

Nitric oxide suppresses EPO-induced monocyte chemoattractant protein-1 in endothelial cells: implications for atherogenesis in chronic renal disease

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Patients with advanced chronic renal disease (CRD) suffer from excessive morbidity and mortality due to complications of accelerated atherosclerosis. Approximately 90% of dialysis-dependent end stage renal disease patients suffer from anemia. Recombinant human erythropoietin (EPO) in combination with iron has become widely used to treat anemic CRD patients. While treatment with EPO results in improved quality of life it may also contribute to the development of atherosclerosis. Recent studies suggest that a reduction in nitric oxide (NO) availability may be linked to EPO-induced vascular dysfunction. Furthermore, CRD *per se* is thought to result in a state of NO deficiency. The present study suggests that EPO may exert proatherogenic activity by augmenting the cytokine-induced expression of monocyte-chemoattractant protein-1 (MCP-1) in human umbilical vein endothelial cells (HUVECs) and by stimulating the proliferation of HUVECs and human vascular smooth muscle cells (HVSMS). Augmentation of MCP-1 expression appears to be linked to EPO-induced downregulation of endothelial NO synthase (eNOS). NO released from a series of synthetic donor compounds suppressed the EPO-mediated augmentation of cytokine-induced MCP-1 expression. *In vitro* studies revealed that EPO reduces eNOS expression at both the protein and mRNA levels and that EPO also mediates a reduction in eNOS enzymatic activity. These observations suggest potential mechanisms through which EPO may contribute to the development of accelerated atherosclerosis, particularly in the setting of CRD where NO availability may already be compromised.

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Cardiovascular complications resulting from accelerated atherosclerosis are the principal cause of morbidity and mortality in patients with advanced CRD.¹ The relative risk of death from myocardial infarction has been estimated as between five to 20 times higher in CRD patients than in the age- and sex-matched general population.^{2,3} Chronic administration of recombinant human erythropoietin (EPO), which is widely used to treat the anemia that accompanies CRD and other nonrenal forms of anemia, can result in hypertension and resistance to the vasodilatory action of NO.^{4,5} EPO also exerts a proliferative effect on vascular wall cells.⁶ These

actions could potentially contribute to the development of atherosclerotic vascular disease.⁷ While little is known about potential mechanisms, a reduction in NO availability has been linked to EPO-induced vascular dysfunction.⁸

Monocyte-chemoattractant protein-1 (MCP-1), a member of the C-C or beta subfamily of chemokines, is pivotal to the recruitment, adhesion, and trans-endothelial migration of monocytes during atherogenesis.^{9,10} Endothelial NO, on the other hand, is a potent endogenous vasodilator and principal mediator of the antiatherogenic properties of intact endothelium.¹¹ Nitric oxide (NO) interferes with monocyte adhesion to the endothelium as well as with platelet–vessel wall interactions.^{12,13} NO decreases endothelial permeability and reduces vascular tone which in turn decrease the flux of lipoproteins into the vessel wall.^{14,15} Exogenous NO as well as NO locally produced by inducible NO synthase (iNOS) in vascular smooth muscle cells

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inhibits oxidation of low-density lipoproteins.¹⁶ Finally, NO inhibits vascular smooth muscle cell proliferation and migration both *in vitro* and *in vivo*.^{17,18}

The results of several *in vitro* and *in vivo* studies aimed at unraveling a possible link between NO- and EPO-induced vascular dysfunction are in conflict. Del Castillo *et al*¹⁹ observed a significant increase in the urinary excretion of NO metabolites (nitrite and nitrate) in rats treated with EPO for 3 weeks. Similar results were reported by Tsukahara *et al*²⁰ and Kanagy *et al*²¹ who treated rats with EPO over a 2-week period. Wu *et al*²² observed that EPO stimulates NO release from resting human umbilical vein endothelial cells (HUVECs) in a time and concentration-dependent manner. In contrast, Wang and Vaziri⁸ reported that EPO downregulates basal and acetylcholine-induced NO production, depresses endothelial NO synthase (eNOS) expression and stimulates proliferation of HUVECs. Studies of EPO-induced hypertension in rats with chronic renal failure (CRF) suggest that EPO may induce resistance to the vasodilatory response of arteries to endogenous NO.²³

In the present study, we observed that exposure of endothelial cells to EPO results in increased endothelial and smooth muscle cell proliferation and downregulation of eNOS at both the protein and mRNA levels. Furthermore, EPO pretreatment induced a significant rise in cytokine-induced MCP-1 concentrations whereas exposure to NO donors immediately following EPO treatment reversed the EPO-mediated increases in cytokine-induced MCP-1 production. These observations suggest that EPO may exert a proatherogenic effect by enhancing the expression of MCP-1 and by stimulating cell proliferation. Coupled with recent reports suggesting that renal failure *per se* results in a state of NO deficiency, our observation that EPO downregulates eNOS expression suggests a possible mechanism through which EPO may exacerbate the accelerated atherosclerosis observed in CRD patients.²⁴

Materials and methods

Recombinant human EPO, human tumor necrosis factor- α (TNF- α), and interleukin-1 β (IL-1 β) were obtained from R&D Systems (Minneapolis, MN, USA). HUVECs, human vascular smooth muscle cells (HVSMEs), and media (EGM-2 and SMGM-2, respectively) were purchased from Clonetics (Walkersville, MD, USA).

Endothelial and Vascular Smooth Muscle Cell Culture

HUVEC and smooth muscle cells were grown at 37°C in 5% CO₂ on 150 × 25 mm² plates as previously described.²⁵ For all experiments, cells were used between first and third passage.

Treatment of HUVECs for Enzyme Immunoassay (EIA) Experiments

HUVECs were grown to partial confluence on 96-well flat bottom polystyrene plates (Costar). The cells were exposed to EPO (5 U/ml) for 24 h. In experiments with NONOates, the medium was changed following this 24 h incubation period. Ten microliters of a concentrated stock solution of NONOate in 0.01 N NaOH (2 mM) was added to 190 μ l of medium to obtain a final concentration of 100 μ M. Following a 30 min incubation with the NONOate, the conditioned medium was replaced by fresh medium containing TNF- α (10 ng/ml) or IL-1 β (10 ng/ml). At the time of cytokine stimulation, all endothelial monolayers were confluent. The conditioned media collected at the end of an 8 h incubation with either TNF- α or IL-1 β were subjected to a MCP-1 EIA. A fraction of the conditioned media was also subjected to cytotoxicity detection (LDH) assays (Roche Applied Science, Indianapolis, IN, USA) to assess cell viability. For all experiments cell viability was greater than 95%.

