Human recombinant erythropoietin inhibits interleukin-1β-stimulated nitric oxide and cyclic guanosine monophosphate production in cultured rat vascular smooth-muscle cells

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Abstract

Background. Recently rat vascular smooth-muscle cells (VSMC) have been shown to possess Epo receptor, and respond to various cytokines for producing nitric oxide (NO). In the present study we examined the effect of pharmacological dose of human recombinant erythropoietin (rHuEpo) on the IL-1β-induced NO and cGMP production as well as inducible nitric oxide synthase (iNOS) in cultured rat VSMC.

Methods. Nitrite, a stable metabolite of NO, and intracellular cGMP contents were assayed by Griess method and enzyme immunoassay. iNOS mRNA expression was analysed by Northern blotting.

Results. RHuEpo inhibited IL-1β-induced nitrite production in a dose- and time-dependent manner with concomitant changes of intracellular cGMP contents. On the other hand, rHuEpo did not inhibit atrial natriuretic peptide- (ANP) or sodium nitroprusside-(SNP)-induced nitrite and cGMP production at all. While rHuEpo inhibited IL-1β-induced iNOS mRNA expression, rHuEpo vehicle did not affect IL-1β-induced iNOS mRNA expression.

Conclusions. It is suggested that a pharmacological dose of rHuEpo inhibits IL-1β-induced NO and cGMP production as well as iNOS mRNA expression, presumably via the Epo receptor.

Key words: cGMP; erythropoietin; erythropoietin receptor; interleukin-1β; nitric oxide; nitric oxide synthase; vascular smooth-muscle cells

Introduction

Recently, numerous studies have shown that nitric oxide (NO) plays several crucial roles in the regulation of blood pressure [1,2]. NO produced by endothelial nitric oxide synthase are thought to be important for the fine adjustments of blood pressure. However, overproduction of NO, as stimulated by bacterial endotoxins or cytokines, may cause the hypotension seen in sepsis [3,4]. NO activates guanylate cyclase in VSMC to generate intracellular cGMP, which causes subsequent muscle relaxation. On the other hand, VSMC themselves produce NO in response to cytokines such as interleukin-1β (IL-1β), tumour necrosis factor, interferon-γ, and lipopolysaccharide through the stimulation of inducible NO synthase [5,6].

Several hormones and autacoids have been found to modulate cytokine-induced NO production. Noradrenaline (NA) via β-receptor suppressed lipopolysaccharide-induced NO production and NO synthase induction [7]. Recently angiotensin II (AII) was shown to inhibit the cytokine-induced NO production via AII type 1 (AT_1) receptor through an activation of protein kinase C and increase of intracellular calcium concentration in cultured rat VSMC [8]. Endothelin (ET) was also shown to inhibit cytokine-stimulated NO production via ET type A (ET_A) receptor in cultured rat mesangial cells [9]. We recently demonstrated that AVP inhibits the IL-1β-induced NO and cGMP production via V₁ receptor in VSMC [10]. It was also recently reported that VSMC possessed Epo receptors and responded to Epo, resulting in an increase of cytosolic calcium concentration [11,12].

The present study was therefore undertaken to examine whether pharmacological doses of rHuEpo, presumably through the Epo receptor, may affect the IL-1β-stimulated NO and cGMP production as well as iNOS mRNA expression in VSMC.

Methods

Cell culture

VSMC were prepared from rat aorta of male Sprague–Dawley rats (150–200 g) as previously described.
In brief, cells were grown in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (Life Technologies, Tokyo, Japan), 100 U/ml penicillin, and 100 mg/ml streptomycin (Life Technology Inc. USA). Cells grown to confluence were detached by a treatment with 0.125% trypsin and 0.02% EDTA and resuspended in secondary cultures. Cells were used between passages 5 and 10. Cells were plated at 1–2 × 10^4 cells/ml in 24-well dishes (Falcon) in DMEM supplemented as described above and allowed to grow subconfluently for 48–72 h. They were then made ‘quiescent’ by a 24-h incubation in serum-free DMEM.

After being rinsed with phosphate-buffered saline (PBS) at pH 7.35, confluent cells were incubated for 24 h with serum-and phenol-red-free DMEM containing 10 ng/ml human recombinant IL-1β with exceptions of the time-course and dose-dependency studies. Agents such as Epo, Epo vehicle were added to the incubation medium concomitantly with IL-1β.

