Nephrol Dial Transplant (1995) 10: 505–508

Nephrology
Dialysis
Transplantation

Original Article

The pressor effect of recombinant human erythropoietin is not due to decreased activity of the endogenous nitric oxide system

D. del Castillo, L. Raij, P. J. Shultz and J. P. Tolins

Department of Medicine, Veterans Affairs Medical Center, and University of Minnesota School of Medicine, Minneapolis, USA

Abstract. In a subset of dialysis patients, erythropoietin (rHuEpo) treatment exacerbates hypertension. The mechanism of this pressor effect is unknown; however, it has been suggested that decreased endogenous nitric oxide (NO) activity may play a role. To explore this hypothesis, Sprague–Dawley rats were given rHuEpo (150 U/kg s.c. three times per week) or corresponding vehicle. Blood pressure, haematocrit, and urinary excretion of the stable NO metabolites, nitrite (NO_2) and nitrate (NO_3), were determined at baseline and 3 weeks. After 3 weeks of rHuEpo treatment there was a significant increase in blood pressure and haematocrit, while in vehicle-treated rats blood pressure and haematocrit remained at basal levels. Urinary excretion of $NO_2 + NO_3$ increased compared to basal in rHuEpo, but not vehicle rats. Thus in normal rats rHuEpo does have a significant pressor effect, but this is not associated with decreased activity of the endogenous NO system. Thus decreased endogenous NO activity is not responsible for rHuEpo-associated hypertension. These data further suggest that endogenous NO activity is increased in rHuEpo-treated rats, perhaps as a counter-regulatory mechanism that limits the pressor effect. Whether this mechanism is active in the setting of rHuEpo-treated chronic renal failure in humans is unknown.

Key words: erythropoietin; hypertension; nitric oxide; NO

Introduction

Recombinant human erythropoietin (rHuEpo) is an effective treatment of the anaemia observed in end-stage renal disease patients maintained on haemodialysis [1–3]. The major side-effect of rHuEpo treatment in dialysis patients is hypertension, which occurs in 20–30% of patients [1–5], and may require initiation or augmentation of antihypertensive medication [6]. In addition it has been suggested that rHuEpo therapy is associated with an increased incidence of vascular thrombosis, especially of the vascular access for dialysis [3].

Because rHuEpo therapy is associated with hypertension and perhaps a thrombotic tendency, it has been hypothesized that rHuEpo treatment may result in a decrease in activity of the endogenous nitric oxide (NO) system [7]. According to this hypothesis, increases in haemoglobin induced by rHuEpo result in increased binding and inactivation of NO, with subsequent development of hypertension [7]. This concept has not been supported by recent animal studies that have suggested that NO activity is in fact increased in normal rats treated with rHuEpo [8,9]. Thus the rHuEpo-induced increase in blood pressure may be limited by a simultaneous increase in NO activity. However, determination of the activity of the endogenous NO system in these studies was indirect, relying on measurement of the pressor responses to nitric oxide synthase inhibitors and measurements of urinary cGMP excretion [8,9]. The former is subject to various interpretations and the latter is non-specific, being influenced by atrial natriuretic peptide as well as NO [10].

Nitric oxide is a very labile substance and direct determinations of NO activity *in vivo* has proved to be technically difficult. However, NO is rapidly degraded in biological solutions into the stable end-products, nitrite (NO_2) and nitrate (NO_3); and the rate of urinary excretion of $NO_2 + NO_3$ has been demonstrated to be an accurate indicator of NO activity in vivo [11–13]. In order to resolve these issues, in the current study we determined the effect of rHuEpo treatment over 3 weeks on haematocrit (Hct), blood pressure (BP), and urinary $NO_2 + NO_3$ excretion rate in normal rats.

