

# Erythropoietin response to hypoxia in patients with diabetic autonomic neuropathy and non-diabetic chronic renal failure

D. R. Bosman, C. A. Osborne\*, J. T. Marsdent, I. C. Macdougall‡, W. N. Gardner\* and P. J. Watkins

King's Diabetes Centre, \*Department of Respiratory Medicine, †Department of Biochemistry and ‡Department of Renal Medicine, King's College Hospital, London, UK

Accepted 14 July 2001

## Abstract

**Aims** An erythropoietin (EPO)-deficient anaemia is recognized in Type 1 diabetic patients with early nephropathy and symptomatic autonomic neuropathy (DN). The aim of this study was to determine whether the EPO response to hypoxia was deficient in order to clarify the mechanisms involved in this process.

**Methods** Five Type 1 diabetic patients DN (age 39 (28–48) years (mean (range))) with EPO-deficient anaemia (haemoglobin, Hb 10.6 (9.5–12.0) g/dl, EPO 5.0 (3.2–6.5) IU/l) and early diabetic nephropathy (persistent proteinuria 1161.6 (130–2835) mg/day, serum creatinine 97.6 (63–123)  $\mu$ mol/l) were compared with nine normal subjects (age 31 (24–39) years, Hb 13.4 (11.8–15.7) g/dl, EPO 7.6 (5.6–10.3) IU/l) and four patients with non-diabetic advanced chronic renal failure RF (proteinuria 2157.5 (571–4578) mg/day, serum creatinine 490.2 (406–659)  $\mu$ mol/l, Hb 10.3 (9.0–11.3) g/dl, EPO 4.6 (2.9–8.5) IU/l). The subjects were exposed to 6 h of hypoxia (inspired oxygen 11.6–12.6%) by breathing a gas mixture via a hood. Hourly serum EPO levels were measured.

**Results** All groups showed a rise in EPO production after 2 h. The diabetic DN group achieved a similar maximal response to the normal subjects at 6 h (EPO  $17.3 \pm 5.4$  vs.  $17.8 \pm 7.9$  IU/l). The renal failure patients mounted an EPO response to hypoxia but at lower EPO levels.

**Conclusions** Although the DN patients have inappropriately low EPO levels for the severity of their anaemia, they can mount an appropriate EPO response to moderate hypoxia. The mechanism underlying the EPO-deficient anaemia present in some diabetic patients remains unclear.

Diabet. Med. 19, 65–69 (2002)

**Keywords** hypoxia, erythropoietin, anaemia, diabetic autonomic neuropathy, nephropathy

**Abbreviations** EPO, erythropoietin; rh, recombinant human; Hb, haemoglobin

## Introduction

Erythropoietin (EPO), a humoral activator of erythropoiesis, was first named by Bonsdorff and Jalavisto in 1948 [1]. EPO

is a monomeric globular acidic glycoprotein containing 165 amino acids. EPO acts via cell surface receptors on target cells in the bone marrow to prevent apoptosis of the erythrocytic progenitors and to trigger erythroid maturation and differentiation to increase the red cell mass in response to tissue hypoxia. In adult life, 90% of EPO production originates in the kidney [2] from peritubular fibroblasts in the renal cortical

Correspondence to: Dr Peter J. Watkins, King's Diabetes Centre, King's College Hospital, Denmark Hill, London SE5 9RS, UK.

interstitium [3]. When chronic renal failure progresses to an extent that the glomerular filtration rate falls below approximately 40 ml/min (equivalent to a serum creatinine of greater than approximately 177  $\mu\text{mol/l}$ ), a chronic anaemia develops, which is associated with inappropriately low serum EPO levels [4,5].