**Determination of nitrite**

Nitrite was measured by the method of Green et al. [14]. In cell culture systems, NO degrades rapidly to nitrite, but in the presence of Fe²⁺ haem, or certain other transition metals, nitrite is converted to the more stable product nitrate. In vivo nitrite seems to be unstable and has a short half-life [15]. However, we have already demonstrated that nitrite production was linearly correlated with nitrate production in cultured rat-VSMC [13]. Therefore we think that the measurement of nitrite may reflect the local generation of NO in the present experimental system. Briefly, assay samples were mixed with an equal volume of the Griess reagent (0.1% naphthylethylenediamine dihydrochloride and 1% sulphanilamide in 3% H₃PO₄) and incubated to yield a chromophore.

Since phenol red interfered with nitrite measurement in some degree, we used phenol-red-free DMEM for incubation. The absorbance at 540 nm was measured, and nitrite concentration was determined using a curve calibrated from sodium nitrite standards.

**Assay for iNOS mRNA**

Inducible NOS mRNA expression in VSMC was analysed by Northern blotting as reported previously [13]. Briefly, VSMC was grown to confluence on 100-mm dishes and lysed using Isogen® (Nippon Gene, Tokyo, Japan), which contains phenol and guanidine isothiocyanate. The lysate was extracted with chloroform/isopropanol, washed with 75% ethanol, and dissolved in 20 ml of diethylylpyrocarbonate (DEPC)-treated water. The RNA was quantitated by ultraviolet absorbance at 260 nm. Total RNA (30 mg) was fractionated on 1% denaturing agarose-formaldehyde gels and capillary blotted onto nylon membranes (Hybond N, Amersham, Bucks, UK) in 20 × standard saline citrate (contains 0.15 M NaCl and 0.0015 M sodium citrate, pH 7.0) overnight.

Filters were prehybridized for 30 min at 68°C before hybridization using Quick-Hyb® (Stratagene, La Jolla, CA, USA). Filters were then hybridized for 1 h at 68°C in the same solution with 10⁶ c.p.m/ml of [γ-³²P]ATP random primer-labelled inducible NO synthase probes from mice [16]. Filters were washed twice for 10 min at room temperature in 2 × standard saline citrate and 0.1% sodium dodecyl sulphate, followed by a 10-min wash at 45°C in 0.1 × standard saline citrate and 0.1% sodium dodecyl sulphate. The hybridized filters were then exposed to Kodak XAR film overnight at −70°C with one intensifying screen. Autoradiography was performed at −70°C and quantified by densitometric scanning (Immunomedica Image Analyser TIF-64).

Measurement of intracellular cGMP contents

cGMP contents were determined as reported previously [10]. Briefly, the phosphodiesterase inhibitor, isobutylmethylnithine (IBMX), was added to each well at a final concentration of 0.5 mM, immediately after measurements of nitrite. After incubation for 15 min at 95% air–5% CO₂ in a humidified incubator at 37°C, the medium was aspirated off and cells were immediately immersed in 0.2 ml of 0.1 N HCl to stop the reaction. Cells were collected into glass tubes with a rubber policeman, boiled for 3 min, and then centrifuged at 2500 g for 15 min at room temperature. The supernatants were decanted and 0.05 ml of 50 mM sodium acetate was added to each tube. These were kept at −30°C until assay for cGMP contents. The pellets were dissolved in 0.2 ml of 1% sodium dodecyl sulphate and kept at −30°C until assay for protein. Intracellular cGMP contents were determined using a cGMP enzyme immunoassay kit (Amersham) and normalized to protein content of each well. The lower limit of detection was 2 fmol/well. Protein content was measured by the method of Lowry [18].

**Materials**

IL-1β was purchased from Genzyme (Cambridge, MA, USA). rHuEpo (epoetin beta) and rHuEpo vehicle were generous gifts from Chugai Pharmaceutical Company (Tokyo, Japan). IBMX and SNP were purchased from Sigma (Tokyo, Japan). ANP was purchased from Peptide Institute Inc., Osaka, Japan. A mouse macrophage iNOS cDNA fragment (nucleotides 1621–2653) prepared by reverse transcription–polymerase chain reaction [16] was kindly donated by Dr Y. Kawahara (Kobe University, Kobe, Japan).

