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Erythropoietin Does Not Affect Nitric Oxide System in Rats with Chronic Renal Failure

We investigated to see whether an altered role of nitric oxide (NO) system is involved in erythropoietin (EPO)-induced hypertension in chronic renal failure (CRF). Male Sprague-Dawley rats were five-sixths nephrectomized to induce CRF. Six weeks after the operation, EPO or vehicle was injected for another 6 weeks. Plasma and urine nitrite/nitrate (NOx) levels were determined. Expression of NO synthase (NOS) proteins in the aortae and kidneys were also determined. In addition, the isometric tension of isolated aorta in response to acetylcholine and nitroprusside was examined. Blood pressure progressively rose in CRF groups, the degree of which was augmented by EPO treatment. Plasma NOx levels did not differ among the groups, while urine NOx levels were lower in CRF groups. Endothelial NOS expression was lower in the kidney and aorta in CRF rats, which was not further affected by EPO-treatment. The inducible NOS expression in the kidney and aorta was not different among the groups. Acetylcholine and sodium nitroprusside caused dose-dependent relaxations of aortic rings, the degree of which was not altered by EPO-treatment. Taken together, EPO-treatment aggravates hypertension in CRF, but altered role of NO system may not be involved.

Key Words: *Renal Failure, Chronic; Erythropoietin; Hypertension; Nitric Oxide*

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INTRODUCTION

The introduction of recombinant human erythropoietin (EPO) into the routine therapy of uremic anemia has reduced or even eliminated the need for blood transfusion (1). However, treatment with EPO is frequently complicated by development of hypertension or exacerbation of preexisting hypertension (2-4). Although the mechanism of EPO-induced hypertension has not been fully understood, it has been hypothesized that increased hemoglobin following EPO treatment results in an increased binding and inactivation of nitric oxide (NO), contributing to the development of hypertension (5).

On the contrary, recent studies have suggested that NO activity is in fact increased by EPO treatment (6, 7). However, determining NO activity in these studies was indirect, dependent on measurement of the pressor response to NO synthase (NOS) inhibitors and measurement of urinary cGMP excretion. Furthermore, the effects of EPO administration on NO system may differ in chronic renal failure (CRF).

The present study was aimed at determining whether the activity of NO system is altered by EPO treatment in CRF. Nitrite/nitrate (NOx) levels in the plasma and urine were measured, and the expression of NOS isozymes in the kidney and aorta were determined in rats induced with CRF. Changes in isometric tension of the isolated thoracic aorta in response to acetylcholine and sodium nitroprusside were also examined.

MATERIALS AND METHODS

Animal preparation

All experiments were performed using male Sprague-Dawley rats weighing 200 to 250 g. The animals were fed standard rat chow and water ad libitum. Systolic blood pressure was measured in a conscious state by the tail-cuff method every other week. To induce CRF, rats were five-sixths nephrectomized under ketamine anesthesia (50 mg/kg, i.p.), consisting of removing the right kidney and inducing infarction of left kidney by ligation of two segmental renal arteries. The animals were kept for 6 weeks to allow the development of CRF, after which they were divided into two groups. In one group

of CRF rats, the recombinant human EPO (Boehringer Mannheim, Mannheim, Germany) was injected (s.c., 200 U/kg, two times weekly) for another 6 weeks (CRF-EPO). In the other, the vehicle alone was treated in the same manner for the 6 weeks. The vehicle (0.9% NaCl) contained (in mg/mL): 2.5 human serum albumin; 5.8 sodium chloride; 5.8 sodium citrate; and 0.06 and citric acid anhydrous. A group of sham-operated rats served as control.

Experimental protocols

On the experiment day, under thiopental anesthesia (50 mg/kg, i.p.), polyethylene catheters were inserted in the right femoral artery for blood pressure monitoring. The urinary bladder was exposed through a small lower midline abdominal incision and was cannulated with polyethylene 50 tubing for urine collection. After urine collection, trunk blood was collected by decapitating to determine hemoglobin, hematocrit, blood urea nitrogen (BUN), creatinine and NOx levels. The kidneys and aortae were also taken to determine tissue expression of NOS isozymes (iNOS and eNOS). The aorta was also examined for its changes of isometric tension in response to acetylcholine, and sodium nitroprusside to discern whether EPO- induced hypertension is associated with an impaired vascular relaxation.

