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Aqueous extract of *Ilex paraguariensis* decreases nucleotide hydrolysis in rat blood serum

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Abstract

Mate is a xanthine-containing beverage, which is prepared as an infusion of the dried and ground leaves of *Ilex paraguariensis* St. Hil. (Aquifoliacea). Previous reports have shown that *Ilex paraguariensis* has the highest levels of caffeine and theobromine when compared to other *Ilex* species. Furthermore, mate is able to interfere in the circulatory system, acting as a diuretic and hypotensive agent. Many processes of vascular injury result in the release of adenine nucleotides, which exert a variety of effects. Nucleoside 5′ tri- and diphosphates may be hydrolyzed by members of the ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) family. The synchronic action of a NTPDase and a 5′-nucleotidase promotes the catabolism of ATP to adenosine, which is able to control the extracellular nucleotides/nucleosides ratio. The chronic ingestion of aqueous extract of *Ilex paraguariensis* by rats during 15 days significantly decreased ATP (55%), ADP (50%) and AMP (40%) hydrolysis in blood serum. These results suggest changes in the balance of purine levels induced by *Ilex paraguariensis* ingestion. Considering the potential effects of *Ilex paraguariensis* in the circulatory system, these results may be relevant since NTPDases are a novel drug target for the treatment of cardiovascular diseases.

Keywords: *Ilex paraguariensis*; NTPDase; Nucleotides; Blood serum; Aquifoliacea

1. Introduction

Mate is a beverage traditionally taken in several Latin American countries, including Southern Brazil, Argentina, Uruguay and Paraguay. Consume of mate in Brazil is approximately 1.2 kg/person/year (reviewed by Fredholm et al., 1999). Mate is prepared as an infusion of the dried and ground leaves of *Ilex paraguariensis* St. Hil (Aquifoliacea) (Schinella et al., 2000). The infusion is drunk for its claimed diuretic, anti-inflammatory and stimulant properties (Cruz, 1982; Mazzafera, 1994). Previous reports have demonstrated that *Ilex paraguariensis* has the highest levels of caffeine and theobromine when compared to other *Ilex* species (Filip et al., 1998). The ingestion of *Ilex paraguariensis* could contribute to the increase of antioxidant defense against the action of free radicals (Schinella et al., 2000). Intake of aqueous extracts of *Ilex paraguariensis* inhibits copper-induced autoxidation of LDL in whole human plasma (Gugliucci, 1996). Furthermore, oral ingestion of *Ilex paraguariensis* infusions was reported to interfere in the circulatory system, acting as a diuretic and hypotensive agent (Mazzafera, 1994).

Circulating nucleotides are released as signaling substances or during pathological events (Zimmermann, 1996; Bodin and Burnstock, 2001a). Many processes of vascular

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injury result in the release of adenine nucleotides which exert a variety of effects (Luthje, 1989; Bodin and Burnstock, 2001b). ATP acts as vasoconstrictor and may be a cytotoxic structure (Opie, 1992), while it could be a potent vasodilator in most vascular beds (Aso et al., 1986; Agteresch et al., 1999). ATP also stimulates cellular production of prostacyclin and nitric oxide, two vasodilators and inhibitors of platelet aggregation (Motte et al., 1995). In contrast, studies have shown that ATP inhibits platelet aggregation acting as a competitive antagonist, whereas ADP is able to promote platelet aggregation (Opie, 1992). There are two distinct families of receptors for purine and pyrimidine nucleotides: P2X and P2Y, which contain eight and seven members, respectively (Ralevic and Burnstock, 1998; Hollopeter et al., 2001; Zhang et al., 2002). These subtypes are expressed with some selectivity on different types of cells (reviewed by Ralevic and Burnstock, 2003), and ATP and ADP may regulate hemostasis through activation of platelet P2Y or P2X receptors (Fredholm, 1994; Jin and Kunapuli, 1998).

Presumably, all tissues have the capacity to metabolize extracellular nucleotides by surface-located enzymes or also by soluble enzymes in the interstitial medium or within body fluids (Zimmermann, 1996, 2001). Nucleoside 5′ tri- and diphosphates may be hydrolyzed by the members of the ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) family (Zimmermann, 2001). Recently, studies from our laboratory have demonstrated a possible soluble NTPDase in rat blood serum (Rücker et al., 2003; Oses et al., 2004). The synchronic action of NTPDase and 5′-nucleotidase promotes the catabolism of ATP to adenosine, which is able to control the extracellular nucleotides/nucleosides ratio. Then, adenosine produced by nucleotide catabolism in the circulation can act as a vasodilator and as an inhibitor of platelet aggregation (Olson and Pearson, 1990; Opie, 1992). Adenosine can exert its functions through four types of P1 receptors: A_1, A_{2A}, A_{2B} and A_3 (Ralevic and Burnstock, 1998). Some of these receptors are expressed in endothelial cells (Bodin and Burnstock, 1995), smooth muscle cells (Giaroni et al., 2002), and sympathetic and sensory nerves (Burnstock, 1996). The action of adenosine in A_1 and A_2 receptors can be blocked by caffeine, an unspecific antagonist of adenosine receptors (Fredholm et al., 2001; da Silva et al., 2003).

