PAF-acetylhydrolase activity in plasma of patients with chronic kidney disease. Effect of long-term therapy with erythropoietin

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Abstract

**Background.** Platelet activating factor acetylhydrolase (PAF-AH) is a Ca²⁺-independent phospholipase A₂ that is secreted mainly from monocytes/macrophages. In human plasma, PAF-AH is associated primarily with low-density lipoprotein (LDL), while a small proportion of enzyme is associated with high-density lipoprotein (HDL). The ratio of HDL–PAF-AH to total plasma enzyme activity may represent a potential marker of atherogenicity. We evaluated possible alterations of lipoprotein-associated enzyme activity in Chronic Kidney Disease (CKD) patients, stages 3–4, and further investigated whether long-term therapy with recombinant human erythropoietin (epoetin) has any influence on the plasma PAF-AH activity in vivo or on the enzyme activity secreted from peripheral blood monocytes (PBMs), in vitro.

**Methods.** Forty-eight patients, 28 men and 20 women, with CKD (stages 3–4) participated in the study. Patients were randomized into groups I and II. Patients of group I (n = 28) were administered subcutaneously epoetin, 50 units/kg once per week. The Hb target was 13 g/dl. In group II (n = 20), epoetin was initiated only when the Hb levels decreased during follow-up to less than 9 g/dl. All patients were seen on an outpatient basis at 2, 4 and 6 months. Twenty-two normolipidemic age- and sex-matched healthy volunteers also participated in the study and were used as controls.

**Results.** The PAF-AH activity in plasma of both patient groups at baseline was higher compared to controls, whereas no difference in the HDL–PAF-AH activity was observed among the studied groups. Thus, the ratio of HDL–PAF-AH to the plasma enzyme activity was significantly lower in both patient groups compared to controls. Epoetin administration in the patients of group I was associated with a significant increase in the plasma PAF-AH and in HDL–PAF-AH activities 2 months after treatment, which remained stable for up to 6 months of therapy, a phenomenon not observed in untreated patients of group II. Thus, the ratio of HDL–PAF-AH to the plasma enzyme activity was significantly increased in patients of group I compared to the baseline values, a phenomenon not observed in patients of group II. In vitro treatment with epoetin of PBMs from patients of group I (undergoing therapy with epoetin) resulted in a dose-dependent increase in total and secreted enzyme activity, a phenomenon not observed in patients of group II who did not receive therapy with epoetin. This suggests that the in vivo increase in lipoprotein-associated PAF-AH observed in patients treated with epoetin may be attributed to the drug-induced enhanced secretion of PAF-AH from PBMs of these patients.

**Conclusions.** CKD patients of stages 3–4 are characterized by an increase in plasma PAF-AH activity and a low ratio of HDL–PAF-AH to total plasma enzyme activity. Long-term therapy with epoetin may improve this atherogenic ratio thus this drug may play an important antiatherogenic role in CKD.

**Keywords:** chronic kidney disease; epoetin; lipoproteins; monocytes; PAF-acetylhydrolase; paraoxonase-1

Introduction

Platelet-activating factor (PAF, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a potent phospholipid mediator of inflammation. Studies in humans and in experimental animals 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 [1]. In the kidney, PAF is synthesized by infiltrating cells as well as by mesangial...
cells [1] and plays an important role in renal haemodynamic changes as well as in the recruitment of inflammatory cells into glomeruli observed in glomerular immune injury [2,3]. A prominent role in the regulation of PAF activity in vivo is played by PAF-acetylhydrolase (PAF-AH), an enzyme that expresses Ca²⁺-independent phospholipase A₂ activity and degrades PAF by catalysing the hydrolysis of the ester bond at the sn-2 position [4]. PAF-AH also hydrolyses phospholipids containing oxidatively fragmented residues at the sn-2 position. Such oxidized phospholipids may play key roles in inflammation and particularly in vascular inflammation and atherosclerosis [4,5].

