Plasma Levels and Metabolism of AcSDKP in Patients With Chronic Renal Failure: Relationship With Erythropoietin Requirements

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● ***N*-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP) is a physiological inhibitor of hematopoiesis that is maintained at stable levels in normal plasma. Its degradation in vivo and in vitro by angiotensin-converting enzyme (ACE) accounts for the high plasma concentrations of AcSDKP in patients treated with ACE inhibitors. Because ACE inhibitors can induce anemia in some patients, we measured plasma AcSDKP concentrations in 176 patients with chronic renal failure: 120 hemodialysis (HD) and 56 nondialysis (nD) patients, 39 of whom were administered ACE inhibitors. We studied the relationships between AcSDKP levels, hematologic parameters, and recombinant human erythropoietin (rHuEPO) requirements in these patients. AcSDKP levels were significantly greater in HD (10.3 ± 3.9 pmol/mL) and nD (3.1 ± 1.8 pmol/mL) patients not administered ACE inhibitors than controls (1.8 ± 0.2 pmol/mL). In all patients, treatment with ACE inhibitors significantly increased these levels fourfold. HD sessions significantly decreased AcSDKP concentrations by 66% and reduced the predialysis in vitro half-life of AcSDKP (270 ± 109 minutes) to values (182 ± 67 minutes) not significantly different from those of controls or nD patients. Most HD patients treated with ACE inhibitors had AcSDKP levels greater than 24 pmol/mL (the greatest concentration found in other nD and HD patients). Only in this group of patients did weekly doses of rHuEPO correlate with AcSDKP levels. Our results show that renal function is essential to maintain stable AcSDKP plasma levels, and at high levels, AcSDKP acts as a uremic toxin causing partial resistance to erythropoietin and inhibiting erythropoiesis.**

INDEX WORDS: *N*-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP); anemia; chronic renal failure (CRF); hemodialysis (HD); erythropoietin (EPO); angiotensin-converting enzyme (ACE) inhibitors.

Editorial, p. 649

N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP) is a physiological negative regulator of the proliferation of hematopoietic stem cells. In vivo, it protects murine hematopoietic stem cells and spleen colony-forming units from the toxicity of irradiation or cytosine arabinoside by blocking their S phase entry.[1-3] In vitro, it inhibits the proliferation of human and murine hematopoietic stem cells and progenitor cells, as well as lymphocytes.[4-6] This tetrapeptide is produced in vitro by multiple cell types and is maintained in normal plasma at stable concentrations.[7,8] Angiotensin-converting enzyme (ACE) degrades AcSDKP both in vitro and in vivo.[9-11]

In healthy volunteers treated for 15 days with an ACE inhibitor (enalapril, 20 mg/d), plasma concentrations and daily urinary excretion of AcSDKP were permanently increased four- to fivefold.[12] Interestingly, this treatment modified concentrations of hematopoietic progenitors physiologically present in blood.[12] High plasma levels of AcSDKP also have been found in hypertensive patients administered an ACE inhibitor.[13] Because the molecular weight of AcSDKP (487 d) is as low as that of creatinine, we measured its concentration in plasma of patients with chronic renal failure (CRF) who were or were not administered an ACE inhibitor.[14] This preliminary study showed that patients with CRF had significantly greater plasma AcSDKP levels than controls, and levels were even greater in ACE inhibitor–treated patients.

Anemia is an almost constant finding in patients with CRF. Although it is mainly related to insufficient erythropoietin (EPO) production,[15] numerous other factors, such as erythropoietic inhibitors,[16-19] have been shown or suspected to

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have a role. Among them, ACE inhibitors, successfully used in the treatment of polycythemia in kidney graft recipients,[20] have been reported to induce anemia in some hypertensive patients,[21] as well as in some renal transplant recipients treated for hypertension.[22] ACE inhibitors are also suspected to be responsible for the anemia and resistance to recombinant human EPO (rHuEPO) therapy in some hemodialysis (HD) patients (reviewed in[23]).

These data led us to measure plasma AcSDKP levels in patients with CRF who were or were not administered an ACE inhibitor and correlate these levels with their biological parameters and rHuEPO requirements.

