

Fig. 1. Effect of acute administration of proline upon ATP, ADP and AMP hydrolysis in rat serum. Data are mean ± SD for 5 independent experiments (animals) performed in triplicate. The values for ATP, ADP and AMP hydrolysis are in nmoles of Pi/min/mg of protein. Different from control, ** $P < 0.01$ (Student's *t* -test).

We also investigated the effect of chronic Pro administration on nucleotide hydrolysis. Figure 2A (rats killed 12 h after the last injection) shows that chronic administration of Pro did not alter ATP [$t(10) = 0.76$; $p > 0.05$], ADP [$t(10) = 0.72$; $p > 0.05$] and AMP [$t(10) = 0.47$; $p > 0.05$] hydrolysis. We also investigated the effect of chronic Pro-treatment on rats killed 3 h after the last injection. Figure 2B shows that chronic Pro-administration decreases NTPDase activity in serum of rats (Pro-treated rats). ATP hydrolysis decreased by (15%) [$t(8) = 2.61$; $p < 0.05$] and ADP hydrolysis decreased by (32%) [$t(8) = 3.42$; $p < 0.01$], when compared to controls. Conversely, chronic Pro-administration did not alter the activity of 5′- nucleotidase, the enzyme that hydrolyzes AMP to adenosine [$t(8) = 0.87$; $p > 0.05$].

In addition, we also investigated the *in vitro* effect of Pro on the same parameters. As can be observed in Figure 3A, B and C, respectively, 1.0 mM Pro added to the incubation medium increased ATP (45%) [$F(4,20) = 7.51$; $p < 0.01$], ADP (55%) [$F(4,20) = 14.8$; $p < 0.01$] and AMP (49%) [$F(4,20) = 7.08$; $p < 0.01$] hydrolysis.

Table 1. Effect of acute administration of proline on ATP, ADP and AMP hydrolysis in rat serum. The animals were sacrificed 3 h or 12 after the injection

	3 h after administration (nmol Pi/min/mg protein)			12 h after administration (nmol Pi/min/mg protein)		
	ATP	ADP	AMP	ATP	ADP	AMP
Control	0.94 ± 0.17	0.91 ± 0.18	0.90 ± 0.21	0.96 ± 0.07	0.96 ± 0.10	1.00 ± 0.07
Proline	0.81 ± 0.13	0.83 ± 0.10	0.92 ± 0.22	1.02 ± 0.04	0.92 ± 0.06	0.99 ± 0.03

Data are mean ± SD of 4 independent experiments performed in triplicate. The values for ATTP, ADP and AMP hydrolysis are in nmoles Pi/min/mg of protein. Data were analyzed statistically by Student's *t*-test

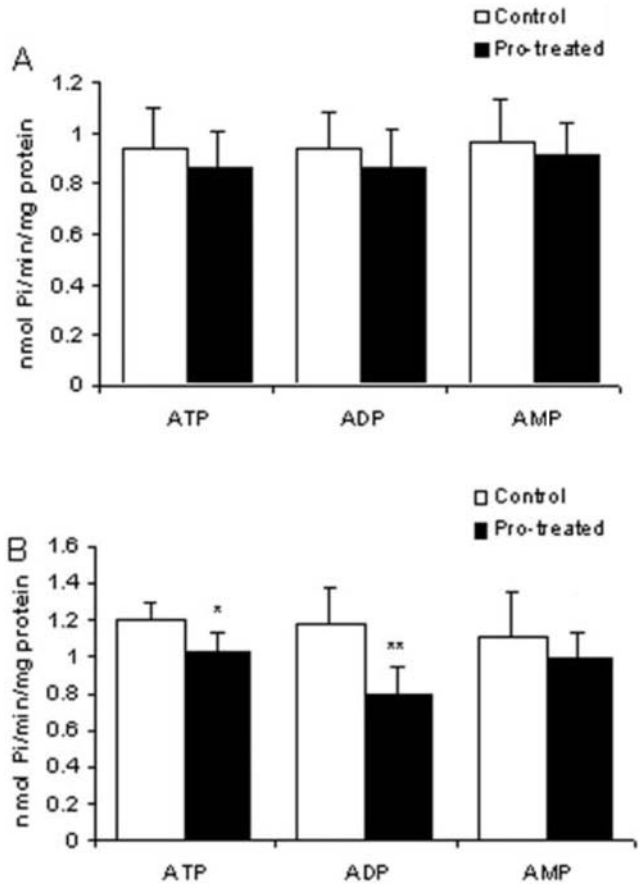

Fig. 2. Effect of chronic administration of proline upon ATP, ADP and AMP hydrolysis in rat serum. The animals were sacrificed 12 h (A) and 3 h (B) after injection. Data are mean ± SD for 5 independent experiments (animals) performed in triplicate. The values for ATP, ADP and AMP hydrolysis are in nmoles of Pi/min/mg of protein. Different from control, $^{*}P < 0.05$; $^{**}P < 0.01$ (Student's *t*-test).

