Erythropoietin Depresses Nitric Oxide Synthase Expression by Human Endothelial Cells

Xiu Q. Wang, Nosratola D. Vaziri

Abstract—We have recently shown that erythropoietin (EPO)-induced hypertension is unrelated to the rise in hematocrit and is marked by elevated cytosolic [Ca$^{2+}$] and nitric oxide (NO) resistance. The present study was done to determine the effect of EPO on NO production and endothelial NO synthase (eNOS) expression by endothelial cells. Human coronary artery endothelial cells were cultured to subconfluence and then were incubated for 24 hours in the presence of either EPO (0, 5, and 20 U/mL) alone or EPO plus the calcium channel blocker felodipine. The experiments were carried out with quiescent (0.5% FCS) and proliferating (5% FCS) cells. Total nitrate and nitrite, eNOS protein, DNA synthesis (thymidine incorporation), and cell proliferation (cell count) were determined. In addition, NO production in response to acetylcholine stimulation was tested. EPO resulted in a dose-dependent inhibition of basal and acetylcholine-stimulated NO production and eNOS protein expression and also led to a significant dose-dependent stimulation of DNA synthesis in endothelial cells. The inhibitory effects of EPO on NO production and eNOS expression were reversed by felodipine. Thus, EPO downregulates basal and acetylcholine-stimulated NO production, depresses eNOS expression, and stimulates proliferation in isolated human endothelial cells. The suppressive effects of EPO on NO production and on eNOS expression are reversed by calcium channel blockade. (Hypertension. 1999;33:894-899.)

Key Words: erythropoietin n endothelium n nitric oxide n endothelium-derived relaxing factor n hypertension n calcium channel blockers

Prolonged administration of recombinant erythropoietin (EPO) can lead to de novo hypertension (HTN) or to exacerbation of preexisting HTN in animals and humans with chronic renal failure (CRF).1-3 We have recently shown that contrary to the prevailing belief, EPO-induced HTN is not related to the correction of anemia in either animals or patients with CRF.4,5 Instead, we have shown that chronic use of EPO raises cytosolic Ca$^{2+}$ ([Ca$^{2+}$]i) concentration, which in turn causes nitric oxide (NO) resistance.1,5 In a recent study, we found no discernible change in either total body NO production or renal or vascular tissue NO synthase (NOS) expression in CRF rats treated with EPO to prevent CRF anemia.5 In contrast, Wilcox et al6 and del Castillo et al7 found increased NO production in normal rats with EPO-induced HTN and erythrocytosis. It should be noted, however, that animals used in the latter studies had intact kidneys, whereas those used in our studies had CRF, which can suppress NOS expression.8 Moreover, EPO-treated CRF rats used in our studies had normal hematocrits, whereas EPO-treated rats with intact kidneys used in the latter studies had severe erythrocytosis.

Elevation of hematocrit, erythrocyte mass, and blood pressure in animals used in the studies reported by Wilcox et al6 and del Castillo et al7 must have caused a marked increase in blood volume, blood viscosity, and shear stress. These secondary events are known to independently stimulate NOS expression and NO production.9-12 As a result of these confounding influences, it is difficult to discern the possible direct effect, if any, of EPO on NO production and NOS expression in vivo. Therefore, the present study was designed to test the possible direct effect of EPO on NO production and NOS expression. To this end, the effect of EPO was tested on cultured human endothelial cells in vitro in which hemodynamic, rheological, and hormonal changes inherent to the in vivo condition were necessarily absent.

Methods

Cell Culture

Human coronary artery endothelial cells were purchased from Bio Whittaker Inc. The cells were suspended in the culture medium (Endothelial Cell Growth System, Bio Whittaker Inc) and placed into 75-cm$^2$ flasks. The flasks were then placed in a humidified incubator at 37°C with 5% CO$_2$. After 2 days, 10 mL of fresh medium was added into the flasks, and the incubation was continued for an additional 2 days. Thereafter, the medium was changed every other day. After a monolayer was formed, the cells were subcultured. The cells were identified by staining with a specific antibody to von Willebrand factor and fluorescent-labeled LDL, as described previously.13

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Study Design

The cells obtained on passages 3 to 4 were used for the experiments. The cells reaching 70% to 80% confluence were subcultured into 6-well plates and incubated for 48 hours after 80% confluence was reached. The cells were then treated with either EPO (5 and 20 U/mL), EPO plus $10^{-7}$ mol/L calcium channel blocker felodipine (Astra Merck Inc), or vehicle in a medium containing 5% FCS for 24 hours. In an attempt to determine the effect of cell growth on the study parameters, the studies were repeated with cells that were made quiescent with a medium containing 0.5% serum for 24 hours before treatment with EPO, EPO plus felodipine, or inactive vehicle. At the conclusion of the 24-hour treatment period, the cells and the supernatants were harvested and saved for the following measurements.