Furthermore, in order to examine the direct effect of EPO on the vasculature, EPO (10 or 100 U/mL) was acutely treated in vitro, and the isometric tension of the aortic ring taken from normal rats was recorded.

Colorimetric assay of nitrite/nitrate

As an index of synthesis of NO, the plasma and urine concentrations of its stable metabolites were measured by a colorimetric NO assay kit (Oxford Biochemedical Research Inc., Oxford, MI, U.S.A.). A microplate was used to perform enzyme reactions in vitro. For spectrophotometric assay of nitrite with Griess reagent, 80 μL MOPS (50 mmol/L)/EDTA (1 mmol/L) buffer and 5-μL urine or plasma samples were added to wells. Nitrate reductase (0.01 U) and 10-μL NADH (2 mmol/L) were added to the reaction mixture, and the plate was shaken for 20 min at room temperature. Color reagents, sulfanilamide, and N-(1-naphthyl) ethylenediamine dihydrochloride were added, then absorbance values at 540 nm were read in a microtiter plate reader (Bio-Rad, Model 3550).

Protein preparation

The kidneys and thoracic aortae were homogenized with a Polytron homogenizer at 3,000 rpm in a solution containing 250 mmol/L sucrose, 1 mmol/L EDTA, 0.1 mmol/L phenylmethylsulfonyl fluoride and 50 mmol/L potassium phosphate buffer at pH 7.6. Large tissue debris and nuclear fragments were removed by two consecutive low speed centrifuge spins (3,000 *g*, 5 min; 10,000 *g*, 10 min). The membrane-bound protein was further centrifuged at 100,000 *g* for 60 min. The pellet was resuspended for protein blotting of eNOS and the supernatant was used for blotting of iNOS. The protein concentrations of the homogenate were determined by Bradford's method (8), with bovine serum albumin as a standard.

Western blot analysis

Protein samples were electrophoretically size-separated with a discontinuous system consisting of a 7.5% polyacrylamide resolving gel and 5% polyacrylamide stacking gel. High-range molecular weight markers (Bio-Rad; Hercules, CA, U.S.A.) were loaded as size standards. An equivalent amount of total tissue protein (100 mg) was loaded on each lane. After separation, the proteins were electrophoretically transferred to a nitrocellulose membrane at 20 V overnight. The membranes were washed in Tris-based saline buffer (pH 7.4) containing 1% Tween-20 (TBST), blocked with 5% nonfat milk in TBST for one hr, and incubated with a 1:2,000 dilution of monoclonal mouse anti-eNOS and anti-iNOS antibodies (Transduction Laboratories; Lexington, KY, U.S.A.) in 2% nonfat milk/TBST for one hr at room temperature. The membranes were then incubated with a horseradish peroxidase-labeled goat anti-mouse IgG (1:1,000) or goat anti-rabbit IgG in 2% nonfat milk in TBST for 2 hr. The bound antibody was detected by enhanced chemiluminescence on X-ray film or hyperfilm (Amersham, Little Chalfont, Buckinghamshire, UK). The membranes were stripped between incubations with different antibodies in Tris-buffered solution, containing 2% sodium dodecyl sulfate and 100 mmol/L-mercaptoethanol at 50℃.

