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Effects of resveratrol and purple grape juice on nucleotide hydrolysis by adult rat serum

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Abstract

ATP, ADP and AMP (adenosine tri, bi and mono-phosphate, respectively) hydrolysis in rat serum were assessed *in vitro* in the presence of certain flavonoids: resveratrol, quercetin and rutin. Also, the effects of purple grape juice (PGJ) and the effect of PGJ before arginine treatment were observed on serum nucleotide hydrolysis. The *in vitro* nucleotide hydrolysis by rat serum was increased in the presence of resveratrol for ATP and ADP hydrolysis. There was a decrease of nucleotide hydrolysis in the presence of other flavonoids tested *in vitro*. The effects of PGJ treatment after 15 days showed that nucleotide hydrolysis increased for ATP, ADP and AMP. We also investigated whether animals that had received injections of arginine and presented reduction of nucleotide hydrolysis activities, would present a retrieval of this reduction by receiving PGJ before injections were administered. Evidently resveratrol may increase nucleotide hydrolysis by serum and PGJ may be capable of preventing the decrease in nucleotide hydrolysis caused by arginine treatment.

Keywords: NTPDase; ATP diphosphohydrolase; Flavonoids; Resveratrol; Quercetin; Nucleotides; Arginine

1. Introduction

Oxidative stress and the production of intracellular reactive oxygen species have been implicated in the pathogenesis of a variety of diseases (Kunsch & Medford, 1999). Oxidative stress has been defined as a disturbance in the equilibrium status of pro-oxidant/antioxidant systems in intact cells, resulting in oxidative damage of lipids, proteins, carbohydrates and nucleic acids, in addition contributing to pathological dysfunctions in the organism (Hassimotto, Genovese, & Lajolo, 2005).

Recent findings have established that cardiovascular disease features similarly to inflammation and is, consequently, amenable to intervention via molecules that comprise anti-inflammatory effects. In addition, research showing adverse effects of oxidants on atherogenesis raises the possibility that antioxidants yield cardio protective effects (Kris-Etherton et al., 2004). Several epidemiological studies have emphasized the importance of the daily consumption of fruits and vegetables for preventing various problems, such as cancers, cardiovascular diseases or stroke (Leontowicz et al., 2002; Manach et al., 1996; Ortiz & Shea, 2004; Rogers, Milhalik, Ortiz, & Shea, 2003; Tsao, Yang, Young, & Zhu, 2003; Walle, 2004). These properties are attributed to a variety of constituents, such as vitamins, minerals, fibre, and numerous phytochemicals, including flavonoids (Hassimotto et al., 2005).

Many studies suggest that flavonoids, a compound family of C_6–C_3–C_6 skeleton structure, display several biological activities, including anti-allergic, antiviral, anti-tumor and anti-inflammatory effects, as well as antioxidant activity. These activities depend mainly upon the number and

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position of hydroxyl groups within the flavonoid structure (Hassimotto et al., 2005). Epidemiologic evidence suggests that moderate daily wine consumption, mainly red wine, is associated with a lower incidence of cardiovascular disease, since wine has a high phenolic compound content (Auger et al., 2004; Hassimotto et al., 2005). More than 4000 phenolic phytochemicals have been identified (King & Young, 1999). The levels of phenolic compounds in wines and grape juices, primarily resveratrol and quercetin (Meng, Maliakal, Lu, Lee, & Yang, 2004), are highly inconsistent depending on the variety of grape, area of cultivation and vinification methods. There are many differences between red and white wines and aged versus young wines (Meng et al., 2004).

Biological mechanisms proposed for red wine-derived phenolic compounds include estrogenic activity, antioxidant/antiradical activity, inhibition of platelet aggregation, modulation of lipid metabolism, inhibition of low-density lipoprotein oxidation, and proliferation of smooth muscle cells (Dillard & German, 2000; Greene et al., 2004; Knekt, Jarvinen, Reunanem, & Maatela, 1996; Lotito & Frei, 2004a, 2004b; Meng et al., 2004). In addition, wine polyphenols produce vasorelaxation effects, mainly through NO-dependent mechanisms (Meng et al., 2004; Mendes, Desgrange, Che'zec, Vercauteren, & Freslon, 2003).

