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Recombinant human erythropoietin (rHuEPO) increases endothelin-1 release by endothelial cells

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Recombinant human erythropoietin (rHuEPO) increases endothelin-1 release by endothelial cells. Hypertension is a major complication of rHuEPO therapy in hemodialysis (HD) patients. We have previously reported that patients receiving rHuEPO intravenously (i.v.) had higher mean arterial pressure (MAP) and plasma endothelin-1 (ET-1) levels than those in which the hormone was administered subcutaneously (s.c.). To test whether the increased serum ET-1 levels associated with i.v. rHuEPO administration are the result of a direct effect of the hormone on ET-1 release by the endothelial cells (EC), we examined the effects of rHuEPO *in vitro*. Bovine pulmonary artery endothelial cells (BPAEC) were exposed to doses of rHuEPO of 0.8; 1.6; 3.3 and 6.6 U/ml. A 24 hour-time course showed maximal ET-1 production at 12 hours for all the doses tested. A significant increase in cell proliferation over controls was observed at 24 hours, for all rHuEPO doses, and no correlation was found between ET-1 values and cell proliferation. Inhibition of protein synthesis by cycloheximide (10 μg/ml) abolished the stimulation of ET-1 release by rHuEPO. Thrombin (4 U/ml) and angiotensin II (10^{-7} M), two potent stimulators of ET-1 release, had additive effects to those of rHuEPO. Specific thrombin and angiotensin II antagonists blocked these additive effects, reducing ET-1 release to the level of rHuEPO stimulation alone. In summary, rHuEPO stimulates vascular EC in culture to increase ET-1 release through an increase in synthesis and in a time dependent fashion. The routes of stimulation seem to differ from other known ET-1 secretogoges. Our data also confirm a significant mitogenic effect of rHuEPO on the endothelial cell.

Within three years of its introduction in clinical medicine, recombinant human erythropoietin (rHuEPO) has gained wide acceptance for the treatment of the anemia of chronic renal failure [1, 2]. Application of this hormone to other anemias unrelated to renal disease is being actively pursued [3–5]. Hypertension is the major complication of rHuEPO therapy arising in at least a third of the hemodialysis (HD) population treated with the hormone [1, 2, 6, 7]. Other complications such as vascular thrombotic events have also been observed [8]. Mechanisms proposed to explain the pathogenesis of rHuEPO-induced hypertension are based on an increase in peripheral vascular resistance. They include increases in blood viscosity [9, 10], increments in cytosolic calcium in vascular smooth muscle and platelets [11, 12], and a direct pressor effect [13]. However, none of these proposed mechanisms have been conclusively demonstrated.

Endothelin (ET-1), a naturally occurring vasoconstrictive peptide, has been purified from vascular endothelial cells (EC) in culture [14, 15]. In animal models, ET-1 infusion results in sustained elevations in the mean arterial pressure (MAP) [16, 17]. We have previously demonstrated that plasma ET-1 levels were higher in HD patients treated with rHuEPO intravenously (i.v.) than in patients treated with rHuEPO subcutaneously (s.c.) [18]. Only the patients treated with i.v. rHuEPO had a significant correlation between ET-1 plasma levels and MAP. We conclude that the administration route may be critical in the rHuEPO-associated hypertension.

The identification of a 45 kDa erythropoietin (EPO) receptor [19] in human umbilical endothelial cell line (HUVEC) suggests that EPO interacts with other cell types in addition to erythroid progenitor cells [20–22]. The vascular endothelium may be another target for the action of the hormone. In order to clarify whether serum ET-1 levels depend on the rHuEPO administration route and whether rHuEPO had direct actions on ET-1 release by EC, we examined ET-1 release from bovine pulmonary artery endothelial cells (BPAEC) stimulated by rHuEPO.