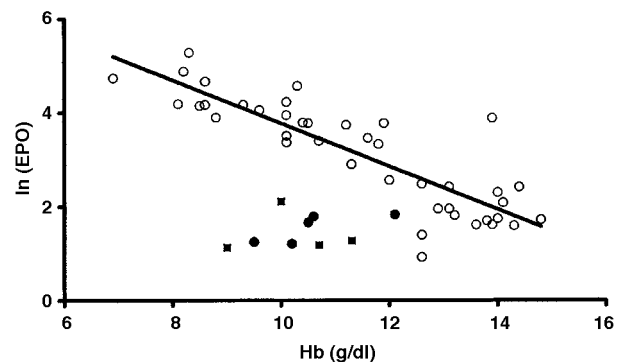
Anaemia occurs in some patients with long-standing diabetic complications. We recently reported the development of an EPO-deficient anaemia in patients with early diabetic nephropathy as defined by persistent proteinuria without renal failure [6], perhaps due to a tubulointerstitial lesion known to be present in diabetic nephropathy [7], which may lead to damage or dysfunction of the EPO producing fibroblasts in the renal interstitium. We and others have shown that patients with severe diabetic autonomic neuropathy may become anaemic with an associated EPO deficiency [8–10]. EPO deficiency in these patients may result from autonomic dysfunction possibly leading to renal denervation, with supporting evidence cited from animal studies [11–14], and observations that patients with primary autonomic failure also suffer an EPO-associated deficiency [15]. However, all these diabetic patients had early diabetic nephropathy and therefore the concomitant renal damage was the most likely cause of the EPO deficiency. The aim of the present study was therefore to elucidate the mechanism of the EPO deficiency in anaemic diabetic patients with early nephropathy and neuropathy to determine whether or not the EPO response to hypoxia was blunted.

## Patients and methods

Patients were recruited from the Diabetes Clinic and the Low Clearance Clinic in the Renal Unit at King's College Hospital. Five Type 1 diabetic patients (DN) with persistent proteinuria and symptomatic diabetic autonomic neuropathy were recruited; and there were four non-diabetic patients with end-stage renal failure and proteinuria (RF). All these patients were anaemic with an associated erythropoietin deficiency and without correctable haematonic deficiencies (normal serum ferritin,

serum B12, and folate levels). EPO deficiency was determined by comparison with results from a group of patients with iron-deficient anaemia recently described by us [10] (Fig. 1). Nine healthy (control) subjects without anaemia were recruited to establish a normal response. The King's Healthcare NHS Trust Research Ethics Committee approved the study and all patients gave written informed consent. The characteristics of the study groups are shown in Table 1.

A detailed medical history was obtained for all subjects, and all patients were questioned for symptoms of diabetic autonomic neuropathy as described by Guy *et al.* [16], including gustatory sweating, postural hypotension, diarrhoea, gastroparesis and bladder paresis. Patients with any clinical contraindication to prolonged hypoxia including significant respiratory, cardiovascular and neurological conditions were excluded. All patients were fully examined including a comprehensive neurological examination involving a battery of peripheral and autonomic function tests. The results of the tests are shown in Table 2.



**Figure 1** The normal relationship between the natural logarithm ( $\ln$ ) of serum erythropoietin (EPO) levels and haemoglobin concentration in non-diabetic iron-deficient anaemic subjects ( $\circ$ ) as recently described by us [10]. The values of  $\ln(\text{EPO})$  against haemoglobin (Hb) are plotted for the diabetic patients ( $\bullet$ ) and renal failure patients ( $\blacksquare$ ) patients in the present study to illustrate that these patients have inappropriately low serum EPO levels.

**Table 1** Clinical, biochemical and haematological characteristics of study groups

	Control	DN	RF	Normal values
No	9	5	4	
M:F	5:4	1:4	4:0	
Age (years)	31 (24–39)	39 (28–48)	43 (34–51)	
DM duration (years)	—	24 (16–32)	—	
Nephropathy (years)	—	7 (1–15)	4 (1–11)	
Creatinine ( $\mu\text{mol/l}$ )	74.7 (65–93)	97.6 (63–123)	490.2 (406–659)	45–120
Proteinuria (mg/day)	—	1161.6 (130–2835)	2157.5 (571–4578)	
HbA <sub>1c</sub> (%)	—	11.1 (8–14.9)	—	
Hb (g/dl)	13.4 (11.8–15.7)	10.6 (9.5–12.0)	10.3 (9.0–11.3)	11.5–15.5
EPO (IU/l)	7.6 (5.6–10.3)	5.0 (3.2–6.5)	4.6 (2.9–8.5)	3.3–16.6
Vitamin B12 (ng/l)	—	533.8 (347–657)	549.5 (398–717)	223–1132
Folate ( $\mu\text{g/l}$ )	—	9.1 (4.2–15.8)	5.2 (4.4–7.1)	3.1–12.4
Ferritin (ng/ml)	—	33.2 (7–111)	203.3 (100–341)	10–200