Subjects and methods

Male Sprague–Dawley rats (275–300 g, Harlan–Sprague–Dawley Inc., Indianapolis, USA) were used for all experi-

Correspondence and offprint requests to: Jonathan P. Tolins MD, Director, Renal Transplant Service and Staff Nephrologist, MVAMC, Assistant Professor of Medicine, U. of Minnesota School of Medicine, Renal Section 111-J, 1 Veterans Dr., Minneapolis, MN. USA 55417.

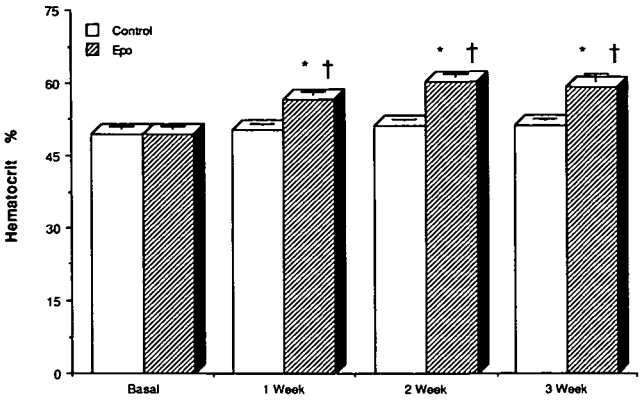

Fig. 1. Effects of treatment with rHuEpo (150 U/kg s.c. three times per week) or vehicle (Control) on haematocrit. $*P<0.05$ versus Basal; $\dagger P<0.05$ rHuEpo versus control.

ments. Rats were housed in temperature-controlled rooms (22°C) with a 12-h light–dark cycle.

Experimental protocol. After baseline studies, rats were randomized into two groups ($n=10$). EPO rats received rHuEpo (Amgen, Inc., Thousand Oaks, California, USA; 150 U/kg s.c. three times per week) for 3 weeks. Control rats received vehicle alone on the same schedule. Blood pressure and Hct were measured at baseline, 1, 2 and 3 weeks. Systolic BP was recorded by the tail-cuff method in conscious animals (Programmed Electro-Sphygmomanometer, Narco Bio-Systems, Inc., Houston, Texas, USA), and blood for the determination of Hct was collected from the distal tail vein under metaphane anaesthesia.

NO_2+NO_3 assay. Urinary excretion of NO_2+NO_3 was determined at baseline and 3 weeks. Samples were assayed for the stable end-products of NO, NO_2 and NO_3, as previously described [11–13]. Briefly, samples were first incubated with *E. coli* nitrate reductase to convert NO_3 to NO_2. To prepare this enzyme, *E. coli* (ATCC 25922) were grown for 18 h under anaerobic conditions, washed, resuspended in PBS, and frozen at −70°C until used. After the enzyme incubation, total NO_2 in the samples (representing both NO_2 and reduced NO_3) was determined using the Griess reagent. Known concentrations of $NaNO_2$ and $NaNO_3$ were used as standards in each assay.

Statistical analysis. Data are presented as mean ± SEM. Parameters between groups were compared by unpaired Student's *t* test. Parameters between basal and subsequent time-points were compared by analysis of variance for repeated measures. All statistical analysis was done with Statview 512 software (Brainpower Inc., Calabasas, California). Differences were considered significant for $P<0.05$.

Results

There was no difference in basal body weight between the two groups (rHuEpo versus control, 283 ± 2 versus 283 ± 3 g, *P* NS), and rats in both groups exibited normal growth over the course of the experiment (final weight: rHuEpo versus control, 351 ± 4 versus 339 ± 5 g, *P* NS). Data for Hct over the time course of the experimental protocol in rHuEpo and control rats is shown in Figure 1. As can be seen, by 1 week there was a significant increase in Hct compared to baseline in rHuEpo rats, while Hct remained unchanged in controls. Final Hct at 3 weeks was significantly greater in rHuEpo compared to control rats. Thus rHuEpo administered by the current protocol was effective in increasing Hct in normal rats.

