Physiological level of norepinephrine increases adenine nucleotides hydrolysis in rat blood serum

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Abstract Extracellular adenosine 5'-triphosphate (ATP) and its breakdown products, adenosine 5'-diphosphate (ADP) and adenosine, have significant effects on a variety of biological processes. NTPDase enzymes, responsible for adenine nucleotides hydrolysis, are considered the major regulators of purinergic signaling in the blood. Previous work by our group demonstrated that ATP and ADP

hydrolysis in rat blood serum are higher during the dark (activity) phase compared to the light (rest) phase. In nocturnal animals (e.g., rats), important physiological changes occur during the dark phase, such as increased circulating levels of melatonin, corticosterone, and norepinephrine (NE). This study investigated the physiological effects, in vivo and in vitro, of melatonin, dexamethasone, and NE upon nucleotides hydrolysis in rat blood serum. For in vivo experiments, the animals received a single injection of saline (control), melatonin (0.05 mg/kg), dexamethasone (0.1 mg/kg), or NE (0.03 mg/kg). For in vitro experiments, melatonin (1.0 nM), dexamethasone (1.0 μ M), or NE (1.0 nM) was added directly to the reaction medium with blood serum before starting the enzyme assay. The results demonstrated that ATP and ADP hydrolysis in both in vitro and in vivo experiments were significantly higher with NE treatment compared to control (in vitro: ATP=36.63%, ADP=22.43%, $P<0.05$; in vivo: ATP=44.1%, ADP=37.28%, $P<0.001$). No significant differences in adenine nucleotides hydrolysis were observed with melatonin and dexamethasone treatments. This study suggests a modulatory role of NE in the nucleotidases pathway, decreasing extracellular ATP and ADP, and suggests that NE might modulate its own release by increasing the activities of soluble nucleotidases.

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Introduction

Adenosine 5'-triphosphate (ATP) is an important extracellular signaling molecule, besides having a well-established role in cellular energy metabolism [8]. Extracellular ATP,

and its breakdown products, adenosine 5'-diphosphate (ADP) and adenosine, have marked effects on a variety of biological processes including, among others, neurotransmission, contraction of smooth muscle, inflammation, platelet aggregation [11, 15], and cell proliferation [7].

ATP can be released into the circulation with norepinephrine (NE) from sympathetic nerves, causing vasoconstriction via P_2X receptors on smooth muscle cells [33, 42]. In addition, there is growing evidence for ATP as a neurotransmitter in perivascular non-adrenergic non-cholinergic, which promote vasodilatation through direct actions on vascular smooth muscle, or indirectly through their effects on the endothelium, mediated by P_2Y receptors [33]. This mediator, together with ADP, affects the vascular endothelium by stimulating cellular production of vasodilators [29]. ADP is a potent platelet aggregator [24], and it also promotes vasoconstriction [14]. Adenine nucleotides and nucleoside promote their physiological effects through binding to purinoceptors, which comprise P2 ionotropic ATP receptors (P_2X_{1-7}) and the G-protein-coupled ATP/ADP receptors ($P_2Y_{1, 2, 4, 6, 11, 12, 13, 14}$), and P1 adenosine receptors ($A_1, 2A, 2B, \text{ and } 3$) [8].

The importance of adenine nucleotides in homeostasis and thrombosis is closely associated with the essential role of an enzymatic system, which provides an adequate control of these signaling molecules in the extracellular medium [45]. The enzymes responsible for the breakdown of adenine nucleotides have been identified as (ecto-)nucleotidases, and they comprise the nucleoside triphosphate diphosphohydrolase family (NTPDases), the nucleotide phosphate/phosphodiesterase family, alkaline phosphatases, and 5'-nucleotidase [47]. The NTPDase family that hydrolyzes ATP and ADP to AMP is composed of eight members: NTPDase 1, 2, 3, and 8, which are expressed on the cell surface with the catalytic site facing the extracellular space, and NTPDase 4, 5, 6, and 7, which all display an intracellular localization [47]. The NTPDase family is ubiquitous in endothelial and hematopoietic cells, and it is considered to be the major regulator of purinergic signaling in blood [34, 46]. Additionally, the soluble NTPDases can be released of from nerve terminals [39, 41] and from vascular endothelial cells [43].

