Nitric Oxide Metabolism in Erythropoietin-Induced Hypertension

Effect of Calcium Channel Blockade

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Abstract—Long-term administration of erythropoietin (EPO) frequently causes hypertension in humans and animals with chronic renal failure (CRF). We recently demonstrated that EPO-induced hypertension is hematocrit independent and accompanied by elevated cytosolic [Ca$^{2+}$], and nitric oxide (NO) resistance. This study was undertaken to examine the effects of therapy with EPO alone or together with calcium channel blockade on NO metabolism. Urinary excretion of NO metabolites (NOx) and thoracic aorta and kidney endothelial and inducible NO synthases (eNOS and iNOS) were studied in 4 groups of 6 nephrectomized rats treated with either placebo, EPO, the calcium channel blocker felodipine, or EPO plus felodipine for 6 weeks. A group of sham-operated placebo-treated animals served as control. The placebo-treated CRF group exhibited moderate hypertension, elevated basal and depressed stimulated [Ca$^{2+}$], reduced urinary NOx excretion, and diminished vascular and renal eNOS and iNOS proteins. EPO therapy further raised blood pressure and increased resting and stimulated [Ca$^{2+}$], but did not change NOx excretion or NOS proteins. Concurrent administration of felodipine abrogated EPO-induced hypertension, normalized resting and stimulated [Ca$^{2+}$], and increased NOx excretion and eNOS and iNOS proteins. Thus, EPO therapy leads to marked increases in blood pressure and resting and stimulated [Ca$^{2+}$]. These abnormalities are ameliorated by calcium channel blockade, which restores [Ca$^{2+}$], to normal and increases vascular and renal NOS expression. (Hypertension. 1998;32:724-729.)

Key Words: nitric oxide synthase ■ renal disease ■ calcium ■ erythropoietin ■ calcium channel blockers ■ hypertension

Introduction of recombinant erythropoietin (EPO) for clinical use has revolutionized the management of anemia in patients with end-stage renal disease. However, long-term administration of EPO frequently results in development of de novo hypertension or exacerbation of preexisting hypertension.1-3 Several factors have been implicated in the pathogenesis of EPO-induced hypertension. Chief among them is the resulting rise in hematocrit, which is thought to raise vascular resistance by increase in blood viscosity, loss of hypoxic vasodilation, and enhanced competition by hemoglobin for endothelium-derived nitric oxide (NO).4,7 Other proposed mechanisms include volume expansion, increased endothelin production,8 enhanced tissue renin-angiotensin activity,9 and direct vasopressor action of EPO.10

In a series of recent studies, we demonstrated that EPO-induced hypertension is not due to the associated increase in hematocrit or erythrocyte mass.11 This was based on the observation that hypertension occurred in both iron-deficient and iron-sufficient uremic rats receiving long-term EPO therapy despite divergent hematocrits. Moreover, repeated red blood cell transfusions did not raise arterial blood pressure despite complete correction of anemia in the uremic animals.11 Accordingly, these experiments provided convincing evidence that EPO-induced hypertension is not related to the erythropoietic action of the hormone. We further found that long-term EPO administration leads to a significant rise in resting cytosolic [Ca$^{2+}$], above the elevated values seen in untreated rats with chronic renal failure (CRF).11 Moreover, EPO therapy normalized the defective stimulated surge in [Ca$^{2+}$], seen in the untreated CRF animals. The study further showed that long-term EPO administration resulted in a marked reduction in hypotensive response in vivo and vasorelaxation response in vitro to administration of the NO donor sodium nitroprusside. These observations pointed to NO resistance as a possible cause of EPO-induced hypertension. Since the vasodilatory action of NO is mediated by cGMP-induced fall in [Ca$^{2+}$], we hypothesized that the observed NO resistance may be due to the demonstrated rise in resting and/or stimulated [Ca$^{2+}$], with EPO therapy.11 The present study was designed to explore the effect of long-term EPO therapy with and without calcium channel blockade on NO production and NO synthase (NOS) expression.

