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L-NAME administration prevents the inhibition of nucleotide hydrolysis by rat blood serum subjected to hyperargininemia

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Summary. The main objective of the present study was to evaluate the *in vivo* and *in vitro* effect of Arg on serum nucleotide hydrolysis. The action of N^{ω}-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthase, on the effects produced by Arg was also examined. Sixty-day-old rats were treated with a single or a triple (with an interval of 1 h between each injection) intraperitoneal injection of saline (group I), Arg (0.8 g/kg) (group II), L-NAME (2.0 mg/kg or 20 mg/kg) (group III) or Arg (0.8 g/kg) plus L-NAME (2.0 mg/kg or 20 mg/kg) (group IV) and were killed 1 h later. The present results show that a triple Arg administration decreased ATP, ADP and AMP hydrolysis. Simultaneous injection of L-NAME (20 mg/kg) prevented such effects. Arg *in vitro* did not alter nucleotide hydrolysis. It is suggested that *in vivo* Arg administration reduces nucleotide hydrolysis in rat serum, probably through nitric oxide or/and peroxynitrite formation.

Keywords: Hyperargininemia – Arginine – ATP diphosphohydrolase – 5′ Nucleotidase – Serum

Introduction

Tissue accumulation of Arg is the biochemical hallmark of hyperargininemia, an inherited metabolic disorder caused by severe deficiency of liver arginase activity (Brusilow et al., 2001). Affected patients present progressive dementia, epilepsy and spasticity, as well as cortical and pyramidal tract deterioration (Iyer et al., 1998). The pathophysiological mechanisms by which these symptoms occur are poorly understood, however, since increases in Arg levels may result in increased nitric oxide (NO) production, a possible role for NO in the pathophysiology of hyperargininemia has been suggested (Buchmann et al., 1996; Wyse et al., 2001; Reis et al., 2002; Delwing et al., 2003; Scaglia et al., 2004). On this respect, it has been reported that rats subjected to an experimental model of hyperargininemia present a significant impairment of learning/memory, indicating neurological damage (Reis et al., 2002). These results were prevented by N^{ω}-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthesis (NOS) inhibitor, suggesting that NO is involved in such effects.

Some evidence supports the hypothesis that other guanidine compounds, such as N-acetylarginine, homoarginine and argininic acid may be involved in the neuropathology of hyperargininemia (De Deyn and Macdonalds, 1990; D'Hooge et al., 1991; Marescau et al., 1992). In agreement with this assumption, epileptogenic properties have been demonstrated for these guanidine compounds (De Deyn et al., 1991; Mori et al., 1971; Mori, 1987; Wierchert et al., 1987; Yokoi et al., 1984). In addition, it has been suggested that they may alter neurotransmission mechanisms, including inhibition of GABA and glycine receptors (De Deyn et al., 1991), Na^+,K^+-ATPase (Silva et al., 1999) and inducing oxidative stress (Wyse et al., 2001). We have also shown that N-α-acetylarginine, argininic acid and homoarginine increase ATP and ADP hydrolysis in rat brain synaptosomes *in vitro* (Balz et al., 2003).

The nucleotidase activity of ATP diphosphohydrolase (EC 3.1.6.5, apyrase, an enzyme able to promote the hydrolysis of the labile phosphates of ATP and ADP) has been proposed to regulate a variety of physiological conditions including cardiac function, hormone secretion, immune responses and platelet aggregation, by modulat-

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ing the nucleotide levels in the blood (Chadwick and Frischauf, 1998; Todorov et al., 1997; Marcus et al., 1997; Gayle et al., 1998). The AMP formed, by the action of the apyrase, is metabolized to adenosine by an ecto-5′-nucleotidase (EC 3.1.3.5) (Zimmermann, 1996). 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 metabolite (Bruno et al., 2002). The enzyme, ATP diphosphohydrolase was recently described (Oses et al., 2004) in rat serum as a soluble enzyme that can be classified in the group of nucleoside triphosphate diphosphohydrolases (NTPDases) (Zimmermann, 2001). The importance of adenosine as a coronary artery vasodilator is well established (Oliveira et al., 1997).

Therefore, the purpose of the present study was to evaluate the *in vivo* and *in vitro* effects of Arg on serum nucleotide hydrolysis. The influence of L-NAME on the effects elicited by Arg was also tested in order to investigate the possible participation of NO on the effects of Arg on nucleotide hydrolysis.

