

## Original Article

## Acute effects of recombinant human erythropoietin on plasma levels of proendothelin-1 and endothelin-1 in haemodialysis patients

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**Background.** The pathogenesis of rHuEpo-induced hypertension in haemodialysis (HD) patients still remains uncertain. Endothelin-1 (ET-1) is produced from proendothelin-1 (proET-1) by an endothelin-converting enzyme. Since proET-1 is known to have approximately 1/100 the potency of ET-1 for contracting an isolated blood vessel, the change in the activity of endothelin-converting enzyme (ECE) has been proposed as an important factor in the pathophysiology of various hypertensive diseases. However there is no report on whether a change in the rate of conversion of proET-1 to ET-1 may be involved in the pathogenesis of rHuEpo-induced hypertension. The purpose of this study was to ascertain the potential role of ECE in the development of rHuEpo-induced hypertension.

**Methods.** The levels of plasma erythropoietin, proET-1, ET-1, and mean arterial blood pressure (MAP) were measured following a single dose of rHuEpo (100 U/kg) in HD patients with 24-h ambulatory blood pressure monitoring. Different routes of administration (19 intravenous group, 10 subcutaneous group) were compared to a placebo-injected control group (10 HD patients).

**Results.** Plasma erythropoietin levels reached maximal value 5 min after i.v. injection of rHuEpo ( $13.1 \pm 2.4$  vs  $2780.9 \pm 290.1$  mU/ml,  $P < 0.01$ ), whereas it was 6 h in the s.c. group ( $14.7 \pm 3.8$  vs  $38.9 \pm 17.7$  mU/ml,  $P < 0.05$ ). A significant increase in MAP was noted 30 min after rHuEpo injection, which lasted for 3 h in the i.v. group. However, no significant changes in MAP were noted in patients given rHuEpo subcutaneously. Both the plasma concentrations of proET-1 and ET-1 started to increase from 10 min after i.v. rHuEpo administration, with the proET-1 reaching a peak level at 30 min ( $13.5 \pm 7.4$  vs  $21.6 \pm 3.8$  pg/ml,  $P < 0.05$ ) and the ET-1 at 1 h ( $4.2 \pm 2.6$  vs  $9.9 \pm 4.8$  pg/ml,  $P < 0.05$ ). In patients with significant interdialysis hypertension following a single i.v. injection of rHuEpo, the molar

ratio of ET-1 over proET-1 (ET-1/proET-1) was significantly higher than in patients without hypertension. In addition, the increase in ET-1 levels was significantly greater in patients with interdialysis hypertension, while changes in proET-1 level were similar in both hypertensive and non-hypertensive groups. Changes in interdialysis MAP ( $\Delta$ IDMAP) was significantly correlated with  $\Delta$ ET-1 during the interdialysis period, but not with  $\Delta$ proET-1.

**Conclusion.** Differences in ET-1/proET-1 ratio in relation to changes in MAP after a single intravenous administration of rHuEpo suggest a potential role for ECE in the pathogenesis of rHuEpo-induced hypertension.

**Key words:** endothelin-1; endothelin-converting enzyme; haemodialysis; proendothelin-1; rHuEpo-induced hypertension

**Introduction**

The positive effects of recombinant human erythropoietin (rHuEpo) treatment on anaemia in chronic renal failure patients have been well documented [1,2]. However, complications such as hypertension, thrombosis of arteriovenous fistula, flu-like symptoms and hyperkalaemia can also develop with rHuEpo therapy [1–3]. Hypertension is an important complication which affects the overall prognosis of end-stage renal disease (ESRD) patients. Twenty to fifty per cent of ESRD patients treated with rHuEpo are at risk of developing a significant rise in blood pressure, even with the occurrence of encephalopathy or seizure [1,2,4]. The exact mechanism of rHuEpo-induced hypertension in haemodialysis (HD) patients still remains uncertain. An imbalance of local endothelial factors such as endothelium-derived relaxation factor and endothelin was postulated as one of the possible mechanisms involved in rHuEpo-induced hypertension [5–8].