Methods

Animal Models

Male Sprague-Dawley rats (Harlan Sprague Dawley Inc) with an average weight of 225 g were used. They were housed in a climate-controlled, light-regulated space with 12-hour light (500 lux) and 12-hour dark (<5 lux) cycles. Animals were allowed free access to water and a low-nitrate rat chow (Purina Rat Chow, Purina Mills...
Inc. The rats were randomized into the following 5 groups: (1) Placebo-treated CRF group: Animals assigned to the placebo-treated CRF group were subjected to surgical resection of the upper and lower thirds of the left kidney followed by right nephrectomy 4 days later. The procedures were performed under general anesthesia with intraperitoneal injection of sodium pentobarbital 50 mg/kg. Strict hemostasis and aseptic measures were observed. The procedures were performed extraperitoneal with the use of a dorsal incision. The animals were then observed for 6 weeks. (2) EPO-treated CRF group: Animals assigned to this group were subjected to renal mass reduction as described above. They were then treated with intraperitoneal injections of recombinant human EPO (Amgen Inc) 150 μU/kg twice a week for 6 weeks. The given dosage was based on our preliminary experiments performed to discern the amount required to prevent or correct anemia of CRF in this model. (3) Felodipine and EPO–treated CRF group: CRF animals assigned to this group were simultaneously treated with EPO and the calcium channel blocker felodipine (Astra Merck Inc). Felodipine was administered by implanted osmotic pumps (Alza Inc) at 7 mg/kg per day. (4) Felodipine-treated CRF group: Animals assigned to this group were treated with felodipine alone. (5) Control group: Animals assigned to this group were sham operated and placebo treated and maintained under conditions identical to those in the other groups. Body weight, tail arterial blood pressure, and hematocrit (microhematocrit method) were measured regularly. At the end of the 6-week observation period, the animals were placed in individual metabolic cages for 24-hour urine collections. Urine was collected in sterilized containers over dry ice. Animals were then killed by exsanguination by means of cardiac puncture performed with the animals under general anesthesia. Thoracic aorta and kidneys were removed immediately, snap-frozen in liquid nitrogen, and stored at −70°C until processed. In addition, plasma was separated and stored at −20°C.

**Blood Pressure Measurement**

Systolic blood pressure was measured weekly by a tail sphygmomanometer (Harvard Apparatus). On each occasion the rats were placed on a heated pad in a climate-controlled room. The rat’s tail was placed inside the cuff, and the cuff was inflated and released several times to condition the animal to the procedure. Thereafter, 3 separate measurements were performed, and the mean of the values obtained was used.

**Measurements of NOx**

The concentration of total nitrates and nitrates (NOx) in the test samples was determined with the use of the purge system of a Sievers model 270B Nitric Oxide Analyzer (NOA, Sievers Instruments Inc) as described previously. Standard curves were constructed with various concentrations of NOx (5 to 100 μmol/L), relating the luminescence produced to the given NOx concentrations of the standard solutions. The amount of NOx in the test sample was determined by interpolation of the result into the standard curve. All samples were run in triplicate, and the mean of the values obtained was used.

**Tissue Preparation**

Thoracic aorta and remnant kidneys were homogenized (25% wt/vol) in 10 mmol/L HEPES buffer, pH 7.4, containing 320 mmol/L sucrose, 1 mmol/L EDTA, 1 mmol/L DTT, 10 μg/mL leupeptin, and 2 μg/mL aprotinin at 0°C to 4°C with a tissue grinder fitted with a motor-driven ground glass pestle. Homogenates were centrifuged at 12 000 g for 20 minutes to remove tissue debris without precipitating plasma membrane fragments. The supernatant was used for determination of NOx protein mass. Total protein concentration was determined with the use of a Bio-Rad kit (Bio-Rad Laboratories).