Biochemical studies have demonstrated that adenine nucleotides are an important potential source of extra cellular adenosine. Once released, these adenine nucleotides are metabolized and rapidly converted to adenosine through the action of (Bruno, Oses, et al., 2002; Zimmermann, 2001) soluble enzymes, such as the enzyme recently described in serum obtained from rats (Oses et al., 2004). ATP diphosphohydrolases (NTPDase1, CD39, apyrase, EC 3.6.1.5) are enzymes that hydrolyze ATP and ADP in nearly the same way.

The physiological role proposed for this enzyme, together with 5′-nucleotidase (EC 3.1.3.5), in the blood stream, is the modulation of the nucleotides/nucleosides ratio. Furthermore, apyrase may inhibit platelet aggregation, promoting ADP hydrolysis, and also controlling the vascular tone, in combination with 5′-nucleotidase, which hydrolyzes adenosine monophosphate (AMP) to adenosine, a vasodilator in the blood stream (Bruno, Bonan, Wolfchuk, Sarkis, & Battastini, 2002; Fontella et al., 2005; Furstenau et al., 2004; Nedeljkovic, Banjac, Horvat, Stojiljkovic, & Nikezic, 2005; Pochmann, Rucker, Battastini, & Sarkis, 2004).

Adenine nucleotides (ATP, ADP and AMP) and their nucleoside derivative, adenosine, are well established as compounds that yield opposing effects. ATP is a vasoconstrictor and may be cytotoxic, while ADP causes platelet aggregation. On the other hand, adenosine, produced by nucleotide degradation, is a vasodilator that inhibits platelet aggregation and presents neuro-modulator effects (Oses et al., 2004). The action of this "enzyme chain" (NTPDase plus 5′-nucleotidase) may regulate the concentrations of ATP, ADP and AMP by increasing/decreasing their hydrolysis, consequently increasing/decreasing adenosine levels, a natural protective metabolite (Bruno, Bonan, et al., 2002; Bruno, Oses, et al., 2002; Delwing, Gonçalves, Sarkis, & Wyse, 2005; Langfort, Czarnowski, Pillis, Wojcik, & Gorski, 1996).

Thus, the purposes of this study were first, to assess the *in vitro* effects of the flavonoids resveratrol, quercetin and rutin, on rat serum nucleotide hydrolysis, second, to evaluate the *in vivo* effects of purple grape juice (PGJ) and, finally, to examine the *in vivo* effects of PGJ on a group of rats subjected to a classical model of argininemia (Delwing et al., 2005).

2. Materials and methods

2.1. Animals and reagents

Nucleotides, flavonoids and arginine were obtained from Sigma Chemical Co. (St. Louis, MO, USA). PGJ was obtained from Suvalan (Tecnovin-Bento Gonçalves, RS, Brazil). All other reagents were of analytical grade. Male Wistar rats from our own breeding stock, weighing around 250 g, were maintained on a 12 h light/12 h dark cycle at a constant room temperature.

2.2. Isolation of blood serum fraction

Blood was drawn after the decapitation of male Wistar rats. Blood samples were centrifuged in plastic tubes for 10 min at 5000*g* at 20 °C, and the obtained serum was kept on ice (Yegutkin, 1997) until immediate use in experiments.

2.3. Treatment with PGJ

The rats were divided into two groups. The control group ($n = 9$) had free access to food (standard lab rat chow) and water. The treated group ($n = 9$) had free access to food, but only received PGJ (purple grape juice) (free access) to drink. The manufactured PGJ was diluted with water (1:2). Each animal from both groups was maintained in an individual cage during all treatment. The volume of liquid consumed by each animal was recorded daily. Three animals from each group ($n = 6$) were sacrificed at three different intervals: after 5 days, 10 days and 15 days of treatment. Blood samples were collected at the time of sacrifice.

2.4. Treatment with PGJ following arginine treatment

The rats were divided into two groups, the control group ($n = 8$) and the treated group ($n = 8$). The treatment with PGJ and diet was the same as that described previously. After 15 days of treatment, each group of eight animals was divided into two groups (each one with four rats), summing up to the total of four groups. One group of four animals that drank only water, and another group of four animals that drank only PGJ, received a triple administra-

tion of saline (0.85% NaCl), with intervals of 1 h between each injection. The other four animals from each group (water or PGJ), received a triple administration of arginine (0.8 g/kg), with intervals of one hour between injections. All the animals were sacrificed 1 h after the last injection. Blood samples were collected at the time of sacrifice.

