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Enzymes that hydrolyze adenine nucleotides in chronic renal failure: Relationship between hemostatic defects and renal failure severity

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Abstract

The activities of the enzymes NTPDase (E.C.3.6.1.5, apyrase, ATP diphosphohydrolase, ecto-CD39) and 5′-nucleotidase (E.C.3.1.3.5, CD73) were analyzed in platelets from patients with chronic renal failure (CRF), both undergoing hemodialysis treatment (HD) and not undergoing hemodialysis (ND), as well as from a control group. The results showed an increase in platelet NTPDase activity in CRF patients on HD treatment (52.88%) with ATP as substrate ($P<0.0001$). ADP hydrolysis was decreased (33.68% and 39.75%) in HD and ND patients, respectively. In addition, 5′-nucleotidase activity was elevated in the HD (160%) and ND (81.49%) groups when compared to the control ($P<0.0001$). Significant correlation was found among ATP, ADP and AMP hydrolysis and plasma creatinine and urea levels ($P<0.0001$). Patients were compared statistically according the time of hemodialysis treatment. We found enhanced NTPDase and 5′-nucleotidase activities between 49 and 72 months on HD patients. Our result suggests the existence of alterations in nucleotide hydrolysis in platelets of CRF patients. Possibly, this altered nucleotide hydrolysis could contribute to hemostasis abnormalities found in CRF.
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Keywords: Chronic renal failure; NTPDase; 5′-nucleotidase; Platelet; Hemodialysis

1. Introduction

Hemostatic abnormalities are commonly encountered in patients with renal failure. Both a bleeding diathesis and the uremic prothrombotic state may be caused by renal disease [1]. Abnormalities of platelet function and platelet–endothelial interactions are probably the major cause of hemostatic failure in uremia [2,3]. Impaired platelet aggregation in response to different agonists has been described [4]. Diminished platelet aggregation in platelet-rich plasma (PRP) induced by ADP, collagen, arachidonic acid and ristocetin in uremic patients when compared to healthy volunteers [5] has also been observed.

Platelets are known to play a major role in the maintenance of endothelial integrity and hemostasis and they adhere to sites of vascular injury, undergo activation, and subsequently release ADP, thromboxane A_2, serotonin and several other biologically active substances. ADP, acting through P2 receptors [6–8], from this release may be responsible for the activation, recruitment, and induction of aggregation of additional platelets in the microenvironment [6]. Thus, the metabolism of ADP in the blood is important for the regulation of platelet functions [9].

NTPDase (E.C.3.6.1.5, ecto-CD39, ecto-apyrase, ATP diphosphohydrolase) is a glycosylated extracellular enzyme that hydrolyzes extracellular adenosine tri- and diphosphate (ATP, ADP) to adenosine monophosphate (AMP), which is subsequently converted into adenosine by 5′-nucleotidase (E.C.3.1.3.5, CD73) [10–13]. This enzyme was characterized by Pilla et al. [13] on human blood platelets, as

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hydrolyzing the nucleosides ATP and ADP equally well [11–15]. In the coagulation cascade NTPDase (CD39), and ecto-5′-nucleotidase (CD73) have an important role in the regulation of platelet aggregation. Published studies [8,11–14] have shown that CD39 inhibits platelet aggregation via two mechanisms: first by scavenging ATP and/or ADP, and secondly by favoring the eventual formation of the anti-aggregatory metabolite adenosine by 5′-ectonucleotidase activity. Recent publications reinforce the growing importance of NTPDase in thromboregulation [15–19]. Furthermore, there are conclusive lines of evidence showing that CD39 could be a potential therapeutic agent for the inhibition of platelet–mediated thrombogenesis [15–18]. It is also interesting to point out that, ATP and ADP levels in CD39 knockout mice were not changed compared to control [20], suggesting that, at least on macro biochemical level, CD39 does not contribute too much to overall ATP and ADP metabolism in circulation. However, those mice were prothrombotic, suggesting that probably on micro level of cell–cell interaction, CD39 plays an important role.

Ecto-5′-nucleotidase is a glycoprotein attached to the extracellular face of the plasma membrane of many cell types, including human platelets, by a glycosylphosphatidyl-inositol (GPI) anchor [21]. A major role of the protein is the extracellular production of adenosine from AMP and the subsequent activation of P1 adenosine receptors [22,23].

