Essential Role of Endothelial Nitric Oxide Synthase in Vascular Effects of Erythropoietin

Livius V. d’Uscio, Leslie A. Smith, Anantha V. Santhanam, Darcy Richardson, Karl A. Nath, Zvonimir S. Katusic

Abstract—Erythropoietin (EPO) fosters tissue oxygenation by stimulating erythropoiesis. More recently, EPO has been recognized as a tissue-protective cytokine. In this study, we tested the hypothesis that endothelial NO synthase (eNOS) plays a key role in the vascular protective effect of EPO. A murine model of wire-induced injury of carotid artery was used to examine the effect of EPO on endothelial repair and arterial wall architecture. Recombinant human EPO (1000 U/kg, SC, biweekly) was administered for 2 weeks in wild-type and eNOS-deficient mice after which reactivity of isolated carotid arteries was studied in vitro, and the vasculature was histologically assessed. Injured arteries exhibited impairment of endothelium-dependent relaxations to acetylcholine ($P<0.05$). This was associated with increased medial cross-sectional area ($P<0.05$). EPO upregulated expression of phosphorylated Ser1177-eNOS and normalized the vasodilator response to acetylcholine ($P<0.05$). Furthermore, EPO prevented the injury-induced increase in medial cross-sectional area ($P<0.05$). The vascular protective effects of EPO were abolished in eNOS-deficient mice. Most notably, EPO significantly increased systolic blood pressure and enhanced medial thickening of injured carotid arteries in eNOS-deficient mice ($P<0.05$). Our results demonstrate that EPO prevents aberrant remodeling of the injured carotid artery. The protective effects of EPO are critically dependent on activation of eNOS. (Hypertension. 2007;49:1142-1148.)

Key Words: nitric oxide synthase ☒ endothelium ☒ vasculature ☒ erythropoietin ☒ mice

Nitric oxide is a potent vasodilator that is generated in endothelial cells from L-arginine by constitutively expressed endothelial NO synthase (eNOS).1,2 It has been recognized that reduced NO bioavailability is a major mechanism responsible for initiation and progression of endothelial dysfunction in vascular disease.3 Furthermore, removal of the endothelium by mechanical vascular injury invariably leads to hyperplasia at the site of injury.4–7 This suggests that the endothelium also regulates vascular structure and that its presence assures quiescence of vascular smooth muscle cells.

Erythropoietin (EPO) is a hypoxia-inducible hormone that is essential for normal erythropoiesis in bone marrow. Administration of recombinant human EPO is an efficient and safe therapeutic approach to anemia associated with chronic renal failure.8 However, EPO receptors are also widely distributed in the cardiovascular system, including endothelial, smooth muscle, cardiac, and other cell types, and nonhematopoietic effects of EPO are increasingly recognized.9–11 For example, it has been reported that EPO has potentially beneficial effects on cardiovascular function.11 Furthermore, EPO increases the number of functionally active endothelial progenitor cells, thus enhancing angiogenesis.12,13 However, little is known about the mechanisms underlying vascular effects of EPO in vivo. The present study was, thus, designed to determine whether EPO prevents pathological repair of injured blood vessel. We hypothesized that eNOS plays a critical role in vascular protective effects of EPO.