MCP-1 EIA

MCP-1 capture plates were prepared by coating sterile 96-well flat bottom ELISA plates (Corning, Corning, NY, USA) with anti-human MCP-1 antibody (R&D Systems, 100 μ l/well, 0.8 μ g/ml in PBS). The plates were incubated at 4°C overnight, washed with Wash Buffer (0.05% Tween-20 in PBS, pH 7.4) and blocked overnight at 4°C with blocking buffer (0.05% Tween-20, 1% BSA in PBS, pH 7.4) to reduce nonspecific binding. Capture plates were stored at 4°C until use. Hundred microliters aliquots of the conditioned cell media and human recombinant MCP-1 standards (R&D systems) were loaded onto capture plates and incubated at 4°C overnight. Biotinylated anti-human MCP-1 detection antibody (R&D Systems) was added to each well (0.1 μ g/ml, 100 μ l per well) and plates were incubated at room temperature for 2 h. After three washes, streptavidin horseradish peroxidase was added (Neutralite avidin, Southern Biotechnology Associates, Birmingham, AL, USA; 100 μ l/well, 1:4000 dilution) and incubated for 30 min at room temperature. Wells were washed to remove unbound conjugated peroxidase and then incubated with 100 μ l/well of peroxidase substrate (ABTS[®], Boehringer Mannheim Biochemica, Germany). After 30 min to 2 h, optical density (OD) at 405 nm was measured using an automated microplate reader (EL₈₀₈, Bio-Tek Instruments, Winooski, VT, USA). MCP-1 concentrations were calculated by 4 parameter curve fitting or linear regression analysis of rhMCP-1 standard curves using KC3 software (Bio-Tek Inc.).

Extraction of Nuclear and Cytosolic Protein

Nuclear extracts were prepared as previously described by a modified method of Dignam *et al*.²⁴

Briefly, HUVECs (5×10^6 – 5×10^7 cells) were harvested by scraping and centrifuged in PBS for 5 min at 1200 r.p.m. The pelleted cells were washed twice with 1 ml of ice-cold buffer A (10 mM Hepes, 1.5 mM MgCl_2 , 10 mM KCl, 0.5 mM dithiothreitol, 0.5 mM PMSF). The pellet was resuspended in 60 μl buffer A + 0.1% Nonidet P-40 and incubated on ice for 5 min. Following centrifugation, the supernatants were diluted in 60 μl buffer D (20 mM Hepes, 50 mM KCl, 20% glycerol, 0.5 mM dithiothreitol, 0.5 mM PMSF), frozen at -80°C , and subsequently utilized for Western blots. The pelleted nuclei were suspended in 60 μl buffer C (20 mM Hepes, 0.42 M NaCl, 1.5 mM MgCl_2 , 25% glycerol, 0.5 mM dithiothreitol, 0.5 mM PMSF). After incubating the suspension on ice for 15 min, it was vortexed gently and centrifuged. The resulting supernatant was diluted in buffer D, aliquoted, and stored at -80°C . Protein determination was carried out by a Micro BCA assay (Pierce, Rockford, IL, USA) using bovine serum albumin as a standard.

Western Blot Analysis

Cytoplasmic extracts (corresponding to 5 μg total protein) were denatured in Tris-glycine sample buffer (Novex, San Diego, CA, USA) and electrophoresed on 4–20% Tris-glycine gels using Tris/Glycine/SDS running buffer (Bio-Rad, Minneapolis, MN, USA). Rainbow markers (Amersham) were used as molecular weight standards. The proteins were transferred to a nitrocellulose membrane using a mini-Trans blot for 6 h at room temperature with 250 mA of current. Blots were blocked overnight with a solution of 1% BSA in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T). After three washes with TBS-T, the eNOS antibody (Transduction Laboratories, N30020) was added at a dilution of 1:2500 and the blots were incubated for 1 h. Blots were washed rapidly three times in TBS-T. The blots were incubated for 20 min in TBS-T containing the detection antibody anti-mouse IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:5000. Immunoreactive bands were visualized using an enhanced chemiluminescence light detecting kit (Pierce).

Ribonuclease (RNase) Protection Assay (RPA)

Total RNA was extracted from endothelial cells using Tri Reagent according to manufacturer's instructions. The RNase protection assays were performed using the RiboQuant Multi-probe kit from Pharmingen (San Diego, CA, USA) and carried out as instructed by the manufacturer. Briefly, chemokine probes were radiolabeled with [γ - ^{32}P] UTP (Amersham) and hybridized with the extracted total RNA at 56°C overnight. RNase digestion was carried out at 30°C for 45 min to digest unhybridized single-

stranded and excess probe and terminated by the addition of proteinase K. After phenol/chloroform extraction and ammonium acetate/ethanol precipitation, the hybridized RNA probes were denatured at 90°C for 3 min and loaded onto a denaturing gel (5% acrylamide, 7 M urea) which was run in $0.5 \times \text{TBE}$ at 55 W constant power for 1.5 h. Gels were vacuum-dried at 50°C for 90 min, placed on Kodak X-OMAT film in an autoradiography cassette with an intensifying screen and developed at -70°C .

mRNA Quantification

MCP-1 mRNA levels were quantified using a commercially available colorimetric micoplate assay kit (Quantikine mRNA; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The lower detection limit is 3.9 amol MCP-1 mRNA/mL.