Together, NTPDase and 5′-nucleotidase, control the availability of ligands (ATP, ADP, AMP and adenosine) for both nucleotide and nucleoside receptors, and, consequently, the duration and extent of receptor activation [24]. These enzymes may also have a protective function by keeping extracellular ATP/ADP and adenosine levels within physiological levels [25].

The progression of chronic renal failure (CRF) is complicated by a complex of hemostatic disorder, which is clinically expressed by both a bleeding tendency and increased thrombotic risk and whose pathogenesis is still poorly understood. Platelet activation during hemodialysis may be due to many factors, including sheer stress, contact activation and agonist activation [26,27]. However, further examination is still needed to elucidate the more precise mechanism of platelet activation during hemodialysis.

The objectives of this study were to explore the relationship between platelet dysfunction in uremia with hemostatic abnormalities and the severity of kidney disease in patients with CRF under conservative treatment (non-dialysed) and hemodialysis treatment. Besides the fact that the NTPDase activity measured on platelets is low compared to that of the endothelial cells and leucocytes, we used platelets because they are intrinsically involved in the nucleotide release (mainly ADP). Consequently, platelet NTPDase might contribute directly in the micro extracellular level control of nucleotides and then in the regulation of platelet reactivity. Thus, biochemistry determinations, platelet aggregation, NTPDase and 5′-nucleotidase activities in platelet-rich plasma (PRP) were determined in these patients.

2. Materials and methods

2.1. Materials

Nucleotides, sodium azide, ethylenediaminetetraacetic acid (EDTA) and hydroxymethyl-aminomethane (Tris) were purchased from sigma (St. Louis, MO). All other reagents used in the experiments were of analytical grade and of the highest purity.

2.2. Patients

Enzyme activities, biochemical and hematological determinations were determined in the blood and plasma of healthy control subjects (group I), patients on hemodialysis treatment (HD, group II), and patients who do not receive hemodialysis (non-dialysed, ND, group III). The patients were selected from the Department of Nephrology, Hospital of the Federal University of Santa Maria. The study protocol was approved by the Human Ethics Committee of the Health Science Center from the Federal University of Santa Maria and informed consent was obtained from the patients (Protocol number 037/2004). Group I comprised 60 healthy subjects considered as control (30 women, 30 men) aged 52.28±11.83 (ranging between 30 and 78) with no clinical history of renal disease. Group II (HD) consisted of 51 patients on hemodialysis treatment (25 women, 26 men), whose mean age was 52.58±13.75 (ranging between 24 and 82 years). Etiological causes of uremia included hypertensive nephropathy (8 patients), pyelonephritis (5), glomerulonephritis (5), diabetic nephropathy (5) and cases of unknown etiology (28). All the patients were clinically stable and were undergoing hemodialysis for an average of 4h, three times a week with hollow-fiber dialyzers equipped with cellulose acetate membranes (Baxter, Brazil). During dialysis, blood flow rates were generally between 200 and 300 mL/min. Anticoagulation was achieved by means of a loading dose and constant infusion of heparin. The dose of heparin varied from patient to patient. Blood samples were collected from arterovenous fistulas before hemodialysis. Group III (ND) comprised 15 patients not undergoing hemodialysis (5 women, 10 men), whose mean age was 54.2±12.48 (ranging between 30 and 70 years). The etiological causes of uremia were hypertensive nephropathy (2 patients), pyelonephritis (6), glomerulonephritis (1), diabetic nephropathy (3) and cases of unknown etiology (3). Patients presenting systemic lupus erythematosus, malignancy, infection, treated with corticosteroids or immunosuppressive drugs were excluded from the study.

2.3. Platelet-rich plasma (PRP) and washed platelet preparation

PRP was prepared from human donors according to Pilla et al. [13]. Briefly, blood was collected with 0.129 M citrate, was pooled and was centrifuged at 160×*g* for 10 min. The platelet-rich plasma was centrifuged at 1400×*g* for 15 min and washed twice by centrifugation at 1400×*g* with 3.5 mM HEPES isomolar buffer containing 142 mM NaCl, 2.5 mM KCl and 5.5 mM glucose. The washed platelets were resuspended in HEPES isomolar buffer and protein was measured according to Bradford [28]. NTPDase and ecto-5′-nucleotidase are ecto-enzymes and thus platelet viability and integrity were confirmed by the measurement of lactate dehydrogenase (LDH) activity using the enzymatic Cobas Integra-400 method (Cobas, Basel, Switzerland).