Methods

Experimental Animals of Carotid Artery Injury
Male C57BL/6j (wild-type) and eNOS-deficient mice (C57BL/6j-Nos3tm1Unc) were obtained from Jackson Laboratory (Bar Harbor, Maine) and were maintained on standard chow with free access to drinking water. All of the experimental protocols were approved by the institutional animal care and use committee of Mayo Clinic. Wire-induced vascular injury was performed in the left common carotid artery.7 Twelve-week-old mice were anesthetized with 90 mg/kg of ketamine and 10 mg/kg of xylazine (IP). Using a dissecting microscope, a midline incision (1 to 1.5 cm) was made, and the salivary glands were moved laterally to access the left carotid artery. Two ligatures were placed proximally and distally around the external carotid artery. A transverse arteriotomy was performed to introduce a 0.003-in stainless steel wire (folded in half to produce a smooth loop to insert into artery). The wire was moved back and forth along the entire length of the artery 3 times using a slight rotation to remove the endothelium. After removal of the wire, the ligatures were tied off, and blood flow was restored. Right uninjured carotid arteries served as controls. Two ligatures were placed proximally and distally around the external carotid artery. A transverse arteriotomy was performed to introduce a 0.003-in stainless steel wire (folded in half to produce a smooth loop to insert into artery). The wire was moved back and forth along the entire length of the artery 3 times using a slight rotation to remove the endothelium. After removal of the wire, the ligatures were tied off, and blood flow was restored. Right uninjured carotid arteries served as controls. Mice were randomly distributed to an injury group (PBS, Gibco) and an injury+EPO group (recombi-
nant human EPO α 1000 U/kg of body weight, biweekly, SC (Amgen). The dose of EPO was selected based on previous pharmaco-kinetic studies in mice. After 14 days of treatment, the animals were euthanized (pentobarbital, 60 mg/kg, IP), and carotid arteries and aortas were harvested. In separate experiments, wild-type mice were treated for 3 days with PBS or EPO (1000 U/kg of body weight, SC) once daily.

**Systolic Blood Pressure**

Mice were trained for blood pressure measurement as described, and systolic blood pressure (SBP) was recorded in quiescent mice by a tail-cuff method (Harvard Apparatus Ltd) before and on day 14 of treatment.

**Blood Cell Count**

Mice were anesthetized in a bell jar containing isoflurane 1%, and blood was quickly drawn from the orbital venous sinus. Blood cell counts were performed with ABAxis VetScan HMT Hematology System. Reticulocytes were enumerated using Ricca New Methylene Blue “N” staining (Ricca Chemical Co).

**Measurement of EPO Levels**

At the end of treatments, EPO levels were measured in plasma by a chemiluminescence immunoassay (Nichols Institute Diagnostics).

**Morphological Analysis of Carotid Arteries**

To evaluate regeneration of the endothelium, 0.5% Evans blue dye was injected into the heart ventricle. Thirty minutes after the injection of Evans blue, carotid arteries were harvested and analyzed. Morphological analyses were performed on perfused and fixed vessels in buffered formalin (10%). Each artery was embedded in paraffin, and cross-sections were continuously cut every 100 μm from 1 edge to the other edge of carotid artery (8 sections). Each section was mounted on slides and subjected to standard Verhoeff–Van Gieson staining. Adobe Photoshop 6.0 was used to analyze the medial cross-sectional area (CSA) of arteries.

**Vascular Reactivity Study**

Reactivity studies of 4-mm-long common carotid arteries were performed in vitro using a video dimension analyzer (Living Systems Instrumentation), as described. Left (injured) and right (control) carotid rings were studied in parallel.

**Western Blot Analysis**

Equal amounts of protein (100 μg) were separated by SDS-PAGE and transferred to nitrocellulose membrane (Amersham). Mouse monoclonal antiphosphorylated Ser1177-eNOS, anti-eNOS (1:250; Transduction Laboratories), and anti-actin (1:50,000; Sigma) were used.

**Calculations and Statistical Analysis**

Results are expressed as mean±SEM, and “n” indicates the number of animals from which tissues were harvested. Relaxations are expressed as a percentage of maximal relaxations induced by papaverine. The concentration–response curves of the different groups were compared by ANOVA for repeated measurements followed by Bonferroni’s correction. Single values were compared by 1-way ANOVA with Bonferroni’s correction for multiple comparisons. For simple comparisons between 2 groups, an unpaired Student’s t test was used where appropriate. A value of P<0.05 was considered significant.

