

## Fluoxetine and nortriptyline affect NTPDase and 5'-nucleotidase activities in rat blood serum

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### Abstract

Depression is a serious condition associated with considerable morbidity and mortality. Selective serotonin reuptake inhibitors and tricyclic antidepressants, such as fluoxetine and nortriptyline, respectively, were commonly used in treatment for depression. Selective serotonin reuptake inhibitors have been associated with increased risk of bleeding complications, possibly as a result of inhibition of platelet aggregation. ATP, ADP and adenosine are signaling molecules in the vascular system and nucleotidases activities are considered an important thromboregulatory system which functions in the maintenance of blood fluidity. Therefore, here we investigate the effect of *in vivo* (acute and chronic) and *in vitro* treatments with the antidepressant drugs on nucleotidases activities in rat blood serum. In acute treatment, nortriptyline decreased ATP hydrolysis (41%), but not altered ADP and AMP hydrolysis. In contrast, fluoxetine did not alter NTPDase and ecto-5'-nucleotidase activities. A significant inhibition of ATP, ADP, and AMP hydrolysis were observed in chronic treatment with fluoxetine (60%, 32%, and 42% for ATP, ADP, and AMP hydrolysis, respectively). Similar effects were shown in chronic treatment with nortriptyline (37%, 41%, and 30% for ATP, ADP, and AMP hydrolysis, respectively). In addition, there were no significant changes in NTPDase and ecto-5'-nucleotidase activities when fluoxetine and nortriptyline (100, 250, and 500  $\mu$ M) were tested *in vitro*. Our results have shown that fluoxetine and nortriptyline changed the nucleotide catabolism, suggesting that homeostasis of vascular system can be altered by antidepressant treatments.

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### Introduction

Adenine nucleotides (ATP, ADP, and AMP) and their nucleoside derivative adenosine are important signaling molecules that mediate diverse biological and pathological processes (Ralevic and Burnstock, 2003). ATP has been suggested to play a role in vascular tone, cardiac function and renal epithelial transport (Ralevic, 2000). It has been observed that micromolar concentra-

tions of ATP inhibit platelet aggregation by both competitive and noncompetitive mechanisms, whereas lower concentrations can be stimulatory (Soslau and Youngprapakorn, 1997). ADP is a nucleotide known to induce changes in platelet shape and aggregation. Several studies have described the important role of these nucleotides in the process of homeostasis and thrombus formation (Ralevic, 2000; Ralevic and Burnstock, 2003). The nucleoside adenosine, produced by nucleotide degradation, is a structure able to act as a vasodilator and cardioprotector (Frassetto et al., 1993; Soslau and Youngprapakorn, 1997).

The levels of extracellular nucleotides can be controlled by the action of ecto- and soluble nucleotidases, including enzymes of the NPP family (nucleotide pyrophosphatase/phosphodiesterase), NTPDase family (nucleoside triphosphate diphosphohydrolase)

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as well 5'-nucleotidase (Zimmermann, 2001). Over the last few years, our group has demonstrated a soluble NTPDase activity in rat blood serum (Osés et al., 2004). This enzyme acts together with 5'-nucleotidase (EC 3.1.3.5, CD73), which hydrolyzes the monophosphonucleoside AMP to inorganic phosphate and adenosine in the circulation. This enzyme cascade regulates the availability of ligands (ATP, ADP, AMP, and adenosine) for both nucleotide and nucleoside receptors and, consequently, the duration and extent of receptors activation (Chen and Guidotti, 2001). Therefore, this cascade constituted by soluble NTPDase and 5'-nucleotidase is an enzyme pathway with a double function of removing a signal of ATP and generating a second signal produced by adenosine (Böhmer et al., 2006). Adenosine also modulates cognitive states and is associated with affective and mood disorders, such as anxiety and depression (Florio et al., 1998; Kaster et al., 2004). According to the World Health Organization, depression is a worldwide mental health problem affecting an estimated 121 million people. Moreover, depression is a multifaceted disease in terms of symptoms, comorbidities and health complications (Rosenzweig-Lipson et al., 2007). Over the last decades, the view that depression is related to a chemical imbalance in the brain has become widely accepted. The earliest treatments for depression were based upon the serendipitous discovery of monoamine oxidase inhibitors (MAOI) and tricyclic antidepressants (TCA), which eventually laid the foundation for further drug strategies. Indeed, the discovery of selective serotonin reuptake inhibitors (SSRIs) and, more recently, inhibitors of both serotonin and norepinephrine reuptake (SNRIs) has changed the face of clinical treatment (Serra et al., 2006; Rosenzweig-Lipson et al., 2007). Nortriptyline, a tricyclic antidepressant, is the leading drug in the treatment for depression. Many investigators have reported that administration of tricyclic antidepressants can result in inhibition of the presynaptic uptake of serotonin (5-HT) and/or noradrenaline (NA) (Morishita and Aoki, 2002). In contrast, fluoxetine, a selective inhibitor of serotonin reuptake, has little effect on other neurotransmitters (Rossi et al., 2004).

