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Effect of 1-week treatment with erythropoietin on the vascular endothelial function in anaesthetized rabbits

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1 Chronic administration of erythropoietin (EPO) is often associated with hypertension in animals and humans. The aim of this study was to estimate whether 1-week treatment with EPO can affect the vascular endothelial function.

2 Rabbits were given with EPO (400 iu kg^{-1} s.c.) or saline each other day for 1 week. Hypotensive responses to intravenously given acetylcholine (ACh), endothelium-independent nitric oxide donors (NOC7, nitroprusside and nitroglycerin) and prostaglandin I_2 were tested before and after administration of N^G-nitro-L-arginine methyl ester (L-NAME), a specific nitric oxide synthase inhibitor, under pentobarbitone anaesthesia.

3 Blood haemoglobin concentration in EPO group was significantly higher than that in control group, whereas baseline values of aortic pressure, heart rate and femoral vascular resistance were similar. The dose of ACh (172 ng kg^{-1}) requiring for a 15 mmHg hypotension from the baseline in EPO group was apparently higher than that (55 ng kg^{-1}) in control group. On the contrary, hypotensive responses to NOC7, nitroprusside, nitroglycerin and prostaglandin I_2 were comparable between two groups. The extent of ACh-induced hypotension did not correlate with haemoglobin concentration.

4 L-NAME significantly inhibited the ACh-induced vasodilating response in control group but did not in EPO group.

5 In another set of rabbits, the same treatment with EPO also decreased vasodilating responses to carbachol, bradykinin and substance P besides ACh as compared with control group.

6 These results indicate that 1-week treatment with EPO selectively attenuates depressor responses to endothelium-dependent vasodilators in anaesthetized rabbits, most likely due to inhibition of endothelial nitric oxide synthase.

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Abbreviations: ACh, acetylcholine; BK, bradykinin; CCh, carbachol; dAoP, diastolic aortic pressure; EPO, erythropoietin; FVR, femoral vascular resistance; Hb, haemoglobin; L-NAME, N^G-nitro-L-arginine methyl ester; NO, nitric oxide; NOC7, 3-(2-hydroxy-1-methylethyl-2-nitrosohydrazino)-N-methyl-1-propanamine; NTG, nitroglycerin; SNP, sodium nitroprusside

Introduction

Erythropoietin (EPO), which is a glycoprotein hormone secreted primarily from the kidney in response to lowered blood O_2 availability due to hypoxia, haemorrhage and anaemia, acts on erythroid progenitor cells in the bone marrow, and then increases peripheral O_2 supply by promoting their proliferation and differentiation (Jelkmann, 1992). Medication of EPO has gained wide acceptance for treatment of the anaemia with chronic renal failure. Development or worsening of hypertension is generally considered as the most serious adverse effect of the treatment of the anaemia with EPO (Raine, 1988). Hypertension usually occurs within several weeks to months after the onset of EPO treatment and is accompanied by the increased haematocrit (Buckner *et al*., 1990). The EPO-induced hypertension is associated with, and primarily due to, an increase in peripheral vascular resistance, which has been explained by the increase in blood viscosity (Raine, 1988; Schaefer *et al*., 1988), and the loss of hypoxia-induced vasodilation (Neff *et al*., 1971). In addition, the increased haemoglobin (Hb) concentration in circulation may theoretically augment vascular resistance by trapping nitric oxide (NO), an endogenous vasodilator substance (Martin & Moncada, 1988). However, these proposed explanations for the mechanism of EPO-induced hypertension remain as yet controversial.

Recently, it has been shown that EPO receptors are found not only on erythroid cells but also on the vascular endothelium (Anagnostou *et al*., 1994; Yamaji *et al*., 1996), and EPO reportedly causes acceleration of endothelial migration and proliferation (Anagnostou *et al*., 1990) and angiogenesis (Carlini *et al*., 1995b), and endothelin-1 release

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in vitro (Carlini *et al*., 1993; Bode Boger *et al*., 1996). The endothelium is known to play an essential role in regulating vascular tone through generation of several potent vasoactive substances such as NO and/or prostaglandin I_2. Indeed, impaired endothelium-dependent vasodilation has been suggested as a causative role of hypertension in experimentally hypertensive rats (Hayakawa *et al*., 1993) and normotensive subjects with a familial history of essential hypertension (Taddei *et al*., 1992). Therefore, it seems important to examine the effect of EPO on release of or responsiveness to endothelium-derived NO. Thus, this study was conducted to estimate whether 1-week repeated administration of EPO can cause endothelial vasodilator dysfunction. The endothelial function *in vivo* was assessed by comparing the responsiveness to the endothelium-dependent vasodilators acetylcholine, carbachol, bradykinin and substance P, and the endothelium-independent NO donors 3-(2-hydroxy-1-methylethyl-2-nitrosohydrazino)-N-methyl-1-propanamine (NOC7), sodium nitroprusside and nitroglycerin in addition to prostaglandin I_2 in anaesthetized rabbits received EPO and its vehicle.

Methods

The animals used in this study were handled in accordance with the Guidelines for Animal Experimentation of the University of the Ryukyus, and the experimental protocol was approved by the Animal Care and Committee of this institution.

Experimental preparation

Male Japanese white rabbits weighing 2.8–4.1 kg were used in this study. After measurement of Hb concentration and haematocrit (Hct) levels, 34 rabbits were divided into two groups. EPO (400 iu kg^{-1}) or normal saline solution (0.2 ml kg^{-1}) was given subcutaneously on four occasions every other day for 1 week. On the day of the experiment, the rabbits were anaesthetized with an initial dose of intravenous pentobarbital sodium (30 mg kg^{-1}). A constant level of anaesthesia was maintained with continuous infusion of pentobarbitone sodium (5 mg kg^{-1} h^{-1}, i.v.) throughout the experimental period *via* an ear vein. Tracheotomy was performed to make spontaneous ventilation easier.

A catheter was inserted into the aorta *via* the right carotid artery or the left femoral artery to measure aortic pressure (AoP). The left or the right internal jugular vein and the left carotid artery were cannulated for intravenous and intra-arterial administration of drugs, respectively. Arterial blood samples were collected from a catheter inserted into the left carotid artery or the left femoral artery for measurement of blood cell count and Hb concentration. AoP was measured by a pressure transducer (TP-200T, Nihon Kohden, Tokyo, Japan), and heart rate (HR) was counted by a cardiotachometer (AT-600G, Nihon Kohden) triggered by R wave of electrocardiogram. The right femoral artery was exposed, and a transonic flow probe was placed around it and connected to a transonic flowmeter (T106, Transonic System Inc., Ithaca, New York, U.S.A.) for continuous femoral arterial blood flow (FABF) monitoring. Femoral vascular resistance (FVR) was calculated as follows: mean AoP/FABF. These cardiovascular parameters were recorded on an eight-channel pen recorder (8K 23, NEC San-ei Instrument Ltd, Tokyo, Japan). Red blood cell (RBC) count, white blood cell (WBC) count, platelet (PLT) count, Hb concentration and Hct level in the blood sample were measured by an automatic blood cell counter (MEK-6158, Nihon Kohden).

Experimental protocol

The rabbits were allowed to stabilize for at least 30 min after completion of the operation. Before drug injection, baseline values of cardiovascular parameters were measured.

Experiment 1 Depressor responses, estimated by the peak reduction in diastolic AoP (dAoP), to intravenous (i.v.) bolus injections of acetylcholine (ACh; 0.05, 0.1, 0.2 and 0.5 μg kg^{-1}), NOC7 (2, 5 and 10 μg kg^{-1}), prostaglandin I_2 (PGI_2; 0.05, 0.1, 0.2 and 0.5 μg kg^{-1}), sodium nitroprusside (SNP; 5, 10 and 20 μg kg^{-1}) and nitroglycerin (NTG; 2, 5, 10 and 20 μg kg^{-1}) were tested, and the results were compared between the rabbits which received EPO (EPO group, $n=10$) and normal saline solution (saline group, $n=10$). Additionally, pressor responses to norepinephrine (NE; 1.0 μg kg^{-1}) were tested in both groups.

To evaluate the effect of pretreatment with N^G-nitro-L-arginine methyl ester (L-NAME), a selective inhibitor of NO synthase (NOS), depressor responses to the above vasodilators were tested again at least 10 min after L-NAME (20 mg kg^{-1}, i.v.). All drugs were injected intravenously in a bolus.

