Erythrocyte PAF-acetylhydrolase activity in various stages of chronic kidney disease: Effect of long-term therapy with erythropoietin

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Background. Erythrocytes represent an important component of the antioxidant capacity of blood, comprising, in particular, intracellular enzymes, including platelet-activating factor acetylhydrolase (PAF-AH) and glutathione peroxidase (Gpx). We evaluated the erythrocyte PAF-AH and Gpx activities in various stages of chronic kidney disease (CKD), and further investigated whether erythropoietin (EPO) administration in these patients has any influence on the enzyme activities.

Methods. Thirty-six patients (19 men and 17 women) with CKD (stages 1 to 5) participated in the study. Thirteen of them presented with CKD stage 1 to 2 (group I), whereas 23 patients presented with CKD stage 3 to 5 and randomized into two groups (i.e., groups II and III). Patients of group II (N = 11) were administered EPO subcutaneously, 50 units per kg once per week. In group III (N = 12), EPO was initiated only when the hemoglobin (Hb) levels decreased during follow-up to less than 9 g/dL. All patients were seen on an outpatient basis at 2 and 4 months. Fifteen normolipidemic age- and sex-matched healthy volunteers also participated in the study and were used as controls. The PAF-AH and Gpx activities were determined in isolated washed erythrocytes.

Results. The erythrocyte-associated PAF-AH and Gpx activities were higher in all CKD patient groups at baseline compared to controls, the groups II and III exhibiting significantly higher enzyme activities compared with group I. In all studied populations, both enzyme activities were negatively correlated with the creatinine clearance values. Importantly, the PAF-AH and Gpx activities were progressively decreased during the follow-up in patients not treated with EPO (group III), a phenomenon not observed in patients receiving EPO (group II), or in patients of group I. This reduction in enzyme activities was positively correlated with the decrease in the creatinine clearance values in patients of group III.

Key words: anemia, chronic kidney disease, erythrocyte, erythropoietin, PAF-acetylhydrolase.

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Conclusion. Significant alterations in the erythrocyte-associated PAF-AH and Gpx activities related to the disease stage are observed in CKD patients. Administration of EPO prevented the reduction in enzyme activities observed during the progression of the renal insufficiency, thus preserving the erythrocyte defense mechanisms against oxidative stress.

Platelet-activating factor (PAF, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a proinflammatory phospholipid mediator that is involved in several physiopathologic conditions, and may have pivotal roles in various syndromes or diseases, including renal diseases [1, 2]. Studies in experimental animals and in humans indicate that PAF may be an important mediator of renal damage, suggesting that its production and action in the kidney may be dysregulated in disease states [2].

Although several enzymes are involved in PAF metabolism, a prominent role in the regulation of PAF activity in vivo plays PAF-acetylhydrolase (PAF-AH) (EC 3.1.1.47), an enzyme that expresses a Ca2+-independent phospholipase A2 activity and degrades PAF and oxidized phospholipids by catalyzing the hydrolysis of the ester bond at the sn-2 position [3]. Four intracellular (Ia, Ib, II, and erythrocyte form) and one secreted (plasma form) isoenzyme have been described. Among the intracellular forms, the erythrocyte PAF-AH is a distinct 25-kD protein [4] that is found primarily in the cytosol, whereas a small proportion of the total enzyme activity exists also in the membrane fraction [4]. In addition to PAF, the erythrocyte PAF-AH hydrolyzes oxidatively fragmented phospholipids, but it does not recognize as substrates structural membrane nonoxidized phospholipids [4]. By acting as a scavenger of oxidized phospholipids, the erythrocyte PAF-AH may play important roles in determining functional properties of these cells. It has been suggested that it may prevent the enhancement of lipid peroxidation and the formation of toxic compounds, thus preventing the damage of cell membrane and maintaining the membrane integrity of the cell [4].
Furthermore, previous studies have shown that PAF-AH may play an important role in maintaining the erythrocyte deformability [5, 6].