Acetylcholine Stimulation Test

The experiments were carried out to determine the effect of EPO on the endothelial cell NO production capacity in response to acetylcholine stimulation. To this end, endothelial cells were incubated in 24-well plates in the presence of either vehicle or EPO for 24 hours. The old medium was then replaced with 0.2 mL of fresh medium. The cells were then treated with either acetylcholine ($10^{-5}$ mol/L) or vehicle for 60 minutes. The medium was used for determination of total nitrite and nitrate (NOx). In addition, the cells were collected for measurement of total protein. The amount of NO produced was normalized against the cellular total protein.

Western Blot Analysis

These measurements were carried out to determine the endothelial NOS (eNOS) protein mass by use of an anti-eNOS monoclonal antibody (Transduction Laboratories), as previously described.8

Cell Proliferation Assay

The endothelial cells were passed onto 96-well, flat bottom, microtiter plates with a density of 1000 cells/0.1 mL per well and were cultured until reaching 70% confluence. The cells were then incubated with EPO or vehicle in the presence of $[^{3}H]$thymidine (1.0 uCi per well) (Dupont NEN). At the end of incubation, the cells were washed with PBS 3 times and harvested onto glass fiber filters with an automatic cell harvester. The filters were placed in 5 mL of Bio-Safe NA, and the radioactivity was measured in a liquid scintillation counter (Model 9000, Beckman Instruments Inc).

These experiments were repeated on 24-well plates for the purposes of cell count and protein measurement. Cells were counted in a hemocytometer, and viability was determined by trypan blue exclusion. Protein was measured with a Bio-Rad kit.

Figure 1. Total NOx generated by human coronary endothelial cells incubated for 24 hours in the presence of vehicle (0) or EPO at concentrations of 5 (5) or 20 U/mL (20). A and B represent data obtained in cells cultured in the presence of 5% and 0.5% FCS, respectively. n=4 to 6 experiments. *P<0.05 vs control (0).

Figure 2. Representative Western blot of eNOS in human coronary endothelial cells incubated for 24 hours in a medium containing 5% (A) or 0.5% FCS (B) in the presence of either vehicle (lanes 1 and 2) or EPO at 5 U/mL (lanes 3 and 4) or 20 U/mL (lanes 5 and 6) for 24 hours. Lane 7 represents positive control.
Measurements of Total NOx

The concentration of total NOx in the culture medium was determined with the purge system of a Sievers Instruments Model 270B Nitric Oxide Analyzer (NOA, Sievers Instruments Inc).\(^5\)

Data Presentation and Analysis

ANOVA and Student’s t-test were used in statistical evaluation of the data, which are given as mean±SEM. \(P<0.05\) was considered significant.

Results

Effect on NO Production

Incubation with EPO (5 and 20 U/mL) for 24 hours resulted in a significant dose-dependent reduction (22.2±1.0% and 33.3±2.0%, respectively; \(P<0.05\)) of NOx production by proliferating human coronary artery endothelial cells. Similarly, NOx production was significantly reduced by EPO in quiescent endothelial cells (15.4±1.1% and 23.1±3.0% in the presence of EPO at 5 and 20 U/mL, respectively; \(P<0.05\)). On each occasion, NOx production by the quiescent cells was significantly lower than that seen in proliferating endothelial cells. A significant inverse correlation was found between EPO concentration and NOx production by endothelial cells (\(r = -0.90, P<0.001\)). Data are shown in Figure 1.

Effect on eNOS Protein Mass

Incubation for 24 hours with EPO at concentrations of 5 and 20 U/mL resulted in marked dose-dependent downregulation of eNOS protein expression by the proliferating human coronary artery endothelial cells (40.8±3.1% and 74.1±1.5%, respectively; \(P<0.01\)). Likewise, addition of EPO depressed eNOS protein expression by quiescent endothelial cells (67.3±2.2% and 88.6±3.1% in the presence of 5 and 20 U/mL of EPO, respectively; \(P<0.01\)). As with NOx production, eNOS protein expression was significantly lower in the quiescent cells than in the proliferating endothelial

![Figure 3. eNOS protein abundance measured by Western blot analysis in human coronary endothelial cells incubated for 24 hours in the presence of vehicle (0) or EPO at concentrations of 5 (5) or 20 U/mL (20). A and B represent data obtained in cells cultured in the presence of 5% and 0.5% FCS, respectively (n=4 to 6 experiments). *\(P<0.01\) vs other groups.](image)

![Figure 4. Basal (control) and acetylcholine-stimulated (stimulated) NOx produced by human coronary endothelial cells incubated with either vehicle (top) or EPO (middle) for 24 hours and stimulated with either acetylcholine or inactive vehicle for 60 minutes. The bottom panel shows the magnitude of acetylcholine-induced rise in NOx production in the EPO- and vehicle-treated cells (n=4 to 6 experiments). *\(P<0.05\) vs basal value. +\(P<0.05\) vs the corresponding value in the upper panel.](image)

![Figure 5. Representative Western blot of eNOS protein in human coronary endothelial cells incubated for 24 hours in the presence of either vehicle (lanes 1 and 2), EPO (20 U/mL, lanes 3 and 4), or EPO plus calcium channel blocker felodipine (10^-7 mol/L, lanes 5 and 6).](image)
cells. eNOS protein abundance was inversely related to EPO concentration \((r = -0.84, P < 0.005)\) and directly related to NOx production \((r = 0.90, P < 0.001)\). Results are shown in Figures 2 and 3.