2.4. Hematological and biochemical determinations

Plasma albumin, creatinine, urea, potassium, inorganic phosphorus, alkaline phosphatase concentration were assayed by spectrophotometric methods, using available commercial kits (Labtest, Minas Gerais, Brazil), with a Cobas Integra-400 method (Cobas, Basel, Switzerland). Calcium concentrations were measured by ion electrode selective (AVL 9180 Series Electrolyte Analyzer-Diagnostic Roche). Platelet aggregation was determined by the technique of Biggs and Douglass [29] consisting of the in vitro macroscopic visualization of aggregates between intervals of 15 to 180 s by the addition of ADP (2 μg), serotonin (10–200 μM) and purified potato apyrase (0.023 μg) to platelets. Purified potato apyrase was pre-incubated for 15 min before to add ADP or 10–200 μM serotonin.

2.5. NTPDase and 5′-nucleotidase determinations

NTPDase activity was determined by the method of Pilla et al. [13] in a reaction medium containing 5.0 mM $CaCl_2$, 100 mM NaCl, 4.0 mM KCl, 50 mM glucose, and 50 mM Tris–HCl buffer, pH 7.4, in a final volume of 200 μL. The reaction was started by the addition of ATP or ADP as substrate at a final concentration of 1.0 mM. 5′-nucleotidase was determined by the method of Heymann et al. [30] in a reaction medium containing 10 mM $MgCl_2$, 100 mM Tris–HCl buffer, pH 7.4, at a final volume of 200 μL. For both assays, 6–10 μg of protein was used per tube to ensure linearity in the enzyme reaction. Inorganic phosphate was measured by the method of Chan et al. [31] using malachite green as the colorimetric reagent and KH_2PO_4 as standard. Enzymes activities were expressed as nmol Pi/min/mg of protein.

2.6. Statistical analysis

All data were expressed as the mean value±S.D. Data were analyzed using the Duncan's multiple test, and also subjected to multivariate analysis of variance (ANOVA). Correlation was evaluated with the Pearson test. Differences were considered significant when the probability was $P<0.05$.

3. Results

The clinical characteristics of the patients are shown in Table 1. The albumin and alkaline phosphatase concentrations were in the normal range in both HD and ND patients. Creatinine and urea were increased ($P<0.0001$), as expected in both HD and ND patients. Potassium and inorganic phosphorus contents were above the normal range ($P<0.0001$).

Platelet integrity was determined by comparing the lactate dehydrogenase (LDH) activity obtained after lysis with Triton X-100 with that of intact platelets. Less than 4% of platelets were disrupted (data not shown), indicating that the platelets preparation was predominantly intact. The platelet aggregation induced by ADP was diminished [$F(2,123)=212.53$, $P<0.0001$] and post hoc comparisons by Duncan's test revealed that platelet aggregation had a significant reduction in regular hemodialysis treatment (HD) and non-dialyzed (ND) patients compared to control subjects (Table 2). Platelet aggregation induced by 10–200 μM serotonin caused a rapid aggregation, followed by disaggregation in all groups studied (data not shown). In all experiments conducted with apyrase, even in the presence of serotonin and ADP (synergistically inductors of platelet aggregation), the value was higher than 180s, time considered as a limit to visualize aggregation (Table 2).

ATP, ADP and AMP hydrolysis were measured in washed platelets, thus it is very unlike that they were contaminated with plasma-derived nucleotidases. The results obtained with NTPDase and 5′-nucleotidase are

Table 1
Clinical parameters of the patients

	Normal range	Control ($n=60$)	HD ($n=51$)	ND ($n=15$)
Creatinine (mg/dL)	0.4–1.3	0.77±0.19	9.16±3.15*	2.82±0.80*
Urea (mg/dL)	15–39	27.05±6.41	145.47±47.50*	108.40±49.16*
Calcium (mmol/L)	1.0–1.3	1.14±0.15	1.25±0.14	1.21±0.03
Inorganic phosphorus (mg/dL)	2.7–4.5	3.49±0.65	5.28±1.84*	3.52±0.79
Alkaline phosphatase (U/L)	26–117	83.03±15.76	113.27±119.56	112.50±40.97
Albumin (g/dL)	3.5–5.5	4.19±0.47	4.02±0.28	4.36±0.40
Potassium (mmol/L)	3.5–5.1	4.33±0.52	5.43±0.80*	4.87±0.62

Values represent mean±standard deviation. Different from the control *($P<0.0001$) Duncan's multiple range test.