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assays (EMSAs) were carried out using a gel shift assay system kit (Promega, Madison, WI, USA). Double-stranded NF- κB consensus oligonucleotide probe (5'-AGT TGA GGG GAC TTT CCC AGC C-3') or AP-1 consensus probe (5'-CGC TTG ATG AGT CAG CCG GAA-3') was end-labeled with [γ - ^{32}P] ATP (3000 Ci/mmol at 10 mCi/ml, Amersham Life Science, Arlington Heights, IL, USA) and polynucleotide kinase (Promega). Following end-labeling, oligonucleotide probe and nuclear protein (5 μg) were incubated for 30 min at room temperature in binding buffer (Promega). Samples were run on a nondenaturing 4% polyacrylamide gel in $0.25 \times \text{TBE}$ buffer at 100 V/10 mA for 3 h. Gels were vacuum dried at 50°C for 90 min and visualized by Kodak X-OMAT film exposure to the gel at -70°C overnight.

Nitric Oxide Synthase Assay

NOS activity was measured using a NOSdetect assay kit (Stratagene, La Jolla, CA, USA) according to manufacturer's directions. Endothelial monolayers were incubated for 24 h either in medium alone or with the addition of EPO (5 U/ml). The cells were washed with PBS, harvested, and homogenized. Protein determination was carried out by a Micro BCA assay (Pierce, Rockford, IL, USA) using bovine serum albumin as a standard.

Bromodeoxyuridine (BrdU) Assay

BrdU Assay was performed using a Cell Proliferation ELISA kit from Roche Diagnostics GmbH (Mannheim, Germany). HUVECs and smooth muscle cells were grown to partial confluence on 96-well plates. The cells were incubated for 24 h in

the presence of medium with or without varying concentrations of EPO or doxorubicin (DOX). The medium was removed and the cells were treated with fresh medium containing 10 μ l of BrdU-labeling reagent. After processing the microtiter plate as instructed by the manufacturer, absorbance at 340 nm with a reference wavelength of 490 nm ($A_{340-490}$) was measured using an automated microplate reader (ELx808, Bio-Tek Instruments, Winooski, VT, USA).

Densitometric Scanning

Autoradiographs from RPAs were scanned using Polaroid PhotoMAX Pro and integrated densities were determined using Scion Image for windows (Scion Corporation, Frederick, MD, USA). To adjust for differences in sample loading between wells, ecNOS mRNA levels were expressed as ratios of integrated densities for ecNOS to GAPDH products.

Statistical Analysis

Statistical analysis was performed using Statistics for Windows, version 2.0. All values are expressed as means \pm s.e. Data were analyzed using Friedman two-way analysis of variance (ANOVA) with Bonferroni comparison of means. Probability (P) values of <0.05 were considered significant.

Results

Recombinant Human EPO Augments Cytokine-Induced MCP-1 Expression in HUVECS

As previously reported, exposure of confluent HUVEC monolayers to either TNF- α or IL-1 β alone results in marked increases in MCP-1 production when compared to unstimulated HUVECs.²⁵ Pretreatment with EPO significantly increased MCP-1 secretion by cytokine-stimulated endothelial cells. OD readings at 405 nm in MCP-1 ELISAs (using ABTS as a substrate) were 2.6-fold elevated in conditioned media from HUVECs that were pretreated with 1 U/ml of EPO prior to cytokine stimulation compared to cells pretreated with medium (control). With 5 U/ml EPO, OD readings were 5.1-fold elevated over control and with 25 U/ml EPO, 4.3-fold elevated over control. Cytokine-induced MCP-1 concentrations were not elevated when HUVECs were pretreated with EPO between 3 and 13 h. Increases in IL-1 induced MCP-1 concentrations were only observed at two later time points that we examined at 24 and 25 h. When HUVECs were pretreated with EPO (5 U/ml) for 24 h, TNF- α and IL-1 β stimulation resulted in 181 and 151%, respectively, increases in MCP-1 secretion (Figure 1). Heat-inactivated EPO (5 U/ml) had no effect on cytokine-induced MCP-1 expression (data not shown).

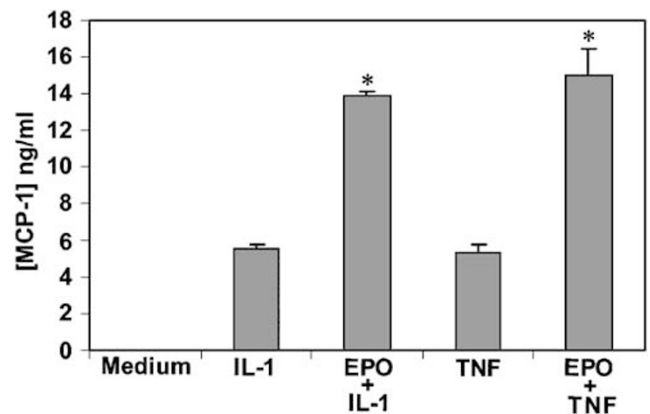


Figure 1 Augmentation of cytokine-induced MCP-1 production by EPO. HUVECs were pretreated for 24 h with either medium or EPO (5 U/ml), followed by a 8 h incubation in the presence of IL-1 β (10 ng/ml) or TNF- α (10 ng/ml). These data represent mean \pm s.e. from three independent experiments, each performed in triplicate. *Statistically significant difference ($P < 0.05$) from positive control (ie IL-1 β or TNF- α alone).

EPO does not Augment MCP-1 Concentrations by Increasing Translocation of NF- κ B in HUVECs

Cytokine-induced MCP-1 gene expression in endothelial cells depends on the cooperative interaction of the redox-sensitive transcription factors NF- κ B and AP-1.^{26,27} Nuclear extracts derived from HUVECs exposed to 5 U/ml of EPO or medium, respectively, for 24 h followed by cytokine exposure for 45 min were subjected to EMSAs with NF- κ B and AP-1 consensus oligonucleotides. As expected, cytokine stimulation of HUVECs resulted in a marked increase in the cytosolic-to-nuclear translocation of NF- κ B (Figure 2). EPO treatment prior to cytokine activation, however, did not affect NF- κ B activation and translocation. While EPO treatment augmented IL-1 β -induced activation of AP-1, it had no effect on TNF- α -induced AP-1 activation (Figure 3).