2.5. Measurement of ATP, ADP and AMP hydrolysis

ATP, ADP and AMP hydrolysis were determined using a method described by Oses et al. (2004). The reaction mixture containing ATP, ADP or AMP as a substrate, in 113 mM Tris–HCl, pH 8.0, was incubated with 0.5–0.7 mg of serum protein at 37 °C for 40 min in a final volume of 0.2 ml. The reaction was blocked by the addition of 0.2 ml 10% TCA. The amount of Pi released was measured by the method of Chan, Delfert, and Junger (1986). Incubation times and protein concentration were chosen to ensure the linearity of the reaction (results not shown). In order to correct non-enzymatic hydrolysis, controls were carried out, adding the serum after the reaction was blocked with TCA. All samples were centrifuged at 5000*g* for 5 min to eliminate protein, and the supernatant was applied for colorimetric assay. All samples were assayed in triplicate. Enzyme activities were generally expressed as nanomoles of Pi released per min per milligram of protein.

2.6. Experiments performed in vitro

To evaluate the effect of flavonoids on serum nucleotide hydrolysis, the *in vitro* experiments were performed using different concentrations of flavonoids (in the range of 50–500 nM) diluted in dimethylsulfoxide (DMSO) in the presence of ATP, ADP and AMP as substrate in the incubation medium as described above. The final concentrations of DMSO, when tested alone in the incubation medium, did not affect the enzyme activity. All the other procedures for enzymatic assays were the same as those described above.

2.7. Protein determination

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

2.8. Statistical analysis

The data obtained are expressed as means ± S.D. of at least four animals. The results were analyzed statistically by Student's *t*-test. A *p* value of less than 0.05 was considered to represent a significant difference.

2.9. Ethics

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

3. Results

3.1. In vitro effect of resveratrol on ATP and ADP hydrolysis by adult rat serum

In experiments using serum from adult rats, ATP hydrolysis was activated *in vitro* by resveratrol, at final concentrations of 50, 100 and 200 nM ($p < 0.05$), when compared to control enzyme activity. The final concentration of 300 and 500 nM, did not yield any effect on hydrolysis of ATP (Fig. 1A). Hydrolysis of ADP, in the same type of experiment, presented a similar profile when compared to ATP (Fig. 1B) although, when ADP was utilized as substrate, an activation effect was also observed with 300 nM of resveratrol. In the range of 50–300 nM, resveratrol had an activation effect ($p < 0.05$) when compared to control enzyme activity (Fig. 1B). Under the same conditions, AMP was used as a substrate for 5′-nucleotidase activity, resulting in no effect of resveratrol at various concentrations, on hydrolysis, when compared to control enzyme activity (Fig. 1C).

3.2. Effects of quercetin and rutin on ATP, ADP and AMP hydrolysis by adult rat serum in vivo

ATP and AMP hydrolysis, in serum, were efficiently inhibited by quercetin at all tested concentrations (50–500 nM) ($p < 0.05$) when compared to control enzyme activity (Fig. 1D and F, respectively). ADP hydrolysis was inhibited only at 300 and 500 nM, ($p < 0.05$) when compared to control enzyme activity (Fig. 1E).

Rutin, another flavonoid tested, inhibited hydrolysis of ATP by rat serum at the concentrations of 300 nM and 500 nM ($p < 0.05$), when compared with control enzyme activity, but did not have any effect in the range 50–200 nM (Fig. 1G). ADP and AMP hydrolysis in serum were inhibited by rutin only at 500 nM ($p < 0.05$), while no effect was seen at any other concentration, when compared with control enzyme activity (Fig. 1H and I, respectively).

It is important to note that controls were performed to correct vehicle (DMSO) interference and no significant differences between vehicle and control enzyme activity were observed. The two highest concentrations (300 nM and 500 nM were tested (Fig. 1).