**Results**

**Mice Characteristics**

Treatment with EPO for 3 or 14 days significantly elevated plasma levels of EPO (P<0.05; Table 1). Numbers of reticuloocytes and red blood cells, along with hematocrit and hemoglobin, were increased after the administration of EPO for 14 days to wild-type mice (P<0.05; Table 1). EPO treatment for 2 weeks did not increase the number of circulating white blood cells (Table 1), suggesting that no systemic inflammation occurred. Platelet counts were unaffected after treatment with EPO after either time point (Table 1). Finally, EPO did not increase SBP in wild-type mice after 14 days of treatment (Table 1).

**Morphology of Injured Carotid Artery**

As demonstrated by Evans blue staining, complete denudation of the endothelium was observed in the carotid artery 1 hour after injury procedure. However, 2 weeks after injury, complete re-endothelialization of injured arteries and those treated with EPO was detected by Evans blue staining (n=6; data not shown). Morphological analysis demonstrated that the number of the carotid arteries increased CSA of the media (Figure 1A and 1B; P<0.05). Interestingly, treatment with EPO for 2 weeks significantly reduced medial CSA of the injured carotid artery (Figure 1C and 1D; P<0.05). In contrast, EPO treatment had no effect on vascular wall parameters in uninjured carotid arteries (Figure 1D).

**Endothelial Function of Carotid Artery**

Two weeks after carotid artery injury, endothelium-dependent relaxations to acetylcholine were impaired compared with controls (P<0.05; Figure 2A). EPO treatment for 14 days normalized endothelium-dependent relaxations of injured arteries in wild-type mice (P<0.05; Figure 2A). On the other hand, EPO did not affect endothelium-dependent relaxations of control arteries in wild-type mice, whereas the relaxations were completely abolished in eNOS-deficient mice (Figure 2B), suggesting that vascular effects of EPO are entirely dependent on NO.

Endothelium-independent relaxations to the NO donor, diethylammonium (z)-1-(N,N-diethylamino)diazen-1-ium-1,2-diolate (Cayman) (DEA-NONOate), were unaltered in injured carotid artery and after treatment with EPO (Figure 2C). Contractions to U46619 (3×10^-8 mol/L) were not significantly different between control and injured carotid.

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**Table 1. Characteristics of Wild-Type Mice and Those Treated With EPO for 3 or 14 Days**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>C57BL/6J</th>
<th>EPO, 3 Days</th>
<th>EPO, 14 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPO, μU/mL</td>
<td>4.6±0.9</td>
<td>284.6±64.9*</td>
<td>162.2±46.6*</td>
</tr>
<tr>
<td>White blood cells, 10^12/mm^3</td>
<td>10.3±0.4</td>
<td>17.3±1.1*</td>
<td>11.8±1.0</td>
</tr>
<tr>
<td>Lymphocytes, 10^9/mm^3</td>
<td>7.5±0.6</td>
<td>12.9±0.7*</td>
<td>8.3±0.7</td>
</tr>
<tr>
<td>Monocytes, 10^9/mm^3</td>
<td>0.31±0.03</td>
<td>0.53±0.03*</td>
<td>0.45±0.08</td>
</tr>
<tr>
<td>Granulocytes, 10^9/mm^3</td>
<td>2.6±0.5</td>
<td>4.0±0.7*</td>
<td>3.1±0.3</td>
</tr>
<tr>
<td>Red blood cells, 10^12/mm^3</td>
<td>7.8±0.2</td>
<td>7.5±0.1</td>
<td>9.1±0.2*</td>
</tr>
<tr>
<td>Reticulocytes, %</td>
<td>1.7±0.2</td>
<td>ND</td>
<td>5.2±0.3*</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>38.0±1.1</td>
<td>40.0±0.5</td>
<td>47.1±1.0*</td>
</tr>
<tr>
<td>Hemoglobin, g/dL</td>
<td>14.5±0.2</td>
<td>15.7±0.1</td>
<td>17.7±0.5*</td>
</tr>
<tr>
<td>Platelets, 10^11/mm^3</td>
<td>664±51</td>
<td>609±42</td>
<td>715±82</td>
</tr>
<tr>
<td>SBP, net increase in mm Hg</td>
<td>4.0±0.28</td>
<td>ND</td>
<td>8.3±2.0</td>
</tr>
</tbody>
</table>

C57BL/6J indicates wild-type mice; ND, not determined. SBP net increase was obtained before and at end of treatment. Data are mean±SEM of 4 to 7 mice. *P<0.05 vs C57BL/6J mice (ANOVA+Bonferroni’s).
artery (36±2% and 44±3%, respectively) and those treated with EPO (44±5% and 50±8%, respectively).