The fact that *Ilex paraguariensis* has a hypotensive role and nucleotides are signaling molecules able to modulate circulatory system, prompted us to examine the effects of acute and chronic ingestion of aqueous extract of *Ilex paraguariensis* in nucleotides hydrolysis in rat blood serum.

2. Materials and methods

2.1. Preparation of Ilex paraguariensis extract

Samples of mate were obtained from commercial products purchased from local supermarkets in Lajeado, Rio Grande do Sul, Brazil. The plant is produced by the manufacturer in Venâncio Aires, Rio Grande do Sul, Brazil.

The extract was prepared as infusions. Herbal commercial samples (5 g) were weighted and put into 100 mL of boiling distilled water and after left to cool down (Schinella et al., 2000). This extract was filtered using filter paper. The solid contents of the aqueous extract was 0.05 g/mL^{-1}. The yield of the extract was 21.54%. The extracts were prepared daily (chronic treatment) and at the time of infusion (acute treatment).

2.2. Animals

Male Wistar rats (weight, 220–260 g; age, 60–90 days) from our breeding colony were used. The animals were housed five to a cage with food ad libitum and maintained on a 12-h light/dark cycle at temperature of 23 ± 1 °C. In all experiments, the “Principles of laboratory animal care” (NIH publication No. 85–23, revised 1996) were strictly followed.

2.3. Chronic treatment

The animals were allotted into two groups, containing 10 rats each. The first group received *Ilex paraguariensis* infusion and a second group tap water during 15 days ad libitum. The infusion of *Ilex paraguariensis* was administered chronically by giving the animals free access to bottles containing this infusion. The proportion of extract and water was given according to Schinella et al. (2000). The intake of *Ilex paraguariensis* was monitored throughout the experiment. Daily fluid intake (mL/day) was estimated once every day based on the fluid consumption by the subjects over a 24-h period and its body weight. Daily fluid intake in control groups with free access to tap water was monitored for comparison. There was no significant weight difference between groups (data not shown).

2.4. Acute treatment

In the acute treatment, two groups of animals ($n = 10$ for each group) were submitted to an oral administration by gavage (0.5–0.6 mL) of water (control group) or *Ilex paraguariensis* infusion (treated group). After 1 hour, blood samples were taken and used for the enzyme assays.

2.5. 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 ADP or ATP as a substrate (at the concentrations indicated), 112.5 mM Tris–HCl, pH 8.0, was incubated with approximately 1.0 mg of serum protein at 37 °C for 40 min in a final volume of 0.2 mL. The reaction was stopped by the addition of 0.2 mL 10% TCA. The samples were chilled on ice and the amount of inorganic phosphate (Pi) released 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). In order to correct non-enzymatic hydrolysis, we performed controls by adding the serum after the reaction was stopped with TCA. All samples were centrifuged at $5000 \times g$ for 5 min to eliminate precipitated protein and the supernatant was used for the colorimetric assay. All samples were assayed in duplicate. Enzyme activities were expressed as nanomoles of Pi released per minute per milligram of protein.

2.6. Protein determination

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

2.7. Statistical analysis

Data were analyzed by Student's *t*-test. Values of $P < 0.05$ were considered significant.

3. Results

As shown in Fig. 1, chronic oral intake of *Ilex paraguariensis* infusion during 15 days induced a significant decrease in ATP hydrolysis (55%, $P < 0.05$) in rat blood serum (0.49 ± 0.12 nmol Pi released min^{-1} mg^{-1} of protein), when compared to the group of animals that received only water (1.11 ± 0.41 nmol Pi released min^{-1} mg^{-1} of protein).

ADP hydrolysis was also significantly decreased (50%, $P < 0.05$) in the chronically-treated group (0.95 ± 0.61 nmol Pi min^{-1} protein), when compared to the control group (1.92 ± 0.95 nmol Pi min^{-1} protein) (Fig. 1).

Similarly, an inhibitory effect of AMP hydrolysis (40%, $P < 0.05$) was observed in the group of rats submitted to the chronic treatment (0.66 ± 0.2 nmol Pi min^{-1} protein), when compared to the animals that received only water (1.10 ± 0.16 nmol Pi min^{-1} protein) (Fig. 1).