Materials and methods

Animals and reagents

Sixty-day-old 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 ($22 \pm 1^{\circ}C$), with free access to water and commercial protein chow. Nucleotides, Arg and L-NAME were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of analytical grade.

Treatment

Male Wistar rats, 60-days old, were treated with a single or triple (three administrations with 1 h intervals between each injection) intraperitoneal injections, as follows: Group I (control): animals received saline (0.85% NaCl), Group II (Arg): animals received Arg (0.8 g/kg), Group III (L-NAME): animals received L-NAME (2.0 mg/kg or 20 mg/kg), Group IV (Arg plus L-NAME): animals received Arg (0.8 g/kg) with simultaneous administration of L-NAME (2.0 mg/kg or 20 mg/kg). The rats were sacrificed 1 h after the injection. The Arg dose was chosen to produce plasma Arg levels similar to those found in hyperarginemic patients (Brusilow et al., 2001), according to the protocol established by Buchmann et al., (1996). The purpose of the triple injection is to prolong plasma Arg levels. The L-NAME doses (2.0 mg/kg and/or 20 mg/kg) were chosen according to the protocols established by Nishikawa et al., (1993) and Zarrindast et al., (2003), respectively.

Isolation of blood serum fraction

Blood serum 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 serum obtained was kept on ice (Yegutkin, 1997). 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 (1997). 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. (1986). Incubation time and protein concentration 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 nmoles of Pi released per minute per milligram of protein.

Experiments performed in vitro

ATP, ADP and AMP hydrolysis were determined using a modification of the method described by Yegutkin (1997). To evaluate the effect of Arg on the serum nucleotide hydrolysis, the *in vitro* experiments were performed using different concentrations of Arg (in the range of 0.1–1.5 mM) in presence of ATP, ADP and AMP as substrate in the incubation medium described above. The control samples were performed without Arg addition. All the other procedures for enzymatic assay were the same described above.

Protein determination

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

Statistical analysis

Data were analyzed by Student's *t*-test or by 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 Arg on ATP, ADP and AMP hydrolysis was measured in the blood serum of control (saline) and treated rats. Figure 1 shows that rats subjected to triple Arg administration present a significant decrease in ATP (34%) [$t(8) = 6.12$; $p < 0.01$], ADP (28%) [$t(8) = 5.08$; $p < 0.01$] and AMP (29%) hydrolysis [$t(8) = 2.87$; $p < 0.05$] when compared to control groups (saline-treated rats). In contrast, a single Arg injection did not alter ATP, ADP and AMP hydrolysis (data not shown).

Considering that the reduction in activities of ATPase, ADPase and 5′-nucleotidase could be attributed to the effect of the Arg itself, we performed experiments in

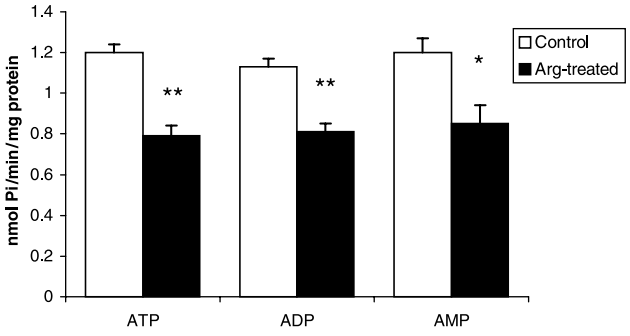

Fig. 1. Effects of acute administration of arginine on ATP, ADP and AMP hydrolysis in rat blood serum. Data are means ± SEM for 5 independent experiments (animals) performed in triplicate. The values for ATP, ADP and AMP hydrolysis are in nmoles of Pi/min/mg of protein. Different from control, $^{*}P<0.05$; $^{**}P<0.01$ (Student's *t* -test)

order to evaluate the *in vitro* effect of different concentrations of this amino acid (in the range of 0.1 mM–1.5 mM) in the nucleotide hydrolysis. No statistically significant alterations in ATP [F(3, 12) = 1.02; p > 0.05], ADP [F(3, 12) = 0.70; p > 0.05] or AMP [F(3, 12) = 0.95; p > 0.05] hydrolysis were observed *in vitro* (Table 1), suggesting that the decreased nucleotide hydrolysis observed *in vivo* is probably induced by an indirect effect of Arg via its metabolites and not by the amino acid itself.