PATIENTS AND METHODS

Patients

One hundred seventy-six patients with CRF and 51 healthy donors used as controls were included in the study after obtaining informed consent. Fifty-six patients with CRF did not require dialysis (nondialysis [nD] patients) and 120 patients underwent HD three times weekly for at least 6 months. Twenty-one of 56 nD patients and 18 of 120 HD patients were administered various daily doses of an ACE inhibitor: benazepril, 2.5 mg (n = 1), 5 mg (n = 4), or 10 mg (n = 3); captopril, 12.5 mg (n = 5), 25 mg (n = 6), or 50 mg (n = 1); enalapril, 5 mg (n = 4) or 10 mg (n = 2); perindopril, 1 mg (n = 2), 2 mg (n = 2), or 4 mg (n = 1); quinapril, 20 mg (n = 1); ramipril, 5 mg (n = 3); or trandolapril, 0.5 mg (n = 1) or 2 mg (n = 3). Causes of CRF in nD patients were chronic glomerulonephritis (18 patients), chronic interstitial nephritis (9 patients), vascular nephropathy (10 patients), diabetic nephropathy (8 patients), polycystic disease (3 patients), and undetermined nephropathy (8 patients). Serum creatinine concentrations of nD patients ranged from 99 to 1,203 μmol/L (median, 310 μmol/L), and creatinine clearances, calculated using Cockroft's formula, ranged from 4 to 82 mL/min (median, 19 mL/min). Creatinine clearance values were not significantly different between nD patients who were or were not administered an ACE inhibitor (21 $\pm$ 16 versus 25 $\pm$ 18 mL/min). No nD patients were administered rHuEPO. Most HD patients (92 of 120 patients) were continuously treated with rHuEPO at a mean dose of 119 IU/kg/wk (range, 20 to 290 IU/kg/wk). Dialysis quality was determined by urea reduction ratio, calculated as:

(Predialysis blood urea nitrogen concentration –
postdialysis blood urea nitrogen concentration)/
predialysis blood urea nitrogen concentration

Plasma Samples

Blood drawn by venipuncture in nD patients and controls and from the arterial needle of the fistula in HD patients was collected in heparinized and EDTA tubes for the determination of AcSDKP concentrations and blood cell counts, respectively. Heparinized plasma was rapidly collected after centrifugation (1,500*g* for 10 minutes at 4°C) of blood samples and immediately stored at –20°C until use.

AcSDKP Assays and Half-Life of Synthetic AcSDKP

As previously described,[7] AcSDKP concentrations were assessed by a specific competitive enzyme immunoassay (limit of detection, 0.1 pmol/mL) on the methanol-extracted plasma fraction. Methanol extraction eliminates high-molecular-weight molecules that could interfere with the immunoassay and concentrates low-molecular-weight peptides, including AcSDKP.

To measure the half-life, freshly collected plasma from 13 controls, 20 nD patients, and 13 HD patients (before and after dialysis), all without treatment with an ACE inhibitor, was supplemented with synthetic AcSDKP (20 pmol/mL) and rapidly aliquoted. Methanol (3/1) was immediately added to one aliquot, whereas the others were incubated at 37°C for 1, 2, and 4 hours before methanol addition and dosage of the methanol-extracted plasma fraction. The percentage of degraded AcSDKP at the different time points was calculated as follows:

100 – (AcSDKP after incubation/
AcSDKP before incubation) $\times$ 100

Results were used to calculate the half-life of AcSDKP in patients and controls, a parameter that reflects plasma ACE biological activity.

Biological Parameters

A blood cell count and differential were performed on all patients. Serum ferritin, parathyroid hormone (PTH), and C-reactive protein (CRP) were measured in HD patients only. We thus selected 76 HD patients who had stable rHuEPO requirements and biological parameters for at least the previous 3 months and hemoglobin (Hb) levels greater than 10 g/dL, serum ferritin levels greater than 100 ng/mL, CRP levels less than 20 mg/L, PTH levels less than 400 ng/L, and urea reduction ratios greater than 65% (Table 1). Among these 76 HD patients, 13 were administered an ACE inhibitor. Their Hb concentration (11.5 $\pm$ 1.08 g/dL) was not significantly different from that of the 63 other HD patients not administered an ACE inhibitor (11.64 $\pm$ 1.2 g/dL).