Increased oxidative stress is a feature characteristic of patients with chronic kidney disease (CKD), as indicated by the increase in various markers of oxidative stress as well as by the reduction in antioxidant levels [7, 8]. Oxidative stress in CKD patients may contribute to malnutrition, anemia, resistance to erythropoietin (EPO), and to increased incidence of atherosclerosis and cardiovascular disease [7, 9–11]. Anemia in CKD is mainly treated with administration of EPO [12, 13], and a number of studies point out that EPO treatment slows the progression of renal disease [14, 15], and this phenomenon can be attributed not only to the correction of anemia, but also to the influence of inflammation and oxidative stress in these patients [7].

Erythrocytes represent an important component of the antioxidant capacity of blood, comprising, in particular, intracellular enzymes, including PAF-AH and glutathione peroxidase (Gpx). Significant changes in the erythrocyte antioxidant systems have been reported in CKD patients [10, 16, 17], whereas EPO administration seems to improve the erythrocyte antioxidant capacity [7, 18, 19]. To the best of our knowledge, there is a paucity of data concerning the erythrocyte-associated PAF-AH activity in CKD patients. By contrast, the plasma or serum enzyme activity in CKD has been previously studied by our group [20] and others [21], and these studies have provided contrasting results showing either that the plasma enzyme activity is significantly higher [20] or it remains unchanged [21] in CKD patients compared with controls. We undertook the present study in order to evaluate possible alterations in the enzyme activity in various stages of CKD and to investigate whether EPO administration in these patients has any influence on the erythrocyte PAF-AH.

METHODS

Study population

Thirty-six patients (19 men and 17 women, mean age 63 years, range 36–81 years) with CKD (stages 1 to 5) participated in the study. Thirteen of them presented with CKD stage 1 to 2 (blood hemoglobin, Hb, levels 14.5 ± 1.3 g/dL, hematocrit, Ht, values 39.5 ± 2.1%) and categorized into patient group I. Twenty-three patients were presented with CKD stage 3 to 5 and exhibited anemia (blood Hb levels 10.0 ± 0.6 g/dL, Ht values 31.00 ± 1.16%). All patients participated in this study were randomly selected from a total population of 88 patients who participated in the recently published randomized controlled trial [15]. Exclusion criteria were diabetic nephropathy, the presence of an easily correctable cause of anemia such as iron deficiency (transferrin saturation <20%), vitamin B12 and folate deficiency, transfusion dependency, hemolysis, the presence of systemic diseases, infections or inflammatory conditions that might inhibit the effect of EPO [7, 9, 11], age <18 years or >85 years, uncontrollable hypertension (blood pressure <130/85 mm Hg), proteinuria >2 g per 24 hours, serum albumin <3.5 g/dL, hepatic insufficiency, active hepatitis, uncontrollable hypothyroidism, chronic alcoholism, congestive heart failure (New York Heart Association class III or IV), severe obesity (body mass index ≥40 kg/m2), nPNA = <0.8 g/kg/day, history of seizures of thrombotic episodes, pregnancy, lactation, known hypersensitivity to EPO alpha, use of hypolipidemic drugs, use of corticosteroids in the previous 6 months, and use of EPO in the previous 6 months. Because of concerns about induced EPO resistance at the time the study was launched [7, 10, 11, 22–24], patients receiving angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers could participate if they were willing to stop these drugs and adequate blood pressure control could be achieved with other drugs for at least 2 months before proceeding with randomization.

Patients of group I with CKD stage 1 to 2 were left untreated, whereas patients with CKD stage 3 to 5 were randomized into two groups (i.e., groups II and III). The subjects of group II (N = 11) were administered EPO (Eprex, Janssen-Cilag Pharmaceutical, Athens, Greece) 50 units per kg subcutaneously once per week. In group III (N = 12), EPO was initiated only when the Hb levels decreased during follow-up to less than 9 g/dL (confirmed on a second measurement within less than 1 week). Details of the protocol regarding visits during the follow-up, dose schedules, and titration of EPO are described in our previous study [15].

