Acute Stimulatory Effect of Erythropoietin on Nitric Oxide Production in Human Aortic Endothelial Cells

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ABSTRACT

A possible role of nitric oxide (NO), an endothelium-derived relaxing factor, in erythropoietin (EPO)-induced hypertension has been implicated. In the present study, we investigated the direct effect of recombinant human erythropoietin (rHuEPO) on NO release in human aortic endothelial cells *in vitro*. Treatment for 4 h with rHuEPO at 10^{-8} to 10^{-7} M increased NO_2^- concentration in the incubation medium of the endothelial cells. The amount of NO synthase (NOS) in the cell lysate determined by the maximal citrulline-generating activity was not changed by rHuEPO. The effect of rHuEPO to stimulate NO release was abolished by anti-EPO antibody. In addition, the effect was inhibited by an intracellular Ca^{2+} -chelating agent, BAPTA-AM. These findings suggest that EPO acutely activates NOS through an increase in intracellular Ca^{2+} level and consequently enhances NO production in the endothelial cells.

Prolonged treatment with recombinant human erythropoietin (rHuEPO) induces hypertension or exacerbate the preexisting hypertension in patients with chronic renal failure (4, 23). The effects have been attributed to an increased blood viscosity associated with a rise in the hematocrit (13), a loss of hypoxic vasodilatation (10) and a direct vasopressor effect of rHuEPO (15). Involvement of such bioactive factors as endothelin (2), prostaglandin (2) and renin-angiotensin (6) has been also suggested. In addition, a possible role of nitric oxide (NO), an endothelium-derived relaxing factor (7), in EPO-induced hypertension has been implicated (5, 12, 21, 22). It was suggested that enhanced generation of NO might prevent hypertension and mediate renal vasodilatation in rats with rHuEPO-induced erythrocytosis (22).

However, the effect of EPO on endothelial

NOS (eNOS) activity and its mechanisms involved remain to be fully elucidated. In the present study, we investigated the possible direct effect of rHuEPO on NO release in human aortic endothelial cells *in vitro*. The involvement of intracellular Ca^{2+} was also studied in a use of an intracellular Ca^{2+} -chelating agent.

MATERIALS AND METHODS

Drugs

rHuEPO and polyclonal anti-EPO antibody were generously provided from Chugai Pharmaceutical Inc. (Tokyo, Japan). BAPTA-AM was from Molecular Probes Inc., Eugene, OR, U.S.A.; Superoxide dismutase (SOD) from bovine liver was from Funakoshi, Tokyo, Japan; Carbacol (CCH) was from Nacalai, Kyoto, Japan. $\text{L-}[^{14}\text{C}]$ citrulline was purchased from New England Nuclear, Boston, MA, U.S.A.. All the other chemicals are of the purest grade available from regular commercial sources.

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Human aortic endothelial cells

Human aortic endothelial cells (Clonetics, San Diego, CA, U.S.A.) were maintained in modified MCDB131 medium supplemented with 2 % fetal calf serum, 12 mg/L bovine brain extract, 1 mg/L hydrocortisone, 10 μ g/L human epidermal growth factor, 50 mg/L gentamycin and 50 μ g/L amphotericin B in a humidified atmosphere at 37°C under 5 % CO₂-95 % air. After 4 to 7 passages, the cells were subcultured into poly-L-lysine coated 24-well plates and submitted to static experiments. The cells were washed three times and preincubated for 30 min in Dulbecco's modified Eagle's medium (DMEM). Then the cells were incubated in fresh DMEM containing CCH, rHuEPO, and anti-EPO antibody for 4 h. In a set of experiments, the cells were preincubated with 2 μ M BAPTA-AM for 90 min. BAPTA-AM were first dissolved in dimethylsulfoxide (DMSO) and diluted with DMEM. The final concentration of DMSO was 0.1 %. The control cells were treated with 0.1 % DMSO. The medium was collected and centrifuged at 10,000 rpm for 10 min. The resulting supernatant was stored at -20°C until assayed for NO₂⁻ and NO₃⁻.