Selective serotonin reuptake inhibitors have been associated with increased risk of bleeding complications, possibly as a result of inhibition of platelet aggregation (Monster et al., 2004). Case reports have described patients with various bleeding disorders treated with SSRIs, while observational studies have focused on upper gastrointestinal bleeding, intracranial bleeding, and bleeding during surgery (Meijer et al., 2004). Considering that ADP is responsible for activation, recruitment and induction of platelet aggregation and the ratio nucleotides/nucleoside in the circulation could evoke responses in both central nervous system and circulatory system, we have here investigated the *in vivo* (acute and chronic) and *in vitro* effects of fluoxetine and nortriptyline on serum nucleotide hydrolysis.

## Materials and methods

### Chemicals

Fluoxetine, nortriptyline, nucleotides, Trizma Base, malachite green, ammonium molybdate, polyvinyl alcohol, EDTA, EGTA,

sodium citrate, Coomassie Blue G, bovine serum albumin, calcium, and magnesium chloride were purchased from Sigma (USA). All other reagents used were of analytical grade.

### Animals

Male Wistar rats (age around 90 days, with 260–320 g) from our breeding stock were housed four to a cage, with food and water *ad libitum*. The animal house temperatures were kept between 22–23 °C with a 12-h light/dark cycle (lights on at 07:00). Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and was approved by the Ethics Committee (CEP 06/03016) of the Pontificia Universidade Católica do Rio Grande do Sul, Brazil.

### Isolation of blood serum fraction

Blood was drawn after decapitation of the male Wistar rats, as described by Osés et al. (2004). Blood samples were centrifuged in plastic tubes at 5000 × *g* for 5 min at 20 °C. The serum samples were maintained in ice throughout the experiments.

### *In vivo* treatments

#### Acute treatment

Animals received one single injection intraperitoneally (i.p.) (10 mg/kg) of fluoxetine and nortriptyline 1 h before they were killed (Zanatta et al., 2001; Borelli et al., 2004; Ejsing and Linnet, 2005; Drapier et al., 2006). Control animals received saline injections (0.9% NaCl) in the same volume as those applied to antidepressant-treated rats.

#### Chronic treatment

The antidepressant drugs were administered daily for 14 days (10 mg/kg, i.p.). The selection of this treatment was based on previous studies showing important neurochemical and antidepressant effects for both drugs (Silva and Brandão, 2000; Zanatta et al., 2001; Borelli et al., 2004; Bonanno et al., 2005; Gameiro et al., 2006). Control animals received saline injections (0.9% NaCl) in the same volume as those applied to antidepressants-treated rats.

### *In vitro* treatments

Antidepressants, fluoxetine or nortriptyline, were added to reaction medium before the preincubation with the rat blood serum and maintained throughout the enzyme assays. Antidepressants were tested at final concentrations of 100, 250 and 500 μM (Dhalla et al., 1980; Zanatta et al., 2001).