Experiment 2 To further examine the effect of endothelium-dependent vasodilator agents, haemodynamic responses to arterial injections of ACh (10, 20, 50 and 100 ng kg^{-1}), bradykinin (BK; 5, 10, 20 and 50 ng kg^{-1}), carbachol (CCh; 20, 50, 100 and 200 ng kg^{-1}) and substance P (Sub P; 0.1, 0.2, 0.5 and 1.0 ng kg^{-1}) in addition to endothelium-independent vasodilators such as PGI_2 (20, 50, 100 and 200 ng kg^{-1}) and NTG (500, 1000, 2000 and 5000 ng kg^{-1}) were tested in separate rabbits which received EPO ($n=7$) or saline ($n=7$) at the same dosage as mentioned above. All drugs were injected into the descending aorta through a catheter inserted from the left carotid artery, since BK is almost inactivated in pulmonary vascular beds when injected intravenously.

In vitro *experiments* Responses of isolated arterial preparations to several vasodilator agents were examined in both groups *in vitro* by using the Magnus method. After completion of *in vivo* experiments, rabbits were killed by bleeding after overdosage of pentobarbitone sodium, then the right carotid artery was isolated and placed rapidly in cold Krebs-Henseleit solution (KHS) to remove fat and connective tissues from the artery. The composition of KHS was (mM): NaCl 120, KCl 4.8, $CaCl_2$ 1.25, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 11.0. The carotid artery was cut into ring segments of 2–3 mm in width. One end of the artery ring was fixed at the base of an organ bath, which was filled with KHS maintained at 37°C and aerated with 95% O_2 and 5% CO_2. The other end was connected by a silk thread to an isometric force-displacement transducer (TB-611T,

Nihon Kohden) which was connected to a polygraph system (RMP-6004, Nihon Kohden). The KHS in the organ bath was exchanged at a 15-min interval during the equilibration of the arterial preparations. After 90 min of stabilization under a resting tension of 1 g (an optimal tension) the ring segments were contracted with KCl (30 mM) in order to test the viability, and thereafter rinsed three times with KHS. For studying relaxing responses of the segments precontracted with KCl (30 mM) to ACh, BK, NTG and SNP in both groups, these vasodilators over a concentration range of 10^{-8}–10^{-4} M were cumulatively added to the bath. Precontraction tensions by KCl were not significantly different between groups. At the end of the experiments, each segment was maximally relaxed by 10^{-4} M papaverine hydrochloride in order to confirm the sufficient relaxant ability of the preparation. Cumulative relaxation data were expressed as a percentage of the precontraction. In additional four rabbits in each group, relaxing responses to the above vasodilators were tested in phenylephrine (10^{-6} M)-precontracted femoral artery rings.

Drugs

EPO (recombinant human erythropoietin, Epogin) was kindly supplied from Chugai Pharmaceutical (Tokyo, Japan). And the following drugs were used: L-NAME (Sigma Chemical, St. Louis, MO, U.S.A.), ACh (Ovisot, Dai-ichi Pharmaceutical, Tokyo, Japan), NOC7 (Dojindo Laboratories, Kumamoto, Japan), PGI_2 sodium salt (Cayman Chemical, Ann Arbor, MI, U.S.A.), SNP (Wako, Osaka, Japan), NTG (Millisrol, Nippon Kayaku, Tokyo, Japan), BK (Peptide Institute Inc., Osaka, Japan), CCh (Wako, Osaka, Japan), Sub P (Peptide Institute Inc., Osaka, Japan), Papaverine hydrochloride (Takeda, Osaka, Japan), phenylephrine hydrochloride (Kowa, Nagoya, Japan) and NE (Sankyo, Tokyo, Japan). NOC7 was dissolved in 0.1 N NaOH. PGI_2 sodium salt was dissolved in ethanol. These stock solutions of NOC7 and PGI_2 were diluted with 50 mM glycine buffer at pH 10.4 just before use. The other drugs were dissolved in and diluted with saline.

An intravenous or intra-arterial injection (0.1 ml kg^{-1}) of normal saline solution or vehicle of NOC7 and PGI_2 in a bolus caused virtually no change in all measured cardiovascular parameters.

Data analysis

Comparisons of dose-response curves were performed by use of two-way analysis of variance. Paired and unpaired data were analysed by paired and unpaired *t*-test, respectively. All values are presented as means ± s.e.mean. Statistical significance was defined as a P value less than 0.05.

Results

On the day of experiments, RBC count, Hb and Hct in EPO group were significantly higher than those in saline group, whereas WBC and PLT counts were similar in both groups (Table 1). Baseline values of cardiovascular parameters such as sAoP, dAoP, HR and FVR except FABF were comparable between two groups (Table 1).

Table 1 Baseline values of haematological and haemodynamic variables in saline and EPO groups

	Saline group (n = 17)	*EPO group* (n = 17)
Haematological data		
WBC count ($\times 10^2$ μl^{-1})	60.4 ± 7.1	72.4 ± 8.7
RBC count ($\times 10^4$ μl^{-1})	664.8 ± 13.2	765.9 ± 15.4**
Hb concentration (g dl^{-1})	13.1 ± 0.3	15.3 ± 0.2**
Haematocrit (%)	40.4 ± 0.8	46.7 ± 0.8**
PLT count ($\times 10^4$ μl^{-1})	33.5 ± 2.7	35.3 ± 2.8
Cardiovascular parameters		
Systolic AoP (mmHg)	126.8 ± 3.4	122.5 ± 3.2
Diastolic AoP (mmHg)	87.6 ± 2.8	87.5 ± 2.8
Heart rate (beats min^{-1})	319.6 ± 7.8	326.8 ± 5.4
FABF (ml min^{-1})	22.0 ± 2.0	15.2 ± 2.1*
FVR (mmHg·min ml^{-1})	5.2 ± 0.5	9.7 ± 2.2

WBC = white blood cell, RBC = red blood cell, Hb = haemoglobin, PLT = platelet, AoP = aortic pressure, FABF = femoral arterial blood flow, FVR = femoral vascular resistance, EPO = erythropoietin. Each value represents mean ± s.e.mean. *P < 0.05, **P < 0.01 between saline and EPO groups.

Responses of blood pressure and vascular resistance to endothelium-dependent and -independent vasodilators

Challenge with each vasodilator used in this study produced dose-dependent hypotension of dAoP in all rabbits irrespective of the route of administration. Nevertheless, depressor responses of dAoP to intravenously given ACh were significantly attenuated in EPO group in comparison with those in saline group (P < 0.01) (Figure 1a), while the endothelium-independent and NO-mediated vasodilators NOC7, SNP and NTG, and the NO-unrelated vasodilator PGI_2 caused equivalent depressor responses between two groups (Figure 1b,c,d,e). The i.v. doses of ACh required for a 15 mmHg reduction of dAoP from baseline ($ED_{15\ mmHg}$) in saline and EPO groups were 55 ng kg^{-1} (95% confidence limit of 50–60 ng kg^{-1}) and 172 ng kg^{-1} (136–217 ng kg^{-1}), respectively, indicating that the responsiveness to ACh in EPO-treated rabbits were 3.1-times less potent than that in control rabbits. Immediately after i.v. injection of ACh as well as other vasodilators, a transient increase in FABF was observed despite hypotension, so FVR decreased dose-dependently. The i.v. ACh-induced decreases in FVR in EPO group were significantly less potent than those in saline group (P < 0.01), while decreases in FVR by NOC7, PGI_2 or SNP except NTG were not significantly different between both groups (Figure 2). When compared at the dose of $ED_{15\ mmHg}$, increases in heart rate (ΔHR at $ED_{15\ mmHg}$) induced by i.v. ACh were comparable in both saline (5.4 ± 1.3 beats min^{-1}) and EPO (7.5 ± 1.7 beats min^{-1}) groups, indicating that the cardiac response would not contribute to the difference in the depressor response. Likewise, there was no significant difference in ΔHR at $ED_{15\ mmHg}$ of intra-arterial injection of ACh between both groups. Additionally, in separate experiments, decreased responses of dAoP and FVR to intra-arterial injections of endothelium-dependent vasodilators including CCh, BK, Sub P in addition to ACh were observed in EPO-treated rabbits, whereas those to PGI_2 were almost equipotent in both groups as in the case of i.v. injection (Figures 3 and 4). The magnitude of depressor