Endothelial cells have receptors for erythropoietin and *in vitro* studies confirmed release of ET-1 when exposed to erythropoietin [9]. Active ET-1 is produced

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from proET-1 or big endothelin (consisting of 38 amino acids) by an endothelin-converting enzyme located in the vascular endothelial cell [10], and proET-1 is an intermediate generated from preproET (203 amino acid). Since the potency of proET-1 for contracting an isolated blood vessel is approximately 100 times less than ET-1, conversion of proET-1 to the active ET-1 is essential for full biological activity [10–12]. Alterations in the change in the activity of ECE have been reported to be a factor in the pathophysiology of certain disease states [13–15]. Increased molar ratio of proET-1/ET-1 with disruption of the endothelin regulatory system appeared to accelerate the development of pre-eclampsia [13] and an elevated proET-1 level with a normal ET-1 level in diabetic patients with microangiopathy was explained by a reduced activity of converting enzyme [14]. However, there has been no study evaluating the role of ECE activity in the pathogenesis of rHuEpo-induced hypertension.

The present study was undertaken to ascertain the potential role of ECE in rHuEpo-induced hypertension by evaluating the acute effects of a single dose of rHuEpo on plasma levels of proET-1 and ET-1 along with the changes in interdialysis blood pressure by 24-h ambulatory blood-pressure monitoring.

## Subjects and methods

### Patients

Thirty-nine clinically stable HD patients (19 males and 20 females) ranging in age from 24 to 67 years (mean 47 years) participated in this study. The study was approved by the institutional ethics committee and informed consent was obtained from each patient. Exclusion criteria were patients with uncontrolled hypertension (blood pressure: systolic >160 mmHg and diastolic >100 mmHg) and those taking antiplatelet agents or anabolic steroids. Twenty (54.1%) patients were treated for hypertension with either calcium-channel blocker (15 cases),  $\alpha$ -adrenergic blocker (8 cases), or angiotensin converting enzyme inhibitor (7 cases). The aetiologies of renal failure were chronic glomerulonephritis (13 patients), diabetic nephropathy (10 patients), hypertensive nephrosclerosis (9 patients), polycystic kidney disease (2 patients), renal tuberculosis (1 patient), lupus nephritis (1 patient), and unknown (3 patients). None of these patients were administered rHuEpo treatment within 1 month of this study.

### Study protocol

Patients were randomized to receive rHuEpo either subcutaneously (10 cases) or intravenously (19 cases) and were compared to an intravenous placebo (2 ml of sterile distilled water) control group (10 HD patients). A single dose of rHuEpo (Recormon<sup>®</sup>, erythropoietin- $\beta$ , Choongwai Co, Seoul, Korea) (100 U/kg body weight) was injected at the end of each dialysis session. Ambulatory blood pressure monitoring was performed using a non-invasive ambulatory blood pressure monitor (BP3, Mediana, USA). Systolic and diastolic blood pressure with pulse rate were recorded every 10 min. Blood pressure monitoring was initiated 1 h before

the end of the HD session until the start of the next HD session. The mean arterial blood pressure (MAP) was calculated as [systolic blood pressure + (2  $\times$  diastolic blood pressure)]  $\times$  1/3. All subjects were hospitalized after rHuEpo administration for blood-pressure monitoring and blood sampling until the next HD session.

Plasma levels of erythropoietin, ET-1 and proET-1 were measured at the following time points; immediately before rHuEpo injection at the end of the HD session, and 5, 10 and 30 min and, 1, 6, 12 and 24 h following rHuEpo injection and before the next HD session. Each blood sample was obtained through a sampling port located in the opposite arm from the vascular access. Plasma concentration of erythropoietin (Diagnostics System Lab., USA), ET-1 (Nichols Institute, USA) and proET-1 (Nichols Institute, USA) were measured by radioimmunoassay. Blood samples for ET-1 and proET-1 were collected into tubes containing aprotinin (500 000 U/l of blood) and EDTA (1 g/l). Plasma was centrifuged at  $-4^{\circ}\text{C}$  and quickly separated. The samples were extracted with 4% acetic acid and the acetone extract was dried with vacuum at  $37^{\circ}\text{C}$  (efficiency 90%). The dried extract was reconstituted and analysed with an RIA using [ $^{125}\text{I}$ ]endothelin and rabbit antiendothelin. To avoid inter-assay variation, all samples were assayed in a single session. The sensitivities of the assay for ET-1 and proET-1 were 2.0 pg/ml and 1.4 pg/ml, respectively. The cross-reactivity of the assay was as follows: ET-1, 100%; ET-2, 52%; ET-3, 96%; and 7% with proET-1.