Table 2 Peripheral and autonomic function test results of patient groups

	DN ( <i>n</i> = 5)	RF ( <i>n</i> = 4)	Normal values [17,18]
Vibration perception	36.2 ± 17.9	10.5 ± 6.5	< 20
Monofilament 10 g (perception +:–)	1:4	4:0	All points perceived
Thermal threshold	24.1 ± 9.7	8.0 ± 4.2	< 10
Resting heart rate (HR) (beats/min)	83 ± 6	78 ± 17	
HR variability	2 ± 1	16 ± 7	> 12
HR increase on standing	4 ± 2	19 ± 2	> 12
Systolic BP drop (mmHg)	17 ± 7	8 ± 15	< 20

All the DN patients had evidence of persistent albuminuria (mean 1161.6 (130–2835) mg/day), though none was in renal failure (mean serum creatinine 97.6 µmol/l; maximum serum creatinine 123 µmol/l). All had evidence of diabetic autonomic neuropathy (DAN) with at least one symptom, and this was confirmed by cardiac autonomic function tests. All had received treatment for proliferative diabetic retinopathy.

All RF patients were in advanced chronic renal failure (mean serum creatinine 490.2 (406–659) µmol/l), not yet requiring dialysis. The causes of renal failure were IgA nephropathy in two patients, Henoch–Schönlein purpura in one patient and hypertensive nephropathy in one patient. None of these patients had symptoms of autonomic dysfunction, and all neurological tests were normal.

#### Peripheral nerve and cardiovascular autonomic function tests

Peripheral nerve tests included assessment with a 10-g Semmes Weinstein monofilament, the biesthesiometer (Biomedical Instruments Co., Newbury, OH, USA) and thermal threshold assessment (Thermotest; RDG Medical, Croydon, UK). A battery of cardiovascular autonomic function tests using standard methods was performed as previously described [6,17]. Postural hypotension was defined as a systolic blood pressure drop of >20 mmHg [18].

#### EPO assay

Serum EPO was measured using an ELISA based on the double antibody sandwich method (Quantikine IVD EPO ELISA; R&D Systems, Minneapolis, MN, USA). The within assay precision was 3.1% (*n* = 30, mean 16.2 IU/l) and between assay coefficient of variation was 6.5% (*n* = 20, mean 10.6 IU/l). The laboratory normal range of EPO for non-anaemic subjects is 3.3–16.6 IU/l.

#### Hypoxia study design

The subjects underwent a 6-h period of hypoxia with an inspired oxygen concentration of 11.6–12.6% equivalent to an altitude of approximately 4000 m. Patients were cannulated and baseline measurements were taken for full blood count, serum EPO and biochemical profile. Normal saline was then infused to replace fluid. Two serum EPO levels were taken

within the first hour of the study and subsequently hourly for the 6-h duration. Whole blood glucose was measured from the diabetic patients hourly (Medisense Card Sensor; Abbott Laboratories Co., UK) to ensure there were no extreme blood glucose excursions.

Patients were monitored continuously using a three-lead ECG monitor, a pulse oximeter, and blood pressure was measured intermittently. Compressed air from a Hydrovane Factair Safe Air cabinet at a continuous flow rate of approximately 30 ml/min, measured via a KDG 200 Flowmeter, was mixed in a wide bore tube with nitrogen flowing from a cylinder at approximately 20 ml/min. The gas mixture was fed into a transparent hood placed over the subject's head and upper body and subsequently evacuated via the bottom of the bag into the atmosphere. The percentage of inspired oxygen concentration was continuously monitored and intermittent expired values were obtained via a mouth piece by using a fine sampling tube attached to a mass spectrometer (Mediflex software, VG Medical Systems, UK). The gas mixture in the hood was adjusted to maintain the desired inspired oxygen levels (11.6–12.6%). As an additional safety measure, the patient's oxygen saturation was not allowed to fall to < 80%. No more than three short breaks (around 5 min) were allowed during the procedure.