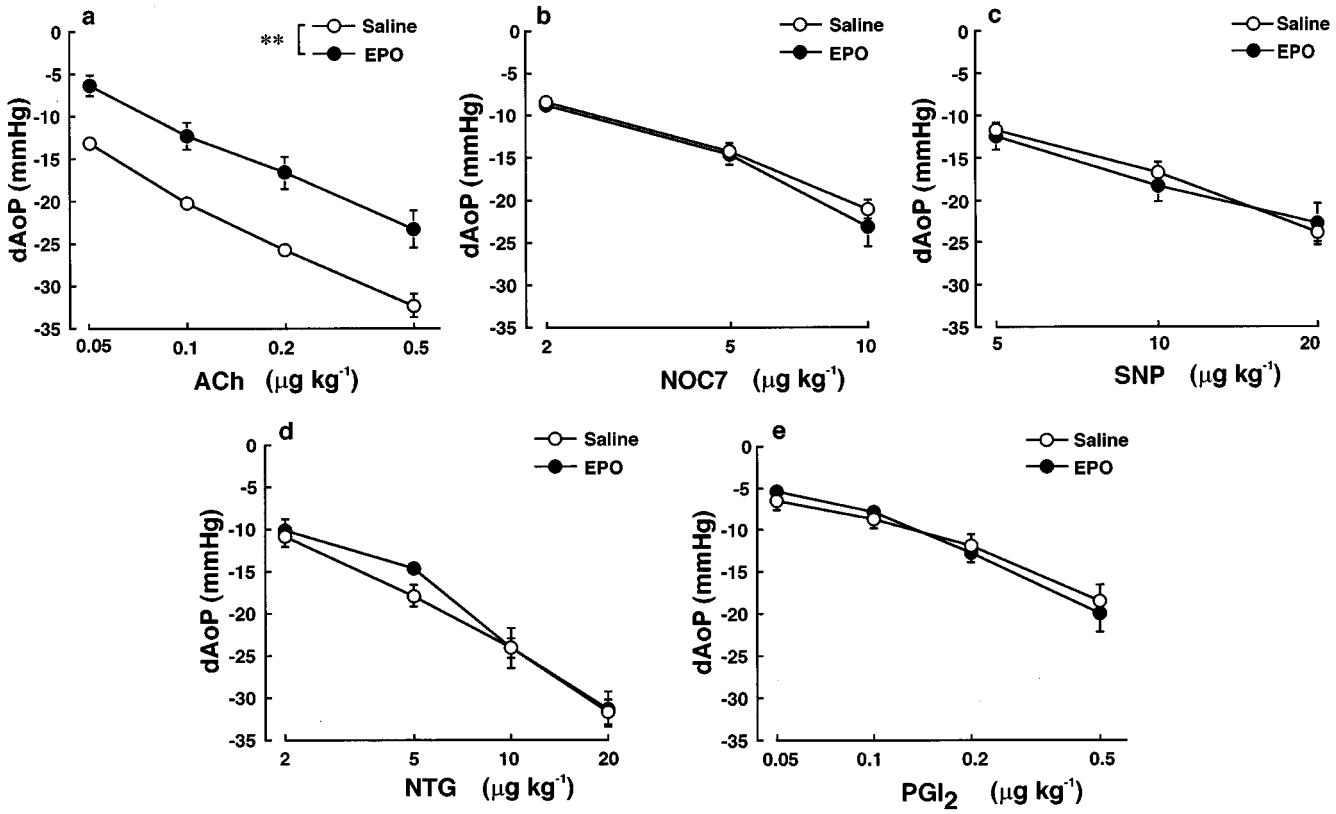

Figure 1 Depressor responses to i.v. injections of (a) ACh, (b) NOC7, (c) nitroprusside (SNP), (d) nitroglycerin (NTG) and (e) PGI_2 in saline ($n=10$) and EPO ($n=10$) groups. Each value represents mean ± s.e.mean. $^{**}P<0.01$ between saline and EPO groups.

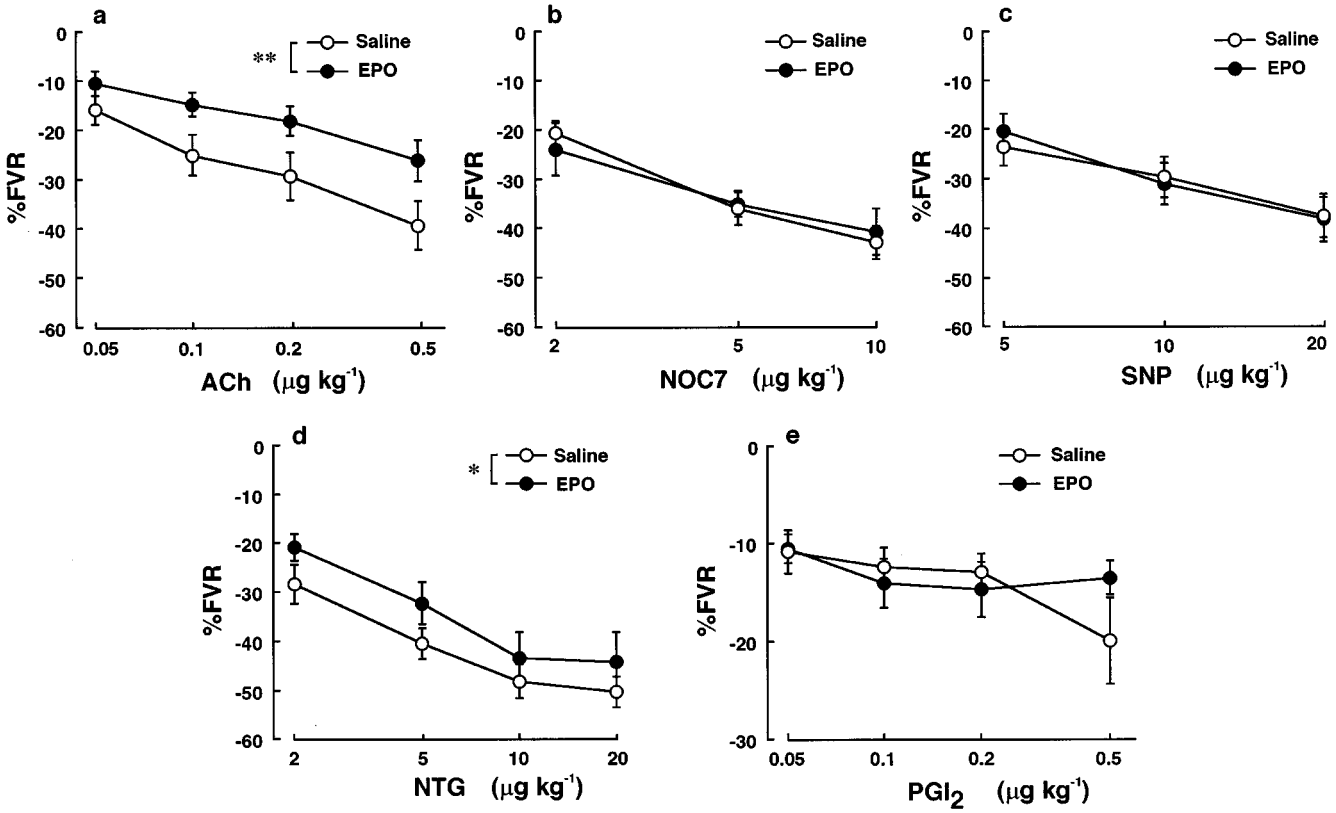

Figure 2 Responses of femoral vascular resistance (FVR) to i.v. injections of (a) ACh, (b) NOC7, (c) nitroprusside (SNP), (d) nitroglycerin (NTG) and (e) PGI_2 in saline ($n=10$) and EPO ($n=10$) groups. Each value represents mean ± s.e.mean. $^{*}P<0.05$; $^{**}P<0.01$ between saline and EPO groups.

response to ACh (0.1 μg kg^{-1}) did not significantly correlate with baseline value of Hb concentration (EPO group: $r=0.40$, $n=17$; saline group: $r=0.19$, $n=17$), and depressor responses to the other doses of ACh and the other agents also did not significantly correlate with Hb concentration in each group. Pressor responses to NE (1 μg kg^{-1}, i.v.) were also not significantly different in saline ($n=17$) and EPO ($n=17$) groups (16.4±1.3 and 18.8±1.7 mmHg, respectively).

In two separate rabbits, acute haemodynamic effect of EPO was examined. I.v. infusion of EPO (1.0, 10 and 100 iu kg^{-1} min^{-1} for about 15 min each) did not cause any detectable changes in AoP, HR and FABF, and did not affect ACh-induced depressor responses.

Effect of L-NAME on depressor and vasodilating responses to vasodilators

Administration of L-NAME elicited significant increases in AoP, FVR, and decreases in FABF and HR in saline and EPO groups, and the per cent changes in these parameters were similar in both groups (Table 2). As shown in Figure 5, treatment with L-NAME significantly suppressed the ACh-induced vasodilating response in saline group but did not in EPO group, although L-NAME significantly inhibited the depressor responses in both groups. In contrast to the case of ACh, depressor responses to endothelium-independent and NO-mediated vasodilators such as NTG, NOC7 and SNP in addition to pressor response to NE in each group were apparently enhanced by treatment with L-NAME (Figure 6), while these responses were not significantly different between two groups both in the presence and the absence of L-NAME (Figure 6b–d,f). L-NAME treatment did not influence the depressor response to PGI_2 in both groups (Figure 6e).