Previous work by our group demonstrated that ATPase and ADPase activities exhibit a circadian temporal pattern in rat blood serum, with the highest enzyme activities during the dark (activity) phase [12]. In contrast, 5'-nucleotidase did not show a temporal pattern. The increase of ATP and ADP hydrolysis during the dark phase is accompanied by an increase in the secretion of melatonin and corticosterone [12], as well as an increase in sympathetic activity, since rodents (e.g., rats) are nocturnal animals and their activity increases during the dark phase.

Considering that previous study of our group showed that hydrolysis of ATP and ADP have circadian rhythms in serum, and that melatonin, corticosterone, and NE have well-defined circadian rhythms, we hypothesized that these rhythms can be related between themselves. In this context, the present study investigated the physiological effects in vivo and in vitro of melatonin, dexamethasone (to mimic endogenous corticosterone levels), or NE (to mimic the effects of sympathetic activation) upon ATP and ADP hydrolysis in rat blood serum.

Methods

Animals and reagents Male Wistar rats aged 50–70 days and weighing 190–240 g ($n=3-6$ per group) were used. The animals were housed in groups of four to five in home cages made of Plexiglass ($65 \times 25 \times 15$ cm) with the floor covered with sawdust. They were maintained under a standard 12-h light/dark cycle [lights on at 0700 hours and lights off at 1900 hours], in a controlled environment ($22 \pm 2^\circ\text{C}$), with rat chow and water ad libitum. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Experimental design Rats were habituated to the maintenance room for 2 weeks before the beginning of the experiments. In order to investigate the role of physiological levels of melatonin, dexamethasone (mimicking endogenous corticosterone), and NE in ATP and ADP hydrolysis, the animals were killed by decapitation at approximately 0800 hours [light (rest) phase], since at this time the activities of the enzymes ATPase and ADPase are low compared to the dark (activity) phase [12], similar to the secretion of the hormones melatonin and corticosterone, and sympathetic activity. For this purpose, individual rats were transferred quickly to a separate room and decapitated within 1 min to obtain the blood samples.

Isolation of blood serum fraction Blood samples were drawn after decapitation, as described previously [43], and were soon centrifuged in plastic tubes at $5,000 \times g$ for 5 min at room temperature without any additive or any anticoagulant. After centrifugation, the supernatant (serum) was separated and immediately used in the experiments.

In vivo treatments Animals received a single intraperitoneal injection of a physiological dose of melatonin (0.05 mg/kg of body weight, an appropriate dose to mimic the nocturnal surge, dissolved in 0.4% ethanol solution) [38], dexamethasone sodium phosphate (0.1 mg/kg of body weight, an appropriate dose of exogenous corticoid to mimic the surge in serum endogenous corticosterone levels, dissolved in

0.9% saline) [18], or NE (0.03 mg/kg of body weight, mimicking circulating levels, dissolved in 0.9% saline) [21]. Controls received a single injection of saline (0.9% NaCl). After 30 min, blood samples were taken and used for the enzyme assays.

In vitro treatments The direct effect of melatonin, dexamethasone, or NE upon soluble nucleotidase activities was tested by incubating rat blood serum samples obtained from untreated animals. Melatonin, dexamethasone, or NE was added directly to the reaction medium before the preincubation and maintained throughout the enzyme assays. The compounds were tested at final physiological concentrations of 1.0 nM [4], 1.0 μ M [36], and 1.0 nM [1], respectively. The compounds were added to all the tubes; however, in control reactions the samples (serum) were added after the enzymatic reaction in order to correct non-enzymatic hydrolysis.

Enzymatic assay (NTPDase activities determination) ATP and ADP hydrolysis were analyzed using a modification of the method described by Oses and colleagues [30]. The reaction mixture, containing 0.5 to 1.0 mg serum protein in 112.5 mM Tris–HCl, pH 8.0, was preincubated for 10 min to equilibrate the mixture. The reaction was started by the addition of ATP or ADP (final concentration of 3.0 mM) and the incubation was performed at 37°C in a final volume of 200 μ l for 40 min. The reaction was stopped by the addition of 200 μ l 10% trichloroacetic acid (TCA). All samples were centrifuged at 5,000 $\times$ g for 5 min to eliminate precipitated protein and the supernatant was used for the colorimetric assay. Inorganic phosphate (Pi) release was measured by the Malachite green method [9].