In human plasma, PAF-AH is associated with lipoproteins, primarily with low-density lipoprotein (LDL), while a small proportion of total enzyme activity is associated with high-density lipoprotein (HDL) [4]. Thus, this enzyme is also denoted as lipoprotein-associated phospholipase A₂ (Lp-PLA₂) [6]. The main cellular source of plasma PAF-AH are cells of haematopoietic origin, primarily monocytes/macrophages [7]. Indeed, peripheral blood monocytes (PBM) synthesize and secrete plasma PAF-AH activity as they differentiate into macrophages [7].

Plasma PAF-AH (which mainly reflects the LDL-associated enzyme) may be an inflammatory marker and data from large Caucasian population studies consistently report a positive association between plasma enzyme mass or activity and the risk for incident cardiovascular disease (CAD) events [8–10]. By contrast, the HDL-associated PAF-AH activity, although present at low levels in plasma, may significantly contribute to the antiatherogenic effects of this lipoprotein [4]. Thus, according to our previously published data, the ratio of HDL–PAF-AH to total plasma enzyme activity may represent a potential marker of atherogenicity [11]. An important role in the antiatherogenic properties of HDL is also played by paraoxonase-1 (PON1), an enzyme present in plasma exclusively associated with HDL [12].

Inflammation plays an important role in uraemia, and several studies have demonstrated the presence of inflammatory mediators in plasma of uraemic patients [13,14]. In this context, we had previously reported that plasma PAF-AH activity is increased in uraemic patients undergoing different dialysis procedures, and this increase is more profound in chronic ambulatory peritoneal dialysis patients [15]. Uraemic patients are at a greatly increased risk for CAD and this is only partly explained by an increased prevalence of conventional risk factors [16]. These patients are usually treated with recombinant human erythropoietin (epoetin) in an effort to correct anaemia [17]. Several studies have shown that epoetin may influence the inflammatory status in uraemic patients although further investigation is also required [18]. In addition to uraemic patients it was recently suggested that patients, with moderate to severe chronic kidney disease (CKD) (stages 3–5) may also exhibit an increased prevalence of inflammation [19,20]. Data from our group and others have suggested that a treatment of these patients with epoetin may slow the progression of renal disease, a subject that needs further investigation [21,22].

We undertook the present study in order to evaluate possible alterations in the PAF-AH activity in CKD patients, stages 3–4, and to investigate whether long-term therapy with epoetin has any influence on the plasma enzyme activity in vivo. We also performed an in vitro study to investigate whether epoetin could influence enzyme secretion from PBMs isolated from these patients, as these cells are the main source of the enzyme in plasma.

Subjects and methods

Study population

Forty-eight patients, 28 men and 20 women (mean age 63 years, range 36–81 years), with CKD (stages 3–4) participated in the study. These patients represent the total population of two centres (Divisions of Nephrology from the University Hospital of Ioannina and Hatzikosta General Hospital of Ioannina) among those participating in the recently published randomized controlled trial, which was completed in November 2002 [22]. All patients exhibited anaemia with haemoglobin (Hb) levels of 9.0–11.6 g/dl, and haematocrit (Ht) values 31.00 ± 1.16%. Serum creatinine levels ranged from 2 to 6 mg/dl. Exclusion criteria are described in details in our previous study [22]. It should be emphasized that none of the patients who participating in the present study received hypolipidaemic drugs (such as statins or fibrates), corticosteroids or epoetin in the previous 6 months. Among the 48 patients who participating in the study, 28 were administered epoetin subcutaneously (Eprex, Janssen–Cilag Pharmaceutical, Athens, Greece) 50 units/kg once per week (group I), whereas according to the protocol of our previous study in 20 patients (group II), epoetin should be initiated only when the Hb levels decreased to less than 9 g/dl during follow-up (confirmed on a second measurement within less than 1 week). The Hb target was 13 g/dl. Details of the protocol regarding visits during the follow-up, dose schedules and titration of epoetin are described in our previous study [22].

Twenty-two normolipidaemic 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 the study participants gave informed consent for the investigation, which was approved by the Ethical Committee of the University Hospital of Ioannina.