EPO does not Augment Cytokine-Induced MCP-1 mRNA Concentrations in HUVECs

Both, RNase protection and colorimetric microplate assays were used to determine whether increased levels of MCP-1 mRNA were present in EPO pretreated endothelial cells (Figure 4). In the RNase protection assays (Figure 4a) no MCP-1 mRNA was detected in HUVECs that had been exposed to medium or EPO alone, respectively, for 24 h. As expected, exposure of HUVECs to either TNF- α or IL-1 β for 4 h resulted in induction of MCP-1 mRNA. When HUVECs were treated with EPO for 24 h prior to cytokine stimulation no increases in MCP-1 mRNA were observed when compared to cells that were pretreated with medium for 24 h prior to IL-1 β or TNF- α stimulation. Results from mRNA quantitation assays depicted in Figure 4b concur

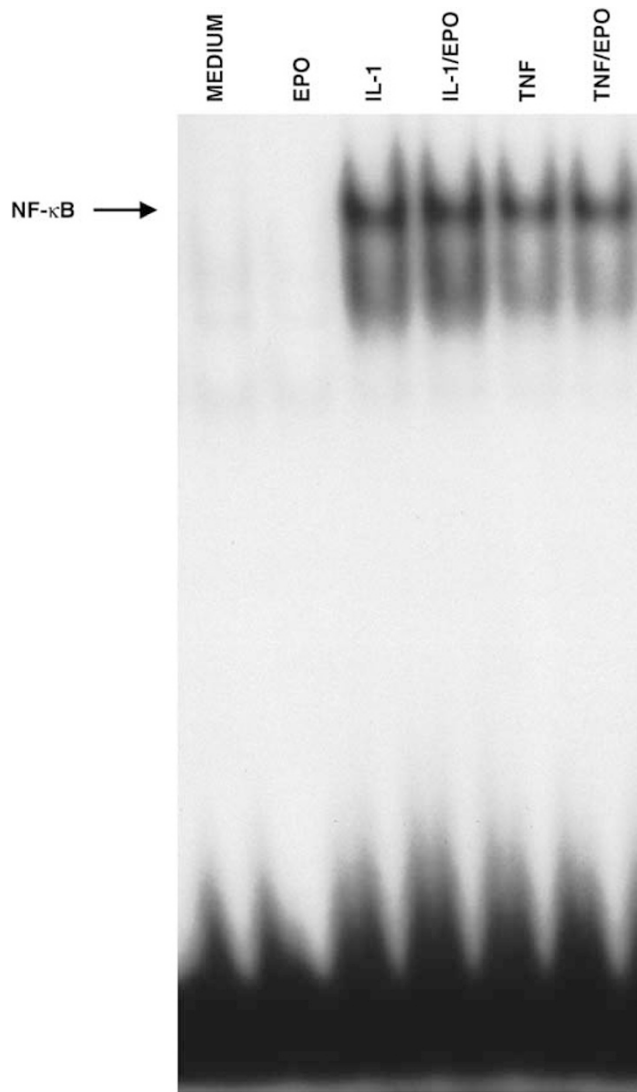


Figure 2 Effect of EPO on cytokine-induced NF- κ B translocation. HUVECs were subjected to a 24 h pretreatment with either medium or EPO (5 U/ml), followed by a 45 min incubation in the presence of IL-1 β (10 ng/ml) or TNF- α (10 ng/ml), respectively. Nuclear extracts were incubated in the presence of a NF- κ B consensus oligonucleotide probe for 30 min at room temperature and samples were run on a nondenaturing 4% polyacrylamide gel. Addition of unlabeled (cold) NF- κ B consensus oligonucleotide to the extract derived from TNF- α -stimulated HUVECs abrogated the gel shift (data not shown). These data are representative of three independent experiments.

with those obtained from the RNase protections assays presented in Figure 4a. Low concentrations of MCP-1 mRNA (between 292 ± 23 and 440 ± 84 amol/ml) were measured in ECs treated with medium or EPO, respectively, for 24 h. Following cytokine stimulation, MCP-1 mRNA concentrations were between eight and nine times higher than in media-treated cells. When ECs were treated with EPO for 24 h prior to cytokine stimulation, no changes in MCP-1 mRNA were measured when compared to cells that were pretreated with medium for 24 h prior to IL-1 β or TNF- α stimulation.

