Levels of Hematopoiesis Inhibitor N-Acetyl-Seryl-Aspartyl-Lysyl-Proline Partially Explain the Occurrence of Anemia in Heart Failure

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Background—Anemia is common in patients with chronic heart failure (CHF) and is associated with a poor prognosis. However, only a minority of patients with CHF have impaired renal function or underlying hematinic deficiencies. It has been shown that inhibition of the renin-angiotensin system is associated with the development of anemia. The aim of the present study was to determine possible mechanisms linking anemia to renin-angiotensin system activity in CHF patients.

Methods and Results—We initially evaluated 98 patients with advanced stable CHF who were treated with ACE inhibitors (left ventricular ejection fraction, 28±1%; age, 69±1 years; 80% male), 10 of whom had an unexplained anemia (normal hematinics and no renal failure). These 10 anemic patients were matched with 10 nonanemic patients in terms of age and left ventricular ejection fraction. Serum ACE activity was 73% lower in anemic CHF patients compared with nonanemic CHF patients (P=0.018). Moreover, serum of these patients inhibited in vitro the proliferation of bone marrow–derived erythropoietic progenitor cells of healthy donors by 17% (P=0.003). Levels of the hematopoiesis inhibitor N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP), which is almost exclusively degraded by ACE, were significantly higher in anemic CHF patients and were clearly correlated to erythroid progenitor cell proliferation (r = −0.64, P=0.001).

Conclusions—Serum ACE activity is markedly lower in anemic CHF patients, and serum of these patients inhibits hematopoiesis. The clear correlation between Ac-SDKP and proliferation of erythroid progenitor cells suggests an inhibitory role of Ac-SDKP on hematopoiesis in CHF patients, which may explain the observed anemia in patients treated with ACE inhibitors. (Circulation. 2005;112:1743-1747.)

Key Words: peptides ■ anemia ■ angiotensin ■ heart failure ■ hematopoiesis

Anemia is present in a substantial part of the chronic heart failure (CHF) population, ranging from 14% to 55%, depending on the definition of anemia and severity of disease.1 Lower hemoglobin levels are also independently associated with an impaired prognosis,2–5 but the cause of anemia in CHF is often unknown. A recent study in the United Kingdom demonstrated that only a minority of CHF patients had renal impairment or underlying hematinic deficiencies.6 This was confirmed by Witte et al,7 who showed that fewer than one third of CHF patients were deficient in iron, folate, or vitamin B12. Therefore, other factors may be involved in the origin of anemia in CHF.

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Since the early 1980s, it has been demonstrated that the use of ACE inhibitors is associated with lower hemoglobin levels.8,9 Possible mechanisms might be related to changes in the renin-angiotensin system (RAS). In vitro, angiotensin II stimulated the proliferation of erythroid progenitor cells, an effect inhibited by angiotensin II receptor blockers.10 Furthermore, it has been shown that N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP), a strong inhibitor of hematopoietic stem cells, was hydrolyzed almost exclusively by ACE. Indeed, ACE inhibitors markedly increased Ac-SDKP levels by 5- to 6-fold and may therefore reduce hematopoietic activity.11

The aim of the present study was to determine possible mechanisms linking anemia to RAS activity in CHF patients. Accordingly, we evaluated the different components of the RAS and studied the effect of serum of anemic CHF patients on the proliferation of erythroid progenitor cells and compared it with findings in nonanemic CHF patients and healthy control subjects.
Methods

Patients
To identify patients with anemia of unknown origin, 98 consecutive patients with advanced systolic CHF were evaluated at our outpatient clinic between February 2003 and November 2003. In all patients, CHF was diagnosed on the basis of symptoms and the presence of left ventricular enlargement or systolic functional impairment by radionuclide ventriculography or echocardiography, according to the European Society of Cardiology guidelines. All patients had a left ventricular ejection fraction (LVEF) <45%, and all patients were in New York Heart Association functional class III. Patients were stable on medication for at least 3 months, and all patients were treated with an ACE inhibitor. The dose of the ACE inhibitor was expressed as a percentage of the recommended dose. Patients using an angiotensin receptor blocker (ARB) or patients with myocardial infarction (within 12 weeks) or malignant or inflammatory diseases were excluded from this study.

To evaluate the hematopoietic response in patients without CHF, we included 8 healthy control subjects without anemia and clinical signs of inflammation or CHF. The study was approved by the institutional review board, and all patients provided written informed consent.