Figure 2 shows awake, systolic BP in rHuEpo and control rats at baseline, 1, 2 and 3 weeks. As can be seen, BP was significantly elevated by 1 week in rHuEpo as compared to baseline, and remained significantly elevated for the duration of the experimental protocol. Thus, treatment of normal rats with rHuEpo results in a sustained and significant increase in BP.

Urinary excretion of NO_2+NO_3 in rHuEpo and control rats is shown in Figure 3. Levels were similar in both groups at baseline. By 3 weeks, rHuEpo rats demonstrated a significant increase in NO_2+NO_3 excretion rate compared to baseline, while excretion rate in control rats remained unchanged. There was a tendency for increased NO_2+NO_3 excretion rate in rHuEpo compared to control rats at 3 weeks, but this did not achieve statistical significance. Thus, treatment with rHuEpo was associated with a significant increase

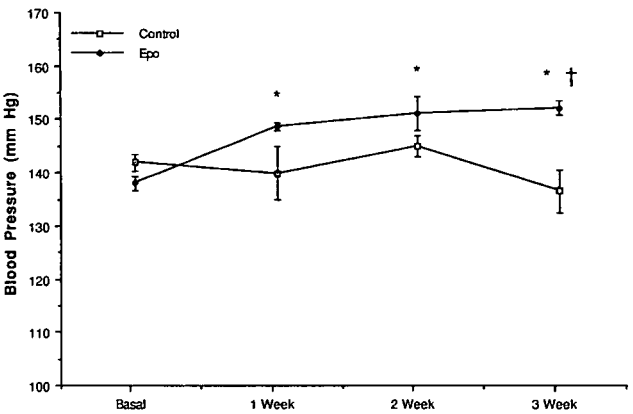

Fig. 2. Awake systolic blood pressure in rats treated with rHuEpo (150 U/kg s.c. three times per week) or vehicle (Control). $*P<0.05$ versus Basal; $\dagger P<0.05$ rHuEpo versus control.

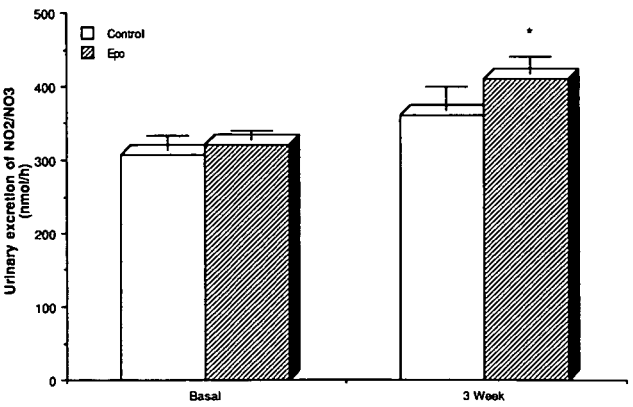

Fig. 3. Urinary excretion of NO_2+NO_3 in rats treated with rHuEpo (150 U/kg three times per week) or vehicle (Control). $*P<0.05$ versus Basal.