Analytical methods

Serum total cholesterol, HDL–cholesterol and triglyceride levels were determined on the Olympus AU560 Clinical Chemistry analyser (Hamburg, Germany) as previously described [11]. Serum LDL–cholesterol levels were calculated using the Friedewald formula. Creatinine levels in serum and urine as well as protein levels in urine were measured by the Jaffé and the biuret methods, respectively, whereas serum albumin was determined by the bromocresol green method.
Histology of hypertension

<table>
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<td>20</td>
</tr>
<tr>
<td>Age (years)</td>
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<td>61 (36–81)</td>
<td>66 (45–79)</td>
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<td>Sex (females/males)</td>
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<td>13/15</td>
<td>7/13</td>
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<td>History of hypertension (%)</td>
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<tr>
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<td>10.2 ± 0.4*</td>
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<td>Creatinine clearance (ml/min)</td>
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<td>226 ± 39**</td>
<td>221 ± 40**</td>
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<td>98 ± 23</td>
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<td>147 ± 32**</td>
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<tr>
<td>HDL-cholesterol (mg/dl)</td>
<td>49 ± 7</td>
<td>43 ± 11</td>
<td>45 ± 10</td>
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Hb, haemoglobin concentration; Ht, haematocrit values; nd, not determined.

Values represent the mean±SD, except for age, which is expressed in terms of median and range.

*P<0.001 and **P<0.05 vs controls.

Assay of PAF-acetylhydrolase activity

PAF-AH activity in plasma and in HDL-rich plasma, after the sedimentation of all apo B-containing lipoproteins with dextran-magnesium chloride (HDL–PAF-AH activity) was measured by the trichloroacetic acid (TCA) precipitation procedure using 1-O-hexadecyl-2-[3H-acetyl]-sn-glycero-3-phosphocholine ([3H]-PAF, DuPont-New England Nuclear, Boston, MA, USA) (100 μM final concentration) as a substrate [23]. Fifty microlitres of total plasma diluted 1:50, v/v with HEPES buffer, pH 7.4, or the HDL-rich plasma (diluted 1:3, v/v with HEPES) were mixed with HEPES in a final volume of 90 μl and used as the source of the enzyme. Incubations were performed for 10min and PAF-AH activity was expressed as nanomole PAF degraded per minute per millilitre of plasma.

PAF-AH activity was also determined by the same method in the supernatant of cultured PBMs (secreted PAF-AH) as well as in the cell lysates (cell-associated PAF-AH).

Isolation and culture of peripheral blood monocytes

To investigate whether epoetin influences the PAF-AH secretion from its cellular sources, we performed an in vitro study in which we investigated the possible effect of epoetin on enzyme secretion from PBMs during their differentiation into macrophages, since these cells represent the main cellular source of the enzyme in plasma [7]. Peripheral blood from eight healthy volunteers as well as from eight patients from each group (before the initiation of therapy with epoetin as well as at 2 months of follow-up) was drawn into EDTA-containing tubes. Blood was centrifuged and PBMs were isolated from the buffy coats as previously described [23]. Cells were cultured and grown in 24-well plastic tissue culture dishes (1 x 10⁶ cells/well) with RPMI medium, containing t-glutamine, 40 μg/ml gentamycin and 1% Nutridoma (Boehringer Mannheim GmbH, Germany) [23]. After 24h of culture, cells were treated for 48h with epoetin at concentrations ranging from 1 to 50IU/ml. After treatment, supernatants were recovered and the cell layers washed twice with PBS and then detached and lysed by the addition of 0.2ml of a lysis solution containing 1% EDTA and 0.1% Triton X-100. Both supernatants and cell lysates were centrifuged (500g for 10min at 4°C), stored at 4°C and analysed for PAF-AH activity and lactate dehydrogenase activity (kit from Boehringer Mannheim GmbH, Germany) within 24h from collection. Cell lysates were further analysed for their protein content, determined with the Lowry method. Viability under all culture conditions was determined by trypan blue dye exclusion and the absence of lactate dehydrogenase release; viability was >95%.

Measurement of paraoxonase-1 activity

PON1 activity towards paraoxon was also determined by the same method in the supernatant of cultured PBMs (secreted PAF-AH) as well as in the cell lysates (cell-associated PAF-AH). Eighty-five microlitres of either supernatant or cell-lysate were used as the source of the enzyme. Incubations were performed for 10min and PAF-AH activity was expressed as nanomole PAF degraded per hour per milligram of cell protein [23].