### Statistics

The data are expressed as mean  $\pm$  standard deviation or median. Differences in plasma ET-1 and proET-1 concentrations were compared by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison. Changes in concentrations of erythropoietin, ET-1, proET-1 and blood pressure were compared by Wilcoxon's test at each time point after rHuEpo administration. Correlation between the changes in blood pressure and plasma levels of ET-1 and proET-1 was analysed by linear regression. Values of  $P < 0.05$  were considered to be significant.

## Results

### Patient characteristics

The clinical characteristics of the subjects are shown in Table 1. There were no significant differences in patient age, duration of HD, and blood pressure among control, i.v., and s.c. groups. Haematocrit, blood urea nitrogen, creatinine and lipid profiles were also similar (Table 2). The median values of erythropoietin in the three groups were 12.4, 13.0 and 13.9 mU/ml respectively. The median values for ET-1 and proET-1 were not different between the three groups.

### Plasma erythropoietin levels after rHuEpo

Figure 1 illustrates the changes in the plasma erythropoietin level in the rHuEpo-injected patients and control group. In the i.v. rHuEpo group, the erythropoietin level rose abruptly and reached a peak at 5 min, whereas in the s.c. group, the peak level was obtained 6 h after the injection. The peak concentration of

**Table 1.** Clinical characteristics of subjects

	Control (n=10)	rHuEpo (n=29)	
		IV (n=19)	SQ (n=10)
Gender (M:F)	5:5	10:9	4:6
Age (years)	43±17	48±25	51±23
Duration of HD (months)	20±7	23±21	27±14
% of Diabetes	24.6	33.9	34.0
% of Anti-HT <sup>a</sup>	30.0	26.3	40.0
SBP (mmHg)	145±34	139±33	149±56
DBP (mmHg)	92±26	90±17	88±23

Values are mean ± SD; <sup>a</sup>% of patients on antihypertensive medications at the time of study; SBP, systolic blood pressure; DBP, diastolic blood pressure.

**Table 2.** Laboratory findings of subjects

	Control (n=10)	rHuEpo (n=29)	
		IV (n=19)	SQ (n=10)
Hct (%) <sup>a</sup>	25.1±5.7	28.1±4.5	27.3±6.1
BUN (mg/dl) <sup>a</sup>	58.9±17.5	67.4±21.1	65.9±30.5
Cr (mg/dl) <sup>a</sup>	10.8±2.1	11.0±4.8	11.2±5.0
Chol (mg/dl) <sup>a</sup>	178±30	180±27	167±38
Tg (mg/dl) <sup>a</sup>	101±14	119±20	98±17
Epo (mU/ml) <sup>b</sup>	12.4	13.0	13.9
ProET-1 (pg/ml) <sup>b</sup>	12.7	13.5	13.0
ET-1 (pg/ml) <sup>b</sup>	4.3	4.2	4.8

<sup>a</sup>Values are mean ± SD; Hct, haematocrit; BUN, blood urea nitrogen; Cr, creatinine; Chol, cholesterol; Tg, triglyceride; Epo, erythropoietin; ProET-1, proendothelin-1; ET-1, endothelin-1; <sup>b</sup>Values are medians.

erythropoietin in the i.v. group was 2780.9 mU/ml, which was significantly higher compared to the value of 38.9 mU/ml in the s.c. group. Plasma erythropoietin level fell gradually after an abrupt peak in the i.v.