For all enzymatic assays, incubation times, substrate, and protein concentrations were chosen in order to ensure the linearity of the reactions. All samples were run in triplicate. In order to correct non-enzymatic hydrolysis, we performed controls by adding the serum after the reaction had been stopped with TCA. Enzyme activities were expressed as nanomoles of Pi released per minute per milligram of protein (nmol Pi min⁻¹ mg⁻¹ of protein). All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Protein determination The protein concentration was measured by the Coomassie Blue method using bovine serum albumin as standard [5].

Statistical analysis Data are expressed as mean $\pm$ standard error of the mean (S.E.M.). Comparisons between groups were made by one-way ANOVA followed by Tukey's test. Differences between groups were considered significant at $P < 0.05$. SPSS 17.0 for Windows was used for statistical analysis.

Results

We examined the in vivo (acute treatment) and in vitro effects of melatonin, dexamethasone or NE on ATP and ADP hydrolysis in rat blood serum.

The in vivo treatments with a physiological dose of melatonin (0.05 mg/kg) or dexamethasone (0.1 mg/kg) did not alter either ATPase or ADPase (Fig. 1) activities. On the other hand, a physiological dose of NE (0.03 mg/kg) caused a significant increase in ATP ($F_{3,19}=8.780$, $P < 0.001$) and ADP ($F_{3,19}=9.561$, $P < 0.001$) hydrolysis. ATP hydrolysis was significantly increased (44.1%) in the NE-treated group (1.17 ± 0.03 nmol Pi min⁻¹ mg⁻¹ of protein), when compared to the control group (0.81 ± 0.10 nmol Pi min⁻¹ mg⁻¹ of protein; Fig. 1). Similarly, acute NE treatment induced a significant increase in ADP hydrolysis (37.28%) in rat blood serum (1.06 ± 0.04 nmol Pi min⁻¹ mg⁻¹ of protein), when compared to the control group (0.77 ± 0.04 nmol Pi min⁻¹ mg⁻¹ of protein; Fig. 1).

In order to verify if melatonin, dexamethasone, or NE treatments could modify the soluble nucleotidase activities by direct mechanisms, in vitro experiments were carried out. Serum samples that were incubated with a physiological concentration of NE (1.0 nM) presented a significant increase in ATP ($F_{3,14}=4.009$, $P < 0.05$) and ADP ($F_{3,14}=7.371$, $P < 0.05$) hydrolysis. ATP hydrolysis increased by 36.63% with NE treatment (0.99 ± 0.01 nmol Pi min⁻¹ mg⁻¹ of protein), compared with control (0.72 ± 0.07 nmol Pi min⁻¹ mg⁻¹ of protein; Fig. 2). ADP hydrolysis was also significantly increased (22.43%) with NE (0.96 ± 0.03 nmol Pi min⁻¹ mg⁻¹ of protein), when compared with control (0.78 ± 0.02 nmol Pi min⁻¹ mg⁻¹ of protein; Fig. 2). ATP and ADP hydrolysis were not affected by melatonin (1.0 nM) or dexamethasone (1.0 μ M) at physiological concentrations (Fig. 2).

Fig. 1 In vivo effects of physiological doses of melatonin, dexamethasone and NE on ATPase (A) and ADPase (B) activities in blood serum. Bars represent mean $\pm$ S.E.M. specific activity (nmol Pi min⁻¹ mg⁻¹ of protein). Number of animals per group=4–6. Asterisk indicates significant difference from other groups; number sign indicates significant difference from control and the ampersand indicates significant difference from melatonin (one-way ANOVA/Tukey, $P < 0.001$)

Fig. 2 Effects of in vitro treatments with physiological concentrations of melatonin, dexamethasone, and NE upon ATP (A) and ADP (B) hydrolysis in blood serum. Bars represent mean $\pm$ S.E.M. specific activity (nmol Pi min⁻¹ mg⁻¹ of protein). Number of animals per group=3–4. Number sign indicates significant difference from control; asterisk indicates significant difference from other groups (one-way ANOVA/Tukey, $P<0.05$)

Accordingly, when these results were expressed as the ratio of ATP/ADP hydrolysis, a value of $\sim 1:1$ was observed with no significant difference between the groups in both in vitro and in vivo experiments ($P>0.05$; Fig. 3). This result indicates the presence of NTPDase 1 in rat blood serum, which hydrolyzes ATP and ADP equally well [45].