### Measurement of ATP, ADP, and AMP hydrolysis

ATP, ADP, and AMP hydrolysis were determined using a modification of the method described by Yegutkin (1997). Briefly, as described by Osés et al., 2004, the reaction mixture containing 3.0 mM ATP, ADP, or AMP as substrate, 112.5 mM

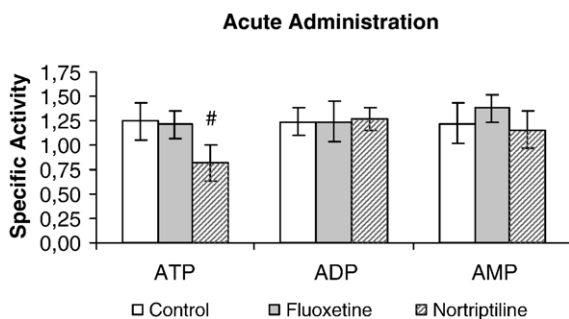


Fig. 1. Effect of acute treatment with fluoxetine or nortriptyline on ATP, ADP, and AMP-hydrolyzing enzyme activities in rat blood serum. Bars represent the mean  $\pm$  S.D. of eight different experiments. The specific enzyme activities are reported as nanomole of inorganic phosphate released per minute per milligram of protein. Data were analyzed by ANOVA followed by a Tukey test (<sup>#</sup> represents difference when compared with control and fluoxetine groups).

Tris-HCl, pH 8.0, was incubated with 0.7 mg to 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 of 10% TCA. The samples were chilled on ice and the amount of inorganic phosphate (Pi) released was measured as previously described (Chan et al., 1986). Incubation times and protein concentrations were chosen to ensure the linearity of the reaction (Oses et al., 2004). Controls to correct for nonenzymatic hydrolysis were performed by adding the serum after the reaction. All samples were centrifuged at 5000  $\times$  g for 5 min to eliminate the precipitate protein and 100  $\mu$ L of supernatant was used for the colorimetric assay. All samples were assayed in duplicate (*in vitro* assays) and triplicate (*in vivo* assays). 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 (Bradford, 1976), using bovine serum albumin as a standard.

#### Statistical analysis

Data were expressed by mean  $\pm$  S.D and analyzed by one-way analysis of variance (ANOVA) followed by the Tukey multiple range test considering  $P \leq 0.05$  as significant difference. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC compatible computer.

#### Results

We evaluated the effect *in vivo* (acute and chronic treatments) and *in vitro* of antidepressant drugs on ATP, ADP and AMP hydrolysis from rat blood serum. NTPDase and ecto-5'-nucleotidase activities were sensitive to antidepressants, fluoxetine and nortriptyline, during chronic treatment. In contrast, in acute treatment only ATP hydrolysis was decreased after administration of nortriptyline. In addition, both drugs did

not alter the enzyme activities in all doses tested for *in vitro* treatment.

In acute treatment, animals received a one single injection (10 mg/kg; i.p.) of fluoxetine or nortriptyline and, after 1 h, the animals were euthanized. Fluoxetine did not alter ATP, ADP and AMP hydrolysis (Fig. 1). However, nortriptyline only changed ATP hydrolysis (41%), when compared to control group (Fig. 1).

The antidepressant drugs were administered daily for 14 days in dose of 10 mg/kg in the chronic treatment. Significant inhibitions of ATP, ADP, and AMP hydrolysis were observed in treatment with fluoxetine (60%, 32%, and 42% for ATP, ADP, and AMP hydrolysis, respectively) (Fig. 2). Similar inhibition was observed in chronic treatment with nortriptyline (37%, 41%, and 30% for ATP, ADP, and AMP hydrolysis, respectively) (Fig. 2).

In addition, for *in vitro* treatment, antidepressants were tested at final concentrations of 100, 250, and 500  $\mu$ M. The results have shown that fluoxetine and nortriptyline did not alter NTPDase (Figs. 3A and 4A) and 5'-nucleotidase (Figs. 3B and 4B) activities from rat blood serum in all concentrations tested (100, 250, and 500  $\mu$ L) when compared to control groups.

#### Discussion

In the present study, there was a significant inhibition of ATP hydrolysis after acute treatment with nortriptyline, but no changes were observed for ADP and AMP hydrolysis in rat blood serum. In contrast, NTPDase and ecto-5'-nucleotidase activities were decreased by chronic treatment with both drugs.

