Erythropoietin-Induced Hypertension in Rat Is Not Mediated by Alterations of Plasma Endothelin, Vasopressin, or Atrial Natriuretic Peptide Levels

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Abstract. Regular administration of recombinant erythropoietin (EPO) in patients with chronic renal failure (CRF) is frequently complicated by a rise in arterial blood pressure. Clinical studies intended to discern the possible role of endothelin (ET) in the pathogenesis of EPO-induced hypertension have produced contradictory results. Given the limitations of the clinical studies, this placebo-controlled study was carried out in CRF (5/6 nephrectomized) rats treated with either EPO, 150 U/kg intraperitoneally, or the vehicle alone twice weekly for 6 wk. Plasma ET was measured at baseline, and weeks 2, 4, and 6. In addition, plasma arginine vasopressin (AVP) and atrial natriuretic peptide (ANP) were determined at the conclusion of the study period. As expected, blood pressure rosemarkedly after 1 wk of EPO therapy as compared with the placebo therapy. However, there was no significant difference in plasma ET levels between the EPO- and placebo-treated groups during the study period. Likewise, EPO therapy had no effect on plasma ANP level but depressed plasma AVP concentration. Thus, this placebo-controlled animal study revealed that EPO therapy markedly raised arterial blood pressure but had no effect on plasma ET in the CRF rats. This observation suggests that EPO-induced hypertension in this model is not mediated by an increased circulating ET level. However, the possible effect, if any, of EPO on local vascular tissue ET level is uncertain and awaits further investigation. (J Am Soc Nephrol 8: 901–905, 1997)

Regular administration of recombinant erythropoietin (EPO) is frequently associated with a mild-to-marked rise in arterial blood pressure (1–4). The EPO-induced hypertension has been variably attributed to the rise in erythrocyte concentration and/or a direct or indirect pressor action of EPO on vascular smooth muscle (4). In a series of recent studies, Vaziri et al. demonstrated that EPO-induced hypertension is hematocrit-independent and is associated with, and perhaps causally related to, increased basal and stimulated cytosolic [Ca2+]i and impaired vasodilatory response to nitric oxide (5,6). In addition, several clinical studies have sought to test the proposition that EPO-induced hypertension may be mediated by increased production of the potent vasoactive peptide endothelin (ET) (7–10). These studies have generally involved comparison of plasma ET levels at baseline with those obtained after a few weeks to several months of regular EPO therapy. The results of these studies have been contradictory. For instance, some investigators have shown a significant rise in plasma ET level in hemodialysis-dependent patients after several months of intravenous EPO therapy (7,8). In contrast, several other investigators have found no rise in plasma ET with EPO administration in either hemodialysis or continuous peritoneal dialysis patients (9–11), whereas still others have found an elevation of plasma ET only in patients exhibiting an EPO-induced blood pressure rise equaling or exceeding 10 mmHg (7). The study presented here was designed to determine the effect, if any, of EPO therapy on plasma ET level in chronic renal failure (CRF; 5/6 nephrectomized) rats. To this end, plasma ET concentration was measured longitudinally in CRF rats during a 6-wk course of EPO or placebo therapies.

Materials and Methods
Animal Models
Male Sprague-Dawley rats (Charles River, Inc., Wilmington, MA) with an average body weight of 350 g were used. The animals were fed a standard laboratory diet (Purina Rat Chow; Purina Mills, Brentwood, MO) and water ad libitum. Under general anesthesia, the animals were subjected to a right nephrectomy followed by a two-thirds left nephrectomy 4 d later (using surgical resection) to produce CRF. The nephrectomies were carried out extraperitoneally through a dorsal incision. Strict hemostasis and aseptic measures were observed during the surgical procedures. The animals were then randomized into two groups.

Group A animals received intraperitoneal injections of recombinant human EPO, 150 U/kg twice weekly for 6 wk. Group B animals received placebo injections at the same frequency. A group of sham-operated rats was included as normal controls (group C).

Systolic arterial blood pressure was monitored once weekly using a tail sphygmomanometer (Harvard Apparatus, South Natick, MA). Body weight and hematocrit (microhematocrit method) were measured once weekly. Blood samples were obtained before and on days 14, 28 and 42 after nephrectomies or sham operation for the measurement of plasma ET level. On each occasion, blood was withdrawn from the orbital sinus (under light anesthesia), and the plasma was
separated and stored at −20°C until measurement. At the end of the 6-wk observation period, the animals were placed in individual metabolic cages for a 24-h urine collection. They were then exsanguinated by cardiac puncture under anesthesia, and plasma was secured for measurements of arginine vasopressin (AVP) and atrial natriuretic peptide (ANP).