**Western Blot Analysis**

The endothelial and inducible NOS (eNOS and iNOS, respectively) proteins were measured with the use of anti-eNOS monoclonal antibody, anti-Mac NOS-I, human endothelial positive control, mouse macrophage positive control, and peroxidase-conjugated goat anti-mouse IgG antibody (Transduction Laboratories). Briefly, tissue preparations (50 μg of protein) were size-fractionated on 4% to 12% Tris-glycine gel (Novex) at 120 V for 3 hours. After electrophoresis, proteins were transferred onto hybond-ECL membrane (Amersham Life Science Inc) at 400 mA for 120 minutes with the Novex transfer system. In preliminary experiments we had found that the given protein concentrations were within the linear range of detection for our Western blot technique. The membrane was prehybridized in 10 mL of buffer A (10 mmol/L Tris-HCl, pH 7.5, 100 mmol/L NaCl, 0.1% Tween 20, and 10% nonfat milk powder) for 1 hour and then hybridized for an additional 1-hour period in the same buffer containing 10 μL of the given anti-NOS monoclonal antibody (1:1000). The membrane was then washed for 30 minutes in a shaking bath, and the wash buffer (buffer A without nonfat milk) was changed every 5 minutes before 1 hour of incubation in buffer A plus goat anti-mouse IgG/horseradish peroxidase at the final titer of 1:1000. Experiments were performed at room temperature. The washes were repeated before the membrane was developed with a light-emitting nonradioactive method with the use of ECL Western blot detection reagent (Amersham Life Science Inc). The membrane was then subjected to autoluminography for 10 seconds. The autoluminographs were scanned with a laser densitometer (model PD1211, Molecular Dynamics) to determine the relative optical densities of the bands. In all instances, the membranes were stained with Ponceau stain, which verified the uniformity of protein load and transfer efficiency across the test samples.

**Platelet Cytosolic Calcium Measurements**

At the end of the 6-week observation period a subgroup of rats was killed, and platelets were isolated by means of a procedure described previously. The isolated platelets were suspended in HEPES-buffered saline with the following composition (mmol/L): NaCl 145, KCl 5.0, NaHPO 4 0.8, KH2PO 4 0.2, MgCl2 1.0, glucose 10, HEPES 10, pH 7.4. This resulted in a platelet suspension containing ~2×108 cells/mL. [Ca2+], was determined with the fluorescent calcium indicator fura 2-AM. The suspended platelets were loaded with 4 μmol fura 2-AM in the presence of 0.02% pluronic F-127 to facilitate entry of the indicator into the cells. In addition, 2 mmol/L probenecid was added to minimize leakage of fura 2 out of the platelets. Cells were incubated for 60 minutes at 37°C, then centrifuged at 400g for 20 minutes. The supernatant was decanted, and an equal volume of HEPES-buffered saline was added. The cells were incubated at 37°C for 30 minutes to allow complete hydrolysis of the AM group. The dye-loaded cells were suspended in HEPES-buffered saline containing 2 mmol CaCl2 and kept under constant magnetic stirring in a thermostatically controlled cuvette of a spectrofluorometer (DMX 1000, SLM Instruments Inc). Alternating excitation wavelengths of 340 and 380 nm were used, with an emission wavelength of 510 nm. Ratios of fluorescence (R~340/380 nm) were measured every second, automatically corrected for autofluorescence, and plotted graphically for each sample analyzed. Values of autofluorescence were <5% of the fluorescence of the dye-loaded cells and were measured for each experiment.

[Ca2+]i was calculated according to the following formula: [Ca2+]i=[(Kd)R(1-R)/Rmax·(Rmax−R)], where B is the ratio of the 380 nm fluorescence in the absence and presence of saturating concentration of calcium, R is the dissociation constant for fura 2 (assumed to be 225 mmol/L), and R is the ratio of fluorescence as defined above. The cells were lysed with Triton (0.5%) to obtain Rmax in the presence of 2 mmol calcium, and R was obtained by the addition of 10 mmol EGTA and sufficient NaOH to raise the pH to 8.5.

**Data Presentation and Analysis**

Data are presented as mean±SEM. ANOVA and Duncan multiple range test were used as appropriate. P values <0.05 were considered statistically significant.