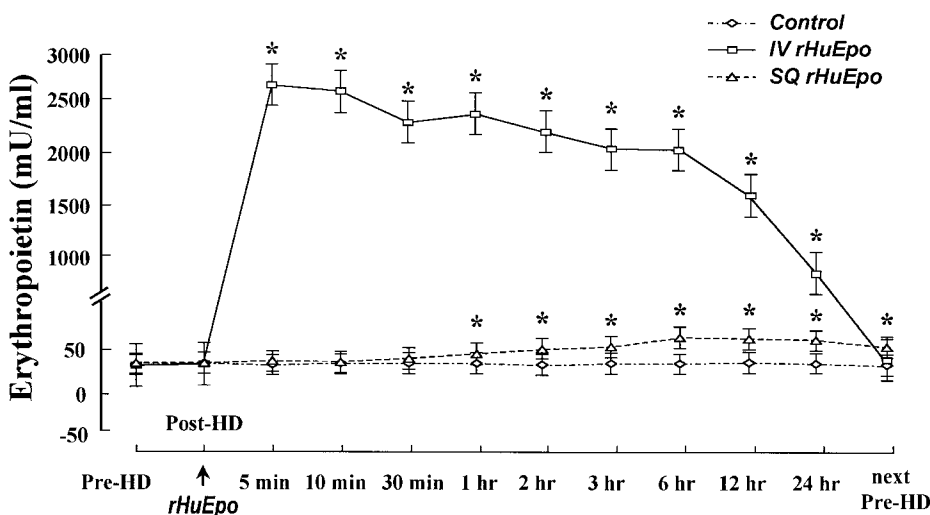
rHuEpo group, returning to the pre-injection level at 48-h post-rHuEpo. In the s.c. group, meanwhile, the changes in erythropoietin level were much slower and substantially smaller compared to the i.v. group, although significantly increased erythropoietin concentrations compared to baseline were observed up to 48 h.

#### Plasma ET-1 and proET-1 after rHuEpo

Plasma ET-1 started to increase 10 min after i.v. rHuEpo, reaching the highest level at 1 h. It remained elevated up to 3 h after rHuEpo. There were no significant changes in plasma ET-1 levels in control and the s.c. rHuEpo group (Figure 2). Similar findings were observed for proET-1 (Figure 3). The proET-1 level rose significantly 10 min after injection, peaking at 30 min and remained elevated up to 3 h after rHuEpo in the i.v. group only.

#### Changes in blood pressure after rHuEpo

MAP increased significantly from 30 min after rHuEpo in the i.v. group (94.2±12.5 vs 119.0±20.3 mmHg,  $P<0.05$ ). However, there were no significant changes in blood pressure in the control and s.c. group (Figure 4). Significant interdialysis hypertension, defined as an increase in interdialysis MAP above 10 mmHg compared to the baseline MAP, was observed in 10 (52.6%) of 19 i.v. rHuEpo-injected patients. In Figure 5, molar ratio of ET-1 over proET-1 (ET-1/proET-1) was plotted against each time point of blood sampling after rHuEpo administration. ET-1/proET-1 ratio significantly increased after i.v. rHuEpo injection compared to baseline value and reached a peak at 1 h of rHuEpo. Thereafter, ET-1/proET-1 gradually returned to the pre-rHuEpo value. In patients with significant interdialysis hypertension, ET-1/proET-1 ratios at 30 min and 1 h were significantly higher compared to the patients without hypertension.



**Fig. 1.** Changes in plasma erythropoietin after the administration of rHuEpo in (□) i.v., (△) s.c., and (◇) control groups. Values are mean ± SD \* $P<0.05$  vs post-HD.