Discussion

In the present study, we have shown for the first time that ATPase and ADPase activities in rat blood serum are sensitive to both in vitro and in vivo NE treatments. With both treatments, a physiological concentration of NE caused an increase in the hydrolysis of adenine nucleotides (ATP and ADP). In contrast, melatonin and dexamethasone treatments did not alter hydrolysis of these nucleotides either in vivo or in vitro. We investigated the possible influence of NE on the nucleotidases pathway through an in vivo approach because serum NE is increased during the nocturnal phase and also because it is released with ATP and soluble nucleotidases. It was observed that a single dose of NE, mimicking circulating NE physiological levels, positively modulates the hydrolysis of ATP and ADP in vivo. This increase can be due to a direct effect of NE on the structure of soluble NTPDases in rat blood serum. It is highlighted that the parallel increase in ATP/ADP hydrolysis suggests an effect on the activity of NTPDase 1, a soluble nucleotidase, since parallel behavior for the hydrolysis of these substrates is a characteristic of this enzyme.

The NTPDase family and ecto-5'-nucleotidase are ubiquitously co-expressed on endothelial and hematopoietic cells, and are considered as the major regulators of purinergic signaling in blood [30, 45]. Previous studies suggested the presence of soluble NTPDases in rat blood

serum [30] and in human blood [43]. Several publications, as well as the present study, as described above, indicate the possibility of soluble form of NTPDase 1/CD39, the exact cellular mechanisms underlying the release of this enzyme remains largely unclear. Study suggests that NTPDase 1 contains two transmembrane intracellular N- and C-terminals domains, and may be released through proteolytic cleavage of these membrane-bound domains [27].

It was previously shown that pharmacological inhibition of the NTPDase 1/CD39 potentiates NE release in porcine cardiac tissue and nerve endings isolated from guinea pig hearts, whereas the administration of soluble CD39, a recombinant form of NTPDase 1/CD39, attenuates it [22]. Recent findings indicate that the level of expression of NTPDase 1/CD39 modulates the autocrine effects of transmitter ATP, thus exerting a key influence on the release of catecholamines from sympathetic terminals, in both normal and ischemic conditions [10]. Moreover, an experiment using isolated nerve endings and whole-organ level clearly demonstrates that NTPDase 1/CD39 plays a major role at sympathetic nerve terminals as a modulator of the release and effects of ATP and NE. This occurs whether ATP is released by neuronal depolarization or ischemia. It has been suggested a new protective role for neuronal NTPDase 1/CD39 in limitation of exaggerated catecholamine release in all of these conditions [10]. In all of these studies, the experiments were done in cell systems. Considering that in the blood serum there are not any cells to release the enzymes, in this study we suggest that NE probably increases the ATPase/ADPase activities by a direct stimulatory effect on the soluble enzymes present in the blood serum.

Previous studies show that, in addition to ATP and NE, soluble nucleotidases are also released from several postganglionic sympathetic nerves [25, 27, 39, 41]. These

Fig. 3 Ratio of ATPase/ADPase activity following in vivo and in vitro treatments with melatonin, dexamethasone, or NE. Bars represent mean $\pm$ S.E.M. specific activity (nmol Pi min⁻¹ mg⁻¹ of protein). The ratio of ATP/ADP hydrolysis showed no differences between groups (one-way ANOVA/Tukey, $P>0.05$)

soluble nucleotidases are released upon sympathetic stimulation and breakdown ATP, as well as ADP, to AMP, thereby exhibiting pharmacological similarities with the NTPDases [27]. The soluble nucleotidases may contribute to counterbalancing the reduced activity of membrane-bound ectonucleotidases in certain inflammatory conditions or trauma where the integrity of the blood vessel is impaired [44]. In addition, studies have demonstrated the possible existence of a soluble NTPDase in rat blood serum [30, 35]. Moreover, NTPDase 1-knockout mice exhibited perturbation of vasculature development and immune system function and presented hemostatic and thromboregulatory disturbances [13], while NTPDase 1-null hearts have been shown to develop thrombotic infarcts [20]. In other work, the recombinant soluble form of human NTPDase 1 has been found to inhibit thrombosis and tissue injury [31], while NTPDase 1 has also been shown to modulate vascular inflammation, cell proliferation, and migration [16, 17]. Taken together, these pieces of evidence suggest that NTPDase 1 is important for hemostasis and thromboregulation.