Recording isometric tension of isolated thoracic aorta

Thoracic aortae were taken under thiopental anesthesia, and were cut into rings, 5-mm long each. Each ring was suspended in a tissue bath containing physiologic salt solution (PSS) at 37℃, while continuously bubbled with 95% O_2-5% CO_2 (pH 7.4). The composition (in mmol/L) of PSS used was NaCl 112, KCl 5, $NaHCO_3$ 25, KH_2PO_4 1.0, $MgSO_4$ 1.2, $CaCl_2$ 2.5 and glucose 11.5. One end of the ring was fixed to the bottom of the bath and the other attached to a force-displacement transducer (Grass FT03) to record its isometric tension. Baseline

load placed on the ring was 2.0 g. Two to three rings were prepared from one animal and their results were averaged to provide a single rat datum. The rings were precontracted with EC_{80} phenylephrine (3.5×10^{-6} mol/L), and the relaxation responses to acetylcholine and sodium nitroprusside were recorded. Relaxation was expressed as percent reductions from the phenylephrine-induced maximum contraction. In the acute treatment study, the vascular responses to acetylcholine and sodium nitroprusside were measured in the aortic rings preincubated with EPO (10 U/mL or 100 U/mL, 1 hr).

Statistical analysis

Results are presented as mean±SEM. One-way analysis of variance was used in analyzing the data. $p<0.05$ was considered statistically significant.

RESULTS

General

Body weight and laboratory parameters are shown in Table 1. The body weight at the conclusion of the study was significantly lower in CRF rats than in the control. The CRF groups showed significant rises in serum creatinine levels and urinary protein levels. CRF groups also exhibited a significant fall in hemoglobin and hematocrit. After 6 weeks of EPO treatment, however, there were significant increases in hemoglobin and hematocrit.

Fig. 1 shows the systolic blood pressure measured by the tail-cuff method during the observation period. Blood pressure progressively rose in the CRF group, which were augmented by EPO treatment. The blood pressure directly measured via femoral artery cannulation on the experiment day was 92±6 mmHg in the control (n=12), 147±7 mmHg in the CRF-vehicle (n=10) and 165±6 mmHg (n=8) in the CRF-EPO group.

NOx levels and NOS expression

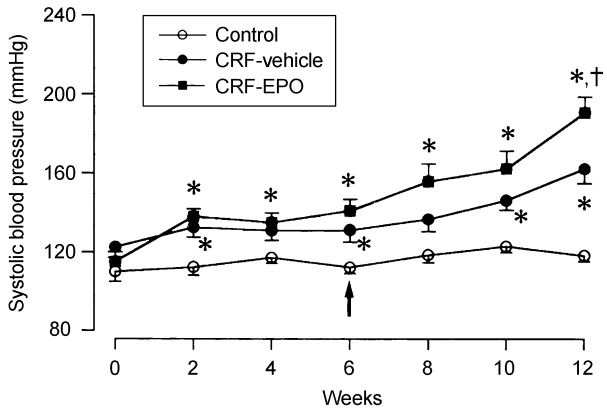

Fig. 1. Systolic blood pressure (SBP) in CRF rats treated with erythropoietin (CRF-EPO) or vehicle (CRF-vehicle). SBP in control rats is also shown. Arrow indicates the beginning of EPO or vehicle treatment. Results are mean±SEM of 8-12 rats. *$p<0.05$, compared with control; †$p<0.05$, compared with CRF-vehicle.

Plasma and urinary NOx concentrations are given in Fig. 2. The plasma levels did not significantly differ among the three groups. However, the urinary levels were significantly lower in CRF groups compared with those in the control. EPO-treatment did not affect nitrite/nitrate levels.

The expression of iNOS and eNOS was determined in the kidney and aorta by Western blot analysis. Fig. 3 and 4 show representative Western blot and their densitometric analysis in the kidney and aorta, respectively. Anti-iNOS and anti-eNOS antibodies recognized protein bands with molecular sizes of 130 and 140 kDa, respectively. eNOS proteins were expressed lower, while iNOS proteins were not significantly altered in CRF groups. EPO-treatment did not further affect either eNOS or iNOS expression.