Hormonal Measurements
Venous blood samples were taken at the outpatient clinic while the patient was in the upright position. The blood samples were centrifuged and stored at −80°C. Serum samples were thawed and analyzed immediately. High-sensitivity C-reactive protein was measured by nephelometry (BNII N, Nade Behring), ACE activity by enzymatic assay (Bühlmann Laboratory AG), and tumor necrosis factor-α by immunoassay (R&D System). Ac-SDKP levels were determined by competitive enzyme immunoassay (Caymann). In the plasma samples, N-terminal pro-brain natriuretic peptide (NT-proBNP) was measured by immunoassay (Roche Diagnostics), erythropoietin (EPO) by Immulite EPO assay (DPC), and angiotensin II levels by specific radioimmunoassay as previously described. All analyses were performed according to the manufacturer’s guidelines.

Renal Function and Anemia
The glomerular filtration rate (GFR) is a standard indicator of renal function. Under steady-state conditions, GFR is estimated from serum creatinine using a formula that accounts for the influence of age and body weight on creatinine production, the Cockcroft Gault equation: GFRc=[140−age in years]×[body weight in kg]/(72×serum creatinine in mg/dL). In women, the value is multiplied by 0.85. This formula has been commonly used in several studies of CHF and renal function. Anemia was defined according to the general accepted definition of the World Health Organization (WHO); hemoglobin ≤12 g/dL for women, ≤13 g/dL for men.

Hematopoiesis Assay
Bone marrow cells of healthy donors were used for the in vitro burst-forming unit–erythroid (BFU-E) assay. Bone marrow mononuclear cells were isolated by Ficoll-Paque gradient centrifugation. Cells were collected, washed, and resuspended in methylcellulose medium (Stemcell Technologies Inc) with 2 U/mL recombinant human EPO and 10% of serum of anemic CHF patients, nonanemic CHF patients, or healthy control subjects or without additional serum. All BFU-E assays were performed in quadruple. BFU-Es were counted after 14 days of incubation by researchers blinded to subgroup allocation. The number of BFU-E colonies in the absence of additional serum was considered the baseline value, and the number of BFU-E colonies was expressed as a percentage from baseline.

Genetic Analysis
Genomic DNA was extracted from white blood cells. The ACE fragment containing the insertion/deletion (I/D) sequence was detected by polymerase chain reaction using oligonucleotide primers flanking the ALU insertion, as previously described. Because of insufficient DNA extraction, no ACE ID polymorphism could be determined in 1 patient.

Statistical Analysis
Data are given as mean±SEM when normally distributed, as median and interquartile range when skewed, and as frequencies for categorical variables. We compared differences between groups with the Student t test, Mann-Whitney U test, or ANOVA with Fisher’s protected least-significant-difference post hoc analysis when appropriate. Correlations between variables were expressed with the Pearson or Spearman correlation coefficient when appropriate. We considered values of P<0.05 statistically significant. For all statistical analysis, SPSS version 11.0 was used.

Results
Baseline characteristics of the total population are shown in Table 1. The average age of the patients was 69±1 years, 80% were men, and the average LVEF was 28±1%. Mean hemoglobin level was 13.6 g/dL (range, 10.1 to 16.8 g/dL; median, 13.6 g/dL). According to the WHO definition, 17 of the 98 CHF patients (17.3%) were anemic. Anemia was associated with lower calculated GFR (P=0.049) and lower systolic blood pressure (P=0.02), possibly reflecting hypoxia-induced vasodilation. When log(EPO) was plotted as a function of hemoglobin, we observed only a weak nonsignificant correlation (r=−0.06, P=0.59).

We found that 4 of the 17 anemic CHF patients (24%) were vitamin B12 deficient (reference range, 170 to 750 pmol/L), 1 patient had low ferritin levels (reference range, 36 to 234 μg/L), and no folate acid deficiencies were observed (refer-
ence range, 4 to 30 nmol/L). Two patients (12%) had a calculated GFR < 30 mL/min.

In most anemic CHF patients (59%, n=10), no explanation for their anemia was found. We matched these 10 anemic patients with 10 nonanemic patients in terms of age and severity of heart failure (LVEF). Serum of anemic CHF patients inhibited the formation of BFU-E by 17% compared with nonanemic CHF patients (P=0.003; Figure 1). There was no difference in BFU-E formation between healthy control subjects and nonanemic CHF patients (P=0.48; Figure 1). The anemic and nonanemic CHF patients were similar with regard to levels of angiotensin II, EPO, NT-proBNP, high-sensitivity C-reactive protein, tumor necrosis factor-α, and GFRc, as well as duration and dose of ACE inhibitor use (Table 2). However, ACE activity was 73% higher in the nonanemic compared with anemic CHF patients (P=0.017; Table 2). Consequently, Ac-SDKP levels were significantly higher in the anemic CHF patients compared with nonanemic CHF patients, whereas healthy control subjects had the lowest Ac-SDKP levels (Figure 2). In addition, there was a strong correlation between Ac-SDKP levels and BFU-E formation (r=-0.64, P=0.001).