Statistics

Results are expressed as mean $\pm$ SD. Student's unpaired and paired *t*-tests or Mann-Whitney test was used when appropriate to compare data. Differences were considered significant at P less than 0.05. Coefficients of correlation were calculated using linear regression.

Table 1. Major Biological Parameters and rHuEPO Requirements of 76 HD Patients

Hb (g/dL)	rHuEPO Requirement (IU/kg/wk)	Serum Ferritin (ng/mL)	CRP (mg/L)	PTH (ng/L)	Urea Reduction Ratio (%)
11.6 ± 1.2	84 ± 64	286 ± 156	12.5 ± 8.5	98 ± 89	72 ± 5.4

NOTE. These patients had stable values for the mentioned biological parameters and rHuEPO treatment for at least the previous 3 months. Results are expressed as mean ± SD.

RESULTS

Relations Between Plasma AcSDKP Levels and Other Biological Parameters in nD Patients

Plasma AcSDKP concentrations were significantly greater ($P < 0.01$) in nD patients not administered an ACE inhibitor (3.1 ± 1.8 pmol/mL) than in controls (1.8 ± 0.2 pmol/mL; Fig 1). ACE inhibitor–treated nD patients had significantly ($P < 0.01$) increased levels of AcSDKP that were fourfold (13.9 ± 9.9 pmol/mL) those of nD patients not administered an ACE inhibitor. This ACE inhibitor–induced increase was similar to that we previously evidenced in healthy volunteers treated with enalapril.[12]

AcSDKP concentration was inversely correlated to creatinine clearance in patients not administered an ACE inhibitor ($r = –0.58$; $P < 0.01$; Fig 2A), but not in those administered an ACE inhibitor ($r = –0.29$; $P = 0.19$; graph not shown). As previously published,[15] we also found a direct correlation between Hb concentration and creatinine clearance in our 56 nD patients (with and without ACE inhibitor treatment; $r = 0.62$; $P < 0.01$). When these two groups of patients were analyzed separately (Fig 2B), this correlation was still significant in both non–ACE inhibitor–treated and ACE inhibitor–treated patients, whereas their AcSDKP plasma levels were significantly different (Fig 1). Because there was an inverse correlation between AcSDKP level and creatinine clearance in nD patients not administered an ACE inhibitor, the inverse correlation that we observed in this group between concentrations of AcSDKP and Hb was expected ($r = –0.53$; $P < 0.01$; Fig 2C).

Between patients with and without ACE inhibitor treatment, no significant differences were found in mean Hb concentration (10.6 ± 2.7 versus 11.0 ± 2.2 g/dL), total white blood cell count (5,270 ± 1,370 versus 4,690 ± 2,100 × 10^9/L), or lymphocyte count (1,840 ± 850 versus 1,850 ± 1,650 × 10^9/L), respectively.

Plasma AcSDKP Levels in HD Patients Before and After HD

Plasma AcSDKP concentrations were significantly greater ($P < 0.01$) in HD patients before HD (10.3 ± 3.9 pmol/mL) than in nD patients or controls and were even greater ($P < 0.01$) in ACE inhibitor–treated HD patients (43.5 ± 32 pmol/mL; Fig 1). In each of the 25 patients tested, postdialysis levels of AcSDKP decreased by 47% to 92% (mean, 66% ± 11%) compared with their predialysis levels. This difference was significant ($P < 0.01$). However, postdialysis