Methods

Endothelial cell culture

BPAEC from American Type Tissue Culture Collection (ATCC, CCL-209, Rockville, Maryland, USA) were grown in Minimum Essential Media Eagle (MEM; Sigma Chemical Co., St. Louis, Missouri, USA) supplemented with 20% fetal bovine serum, antibiotic free. The cells were incubated at 37°C in a humidified 5% CO_2: 95% air atmosphere. The cells were passaged weekly at a split ratio of 1:3 using the trypsin/EDTA method. Confluent, monolayer cultures between the 18th to 24th passage were used. The cells were plated into 6 well clusters (Costar Corp., Cambridge, Massachusetts, USA) at a density of 3×10^4. The media were changed twice per week. On day five, 24 hours prior to the experiment, when over 80% cell confluency was achieved, the media were removed and 3 ml of serum-free media (SFM) were added to each well.

Experimental groups

The SFM was removed and the cells were washed twice with phosphate buffer saline (PBS) (pH 7.4), and reincubated (37°C)

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Table 1. Time course and dose response for the effect of rHuEPO on ET-1 production

Doses	Endothelin-1 pg/μg DNA			
	1 hr	4 hr	12 hr	24 hr
Control	2.4 ± 0.7	2.8 ± 0.6	17.7 ± 2.2	19.0 ± 2.3
0.8 U/ml	2.9 ± 0.2	9.2 ± 0.5[a]	47.3 ± 0.4[a]	36.4 ± 1.4[b]
1.6 U/ml	2.9 ± 0.4	24.6 ± 3.5[b]	51.9 ± 12.6[a]	52.2 ± 1.1[b]
3.3 U/ml	2.5 ± 0.1	18.9 ± 0.6[c]	58.4 ± 8.6[b]	39.0 ± 1.9[b]
6.6 U/ml	7.4 ± 1.9[a]	19.3 ± 1.1[c]	45.2 ± 2.0[a]	37.8 ± 0.7[b]

BPAEC were incubated in serum-free media (MEM) with 0.8; 1.6; 3.3 and 6.6 U/ml rHuEPO for 1; 4; 12 and 24 hours. Control cells were exposed to rHuEPO vehicle alone. ET-1 levels are expressed as pg of ET-1/μg of DNA. Results represent mean ± SD of three experiments performed in duplicate. Each ET-1 level was compared with its respective time point control.

[a] $P < 0.05$

[b] $P < 0.001$

[c] $P < 0.01$

with 3 ml SFM and the various reagents detailed below. For time-course experiments, the cells were exposed to rHuEPO concentrations of 0.8, 1.6, 3.3, and 6.6 U/ml Epoetin Alfa (Amgen, Inc., Thousand Oaks, California, USA) for one, four, 12, and 24 hours.

In the other experiments, cells were exposed for four hours to a rHuEPO dose of 3.3 U/ml and to the following reagents (from Sigma Chemical Co.): cycloheximide: 10 μg/ml; thrombin (Human): 4 U/ml; recombinant hirudin: 0.1 μg/ml; angiotensin II: 10^{-7} M; and [Sar^1 Ala^8]-Ang II: 10^{-6} M. Control wells were incubated with rHuEPO vehicle alone.

Endothelin RIA

After incubation, the media were aspirated and centrifuged at 2000 rpm for 10 minutes. Supernatant was then stored at −70°C. ET-1 was extracted using Sep-Pak C_{18} cartridges (Water Assoc., Milford, Massachusetts, USA) and ET-1 determinations were performed utilizing the ET-1 radioimmunoassay (RIA) kit (Peninsula Lab, Inc., Belmont, California, USA). The recovery capability of the assay is 85% with a 100% specificity for ET-1.

DNA assay

Wells were washed twice with 2 ml acetonitrile-water (1:1), and the cells were then scraped and pooled. Samples were vortexed for 30 seconds and centrifuged at 1500 g for 15 minutes. Total DNA in each well was assayed in the pellet using the ethidium bromide method [23]. ET-1 levels were corrected for the DNA content of the well.