3.3. Effect of administration of PGJ on ATP, ADP and AMP hydrolysis in vivo by adult rat serum

In experiments using serum obtained from rats subjected to a treatment with PGJ, we evaluated the effect of the juice on ATP, ADP and AMP hydrolysis (Fig. 2). The rats were killed at different times after the beginning of the administration. After 5 and 10 days of treatment, we did not observe any change in nucleotide hydrolysis. In contrast, after 15 days of treatment, a significant increase was observed in the rate of hydrolysis by rat serum for the three

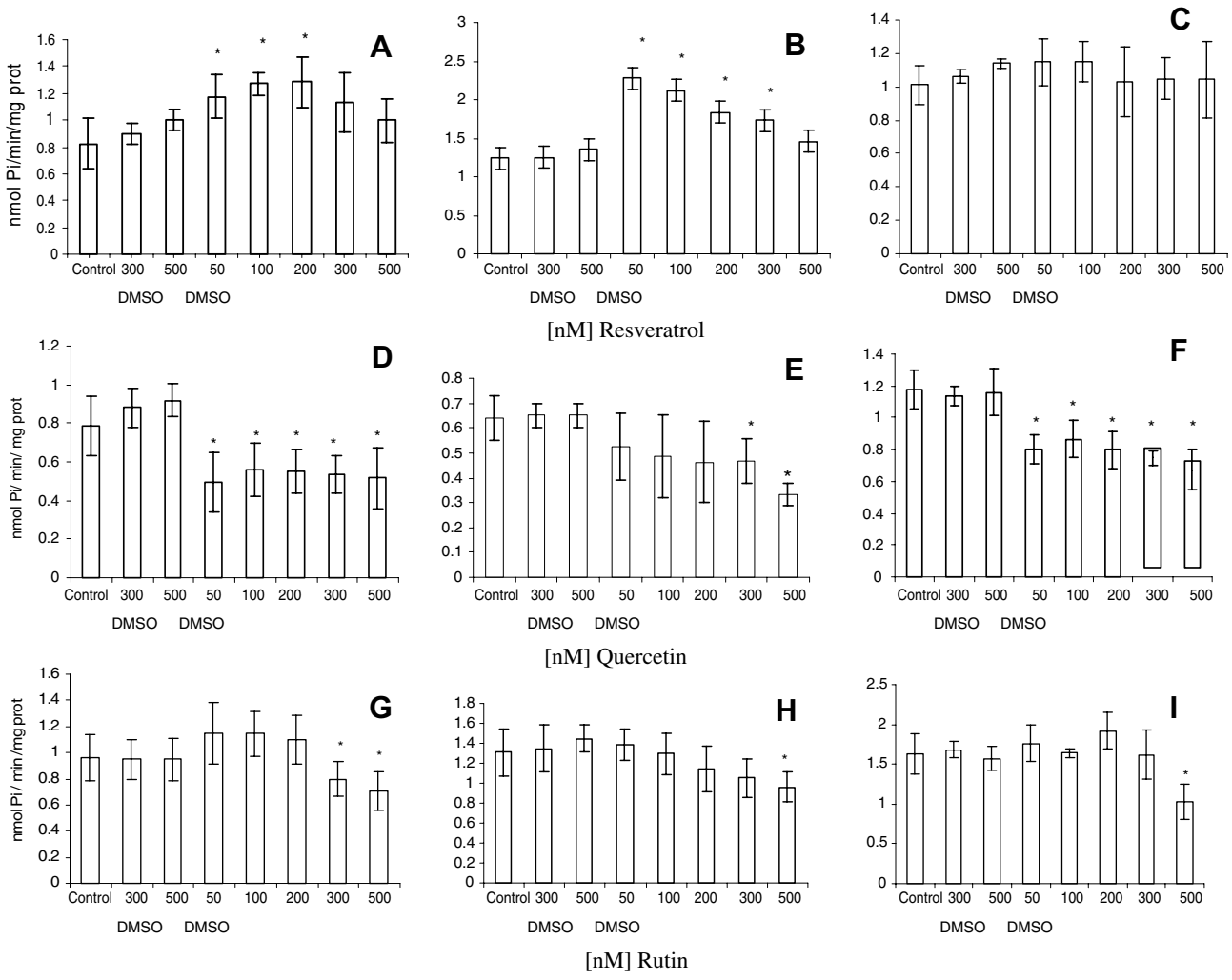

Fig. 1. Effects of flavonoids: resveratrol on ATP (A),ADP (B) and AMP (C); quercetin on ATP (D), ADP (E), and AMP (F) and rutin on ATP (G), on ADP (H), and on AMP (I) hydrolysis in rat serum. The first bars, in all graphics, represent the control; second and third bars represent control of vehicle DMSO in 300 and 500 nM, respectively. The other bars in all graphics represent different concentrations of each flavonoid in nM. Bars represent mean ± SD for four independent experiments. Results are expressed as nmol Pi released/min/mg protein. * indicates significant difference from control enzyme activity ($p < 0.05$). Data were statistically analyzed by Student's *t*-test.

nucleotides, demonstrating a clear effect of PGJ on the enzymatic chain, which was able to promote the hydrolysis of ATP to adenosine.