Several studies have found an increased risk of myocardial infarction among depressed patients (Schlienger and Meier, 2003). Tricyclic antidepressants are not recommended in patients with cardiovascular disease owing to their arrhythmic effects (Cohen et al., 2000, Roose and Spatz, 1998; Roose et al., 1998). Selective serotonin reuptake inhibitors (SSRI), on the other hand, appear to lack the adverse cardiovascular effects of other antidepressants (Roose and Spatz, 1998; Roose et al., 1998). Moreover, SSRIs have been shown to inhibit platelet function both *in vitro* (Serebruany et al., 2001) and *in vivo* (Hergovich et al., 2000), and may thus lower the risk of

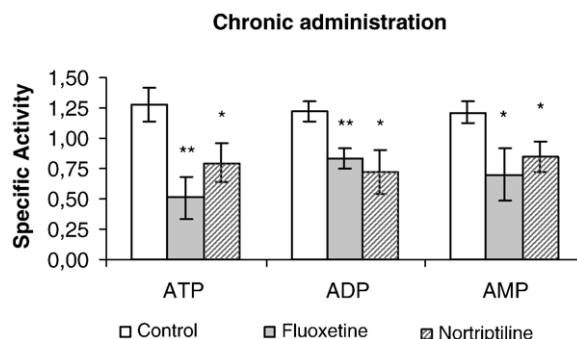


Fig. 2. Effect of chronic treatment with fluoxetine or nortriptyline on ATP, ADP, and AMP-hydrolyzing enzymes activities in rat blood serum. Bars represent the mean  $\pm$  S.D. of eight different experiments. The specific enzyme activities are reported as nanomole of inorganic phosphate released per minute per milligram of protein. Data were analyzed by ANOVA followed by a Tukey test ( $*P \leq 0.01$ ,  $**P \leq 0.001$  when compared to control group).

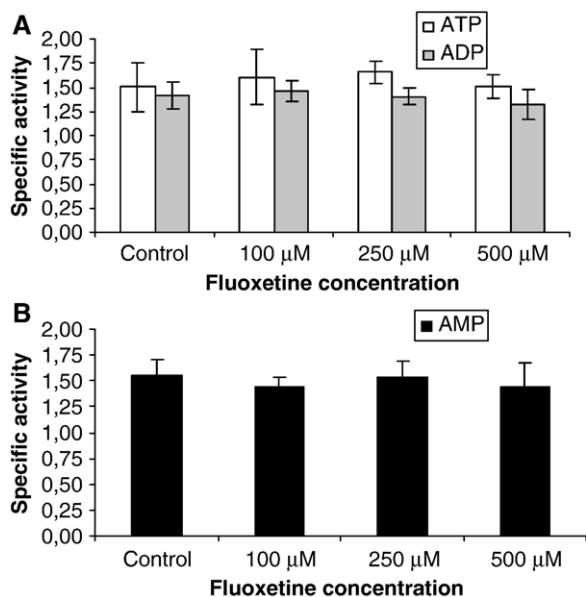


Fig. 3. *In vitro* effect of fluoxetine on NTPDase (A) and ecto-5'-nucleotidase (B) activities in rat blood serum. Bars represent the mean  $\pm$  S.D. of eight different experiments. The specific enzyme activities are reported as nanomole of inorganic phosphate released per minute per milligram of protein. Data were analyzed by ANOVA followed by a Tukey test.

myocardial infarction (Serebruany et al., 2001, Hergovich et al., 2000). Studies have described patients with various bleeding disorders treated with SSRIs, while observational studies have focused on upper gastrointestinal bleeding, intracranial bleeding, and bleeding during surgery (Meijer et al., 2004). The suggested mechanism underlying these adverse effects is that SSRIs limit uptake of blood serotonin by platelets. Since platelets are unable to synthesize serotonin, this leads to a lower concentration of serotonin within the platelets, and because one of the functions of serotonin within the platelets is to promote platelet aggregation, a decreased amount of serotonin in the platelets may increase the risk of abnormal bleeding (Hergovich et al., 2000).