Determination of medium NO₂⁻ and NO₃⁻ levels

NO₂⁻ and NO₃⁻ concentrations in the medium were determined as previously described (9), using a reverse phase HPLC system combined with a cadmium-reducing column, reaction with Griess reagent and a spectrophotometer (NOD-10, Eicom, Kyoto, Japan). Briefly, standard preparations containing NO₂⁻ and NO₃⁻ (0.024 to 1.563 μ M) in DMEM and samples (50 μ L) were injected by an autosampler (AS2000, Hitachi, Tokyo, Japan). The standard preparations and the samples were kept at 4°C throughout the measurement. NO₂⁻ and NO₃⁻ were separated by a 4.6 $\times$ 50 mm polystyrene polymer column. Mobile phase was 0.15 M NaCl, 0.15 M NH₄Cl, 10 % methanol containing 0.5 g/L EDTA disodium salt (flow rate: 300 μ L/min). After separation, NO₃⁻ was converted to NO₂⁻ through a reduction column packed with fine particles of copper-plated cadmium. The effluent was reacted with Griess reagent (5 g/L sulfanilamide, 0.25 g/L naphthylethylenediamine, 1.25 % HCl) (flow rate: 100 μ L/min), and absorbance at the wavelength of 540 nm was detected by a flow-through spectrophotometer.

The cells were washed with phosphate buffered saline (PBS, pH 7.4) and collected with 0.5 mL of 50 mM Tris•HCl (pH 7.4) containing 1 mM dithiothreitol, 1 mM EDTA, 10 μ g/mL leupeptin, 10 μ g/mL aprotinin and 1 mM phenylmethylsulfonyl fluoride. After sonication, cells were centrifuged at 100,000 $\times g$ for 30 min at 4°C. Resulting supernatant was stored at -80°C until assayed for NOS activity.

Assay of NOS activity

NOS activity was measured by production of L-[³H] citrulline from L-[³H] arginine. Supernatant (50 μ L) of cell lysate was incubated at 37°C for 20 min in the presence of 50 mM Tris • Cl (pH 7.5), 1 mM NADPH, 10 μ M flavin adenine dinucleotide, 10 μ M tetrahydrobiopterin, 40 μ M L-arginine, 3.7 MBq of L-[³H] arginine and 4 mM CaCl₂ in a total volume of 100 μ L. The reaction was halted by the addition of 1.25 mL of ice-cold 20 mM HEPES (pH 5.5), and the total volume was applied on a Dowex-50W $\times$ 8 (200-400 mesh, Na⁺ form) column preequilibrated with 20 mM HEPES (pH 5.5). The flow-through and 2 mL wash of distilled water were collected and the radioactivity was measured by a liquid scintillation counter. The recovery rate of citrulline determined by applying L-[¹⁴C] citrulline solution were 80-90 %. The L-[³H] citrulline concentration produced was corrected by the recovery rate.

Protein content

Protein content was measured using Bio-Rad protein assay with bovine serum albumin as the standard (3).

Statistics

All the results were expressed as means $\pm$ SEM of 4-8 different experiments. The statistical difference was analyzed by ANOVA and Fisher's test. A *P* value less than 0.05 was considered significant.

RESULTS

As shown in Fig. 1, CCH (10⁻⁵ M) increased medium NO₂⁻ concentrations in human aortic endothelial cells. Treatment with rHuEPO (10⁻⁸ and 10⁻⁷ M) elicited a dose-related increase in

Fig. 1 Effect of recombinant human erythropoietin (rHuEPO) and carbacol (CCH) on medium NO₂⁻ accumulation in human aortic endothelial cells. The cells were incubated for 4 h in the presence of rHuEPO (10⁻⁸ and 10⁻⁷ M) or CCH (10⁻⁵ M). Medium NO₂⁻ levels were determined by HPLC. Mean ± SE values are shown. *: $P < 0.05$, **: $P < 0.01$ vs. control.