Fifteen normolipidemic age- and sex-matched healthy volunteers also participated in the study and were used as controls. The clinical and laboratory characteristics of the study population at baseline are shown in Table 1. All study participants gave informed consent for the investigation, which was approved by the Ethical Committee of the University Hospital of Ioannina.

Preparation of cytosol and membranes of erythrocytes

Venous blood samples (12 mL) were collected after a 14-hour overnight fast in polystyrene vacuum tubes with acid citrate as coagulant. Blood cells were sedimented by centrifugation at 1500g for 20 minutes, and the supernatant plasma, along with the buffy coat, was removed by aspiration. The remaining erythrocyte pellet was then washed 3 times with a 4.2 mmol/L HEPES buffer solution, pH 7.4. A small portion of this suspension was used for erythrocyte count. The remaining suspension was centrifuged as above and the sedimented erythrocytes were lysed by mixing with 5 volumes of a 7 mmol/L sodium phosphate buffer solution, pH 7.4, for 20 minutes
Table 1. Clinical and laboratory characteristics of the study population

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>15</td>
<td>13</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>Age years (±SD)</td>
<td>59 (32–79)</td>
<td>63 (37–76)</td>
<td>61 (36–81)</td>
<td>66 (45–79)</td>
</tr>
<tr>
<td>Females/males</td>
<td>12/10</td>
<td>5/8</td>
<td>5/6</td>
<td>7/5</td>
</tr>
<tr>
<td>Weight kg</td>
<td>69.2 ± 13.5</td>
<td>68.2 ± 10.3</td>
<td>72.3 ± 8.9</td>
<td>70.4 ± 9.4</td>
</tr>
<tr>
<td>History of hypertension, N (%)</td>
<td>–</td>
<td>68</td>
<td>93</td>
<td>84</td>
</tr>
<tr>
<td>Hb g/dL</td>
<td>15.4 ± 1.3</td>
<td>14.5 ± 1.3</td>
<td>10.0 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.2 ± 0.4&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ht%</td>
<td>40.6 ± 2.6</td>
<td>39.5 ± 2.1</td>
<td>30.8 ± 2.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31.2 ± 1.1&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Serum creatinine mg/dL</td>
<td>0.8 ± 0.12</td>
<td>1.00 ± 0.50</td>
<td>3.27 ± 0.99&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>3.39 ± 0.89&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Creatinine clearance mL/min</td>
<td>109.0 ± 6.2</td>
<td>87.2 ± 7.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.2 ± 6.5&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>23.9 ± 6.6&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>24-hour protein g</td>
<td>6.2 ± 0.15</td>
<td>0.65 ± 0.39</td>
<td>0.66 ± 0.39</td>
<td>0.57 ± 0.36</td>
</tr>
<tr>
<td>Total cholesterol mg/dL</td>
<td>202 ± 33</td>
<td>206 ± 30</td>
<td>232 ± 33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>231 ± 37&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Triglycerides mg/dL</td>
<td>104 ± 55</td>
<td>150 ± 20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>149 ± 41&lt;sup&gt;a&lt;/sup&gt;</td>
<td>153 ± 47&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>LDL-cholesterol mg/dL</td>
<td>135 ± 32</td>
<td>139 ± 32</td>
<td>160 ± 33</td>
<td>156 ± 33</td>
</tr>
<tr>
<td>HDL-cholesterol mg/dL</td>
<td>46 ± 13</td>
<td>45 ± 15</td>
<td>42 ± 10</td>
<td>45 ± 10</td>
</tr>
</tbody>
</table>

Abbreviations are: Hb, hemoglobin concentration; Ht, hematocrit values; nd, not determined. Values represent the mean ± SD, except for age, which is expressed in terms of median and range.