Vascular isometric tension responses

Fig. 5 shows the responses of the isolated aorta to acetylcholine and sodium nitroprusside. Acetylcholine and sodium nitroprusside induced dose-dependent relax-

Table 1. Body weights and laboratory parameters

	Control (n=8)	CRF-vehicle (n=10)	CRF-EPO (n=10)
Body weight (g)	490±6	437±15*	435±18*
Hemoglobin (g/dL)	14.1±2.3	11.6±1.2†	13.9±2.6§
Hematocrit (%)	42.8±0.6	34.9±0.9†	41.6±2.9§
Plasma creatinine (mg/dL)	0.41±0.1	0.87±0.2†	0.82±0.2†
Proteinuria (mg/dL)	7.0±1	317±133†	330±157†

Data are presented as mean±SEM. CRF, chronic renal failure; EPO, erythropoietin
*$p<0.05$, †$p<0.01$, ‡$p<0.001$, compared with control; §$p<0.05$, compared with CRF-vehicle

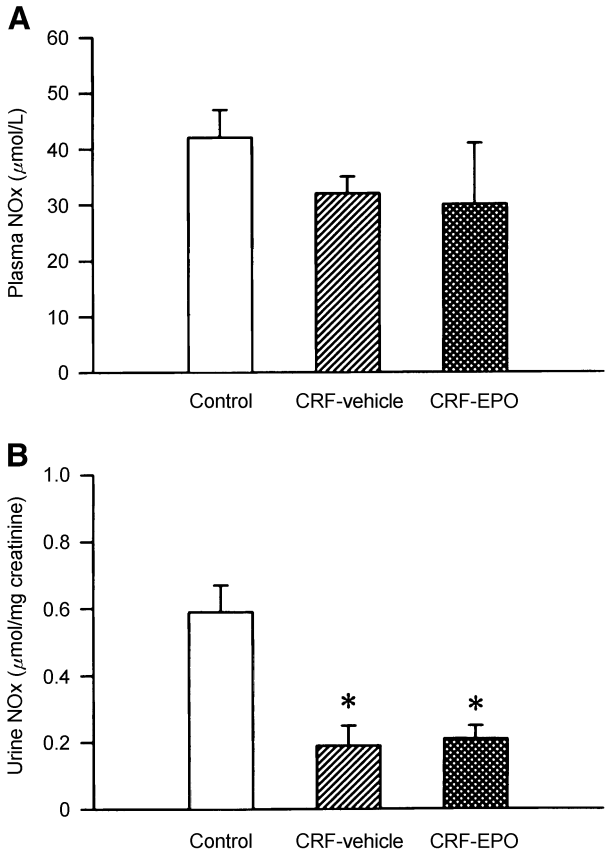

Fig. 2. Plasma (A) and urinary (B) levels of NO metabolites. Each column represents mean±SEM of 6 experiments. Abbreviations as in Fig. 1. *$p<0.05$, compared with control.

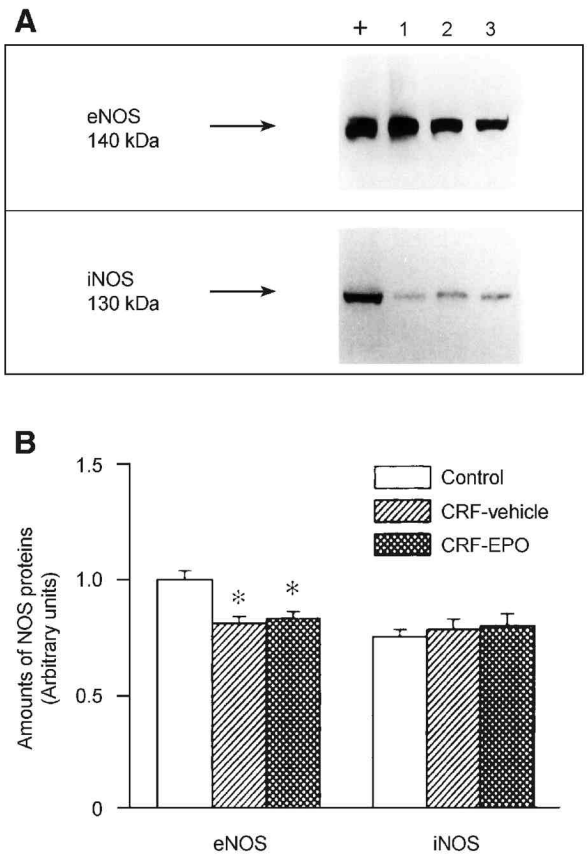

Fig. 3. NOS protein expression in the kidney. Representative Western blot analysis of eNOS and iNOS (A), and their densitometric analysis of 8 rats are each shown (B). +, positive control; 1, control; 2, CRF-vehicle; 3, CRF-EPO; *$p<0.05$, compared with control.