Table 2
Platelet aggregation time obtained from controls, HD and ND patients

Groups	Control ($n=5$)	HD ($n=5$)	ND ($n=5$)
ADP 2 μg	$37\pm1^{a,b}$	49 ± 4^{a}	60 ± 7^{a}
Apyrase+ADP	↑ 180	↑ 180	↑ 180
Serotonin 10 μM+apyrase	↑ 180	↑ 180	↑ 180
Serotonin 50 μM+apyrase	↑ 180	↑ 180	↑ 180
Serotonin 100 μM+apyrase	↑ 180	↑ 180	↑ 180
Serotonin 200 μM+apyrase	↑ 180	↑ 180	↑ 180
Serotonin 10 μM+apyrase+ADP	↑ 180	↑ 180	↑ 180
Serotonin 50 μM+apyrase+ADP	↑ 180	↑ 180	↑ 180
Serotonin 100 μM+apyrase+ADP	↑ 180	↑ 180	↑ 180
Serotonin 200 μM+apyrase+ADP	↑ 180	↑ 180	↑ 180

Values represent mean±standard deviation ($n=5$). Serotonin concentration varied from 10 to 200 μM, apyrase was 0.0023 μg and ATP was 2 μg per test.

[a] Different from the others in the same column ($P<0.0001$).

[b] Different from the others in the same line ($P<0.01$). Duncan's multiple range test.

shown in Fig. 1. As can be observed, ATP hydrolysis was enhanced [$F(2,120)=25.13$, $P<0.0001$] and post hoc comparisons by Duncan's test revealed that ATP hydrolysis was significantly higher in hemodialysis patients (52.88%) compared to control subjects. ADP hydrolysis was decreased [$F(2,118)=53.63$, $P<0.0001$] and post hoc comparisons in hemodialysis patients showed that ADP hydrolysis was significantly lower in patients with HD (33.68%) and ND (39.75%) compared to control subjects. 5′-nucleotidase activity was also altered [$F(2,114)=24.67$, $P<0.0001$] and post hoc comparisons by Duncan's test revealed that AMP hydrolysis was significantly higher in HD (160%) and ND (81.49%) patients compared to control subjects.

Significant correlation was found between ATP hydrolysis and plasma creatinine level ($r=0.51$, $P<0.0001$), Fig. 2 (ATP). Similarly, significant correlation coefficients were found between plasma creatinine and ADP ($r=0.45$, $P<0.0001$) and AMP ($r=0.53$, $P<0.0001$), Fig. 2. In addition an association was found between plasma urea and ATP ($r=0.41$, $P<0.0001$), ADP ($r=-0.56$, $P<0.0001$) and AMP hydrolysis ($r=0.44$, $P<0.0001$), Fig. 3.

ATP hydrolysis was enhanced as a function of length of hemodialysis [$F(5,117)=9.32$, $P<0.0001$] and post hoc comparisons by Duncan's test revealed that ATP hydrolysis was significantly higher in regular hemodialysis patients being treated from 49 to 72 months (62.97%), Fig. 4 (ATP). Moreover, a decrease can be observed in the hydrolysis of ADP as a function of length of hemodialysis [$F(5,12)=23.10$, $P<0.0001$] and post hoc comparisons by

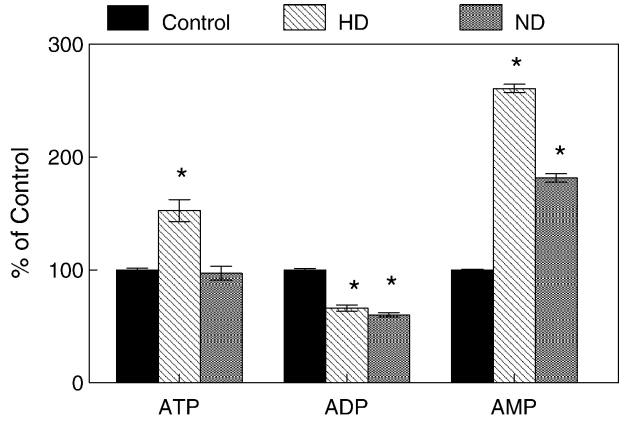

Fig. 1. NTPDase and 5′-nucleotidase activities in platelets obtained from CRF patients. Control ($n=60$), hemodialysis patients (HD) ($n=51$), non-dialyzed patients (ND) ($n=15$). Control values with ATP (15.97±1.80), ADP (11.22±1.38) and AMP (2.27±0.73) as substrate. Values represent mean±standard deviation. *Different from the control ($P<0.0001$) Duncan's multiple test.