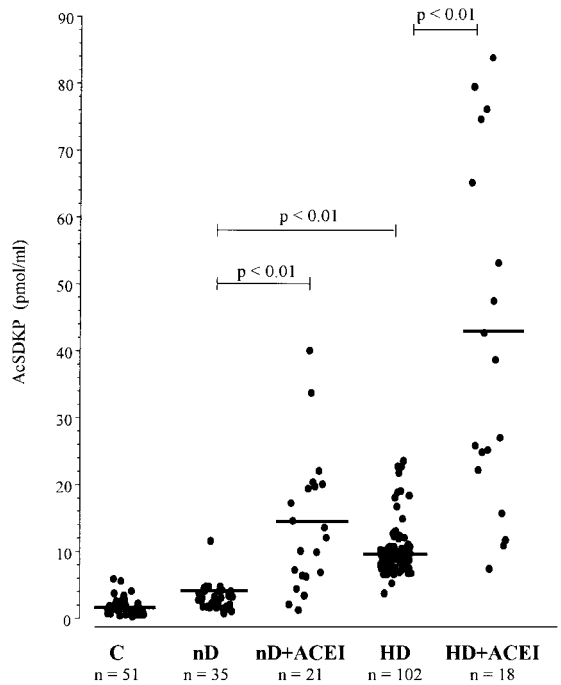

Fig 1. Plasma AcSDKP concentrations in controls (C) and nD or HD patients before dialysis sessions with or without treatment with an ACE inhibitor (ACEI) were measured by enzyme immunoassay. Horizontal bars represent the mean of each group. Differences between controls and all other groups were significant ($P < 0.01$). The significance of other differences is indicated on the graph.

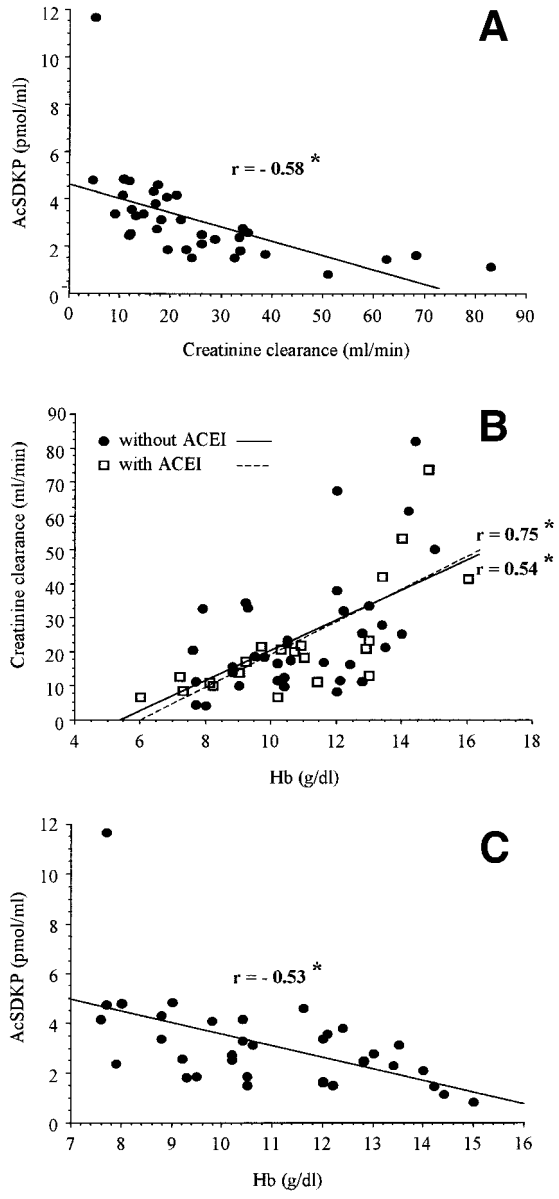

Fig 2. Correlation between (A) plasma AcSDKP concentration and creatinine clearance, (B) creatinine clearance and Hb concentration in 35 nD patients not administered an ACE inhibitor (ACEI; ●; solid line) and 21 other nD patients administered an ACE inhibitor (□; dashed line), and (C) plasma AcSDKP and Hb concentrations in the 35 nD patients not administered an ACE inhibitor. Creatinine clearance was calculated using Cockroft's formula. AcSDKP concentrations were measured by enzyme immunoassay. Correlation was studied by linear regression analysis (*$P < 0.01$).

levels (6.0 ± 1.9 pmol/mL) remained significantly greater ($P < 0.01$) than those of controls and nD patients.

Half-Life of Synthetic AcSDKP

The half-life of synthetic AcSDKP in plasma was similar in nD patients not administered an ACE inhibitor (135 ± 37 minutes) and controls (138 ± 40 minutes; Fig 3). Conversely, its half-life was significantly longer ($P < 0.05$) before dialysis (270 ± 109 minutes) in HD patients not administered an ACE inhibitor than in controls. After dialysis, it diminished to levels (182 ± 67 minutes) not significantly different from those of controls or nD patients (Fig 3). Because we had previously shown that long-term treatment with an ACE inhibitor (enalapril) dramatically increased the half-life of AcSDKP in plasma of healthy controls,[12] we did not perform this experiment in the ACE inhibitor–treated nD and HD patients.