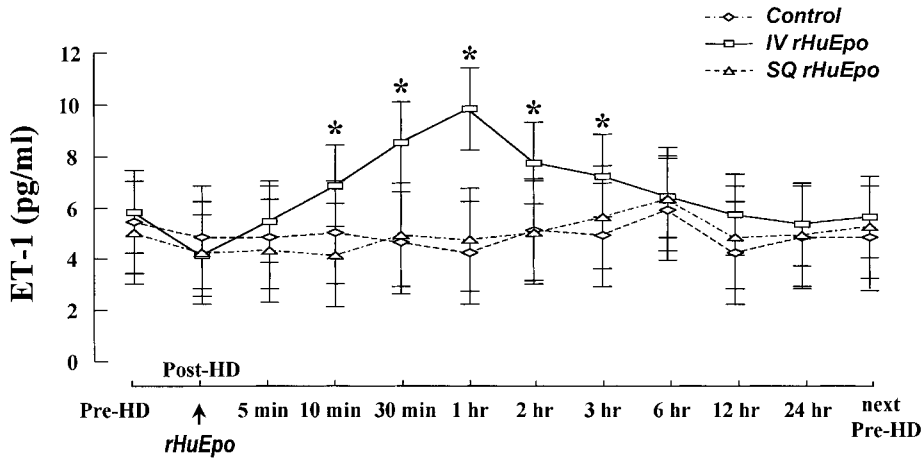


Fig. 2. Changes in plasma endothelin-1 level after the administration of rHuEpo in (□) i.v., (△) s.c., and (◇) control groups. Values are mean ± SD \**P* < 0.05 vs post-HD.

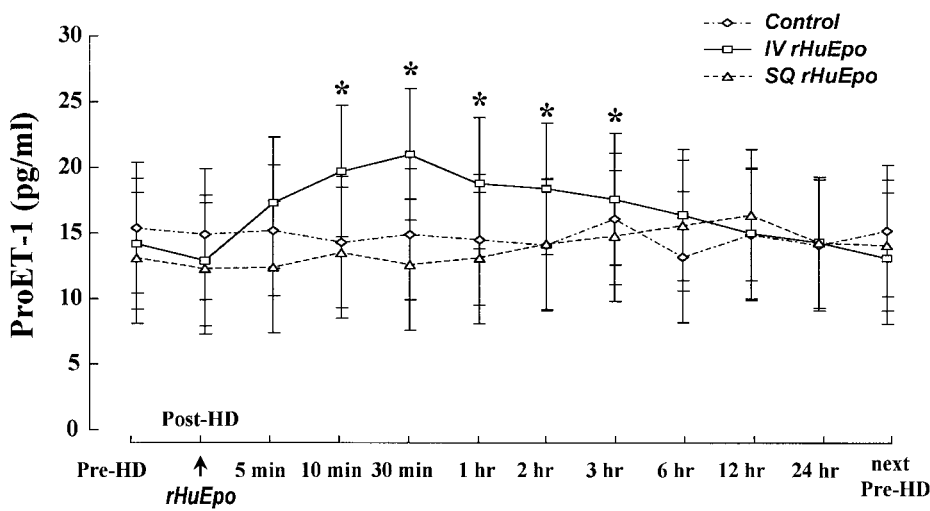


Fig. 3. Changes in plasma proendothelin-1 after the administration of rHuEpo in (□) i.v., (△) s.c., and (◇) control groups. Values are mean ± SD \**P* < 0.05 vs post-HD.

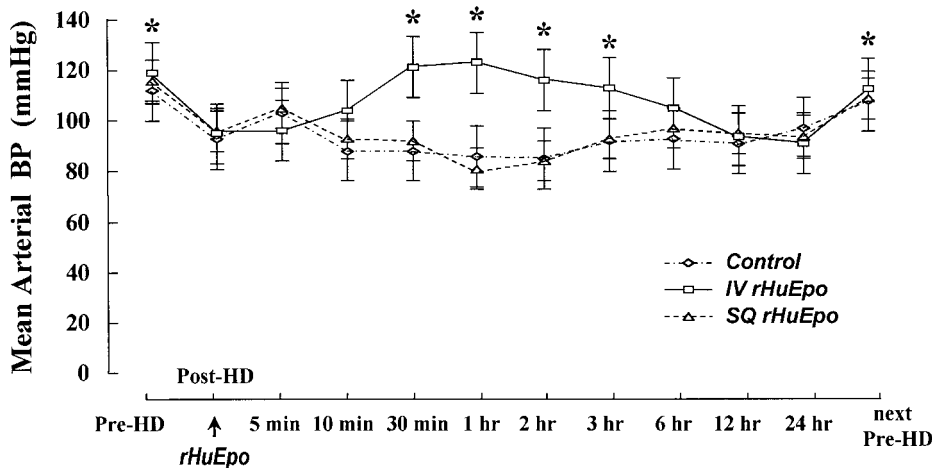


Fig. 4. Changes in mean arterial blood pressure after the administration of rHuEpo in (□) i.v., (△) s.c., and (◇) control groups. Values are mean ± SD \**P* < 0.05 vs post-HD.