In vitro *experiments*

As shown in Figure 7, results with isolated carotid arterial segments were obviously different from those with the *in vivo* experiments described above. Vasorelaxations produced by ACh and BK in KCl-precontracted arterial segments obtained from saline- and EPO-treated rabbits were not significantly different between two groups (Figure 7a,b). On the other hand, relaxing responses to NTG and SNP were significantly enhanced in EPO groups as compared with those in saline group (Figure 7c,d). In phenylephrine-precontracted arterial preparations of the two groups ($n=4$ each), there were no significant differences in vasorelaxations by ACh and BK between groups.

Discussion

One-week administration of EPO did not develop hypertension despite that significant increases in Hb and Hct occurred. Since it is well known that free Hb can eliminate the biological actions of NO *in vitro* (Moncada *et al.*, 1991a), increased Hb concentration in blood was expected to

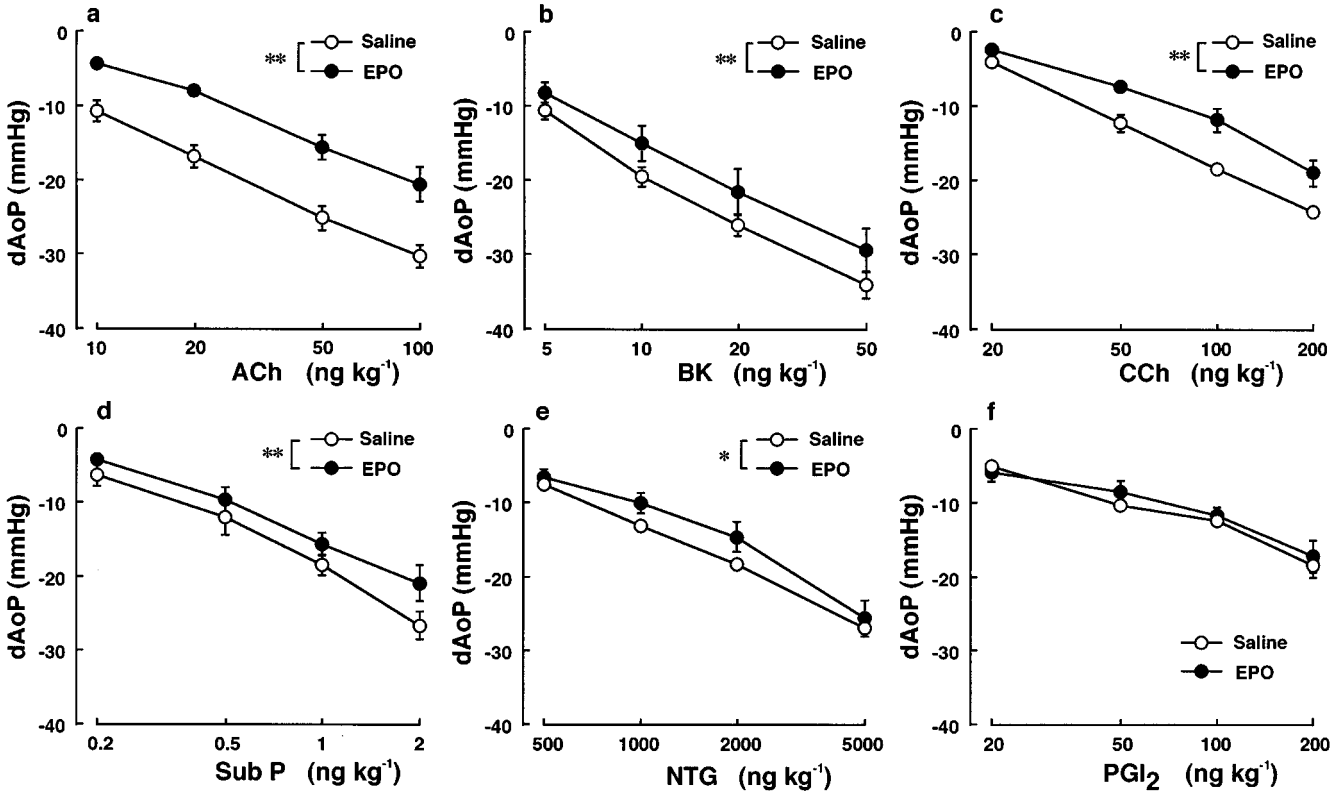

Figure 3 Depressor responses to intra-arterial injections of (a) ACh, (b) bradykinin (BK), (c) carbachol (CCh), (d) substance P (Sub P), (e) nitroglycerin (NTG) and (f) PGI_2 in saline ($n=7$) and EPO ($n=7$) groups. Each value represents mean±s.e.mean. $^*P<0.05$; $^{**}P<0.01$ between saline and EPO groups. Agents were given into the descending aorta in a bolus.

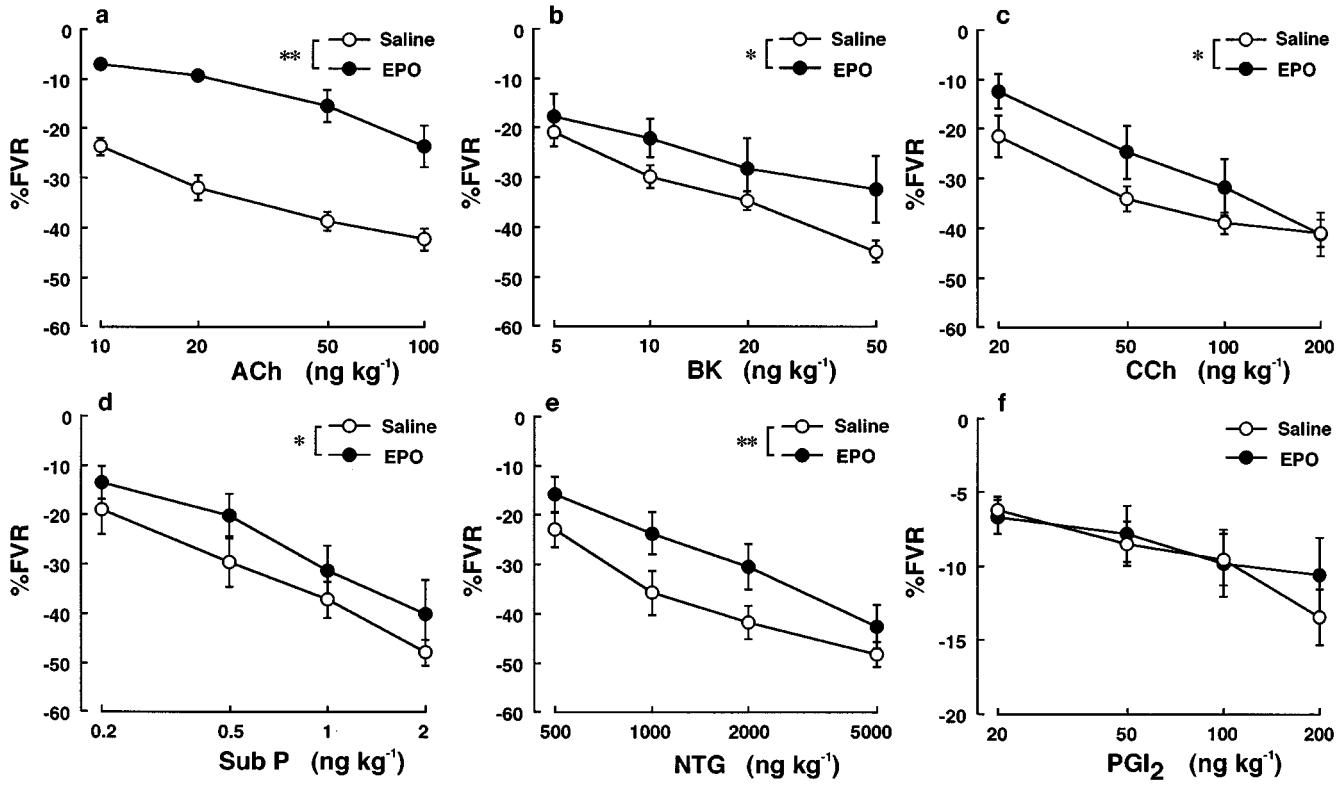

Figure 4 Responses of femoral vascular resistance (FVR) to intra-arterial injections of (a) ACh, (b) bradykinin (BK), (c) carbachol (CCh), (d) substance P (Sub P), (e) nitroglycerin (NTG) and (f) PGI_2 in saline ($n=7$) and EPO ($n=7$) groups. Each value represents mean ± s.e.mean. $^*P<0.05$; $^{**}P<0.01$ between saline and EPO groups. Agents were given into the descending aorta in a bolus.