#### Statistical analysis

All patient baseline data are expressed as the mean and range or mean ± SD. Baseline EPO values were determined from the mean of two values taken within the first half hour of the study for each subject. All EPO data were transformed using the natural logarithm. Individual regression lines were fitted to ln(EPO) against time (1 h to 6 h) for each study. Individual slopes, baseline ln(EPO), maximal ln(EPO) values, inspired percentage oxygenation and oxygen saturation were compared among the three groups using ANOVA followed by *post hoc* comparisons. According to the Bonferroni correction, pairwise group comparisons were significant at the 5% level if their *P*-value was < 0.017.

#### Results

Both the DN group and the RF group were anaemic with inappropriately low levels of EPO for the severity of the anaemia. This is illustrated in Fig. 1, which compares the results from these patients plotted on a graph of the ln(EPO)

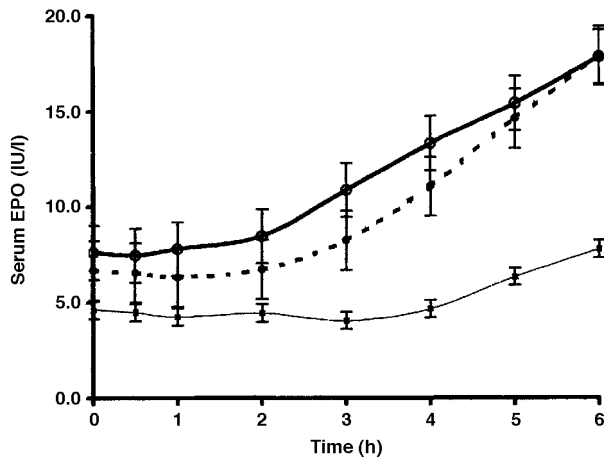


Figure 2 Serum EPO levels (mean  $\pm$  SEM) in response to hypoxia in diabetic patients (DN), renal failure patients (RF) and healthy control subjects. —, Mean control; - - - -, mean DN; ·····, mean RF.

vs. the haemoglobin concentration against the normal relationship of  $\ln(\text{EPO})$  with haemoglobin in a group of patients with iron-deficient anaemia. The data from all the DN and RF patients fall below the line.

The response to the hypoxic stimulus is illustrated in Fig. 2. There was no difference between the three groups in terms of percentage inspired oxygen levels (controls  $11.8 \pm 0.33\%$ , DN  $12.2 \pm 0.23\%$ , RF  $12.2 \pm 0.32\%$ ,  $F = 3.071$ ,  $P = 0.076$ ) or oxygen saturation (controls  $84.3 \pm 3.0\%$ , DN  $83.0 \pm 2.3\%$ , RF  $85.8 \pm 2.5\%$ ,  $F = 1.122$ ,  $P = 0.352$ ). It can be seen that the DN patients were able to achieve a similar rise in serum EPO over 6 h compared with the control group despite the fact that they had a lower EPO level for the degree of anaemia. The control results compare favourably with previous altitude studies performed by Eckardt [19]. There was no statistical difference in the maximal  $\ln(\text{EPO})$  level between the DN and control groups. The RF group started with a lower baseline EPO level than the controls ( $P = 0.008$ ) and the maximal  $\ln(\text{EPO})$  level at 6 h was statistically lower than both the control ( $P = 0.001$ ) and the DN groups ( $P = 0.002$ ).

After the first hour, the control group began to show a rise in  $\ln(\text{EPO})$  with a steady rise during the following 5 h. For each hour the  $\ln(\text{EPO})$  increased by 0.17 (SD 0.0592, confidence interval (CI) 0.126–0.217), i.e. serum EPO was estimated to change by a factor of 1.18 per hour. When comparing the slopes of the rise in  $\ln(\text{EPO})$  with time between all three groups there was no statistical difference ( $F = 3.3$ ,  $P = 0.06$ ).

Thus, although the DN patients had an EPO-deficient anaemia, they were able to mount an appropriate response to hypoxia comparable with normal controls. However, the RF patients had a lower baseline level of EPO, and produced lower levels of EPO at 6 h. Despite this, however, the production rate per hour for the RF group did not differ from the control or DN groups.

## Discussion

This study illustrates that, although diabetic patients with early nephropathy associated with symptomatic autonomic neuropathy may become chronically anaemic with an associated EPO deficiency, these patients are able to produce EPO appropriately when challenged by moderate hypoxia. We may assume that if hypoxia in simulated altitude can produce an EPO response, other conditions causing acute tissue hypoxia such as significant haemorrhage or acute respiratory dysfunction may also stimulate appropriate EPO production. This observation contrasts with that seen in patients with end-stage renal failure, where although the EPO production rate is similar the EPO levels remain lower throughout the 6 h.