The degradation of AcSDKP by ACE leads to the formation of the dipeptide Lys-Pro, shown to be a potential inhibitor of ACE activity.[24] Considering its low molecular weight, the dipeptide Lys-Pro probably is cleared mainly by the kidney. When its concentration increases between two dialysis sessions, it could then act as an ACE inhibitor in predialysis plasma, thus accounting for the longer half-life of synthetic AcSDKP in HD patients before dialysis. To show a greater inhibitory activity of this dipeptide in patients before dialysis, we prepared methanol-extracted plasma fractions (that contained AcSDKP and

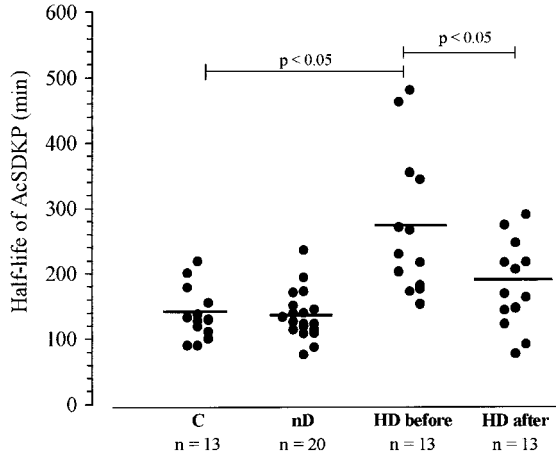

Fig 3. In vitro half-life of synthetic AcSDKP in plasma of healthy controls (C), non–ACE inhibitor–treated nD patients, and HD patients before or after dialysis. Horizontal bars represent the mean of each group. Half-life was measured as described in Patients and Methods.

the dipeptide Lys-Pro) from three controls and three patients before and after dialysis. Serial dilutions of these fractions were added to a control sample of plasma containing 20 pmol/mL of synthetic AcSDKP to study their effect on the half-life of synthetic AcSDKP (see Patients and Methods). All methanol-extracted fractions of controls and HD patients similarly increased the half-life of synthetic AcSDKP (data not shown).

Relationships Between Plasma AcSDKP Levels and Weekly rHuEPO Requirements in HD Patients

In the group of 76 HD patients selected for their stable biological parameters for at least the previous 3 months, the correlation, even if statistically significant ($r = 0.39$; $P < 0.01$) was weak between the weekly requirement of rHuEPO and AcSDKP concentration. If patients were classified according to treatment with an ACE inhibitor, this correlation was good ($r = 0.69$; $P < 0.01$) in patients administered an ACE inhibitor (n = 13), whereas it was absent ($r = 0.13$; $P = 0.32$) in those not administered an ACE inhibitor (n = 63; Fig 4). ACE inhibitor–treated patients had significantly greater ($P < 0.01$) plasma levels of AcSDKP and significantly greater ($P < 0.05$) weekly rHuEPO requirements than untreated patients (AcSDKP, 38.8 ± 25 versus 10.3 ± 3.6 pmol/mL; rHuEPO, 123 ± 60 versus 77 ± 63 IU/kg/wk); Hb concentrations were not statistically different. When the 76 HD patients were divided into two groups according to weekly rHuEPO requirements (Fig 4, inset), those requiring more than 100 IU/kg/wk (n = 30) had significantly greater ($P < 0.01$) AcSDKP levels than those requiring less than 100 IU/kg/wk (n = 46; 22.4 ± 21 and 10.4 ± 3.9 pmol/mL, respectively). All patients (with or without ACE inhibitor treatment) administered less than 100 IU/kg/wk of rHuEPO had AcSDKP levels less than 24 pmol/mL (Fig 4, inset). This group included only 9% of ACE inhibitor–treated patients. In the group of patients requiring more than 100 IU/kg/wk, 30% were treated with an ACE inhibitor. Among them, 89% had plasma AcSDKP concentrations greater than 24 pmol/mL.