Table 2 Effect of L-NAME on haemodynamic parameters in saline and EPO groups

	Saline group (n = 10)		*EPO group* (n = 10)	
	Before	*After*	*Before*	*After*
sAoP (mmHg)	131.4 ± 3.2	136.9 ± 3.1**	126.2 ± 3.9	133.7 ± 4.4*
%Δ		4.3 ± 1.0		6.0 ± 2.0
dAoP (mmHg)	100.3 ± 2.8	111.0 ± 2.5**	96.2 ± 3.8	110.5 ± 3.7**
%Δ		11.0 ± 1.9		15.3 ± 2.4
FABF (ml min^{-1})	20.1 ± 2.6	13.6 ± 1.8**	16.3 ± 1.8	9.7 ± 1.5**
%Δ		−32.6 ± 3.3		−41.9 ± 5.1
HR (beats min^{-1})	282.6 ± 7.5	228.6 ± 7.7**	283.2 ± 9.0	227.3 ± 8.7**
%Δ		−19.2 ± 1.4		−19.8 ± 1.5
FVR (mmHg·min ml^{-1})	6.47 ± 0.93	10.70 ± 1.67**	7.23 ± 0.79	15.44 ± 2.60**
%Δ		64.4 ± 8.8		105.7 ± 17.9

Values are before and 10 min after L-NAME (20 mg kg^{-1} i.v.), sAoP = systolic aortic pressure, dAoP = diastolic aortic pressure, FABF = femoral blood flow, FVR = femoral vascular resistance. %Δ means per cent change from the value before L-NAME treatment. Each value represents mean ± s.e.mean. $^*P<0.05$, $^{**}P<0.01$ *vs* before L-NAME treatment (paired *t*-test). Note that the corresponding values and per cent changes in any parameters are not significantly different between groups.

cause vasoconstriction by trapping NO as suggested by Martin & Moncada (1988). In the present study, however, rabbits treated with saline and EPO showed no significant differences in baseline blood pressure and peripheral vascular resistance, and exhibited similar depressor responses to NOC7 that releases NO spontaneously in neutral solutions (Zhang *et al*., 1996), and to SNP, a NO donor that does not need cofactors to generate NO unlike NTG, and the magnitudes of hypotension induced by these NO-releasing vasodilators did not correlate with the level of Hb. These results suggest that a prolonged increase in blood Hb concentration appears primarily not to affect NO availability to the vascular smooth muscle *in vivo*. Previous findings concerning the hypothesis that the increment of Hb in intact erythrocytes is capable of trapping NO in circulation and therefore of increasing vascular tone are contradictory (Vaziri *et al*., 1995; Casadevall *et al*., 1996). The varied results may be due to the differences in the extent of the increase in Hb, preexisting NO levels or the employed experimental model.

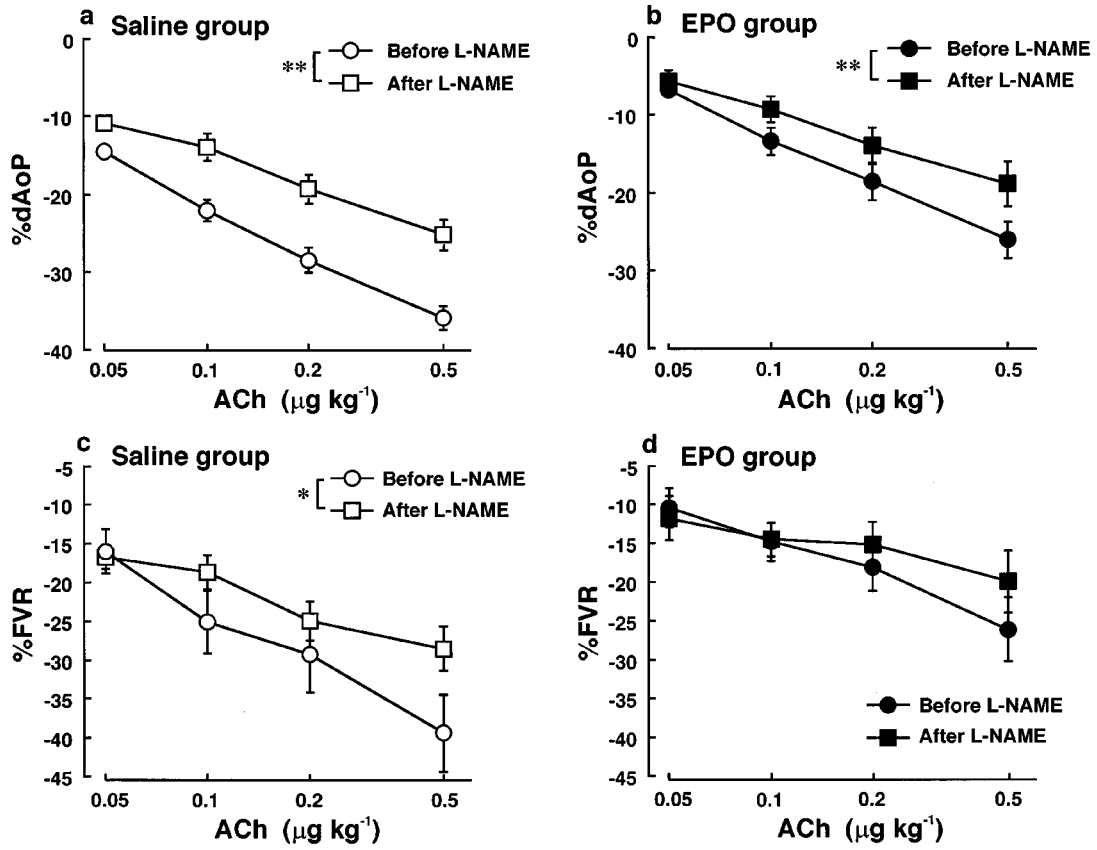

Figure 5 Responses of diastolic aortic pressure (dAoP) and femoral vascular resistance (FVR) to i.v. injections of ACh in saline ((a) and (c), $n=10$) and EPO ((b) and (d), $n=10$) groups in the absence and presence of N^G-nitro-L-arginine methyl ester (L-NAME). Each value represents mean ± s.e.mean. $*P<0.05$; $**P<0.01$ between saline and EPO groups.

Responses of blood pressure and vascular resistance to endothelium-dependent vasodilators including ACh, CCh, BK and Sub P were significantly lessened by EPO treatment, whereas those to PGI_2 and the endothelium-independent NO donors NOC7 and SNP were not affected. Furthermore, significant inhibitory action of L-NAME, a specific NOS inhibitor, on responses of vascular resistance to ACh was not observed in EPO received rabbits, while the inhibitor-induced rises in basal blood pressure and vascular resistance were comparable in EPO- and saline-treated animals. The latter data apparently implicate that the tonic release of NO might be preserved, although agonist-stimulated release of NO was impaired by treatment with EPO. The difference in the inhibitory effect of L-NAME may suggest a different degree of activity of the arginine/NO pathway between groups. In addition, no direct vascular effect nor inhibition of ACh-induced hypotension was found when i.v. EPO was acutely given, as demonstrated consistently by most previous investigators (Hon *et al*., 1995; Vaziri *et al*., 1995; Casadevall *et al*., 1996). These observations thus indicate that administration of EPO for 1 week may attenuate agonist-stimulated endothelium-derived NO activity probably through suppression of endothelial NOS activity rather than facilitation of scavenging ability or moderation of the soluble guanylate cyclase cyclic-GMP system of the vascular smooth muscle. In contrast, Wilcox *et al*. (1993) and del Castillo *et al*. (1995) have reported that increased NO production was associated with EPO-induced hypertension and erythrocytosis in normal rats. On the other hand, Ni *et al*. (1998) have shown that administration of EPO changed neither total body NO production nor renal or vascular tissue NOS expression in rats with chronic renal failure. In these previous investigations, however, marked rises in blood pressure and Hct occurred with administration of EPO, which should obscure the direct effect of EPO on NO production and NOS activity *in vivo*, because increases in blood viscosity and shear stress due to the hyperdynamic circulation are known to stimulate endothelial NOS expression and NO production (Awolesi *et al*., 1995; Noris *et al*., 1995). In this study, fortunately, the effect of EPO on the endothelial function *in vivo* was estimated under a condition that was associated with unchanged blood pressure. Thus the secondary influence of EPO administration on NO release resulting, at least, from hypertension can be excluded from the present findings. In harmony with our results, Wang & Vaziri (1999) have recently demonstrated that incubation of EPO for 24 h downregulates basal and ACh-stimulated NO production, and depresses NOS expression in isolated human endothelial cells, implying that EPO may possess a direct inhibitory action on the endothelial NO production.