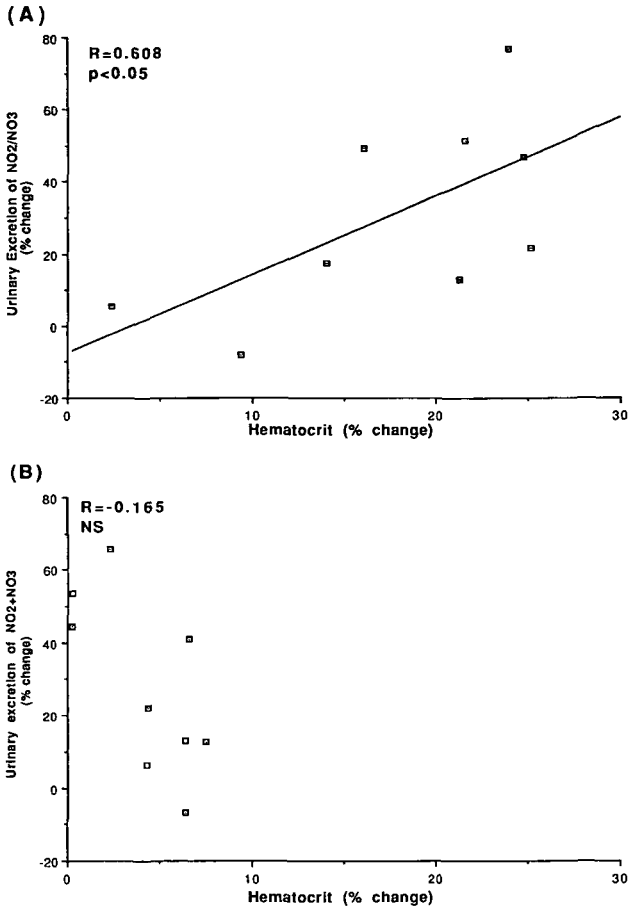

Fig. 4. Correlation between percent change in urinary excretion of NO_2+NO_3 and percent change in haematocrit, from basal to three weeks. A significant positive correlation was found between the increase in haematocrit and the increase in urinary NO_2+NO_3 excretion in the rHuEpo-treated rats (**a**), but not vehicle-treated rats (**b**).

in urinary excretion of the NO metabolites, NO_2+NO_3. Furthermore, as shown in Figure 4, in rHuEpo-treated rats, the change in urinary NO_2+NO_3 excretion rate from baseline to 3 weeks was significantly correlated with the change observed in Hct. In control rats there was no significant correlation between the changes in NO_2+NO_3 excretion and Hct (Figure 4). There was no significant correlation between the change in blood pressure and the change in urinary excretion of NO_2+NO_3 from baseline to 3 weeks in either group of rats.

Discussion

The mechanism by which treatment with rHuEpo increases BP in haemodialysis patients remains unclear. Potential mechanisms for this adverse effect include increased blood viscosity associated with increased Hct [14,15], reversal of hypoxic vasodilatation [6,16], and a vasoconstrictor effect of rHuEpo [17]. Recently, Caravaca *et al.* [18] reported that in dialysis patients treated with rHuEpo, use of antiplatelet agents markedly decreased the incidence of hypertension. They postulated that rHuEpo may effect platelet aggregability and the balance between vasoconstrictor and vasorelaxing prostanoids or other factors produced by the endothelium, in such a way as to favour vasoconstriction and hypertension [18]. Along these same lines, Carlini *et al.* [19] have reported that rHuEpo increased production and release of the vasoconstrictor, endothelin-1, by endothelial cells *in vitro*.

In patients treated with rHuEpo for the anaemia of ESRD the hypertension that develops or worsens in about one-third of patients does not seem to be related to the dose of rHuEpo or the rate of rise in Hct [6]. The hypertension in these patients appears to be most directly related to an increase in systemic vascular resistance, as cardiac output and blood volume are unchanged [6]. It has recently been hypothesized that this increase in vascular resistance could be due to increased binding and inactivation of NO, as a consequence of the rise in haemoglobin during rHuEpo treatments [7]. The resulting decrease in NO activity would favour vasoconstriction and increased systemic vascular resistance. This hypothesis is based on the well-described binding of NO by haemoglobin *in vitro*. However, there is no evidence that the changes in haemoglobin observed clinically in patients treated with rHuEpo would effect endogenous NO activity. Furthermore two recent studies in animals have suggested that NO activity is increased when the haematocrit is raised in normal animals by treatment with rHuEpo [8,9].