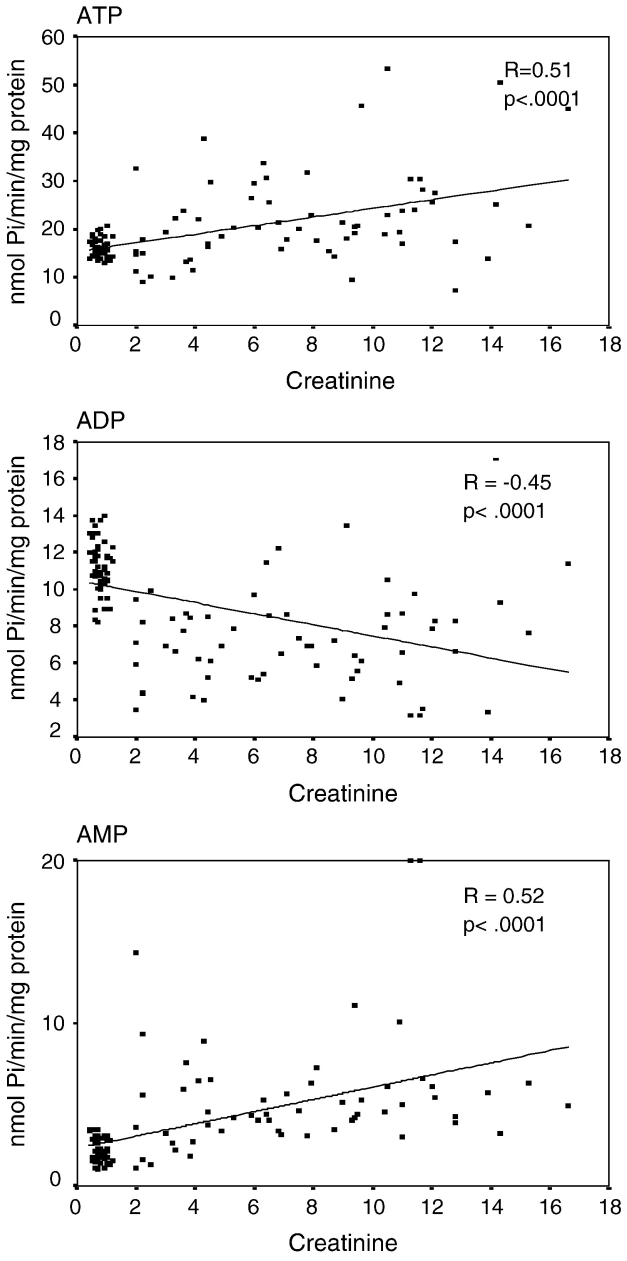

Fig. 2. Pearson's correlation between plasma levels of creatinine and ATP, ADP, AMP hydrolysis in HD, ND and control subjects.