Statistics

Values are expressed as the mean ± standard deviation. Analysis of variance (ANOVA) by Instat (GraphPAD Software, Inc., USA) was used to compare differences between experimental groups.

Results

Effects of rHuEPO on ET-1 release

Table 1 depicts ET-1 release by BPAEC during a 24 hour period of treatment with varying rHuEPO concentrations from 0.0 to 6.6 U/ml. A time-dependent increase in ET-1 production was observed in control and treated cells. Maximal levels of ET-1 release were observed at 12 hours in all the conditions tested.

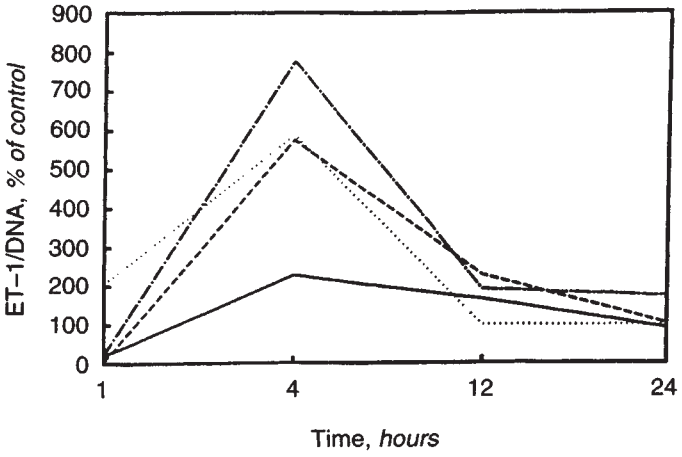

Fig. 1. *Percent increase of ET-1 levels over control.* BPAEC were incubated in serum-free media (MEM) for 1; 4; 12 and 24 hours with 0.8 (——); 1.6 (· – · – ·); 3.3 (– – – –); and 6.6 (·····) U/ml rHuEPO, respectively.

Figure 1 depicts the percent increase of ET-1 release over control levels. At all doses, maximal rHuEPO stimulation was observed at four hours. At the highest dose of 6.6 U/ml, there was a 210% increase in one hour. After 24 hours exposure, the increase in ET-1 levels above control (86 to 167%) was not statistically significant for the different doses except for the 1.6 U/well ($P < 0.01$).

To assess whether ET-1 stimulation by rHuEPO is produced through new protein synthesis, we tested the effect of cyclohexamide, a protein synthesis inhibitor. As demonstrated in Figure 2, incubation of EC with 3.3 U/ml rHuEPO and 10 μg/ml cycloheximide (a dose which blocked ^{3}H-leucine incorporation) failed to stimulate ET-1 release above control values. This demonstrated that the stimulation of ET-1 release by rHuEPO was dependent on new protein synthesis.

To further identify the mechanisms by which rHuEPO stimulates ET-1 secretion, we studied the effects of angiotensin II and thrombin. A dose-response for the effect of angiotensin II (from 10^{-5} to 10^{-8} M) on ET-1 release showed maximal stimulation at the 10^{-7} M dose (data not shown). After a four hour incubation, both angiotensin II (10^{-7} M) and rHuEPO (3.3 U/ml) significantly stimulated ET-1 release over controls (Fig. 3). Simultaneous incubation of rHuEPO with angiotensin II revealed an additive effect. When the angiotensin II receptor antagonist [Sar^1-Ala^8]-Ang II was introduced, it completely abolished the effects of angiotensin II without alterations in the rHuEPO-promoted ET-1 secretion. Using a similar experimental design, thrombin was utilized as a co-stimulator of ET-1 release by rHuEPO. Increasing thrombin concentrations from 3 U/ml to 5 U/ml revealed maximal ET-1 stimulation at 4 U/ml. Figure 4 demonstrates an additive thrombin effect on rHuEPO stimulation which was blocked by hirudin, a specific thrombin inhibitor, which does not affect the response to rHuEPO.