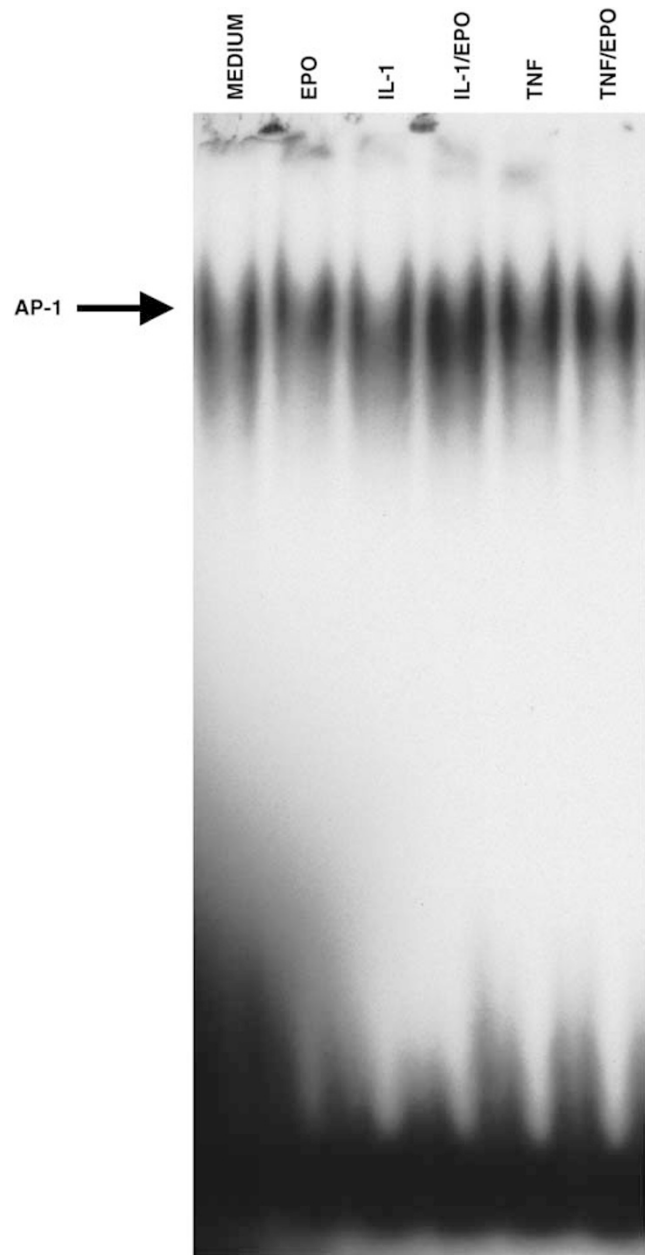


Figure 3 Effect of EPO on cytokine-induced AP-1 activation. HUVECs were subjected to a 24 h pretreatment with either medium or EPO (5 U/ml), followed by a 45 min incubation in presence of IL-1 β (10 ng/ml) or TNF- α (10 ng/ml), respectively. Nuclear extracts were incubated in presence of an AP-1 consensus oligonucleotide probe for 30 min at room temperature and samples were run on a nondenaturing 4% polyacrylamide gel. Addition of unlabeled (cold) AP-1 consensus oligonucleotide to the extract derived from TNF- α -stimulated HUVECs abrogated the gel shift (data not shown). These data are representative of three independent experiments.

NO Suppresses EPO-Mediated Augmentation of Cytokine-Induced MCP-1 Expression in HUVECs

It is currently believed that tonic production of NO by vascular endothelial cells plays an important role in the regulation of vascular tone and in retarding atherogenesis.¹¹ Dose-response and

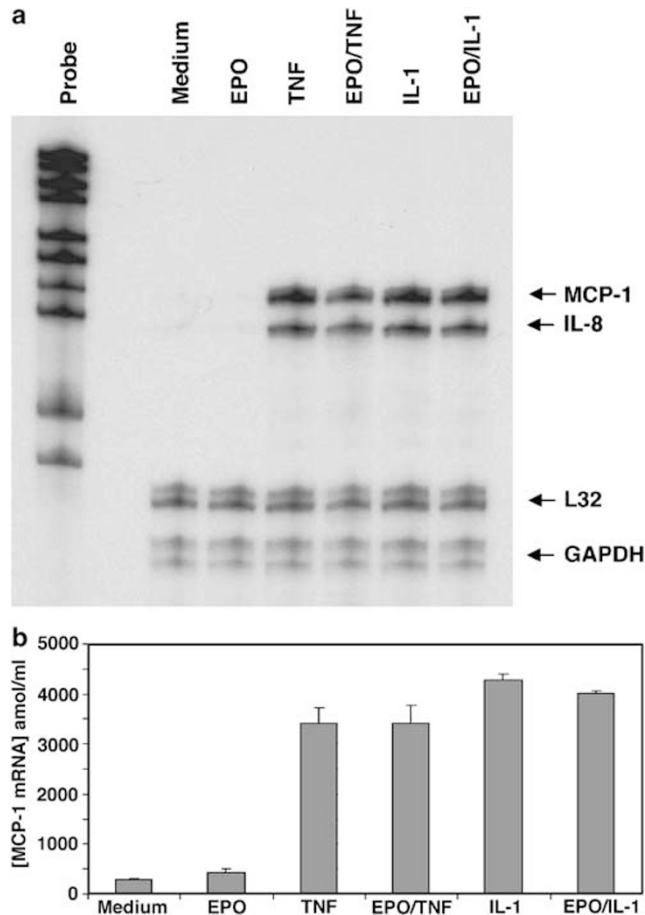


Figure 4 MCP-1 mRNAs in cytokine-activated HUVECs detected by (a) ribonuclease protection assays and (b) colorimetric mRNA quantitation assays. Cells were exposed to 5 U/ml EPO or medium for 24 h followed by cytokine (TNF- α or IL-1 β at 10 ng/ml, respectively) or medium, respectively, for 4 h. In the experiments depicted in (a), specific transcripts for MCP-1 and the 'house-keeping' genes, L32 and GAPDH as controls were detected by ribonuclease protection assays. In the experiments shown in (b), total mRNA was hybridized with MCP-1 gene-specific biotin-labeled capture oligonucleotide probes and digoxigenin-labeled detection probes. Both data sets are representative of two independent experiments. In the mRNA quantitation assay each sample was analyzed in duplicate.

time course experiments employed chemical NO donors (NONOates) with varying half-lives (PAPA NONOate ($t_{1/2}$ = 15 min at pH 7.4, 37°C), diethylamine NONOate ($t_{1/2}$ = 2 min at pH 7.4, 37°C), and spermine NONOate ($t_{1/2}$ = 39 min at pH 7.4, 37°C)). Compared to cells that were treated with TNF- α or IL-1 β alone, EPO pretreatment (followed by either TNF- α or IL-1 β) induced significant increases in MCP-1 concentrations (Figure 5). When EPO-pretreated cells were incubated with PAPA NONOate for 30 min prior to TNF- α or IL-1 β stimulation, a significant decrease in MCP-1 concentration was observed when compared to cells that were treated with EPO and cytokine (IL-1 β or TNF- α , respectively). Similar results were observed when the NO donors diethylamine and spermine NONOate (data not shown) were employed.