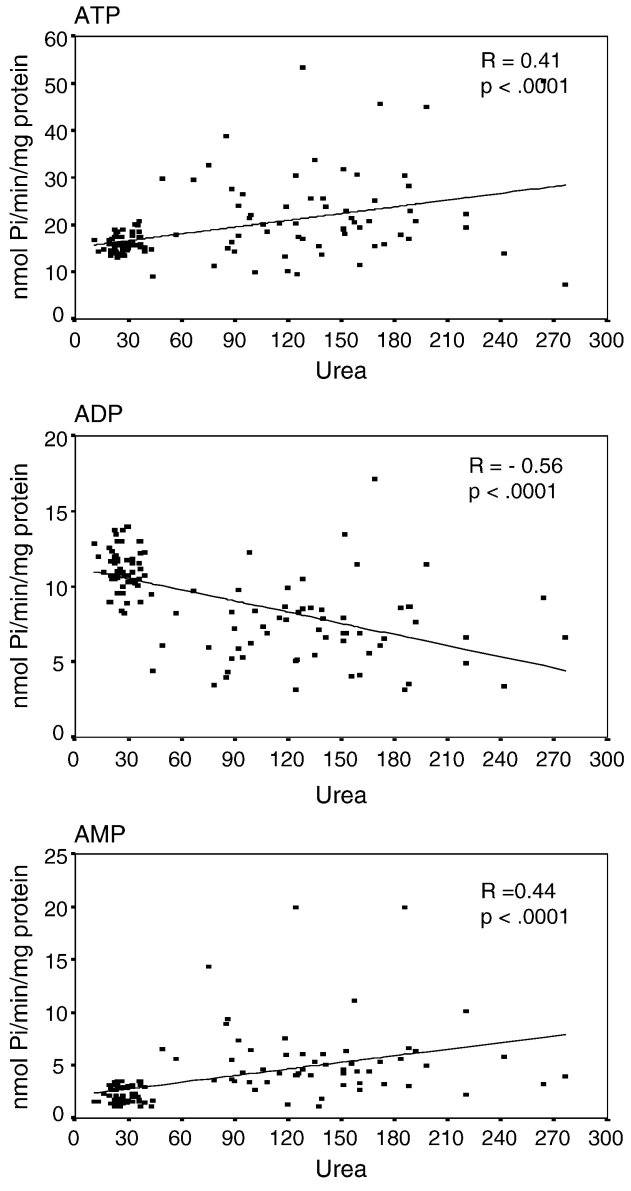

Fig. 3. Pearson's correlation between plasma levels of urea and ATP, ADP, AMP hydrolysis in HD, ND and control subjects.

Duncan's test revealed that ADP hydrolysis was significantly lower in regular hemodialysis patients being treated from 49 to 72 months (30%), Fig. 4 (ADP). Furthermore, an increase was observed in 5′-nucleotidase activity as function of length of hemodialysis treatment [$F(5,1)=15.67$, $P<0.0001$] and post hoc comparisons by Duncan's test revealed that AMP hydrolysis was significantly higher in regular hemodialysis patients being treated from 49 to 72 months (121.7%), Fig. 4 (AMP).

4. Discussion

Biochemical parameters obtained from the patients confirmed their clinical condition. As expected, creatinine and urea levels were elevated in both HD and ND groups. In relation to platelet function, it was observed that HD and ND patients presented a lower platelet aggregation capacity induced by ADP when compared to the control group (Table 2). These results are in agreement with Malyszko et al. [5] and indicate a more delayed aggregation response. As expected, the presence of purified apyrase enhanced the aggregation time to values higher than 180s, confirming its activity as an antithrombotic agent, even when platelets were co-stimulated with ADP and 10–200 μM serotonin (Table 2).

Here, for the first time, we have shown that platelet ATP hydrolysis was enhanced in HD patients, conflicting with the reduction of ADP hydrolysis in HD and ND patients. The inhibition of extracellular ADP hydrolysis, as observed in CRF patients, could indicate a decrease in adenosine production from the extracellular ADP breakdown, and an accumulation of ADP, a proaggregatory agent. Perhaps, the increase in the activity of 5′-nucleotidase observed in HD and ND patients could be a compensatory mechanism once it culminates with adenosine production. Considering the results obtained in this study, perhaps the enhancement in