Effects of rHuEPO on cell proliferation

rHuEPO effects on ET-1 release could have been mediated by an increase in cell number. Thus DNA levels in the 6-well

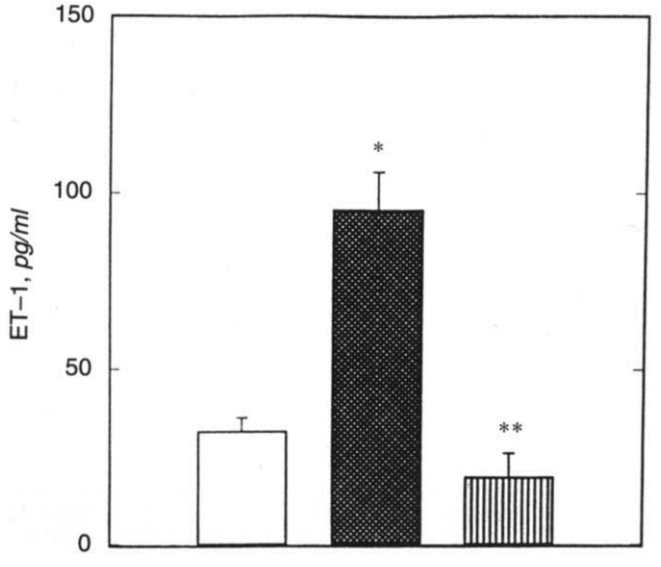

Fig. 2. *Effect of cycloheximide on rHuEPO stimulation of ET-1 release.* BPAEC were incubated in serum-free media (MEM) for 4 hours with rHuEPO vehicle control (□); 3.3 U/ml rHuEPO (▩); 3.3 U/ml rHuEPO and 10 μg/ml cycloheximide (▥). Bar and error bars represent the mean ± SD of three experiments performed in duplicate. *$P < 0.01$ control vs. rHuEPO; **$P < 0.001$ rHuEPO vs. rHuEPO + cycloheximide.

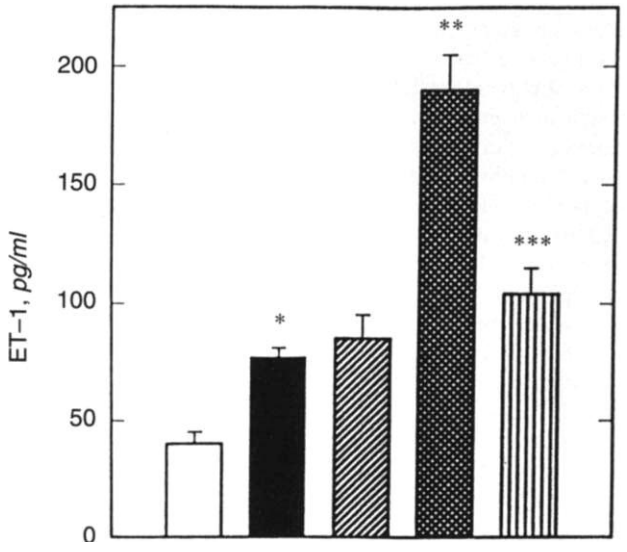

Fig. 3. *Effect of angiotensin II and [Sar¹-Ala⁸]-ANG II on rHuEPO stimulation of ET-1 release.* BPAEC were incubated in serum-free media (MEM) for 4 hours with rHuEPO vehicle control (□); 3.3 U/ml rHuEPO (■); 10^{-7} M angiotensin II (▨); 3.3 U/ml rHuEPO + 10^{-7} M angiotensin II (▩); 3.3 U/ml rHuEPO + 10^{-7} M angiotensin II + 10^{-6} M [Sar¹-Ala⁸]-ANG II (▥). Bar and error bars represent mean ± SD of three experiments performed in duplicate. *$P < 0.05$ control vs. rHuEPO; **$P < 0.001$ rHuEPO vs. rHuEPO + angiotensin II; ***$P < 0.01$ control vs rHuEPO + angiotensin II + [Sar¹-Ala⁸]-Ang II.