<sup>a</sup>P < 0.05 and <sup>b</sup>P < 0.001 vs. controls; <sup>c</sup>P < 0.001 vs. group I.

at 4°C [25]. The erythrocyte lysate was then centrifuged at 20,000g for 10 minutes at 4°C to obtain the supernatant (cytosol) and the sedimented erythrocyte membranes. The membrane fraction was then washed up to 4 times with the 7 mmol/L sodium phosphate buffer solution, until a colorless one was obtained. The membrane fraction was suspended in 200 μL of the 4.2 mmol/L HEPES buffer solution and stored along with the cytosolic fraction at −80°C.

Assay for PAF-acethylhydrolase activity

PAF-AH activity was measured by the trichloroacetic acid precipitation procedure using 90 μL of the erythrocyte total lysate or the cytosol or the erythrocyte membrane suspension, as the source of the enzyme, and [3H]-PAF (100 μmol/L final concentration) as a substrate. Incubations were performed for 10 minutes at 37°C. PAF-AH activity in both the cytosolic and membrane fraction was expressed as nmol PAF degraded per min per 10<sup>9</sup> cells or per g of Hb (total lysate or cytosolic fraction).

Assay for glutathione peroxidase activity

Gpx activity was determined spectrophotometrically as previously described [26], using a commercially available kit (Calbiochem, San Diego, CA, USA). Gpx activity was quantitated by measuring the change in absorbance at 340 nm caused by the oxidation of NADPH. One unit (IU) is defined as the amount of enzyme that will catalyze the oxidation of 1.0 mmol/L of NADPH to NADP per minute at 37°C, measured at 340 nm [26]. Fifteen μL of the total lysate or the cytosol (diluted 1/10 v/v with assay buffer) was used as the source of the enzyme. Gpx activity was expressed as IU per minute per 10<sup>9</sup> cells or per g of Hb.

Oxidation of intact erythrocytes in vitro

Washed erythrocytes were diluted with an equal volume of phosphate-buffered saline (PBS) buffer solution, pH 7.4. Two mL of this suspension corresponding to 252 mg of Hb or 372 × 10<sup>10</sup> erythrocytes, was submitted to oxidative stress in the presence of 0.2 mmol/L CuSO<sub>4</sub> and 1 mmol/L ascorbic acid in a shaking water bath for 1 hour at 37°C. Oxidation was performed in the absence or in the presence of EPO at various concentrations ranging from 1 to 8 IU/mL. The oxidation was terminated by addition of 10 μL 10 mmol/L EDTA. The copper-ascorbate treated erythrocytes were washed twice with PBS buffer solution, pH 7.4. The packed cells were lysed with 2 volumes of 7 mmol/L sodium phosphate buffer (pH 8.0) at −4°C for 20 minutes [25]. Lysates from untreated washed packed erythrocytes were also prepared in a similar way and served as the respective controls [27]. All lysates were used for the determination of the Hb concentration, as well as the activities of PAF-AH and Gpx.

Analytical methods

Products of lipid peroxidation were detected through the measurement of thiobarbituric acid–reactive substances (TBARS). TBARS were determined in EDTA-anticoagulated plasma by using a spectrophotometric assay, essentially as previously described [28]. Serum total cholesterol, high-density lipoprotein (HDL)-cholesterol, and triglyceride levels were determined on the Olympus AU560 Clinical Chemistry analyzer (Hamburg, Germany) as previously described [29]. Serum low-density lipoprotein (LDL)-cholesterol levels were calculated using the Friedewald formula. Creatinine and protein levels in serum and urine were measured by the Jaffé and the biuret methods, respectively, whereas albumin was determined by the bromocresol green method.
Table 2. Hb levels and Ht values in EPO-treated patients of group II and in untreated patients of group III