In the current study we sought to resolve this controversy by determining endogenous NO activity in normal rats treated with rHuEpo in doses sufficient to significantly raise haematocrit. After 3 weeks of treatment with rHuEpo normal rats demonstrated a significant increase in blood pressure and haematocrit, changes that were not observed in control rats. Rats treated with rHuEpo also demonstrated a significant increase in the urinary excretion of the NO metabolites, NO_2+NO_3, when compared to basal levels, while NO_2+NO_3 excretion rates remained unchanged in control rats. Inspection of Figure 3 reveals that there was a numerical increase in the urinary excretion of NO metabolites in control rats that was not statistically significant. We have previously reported that otherwise unmanipulated control rats do tend to increase excretion of NO_2+NO_3 in the urine over time [12,23]. This finding is most likely due to increased body weight and increased food consumption. These data clearly demonstrate that activity of the endogenous NO system is increased, not decreased, in response to rHuEpo. Furthermore, as shown in Figure 4 there was a significant correlation between the increase in haematocrit and the increase in urinary NO_2+NO_3 excretion in rHuEpo-treated rats.

Urinary excretion of NO_2+NO_3 is currently the best available *in-vivo* marker of activity of the endogenous NO system [11–13]. Tolins *et al.* [20] first suggested that activity of the endogenous NO system could be

quantitated *in vivo* by measuring urinary excretion rate of cGMP, the second messenger of NO *in vivo*. Wilcox and co-workers [9] indeed demonstrated increased urinary cGMP excretion rates in rHuEpo-treated rats. However, urinary cGMP excretion is also increased by other factors, such as atrial natriuretic factor [10], and this can complicate the interpretation of data. Other studies [12], have suggested that the haemodynamic response to inhibition of NO synthase can provide information on basal activity of the endogenous NO system. In this situation it is proposed that an enhanced vasoconstrictor or pressor response to acute and preferably maximal inhibition of NO synthase indicates increased NO activity in the baseline [12]. Reports of this type of experiment in rHuEpo-treated rats [8,9] have also suggested that NO activity is increased rather than decreased in response to rHuEpo treatment. Thus our data is complementary to previous work in this model, and confirms by specific assay that activity of the endogenous NO system is increased in rHuEpo-treated rats.

Previous work has demonstrated that stimulation of the endogenous NO system results in vasodilatation and natriuresis [20,21], while inhibition of NO synthase *in vivo* induces hypertension and vasoconstriction, and decreases sodium excretion [12,20,22]. Thus it is reasonable to propose that the increase in NO activity observed in rHuEpo-treated rats has a modulating effect on the pressor response, limiting the increase in blood pressure. It is also reasonable to speculate that patients with a deficient NO response to rHuEpo treatment might be particularly susceptible to the hypertensive effects of this agent. Perhaps in future studies, levels of NO_2+NO_3 in serum of dialysis patients at baseline and during rHuEpo treatment could be determined and correlated with changes in blood pressure.

In summary, in normal rats treated for 3 weeks with rHuEpo, haematocrit and blood pressure were increased. Activity of the endogenous NO system was also increased during rHuEpo treatment. We conclude that decreased activity of the endogenous NO system does not underlie the pressor effect of rHuEpo in normal animals. Furthermore increased NO activity during rHuEpo treatment may have a modulating effect, limiting the development of hypertension. The relevance of these findings to rHuEpo-induced hypertension in dialysis patients awaits further study.

Acknowledgements. This study was supported by research funds from the US Dept of Veterans Affairs. We thank B. Nyguyen, K. Lewis and K. Anderson for technical assistance.