The mechanism underlying the unexpected results that the vascular resistance response to NTG was blunted by EPO-treatment is unknown at present, although there is a clinical investigation reporting that the vasodilating response to NTG

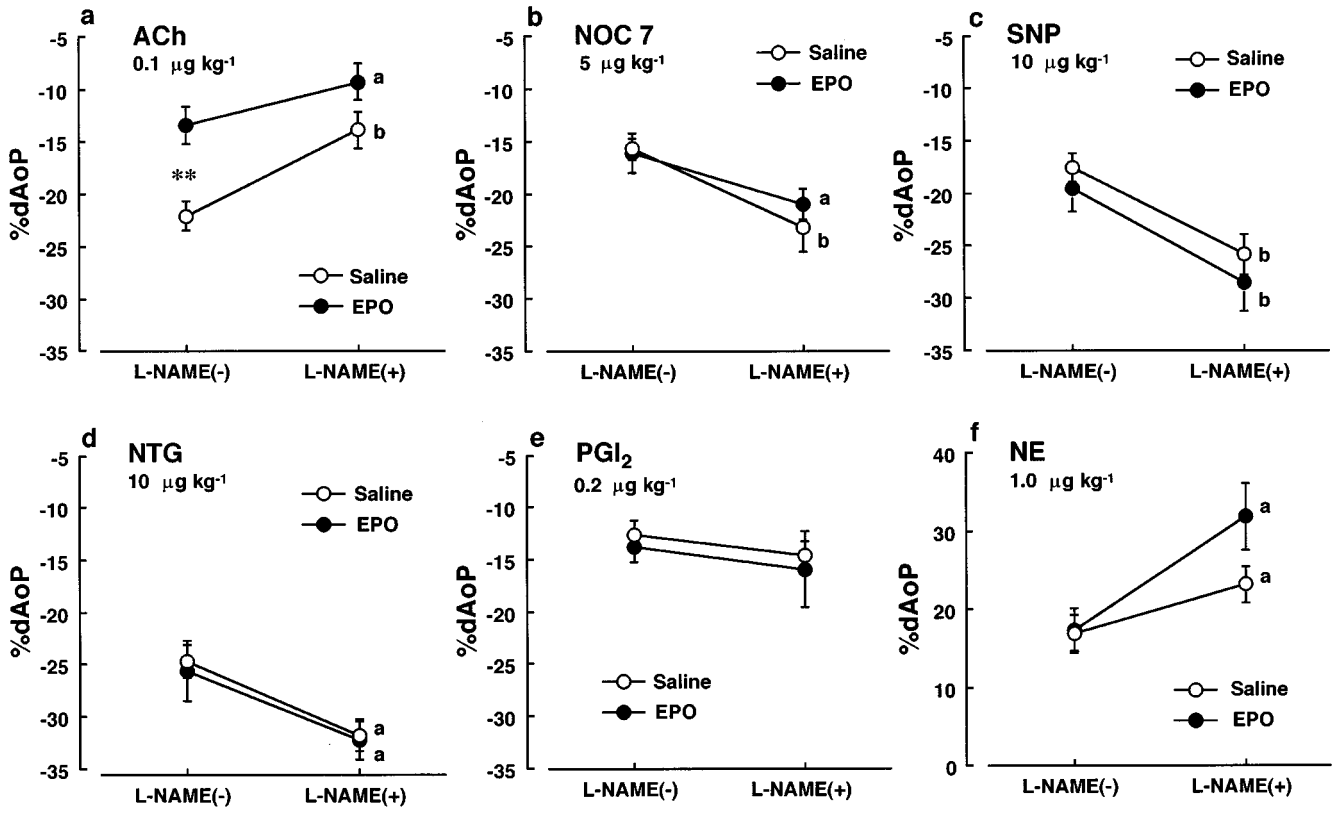

Figure 6 Depressor responses to i.v. (a) ACh (0.1 μg kg^{-1}), (b) NOC7 (5 μg kg^{-1}), (c) nitroprusside (SNP, 10 μg kg^{-1}), (d) nitroglycerin (NTG, 10 μg kg^{-1}) and (e) PGI_2 (0.2 μg kg^{-1}), and pressor response to i.v. (f) norepinephrine (NE, 1.0 μg kg^{-1}) in the absence and presence of N^G-nitro-L-arginine methyl ester (L-NAME) in saline ($n=10$) and EPO ($n=10$) groups. Each value represents mean$\pm$s.e.mean. $^{**}P<0.01$ between saline and EPO groups. $^{a}P<0.05$; $^{b}P<0.01$ *vs* before L-NAME.

in addition to the flow-dependent vasodilation was significantly decreased in normotensive haemodialyzed patients treated with EPO as compared to those in healthy controls (Joannides *et al*., 1997). NTG, differently from NOC7 or SNP, is thought to require bioconversion to NO for vasodilating activity (Ahlner *et al*., 1991). Considering the present results with the *in vitro* experiments that vasorelaxations of isolated arteries of EPO-treated rabbits to NTG as well as SNP were even enhanced, a blood-derived factor(s) such as Hb is most likely involved in the blunted *in vivo* responses to NTG in EPO group, probably through the mechanism relating to the bioconversion. Interestingly, it has previously been shown that the biotransformation of NTG occurs not only in the vascular tissues but also in the red blood cell by haemoglobin-mediated enzymatic processes (Bennett *et al*., 1984). Recently, NO itself has been demonstrated to have an inhibitory action on the vascular bioactivation of NTG (Kojda *et al*., 1998). Theses findings suggest that an increase in blood Hb results in a certain increase in Hb-mediated NTG metabolism in blood, but the resultant increase in NO in plasma inhibits the vascular conversion of NTG to NO, which may lead to the attenuated vasodilation to NTG.

It is now generally believed that endothelium-dependent vasodilation induced by ACh and other autacoids such as BK and Sub P is mediated by NO, PGI_2 and an as yet unidentified hyperpolarizing factor (EDHF), although the relative proportions and significance of each factor appear rather variable depending on the amount of stretch, on the artery and on the species of animal (Nagao & Vanhoutte, 1993; Parkington *et al*., 1993). Taking into account the previous findings that the contribution of EDHF to endothelium-dependent vasodilation may be major in resistance arteries (Nagao *et al*., 1992; Shimokawa *et al*., 1996; Bolz *et al*., 1999), and EDHF may act as a back-up mechanism when the endothelial production of NO is impaired (Kilpatrick & Cocks, 1994), it will be necessary to consider the possibility that EDHF might play a part in the suppressive effect of EPO on depressor responses to endothelium-dependent vasodilators observed in this study. However, it seems less likely that treatment with EPO primarily modified ACh-induced endothelial production of EDHF or PGI_2 instead of NO. The rationale for this is based on the following data: firstly, ACh-induced vasorelaxation has been shown to be unaltered by indomethacin in isolated rabbit (Dong *et al*., 1997) and rat (Shimokawa *et al*., 1996) arteries, indicating that a role of PGI_2 is probably minimal; secondly, reduced inhibitory effect of L-NAME on ACh-induced vasodilating action was observed in the EPO group as compared to the control group, indicating that NOS activity had already been inhibited or a sensitivity of NOS to L-NAME was decreased in EPO treated rabbits; thirdly, relatively prominent attenuation of depressor responses to ACh or CCh by EPO treatment was seen in comparison with

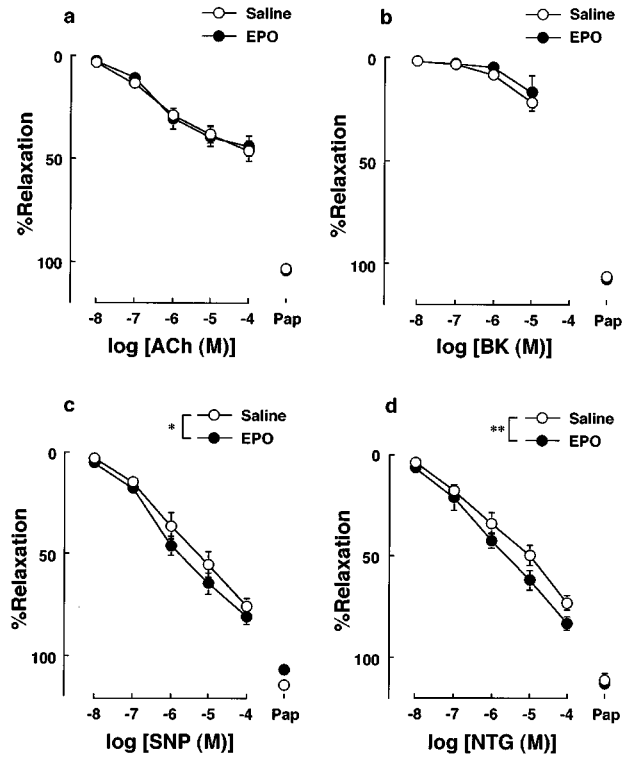

Figure 7 Vasorelaxation to (a) ACh, (b) bradykinin (BK), (c) nitroprusside (SNP) and (d) nitroglycerin (NTG) in KCl-precontracted carotid arterial segments isolated from saline ($n=8$)- and EPO ($n=8$)-treated rabbits. Relaxation exhibits per cent relaxation from KCl (30 mM)-induced contraction. Pap = relaxation by papaverine hydrochloride at 10^{-4} M. Each value represents mean ± s.e.mean. $^{*}P<0.05$; $^{**}P<0.01$ between saline and EPO groups.

those to BK or Sub P, suggesting the possibility that the difference in the endothelium-dependent vasodilator effect of these agents between the two groups may reflect the difference in the extent of endothelial NO release, since vasorelaxation of microvessels by both BK and Sub P, differently from ACh, has been shown to be mediated predominantly by EDHF (Hozumi *et al*., 1997; Miura *et al*., 1999). Finally, the *in vitro* studies showing that vasorelaxations by ACh of isolated arteries precontracted with irrespective of whether potassium or phenylephrine were quite similar in saline and EPO groups, suggest that the ability of EDHF generation would be indistinguishable between the two groups. Therefore, it is most likely that the impaired vasodilator responses to endothelium-derived NO releasing agents by EPO administration seen in our study is attributable mainly to suppression of endothelial NOS activity.