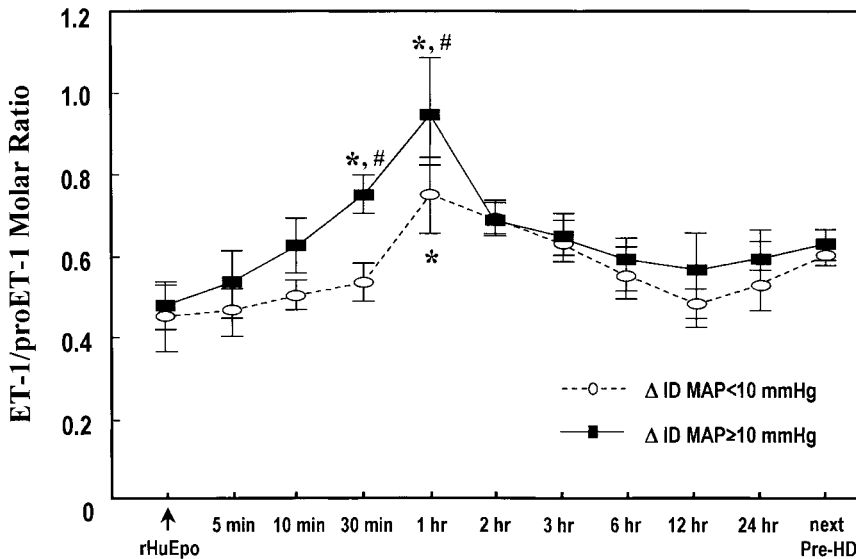


Fig. 5. Changes in ProET-1 to ET-1 ratio after IV administration of rHuEpo for the groups with a change in interdialysis mean arterial blood pressure (IDMAP)  $>10$  mmHg and  $<10$  mmHg. Values are mean  $\pm$  SD \* $P < 0.05$  vs pre-rHuEpo value, # $P < 0.05$  vs patients with IDMAP  $<10$  mmHg.

The changes in proET-1 ( $\Delta$ proET-1) and ET-1 ( $\Delta$ ET-1) are compared in Table 3.  $\Delta$ ET-1 and changes in interdialysis MAP,  $\Delta$ IDMAP, represent the difference between the mean value of all measurement during the interdialysis period and the values prior to i.v. rHuEpo injection of the preceding dialysis session.  $\Delta$ ET-1 was significantly greater in the group of patients with  $\Delta$ IDMAP  $>10$  mmHg than in those with  $\Delta$ IDMAP  $<10$  mmHg (7.4 vs 3.2 pg/ml,  $P < 0.05$ ). However, the changes in  $\Delta$ proET-1 were similar in both patient groups. Interdialysis weight gains were similar in both groups, and therefore a difference in volume status was unlikely to be a cause of the rHuEpo-induced hypertension.

#### Correlation between the changes of blood pressure and ET-1

There was no significant correlation between  $\Delta$ IDMAP and  $\Delta$ proET-1 in the i.v. rHuEpo patients (Figure 6). In contrast, there was a significant positive correlation between  $\Delta$ IDMAP and  $\Delta$ ET-1 in the i.v. rHuEpo group with a  $r$ -value of 0.78 (Figure 7). We could not find any significant correlation between  $\Delta$ proET-1 and  $\Delta$ ET-1.