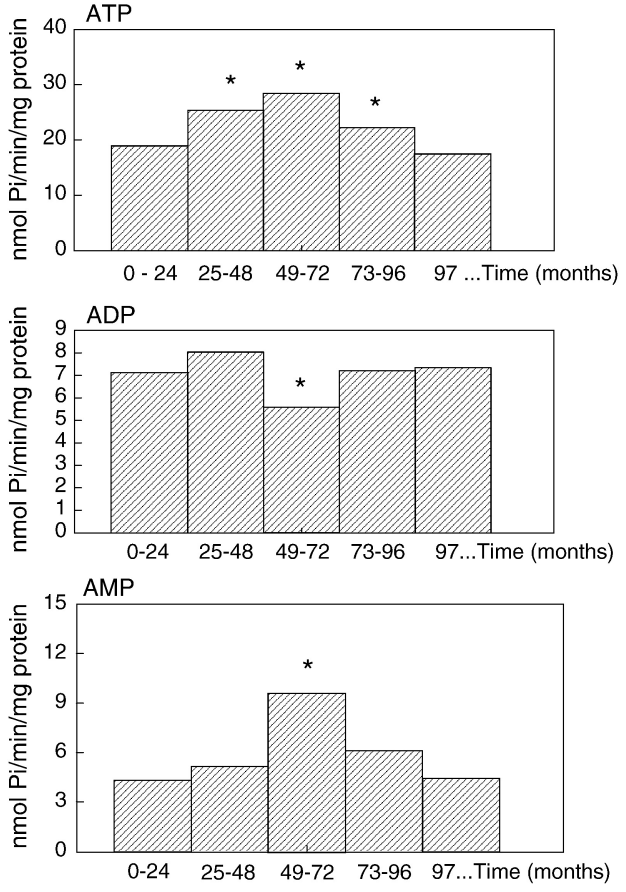

Fig. 4. Effect of hemodialysis length on NTPDase and 5′-nucleotidase activities in hemodialysis patients, ATP, ADP and AMP as substrate. Length hemodialysis in months 0–24, 25–48, 49–72, 73–96, 97 months. Values represent mean±standard deviation. *Different from the others $P<0.0001$ Duncan's multiple test.

ATP hydrolysis demonstrates the presence of other NTPDase (preferentially hydrolyzing ATP).

In the context, the change of the ATP, ADP and AMP hydrolysis observed in the present study is very interesting. Since premature atherosclerosis is one of the most important causes of death at the end-stage of renal failure patients [32,33], and ADP is a signaling molecule, which activates platelet aggregation, we suggest that decreased local ability of platelet to metabolize ADP might result in higher local exposure of platelet P2Y1 receptors to ADP. Possibly, indicating a role of this factor in the etiology of atherosclerosis.

ATP can be an inducer of platelet aggregation because it induces platelet aggregation in the presence of norepinephrine (NE) or epinephrine [34] and it is known that in CRF there is an increase in NE release. Furthermore, ATP can be metabolized to ADP by CD39 and CD39-like enzymes [30]. At the initiation of vascular damage, ATP is hydrolyzed to ADP for ecto-CD39, resulting in platelet aggregation and the prevention of bleeding. Perhaps, the prevention of bleeding is being achieved by the inhibition of ADP hydrolysis. Relating the diminished platelet aggregation induced by ADP (Table 2) with the inhibition of ADP hydrolysis (Fig. 1), one can speculate that there could be a lower cellular response to ADP, as the inhibition of ADP hydrolysis causes an elevation on ADP levels and consequently the expected rapid aggregation response was not observed. Probably, a potential P2Y1 desensitization could occur in the platelets from renal disease patients by the heightened presence of ADP in the microenvironment of platelets from CRF.

The metabolism of extracellular nucleotides is an important regulatory tool to maintain a coagulant state as well as a homeostasis of platelet aggregation. Three main anti-aggregatory systems are involved in the control of platelet reactivity: prostaglandin production, nitric oxide and ecto-nucleotidase activity, respectively. The role of ecto-nucleotidases in platelet aggregation has been known for many years [8–13,35]. Recently, the importance was confirmed in CD39-deficient mice, which had prolonged bleeding times and platelet hypofunction [20]. Under a resting condition, the efficient turnover of ATP and ADP to AMP prevents the onset of an amplification cascade of platelet recruitment and activation induced by ADP and thus the formation of a hemostatic plug. Moreover, ecto-5′-nucleotidase may enhance the role of NTPDase by converting AMP to adenosine, a known inhibitor of platelet aggregation [36]. Recently, platelet altered nucleotide hydrolysis was observed by our laboratory in diabetic, hypertensive, diabetic/hypertensive [19] and breast cancer patients [37], suggesting that it could be an important physiological and pathological parameter.

In our study, we found that plasma creatinine and urea levels were positively correlated with ATP and AMP hydrolysis and negatively correlated with ADP hydrolysis. We then speculated that changes in NTPDase and 5′-nucleotidase activities could play a role in the accelerated atherosclerosis and increased thrombotic risks of uremic patients, which correlates with the accumulation of metabolites.

We found enhanced ATP and AMP hydrolysis mainly between 25–96 and 49–72 months of hemodialysis treatment, respectively. After 97 months of hemodialysis, there is no change in nucleotide hydrolysis, indicating a normalization of such activities.

Ours results suggest the existence of defects in nucleotide hydrolysis in platelets, which might contribute to abnormal homeostasis in renal failure patients, thus contributing to the enhanced risk of thromboembolic complication and accelerated atherosclerosis in patients with renal failure. However, ours is the first study to investigate NTPDase and 5′-nucleotidase activity in CRF patients and more studies are necessary to elucidate the altered level of nucleotides in CRF patients.

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