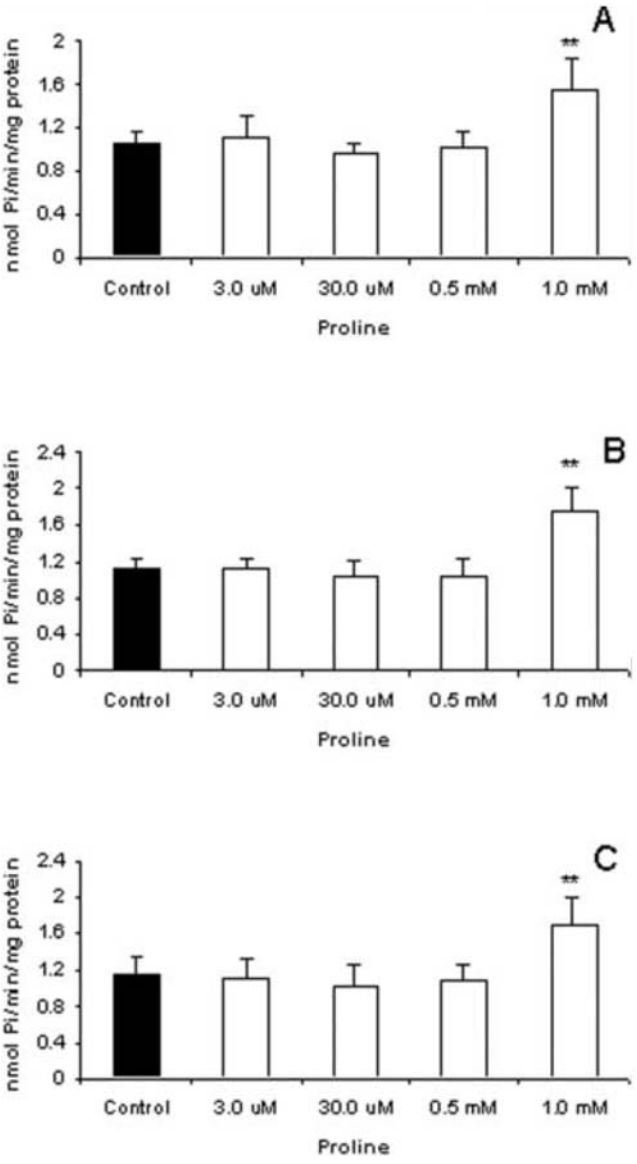

Fig. 3. In vitro effect of increasing concentrations of proline (3.0 μM–1.0 mM) on ATP (A), ADP (B) and AMP (C) hydrolysis in rat blood serum. Data are mean ± SD for 5 independent experiments performed in triplicate. The values for ATP, ADP and AMP hydrolysis are in nmoles Pi/min/mg of protein. Different from control, $^{**}P < 0.01$. Data were analyzed statistically by one-way ANOVA.

Discussion

Hyperprolinemia type II is an inherited disorder caused by a deficiency of Δ-1-pyrroline-5-carboxilic acid dehydrogenase, whose biochemical hallmark is Pro accumulation in plasma and tissues. Most patients experience neurological dysfunctions such as seizures and mental retardation, the pathophysiology of which is poorly understood [17].

Data from our laboratory have shown that Pro administration decreases the activities of (Na^+, K^+)-ATPase, creatine kinase and acetylcholinesterase, which are considered critical enzymes for normal CNS function [24–26]. In addition, Pro impairs memory [27] and induces free radical generation and reduces antioxidant defenses in rat brain, suggesting that Pro elicits oxidative stress [28, 29]. Furthermore, pretreatment with α-tocopherol and ascorbic acid prevents the reduction of (Na^+, K^+)-ATPase and acetylcholinesterase activities in rat brain caused by Pro administration [19, 30]. On the other hand, it has been shown that Pro provokes alteration in the cardiovascular system via actions in the brainstem [20].