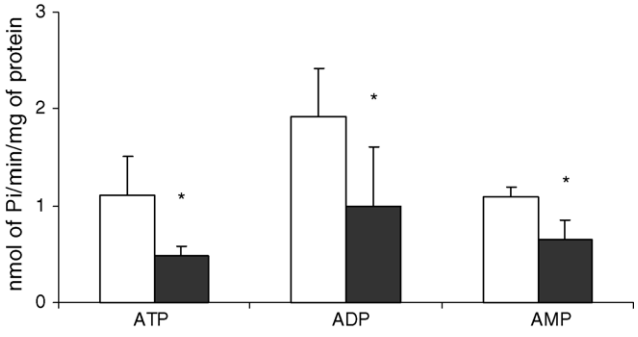

Fig. 1. Effect of chronic treatment with aqueous extract of *Ilex paraguariensis* on ATP, ADP and AMP hydrolysis in rat blood serum. White (control) and black bars (treated group) represent mean ± S.D. *N* per group was 5–7 animals. * Significantly different from the respective control group (Student's *t*-test, $P < 0.05$).

Acute oral treatment with *Ilex paraguariensis* infusion by gavage was unable to modify ATP, ADP and AMP hydrolysis when compared to the control (oral administration of water) in rat blood serum (data not shown).

4. Discussion

Purinergic signaling in the vasculature has the potential to influence vasomotor responses, cardiac function and inflammatory process (Maliszewski, 1994). Recently, our laboratory has demonstrated the occurrence of a possible circulating-soluble ecto-enzyme in rat blood serum with characteristics of NTPDase (Oses et al., 2004). This enzyme may modulate the levels of the nucleotides and presents an important role in the maintenance of normal physiology and health (Oses et al., 2004). It has been shown that platelet aggregation is inhibited by ATP, acting as competitive antagonists of the platelet $P2Y_{12}$ receptor. Thus, high local concentrations of ATP arising as a result of degranulation could serve to control the extent of platelet aggregation (Ralevic and Burnstock, 2003). However, ADP, an agonist of $P2Y_{12}$ receptor, is the principle platelet-recruiting factor originated from platelet-dense granules during activation (Luthje, 1989). The chronic treatment with *Ilex paraguariensis* infusion was able to decrease the nucleotide hydrolysis in rat blood serum. This effect suggests a modulatory role of *Ilex paraguariensis* on nucleotidase pathway, possibly inducing an enhancement of extracellular ATP concentrations and, consequently, a decrease in the ADP, AMP and adenosine levels in the circulation.

It has been reported that *Ilex paraguariensis* exerts a hypotensive role probably by its diuretic properties (Mazzafera, 1994). The ingestion of *Ilex paraguariensis* is recommended for people with weak circulation and venous leg ulcers (Cruz, 1982). The aqueous extract of *Ilex paraguariensis* was able to induce endothelium-dependent vasorelaxation on mesenterical arterial bed, probably involving the action of endothelial nitric oxide (Baisch et al., 1998). ATP can stimulate cellular production of prostacyclin and nitric oxide, two vasodilators and inhibitors of platelet aggregation (Motte et al., 1995). Taken together, there is evidence supporting the possible relation between the hypotensive effect of *Ilex paraguariensis* and the increased level of ATP, which can induce vasodilatation.

Some of the pharmacological activities from *Ilex paraguariensis* are attributed to the high content of caffeoyl derivatives and flavonoids (Filip et al., 2001). Among the important biological activities exerted by flavonoids, it is important to highlight its inhibitory effects on the enzyme systems involved in the initiation and maintenance of the inflammatory and immune response (Ferriola et al., 1989; Havsteen, 1983). A number of flavonoids have also been found to scavenge free radicals directly and to inhibit lipid peroxidation (Baumann et al., 1980). Studies in vitro have demonstrated that some flavonoids were able to inhibit 5′-nucleotidase activity, being quercetin were considered one

of the most potent inhibitory agents of this enzyme activity (Kavutcu and Melzig, 1999). Recently, it has been shown that *Ilex paraguariensis* present a higher content of flavonoids, such as quercetine, when compared to other assayed species (Filip et al., 2001). Therefore, it is possible to suggest that the decrease in AMP hydrolysis promoted by chronic ingestion of *Ilex paraguariensis* could be due to the action of flavonoids.

In conclusion, we have shown that chronic treatment with *Ilex paraguariensis* infusion promoted a decrease of ATP, ADP and AMP hydrolysis in rat blood serum. Thus, it seems that this treatment can alter the nucleotidase pathway, modulating the balance in the purine levels which can induce relevant effects, for example in the cardiovascular system. The effects of *Ilex paraguariensis* administration on the nucleotidase pathway contribute to the current challenge in this field, which is to propose these enzymes as important drug targets for the therapeutic of cardiovascular diseases.

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