3.4. Arginine treatment and the protective effect of PGJ

Previous data from our laboratory (Delwing et al., 2005) demonstrated that argininemia, *in vivo*, promotes an inhibition of nucleotide hydrolysis by rat blood serum. As such, we evaluated whether the effect of the treatment with arginine could be reversed by the administration of PGJ. ATP, ADP and whether AMP hydrolysis by serum of rats was inhibited in animals that received drinking water during 15 days before receiving injections of arginine (Group II), in comparison to the control group (Group I) that received drinking water during 15 days before receiving injections of saline (Fig. 3). These results are in agreement with previous results obtained by us in another study (Delwing et al., 2005). The effect of the PGJ may be observed when comparing rats that received PGJ plus saline (Group III) and rats that received PGJ plus arginine (Group IV) with the Group II (water plus arginine). The juice was able to reverse and prevent the inhibitory effect of arginine. The juice administration activated the hydrolysis of the three nucleotides when comparing Groups III and IV to the control (Group I). These results are in accordance with the results obtained from experiments with resveratrol *in vitro* (Fig. 1A and B) and with the administration of PGJ *in vivo* (Fig. 2). The physiological significance of these results will be further discussed.

4. Discussion

Circulating nucleotides are known to be important signalling molecules that render (potentially) a variety of physiological responses. The role of adenine nucleotides (ATP, ADP) and their derivative nucleoside, adenosine, as compounds with opposite effects is well established (Delwing

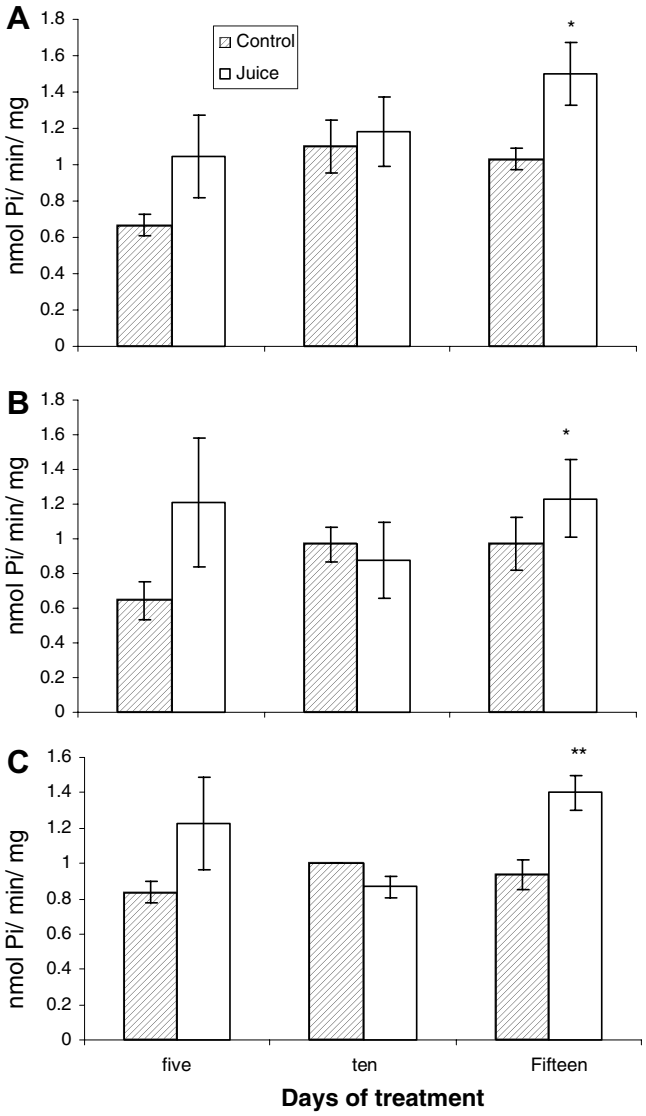

Fig. 2. Effects of purple grape juice on ATP (A), on ADP (B) and on AMP (C) hydrolysis, in rat serum. Bars represent means ± SD for four independent experiments. Results are expressed as nmol Pi released/min/mg protein. * indicates significant difference from control enzyme activity ($p < 0.05$) or ** ($p < 0.01$). Data were statistically analyzed by Student's *t*-test.