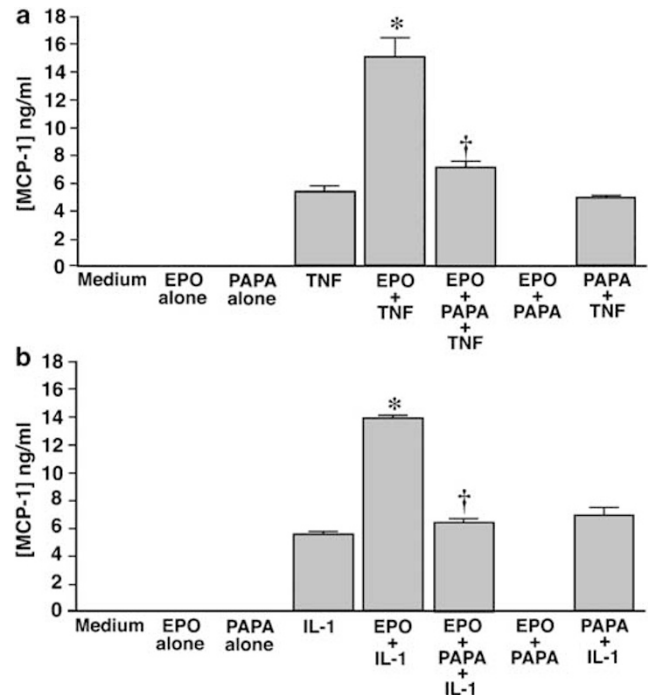


Figure 5 (a) Suppression of the EPO-mediated augmentation of TNF- α -induced MCP-1 expression by PAPA NONOate. HUVECs were pretreated for 24 h with either medium or EPO (5 U/ml), followed by a 30 min incubation with medium or PAPA NONOate (100 μ M). The cells were subsequently exposed to either medium or TNF- α (10 ng/ml) for 8 h. These data represent mean \pm s.e. from three independent experiments, each performed in triplicate. *Significant difference from positive control (TNF- α alone). †Statistically significant difference ($P < 0.05$) from HUVECs treated with a combination of EPO and TNF- α . (b) Suppression of the EPO-mediated augmentation of IL-1 β -induced MCP-1 expression by PAPA NONOate. HUVECs were pretreated for 24 h with either medium or EPO (5 U/ml), followed by a 30 min incubation with medium or PAPA NONOate (100 μ M). The cells were subsequently exposed to either medium or IL-1 β (10 ng/ml) for 8 h. These data represent mean \pm s.e. from three independent experiments, each performed in triplicate. *Significant difference from positive control (IL-1 β alone). †Statistically significant difference ($P < 0.05$) from HUVECs treated with a combination of EPO and IL-1 β .

Recombinant Human EPO Downregulates the Expression of eNOS in HUVECs

HUVECs maintained in medium for 24 h expressed high concentrations of eNOS as measured by Western blot (Figure 6, lane 2). No differences in eNOS expression were seen in HUVECs grown in the presence of 1 or 25 U/ml, respectively, of EPO for 24 h. Marked suppression of eNOS expression occurred after a 24 h incubation in the presence of 5 U/ml EPO (Figure 6, lane 4).

EPO Treatment Reduces eNOS mRNA Concentrations in HUVECs

RNase protection experiments revealed that a 24 h treatment of HUVECs with EPO (5 U/ml) resulted in

a 64% reduction in ecNOS mRNA when compared to HUVECs incubated in medium alone (Figure 7, see integrated density ratios).

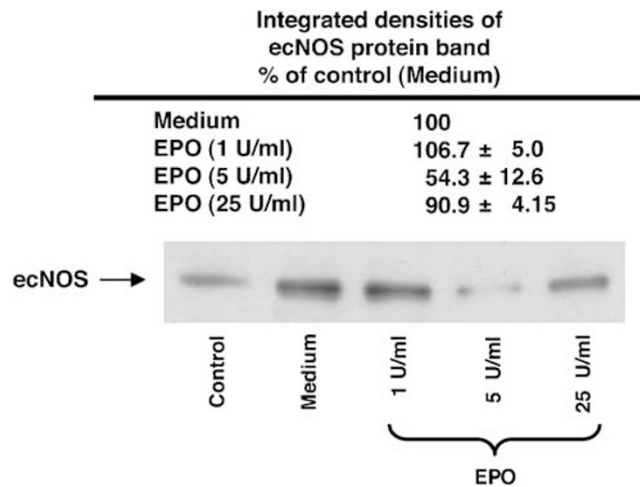


Figure 6 Downregulation of ecNOS protein expression by EPO. HUVECs were treated with the specified concentrations of EPO or medium for 24 h. The protein expression of ecNOS was measured by Western blot. Control (lane 1) represents a human endothelial lysate derived from an aortic endothelial cell line that was provided with the ecNOS antibody. All lanes in the gel were loaded with equal amounts of total protein and integrated densities of the ecNOS protein bands obtained from scannings of autoradiographs from two independent experiments are displayed in the table.

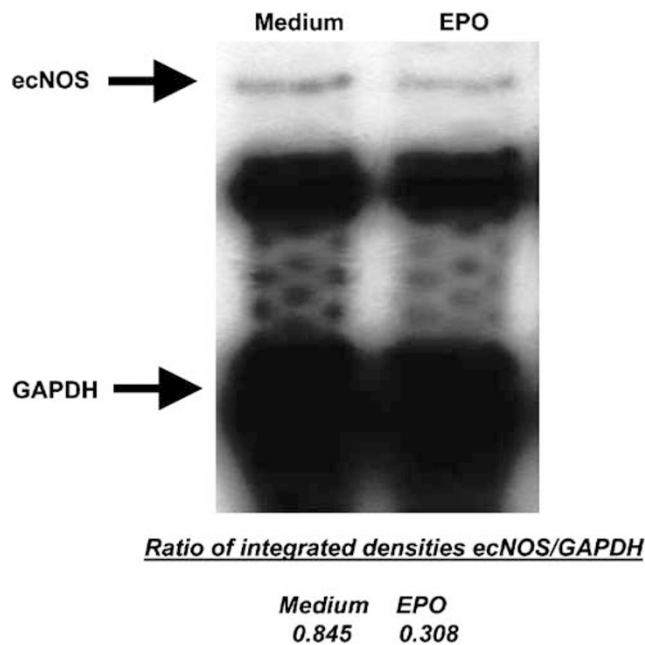


Figure 7 Downregulation of ecNOS mRNA expression by EPO. HUVECs were treated with medium or EPO (5 U/ml) for 24 h. Total RNA was extracted and utilized in a ribonuclease protection assay. Integrated densities of the ecNOS mRNA bands are displayed below. These data are representative of three independent experiments.