The EPO response to tissue hypoxia in renal failure patients has been reported previously to be either absent or blunted. One study showed no response to 3.5 h of acute hypobaric hypoxia at 4560-m altitude equivalent [20], although this duration of hypoxia was probably insufficient to elicit a response. Renal failure patients exposed to acute hypoxic stress (e.g. pulmonary oedema, acute haemolysis, congestive cardiac failure and hypotension from sepsis) have been shown to mount a several-fold increase in serum EPO levels from previous steady state values [5,21,22]. End-stage kidneys are capable of increasing EPO production sufficiently to produce an improvement in the anaemia following the introduction of continuous ambulatory peritoneal dialysis [23]. These studies suggest that although EPO production is possible, the levels of EPO remain inappropriate, and it is therefore unable to correct the anaemia resulting from various acute insults.

The severity of anaemia in end-stage renal failure can be variable, suggesting that the pathogenesis is multifactorial. A number of explanations have been proposed for the failure of adequate EPO production in renal failure, which may also account for the anaemia with EPO deficiency observed in diabetic patients with early diabetic nephropathy and neuropathy. Renal damage may lead to an inability to sustain the required increased EPO levels due to a limited renal mass, damage of the fibroblasts or inadequate extrarenal supply of EPO [5]. However, as the diabetic patients have a normal EPO response to hypoxia, it seems unlikely that the EPO-producing fibroblasts are destroyed by the tubulointerstitial damage of early diabetic nephropathy [7]. The renal fibroblasts seem to be able to produce EPO appropriately in response to hypoxia. We cannot disprove the theory that the increased EPO production in the diabetic patients is from the liver, which is known to produce around 10% of the total production in adults [3]. Alternative explanations for the low EPO levels associated with an anaemia might include a reduced oxygen affinity of haemoglobin, a decreased sensitivity of the renal oxygen sensor, or an increased EPO metabolism and malnutrition. Inhibitors of erythropoiesis may also contribute to the anaemia [24]. Cytokines such as IL-1 ( $\alpha$  or  $\beta$ ) and tumour necrosis factor ( $\alpha$  or  $\beta$ ) [25] are thought to inhibit both EPO action and EPO production, causing the anaemia of chronic disease and

end-stage renal failure. It seems possible that cytokine involvement may contribute to the anaemia of early diabetic nephropathy but that these inhibitors can be overcome by moderate hypoxic stimulation.

Thus, the pathogenesis of the EPO-associated chronic anaemia of diabetic patients with renal and neurological complications remains unclear. Inhibition of EPO production by cytokines, decreased sensitivity of the oxygen sensor in the kidney, reduced oxygen demand of the diabetic kidney, and the role of dysautonomia need further investigation in diabetes.

### Acknowledgements

The authors would like to thank Sabina Landau, Department of Biostatistics and Computing, Institute of Psychiatry, Decrespigny Park, London for statistical advice. This study was supported by a grant from Diabetes UK.