**Statistics**

The results are expressed as mean ± SEM (number of samples). Data were analysed by analysis of variance combined with Fisher’s PLSD. Differences with P < 0.05 were considered to be significant. Each figure is a representative one of at least three separate experiments except for Figure 4a and b.

**Results**

VSMC produced nitrite in response to 1.25–20 ng/ml IL-1β in a dose-dependent manner with a submaximum dose of 10 ng/ml. In the presence of 10 ng/ml of IL-1β, nitrite production was increased in a time-dependent fashion for 48 h, as reported previously [13].

**Time course of rHuEpo effect on IL-1β-induced nitrite and cGMP production**

First we examined the time course of rHuEpo effect on IL-1β-induced nitrite and cGMP production. As
shown in Figure 1a, IL-1β alone linearly increased nitrite production with significance over 24-h incubation. 250 U/ml rHuEpo inhibited IL-1β-induced nitrite production significantly over 24-h coincubation. The average (28.8%) inhibition by rHuEpo was obtained at 24-h coincubation. On the other hand, cGMP production by DMEM, IL-1β, and IL-1β plus rHuEpo were monophasic, reaching maximal levels at 24 h of exposure (Figure 1b); 250 U/ml rHuEpo significantly inhibited IL-1β-induced cGMP production at 24-h coincubation. Therefore we incubated VSMC with the test agents for 24 h in the subsequent experiments for measurements of nitrite and cGMP production.  

**Effect of graded concentration of rHuEpo on IL-1β-induced nitrite and cGMP production**

As shown in Figure 2a, nitrite production is fairly low (36.1 ± 2.7 nmol/mg protein) in the absence of IL-1β, whereas addition of 10 ng/ml IL-1β markedly enhanced nitrite production (537.2 ± 21.2 nmol/mg protein). In the presence of IL-1β, rHuEpo inhibited nitrite production in a dose-dependent manner with significance at concentration of 250 U/ml. On the other hand, in the absence of IL-1β, 250 U/ml rHuEpo did not affect nitrite production at all. Like nitrite, rHuEpo inhibited cGMP production in a dose-dependent fashion with significance at concentration of 250 U/ml (Figure 2b).

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**Fig. 1.** (a) Time course of IL-1β, IL-1β + rHuEpo-induced nitrite production and IL-1β-independent nitrite production. VSMC were incubated with either IL-1β (10 ng/ml) alone, IL-1β plus rHuEpo (250 U/ml) or DMEM for the time indicated. Each point is mean ± SEM of four samples. *1 P < 0.01 vs IL-1β + rHuEpo; *2 P < 0.01 vs DMEM. (b) Time course of IL-1β, IL-1β + rHuEpo-induced cGMP production and IL-1β-independent cGMP production. After measuring nitrite, VSMC were incubated with 0.5 mM IBMX for 15 min. Each point is mean ± SEM of four samples. *1 P < 0.01 vs IL-1β + Epo; *2 P < 0.01 vs DMEM.

**Fig. 2.** (a) Dose-response of rHuEpo on IL-1β-dependent or-independent nitrite production. VSMC were incubated with 25 and 250 U/ml of rHuEpo for 24 h in the presence or absence of 10 ng/ml IL-1β. Column and bar represent mean ± SEM of four samples. *1 P < 0.01 vs DMEM; *2 P < 0.01 vs IL-1β alone. (b) Dose response of rHuEpo on IL-1β-dependent or -independent cGMP production. After measuring nitrite, VSMC were incubated with 0.5 mM IBMX for 15 min. Column and bar represents mean ± SEM of four samples. *1 P < 0.01 vs DMEM; *2 P < 0.01 vs IL-1β alone.
In the absence of IL-1β, 250 U/ml rHuEpo did not affect cGMP production at all.