**Endothelin Determination**

Plasma ET was measured by RIA using reagents obtained from Nichols Institute Diagnostics (San Juan Capistrano, CA). EDTA-treated plasma samples were extracted with acetone, and the acetone extract was dried under nitrogen (extraction efficiency was 90%). The dried extract was reconstituted and assayed in an RIA using 125I-ET and rabbit anti-ET. The bound/free separation was achieved using second antibody separation procedure (donkey anti-rabbit gamma globulin). To avoid interassay variations, all samples were assayed in a single session. The intra-assay variation for this test was less than 8%. Sensitivity of the assay was 1 pg/ml for a 1-ml sample size. The specificity of the antibody was as follows: ET, 100%; ET-2, 52%; and specificity of the antibody was 9%. The assay had minimal crossreactivity with big ET (7%) and no crossreactivity with ANP, AVP, angiotensin II, or ACTH.

**Arginine Vasopressin Determination**

Plasma AVP was measured by RIA after bentonite extraction as described by Skowsky et al. (12). In a typical assay, 1 ml of plasma was extracted with bentonite (3 mg), and the AVP bound to the bentonite was eluted using acidified acetone. The eluate was dried under nitrogen. The AVP-containing residue was reconstituted and assayed by RIA. In the RIA, an aliquot of the reconstituted material was mixed with rabbit anti-AVP and incubated for 72 h at 4°C. 125I AVP was added, and the incubation continued for another 24 h. Bound/free separation was achieved using a second antibody (goat anti-rabbit gamma globulin). Extraction efficiency was monitored routinely, and the final results were corrected for extraction efficiency (70%). The sensitivity of the assay was 1 pg/ml. All samples were assayed in a single session. The intra-assay variation of the assay is 9%.

**Atrial Natriuretic Peptide Determination**

Plasma samples were extracted using Sep-Pak column (Waters Inc., Milford, MA) as described by Yandle et al. (13). The antibody used in the assay recognizes the amino-terminal end of the ANP molecule (14). In a typical assay, 1 ml of plasma was acidified with 0.1 ml of 1 N HCl, and the mixture was loaded onto a C18 Sep-Pak column. The ANP bound to the column was eluted with 80% methanol in triethanolamine buffer (vol/vol), pH 4.0. The eluate was then dried under nitrogen and the residue reconstituted in RIA buffer (pH 7.1, containing 0.01 M sodium phosphate, 0.85% sodium chloride, 0.05 M ethylenediaminetetraacetate, and 0.05% bovine serum albumin). The ANP-containing solution was mixed with rabbit anti-ANP and incubated for 24 h at 4°C. 125I ANP was added, and the incubation continued for an additional 48 h. At the end of the incubation, a second antibody (goat anti-rabbit gamma globulin) was added to separate the free from bound ET. The extraction efficiency of the assay was 75%. The intra-assay variation of this test is 8.4%, and the assay is sensitive to 10 pg/ml. All samples were assayed in a single session.

**Statistical Analyses**

Data are presented as mean ± SEM. ANOVA, Duncan’s multiple range test, and paired t test were used in analysis of the data, as appropriate. P values equal to or less than 0.05 were considered statistically significant.

**Results**

**General Data**

The body weight obtained at the conclusion of the study was significantly lower in the CRF groups as compared with the normal control group despite comparable values at the time of randomization (Table 1). As expected, the CRF groups showed a significant decline in creatinine clearance rate. The placebo-treated CRF animals exhibited a significant reduction in hematocrit value after induction of CRF. In contrast, EPO-treated animals maintained normal hematocrit values.

**Arterial Blood Pressure**

During the observation period, arterial blood pressure rose modestly in the CRF/placebo group compared with both the baseline value and that found in the control group, consistent with the known effect of CRF (Figure 1). The CRF-associated rise in arterial blood pressure was markedly augmented by regular EPO administration. The effect of EPO on blood pressure became evident during the second week of therapy, with no discernible effect observed during the first week.

**Plasma Endothelin Level**

Plasma ET levels were comparable among the study groups throughout the study period (Table 2). However, all groups of rats showed a slight but significant increase in plasma ET level at the end of the 6-wk observation period.