**Results**

**Effects of CRF**

As expected, the placebo-treated CRF group showed a significant increase in serum creatinine concentration and a significant fall in creatinine clearance compared with the normal control.
group. In addition, the placebo-treated CRF group exhibited a significantly lower weight gain than that seen in the control animals (Table). CRF resulted in a significant fall in hematocrit and a significant rise in blood pressure during the observation period (Figures 1 and 2). This was associated with a significant increase in basal cytosolic \([\text{Ca}^{2+}]_i\) and a significant reduction in stimulated cytosolic \([\text{Ca}^{2+}]_i\) in the platelets (Figure 3), consistent with our earlier observations. \(^\text{11}\) The CRF-associated hypertension was accompanied by a significant reduction in urinary NOx excretion together with depressed thoracic aorta and remnant kidney tissue eNOS and iNOS protein abundance (Figures 4 through 8). These observations are in agreement with our recent studies demonstrating downregulation of \(\text{L-arginine–NO pathway}\) in this model. \(^\text{16}\)

### Effects of EPO

EPO therapy did not lead to a discernible change in either serum creatinine, creatinine clearance, or weight gain compared with values seen in the untreated CRF animals (Table). EPO therapy completely prevented the CRF-associated anemia (Figure 1) but led to a marked rise in arterial blood pressure, which began \(\approx 1\) week after the onset of EPO administration (Figure 2).

**Body Weight, Systolic Blood Pressure, Hematocrit, and Creatinine Clearance in Sham-Operated Normal Control Group and Rats With CRF Treated With Placebo, EPO Alone, Felodipine Alone, and EPO Plus Felodipine**

<table>
<thead>
<tr>
<th>Group</th>
<th>Weight, g</th>
<th>Blood Pressure, mm Hg</th>
<th>Hematocrit, %</th>
<th>Creatinine Clearance, mL/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>402±12†</td>
<td>125.5±0.1†</td>
<td>46.4±1.2†</td>
<td>3.3±0.26‡</td>
</tr>
<tr>
<td>Placebo*</td>
<td>345±16</td>
<td>146±4.2</td>
<td>40.0±0.8</td>
<td>1.9±0.2</td>
</tr>
<tr>
<td>EPO*</td>
<td>352±8</td>
<td>175±8.2†</td>
<td>52.6±2.9†</td>
<td>2.1±0.4</td>
</tr>
<tr>
<td>EPO+ felodipine*</td>
<td>361±17</td>
<td>130±3.3†</td>
<td>51.6±2.2†</td>
<td>2.0±0.2‡</td>
</tr>
<tr>
<td>Felodipine*</td>
<td>367±10</td>
<td>125±5.4†</td>
<td>38.0±3.6</td>
<td>2.0±0.25</td>
</tr>
<tr>
<td>(P) (ANOVA)</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

*Rats with CRF.
†\(P<0.05\), ‡\(P<0.01\) vs group with CRF treated with placebo.

The EPO-induced hypertension was accompanied by a significant rise in basal \([\text{Ca}^{2+}]_i\), above the elevated value found in the CRF group. In addition, EPO therapy resulted in a significant increase in stimulated \([\text{Ca}^{2+}]_i\), to a value that was similar to that of the control group. Thus, EPO therapy resulted in complete correction of the
CRF-induced defective surge in platelet [Ca$^{2+}$]i after thrombin stimulation (Figure 3). This observation is consistent with our earlier studies in this model. However, EPO therapy failed to alter either urinary NOx excretion or thoracic aorta eNOS or iNOS protein abundance (Figures 4 through 6). Likewise, EPO therapy had no effect on either eNOS or iNOS protein abundance of the remnant kidney in the CRF group treated with EPO alone (Figures 7 and 8).

Effects of Calcium Channel Blockade

Coadministration of felodipine with EPO completely abrogated the EPO-induced hypertension (Figure 2). This was accompanied by a reduction in resting [Ca$^{2+}$]i to a level that was similar to that seen in the normal control group (Figure 3). Interestingly, felodipine ad-

ministration did not affect the thrombin-stimulated rise in [Ca$^{2+}$]i, (Figure 3). Thus, the combination of EPO therapy and calcium channel blockade resulted in normalization of both basal and stimulated [Ca$^{2+}$]i. In addition, calcium channel blockade led to a significant rise in urinary NOx excretion, suggesting increased total body NO production (Figure 4). The rise in urinary NOx excretion with calcium channel blockade was accompanied by a significant

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Figure 4. Urinary excretion of NOx in the sham-operated normal control group (NL) and rats with chronic renal failure treated with either placebo (CRF), erythropoietin alone (CRF/EPO), felodipine alone (CRF/F), or a combination of EPO plus felodipine (C/E/F). n=6 in each group. **P<0.05 vs control, felodipine-treated, and EPO plus felodipine-treated groups.