EPO Decreases NOS Activity in HUVECs

To elucidate whether the decrease in NOS-specific mRNA and protein concentrations also resulted in reduced enzymatic activity, we performed a NOS detect assay. This assay is based on the NOS-catalyzed conversion of radiolabeled L-arginine to L-citrulline. In HUVECs incubated in the presence of 5 U/ml EPO for 24 h, NOS activity was reduced by 28% when compared to media-treated control cells.

EPO Stimulates Cell Proliferation in HUVECs and HVSMSCs

Atherosclerosis is a fibroproliferative disease of the arterial intima.²⁸ The effect of EPO treatment on endothelial cell and smooth muscle cell proliferation was assessed using a BrdU assay. EPO treatment alone (at 1 and 5 U/ml) significantly increased HUVEC proliferation (Figure 8a). Nearly identical results were observed in HVSMSCs (Figure 8b). Treatment with 1 μ M DOX inhibited proliferation of both HUVECs and HVSMSCs.

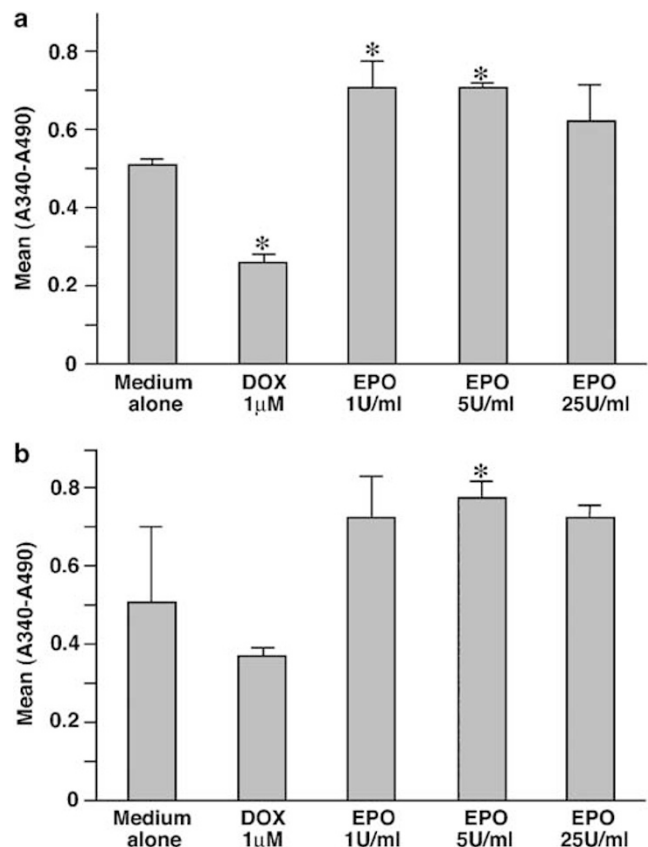


Figure 8 Stimulation of endothelial and smooth muscle cell proliferation by EPO. HUVECs (a) and HVSMSCs (b) were exposed to the specified concentrations of EPO or 1 μ M doxorubicin for 24 h and then incubated in the presence of BrdU for 20 h. Incorporation of BrdU into proliferating cells was quantitated by immunoassay. These data represent mean \pm s.e. from three independent experiments, each performed in triplicate. *Significant difference from control (medium alone) as determined by Bonferroni comparison of means.

Discussion

The advents of both dialysis and renal transplantation in patients with chronic renal disease (CRD) have reduced the mortality rate due to uremia. Accelerated cardiovascular disease has become the leading cause of death in this patient population. EPO, currently the drug with the highest sales worldwide is routinely used to treat the anemia of CRD patients and those with other nonrenal forms of anemia.²⁹ While treatment with EPO has severely reduced the transfusion requirements and clearly results in an improved quality of life in CRD patients it may also contribute to vascular dysfunction.^{4,5} Several studies have suggested that EPO may contribute to the development of hypertension and thrombosis.^{3,30} Resistance to the vasodilatory effects of NO has been implicated in these processes.²³ Research aimed at elucidating a possible link between NO and EPO-induced vascular dysfunction has yielded conflicting results.^{8,19,20,22,23,31}

The present study indicates that EPO may exert proatherogenic activity by stimulating endothelial and smooth muscle cell proliferation and by augmenting cytokine-induced expression of MCP-1 in ECs. Exogenous NO suppresses the EPO-mediated increases in cytokine-induced MCP-1 expression. EPO downregulates eNOS expression at both the protein and mRNA level and suppresses NOS activity. These observations suggest that there is an interplay between NO and EPO in the expression of MCP-1 by vascular wall cells and that NO can prevent the EPO-mediated augmentation of cytokine-induced endothelial MCP-1 production. The EPO-mediated upregulation of MCP-1 expression detected by EIA is not accompanied by increased cytosolic-to-nuclear translocation of NF- κ B. Whether the EPO-mediated augmentation of IL-1 β -induced MCP-1 expression is a result of increased AP-1 activation is unclear. However, since RNase protection and mRNA quantitation assays failed to reveal increases in cytokine-induced MCP-1 mRNA concentrations following EPO pretreatment, the marked rise in MCP-1 protein concentrations detected by EIA does not appear to occur at the transcriptional level. While our study does not address mechanistic aspects of the EPO-induced upregulation of MCP-1 expression, post-transcriptional regulation of MCP-1 gene expression may present a plausible explanation. In this regard, recent evidence suggests that gene expression is regulated, at least in part, at a post-transcriptional level by factors inducing the extremely rapid degradation of messenger RNAs.³² In fact, Brueckmann *et al*³³ recently observed that activated protein C upregulates MCP-1 expression in HU-VECs. Similar to our observations, MCP-1 upregulation in their experiments was not accompanied by increased NF- κ B and AP-1 activation. Using the same type of colorimetric mRNA quantitation assays employed in our studies, the authors concluded that

upregulation of MCP-1 occurred most probably by increasing MCP-1 mRNA stability rather than through transcriptional activation via the NF- κ B or AP-1 pathway.