It is well-known that ATP, substrate of NTPDase 1, is released as a cotransmitter together with NE from perivascular sympathetic nerves. ATP released in this way causes rapid vasoconstriction via ionotropic P_2X_1 receptors, while that the vasoconstriction produced by NE is slower because it results of binding G-protein-coupled receptors, and the end result is a synergistic effect [32]. An added note of adenosine formed through ATP breakdown by (ecto-) nucleotidase enzymes acts via presynaptic A_1 receptors to inhibit the release of ATP and NE [6, 19]. Further investigation by Sesti and colleagues [37] showed that the ATP-induced release of NE was potentiated by a nucleotidase inhibitor and inhibited by the soluble form of human NTPDase 1 detected in cardiac sympathetic nerve terminals, leading to the suggestion that nucleotidases control the release of NE by decreasing the extracellular concentration of ATP. Interestingly enough, the modulating action of ATP upon NE release from sympathetic nerves involves P_2X (positive modulation) and P_2Y (negative modulation) receptors [37], where lower concentrations of ATP activate the P_2Y receptor, while higher concentrations of ATP activate the P_2X receptor [2]. In this context, a number of studies indicate the existence of an interaction between the purinergic signaling system and the sympathetic autonomic system.

It is interesting to note that a recent study from our group examining a possible temporal variation in the nucleotidases activities found an increase in ATP and ADP hydrolysis in rat blood serum during the dark (activity) phase [12]. During this phase, which is the time of greatest activity in rodents, there is an increase in sympathetic activity compared with the light (rest) phase, and conse-

quently, increased circulating NE. Enhanced sympathetic activity was confirmed by plasma levels of melatonin [12], which is considered a marker of sympathetic activity [26, 28]. The increased ATP and ADP hydrolysis during the dark (activity) phase of rats may be due to direct modulation of nucleotidase (probably NTPDase 1-like) activities by NE.

Enhanced adrenergic activity and NE release are recognized as causes of clinical cardiac dysfunction such as arrhythmias, myocardial ischemia, sudden cardiac death, and hypertension [3, 23, 40]. It is believed that NTPDase may exert a cardioprotective action by reducing NE release via ATP breakdown [37]. With this in mind, the NE-induced increase in activities of soluble nucleotidases observed in this study raises the possibility that in states of sympathetic hyperactivation this enzymatic modulation could be of great interest in the development of novel therapeutic approaches to cardiovascular disease.

Conclusion

In summary, this study found that treatment with exogenous NE at a physiological concentration, in order to mimic sympathetic activation, stimulated the hydrolysis of adenine nucleotides. This positive modulatory effect suggests a role for circulating NE in the regulation of the nucleotidase (probably soluble NTPDase 1) pathway, where it may induce a decrease in extracellular ATP and ADP in the circulation. This modulatory effect of NE could be involved in the maintenance of extracellular nucleotides at physiological levels and, consequently, contribute to the inhibition of platelet aggregation and thrombus formation induced by ATP and ADP. Additionally, these findings raise the possibility that NE might modulate its own release by increasing the activities of soluble nucleotidases, reducing the concentration of circulating ATP, in turn resulting in P_2Y receptor activation, and consequently, the inhibition of NE release. Placing this in context, our study suggests a novel pathway for the regulation of soluble nucleotidases activities by NE circulating.

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Conflict of interest The authors declare that they have no conflict of interest. The authors alone are responsible for the content and writing of the paper.

Ethical standard All experiments were performed in accordance with the Brazilian Community's Council Directive of October 8, 2008

(Law No. 11.794) with all efforts made to minimize animal suffering and using the number of animals necessary to produce reliable scientific data. The experimental protocol was approved by the Ethics Committee at the Hospital de Clínicas de Porto Alegre.

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