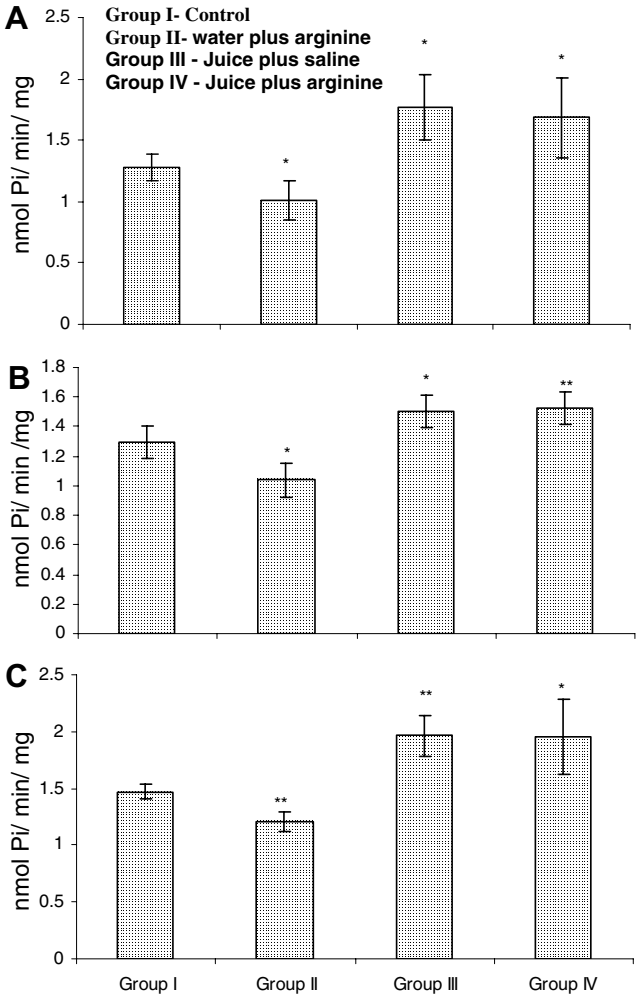

Fig. 3. Effects of purple grape juice and arginine on ATP (A), on ADP (B) and on AMP (C) hydrolysis, in rat serum. Group I represents control with water and saline. Group II, water plus arginine. Group III, Juice plus saline. Group IV, Juice plus arginine. Bars represent means ± SD for four independent experiments. Results are expressed as nmol Pi released/min/mg protein. * indicates significant difference from control enzyme activity ($p < 0.05$) or ** ($p < 0.01$). Data were statistically analyzed by Student's *t*-test.

et al., 2005), and the importance of adenosine as a coronary artery vasodilator is also well recognized (Kaneider, Mosheimer, Reinisch, Patsch, & Wiedermann, 2004).

Nitric oxide (NO) appears to have a number of important physiological roles under normal conditions, including neurotransmitter release, gene expression, pain perception, synaptic plasticity and learning acquisition (Delwing et al., 2005). Several studies have suggested that consumption of red wine, in moderation, is associated with a reduction in the risk of coronary heart disease and cancer (Manach et al., 1996; Ortiz & Shea, 2004; Rogers et al., 2003; Walle, Hsieh, DeLegge, Oatis, & Walle, 2004; Leontowicz et al., 2002; Tsao et al., 2003). PGJ, which is available to a broader range of the population, may also have similar beneficial health effects to those of wine (Meng et al., 2004).

Although many studies have implicated the roles of resveratrol and quercetin (flavonoids present in PGJ) in cardiac disease prevention, their biological effects *in vivo* and details about their action mechanism are unclear (Meng et al., 2004; Lotito & Frei, 2004a). Previous studies also have established that polyphenols present in wine can induce a NO-dependent relaxation in isolated rat aortic rings and that extracellular ATP and P2Y purinoceptors could be involved in this relaxing effect. The vasorelaxation induced by wine polyphenols is now well documented; polyphenolic compounds, obtained from wine extract, demonstrated a relatively great relaxation that is probably mediated by NO release (Mendes et al., 2003).