ations of phenylephrine-precontracted aortic rings. The degree of vasorelaxation was not altered in CRF groups, regardless of whether EPO was treated or not. An acute treatment with EPO (10 U/mL or 100 U/mL) in vitro also did not affect vascular responses to acetylcholine or sodium nitroprusside (data not shown).

DISCUSSION

NO metabolism in CRF has been the subject of controversy. Both increased and decreased activities of NO system have been suggested (9-11). In the present study, urinary nitrite/nitrate levels were significantly decreased in CRF along with diminished eNOS protein expression in the vasculature and remnant kidney. These findings agree with previous observations in which progressive nephropathies developed in association with a reduced capacity of the kidney to generate NO (12, 13). It has also been shown that progressive deterioration of renal function and structure was retarded by a chronic administration of NO donors in experimental CRF (12). Furthermore, NO precursor L-arginine ameliorated renal hemodynamics, reduced proteinuria and preserved renal morphology in partial nephrectomized rats (13).

Along with decreased urinary nitrite/nitrate levels, the expression of eNOS proteins was diminished in the kidney and aorta, while iNOS protein expression was not altered in CRF. This finding suggests that eNOS expression is specifically inhibited in CRF. It is likely that loss of functional endothelium by a glomerular and vascular sclerosis reduces eNOS expression in CRF. Vaziri et al. (10) also demonstrated decreases in NOS protein expression and NOS activity in the kidney and aorta in rats with CRF.

However, plasma NOx levels were normal in CRF in the present study. If systemic NO generation was maintained normally despite decreased NOS expression in CRF, the plasma NOx would have piled up. Therefore, the normality of the plasma levels of NO metabolites suggests a decreased formation of NO.

The aggravation of pre-existing hypertension or the

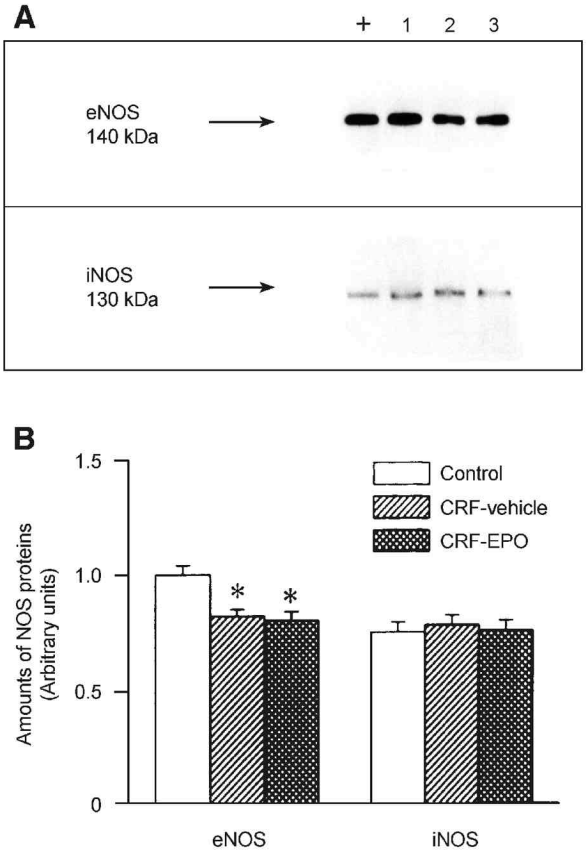

Fig. 4. NOS protein expression in the thoracic aorta. Legends as in Fig. 3. *$p<0.05$, compared with control.