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group II Baseline</th>
<th>2 months</th>
<th>4 months</th>
<th>Group III Baseline</th>
<th>2 months</th>
<th>4 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb (g/dL)</td>
<td>10.0 ± 0.6</td>
<td>11.7 ± 0.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.2 ± 0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.2 ± 0.4</td>
<td>10.6 ± 0.5</td>
<td>10.4 ± 0.6</td>
</tr>
<tr>
<td>Ht (%)</td>
<td>30.8 ± 2.2</td>
<td>36.0 ± 2.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>37.6 ± 2.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31.2 ± 1.1</td>
<td>31.6 ± 1.0</td>
<td>32.1 ± 1.5</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>81.1 ± 8.1</td>
<td>80.4 ± 7.8</td>
<td>81.1 ± 8.1</td>
<td>87.7 ± 2.7</td>
<td>87.4 ± 3.2</td>
<td>86.4 ± 2.3</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>26.3 ± 3.2</td>
<td>26.2 ± 3.2</td>
<td>26.2 ± 3.0</td>
<td>28.7 ± 1.4</td>
<td>28.7 ± 1.1</td>
<td>28.5 ± 1.3</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>32.4 ± 1.2</td>
<td>32.5 ± 1.3</td>
<td>32.4 ± 1.0</td>
<td>32.8 ± 0.7</td>
<td>32.9 ± 0.9</td>
<td>33.0 ± 0.8</td>
</tr>
<tr>
<td>Creatinine clearance (mL/min)</td>
<td>20.2 ± 6.5</td>
<td>20.4 ± 7.2</td>
<td>19.2 ± 7.0</td>
<td>23.9 ± 6.6</td>
<td>22.8 ± 7.3</td>
<td>18.3 ± 5.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Abbreviations are: MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration. Values represent the mean ± SD.

<sup>a</sup> P < 0.05 and <sup>b</sup> P < 0.001 vs. baseline values.

**Statistical analysis**

Values are expressed as mean ± SD, except for age, which is expressed in terms of median and range. A comparison of continuous variables was performed by unpaired two-tailed t test, as well as by one-way analysis of variance with multiple Scheffe-type comparisons. The distribution of erythrocyte PAF-AH and Gpx activities was tested for normality using the Kolmogorov-Smirnov test. Because the enzyme activity values did not significantly differ from normal distribution in all studied groups, one-way analysis of variance (with Fisher least significant difference method for multiple comparisons) was used to compare enzyme activities among groups. Correlations between enzyme activities and covariates were estimated using Pearson’s correlation coefficients. Pearson’s χ² test was used to assess the difference in prevalence between the studied groups. Significance levels were set at 0.05 in all cases.

**RESULTS**

**Characteristics of the study population**

As shown in Table 1, no difference in the age, sex distribution, and weight values was observed among the studied groups. Patients of group I exhibited significantly lower creatinine clearance values at baseline compared with controls, whereas no significant differences were observed in the Hb and Ht values or in the lipid parameters between these two groups. As expected, patient groups II and III exhibited significantly lower Ht and Hb levels at baseline compared either with controls or with patients of group I. Baseline serum creatinine levels, as well as total cholesterol, LDL-cholesterol, and triglyceride levels were significantly higher in groups II and III, whereas creatinine clearance levels were significantly lower compared either to group I or to controls. As expected, the Hb values were positively correlated with the creatinine clearance values (r = 0.813, P < 0.001) in the whole studied population. Finally, no difference was observed in all studied parameters between groups II and III.

**Hematologic and biochemical parameters during the follow-up**

All patients were seen on an outpatient basis at 2 and 4 months. There were no significant changes compared with the baseline values in the Hb and Ht levels in the erythrocyte markers, MCV, MCH, and MCHC, as well as in the creatinine clearance values in patients of group I, either at 2 or at 4 months after the onset of the study (data not shown). No significant changes were also observed in all hematologic parameters in untreated patients of group III, whereas the creatinine clearance values were progressively reduced at 2 or at 4 months, reaching a statistical significance at 4 months from the onset of the study (Table 2). EPO administration in patients of group II caused a significant progressive increase in Hb and Ht levels during the follow-up compared to the baseline values, whereas the creatinine clearance values remained unaltered (Table 2). Finally, no significant changes in the erythrocyte markers were observed in this patient group during the follow-up (Table 2).