Fig. 2 Effect of pretreatment with BAPTA-AM on rHuEPO-induced NO₂⁻ accumulation in human aortic endothelial cells. The cells were preincubated for 90 min in the presence or absence of 2 μM BAPTA-AM, then incubated for 4 h with or without rHuEPO (10⁻⁷ M). *: $P < 0.05$ vs control. #: $P < 0.05$ vs BAPTA-AM (-).

medium NO₂⁻ levels. Medium NO₃⁻ levels were not detectable in all the samples. The NOS activ-

ities in the cell lysates obtained after 4 h incubation with rHuEPO (10⁻⁷ M) (14.03 ± 1.63 nmol/mg protein/10 min, $n=4$) were not different from control (13.40 ± 0.68 nmol/mg protein/10 min, $n=4$).

The NO-producing activity of rHuEPO (10⁻⁷ M) was inhibited in the presence of anti-EPO antibody (130 ± 16 % of basal vs. rHuEPO alone: 380 ± 53 % of basal, $n=6$, $P < 0.01$). As shown in Fig. 2, pretreatment with 2 μM BAPTA-AM to chelate intracellular Ca²⁺ inhibited the rHuEPO (10⁻⁷ M)-induced NO production.

DISCUSSION

Several lines of evidence suggest that EPO stimulates NO synthesis *in vivo*. It was reported that urinary NO₂ + NO₃ excretion was increased after the treatment with rHuEPO in rats (5, 17). Urinary cGMP excretion, a parameter of NO action, was also increased in EPO-treated animals (22). The mechanisms mediating the *in vivo* effect of EPO to stimulate NO synthesis remain to be fully elucidated. It was suggested that a rise in hematocrit induced by rHuEPO could increase blood viscosity and the vessel wall shear stress (22). The shear stress might stimulate the expression of NOS (14) and the release of NO (8). However, there have been some reports speaking against above explanation. Ni *et al.* (11) demonstrated in uremic rats that EPO-induced hypertension was independent on hematocrit or erythrocyte mass. Vaziri *et al.* (19) found a rise in plasma cGMP levels after a single injection of rHuEPO in rats. In the present study, we first found that rHuEPO raised NO₂⁻ concentration in the culture medium of human aortic endothelial cells, NO₂⁻ is a major metabolite of NO in serum-free aqueous solution (18). The stimulating effect of rHuEPO on NO release was inhibited by anti-EPO antibody, suggesting a specific action of EPO. Our present observation provides the first evidence that EPO directly stimulates NO production in endothelial cells. Expression of functional EPO receptor in human endothelial cells (1) supports our present observation.

There have been conflicting reports on the effect of rHuEPO on endothelial NOS (eNOS) expression. Ongil *et al.* (12) reported that rHuEPO did not modify eNOS mRNA expression in cultured bovine aortic endothelial cells. Wang and Vaziri (21) found a decrease in eNOS protein level in human endothelial cells treated with

rHuEPO for 24 h. In this study, we clearly demonstrated that a short-term treatment with rHuEPO increased NO production without changing the NOS amount determined by the maximal citrulline-generating activity in human aortic endothelial cells. Thus EPO, by acutely increasing the enzyme activity and chronically decreasing the protein abundance of NOS, might dually modulate NO production and might participate in complicated mechanisms in the regulation of vascular function.

In this study, the acute effect of rHuEPO to increase NO synthesis was inhibited by BAPTA-AM, an intracellular Ca^{2+} -chelating agent. Indeed Vogel *et al.* (20) demonstrated that rHuEPO raised $[\text{Ca}^{2+}]_i$ in porcine endothelial cells. The eNOS activity depends on Ca^{2+} and calmodulin, and insulin (24) and IGF-I (16) are known to activate eNOS in the endothelial cells through an increase in $[\text{Ca}^{2+}]_i$. Thus an increase in $[\text{Ca}^{2+}]_i$ is the common pathway to activate eNOS for EPO and other hormones or growth factors. Interestingly, Wang *et al.* (21) reported that a sustained elevation of $[\text{Ca}^{2+}]_i$ induced by rHuEPO depressed eNOS expression, which was restored by a Ca channel blocker. Thus intracellular Ca^{2+} might be involved in the actions of rHuEPO to modulate both eNOS activity and expression.

In conclusion, our present findings suggest that EPO acutely activates eNOS through an increase in $[\text{Ca}^{2+}]_i$ and consequently enhances NO production in the endothelial cells.

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