### References

- Bonsdorff E, Jalavisto E. A humoral mechanism in anoxic erythrocytosis. *Acta Physiol Scand* 1948; **16**: 150–170.
- Jacobson LO, Goldwasser E, Fried W, Plzak L. Role of the kidney in erythropoiesis. *Nature* 1957; **179**: 633–634.
- Bachmann S, Le Hir M, Eckardt KU. Co-localisation of erythropoietin mRNA and ecto-5' nucleotidase immunoreactivity in peritubular cells of rat renal cortex indicates that fibroblasts produce erythropoietin. *J Histochem Cytochem* 1993; **41**: 335–341.
- Ratke HW, Claussner A, Erbes PM, Scheuermann EH, Scheppe W, Koch KM. Serum erythropoietin concentration in chronic renal failure: relationship to degree of anaemia and excretory renal function. *Blood* 1979; **54**: 877–884.
- Chandra M, Clemons GK, McVicar MI. Relation of serum erythropoietin levels to renal excretory function: evidence for lowered set point for erythropoietin production in chronic renal failure. *J Pediatr* 1988; **113**: 1015–1021.
- Bosman DR, Winkler AS, Marsden J, Macdougall IC, Watkins PJ. Anaemia associated with erythropoietin deficiency in early diabetic nephropathy. *Diabetes Care* 2001; **24**: 495–499.
- Fiorotto P, Mauer M, Brocco E, Velussi M, Frigato F, Muollo B *et al.* Patterns of renal injury in NIDDM patients with microalbuminuria. *Diabetologia* 1996; **39**: 1569–1576.
- Rarick MU, Espina BM, Colley DT, Chrusoskie A, Gandara S, Feinstein DI. Treatment of a unique anemia in patients with IDDM with Epoetin Alfa. *Diabetes Care* 1998; **21**: 423–426.
- Ricerca BM, Todaro L, Caputo S, Cotroneo P, Damiani P, Manto A *et al.* Blunted erythropoietin response to anaemia in type 1 diabetic patients. *Diabetes Care* 1999; **22**: 647.
- Winkler AS, Marsden J, Chaudhuri KR, Hambley H, Watkins PJ. Erythropoietin depletion and anemia in diabetes mellitus. *Diabet Med* 1999; **16**: 813–819.
- Takaku F, Hirasima K, Okinaka S. Effects of the bilateral section of the splanchnic nerve in erythropoiesis. *Nature* 1961; **191**: 500–501.
- Beynon G. The influence of the autonomic nervous system in the control of the erythropoietin secretion in the hypoxic rat. *J Physiol* 1977; **266**: 347–360.
- Fink GD, Fisher JW. Stimulation of erythropoiesis by beta adrenergic agonists. II. Mechanisms of action. *J Pharmacol Exp Therapeut* 1977; **202**: 199–208.
- Zivny J, Ostadal B, Neuwirt J, Prtochazka J, Pelouch V. Effect of beta adrenergic blocking agents on erythropoiesis in rats. *J Pharmacol Exp Therapeut* 1983; **226**: 222–225.
- Biaggioni I, Robertson D, Krantz S, Jones M, Haile V. The anemia of primary autonomic failure and its reversal with erythropoietin. *Ann Intern Med* 1994; **121**: 181–186.
- Guy RJC, Richards F, Edmonds ME, Watkins PJ. Diabetic autonomic retinopathy and iritis: an association suggesting an immunological cause. *Br Med J* 1984; **189**: 343–345.
- Purewal TS, Edmonds ME, Watkins PJ. Clinical assessment of neuropathy, vascular disease and the leg in diabetes. In Mogensen CE, Standl E, eds. *Diabetes Forum V: Research Methodologies in Human Diabetes*, Part 2. Berlin: Wide Gruyter, 1995: 247–287.
- American Autonomic Society and the American Academy of Neurology. Consensus statement on the definition of orthostatic hypotension, pure autonomic failure, and multiple system atrophy. In Robertson D, Low PA, Polinsky RJ eds. *Primer on the Autonomic Nervous System*, 1st edn. San Diego: Academic Press, 1996: 334–336.
- Eckardt KU, Boutellier URS, Kurtz A, Schopen M, Koller EA, Bauer C. Rate of erythropoietin formation in humans in response to acute hypobaric hypoxia. *J Appl Physiol* 1989; **66**: 1785–1788.
- Quick J, Eichenberger A, Binswanger U. Stimulation of erythropoietin in renal insufficiency by hypobaric hypoxia. *Nephrol Dial Transplant* 1992; **7**: 1002–1006.
- Kato A, Hishida A, Kumagai H, Furuya R, Nakajima T, Honda N. Erythropoietin production in patients with chronic renal failure. *Ren Fail* 1994; **16**: 645–651.
- Ross RP, McCrea JB, Besarab A. Erythropoietin response to blood loss in haemodialysis patients is blunted but preserved. *ASAIO J* 1994; **40**: M880–M885.
- Morris K, Coulthard M. End-stage kidneys are capable of increased erythropoietin production. *Pediatr Nephrol* 1993; **7**: 273–275.
- De Klerk G, Wilmink JM, Rosengarten PCJ, Vet RJWM, Goudsmit R. Serum erythropoietin (ESF) titers in anemia of chronic renal failure. *J Lab Clin Med* 1982; **100**: 720–734.
- Means RT, Krantz SB. Progress in understanding the pathogenesis of the anemia of chronic disease. *Blood* 1992; **80**: 1639–1647.