References

1. Eschbach JW, Egrie JC, Downing MR, Browne JK, Adamson JW. Correction of the anemia of end-stage renal disease with recombinant human erythropoietin. *N Engl J Med* 1987; 316: 73–78
2. Eschbach JW, Abdulhadi MH, Browne JK *et al.* Recombinant human erythropoietin in anemic patients with end-stage renal disease. *Ann Intern Med* 1989; 111: 992–1000
3. Canaud B, Polito-Bouloux C, Garred LJ *et al.* Recombinant human erythropoietin: 18 months' experience in hemodialysis patients. *Am J Kidney Dis* 1990; 15: 169–175
4. Eschbach JW. *Anemia, Hypertension and Hemodynamics in Patients on Dialysis.* Global Medical Communications, New York, 1990
5. Van de Borne P, Tielemans C, Vanherweghem J-L, Degaute J-P. Effect of recombinant human erythropoietin therapy on ambulatory BP and heart rate in chronic haemodialysis patients. *Nephrol Dial Transplant* 1992; 7: 45–49
6. Raine AEG, Roger SD. Effects of erythopoietin on blood pressure. *Am J Kidney Dis* 1991; 18: 76–83
7. Martin J, Moncada S. Blood pressure, erythropoietin and nitric oxide. *Lancet* 1988; 1: 644
8. Muntzel M, Hannedouche T, Lacour B, Drueke T. Effect of erythropoietin on hematocrit and blood pressure in normotensive and hypertensive rats. *J Am Soc Nephrol* 1992; 3: 182–187
9. Wilcox CS, Deng X, Doll AH, Snellen H, Welch WJ. Nitric oxide mediates renal vasodilation during erythropoietin-induced polycythemia. *Kidney Int* 1993; 430–435
10. Arnal JF, Warin L, Michel JB. Determinants of aortic cyclic guanosine monophosphate in hypertension induced by chronic inhibition of nitric oxide synthase. *J Clin Invest* 1992; 90: 647–652
11. Tolins JP, Shultz PJ, Raij L, Brown DM, Mauer SM. Abnormal renal hemodynamic response to reduced renal perfusion pressure in diabetic rats: Role of NO. *Am J Physiol* 1993; 265: F886–F895
12. Shultz PJ, Tolins JP. Adaptation to increased dietary salt intake in the rat. Role of endogenous nitric oxide. *J Clin Invest* 1993; 91: 642–650
13. Shultz PJ, Raij L. Endogenously synthesized nitric oxide prevents endotoxin-induced glomerular thrombosis. *J Clin Invest* 1992; 90: 1718–1725
14. Pirofsky B. The determination of blood viscosity in man by a method based on Poiseuille's Law. *J Clin Invest* 1953; 32: 292–298
15. Steffen HM, Brunner R, Muller R *et al.* Peripheral hemodynamics, blood viscosity, and the renin–angiotensin system in hemodialysis patients under therapy with recombinant human erythropoietin. *Contrib Nephrol* 1989; 76: 292–298
16. Raine AEG. Hypertension, blood viscosity, and cardiovascular morbidity in renal failure: implications of erythropoietin therapy. *Lancet* 1988; 1: 97–100
17. Heidenreich S, Rahn K-H, Zidek W. Direct vasopressor effect of human erythropoietin on renal resistance vessels. *Kidney Int* 1991; 39: 259–265
18. Caravaca R, Pizarro JL, Arrobas M, Cubero JJ, Garcia MC, Perez-Miranda M. Antiplatelet therapy and development of hypertension induced by recombinant human erythropoietin in uremic patients. *Kidney Int* 1994; 45: 845–851
19. Carlini RG, Dusso AS, Obialo CI, Alvarez UM, Rothstein M. Recombinant human erythropoietin increases endothelin-1 release by endothelial cells. *Kidney Int* 1993; 43: 1010–1014
20. Tolins JP, Palmer RMJ, Moncada S, Raij L. Role of endothelium-derived relaxing factor in regulation of renal hemodynamic responses. *Am J Physiol* 1990; 258: H655–H662
21. Lahera V, Salom MG, Fiksen-Olsen J, Raij L, Romero JC. Effects of NG-monomethyl-L-arginine and L-arginine on acetylcholine renal response. *Hypertension* 1990; 15: 659–663
22. Tolins JP, Raij L. Effects of aminoacid infusion on renal hemodynamics. Role of endothelium-derived relaxing factor. *Hypertension* 1991; 17: 1045–1051
23. Tolins JP, Shultz PJ. Endogenous nitric oxide synthesis determines sensitivity to the pressor effect of salt. *Kidney Int* 1994; 46: 230–236

Received for publication 14.4.94
Accepted in revised form 24.11.94