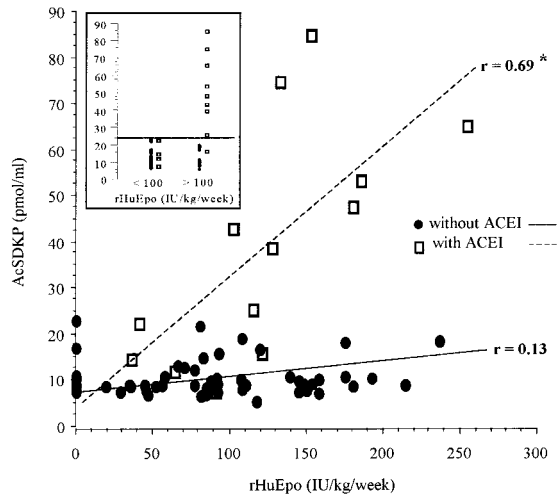

Fig 4. Correlations between weekly requirement of rHuEPO and AcSDKP concentration in 76 HD patients with stable biological parameters and stable rHuEPO requirements not administered (●; solid line; n = 63) or administered an ACE inhibitor (□; dashed line; n = 13). (Inset) The 76 HD patients were classified according to weekly rHuEPO requirement: less than 100 IU/kg/wk (n = 46) and more than 100 IU/kg/wk (n = 30). The horizontal bar represents the highest value of AcSDKP for patients with a rHuEPO requirement less than 100 IU/kg/wk. AcSDKP concentrations were measured by enzyme immunoassay. Correlation was studied by linear regression analysis (*$P < 0.01$).

DISCUSSION

AcSDKP inhibits the proliferation of hematopoietic stem cells and progenitors by blocking their S phase entry.[1] This 487-d tetrapeptide is maintained at stable concentrations in normal plasma.[6] Because AcSDKP is physiologically degraded by the N-terminal active site of ACE[10] and partially eliminated in urine,[12] its plasma concentrations are a result of a complex balance between its production, degradation by ACE, and renal elimination.

Confirming previous preliminary results,[14] we detected a substantial increase in plasma AcSDKP concentrations in both nD and HD patients. In nD patients not administered ACE inhibitors, we found an inverse relationship with creatinine clearance ($r = –0.58$; $P < 0.01$). The absence of correlation between these two parameters in ACE inhibitor–treated nD patients was probably caused by individual variation in type, dose, and metabolism of ACE inhibitor (in addition to individual differences in residual renal clearance of creatinine and AcSDKP). Because the in vitro half-life of synthetic AcSDKP in plasma was identical in nD patients and controls, we thus confirmed previous personal studies[12,14] establishing that its

glomerular filtration has a major role in the regulation of its plasma level. The significantly greater levels of AcSDKP in HD as opposed to nD patients were caused partly by the accumulation of AcSDKP between dialysis sessions. The longer predialysis half-life of the tetrapeptide (which returns to normal values after dialysis) may also be a contributing factor. This longer half-life reflects the decreased functional activity of plasma ACE in HD patients. Our hypothesis that the dipeptide Lys-Pro (a degradation product of AcSDKP) was responsible for the reduced activity of ACE in the predialysis plasma of HD patients was not confirmed by the experimental method that we used (see Results).

Decreased EPO synthesis is the major pathogenic factor in the anemia of patients with CRF.[15] Although chronic rHuEPO therapy corrects anemia in most patients by increasing the reticulocytosis and life span of red blood cells,[15,25] some patients are resistant to high doses of rHuEPO.[26] Hemolysis, occult bleeding and iron deficiency,[15] elevated PTH levels,[19,27] inflammatory states,[28] and inadequate dialysis[29,30] are potential causes of this rHuEPO resistance. Interestingly, treatment with an ACE inhibitor can induce anemia and neutropenia in some hypertensive patients[21,22] and is also suspected to induce rHuEPO resistance in HD patients,[23] although some studies are contradictory.[30-33] Finally, treatment with ACE inhibitors or angiotensin II type 1 receptor antagonists has cured polycythemia occurring in some kidney graft recipients.[20,34] These clinical observations indicate that the renin-angiotensin system has a role in the regulation of hematopoiesis; however, the mechanisms are poorly understood. ACE inhibitors reduce the production of EPO in animal models and renal transplant recipients.[35-37] It recently was shown that angiotensin II directly increases in vitro proliferation of erythroid progenitors.[38] Because AcSDKP has an inhibitory effect on the cycling of hematopoietic progenitors,[1-5] it is reasonable to believe that its accumulation in plasma of patients with CRF (particularly those administered ACE inhibitors) could partially account for their deficient erythropoiesis. Our recent work showing that the administration of an ACE inhibitor significantly reduced the number of committed hematopoietic progenitors (burst-forming unit–erythroid and colony-forming unit granulocyte mononocytes) in the blood of healthy volunteers supports this hypothesis.[12]