It should be noted that adenosine, produced from ATP by an enzymatic cascade (NTPDase plus 5′-nucleotidase) by rat serum (Bruno, Bonan, et al., 2002; Bruno, Oses, et al., 2002; Delwing et al., 2005) is an important substance,

capable of promoting vasodilatation and, as such, is considered to be a neuro and a cardio-protector. In this study, we evaluated the ability of flavonoids to interfere with/modulate the nucleotide hydrolysis by the serum fraction obtained from rats. This study was performed *in vitro* and *in vivo*, using flavonoids, such as resveratrol, quercetin and rutin and PGJ.

The first flavonoid tested *in vitro* was resveratrol, which augmented the hydrolysis of ATP and ADP by rat serum *in vitro*, while 5′-nucleotidase was not affected. We postulate that this flavonoid may promote the activity of this enzyme, in turn, increasing the production of adenosine from ATP in the circulation, *in vivo*.

Quercetin and rutin – the latter known as a glycoside derivative (Alía et al., 2006) – showed contrasting effects *in vitro*, when compared to resveratrol. A previous study demonstrated that rutin may have similar effects to quercetin, but is less active, possibly due to its lower bioavailability in growing cells (Alía et al., 2006). In our study, the effects of these compounds were very similar. A previous study questioned whether resveratrol and quercetin could inhibit the signal transduction of thrombin, concluding that there is no known specific receptor for either polyphenol (Kaneider et al., 2004).

From the results, which demonstrate that quercitin and rutin inhibit NTPDase and 5′-nucleotidase and that resveratrol activates NTPDase *in vitro*, we may consider that these flavonoids act via different mechanisms on the enzymes. We therefore evaluated the possible effects of the administration of PGJ (containing flavonoids) instead of water, while comparing another group of animals receiving only drinking water. After 15 days, an activation of the NTPDase and 5′-nucleotidase enzymes in rat serum was observed in animals that drank PGJ. Although quercitin and resveratrol are present in PGJ, the effects of resveratrol on the enzyme seem to be more efficient than those of quercitin (see the *in vitro* effects of resveratol, Fig. 1A–C). As such, an increased consumption of PGJ may lead to augmentation of adenosine levels, supporting theories that the daily consumption of fruits and vegetables, or derived products containing resveratrol, among other flavonoids, may reduce the risks of a number of diseases. Therefore, augmentation in adenosine levels may promote vasodilatation, may induce nucleotide hydrolysis, avoiding ADP levels in the circulation, and consequently a pro-thrombotic condition.

After the outcome of a satisfactory result subsequent to the administration of PGJ to the animals, and taking into account previous results (Delwing et al., 2005) from our laboratory, demonstrating that a model of hyperargininemia promotes a reduction in ATP, ADP and AMP hydrolysis by rat serum (effect that can be deleterious), we also tested the effects of the treatment with PGJ. Based on previous results, the treatment with PGJ was administered, 15 days before the arginine treatment, since arginine inhibits nucleotide hydrolysis in rat serum, probably via nitric oxide (NO) formation (Delwing et al., 2005). Our data demonstrate that PGJ seems to reverse and prevent the inhibition of nucleotide hydrolysis induced by hyperargininemia. A number of studies suggest that polyphenols may inhibit free radical production; in particular one report suggests that resveratrol treatment reduces reactive oxygen species production (Floreani, Napoli, Quintieri, & Palatini, 2003), providing evidence that flavonoids may inhibit NO generation, and consequently may decrease the effect of arginine on nucleotide hydrolysis; such an effect may ameliorate or decrease the deleterious physiopathological characteristics on hyperargininemic patients.

Tissue accumulation of arginine is the biochemical hallmark of hyperargininemia, an inherited metabolic disorder caused by severe deficiency of liver arginase activity. Affected patients present progressive dementia, epilepsy, as well as cortical and pyramidal tract deterioration. Rats subjected to an experimental model of hyperargininemia, mimicking the human disease, showed a significant impairment of learning and memory, indicating neurological damage (Delwing et al., 2005).

Of particular interest is the finding that an improvement in vascular function may be obtained without alcohol (Coimbra, Lage, Brandizzi, Yoshida, & Luz, 2005). This issue is very important from the clinical point of view, regarding patients with risk of cardiovascular events, for whom even moderate alcohol intake is not advisable.

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