Figure 5. A, Representative Western blots of thoracic aorta eNOS protein in 2 sham-operated normal control rats (NL), 2 placebo-treated rats with chronic renal failure (CRF), and 2 erythropoietin-treated (CRF/E, CRF/EPO), 2 felodipine-treated (CRF/F, CRF/FEL), and 2 EPO plus felodipine–treated rats with chronic renal failure (C/E/F). B, Group data depicting relative optical densities of thoracic aorta eNOS protein bands in the study groups. n=6 in each group. *P<0.01 vs control and felodipine-treated groups, **P<0.05 vs other groups.

Figure 6. A, Representative Western blots of thoracic aorta iNOS protein in 2 sham-operated normal control rats (NL), 2 placebo-treated rats with chronic renal failure (CRF), and 2 erythropoietin-treated (CRF/E, CRF/EPO), 2 felodipine-treated (CRF/F, CRF/FEL), and 2 EPO plus felodipine–treated rats with chronic renal failure (C/E/F). B, Group data depicting relative optical densities of thoracic aorta iNOS protein bands in the study groups. n=6 in each group. *P<0.01 vs control and felodipine-treated groups, **P<0.05 vs other groups.

Figure 7. A, Representative Western blots of kidney eNOS protein in 2 sham-operated normal control rats (NL), 2 placebo-treated rats with chronic renal failure (CRF), and 2 erythropoietin-treated (CRF/E, CRF/EPO), 2 felodipine-treated (CRF/F, CRF/FEL), and 2 EPO plus felodipine–treated rats with chronic renal failure (C/E/F). B, Group data depicting relative optical densities of kidney eNOS protein bands in the study groups. n=6 in each group. *P<0.01 vs control and felodipine-treated groups, **P<0.05 vs other groups.
increase in thoracic aorta eNOS and iNOS proteins (Figures 5 and 6). Likewise, felodipine administration increased eNOS and iNOS protein abundance in the remnant kidneys of the CRF group treated with EPO and felodipine. Administration of felodipine in the CRF group resulted in amelioration of CRF-associated hypertension, reduction in basal \([\text{Ca}^{2+}]\), no change in stimulated \([\text{Ca}^{2+}]\), increased urinary NOx excretion, and enhanced renal and vascular eNOS and iNOS protein expression, confirming our earlier observations (Figures 7 and 8).  

**Discussion**

The CRF animals included in the present study exhibited an expected fall in hematocrit and a modest rise in arterial blood pressure. This was accompanied by a significant decline in urinary excretion of total NOx together with depressed urinary excretion of total NOx. These observations confirm the results of our previous studies and point to downregulation of L-arginine–NO system in normal rats. For instance, del Castillo et al showed a significant rise in urinary excretion of NOx in normal rats treated with EPO 150 U/kg thrice weekly for 3 weeks. This was associated with a significant rise in arterial blood pressure and severe erythrocytosis. In another study, induction of severe erythrocytosis with EPO therapy in normal rats resulted in hypertension and increased renal blood flow. Administration of NO inhibitor abrogated the associated rise in renal blood flow and led to an exaggerated hypertensive response in animals with erythrocytosis. These findings were taken as indirect evidence that EPO-induced erythrocytosis results in a compensatory stimulation of L-arginine–NO system in normal rats.  

Several factors can account for the different effects of EPO therapy on NO production in CRF animals used here and normal animals used in the studies of Wilcox et al, del Costillo et al, and Tsukahara et al. First, the animals used here had CRF, which as shown earlier and confirmed here, results in marked downregulation of NOS expression and NO production. Second, the EPO dosage used here was gauged to correct and prevent anemia. Thus, our EPO-treated animals had normal hematocrit and erythrocyte mass. In contrast, normal animals used in the former studies exhibited severe erythrocytosis with long-term EPO administration. This was inevitably accompanied by a marked rise in blood viscosity, volume expansion, and increased shear stress, which can upregulate vascular and renal NOS expression. These factors were clearly absent in our CRF group treated with EPO alone. Similarly, the goal of EPO replacement in patients with renal disease is the amelioration of CRF anemia as opposed to the induction of erythrocytosis.  