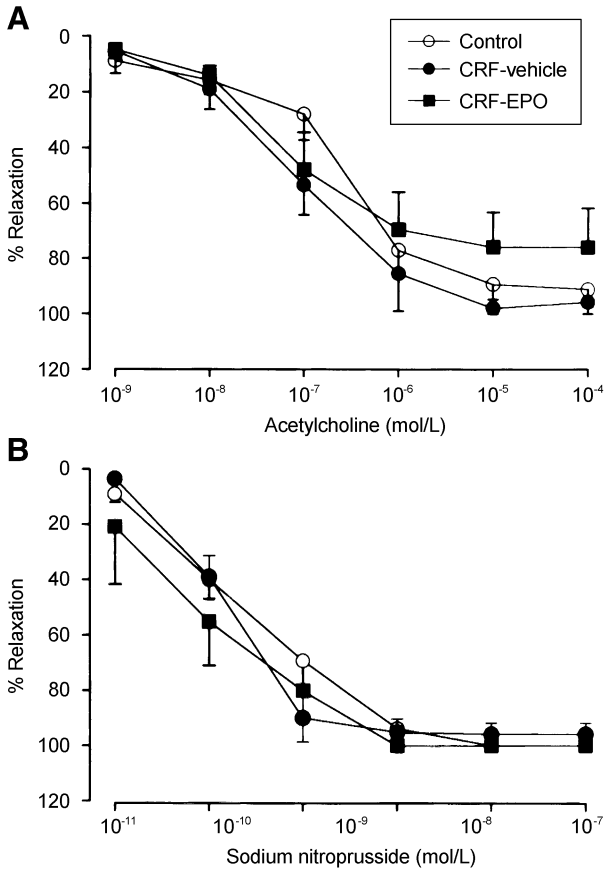

Fig. 5. Cumulative concentration-responses of isolated thoracic aortic rings precontracted with phenylephrine to acetylcholine (A) and sodium nitroprusside (B). Each point represents mean±SEM of 6 experiments. Abbreviations as in Fig. 1.

development of de novo hypertension in uremic patients was recognized soon after the introduction of EPO into clinical use (14, 15). It has been suggested that hypertension associated with EPO therapy is due to increased trapping of NO by the high levels of hemoglobin (5, 16). Although NO system may not be directly affected by EPO therapy, its role may be decreased due to an increased quenching as a consequence of the increased hemoglobin. In the present study, CRF animals exhibited a significant rise in arterial pressure, which was aggravated by EPO-treatment in association with correction for anemia. However, EPO therapy did not affect either NO production or NOS protein abundance in CRF. Furthermore, urinary and plasma nitrite/nitrate concentrations were not altered by EPO. These findings do not support the previous observations made by Muntzel et al. (6) and Wilcox et al. (7), in which an increased action of vascular NO was noted in the rat with EPO-induced polycythemia. A rise in hematocrit induced by EPO therapy will increase blood viscosity and thus vessel wall shear stress, resulting in a stimulation of NO system. In addition, del Castillo et al. (17) showed a significant rise in urinary excretion of NO metabolites along with increased arterial pressure and severe erythrocytosis in normal rats when treated with moderate doses of EPO for 3 weeks. However, these studies used normal rats. An erythrocytosis may develop as a consequence of EPO therapy in normal subjects (7, 17, 18), which inevitably culminates in marked rises in blood viscosity and shear stress with subsequent upregulation of vascular NOS expression (19, 20). Furthermore, a recent in vitro study using bovine aortic endothelial cells showed that EPO did not modify the synthesis of NO and expression of NOS (21). It is therefore likely that the effect of EPO may differ between normal and CRF subjects.

Acetylcholine and sodium nitroprusside elicited dose-dependent relaxations of isolated aortic rings. However, the degree of relaxation was not affected by chronic treatment with EPO, nor was the relaxation response to acetylcholine and sodium nitroprusside affected by acute treatment with EPO in vitro. These findings also indicate that the pressor effect of EPO may not be attributed to impaired relaxation response of the vasculature.

In summary, our study showed that EPO aggravated

hypertension in experimental CRF, in which an altered role of NO system may not be involved.

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