**Effect on Acetylcholine Stimulation**
Addition of acetylcholine resulted in a marked increase in NOx production by the endothelial cells in the control experiments. However, acetylcholine-stimulated NOx production by EPO-treated cells was significantly lower than that seen in untreated cells. Data are shown in Figure 4.

**Effect of Calcium Channel Blockade**
Calcium channel blockade with felodipine abrogated the EPO-induced downregulation of NOx production and eNOS expression by cultured endothelial cells. Data are shown in Figures 5 and 6.

**Effect on Endothelial Cell Growth**
At concentrations of 5 and 20 U/mL, EPO caused a significant dose-dependent rise in DNA synthesis (measured as thymidine incorporation) in the quiescent human coronary artery endothelial cells. In addition, EPO significantly augmented the stimulatory effect of 5% FCS in proliferating endothelial cells. On each occasion, the EPO-induced stimulation of DNA synthesis was accompanied by a parallel increase in cell replication as determined by cell count. A significant direct correlation was found between EPO concentration and DNA synthesis \((r = 0.87, P < 0.005)\) and endothelial cell proliferation \((r = 0.87, P < 0.005)\). Data are shown in Figures 7 and 8.

**Discussion**
Incubation with EPO resulted in a dose-dependent decline in basal NO production by endothelial cells. In addition, acetylcholine-stimulated rise in NO production was significantly reduced by prior incubation with EPO. The fall in NO production was accompanied by a parallel reduction in eNOS protein abundance in EPO-treated endothelial cells. The latter can account for the observed reduction in basal and acetylcholine-stimulated production of NO by endothelial cells treated with EPO. Accordingly, EPO exerts a downregulatory effect on NO production and eNOS expression by endothelial cells in vitro. Interestingly, we have previously shown that regular administration of EPO at a dosage sufficient to correct anemia causes HTN but
does not change either total body NO production or NOS expression in the kidney or vascular tissue. Moreover, extended EPO therapy leading to marked erythrocytosis and HTN in normal rats has been shown to increase urinary excretion of NO metabolites, suggesting enhanced renal and systemic NO production. Thus, the direct effect of EPO on isolated endothelial cells in vitro seems to be different from that observed with chronic EPO administration in the whole animal. It should be noted, however, that EPO administration in the CRF animals used in our earlier study led to a marked rise in blood pressure. The associated rise in blood pressure can independently upregulate eNOS expression and NO production. This upregulatory effect of the associated HTN may have masked the direct downregulatory action of EPO on the endothelial cells in the EPO-treated CRF animals used in our earlier study. Similarly, increased NO production in rats with EPO-induced HTN and erythrocytosis reported by Wilcox et al and del Castillo et al could be explained by the predominant stimulatory effects of increased blood pressure, intravascular volume, and shear stress that obscured the direct inhibitory action of EPO per se. Thus, the direct inhibitory action of EPO on the L-arginine–NO pathway can be offset or overridden by the stimulatory actions of the rise in blood pressure, blood volume, and viscosity (increased erythrocyte mass) resulting from EPO therapy in vivo. Consequently, the net effect of EPO on the L-arginine–NO pathway is the sum of its opposing direct and indirect influences.

Several earlier studies have shown that chronic EPO administration leads to a rise in $[\text{Ca}^{2+}]$, as well as that normalization of $[\text{Ca}^{2+}]$, with either parathyroid ablation or calcium channel blockade leads to reversal of depressed NO production and NOS expression in rats with experimental CRF. In another study, we showed that restoration of normal $[\text{Ca}^{2+}]$, by calcium channel blockade enhances NO production and NOS expression in EPO-treated CRF rats that exhibit marked elevation of $[\text{Ca}^{2+}]$. On the basis of these observations, we hypothesized that sustained elevation of basal $[\text{Ca}^{2+}]$, may depress eNOS expression. This viewpoint is supported by the observation that EPO, which is known to raise $[\text{Ca}^{2+}]$, downregulated NO production and eNOS expression and that this effect was reversed by calcium channel blockade. In addition, we have recently shown upregulation of NO production and eNOS expression by calcium channel blockade in cultured endothelial cells, pointing to the likely role of $[\text{Ca}^{2+}]$, in regulation of eNOS expression.

Addition of EPO to the culture medium led to a marked stimulation of DNA synthesis and to proliferation in the quiescent endothelial cells. The magnitude of the stimulatory action of EPO on endothelial cell growth was comparable to that seen with 5% FCS. This observation points to the potent growth-promoting effect of EPO in this system. The stimulatory action of EPO on endothelial cell growth shown here confirms the results of several earlier studies conducted by other investigators.

References


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