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 plasma PAF-AH and PON1 activities was tested for normality using the Kolmogorov–Smirnov test. Since the enzyme activity values did not significantly differ from normal distribution in all studied groups, one-way analysis of variance (with Fisher’s least significant difference method for multiple comparisons) was used to compare enzyme activities among groups. 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. As expected, patient groups I and II exhibited significantly lower Ht and Hb levels at baseline compared with controls. Baseline serum creatinine levels as well as total cholesterol, LDL–cholesterol and triglyceride levels were significantly higher in groups I and II, whereas creatinine clearance levels were significantly lower, compared to controls. Finally, no difference was observed in all studied parameters between groups I and II.

Haematological and biochemical parameters during the follow-up

All patients were seen on an outpatient basis at 2, 4 and 6 months. There were no significant changes, compared with the baseline values, in the Hb and Ht levels, in epoetin-untreated patients of group II, either at 2, 4 or at 6 months after the onset of the study, whereas the creatinine clearance values were progressively reduced during follow-up, reaching a statistical significance at 4 months from the onset of the study (Table 2). It should be noted that none of patients of group II needed epoetin therapy during follow-up. Epoetin administration in patients of group I caused a significant progressive increase in Hb and Ht levels during follow-up, compared to baseline values, whereas the creatinine clearance values remained unaltered (Table 2). Details about renal function have already been reported in our previously published randomized controlled trial [22]. Importantly, a gradual significant increase in HDL–cholesterol levels, from baseline to 6 months of therapy, was observed in group I patients treated with epoetin, a phenomenon not observed in untreated patients (Table 2).

Effect of epoetin on PAF-AH and PON1 activities

PAF-AH activity was determined in plasma and in HDL-rich plasma. As shown in Figure 1A, the enzyme activity in plasma of both patient groups at baseline was significantly higher compared to controls, whereas no difference in the HDL–PAF-AH activity was observed among the studied groups (Figure 1B). Thus, the ratio of HDL–PAF-AH to the plasma enzyme activity was 0.068±0.029 in controls and 0.048±0.019 in either group I or group II (P<0.05). These findings are in accordance with our previously published results [15]. Interestingly, epoetin administration in patients of group I resulted in a significant increase by 13% in the plasma PAF-AH activity, 2 months after treatment (P<0.001, compared to baseline values), which remained stable for up to 6 months of therapy, a phenomenon not observed in untreated patients of group II (Figure 2A). Similarly to total plasma enzyme activity, epoetin therapy led to a significant increase in HDL–PAF-AH activity by 27%, at 2 months after treatment (P<0.001, compared to baseline values) which also remained stable for up to 6 months of therapy, a phenomenon not observed in untreated patients of group II (Figure 2B). Thus, the ratio of HDL–PAF-AH to the plasma enzyme activity was significantly increased from 0.048±0.019 at baseline to 0.058±0.022 at 2 months of follow-up (P<0.05), a phenomenon not observed in patients of group II.

The serum PON1 activity towards paraoxon (in IU/l) was similar among the studied groups (62±42 and 82±54 for group I and II, respectively, 75±44 for controls) and the same phenomenon was observed for the enzyme activity towards phenylacetate (in IU/ml) (54±17 and 64±15 for group I and II, respectively, 64±22 for controls). Both PON1 activities were not significantly altered during the study period in either patient group (data not shown).

Effect of epoetin on PAF-AH secretion from monocytes in culture

PBMIs were isolated and incubated for 48 h with various concentrations of epoetin ranging from 1 to 50 IU/ml. There was a spontaneous increase in total (secreted plus cell-associated) PAF-AH activity in untreated cells (incubated in the absence of epoetin) from controls, attaining 96.4±14.1 nmol/mg cell protein/h at 24 h and

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<th>Parameters</th>
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<tr>
<td></td>
<td>Baseline</td>
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<tr>
<td>Hb (g/dl)</td>
<td>10.0±0.6</td>
<td>11.7±0.8**</td>
</tr>
<tr>
<td>Ht (%)</td>
<td>30.8±2.2</td>
<td>36.0±2.4**</td>
</tr>
<tr>
<td>Creatinine clearance (ml/min)</td>
<td>21.2±6.8</td>
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<td>Total-cholesterol (mg/dl)</td>
<td>226±39</td>
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<tr>
<td>Triglycerides (mg/dl)</td>
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<tr>
<td>LDL-cholesterol (mg/dl)</td>
<td>155±36</td>
<td>160±42</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dl)</td>
<td>43±11</td>
<td>47±11**</td>
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Hb, haemoglobin concentration; Ht, haematocrit values. Values represent the mean±SD.