The mechanism underlying the decreased endothelium-dependent vasodilator function by 1-week administration of EPO is not defined in the present study. EPO has been shown to raise cytosolic calcium concentration in vascular smooth muscle and endothelial cells (Neusser *et al*., 1993; Carlini *et al*., 1995a) besides erythroid precursors (Miller *et al*., 1988), and thereby possibly increases endothelin-1 synthesis (Vogel *et al*., 1997). It is conceivable that endothelin-1 might involve in the vascular effect of EPO observed in this study, as recently proposed by Mombouli & Vanhoutte (1999) that increased generation of endothelin-1 as well as several other causes is implicated in endothelial dysfunction. Also, decreased basal femoral blood flow observed in EPO group may relate to the altered endothelial function, since a prolonged reduction in peripheral blood flow must lessen shear stress and may lead to a decrease in endothelial NO production and NOS expression in view of previous observations (Miller *et al*., 1986; Wang *et al*., 1993; Sessa *et al*., 1994).

In contrast to results with the *in vivo* experiments, vasorelaxations by ACh and BK were comparable in arterial segments isolated from rabbits of control and EPO groups. Moreover, relaxing responses to NTG and SNP were significantly greater in EPO group as compared with those in saline group, indicating that the sensitivity of the artery from EPO-treated rabbits to NO may be enhanced. These data seem to correspond with the results of the *in vivo* study that hypotensive effects of endothelium-independent NO-donors but not PGI_2 were all potentiated after L-NAME administration, as previously described (Moncada *et al*., 1991b). Recently, the supersensitivity to NO after NOS inhibition has been suggested to attribute to upregulation of soluble guanylate cyclase (Hussain *et al*., 1999). Furthermore, it is possible that, in the whole body, increased Hb concentration in circulation of EPO-treated rabbits might conceal the enhanced NO-sensitivity of blood vessels by scavenging both endothelium- and agents-derived NO. This may also explain the seemingly puzzled result that the pressor action of L-NAME was similar in both groups. Otherwise, suppression of agonist-stimulated endothelial NO release in EPO treated animals may arise from alterations in certain factor(s) other than vascular smooth muscle or the endothelium itself. Possible factors may include an increase in circulating endogenous NOS inhibitors such as N^G-monomethyl-L-arginine and N^G, N^G-dimethyl-L-arginine (Vallance *et al*., 1992). In addition, the discrepancy between the *in vivo* and *in vitro* data may be explainable by the fact that these data were based on entirely different vessels in size. Finally, it should be noted that biochemical studies such as assessment of vascular cyclic-GMP levels, plasma nitrite/nitrate levels or endothelial NOS activity would aid a further understanding of mechanisms underlying the effects of EPO.

In conclusion, the present results indicate that administration of EPO for 1 week selectively inhibits *in vivo* endothelium-dependent vasodilation, especially by ACh, without changing the responses to endothelium-independent vasodilators such as spontaneous NO releasing agents and PGI_2 in anaesthetized rabbits, probably through inhibition of endothelial NOS. Moreover, these findings suggest that in chronic EPO therapy a decrease in endothelial vasodilator function might occur even before development of hypertension, and might contribute, at least in part, to the future increase in vascular resistance.

References

AHLNER, J., ANDERSSON, R.G.G., TORFGÅRD, K. & AXELSSON, K.L. (1991). Organic nitrate esters: Clinical use and mechanisms of actions. *Pharmacol. Rev.,* **43,** 351 – 423.

ANAGNOSTOU, A., LEE, E.S., KESSIMIAN, N., LEVINSON, R. & STEINER, M. (1990). Erythropoietin has a mitogenic and positive chemotactic effect on endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.,* **87,** 5978 – 5982.

ANAGNOSTOU, A., LIU, Z., STEINER, M., CHIN, K., LEE, E.S., KESSIMIAN, N. & NOGUCHI, C.T. (1994). Erythropoietin receptor mRNA expression in human endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.,* **91,** 3974 – 3978.

AWOLESI, M.A., SESSA, W.C. & SUMPIO, B.E. (1995). Cyclic strain upregulates nitric oxide synthase in cultured bovine aortic endothelial cells. *J. Clin. Invest.,* **96,** 1449 – 1454.

BENNETT, B.M., NAKATSU, K., BRIEN, J.F. & MARKS, G.S. (1984). Biotransformation of glyceryl trinitrate to glyceryl dinitrate by human hemoglobin. *Can. J. Physiol. Pharmacol.,* **62,** 704 – 706.

BODE BOGER, S.M., BOGER, R.H., KUHN, M., RADERMACHER, J. & FROLICH, J.C. (1996). Recombinant human erythropoietin enhances vasoconstrictor tone via endothelin-1 and constrictor prostanoids. *Kidney Int.,* **50,** 1255 – 1261.

BOLZ, S.S., DE WIT, C. & POHL, U. (1999). Endothelium-derived hyperpolarizing factor but not NO reduces smooth muscle Ca^{2+} during acetylcholine-induced dilation of microvessels. *Br. J. Pharmacol.,* **128,** 124 – 134.

BUCKNER, F.S., ESCHBACH, J.W., HALEY, N.R., DAVIDSON, R.C. & ADAMSON, J.W. (1990). Hypertension following erythropoietin therapy in anemic hemodialysis patients. *Am. J. Hypertens.,* **3,** 947 – 955.

CARLINI, R.G., DUSSO, A.S., OBIALO, C.I., ALVAREZ, U.M. & ROTHSTEIN, M. (1993). Recombinant human erythropoietin (rHuEPO) increases endothelin-1 release by endothelial cells. *Kidney Int.,* **43,** 1010 – 1014.

CARLINI, R.G., GUPTA, A., LIAPIS, H. & ROTHSTEIN, M. (1995a). Endothelin-1 release by erythropoietin involves calcium signaling in endothelial cells. *J. Cardiovasc. Pharmacol.,* **26,** 889 – 892.

CARLINI, R.G., REYES, A.A. & ROTHSTEIN, M. (1995b). Recombinant human erythropoietin stimulates angiogenesis in vitro. *Kidney Int.,* **47,** 740 – 745.

CASADEVALL, M., PIQUE, J.M., CIRERA, I., GOLDIN, E., ELIZALDE, I., PANES, J., MARTINEZ CUESTA, M.A., BOSCH, J., TERES, J. & RODES, J. (1996). Increased blood hemoglobin attenuates splanchnic vasodilation in portal-hypertensive rats by nitric oxide inactivation. *Gastroenterology,* **110,** 1156 – 1165.

DEL CASTILLO, D., RAIJ, L., SHULTZ, P.J. & TOLINS, J.P. (1995). The pressor effect of recombinant human erythropoietin is not due to decreased activity of the endogenous nitric oxide system. *Nephrol. Dial. Transplant.,* **10,** 505 – 508.

DONG, H., WALDRON, G.J., GALIPEAU, D., COLE, W.C. & TRIGGLE, C.R. (1997). NO/PGI_2-independent vasorelaxation and the cytochrome P450 pathway in rabbit carotid artery. *Br. J. Pharmacol.,* **120,** 695 – 701.

HAYAKAWA, H., HIRATA, Y., SUZUKI, E., SUGIMOTO, T., MATSUOKA, H., KIKUCHI, K., NAGANO, T., HIROBE, M. & SUGIMOTO, T. (1993). Mechanisms for altered endothelium-dependent vasorelaxation in isolated kidneys from experimental hypertensive rats. *Am. J. Physiol.,* **264,** H1535 – H1541.