In a recent study we demonstrated that EPO-induced hypertension in rats with CRF is unrelated to changes in erythrocyte mass and hematocrit. This was based on the demonstrated occurrence of hypertension with regular EPO administration to CRF rats with iron deficiency in the face of persistent EPO-resistant anemia. Furthermore, repeated blood transfusions, sufficient to prevent anemia, in a separate group of CRF rats failed to raise arterial blood pressure. These observations pointed to the role of regular EPO administration as opposed to the correction of anemia per se in the genesis of EPO-induced hypertension. We further showed that EPO-induced hypertension was associated with resistance to the hypotensive action of the NO donors sodium nitroprusside and S-nitroso penicillamine in vivo and to their vasodilatory actions in vitro. We hypothesized that the resistance to the vasodilatory action of NO may be due to
EPO-induced elevations of resting and stimulated [Ca\(^{2+}\)]. This hypothesis was based on the fact that the vasodilatory action of NO is mediated by the cGMP-induced reduction in [Ca\(^{2+}\)], Hence, elevated [Ca\(^{2+}\)], can naturally contribute to NO resistance and hypertension. On the basis of these observations, in the present study we included a group of CRF animals receiving concurrent EPO treatment and the calcium channel blocker felodipine.

Concurrent administration of felodipine normalized resting [Ca\(^{2+}\)], and abrogated the EPO-induced hypertension. This was accompanied by a rise in urinary excretion of NOx, suggesting increased total body NO production. The rise in NO production was coupled with increased vascular and remnant kidney tissue NOX protein expression in EPO-treated CRF animals. Similarly, calcium channel blockade normalized blood pressure and reversed downregulation of l-arginine–NO pathway in the CRF group, confirming our earlier observations.\(^{16}\) The mechanism by which EPO raises [Ca\(^{2+}\)], in erythroid progenitor and contractile cells has been recently elucidated.\(^{25,26}\) It has been demonstrated that EPO binding to its receptor results in the activation of a cytosolic tyrosine kinase that, in turn, catalyzes the tyrosine phosphorylation and activation of phospholipase Cγ-1. The latter causes hydrolysis of phosphatidylinositol 4,5-bisphosphate and generation of inositol 1,4,5-triphosphate. These events lead to a biphasic rise in [Ca\(^{2+}\)], associated with an initial Ca\(^{2+}\) release from intracellular stores, followed by influx of Ca\(^{2+}\) through EPO receptor-operated, voltage-independent channels.\(^{26}\) Interestingly, AT\(_1\) receptor stimulation by angiotensin II also involves tyrosine kinase-dependent phosphorylation/activation of phospholipase Cγ-1.\(^{27,28}\) This is particularly noteworthy since administration of angiotensin-converting enzyme (ACE) inhibitor has been shown to prevent EPO-induced hypertension in rats with CRF. Given the dual role of ACE in catalyzing the production of angiotensin II and degradation of bradykinin (kininase activity), its inhibition by ACE inhibitors can contribute to the reduction of [Ca\(^{2+}\)], by 2 mechanisms: (1) by mitigating the angiotensin II–mediated activation of phospholipase Cγ-1 and (2) by bradykinin-mediated activation of eNOS, leading to increased NO production. In addition, EPO has been shown to upregulate tissue renin-angiotensin system in the rat.\(^{29}\) These observations form the basis for the demonstrated benefit of ACE inhibition in animals with EPO-induced hypertension.\(^{3}\)

In conclusion, the results of the present study point to the possible role of dysregulation of [Ca\(^{2+}\)] in the pathogenesis of depressed l-arginine–NO pathway and NO resistance in EPO-treated CRF animals. If true, calcium channel blockade may be a logical approach to treatment of hypertension in this setting.

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