**Vascular eNOS Protein Expression**
To evaluate the mechanisms underlying the observed beneficial effects of EPO, we determined eNOS expression by Western blot analysis. Three or 14 days of treatment with EPO did not affect protein expressions of eNOS in the aorta (data not shown). However, EPO treatment increased expressions of phosphorylated eNOS in the arterial wall independent of the length of treatment (Figure 3; *P* < 0.05).

**Effect of EPO in eNOS-Deficient Mice**
To further determine the role of eNOS in the observed protective effects of EPO, we performed studies on eNOS-deficient mice. Medial CSA was significantly increased in injured arteries as compared with controls (Figure 4B; *P* < 0.05). Most interestingly, in contrast to wild-type mice, treatment with EPO for 14 days further increased the medial CSA of injured carotid arteries in eNOS-deficient mice (Figure 4C and 4D; *P* < 0.05). EPO tended to increase CSA in eNOS-deficient mice uninjured carotid arteries (Figure 4D; *P* = 0.14). In addition to structural vascular changes, EPO further enhanced SBP in hypertensive eNOS-deficient mice (Table 2; *P* < 0.05).

**Discussion**
This is the first study to comprehensively investigate the in vivo effect of EPO on structural and functional alterations of
arteries after injury. We report several novel findings. First, treatment of wild-type mice with EPO for 14 days abrogated the impairment in endothelium-dependent relaxations to acetylcholine, which occurs after injury and attenuated the expansion in the medial CSA in injured carotid arteries. Second, vascular protein expression of phosphorylated eNOS was increased to a similar degree after 3 or 14 days of treatment with EPO, thus demonstrating that the stimulatory effect of EPO is independent of increased shear stress caused by elevation of hematocrit. Third, the observed protective effects of EPO were abolished in eNOS-deficient mice. Indeed, deletion of eNOS gene unmasked the stimulatory effect of EPO on vascular medial thickness. Fourth, EPO increased SBP in eNOS-deficient mice.