**Effect of rHuEpo on ANP- or SNP-stimulated nitrite and cGMP production**

We also examined the effects of rHuEpo on ANP- or SNP-stimulated nitrite and cGMP production in the absence of IL-1β to confirm that the effects of rHuEpo on nitrite and cGMP production are linked. As shown in Figure 3a, 10⁻⁶ M ANP itself did not stimulate nitrite accumulation in the absence of IL-1β, whereas addition of rHuEpo to ANP did not affect nitrite production. On the other hand, 10⁻⁴ M SNP markedly stimulated nitrite production in the absence of IL-1β, whereas addition of rHuEpo to SNP did not affect nitrite production. As for cGMP production shown in Figure 3b, ANP stimulated cGMP production, whereas addition of rHuEpo to ANP did not modify the cGMP accumulation. Similar to ANP, SNP also stimulated cGMP production, whereas addition of rHuEpo to ANP did not modify the cGMP accumulation.

**Effect of rHuEpo and rHuEpo vehicle on IL-1β-induced nitrite and cGMP production**

Next we investigated whether the inhibition by rHuEpo may be due to some components of rHuEpo solvent. For this we incubated VSMC with rHuEpo vehicle that contained the same solution as the active rHuEpo vial. As shown in Figure 4a, rHuEpo vehicle did not inhibit IL-1β-dependent nitrite production, whereas 250 U/ml rHuEpo did inhibit IL-1β-induced nitrite production. Identical results were obtained for cGMP production (Figure 4b). This indicates that rHuEpo itself, and not its solvent, inhibits IL-1β-induced nitrite and cGMP production.

**Effect of rHuEpo and rHuEpo vehicle on iNOS mRNA expression**

We also examined the effects of rHuEpo and rHuEpo vehicle on iNOS mRNA expression. As shown in Figure 5, 10 ng/ml of IL-1β markedly induced iNOS mRNA expression; 250 U/ml rHuEpo showed inhibition of iNOS mRNA expression induced by IL-1β, whereas rHuEpo vehicle did not affect iNOS mRNA expression.

**Discussion**

The present study clearly demonstrates for the first time that a pharmacological dose of rHuEpo inhibits IL-1β-induced NO and cGMP production as well as iNOS mRNA expression, presumably via Epo receptor.

Ammarguellat et al. recently showed Epo receptor mRNA expression in cultured rat VSMC by using Northern blot analysis. From these results, it is reasonable to speculate that Epo may directly affect VSMC function through Epo receptor. Indeed, there are several reports demonstrating the direct effects of Epo on VSMC, showing mitogenic and vasoconstrictive effects of Epo in VSMC [11,12].

Although the rHuEpo concentrations used in this study are much higher than the plasma concentrations reached with therapeutic doses, such differences in concentrations between in vivo and in vitro are not exceptional. Further, a lower sensitivity of cultured VSMC can be expected in vitro than in vivo. In addition, in vivo hormone concentrations at the target sites may be much higher than the circulating concentration of the hormone. Indeed, it is reported that Epo concentrations in several tissues were found to be higher than serum concentration after treatment with Epo [17]. Since it is reported that there is 82% homology between human and rat Epo receptor, the possibility that such a difference may contribute to the low
It is a well-known fact that various vasoconstrictors such as NA, AII, ET and AVP cause vascular contraction in a direct manner. Recently, it has been shown that these substances may cause vasoconstriction indirectly through inhibiting cytokine-induced NO production [7–10]. In contrast to these vasoconstrictors, vasodilating hormones such as ANP, PGI2 and PGE1 were shown to stimulate cytokine-induced NO production in VSMC [16,21]. Therefore it is plausible that both vasoconstrictors and vasodilators seem to enhance their original functions through cytokine-induced NO production.

NO is known to activate soluble guanylate cyclase (a) and subsequently intracellular cGMP production. VSMC were incubated with rHuEpo (250 U/ml) or rHuEpo vehicle for 24 h in the presence or absence of 10 ng/ml IL-1β. Column and bar represent mean ± SEM of 12 individual samples from three separate experiments. Values were expressed as the percentage of mean value of condition 3. *1 P < 0.01 vs condition 1; *2 P < 0.05 vs condition 3.