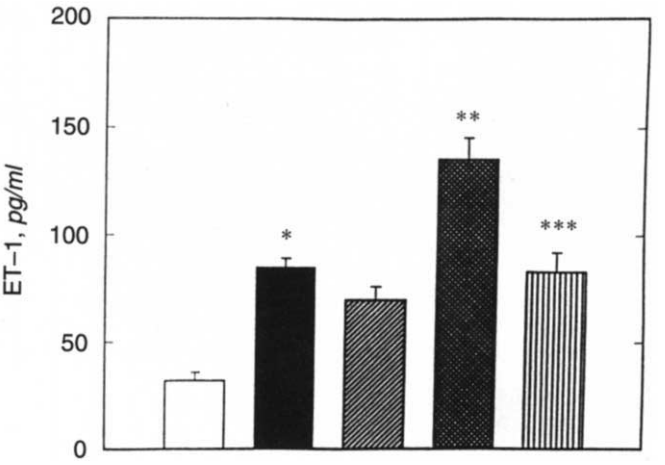

Fig. 4. *Effect of thrombin and hirudin on rHuEPO stimulation of ET-1 release.* BPAEC were incubated in serum-free media (MEM) for 4 hours with rHuEPO vehicle control (□); 3.3 U/ml rHuEPO (■); 4 U/ml thrombin (▨); 3.3 U/ml rHuEPO + 4 U/ml thrombin (▩); 3.3 U/ml rHuEPO + 4 U/ml thrombin + 0.1 μg/ml hirudin (▥). Bar and error bars represent the mean ± SD of three experiments performed in duplicate. *$P < 0.01$ control vs. rHuEPO; **$P < 0.01$ rHuEPO vs. rHuEPO + thrombin; ***$P < 0.01$ control vs. rHuEPO + thrombin + hirudin.

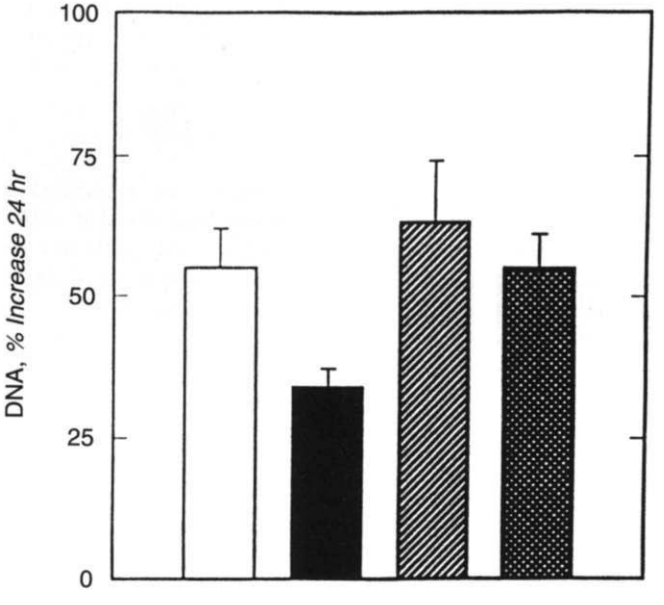

Fig. 5. *Effect of rHuEPO on cell proliferation.* BPAEC were incubated in serum-free media (MEM) for 24 hours with 0.8 (□); 1.6 (■); 3.3 (▨) and 6.6 (▩) U/ml of rHuEPO. DNA is expressed as % increase over controls. Bar and error bars represent the mean ± SD of three experiments performed in duplicate.

cluster plates were measured. We observed that after 12 hours, rHuEPO began to increase the BPAEC number at 6.6 U/ml (data not shown). After 24 hours exposure, all rHuEPO doses stimulated cell proliferation 35 to 60% above controls (Fig. 5) with the highest dose, 6.6 U/ml, exerting a significant proliferative stimulus by 12 hours (not shown). There was no correlation between ET-1 and DNA levels.