HON, G., VAZIRI, N.D., KAUPKE, C.J., TEHRANZADEH, A. & BARTON, C. (1995). Lack of a fast-acting effect of erythropoietin on arterial blood pressure and endothelin level. *Artif. Organs,* **19,** 188 – 191.

HOZUMI, T., FUKUTA, H. & SUZUKI, H. (1997). Comparison of the relaxing actions of acetylcholine and substance P in smooth muscle of the guinea-pig aorta. *J. Smooth Muscle Res.,* **33,** 67 – 77.

HUSSAIN, M.B., HOBBS, A.J. & MACALLISTER, R.J. (1999). Autoregulation of nitric oxide-soluble guanylate cyclase-cyclic GMP signalling in mouse thoracic aorta. *Br. J. Pharmacol.,* **128,** 1082 – 1088.

JELKMANN, W. (1992). Erythropoietin: structure, control of production, and function. *Physiol. Rev.,* **72,** 449 – 489.

JOANNIDES, R., BAKKALI, E.H., LE ROY, F., RIVAULT, O., GODIN, M., MOORE, N., FILLASTRE, J.P. & THUILLEZ, C. (1997). Altered flow-dependent vasodilatation of conduit arteries in maintenance haemodialysis. *Nephrol. Dial. Transplant.,* **12,** 2623 – 2628.

KILPATRICK, E.V. & COCKS, T.M. (1994). Evidence for differential roles of nitric oxide (NO) and hyperpolarization in endothelium-dependent relaxation of pig isolated coronary artery. *Br. J. Pharmacol.,* **112,** 557 – 565.

KOJDA, G., PATZNER, M., HACKER, A. & NOACK, E. (1998) Nitric oxide inhibits vascular bioactivation of glyceryl trinitrate. A novel mechanism to explain preferential venodilation of organic nitrates. *Mol. Pharmacol.,* **53,** 547 – 554.

MARTIN, J. & MONCADA, S. (1988). Blood pressure, erythropoietin, and nitric oxide [letter]. *Lancet,* **1,** 644.

MILLER, B.A., SCADUTO, JR, R.C., TILLOTSON, D.L., BOTTI, J.J. & CHEUNG, J.Y. (1988). Erythropoietin stimulates a rise in intracellular free calcium concentration in single early human erythroid precursors. *J. Clin. Invest.,* **82,** 309 – 315.

MILLER, V.M., AARHUS, L.L. & VANHOUTTE, P.M. (1986). Modulation of endothelium-dependent responses by chronic alterations of blood flow. *Am. J. Physiol.,* **251,** H520 – H527.

MIURA, H., LIU, Y. & GUTTERMAN, D.D. (1999). Human coronary arteriolar dilation to bradykinin depends on membrane hyperpolarization: contribution of nitric oxide and Ca^{2+}-activated K^+ channels. *Circulation,* **99,** 3132 – 3138.

MOMBOULI, J.V. & VANHOUTTE, P.M. (1999). Endothelial dysfunction: from physiology to therapy. *J. Mol. Cell. Cardiol.,* **31,** 61 – 74.

MONCADA, S., PALMER, R.M. & HIGGS, E.A. (1991a). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.,* **43,** 109 – 142.

MONCADA, S., REES, D.D., SCHULZ, R. & PALMER, R.M. (1991b). Development and mechanism of a specific supersensitivity to nitrovasodilators after inhibition of vascular nitric oxide synthesis in vivo. *Proc. Natl. Acad. Sci. U.S.A.,* **88,** 2166 – 2170.

NAGAO, T., ILLIANO, S. & VANHOUTTE, P.M. (1992). Heterogeneous distribution of endothelium-dependent relaxations resistant to N^G-nitro-L-arginine in rats. *Am. J. Physiol.,* **263,** H1090 – H1094.

NAGAO, T. & VANHOUTTE, P.M. (1993). Endothelium-derived hyperpolarizing factor and endothelium-dependent relaxations. *Am. J. Respir. Cell. Mol. Biol.,* **8,** 1 – 6.

NEFF, M.S., KIM, K.E., PERSOFF, M., ONESTI, G. & SWARTZ, C. (1971). Hemodynamics of uremic anemia. *Circulation,* **43,** 876 – 883.

NEUSSER, M., TEPEL, M. & ZIDEK, W. (1993). Erythropoietin increases cytosolic free calcium concentration in vascular smooth muscle cells. *Cardiovasc. Res.,* **27,** 1233 – 1236.

NI, Z., WANG, X.Q. & VAZIRI, N.D. (1998). Nitric oxide metabolism in erythropoietin-induced hypertension: effect of calcium channel blockade. *Hypertension,* **32,** 724 – 729.

NORIS, M., MORIGI, M., DONADELLI, R., AIELLO, S., FOPPOLO, M., TODESCHINI, M., ORISIO, S., REMUZZI, G. & REMUZZI, A. (1995). Nitric oxide synthesis by cultured endothelial cells is modulated by flow conditions. *Circ. Res.,* **76,** 536 – 543.

PARKINGTON, H.C., TARE, M., TONTA, M.A. & COLEMAN, H.A. (1993). Stretch revealed three components in the hyperpolarization of guinea-pig coronary artery in response to acetylcholine. *J. Physiol. Lond,* **465,** 459 – 476.

RAINE, A.E. (1988). Hypertension, blood viscosity, and cardiovascular morbidity in renal failure: implications of erythropoietin therapy. *Lancet,* **1,** 97 – 100.

SCHAEFER, R.M., LESCHKE, M., STRAUER, B.E. & HEIDLAND, A. (1988). Blood rheology and hypertension in hemodialysis patients treated with erythropoietin. *Am. J. Nephrol.,* **8,** 449 – 453.

SESSA, W.C., PRITCHARD, K., SEYEDI, N., WANG, J. & HINTZE, T.H. (1994). Chronic exercise in dogs increases coronary vascular nitric oxide production and endothelial cell nitric oxide synthase gene expression. *Circ. Res.,* **74,** 349 – 353.

SHIMOKAWA, H., YASUTAKE, H., FUJII, K., OWADA, M.K., NAKAIKE, R., FUKUMOTO, Y., TAKAYANAGI, T., NAGAO, T., EGASHIRA, K., FUJISHIMA, M. & TAKESHITA, A. (1996). The importance of the hyperpolarizing mechanism increases as the vessel size decreases in endothelium-dependent relaxations in rat mesenteric circulation. *J. Cardiovasc. Pharmacol.,* **28,** 703 – 711.

TADDEI, S., VIRDIS, A., MATTEI, P., ARZILLI, F. & SALVETTI, A. (1992). Endothelium-dependent forearm vasodilation is reduced in normotensive subjects with familial history of hypertension. *J. Cardiovasc. Pharmacol.,* **20,** S193 – S195.

VALLANCE, P., LEONE, A., CALVER, A., COLLIER, J. & MONCADA, S. (1992). Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. *Lancet,* **339,** 572 – 575.

VAZIRI, N.D., ZHOU, X.J., SMITH, J., OVEISI, F., BALDWIN, K. & PURDY, R.E. (1995). In vivo and in vitro pressor effects of erythropoietin in rats. *Am. J. Physiol.,* **269,** F838 – F845.

VOGEL, V., KRAMER, H.J., BACKER, A., MEYER LEHNERT, H., JELKMANN, W. & FANDREY, J. (1997). Effects of erythropoietin on endothelin-1 synthesis and the cellular calcium messenger system in vascular endothelial cells. *Am. J. Hypertens.,* **10,** 289 – 296.

WANG, J., WOLIN, M.S. & HINTZE, T.H. (1993). Chronic exercise enhances endothelium-mediated dilation of epicardial coronary artery in conscious dogs. *Circ. Res.,* **73,** 829 – 838.

WANG, X.Q. & VAZIRI, N.D. (1999). Erythropoietin depresses nitric oxide synthase expression by human endothelial cells. *Hypertension,* **33,** 894 – 899.

WILCOX, C.S., DENG, X., DOLL, A.H., SNELLEN, H. & WELCH, W.J. (1993). Nitric oxide mediates renal vasodilation during erythropoietin-induced polycythemia. *Kidney Int.,* **44,** 430 – 435.

YAMAJI, R., OKADA, T., MORIYA, M., NAITO, M., TSURUO, T., MIYATAKE, K. & NAKANO, Y. (1996). Brain capillary endothelial cells express two forms of erythropoietin receptor mRNA. *Eur. J. Biochem.,* **239,** 494 – 500.

ZHANG, P., OHARA, A., MASHIMO, T., SUN, J., SHIBUTA, S., TAKADA, K., KOSAKA, H., TERADA, M. & YOSHIYA, I. (1996). Cardiovascular effects of an ultra-short-acting nitric oxide-releasing compound, zwitterionic diamine/NO adduct, in dogs. *Circulation,* **94,** 2235 – 2240.

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