**Erythrocyte PAF-acetylhydrolase activity**

PAF-AH activity was determined in the erythrocyte cytosolic and membrane fractions. As shown in Figure 1A, the enzyme activity in the erythrocyte cytosol of all patient groups at baseline was significantly higher compared to controls. Importantly, CKD stage 3 to 5 patient groups (groups II and III) exhibited significantly higher enzyme activity compared with group I. Similar results were obtained when the enzyme activity was expressed in nmol/g Hb/min (198 ± 45, 277 ± 29, 440 ± 90, and 430 ± 116 for controls, group I, group II, and group III, respectively, P < 0.05 for comparisons between group I and controls or groups II and III, P < 0.001 for comparisons between group II or III and controls). Similarly, the PAF-AH activity associated with the membrane fraction in all patient groups was significantly higher compared with controls, group I having significantly lower enzyme activity compared with either group II or group III (Fig. 1B). In the control group, as well as in each patient group, the enzyme activity associated with the membrane fraction comprises 0.83 ± 0.07% of the total.
activity in erythrocytes (the sum of cytosolic and membrane-associated PAF-AH activity), and this observation is in accordance with previously published results [4, 30]. We should note that the PAF-AH activity in the cytosol was negatively correlated with the creatinine clearance values in all studied population (Fig. 2). Similarly, a negative correlation was observed between the membrane-associated enzyme activity and creatinine clearance values in all studied population, \( r = -0.799, P < 0.001 \). In all studied groups, the Gpx activity was negatively correlated with the creatinine clearance values, \( r = -0.439, P < 0.05 \). During the follow-up, the Gpx activity was progressively decreased at 2 and 4 months in untreated patients of group III, whereas it did not significantly change in patients of group II (Fig. 5B). The reduction in Gpx activity in group III was positively correlated with the decrease in the creatinine clearance values, \( r = 0.751, P < 0.05 \).

**Erythrocyte glutathione peroxidase activity**

The baseline values of the Gpx activity in the total cell lysate were significantly higher in both patient groups compared to controls, the group I patients presenting significantly lower enzyme activity compared with either group II or group III (Fig. 5A). Similar results were obtained when the enzyme activity was expressed in IU/g Hb (9.98 ± 3.33, 11.30 ± 2.88, 15.20 ± 4.70, and 15.50 ± 5.00 for controls, group I, group II, and group III, respectively). In all studied groups, the Gpx activity was negatively correlated with the creatinine clearance values, \( r = -0.439, P < 0.05 \). During the follow-up, the Gpx activity was progressively decreased at 2 and 4 months in untreated patients of group III, whereas it did not significantly change in patients of group II (Fig. 5B). The reduction in Gpx activity in group III was positively correlated with the decrease in the creatinine clearance values, \( r = 0.751, P < 0.05 \).

**Plasma TBARS levels**

To investigate whether there is any role of oxidative stress in the alterations described above of the erythrocyte PAF-AH and Gpx activities, we determined the concentration of TBARS in plasma as an indicator of free radical-induced lipid peroxidation. TBARS levels were determined in all studied groups and expressed as nmol of malondialdehyde (MDA) equivalents/mL of plasma. Patients of group I exhibited higher TBARS levels at baseline compared with controls; however, these did not reach statistical significance. By contrast, patients of groups II and III exhibited significantly increased baseline plasma
TBARS levels compared either to controls or to patients of group I (Fig. 6A). Finally, no significant differences were observed during the follow-up in patients of either group II or group III (Fig. 6B).