Several reports indicate that treatment of endothelial and smooth muscle cells with EPO results in an augmentation of intracellular calcium concentrations and in the activation of tyrosine kinase-mediated signaling pathways.^{34,35} However, the EPO concentrations used in these studies are significantly higher (up to 250 U/ml) than those used in our experiments. In the present study, EPO was used at a concentration of 5 U/ml in all experiments except the dose-response effect on eNOS protein expression. Even in this experiment 5 U/ml was found to be the optimal concentration required to downregulate eNOS expression (Figure 6). Although we do not have an explanation for this dose-response effect, our observations are supported by reports from other laboratories. Anagnostou *et al*³⁶ serendipitously noticed the mitogenic effects of EPO on endothelial cells and were the first to report optimal stimulation of cell proliferation by EPO at 5 U/ml when concentrations up to 20 U/ml were tested. Subsequently, a number of published studies have documented biological effects of EPO on vascular smooth muscle and endothelial cells.^{8,34-37} In all of these *in vitro* studies the concentrations of EPO used range from 4 to 250 U/ml.^{6,8,31,37} While the physiological concentrations of EPO in most healthy animals and humans are in the range of 4–30 mU/ml, up to 100-fold increases can be seen in severe anemias (thus resulting in plasma concentrations as high as 3 U/ml).^{36,38} Furthermore, relatively high EPO plasma concentrations in the order of 1–5 U/ml may be obtained intermittently in patients immediately after intravenous injection of the hormone.^{6,39,40}

Our observation that EPO stimulates proliferation of endothelial and vascular smooth muscle cells is particularly interesting in view of the fact that an increased intima/media thickness of the carotid artery has been observed in hemodialysis patients when compared to age-matched healthy controls but to our knowledge no published study to date has directly addressed the effect of EPO therapy on vessel wall cell proliferation *in vivo*.⁴¹

It is currently believed that early atherosclerosis is marked by a reduction in endothelial NO production.¹¹ Schmidt and Baylis²⁴ reported that patients with CRF are in a NO-deficient state. Reduced vascular NOS activity and eNOS and iNOS expression were also observed in rats with surgically induced CRF.⁴² In these animals, calcium channel blockade resulted in a significant rise in urinary excretion and plasma concentrations of nitrite and nitrate to supranormal values. This was accompanied by a complete restoration of renal and vascular tissue eNOS and iNOS proteins. These data suggest that the CRF-associated downregulation of vascular eNOS and iNOS may be due to dysregulation of

intracellular calcium. Since EPO has been shown to augment intracellular calcium stores both *in vivo* and *in vitro*, EPO-mediated downregulation of NOS expression may result from a dysregulation of calcium homeostasis.⁴³ In the context of these findings, our observation that exogenous NO reverses the EPO-mediated augmentation of cytokine-induced MCP-1 expression and that EPO downregulates eNOS, suggests a possible mechanism through which EPO may contribute to the accelerated atherosclerosis of CRD patients. However, the results from our *in vitro* studies must be extrapolated to the CRD population with great caution since our observations have not yet been confirmed *in vivo* and EPO is not only administered to patients with CRD but also to those that suffer from various nonrenal forms of anemia. It is noteworthy that our results are supported by a recent study by Wang and Vaziri⁸ who examined the effect of EPO on total NO (nitrate and nitrite) in human coronary artery endothelial cells. As in our experiments, these researchers found that a 24 h incubation with EPO at 5 U/ml resulted in downregulation of eNOS protein expression.⁸ Furthermore, EPO significantly reduced NO_x production by both quiescent ($15.4 \pm 1.1\%$) and proliferating ($22.2 \pm 1.0\%$) endothelial cells. Banerjee *et al*³¹ observed that short-term exposure of ECs had little or no effect on NOS transcript. However, in contrast to our results and those of Wang and Vaziri, these researchers detected a significant increase in NOS mRNA after 24 h of exposure to EPO but cells needed to be exposed to EPO for several days in order to increase NO production.^{8,31}

A cross-sectional 3-year prospective placebo-controlled study in a small number of hemodialysis patients revealed that EPO significantly increased plasma MCP-1 concentrations.⁴³ After EPO withdrawal MCP-1 concentrations returned to pretreatment levels. Papayianni *et al*^{44,45} compared MCP-1, ICAM-1 and VCAM-1 concentrations in hemodialysis patients. The dialysis session resulted in a significant increase in the plasma concentrations of all three proinflammatory mediators. These observations support the hypothesis that the increased oxidative stress that is known to result from dialysis treatment *per se* may contribute to the increased expression of proinflammatory mediators.^{46,47}

Interestingly, in the study by Papayianni *et al*⁴⁵ predialysis concentrations of MCP-1, ICAM-1, and VCAM-1 were also significantly elevated in hemodialysis patients when compared to healthy controls. It is possible that certain disease-associated factors (eg the uremic state *per se*) may be responsible for the significant difference in predialysis and control MCP-1 concentrations but a contributing effect by EPO cannot be ruled out since more than 86% of the patients enrolled in this study were treated with EPO. However, since plasma EPO concentrations were not assessed in these two studies and due to other confounders, we cannot

conclusively attribute the increased MCP-1 concentrations to EPO therapy.

Nevertheless, these two clinical studies in combination with our *in vitro* data may provide indirect evidence that EPO may augment endothelial MCP-1 elaboration *in vivo* which, in turn, may result in the accelerated recruitment of monocytes to atherosclerotic lesions. Probably the most convincing evidence to date for the potential atherogenic effects of EPO is provided by a very intriguing study by Tous *et al*⁴⁸ who recently observed that continuous administration of EPO to apolipoprotein E-deficient mice resulted in enhanced lesion size. Unfortunately, the potentially confounding effects of EPO on increased blood pressure are not addressed in this study. Considering the vast number of anemic patients being treated with EPO, our *in vitro* data in combination with the *in vivo* observations by Tous *et al*⁴⁸ clearly warrant further studies aimed at elucidating the role of EPO in atherogenesis. In the long run, a better understanding of the pro- and antiatherogenic effects of EPO may lead to the development of novel therapeutic agents that correct anemia with maximum vasculoprotective effects.

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Duality of interest

The authors have no duality of interest to declare.

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