With this idea in mind, we examined the possible action of L-NAME on the effects produced by Arg on the nucleotide hydrolysis. We observed that rats treated with a single or triple injection of L-NAME (2.0 mg/kg), did not present any reduction in ATP, ADP and AMP hydrolysis caused by Arg (data not shown). However, as observed in Fig. 2A, B and C, respectively, a single injection of a higher dose of L-NAME (20 mg/kg) prevented the inhibition of ATP [F(3, 12) = 12.70; p < 0.01], ADP [F(3, 12) = 43.93; p < 0.01] and AMP [F(3, 12) = 11.91; p < 0.01] hydrolysis in rat blood serum caused by Arg.

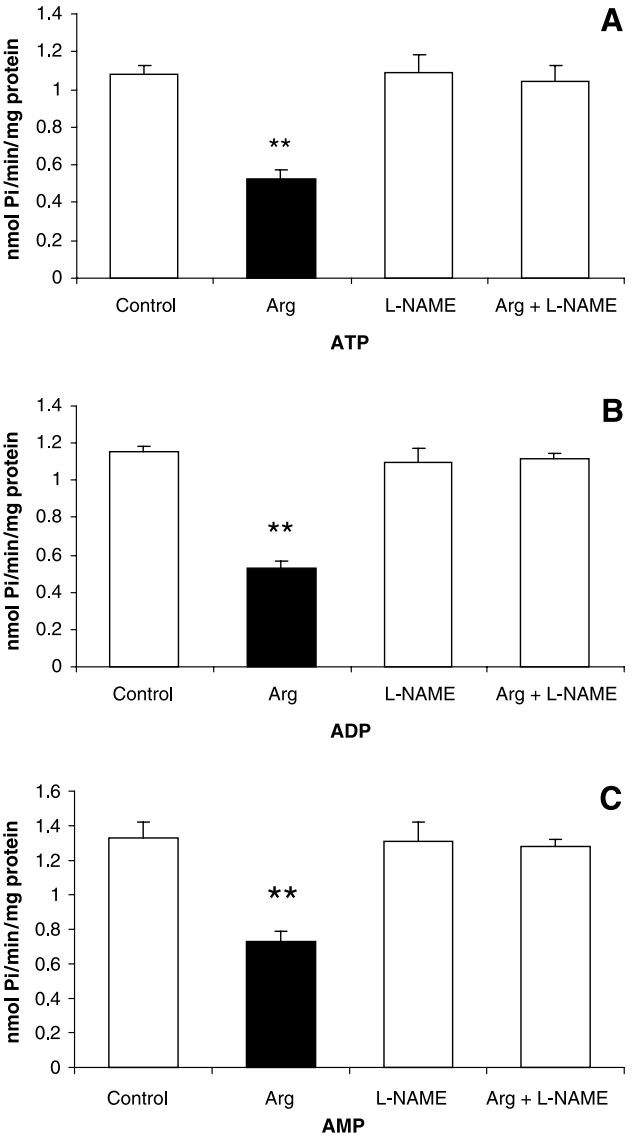

Fig. 2. Effects of acute administration of arginine, L-NAME and arginine plus L-NAME on ATP (A), ADP (B) and AMP (C) hydrolysis in rat blood serum. Data are means ± SEM for 4 independent experiments (animals) performed in triplicate. The values for ATP, ADP and AMP hydrolysis are in nmoles of Pi/min/mg of protein. Different from control, $^{**}P<0.01$ (Duncan multiple range test)

Table 1. *In vitro* effect of increasing concentrations of arginine on ATP, ADP and AMP hydrolysis in rat blood serum. Data are means ± SEM for 4 independent experiments performed in triplicate. The values for ATP, ADP and AMP hydrolysis are in nmoles Pi/min/mg of protein. Data were analyzed statistically by one-way ANOVA

Nucleotidases activities (nmol Pi/min/mg protein)	Arginine concentration			
	0.0	0.1 mM	0.5 mM	1.5 mM
ATP (n = 4)	1.52 ± 0.05	1.46 ± 0.10	1.69 ± 0.10	1.60 ± 0.13
ADP (n = 4)	1.84 ± 0.12	1.75 ± 0.13	1.66 ± 0.12	1.90 ± 0.12
AMP (n = 4)	1.79 ± 0.10	1.58 ± 0.15	1.84 ± 0.11	1.89 ± 0.17