Effect of erythrocyte oxidation on PAF-AH and GPX activities in vitro

To further investigate whether oxidative stress plays any role in the alterations described above of the erythrocyte-associated enzyme activities, as well as the possible effect of EPO, we performed experiments in vitro in which we studied the effect of erythrocyte oxidation on the PAF-AH and Gpx activities in the absence or in the presence of EPO. Erythrocytes from all studied groups, prepared at baseline, were submitted to oxidation in vitro, as described in Methods. Erythrocyte oxidation resulted in the cell lysis, which was determined as an increase in Hb concentration in the supernatant after the cell sedimentation. No difference in the Hb concentration in the supernatant of erythrocytes after oxidation was observed among the studied groups (data not shown). In addition to hemolysis, oxidation of erythrocytes from all studied groups leads to a significant decrease in the PAF-AH and Gpx activities, measured in the total lysate, which was similar in all studied groups. Figure 7A and B demonstrates the results from the oxidation experiments using
erythrocytes from the control group. It should be noted that the decrease in both enzyme activities could not be attributed in enzyme loss due to hemolysis because both enzyme activities were expressed per g of Hb. Addition of EPO before the initiation of the erythrocyte oxidation at various concentrations ranging from 1 to 8 IU/mL did not prevent either the hemolysis or the reduction in both enzyme activities. Figure 7A and B demonstrates the lack of any effect of 2 IU/mL of EPO on the oxidation parameters of erythrocytes from the control group.

**DISCUSSION**

The results of the present study show for the first time that the erythrocyte-associated PAF-AH activity in patients with CKD is progressively increased as a function of the disease stage, the patients in stages 3 to 5 having significantly higher enzyme activity compared to those of stage 1 to 2. Furthermore, a negative correlation between the enzyme activity and the degree of renal insufficiency (expressed as creatinine clearance values) was observed, suggesting a close relationship between the above-mentioned enzyme alterations and renal insufficiency. However, in CKD patients (stages 3 to 5), a progressive decrease in the enzyme activity is observed in parallel to the progress of renal insufficiency, which is significantly attenuated by EPO treatment. Similar results were obtained for the erythrocyte-associated Gpx activity, and these are in accordance to previously published data for the erythrocyte antioxidant systems [16].

It has been suggested that the erythrocyte PAF-AH is important for the cell physiology because it protects the cell against oxidation-induced damage by degrading the toxic products of phospholipid peroxidation without acting on structural membrane nonoxidized phospholipids [4]. Such cytotoxic phospholipids can be generated during erythrocyte peroxidation, and are capable of damaging the erythrocyte membranes [31–33]. Furthermore, the hydrolysis of oxidized phospholipids by PAF-AH allows the subsequent restoration of membrane integrity by reacylation of the lyso derivatives with long chain fatty acyl groups [34, 35]. It is known that damage of erythrocyte membranes by lipid peroxidation leads to deterioration of the rheologic properties of these cells [36], a phenomenon that is observed in CKD patients and may contribute to the reduced erythrocyte survival observed in these patients [37]. Thus, by acting as a scavenger of oxidized phospholipids, the erythrocyte PAF-AH may contribute in maintaining the membrane integrity and the
erythrocyte deformability. This is also supported by previously published results showing that the erythrocyte PAF-AH activity is positively correlated with erythrocyte deformability indices in stroke patients [5]. Oxidized phospholipids formed on the membrane of erythrocytes may also represent a signal for the cell recognition by scavenger receptors of macrophages, primarily the CD36, thus leading to their subsequent elimination from the circulation and to a decrease in their life span in blood [33, 38]. In this context, it has been demonstrated that in chronic uremia the percentage of annexin V-positive erythrocytes (annexin V recognizes phosphatidylserine molecules as well as oxidized phospholipids) increases with the progressive decline in renal function, and may contribute to the reduced erythrocyte life span and to the anemia that characterizes these patients [39]. Consequently, PAF-AH could be an important enzyme for erythrocytes, which may work in concert with the well-known intracellular antioxidant systems to protect them from oxidative damage, to conserve the cell functionality, and to prolong their life span in blood, which is reduced in CKD. Overall, we may suggest that the augmentation of erythrocyte PAF-AH observed at baseline in all groups of our CKD patients represents an adaptive mechanism of these cells to the increased oxidative stress, which has been previously shown to occur in CKD patients [40].