With regard to the ACE I/D polymorphism, patients homozygous for the I allele were present only in the anemic subgroup (P=0.07; Table 2). Furthermore, the ACE I/D polymorphism was related to BFU-E formation. Patients homozygous for the I allele showed significant lower proliferation of erythroid progenitor cells compared with patients with DD or ID genotypes (P=0.046 and P=0.026, respectively).

**Discussion**

In the present study, we show for the first time that CHF patients without identifiable cause of anemia have lower ACE activity and that their serum inhibits the proliferation of bone marrow–derived erythropoietic cells. We found that levels of Ac-SDKP, a strong hematopoiesis inhibitor, were signifi-

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**TABLE 2. Baseline Characteristics of the Subpopulation**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Anemia (n=10)</th>
<th>Nonanemia (n=10)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin levels, g/dL</td>
<td>11.5±0.3</td>
<td>14.9±0.3</td>
<td>...</td>
</tr>
<tr>
<td>Age, y</td>
<td>68±3</td>
<td>70±4</td>
<td>...</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>33±4</td>
<td>33±3</td>
<td>...</td>
</tr>
<tr>
<td>Male sex, n</td>
<td>8</td>
<td>9</td>
<td>0.53</td>
</tr>
<tr>
<td>Ischemic origin, n</td>
<td>7</td>
<td>7</td>
<td>1.00</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>115±4</td>
<td>140±7</td>
<td>0.005</td>
</tr>
<tr>
<td>GFRc, mL/min</td>
<td>61±5</td>
<td>72±9</td>
<td>0.32</td>
</tr>
<tr>
<td>Erythropoietin, mU/mL</td>
<td>15.5±2.7</td>
<td>19.0±3.4</td>
<td>0.43</td>
</tr>
<tr>
<td>NT-proBNP, pmol/L</td>
<td>83 (38–161)</td>
<td>96 (57–313)</td>
<td>0.55</td>
</tr>
<tr>
<td>Angiotensin II, pmol/L</td>
<td>10.1 (4.2–23.4)</td>
<td>13.0 (4.3–54.9)</td>
<td>0.74</td>
</tr>
<tr>
<td>ACE activity, U/L</td>
<td>13.7 (11.3–21.4)</td>
<td>23.8 (18.9–61.2)</td>
<td>0.017</td>
</tr>
<tr>
<td>hs-CRP, mg/L</td>
<td>3.1 (1.8–7.3)</td>
<td>4.6 (1.1–11.0)</td>
<td>0.53</td>
</tr>
<tr>
<td>TNF-α, pg/mL</td>
<td>1.5 (1.0–2.5)</td>
<td>2.0 (1.0–3.6)</td>
<td>0.52</td>
</tr>
<tr>
<td>Medication, % use</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACEI</td>
<td>100</td>
<td>100</td>
<td>...</td>
</tr>
<tr>
<td>Duration of ACEI use, mo</td>
<td>23 (8–46)</td>
<td>43 (8–46)</td>
<td>0.22</td>
</tr>
<tr>
<td>Recommended dose of ACEI, %</td>
<td>50 (44–100)</td>
<td>63 (25–100)</td>
<td>0.75</td>
</tr>
<tr>
<td>β-Blocker</td>
<td>90</td>
<td>70</td>
<td>0.48</td>
</tr>
<tr>
<td>Diuretics</td>
<td>90</td>
<td>90</td>
<td>1.00</td>
</tr>
<tr>
<td>ACEI genotype, %</td>
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<td></td>
</tr>
<tr>
<td>D/D+I/D</td>
<td>70</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>ILI</td>
<td>30</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

SBP indicates systolic blood pressure; TNF, tumor necrosis factor; hs-CRP, high-sensitivity C-reactive protein; and ACEI, ACE inhibitor.

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**Figure 1.** Comparison of the effect of serum on the formation of BFU-E as a percentage of the baseline value (incubation without serum) in anemic, nonanemic, and healthy control subjects. Box plot show the median with 25% to 75% range of the BFU-E colonies present in culture.