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight (g)</th>
<th>Hct (%)</th>
<th>Ccr (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Week 6</td>
<td>Baseline</td>
</tr>
<tr>
<td>CRF/EPO</td>
<td>352 ± 5</td>
<td>411 ± 7b</td>
<td>50.2 ± 0.7</td>
</tr>
<tr>
<td>CRF/placebo</td>
<td>349 ± 5</td>
<td>406 ± 14b</td>
<td>51.0 ± 0.5</td>
</tr>
<tr>
<td>Control</td>
<td>351 ± 4</td>
<td>445 ± 6bc</td>
<td>49.7 ± 0.6</td>
</tr>
</tbody>
</table>

* Data are presented as means ± SEM. Each group represents the mean value obtained from eight rats.

b P < 0.05 versus baseline.

c P < 0.05 versus any other groups (ANOVA).
Figure 1. Sequential measurements of arterial blood pressure in rats with chronic renal failure treated with EPO (CRF/EPO) or placebo (CRF/placebo) as well as normal controls. Each data point represents mean values obtained from eight rats. Bars denote SEM. *P < 0.05 (ANOVA).

Table 2. Plasma endothelin levels obtained at baseline and at weeks 2, 4, and 6 in rats with chronic renal insufficiency treated with EPO (CRF/EPO) or placebo (CRF/placebo) and normal control rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Endothelin (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
</tr>
<tr>
<td>CRF/EPO</td>
<td>15.3 ± 1.7</td>
</tr>
<tr>
<td>CRF/placebo</td>
<td>17.3 ± 2.2</td>
</tr>
<tr>
<td>Control</td>
<td>17.2 ± 0.9</td>
</tr>
</tbody>
</table>

* Data are presented as means ± SEM. Each group represents the mean value obtained from five to six rats.
*b P < 0.05 versus other times.

Plasma Vasopressin and ANP Levels

Plasma vasopressin concentration in the anemic placebo-treated CRF group was comparable with that of the control group and was significantly (P < 0.002 ANOVA) higher than that in the nonanemic EPO-treated CRF group (Figure 2). Plasma ANP levels in the EPO- and placebo-treated CRF animals were comparable with one another and significantly lower (P < 0.001) than that found in the normal control group.

Correlations

No correlation was observed between arterial blood pressure and plasma ET, AVP, or ANP levels in the entire study population.

Discussion

The results of this study revealed that the rise in arterial blood pressure in EPO-treated rats is not associated with increased plasma ET level. This observation suggests that EPO-induced hypertension in CRF rats must be mediated by factor(s) other than circulating ET. This finding seems to contradict those reported by some investigators (7,8), who found increased plasma ET concentration in hemodialysis patients who received intravenous EPO therapy for several months. The reason for the disparity between the results of the placebo-controlled animal study presented here with those of the above-mentioned clinical studies is uncertain. However, it may be a result of the species differences. In addition, the animal model utilized here was produced by a measured and largely uniform renal mass reduction with no other underlying renal or systemic disorders in a genetically homogeneous population of animals of the same age and gender. Furthermore, anemia and acid-base and electrolyte disorders are not pronounced in experimental CRF. In contrast, clinical cases of CRF represent a heterogeneous mixture of underlying disor-
It should be noted that the lack of demonstrable difference in plasma ET level among EPO- and placebo-treated animals does not entirely exclude a possible effect on tissue ET production. In this regard, Wagner et al. recently demonstrated that ET secretion by cultured umbilical vein endothelial cells is polarized in the abluminal direction (19). This phenomenon, together with the short plasma half-life of ET (20) and relative resistance of endothelial barrier to ET diffusion (21), tends to diminish the value of plasma ET level as a definitive index of its local production. Thus, further studies are required to determine the possible effect, if any, of EPO therapy on vascular tissue ET production and concentration.

The effect of EPO on plasma concentration of ANP is controversial. Increased (22,23), unchanged (24), and decreased (25) plasma ANP levels have been reported in hemodialysis patients treated with EPO. The study presented here revealed that EPO therapy had no direct effect on plasma ANP level in CRF rats. Thus, EPO-induced hypertension does not appear to be a result of alteration in the circulating ANP level.

EPO-treated CRF animals exhibited a significant reduction in the circulating vasopressin level, confirming previous clinical observations (19,20). This phenomenon is most likely a result of the associated rise in erythrocyte mass and blood volume, modulating vasopressin production and release.

In conclusion, EPO-induced hypertension in rats with CRF is independent of the circulating endothelin level. Moreover, measurements of the ANP and AVP levels in the study groups revealed no evidence for the direct role of these hormones in the genesis of EPO-induced hypertension.

References
8. Carlini R, Obialo CI, Rothstein M: Intravenous erythropoietin...
administration increases plasma endothelin and blood pressure in hemodialysis patients. Am J Hypertens 6: 103–107, 1993