The increased oxidative stress in CKD patients is also supported by the results of the present study showing a significant elevation of TBARS in the plasma of patients with CKD stage 3 to 5. Because erythrocytes are anucleate cells and contain only preformed proteins, the enhancement in PAF-AH and Gpx activities could be attributed to exposure of the erythropoietic stem cells to increased oxidative stress with a consequent adaptive response. Despite the increase in the erythrocyte PAF-AH activity, and the important role that it may play for erythrocyte deformability and life span in CKD, it did not prevent the anemia observed in our patients of groups II and III, thus suggesting that the low rate of the erythrocyte production, rather than the decrease in the cell life span in blood, could be the major determinant of the anemia observed in CKD.

An important observation of the present study is that during the follow-up of patients with CKD stage 3 to 5 who were not treated with EPO, the erythrocyte PAF-AH activity was significantly decreased, and this reduction was positively correlated with the progression of renal insufficiency (decrease in creatinine clearance values). Similar results were obtained for the Gpx activity. Based on this data we may suggest that there is a critical point during the disease progression in which the erythropoietic stem cells of predialysis CKD patients lose their adaptive mechanisms, which were responsible for the increase in the erythrocyte PAF-AH and Gpx activities. Consistent with this hypothesis are the previously reported results showing that CKD patients before hemodialysis, or CKD patients receiving either hemodialysis or continuous ambulatory peritoneal dialysis, exhibit lower erythrocyte SOD and Gpx activities, as well as glutathione levels [16, 17, 41, 42].

We should emphasize that the above-mentioned reduction in PAF-AH and Gpx activities was not observed in patients treated with EPO, neither of these patients exhibited a further decrease in creatinine clearance values. According to our recently published results, EPO administration in these patients significantly slows the progression of renal insufficiency, and delays the initiation of renal replacement therapy [15]. Thus, based on the important role that the erythrocyte PAF-AH may have for the cell functionality and life span in CKD, we may suggest that the EPO-induced inhibition of the progressive inactivation of the erythrocyte PAF-AH could significantly contribute to the retardation of the progression of renal insufficiency observed in CKD patients receiving EPO [15].

To investigate whether the reduction in erythrocyte PAF-AH during the follow-up was due to any effect that oxidative stress may have on the enzyme activity, we submitted erythrocytes to oxidation in vitro. Under our experimental conditions, oxidation induced a significant decrease in the PAF-AH activity, and this finding is in accordance with previously published results suggesting that this enzyme is sensitive to oxidation [4]. EPO at any concentration used did not prevent the oxidation-induced reduction in the enzyme activity in vitro. Based on the above results, the decrease in enzyme activity observed in CKD patients could be attributed to the erythrocyte exposure in an increased oxidative stress. However, according to our results, the plasma TBARS concentration did not increase either at 2 months or at 4 months of follow-up; thus, this hypothesis is unlikely. Furthermore, the prevention of the enzymatic activity reduction by EPO could not be attributed to any antioxidant effect of this hormone because neither had it protected the enzyme from the oxidation-induced inactivation in vitro, nor had it reduced the plasma levels of TBARS during the follow-up. It has been reported that the erythrocyte PAF-AH activity is reduced as erythrocyte density is increased (i.e., with in vivo aging of these cells). This may be due to enzyme consumption induced by a mechanical stress of repeated change in cell shape, or by its release with vesicles through microvesiculation [43, 44]. Thus, we may hypothesize that the EPO-induced prevention of the PAF-AH inactivation could be due to the hormone-induced production of new erythrocytes, thus resulting in the increase of the number of young cells in the circulation, a phenomenon that does not occur in patients not treated with EPO. The same phenomenon may occur for the Gpx, which is not decreased in our EPO-treated patients, since previous studies have demonstrated that young erythrocytes contain increased...
CONCLUSION

CKD patients exhibit significant alterations in the erythrocyte-associated PAF-AH activity that are related to the disease stage and to the progression of the renal insufficiency, and may represent a defensive mechanism against the erythrocyte deformability and decreased life span in blood observed in these patients. Administration of EPO prevented the decrease in the erythrocyte PAF-AH activity observed during the progression of the renal insufficiency; thus, it preserves the erythrocyte protection mechanisms against oxidative stress.

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