**Figure 2.** Levels of Ac-SDKP in anemic, nonanemic, and healthy control subjects. Box plot show the median with 25% to 75% range of Ac-SDKP (in nmol/L).
effects were also observed in CHF patients. A recent substudy of the Studies of Left Ventricular Dysfunction (SOLVD) demonstrated convincingly that enalapril significantly increased the odds of developing anemia by 56% in patients with CHF. The reduction in hematocrit levels occurs within several months after the start of enalapril and is sustained for at least several years. However, the observed effect on hematocrit seems to be modest and might affect only a selected population. In addition, ACE inhibitor use was associated with better survival even after adjustment for episodes of anemia and in patients with prevalent anemia.

Various mechanisms might play a role in the negative effects of ACE inhibitors on hematopoiesis. ACE inhibitor therapy may directly decrease the production of EPO in kidney, probably by inhibiting angiotensin II formation. Recently, Ac-SDKP, a natural inhibitor of pluripotent hematopoietic stem cell proliferation, has been found to be associated with ACE inhibitor therapy. Ac-SDKP is a tetrapeptide that reversibly prevents the recruitment of hematopoietic stem cells into the S phase of the cell cycle by maintaining them in the G0 phase. This tetrapeptide was found to be degraded almost exclusively by ACE, which can be blocked by ACE inhibitors. Already, a single dose of the ACE inhibitor captopril resulted in a 5.5-fold increase in the levels of Ac-SDKP. In our study, we found that Ac-SDKP levels were 2 times higher in anemic CHF patients. This was associated with a marked lower ACE activity in anemic compared with nonanemic CHF patients. The observed differences in ACE activity and therefore Ac-SDKP levels might be related to the ACE I/D genotype. We observed a lower hematopoietic activity in CHF patients homozygous for the ACE I allele, which is linked to lower ACE activity. This is in line with previous findings of Varagunam et al, who showed that dialysis patients with the ACE ID/I genotype required significantly higher dosages of human recombinant EPO for the treatment of their renal anemia compared with the patients homozygous for the D allele.

Several studies found a clear correlation between renal function and Ac-SDKP levels. Comte et al found that besides degradation by ACE, Ac-SDKP is partially eliminated in the kidney. We found that GFRc was lower in anemic CHF patients compared with nonanemic CHF patients, which might have influenced Ac-SDKP levels. The clinical relevance of Ac-SDKP levels was further explored by Le Meur et al, who studied the relation between Ac-SDKP levels and the weekly dose of recombinant human EPO for the treatment of renal anemia. They observed that dialysis patients required significantly more recombinant human EPO when their Ac-SDKP levels were higher, indicating an inhibitory effect of Ac-SDKP on hematopoiesis.

The study by Mrug et al showed that angiotensin II levels stimulate the proliferation of hematopoietic cells through activation of the angiotensin II type I receptor; this effect was abolished by the addition of an ARB. One may speculate that ACE inhibitors lower angiotensin II levels and therefore inhibit the hematopoietic proliferation. In our study, we included only patients using ACE inhibitors without concomitant use of ARBs. Because there were no significant differences in angiotensin II levels between anemic and nonanemic CHF patients, our study does not suggest a major contribution of angiotensin II levels to the observed decreased hematopoietic activity in CHF patients. However, because patients receiving ARBs were not included in our study, we are unable to draw conclusions on the possible effect of angiotensin II on hematopoiesis in CHF patients. Studies in larger groups of patients that compare the effects of ACE inhibitor and ARB treatment should address this issue.

Several limitations have to be acknowledged. Hemodilution might have influenced the degree of anemia, although all patients were stable on medication for at least 3 months. Inflammation in patients with CHF might lower hemoglobin levels and induce anemia. Inflammatory parameters measured in our study were not different in anemic and nonanemic CHF patients. However, other proinflammatory cytokines might play a role in the pathogenesis of anemia in CHF. Furthermore, because of the relatively small sample size, one should cautiously interpret the link between the ACE I/D polymorphism and hematopoiesis. Studies analyzing larger groups of patients should clarify the possible relationship between anemia and ACE genotype in CHF patients. Because of these limitations, we regard our study primarily as hypothesis generating.

In summary, ACE activity in CHF patients without an identifiable cause of anemia is significantly lower compared with nonanemic CHF patients, and serum of these patients inhibits hematopoiesis. The correlation between Ac-SDKP and proliferation of erythropoietic progenitor cells suggests an inhibitory role of Ac-SDKP in hematopoiesis, linking the RAS to hematopoiesis in CHF patients.

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References

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