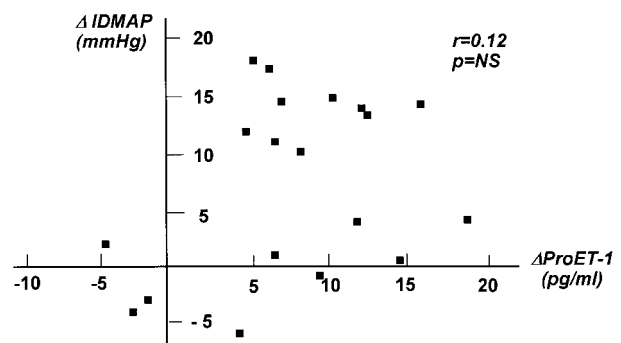


Fig. 6. Relationship between  $\Delta$ IDMAP and  $\Delta$ ProET-1 in i.v. rHuEpo patients ( $n=19$ ) showing no significant correlation between  $\Delta$ IDMAP and  $\Delta$ ProET-1. NS, not significant.  $\Delta$ IDMAP and  $\Delta$ ProET-1 represent the difference between the value of all measurements during the 48-h interdialytic period and the value obtained after the preceding haemodialysis session before the i.v. rHuEpo injection.

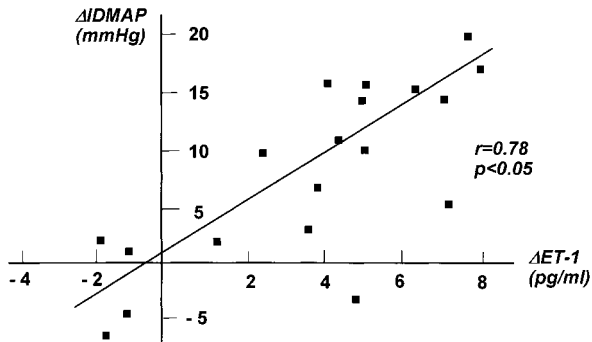
#### Discussion

The pathogenesis of rHuEpo-induced hypertension in HD patients still remains uncertain. Increased blood viscosity and loss of hypoxic vasodilatation subsequent

Table 3. Changes in ProET-1 & ET-1 according to the changes in interdialytic MAP in IV rHuEpo patients ( $n=19$ )<sup>a</sup>

	$\Delta$ IDMAP $<10$ mmHg <sup>b</sup> ( $n=9$ )	$\Delta$ IDMAP $>10$ mmHg ( $n=10$ )
$\Delta$ ProET-1	7.9 $\pm$ 2.1	8.5 $\pm$ 4.3
$\Delta$ ET-1	3.2 $\pm$ 2.8	7.4 $\pm$ 2.4 <sup>c</sup>
Interdialysis weight gain (kg)	2.7 $\pm$ 1.9	2.6 $\pm$ 1.7

<sup>a</sup>Values are mean  $\pm$  SD; MAP, mean arterial blood pressure; <sup>b</sup> $\Delta$ IDMAP, Changes in MAP during interdialysis period = MAP during interdialysis period - postHD, preEpo MAP of preceding dialysis session; <sup>c</sup> $P < 0.05$  vs  $\Delta$ IDMAP  $<10$  mmHg group.



**Fig. 7.** Correlation between  $\Delta$ IDMAP and  $\Delta$ ET-1 in i.v. rHuEpo patients ( $n=19$ ) showing significant correlation ( $r=0.7843$ ,  $P<0.001$ ).  $\Delta$ IDMAP and  $\Delta$ ET-1 represent the difference between the value of all measurements during the 48-h interdialytic period and the value obtained after the preceding haemodialysis session before the i.v. rHuEpo injection.

to the correction of anaemia, and mobilization of vascular cytosolic calcium [16–18] are the postulated pathogenetic mechanisms. Increased circulating levels of vasoactive substances including endothelin have also been reported to play a role in the increase in blood pressure in rHuEpo-treated patients [19,20]. Incubation with rHuEpo has been shown to increase the ET-1 synthesis in endothelial cells from human umbilical vein [21] and bovine pulmonary artery [7]. However, there are controversies regarding a direct vasoconstrictor effect of rHuEpo [17,21,22]. Bode-Böger *et al.* [21] demonstrated that rHuEpo had no direct vasoconstrictor effect, but it enhanced noradrenaline-induced vasoconstriction while others observed constant contraction of caudal artery rings with rHuEpo [22]. In the study of Bode-Böger *et al.*, the selective endothelin-A receptor antagonist partly inhibited rHuEpo-induced enhancement of vascular responsiveness to noradrenaline [21].