Considering that Pro provokes biochemical and behavioral alterations and that the ratio nucleotides/nucleoside in the circulation could present some changes that could evoke responses in both circulatory system and CNS, in the present study, we investigate the possible *in vivo* (acute and chronic) and *in vitro* effects of Pro on ATP, ADP and AMP hydrolysis

in the serum of rats. We verified that acute administration of Pro significantly reduced ATP and ADP hydrolysis, but did not alter AMP hydrolysis, suggesting an effect promoted by secondary metabolites and/or by a direct interaction of Pro. However, since chronic administration of Pro did not alter ATP, ADP and AMP hydrolysis, it appears that the presence of Pro is necessary for its action, since in the acute treatment, the animals were sacrificed 1 h after injection when Pro levels were high, whereas in the chronic treatment, they were killed 12 h after the last injection of Pro, when levels of this amino acid had probably returned to normal.

The next set of experiments was performed in order to evaluate whether rats sacrificed 3 h or 12 h after acute administration would be able to alter nucleotide hydrolysis. We observed that rats killed 3 h or 12 h after acute administration of Pro did not alter nucleotide hydrolysis in rat serum, explaining why chronic treatment did not alter these parameters, suggesting that high Pro levels are essential for these actions. In contrast, rats killed 3 h after the last injection of chronic Pro-treatment, interestingly, decreased ATP and ADP hydrolysis, demonstrating an effect caused by the presence of Pro plus an indirect long-term effect of Pro.

We also tested the *in vitro* effect of different concentrations of Pro on ATP, ADP and AMP hydrolysis in the serum of rats and observed that 1.0 mM Pro significantly increased ATP, ADP and AMP hydrolysis, suggesting a direct interaction of the amino acid with ATP diphosphohydrolase and 5′-nucleotidase, increasing its activities. These results are in agreement with previous results obtained from our laboratory showing that other amino acids such as phenylalanine [14] and arginine [16] reduce ATP diphosphohydrolase activity in rat blood serum. In addition, this enzyme could be relevant as a marker for several central and peripheral diseases.

Nucleoside triphosphate diphosphohydrolase ATP diphosphohydrolases, now included in the class of NTPDases [10], is the general designation for enzymes that hydrolyse ATP, ADP and other triphospho- and diphosphonucleosides to their equivalent monophosphonucleosides and inorganic phospate. This enzyme was first named by Meyerhof [1]. It has been demonstrated that, in CNS, the neurotransmitter ATP is hydrolyzed to adenosine by the conjugated action of an ATP diphosphohydrolase and a 5′-nucleotidase [31, 32]. Thus, the action of this "enzyme chain" may regulate the concentrations of ATP, ADP and AMP by increasing/decreasing their hydrolysis with a consequent increase/decrease in adenosine levels, a natural protective and neuromodulator metabolite. Although it is well established that the breakdown of ATP is mediated by the membrane-bound ectonucleotidases, recent studies indicate that soluble nucleotidases, probably released from sympathetic nerves, are also involved in ATP degradation to adenosine [6, 33]. In addition, circulating soluble ecto-enzymes, such as a nucleotidase may reduce the excess of the levels of these molecules and play an important role in maintaining normal physiology. Furthermore, CD 39, the first human gene reported to encode a protein with ecto-ATP diphosphohydrolase activity [34, 35], is expressed in macrophages, suggesting that this protein is present in the circulation [35]. In a recent study from our laboratory we described a nucleotidase, in fact an ATP diphosphohydrolase activity in rat blood serum [9], that together with 5′-nucleotidase reinforces the effect of the nucleotides/nucleoside ratio in the circulation and in this way modulates platelet aggregation and the vascular response.

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 [36], while adenosine triphosphate (ATP) competitively inhibits ADP-induced platelet aggregation [37]. Several authors have described the important role of these nucleotides in the process of homeostasis and thrombus formation [37, 38] and it is well established that ATP and ADP can promote contraction of smooth muscle cells [39].

In conclusion, if the role of ATP in the vascular system as a vasoconstrictor is well established and the ADP nucleotide is demonstrated to induce changes in platelet shape and aggregation [37, 39] and that there is data showing that Pro causes change in the cardiovascular system [20]**,** according to our results showing that Pro alters nucleotide hydrolysis, it seems reasonable postulate that untreated hyperprolinemic patients may present some types of circulatory problem and in this case, the activity of serum NTPDase could be determined. Further experiments will be necessary to obtain more detailed data regarding the possible consequences of increases in the levels of Pro.

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