*P<0.01 and **P<0.001 vs baseline values of the same group.
334.7 ± 46.6 nmol/mg cell protein/h at 72 h of culture. This increase reflected mainly the secreted enzyme activity, which was 58.0 ± 14.3 nmol/mg cell protein/h (60% of total) at 24 h and 230.3 ± 14.6 nmol/mg cell protein/h (69% of total) at 72 h of culture. A similar phenomenon was observed in cells from patients of either group I or II, at baseline. Thus, there were no observed differences in the total or the secreted enzyme activity among studied groups either at 24 h or at 72 h of culture (Figure 3A and B). Treatment of PBMs with epoetin in vitro, resulted in a dose-dependent increase of the total enzyme activity in cells from controls (13% at 1 IU/ml, 34% at 10 IU/ml and 63% at 50 IU/ml compared to the spontaneous increase in untreated cells). A similar dose-dependent increase was observed in the secreted enzyme activity (data not shown). Importantly, this phenomenon was not observed in cells from either patient group at baseline. Thus, the total PAF-AH activity (in nmol/mg cell protein/h) in untreated cells of groups I and II was 336.7 ± 100.0 and 341.6 ± 65.3, respectively, while in cells treated with 50 IU/ml epoetin the enzyme activity was 355.2 ± 151.0 and 360.2 ± 14.0, respectively. The secretion of PAF-AH was also studied from PBMs isolated from patients at 2 months of follow-up. As shown in Figure 4 (A and B), a spontaneous increase in total and secreted enzyme activity from PBMs was similar among the studied groups. However, when cells were treated with epoetin, a dose-dependent increase in total and secreted enzyme activity was observed in controls and patients of group I, undergoing therapy with epoetin, but not in patients of group II who did not receive therapy with epoetin (Figure 4A and B).

Discussion

The results of the present study show that the PAF-AH activity in plasma of patients with CKD stages 3–4 is significantly higher compared to controls, a phenomenon which was not observed for the HDL–PAF-AH. This finding is in accordance with our previously published data [15]. The higher enzyme activity in plasma of CKD patients could be attributed to an enhancement in the enzyme secretion from its main
Indeed PBMs do not express PAF-AH; however, upon including the differentiation state of these cells and the PAF-AH in monocytes is regulated by various factors, the cells/macrophages represent the major source of cellular sources and it is well established that monocytes/macrophages represent the major source of PAF-AH activity in plasma [7]. The expression of PAF-AH in monocytes is regulated by various factors, including the differentiation state of these cells and the degree of activation by proinflammatory mediators. Indeed PBMs do not express PAF-AH; however, upon differentiation into macrophages in vitro, PAF-AH mRNA becomes detectable and enzyme activity is secreted into the culture medium [7,23]. However, according to our results, the spontaneous secretion of enzyme activity from PBMs of CKD patients at baseline is similar to that of controls, thus, it is unlikely that this mechanism contributes to the increase in plasma PAF-AH activity observed in these patients. Recent data suggest that inflammation may play a role in moderate to severe CKD (stages 3–5) and these patients exhibit increased levels of inflammatory biomarkers such as C-reactive protein (CRP), interlukin-6, vascular cell adhesion molecule-1 and others [19,20,24]. Thus, we may hypothesize that the increase in plasma PAF-AH activity observed in the present study is attributed to the effect of the proinflammatory mediators on enzyme secretion by monocytes. However, this possibility is unlikely since most of the proinflammatory mediators inhibit PAF-AH expression by macrophages in vitro [25]. Furthermore, reduced PAF-AH activity has been observed in inflammatory diseases such as active juvenile rheumatoid arthritis, systemic lupus erythematosus, sepsis and Crohn’s disease as reviewed by Tselepis et al. [4]. It has been previously shown by our group [11,23] and others [26], that one of the important factors that determine plasma levels of PAF-AH is the rate of removal of LDL from the circulation. According to our results, CKD patients exhibited higher LDL–cholesterol levels compared to controls; thus the elevation in LDL–cholesterol levels could represent the major mechanism for the elevation of plasma PAF-AH activity in CKD, observed in the present study.