Nucleotides have been shown to act in blood serum (Torres et al., 2002). Extracellularly, the nucleotide ATP has important vascular actions (Burnstock, 1990), can be released by cell lysis and/or cell death as well as exocytosis. 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 (Mills, 1996; Ralevic, 2000). Their biological effects are mainly determined by their rate of release in the extracellular medium, the activity of nucleotidases and their binding affinity to specific receptors. In elevated concentrations, it induces vasoconstriction of the vascular wall, and promotes its own-stimulated release from endothelial cells. On the other hand, ADP is potent platelet-recruiting factor inducing platelet aggregation via interaction of platelet P2Y<sub>12</sub> receptors (Gachet, 2001). Circulating soluble ecto-enzymes such as nucleotidases may reduce the excess of the levels of these molecules and play an important role in maintaining normal physiology. Our laboratory has described a nucleotidase, in fact, a NTPDase activity in rat blood serum (Osés et al., 2004), that together with a 5'-

nucleotidase reinforces the effect of nucleotides/nucleoside ratio in the circulation, modulating platelet aggregation and vascular response. Inhibition of nucleotidases may prolong the effect of nucleotides ATP, ADP and AMP at their respective receptors (Gendron et al., 2002). Therefore, changes in enzyme activities involved in the nucleotide levels in rat blood serum could contribute to abnormal bleeding observed for antidepressant therapy. Vascular disorders can be also associated to an unbalance in the ratio nucleotides/nucleoside in the circulation. Chronic treatment with antidepressant drugs was able to decrease the nucleotide hydrolysis in rat blood serum. This effect suggests a modulatory role of fluoxetine and nortriptyline on nucleotidase pathway and a possible consequence of the decrease in the ATP, ADP and AMP hydrolysis is an increase of the levels of these nucleotides and a decrease in the levels of adenosine in the circulation.

We also evaluated the direct effect of antidepressants in ecto-nucleotidases. Previous studies from our laboratory have shown that NTPDase, but not ecto-5'-nucleotidase, activities from cerebral cortex and hippocampus are decreased by the antidepressants sertraline and clomipramine after *in vitro* exposure (Pedraza et al., 2007). It has been suggested that changes in membrane bilayer environment promoted by the interaction with clomipramine and sertraline may be able to promote the inhibitory effect observed on NTPDase activity. The different effects promoted by antidepressant drugs on NTPDase and ecto-5'-nucleotidase activities can be related to the differences in membrane anchorage of these enzymes. Here our results have shown that exposure *in vitro* to antidepressant drugs are not able to alter enzyme activities in rat blood serum,

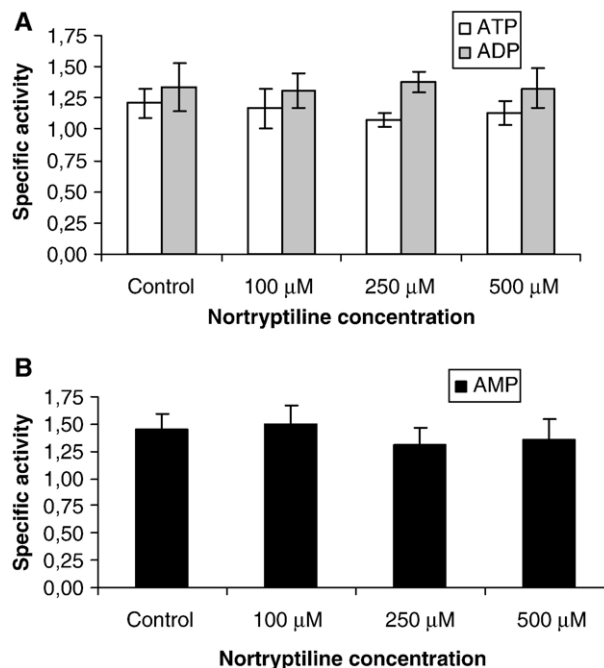


Fig. 4. *In vitro* effect of nortriptyline on NTPDase (A) and ecto-5'-nucleotidase (B) activities in rat blood serum. Bars represent the mean  $\pm$  S.D. of eight different experiments. The specific enzyme activities are reported as nanomole of inorganic phosphate released per minute per milligram of protein. Data were analyzed by ANOVA followed by a Tukey test.

probably because these enzymes are not attached to membrane, but present a soluble form.

In summary, our findings have shown that the *in vivo* treatment with antidepressants alter nucleotide hydrolysis in rat blood serum, suggesting that homeostasis of vascular system can be influenced by these drugs. This alteration on the nucleotide pathway could be considered one of side effects promoted by the chronic treatment with antidepressant drugs, which could induce relevant actions on vascular system.

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