Discussion

This study demonstrates the rHuEPO effect on ET-1 release by BPAEC. We found that rHuEPO has a direct stimulatory effect on ET-1 release which was time-dependent. Stimulation of ET-1 release by rHuEPO reached a plateau at 12 hours. This

could be due to a decrease in cell receptors as a result of cell confluency. A decrease in receptor binding with increments in cell density has been described for several other growth factors [24]. The inhibitory effect of cycloheximide on ET-1 release in response to rHuEPO suggests that the hormone stimulates *de novo* ET-1 synthesis. We have confirmed the known effects of angiotensin II and thrombin-stimulated ET-1 release by BPAEC [25–27], and also demonstrated that both compounds have a synergistic effect with rHuEPO on ET-1 release. rHuEPO co-incubation with specific inhibitors or antagonists of both compounds did not interfere with rHuEPO effects. This makes it unlikely that stimulation of ET-1 release by rHuEPO is mediated by either angiotensin II or thrombin.

The ability of rHuEPO to increase ET-1 release beyond the maximal effect of the two potent stimulants, angiotensin II and thrombin, suggests that the hormone promotes ET-1 release through a different pathway. Preliminary studies in our laboratory seem to indicate the calcium dependency of rHuEPO stimulation on ET-1 release (data not shown).

It is well known that rHuEPO interacts with its target cells through specific surface receptors. Until recently, it was thought that their expression was limited to erythroid precursors [20–22]. Anagnostou et al [19] have described a novel rHuEPO receptor on HUVEC with a molecular weight different from the ones expressed in cells in which this hormone exerts a differentiation/maturation effect. They also reported a dose-dependent increase in cell proliferation after a seven day incubation, demonstrating for the first time the mitogenic effect of rHuEPO on non-hematopoietic cells. In our short-term incubation studies (24 hours), we have also observed an average 50% increase in cell proliferation over controls, although dose-dependency was not seen over that time period. It remains to be clarified whether the mitogenic rHuEPO effects on BPAEC are a direct action of the hormone, as seen in the erythroid progenitors, or are mediated by the release of ET-1 acting in an autocrine fashion. It is possible that both rHuEPO and ET-1 act synergistically promoting mitogenesis.

The possible chronic ET-1 effects in vascular remodeling and regulation of cell proliferation have yet to be explored. ET-1 is a powerful mitogen for cultured vascular smooth muscle cell [28] and glomerular mesangial cells [29]. Its effects are mediated through an increase of the protooncogenes, c-*fos* and c-*myc* mRNA levels [30]. Indeed, chronically enhanced local production by the macro- and microvascular endothelium may lead to vascular smooth muscle hyperplasia and hypertrophy, potentially leading to the acceleration of atherosclerosis. Clinical studies that correlate atherosclerotic sites and ET-1 levels are suggestive of this hypothesis [31].

Hypertension and hypercoagulability have been recognized as complications of rHuEPO therapy in HD patients [1, 2, 6–8]. Until now, no single cause-effect relationship has been found to explain this problem. Although the pathophysiological role of endothelin remains unclear, high ET-1 levels have been described in essential hypertension [32]. Uremia has also been found to increase ET-1 levels [33–36].

Earlier we had also shown that HD patients receiving i.v. rHuEPO had a significant correlation between ET-1 plasma levels and MAP [18]. rHuEPO i.v. administration results in very high serum levels of the hormone [37, 38], exposing the vascular endothelium to supraphysiological rHuEPO concentrations. Such high rHuEPO plasma levels could induce ET-1 release by EC.

In summary, the present work provides an *in vitro* explanation for the vascular and hypertensive effects of pharmacological rHuEPO administration.

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