Impact of rHuEpo on plasma ET-1 level and its relation with rHuEpo-induced hypertension remains controversial. Most of the previous studies revealed that chronic administration of rHuEpo was not associated with an increase in ET-1 levels in predialysis renal failure [23,24], HD [25,26], and CAPD [27] patients. Takahashi *et al.* [28] reported that plasma ET-1 level in HD patients increased only in patients whose MAP increased by more than 10 mmHg after 8 weeks of rHuEpo. Our data, as well as another report [6], clearly indicate that a single intravenous injection of rHuEpo can acutely increase both blood pressure and plasma ET-1. However, Hon *et al.* [29] reported no changes in blood pressure and ET-1 concentration after a single i.v. rHuEpo injection in their double-blind cross-over study with nine HD patients. The observed differences in blood pressure and ET-1 levels after a single injection of rHuEpo may be due to the timing of blood pressure and ET-1 measurements. We measured blood pressure after HD, while Hon *et al.* performed this measurement prior to HD. In addition, they administered a lower amount of rHuEpo (50 U/kg) and there was no information regarding plasma

erythropoietin level after rHuEpo administration. Our study revealed that there were no changes in both blood pressure and the levels of ET-1 in the s.c. rHuEpo group, which is concordant with the result of others [6,30].

The ratio of ET-1/proET-1, a surrogate marker of ECE activity, increased significantly after rHuEpo administration only in the patients with significant interdialysis hypertension and its value was significantly higher than in patients without a significant elevation in blood pressure. In both groups the ratio of ET-1/proET-1 was highest at 1 h following rHuEpo administration, which coincided with the maximal increment in MAP. The changes in ET-1 after i.v. rHuEpo administration were significantly greater in the patients with significant interdialysis hypertension compared to the patients without hypertension, while their changes in proET-1 were similar (Table 3). Moreover, changes in interdialysis blood pressure was positively correlated with changes in ET-1 (Figure 7) whereas it was not correlated with  $\Delta$ proET-1 (Figure 6). These findings suggest that ECE activity may play a role in the development of hypertension following acute injection of rHuEpo in HD patients. Intravenous rHuEpo may stimulate the production or release of proET-1 from vascular endothelial cells, with hypertension developing only in a specific group of patients who have an increased activity of ECE resulting in the elevated ET-1 and blood pressure. It should be stressed that this interpretation is very presumptive since direct measurement of ECE activity was not carried out and any differences in ECE activity in relation to the interdialysis hypertension has not been proved. Other possibilities include preferential excretion of ET-1 from endothelial cells to the circulation or diminished break-down of ET-1 in patients with interdialysis hypertension. To elucidate a potential role of ECE in rHuEpo-induced hypertension, experiments with ECE blocker are necessary.

After i.v. rHuEpo, plasma erythropoietin concentration remained high for about 6–12 h, whereas the effect on ET-1 and blood pressure lasted only 3–6 h. The initial non-physiological rise in plasma erythropoietin level seems to be important in raising blood pressure; the exact cause for this discrepancy in the time course is unclear. The other limitation of this study was that it is not a double-blind cross-over study. However, it would be rather difficult to keep various clinical parameters such as interdialytic period, fluid status, and medications similar between two occasions of rHuEpo or placebo administration. Therefore we selected subjects with similar clinical characteristics to overcome this problem.

In conclusion, plasma levels of proET-1 and ET-1 increase after a single intravenous administration of rHuEpo which can be associated with an acute elevation of blood pressure in haemodialysis patients. Molar ratios of ET-1/proET-1 at the time of maximal increment of blood pressure were higher in patients with a significant interdialysis hypertension, suggesting a possibility of potential role of ECE in the genesis of

rHuEpo-induced hypertension. Further studies with direct measurement of ECE activity in endothelial cells will be required in order to definitely ascertain the role of proET-1 and ET-1 in rHuEpo-induced hypertension.

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