Discussion

Circulating nucleotides are known to be important signaling molecules, potentiating a variety of physiological responses. The role of the adenine nucleotides (ATP, ADP) and their derivative nucleoside, adenosine, as compounds with opposite effects is well established. Accordingly, ATP is a vasoconstrictor and may be cytotoxic, while ADP causes platelet aggregation. Adenosine produced by nucleotide degradation is a vasodilator, inhibits platelet aggregation and presents neuromodulatory effects (Soslau and Youngprapakorn, 1997). It is important to note that the adenosine receptors could convey different effects, such as to activate K^+ channels or to inhibit Ca^{2+} channels, but can also stimulate several phosphatidylinositolpathaways (Ribeiro et al., 2002). However in other tissues, activation of A_1 receptors could result in synergistic activation of the phosphatidylinositol pathway in concert with Ca^{2+}-mobilizing hormones or neurotransmitters (Ralevic and Burnstock, 1998). The enzymatic chain (ATP diphosphohydrolase plus 5′-nucleotidase) is present on the endothelial cell surface of bovine aorta and in smooth muscle cells (Liebermann et al., 1977; Yagi et al., 1991) and may represent a critical mechanism for the control of vascular homeostasis, contributing to the inhibition of the thrombogenic event. More recently, we have described a nucleotidase (ATP diphosphohydrolase) activity in rat blood serum (Oses et al., 2004) that, together with a 5′-nucleotidase, reinforces the role of the nucleotides/nucleoside ratio in the circulation and, in this manner, could modulate the vascular response.

NO appears to have a number of important physiological roles under normal conditions, including neurotransmitter release, gene expression, pain perception, synaptic plasticity and learning (Dawson and Dawson, 1996; Dinerman et al., 1994; Lincoln et al., 1997). On the other hand, NO is considered to be an important mediator of neurotoxicity in a variety of neurodegenerative disorders, such as stroke, Alzheimer's and Parkinson diseases (Dawson and Dawson, 1996; Law et al., 2001). Considering that an increase in Arg levels may result in increased NO production, a possible role for NO in the pathophysiology of hyperargininemia is emerging (Buchmann et al., 1996; Wyse et al., 2001; Reis et al., 2002; Delwing et al., 2003; Scaglia et al., 2004).

In the present study, we investigated the *in vitro* and *in vivo* effect of Arg on ATP, ADP and AMP hydrolysis in rat serum. It is shown that acute administration of Arg (triple injection) significantly reduced ATP, ADP and AMP hydrolysis and, taken together, they suggest an effect promoted by Arg metabolites, since Arg *in vitro* did not significant alter ATP, ADP and AMP hydrolysis.

We also tested the influence of L-NAME on the effects produced by Arg administration on nucleotide hydrolysis in order to evaluate the participation of NO or/and its derivate, peroxynitrite, in such effects. We observed that L-NAME (20 mg/kg) administration *per se* did not alter ATP, ADP and AMP hydrolysis, but when administered simultaneously with Arg prevented the inhibition of nucleotide hydrolysis in rat serum, suggesting that this inhibition is probably caused by Arg administration via NO formation. These results are in agreement with previous findings showing that L-NAME prevents the induction of oxidative stress (Wyse et al., 2001), decreasing the activity of Na^+, K^+-ATPase (Wyse et al., 2001; Reis et al., 2002), catalase (Delwing et al., 2002), energy metabolism (Delwing et al., 2003) and impairment of memory in rats subjected to hyperargininemia (Reis et al., 2002). Taking together these observations and our present results, it may be concluded that Arg administration reduces ATP, ADP and AMP hydrolysis in rat blood serum, possibly by NO and/or its derivate peroxynitrite formation. However, we do not discard other possibilities. For instance, guanidine compounds may reduce the ectonucleotidase and 5′-nucleotidase activities elicited by Arg administration. Furthermore, the increased production of NO could have a modulatory role in the ectonucleotidase activity (Kirchner et al., 2001). In addition, it is tentative to speculate that nucleotide ADP may cause platelet aggregation in patients with hyperargininemia, as a function of the inhibition of ATP, ADP and AMP hydrolysis.

In summary, the present study demonstrated that Arg administration significantly decreases ATP, ADP and AMP hydrolysis via NO formation, and that L-NAME prevents these effects. It is difficult to extrapolate our findings to the human condition, but our results may be associated, at least in part, with the pathophysiological characteristics of hyperargininemic patients. Whether this or other abnormalities are responsible for symptoms in hyperargininemic patients is a matter for further investigation.

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