Importantly, the present study showed for the first time that in patients receiving therapy with epoetin, the plasma PAF-AH activity was further increased at 2 months after the initiation of therapy, despite the fact that the LDL–cholesterol levels remained unchanged. Consequently, the epoetin-induced increase in plasma PAF-AH activity could not be attributed to any drug effect on LDL metabolism. Thus, we asked whether the above phenomenon could be due to any drug effect on the enzyme secretion from PBMs. Indeed several studies have shown that aside from the stimulating effect on erythrocyte production, epoetin influences the production of various cytokines by whole blood cells or monocytes from haemodialysis patients, in vitro [27,28]. Furthermore, therapy with epoetin alters the plasma levels of several inflammatory markers in dialysis patients [18]. Our results showed for the first time that epoetin stimulates PAF-AH secretion from PBMs from controls, in vitro; however, this phenomenon was not observed in PBMs from CKD patients at baseline. Importantly, therapy with epoetin for 2 months restores the ability of PBMs to respond to the epoetin stimulation in vitro for PAF-AH secretion, without influencing the spontaneous PAF-AH secretion. Consequently, we may suggest that PBMs from CKD patients (stages 3–4) are defective in their ability to respond to epoetin stimulation for PAF-AH secretion in vitro, but not as concern their ability to spontaneously secrete the enzyme during culture. This difference in the cell behaviour could be explained by the fact that the promoter of the PAF-AH gene contains seven MS2 and 11 STAT binding consensus sequences [25]. The multiple MS2 binding sites may be primarily responsible for the gene expression during cell differentiation (spontaneous PAF-AH secretion), whereas the STAT consensus sequences may mediate the effects of inflammatory mediators on PAF-AH gene expression [4]. Based on the aforesaid results, we may suggest that the increase in PAF-AH activity observed in plasma of CKD patients treated with epoetin compared to the baseline values, is due to the restoration of the PBMs ability to respond to epoetin stimulation, as well as to the fact that epoetin does not influence the increased serum LDL–cholesterol levels.

We have previously shown that the ratio of HDL–PAF-AH to total plasma enzyme activity might be useful as a potential marker of atherogenicity [11].
According to our results, this ratio in CKD patients is significantly increased (improved) by epoetin administration, a phenomenon primarily due to the higher percent increase in HDL–PAF-AH activity compared to that of total plasma enzyme activity. The increase in HDL–PAF-AH activity observed in epoetin-treated patients could be attributed to the increased secretion of PAF-AH from monocytes induced by epoetin therapy, as well as to the increased levels of serum HDL–cholesterol. The latter phenomenon is currently under investigation in our laboratory. However, the PON1 activity, which is exclusively associated with HDL in plasma, is not affected by epoetin administration; thus we may suggest that the increase in HDL–PAF-AH activity is primarily due to the enhancement of enzyme secretion from PBMs. Several lines of evidence suggest that HDL–PAF-AH activity may contribute substantially to the anti-oxidant and anti-inflammatory effects of HDL; thus this activity may be an important component of the multiple mechanisms by which HDL slows the progression of atherosclerosis [4]. Consequently, the increase in HDL–PAF-AH activity by epoetin could represent an important antiatherogenic effect of this drug.

In conclusion, the present study shows that CKD patients of stages 3–4 are characterized by an increase in plasma PAF-AH activity and a low ratio, HDL–PAF-AH to total plasma enzyme activity. Long-term therapy with epoetin significantly improves this athero- genic ratio, thus this drug may play an important antiatherogenic role in CKD.

Conflict of interest statement. None of the authors have conflicts of interest to declare.
Plasma PAF-AH in CKD. Effect of epoetin

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