(b) Effects of rHuEpo and rHuEpo vehicle on IL-1β-dependent or -independent nitrite production. VSMC were incubated with rHuEpo (250 U/ml) or rHuEpo vehicle for 24 h in the presence or absence of 10 ng/ml IL-1β. Column and bar represent mean ± SEM of 20 individual samples from five separate experiments. Values were expressed as the percentage of mean value of condition 3. *1 P < 0.01 vs condition 1; *2 P < 0.05 vs condition 3.

sensitivity of rHuEpo on nitrite production cannot be denied [19]. In clinical settings, relatively high rHuEpo plasma concentrations, of the order of 1–5 U/ml, may be obtained intermittently in chronic haemodialysis patients, at least immediately after intravenous injection of the hormone [20]. Therefore it is reasonable to speculate that even pharmacological doses of rHuEpo may affect cytokine-induced nitrite metabolism in some patients undergoing chronic HD.

Fig. 4. (a) Effects of rHuEpo and rHuEpo vehicle on IL-1β-dependent or -independent nitrite production. VSMC were incubated with rHuEpo (250 U/ml) or rHuEpo vehicle for 24 h in the presence or absence of 10 ng/ml IL-1β. Column and bar represent mean ± SEM of 20 individual samples from five separate experiments. Values were expressed as the percentage of mean value of condition 3. *1 P < 0.01 vs condition 1; *2 P < 0.05 vs condition 3. (b) Effects of rHuEpo and rHuEpo vehicle on IL-1β-dependent or -independent cGMP production. VSMC were incubated with rHuEpo (250 U/ml) or rHuEpo vehicle for 24 h in the presence or absence of 10 ng/ml IL-1β. Column and bar represent mean ± SEM of 12 individual samples from three separate experiments. Values were expressed as the percentage of mean value of condition 3. *1 P < 0.01 vs condition 1; *2 P < 0.05 vs condition 3.

Fig. 5. Effects of rHuEpo and rHuEpo vehicle on IL-1β-induced iNOS mRNA expression. VSMC were incubated with or without IL-1β for 24 h. Lane 1, control; Lane 2, rHuEpo (250 U/ml); Lane 3: IL-1β (10 ng/ml) alone; Lane 4, IL-1β + rHuEpo vehicle; Lane 5, IL-1β + rHuEpo (250 U/ml).
tion, especially in patients with chronic renal failure or those undergoing HD treatment with bioincompatible dialysis membranes where blood cytokines levels are thought to be elevated [22,23].

Concerning the mechanism of inhibition by rHuEpo on IL-1β-induced NO production and iNOS mRNA expression, Epo-receptor-mediated phosphoinositide hydrolysis might be responsible. Since AI was shown to inhibit cytokine-stimulated NO production and iNOS mRNA expression via activation of protein kinase C and increase of intracellular calcium in VSMC [8], the same mechanism may operate for Epo. Indeed, Neussler et al. demonstrated that Epo increased cytosolic free calcium concentration in cultured rat vascular smooth-muscle cells [12]. In addition Gogusey et al. showed that Epo acts on rat VSMC by enhancing proto-oncogene expression and phospholipase C activity, indicating that Epo may function as a vascular smooth-muscle cell growth promoting factor through activation of the phospholipase C cascade and a modulation of proto-oncogene expression [24]. However, the precise association between inhibitory action of Epo on IL-1β-induced NO production and vascular smooth-muscle cell growth remains to be elucidated. Since rHuEpo did not affect ANP- or SNP-induced nitrite and cGMP production, the inhibitory action of rHuEpo may not involve the activation of either membrane-bound or soluble guanylate cyclase.

As for the clinical relevance, there is a possibility that this NO-inhibiting action of Epo may partly be related to arterial hypertension in uraemic patients undergoing chronic HD. Since HD patients have high blood levels of cytokines, NO production is usually stimulated [25]. Indeed, we observed that blood NO levels are much higher in HD patient than in healthy volunteers [26]. Therefore it is reasonable to speculate that Epo administration inhibits cytokine-stimulated NO production, thereby inducing elevation of blood pressure in these patients. However, clinical studies are needed to determine whether Epo may inhibit NO production and induce arterial hypertension in uraemic patients.

In conclusion, the present study suggests that pharmacological doses of rHuEpo may cause vascular contraction presumably via Epo receptor not only by a direct action but also through indirect action by inhibiting NO and cGMP production under some cytokine elevated conditions.

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