We thus investigated in nD and HD patients the possible relationships between high plasma AcSDKP levels (related to CRF itself, an ACE inhibitor, or both) and Hb concentrations or rHuEPO requirement. In nD patients, Hb levels were similar regardless of whether the patients were treated with an ACE inhibitor, whereas AcSDKP concentrations were significantly different. Moreover, in these patients, the relationship between Hb concentration and creatinine clearance was not modified by ACE inhibitor treatment. The variability of other factors, such as PTH level, ferritin level, and residual EPO production (an essential factor), in nD patients can account for these results and suggests that these factors have a major effect on erythropoiesis.

Conversely, we studied the relationship between high levels of AcSDKP and EPO requirement in a selected population of HD patients in which these biological factors and rHuEPO treatment were stable for more than 3 months. In this population, we found a significant correlation between individual rHuEPO requirement and AcSDKP level. Furthermore, HD patients requiring smaller doses of rHuEpo ($<$100 IU/kg/wk) had a statistically significant twofold reduction in AcSDKP levels than those requiring greater doses ($>$100 IU/kg/wk). These results suggest that at less than a certain threshold ($\sim$24 pmol/mL; Fig 4, inset), which is much greater than in healthy individuals, AcSDKP does not modify rHuEPO requirements. Above that threshold, the significantly greater rHuEPO requirements suggest an inhibitory effect of the tetrapeptide on erythropoiesis. If verified in a larger number of patients, these data could account for the absence of correlation between AcSDKP and Hb levels in nD patients and HD patients not administered an ACE inhibitor.

Nevertheless, some HD patients (n = 22; Fig 4, inset) treated with more than 100 IU/kg/wk of rHuEPO had AcSDKP levels less than 24 pmol/mL, suggesting that this tetrapeptide is one among other factors able to induce anemia in CRF. A threshold level of AcSDKP leading to EPO resistance could explain the apparent contradictory

results of some previous articles studying rHuEPO resistance in HD patients.[30-33] This hypothesis is supported by our two complementary results showing: (1) all HD patients that had recieved more than 100 IU/kg/wk of rHuEPO had more than 24 pmol/mL of AcSDKP; and (2) a significant correlation of plasma AcSDKP with rHuEPO treatment was present only in patients with very high plasma AcSDKP concentrations, who in most cases were those administered an ACE inhibitor. In these previous publications, AcSDKP levels of HD patients were not considered. The AcSDKP threshold was probably also reached by only a few patients who were not identified and thus not included in a specific group for statistical analysis. It could explain the apparent discrepancies with our present results.

Our results in HD patients indicate that AcSDKP is a uremic toxin, similar to other erythropoietic inhibitors described in the literature[16-18]; accumulation can lead to rHuEPO resistance. Inadequate dialysis in addition to treatment with an ACE inhibitor permanently increases plasma and probably bone marrow AcSDKP levels, which thus could inhibit erythropoiesis and reduce the response of erythroid progenitors to usual doses of EPO. Mrug et al[38] recently showed that angiotensin II increases erythropoiesis in vitro. If these results apply to in vivo situations, they suggest that treatment with ACE inhibitors could inhibit erythropoiesis and reduce EPO sensitivity by two different and complementary pathways: increasing concentrations of AcSDKP and reducing levels of angiotensin II. This could explain more appropriately why angiotensin II receptor antagonists exert less of an effect on erythropoiesis than ACE inhibitors[39]; they reduce the functional activity of angiotensin II on its target cells without increasing AcSDKP levels that inhibit cell cycling. Direct evidence to support these hypotheses is still lacking. This could be obtained by first measuring in vivo the percentage of cycling bone marrow hematopoietic progenitors (S phase) in animal models, and later, in nD and HD patients, with or without ACE inhibitor or angiotensin II receptor antagonist treatment and with high or low levels of AcSDKP.

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