Endothelial cell loss is a major contributing factor to the pathological repair of the injured blood vessel.6 As reported in previous studies,16,19,20 wire-induced injury of the carotid artery caused a significant medial thickening without formation of neointima. The genetic background of the mouse is of critical importance in pathological repair of the injured blood vessel. For instance, Swiss Webster mice used in the original wire injury model described by Lindner et al7 develops neointima after endothelial denudation. Because our focus has been on endothelial dysfunction of regenerated carotid endothelium rather than neointima formation, we examined the ability of regenerated endothelium to produce NO by studying endothelium-dependent relaxation to acetylcholine. We and others have demonstrated that, in the mouse carotid artery, endothelium-dependent relaxation is mainly mediated by production and release of NO from endothelial cells via eNOS.17,21 Despite complete re-endothelialization, the regenerated endothelium in the carotid artery is dysfunctional as
remarkable that, in eNOS-deficient mice, EPO increased SBP
information regarding the effect of EPO on vascular wall
has reduced the population of eNOS-positive cells.23 Thus,
cytokine.11,24,25 In the present study, we showed that EPO
independent relaxations to NO were not affected in injured
This conclusion is supported by the fact that endothelium-
caused by reduced production and/or bioavailability of NO
rather than reduced reactivity of smooth muscle cells to NO.
This conclusion is supported by the fact that endothelium-
relaxations to NO were not affected in injured
arteries.
EPO is increasingly regarded as a potent tissue-protective
cytokine.11,24,25 In the present study, we showed that EPO
significantly reduced medial CSA and improved endothelium-
dependent relaxations to acetylcholine in carotid arteries
after injury. This is consistent with previous studies demonstrating increased vascular NO production and augmented
NO-mediated endothelium-dependent relaxations in experimental animals with high plasma levels of EPO.26–29 Because an increased number of circulating red blood cells and subsequent increase in shear stress is a powerful stimulus for eNOS and NO production in endothelial cells,26,30 it is possible that the protective effect of EPO may depend in part on this effect. However, in our experiments, administration of EPO for 3 days, although not affecting the number of circulating red blood cells, stimulated phosphorylation of eNOS to a similar degree as did treatment with EPO for 14 days; these findings indicate that a direct stimulatory effect of EPO on vascular endothelium, and one that is independent of increases in hematocrit, is responsible for upregulation of phosphorylated eNOS. The exact molecular mechanism of EPO-induced eNOS activation remains to be resolved. Most recently, it was demonstrated that activation of eNOS by hypoxia was abolished in EPO-receptor–deficient mice, the latter mutants also exhibiting an exacerbation of pulmonary hypertension and vascular injury; these findings suggest that the vascular protective effects of EPO are dependent on NO production via activation of the EPO receptor in endothelial cells.31,32 This conclusion is entirely consistent with our observations that EPO does not prevent the increase in medial CSA of injured carotid arteries in eNOS-deficient mice. Because recovery of NO-mediated endothelial function is a major therapeutic goal in the prevention and treatment of vascular disease,33 we speculate that the observed effects of EPO may have important therapeutic implications.
As clinically recognized, systemic hypertension is one of the major adverse effects observed in patients treated with EPO.34 The results of our study offer novel and important information regarding the effect of EPO on vascular wall architecture that may elucidate the pressor effect of EPO. It is remarkable that, in eNOS-deficient mice, EPO increased SBP and exacerbates medial thickening of injured carotid arteries. We regard this observation as an important one, because it underscores the importance of eNOS activation as the vascular adaptations to increased circulating levels of EPO. Our findings are the first to demonstrate that loss of eNOS in vivo predisperses the blood vessel wall to maladaptive (prohypertensive or proatherogenic) remodeling in response to EPO. In agreement with our findings, the inhibitory effect of NO on smooth muscle cell proliferation and preservation of vascular architecture is well documented.35 The exact mechanism of the hypertensive effect of EPO in eNOS-deficient mice remains to be determined. However, a previous study showed that EPO can induce production of endothelin-1.36 Contractions to endothelin-1 are enhanced in aortas of EPO transgenic mice treated with the NO synthase inhibitor, thus suggesting that in the absence of NO endothelin-1 may contribute to the hypertensive effect of EPO.36 Consistent with this concept, studies on cultured endothelial cells demonstrated that inactivation of NO synthesis caused increased production of endothelin-1.37

Previous study by Rudic et al demonstrated abnormal vascular remodeling of ligated eNOS-deficient carotid arteries resulting in increased vascular CSA. At variance with this observation, we did not detect a significant difference in medial CSA between wild-type and eNOS-deficient arteries after vascular injury. The exact reason for this discrepancy is not immediately apparent. However, methodologic differences offer one possible explanation. Wire-induced injury mechanically removes the endothelial layer, thus abolishing endothelial influence on medial smooth muscle cells. In contrast, ligation of the carotid artery does not cause abrupt cessation of production and release of endothelium-derived vasoactive factors, including NO. We speculate that, in the ligation model, a more pronounced difference in the vascular concentration of NO between wild-type and eNOS-deficient arteries may account for a significant difference in vascular remodeling. On the other hand, mechanical removal of endothelium abolishes the major difference between wild-type and eNOS-deficient mice. In wild-type mice, loss of NO persists during endothelial repair (as demonstrated by impairment of endothelium-dependent relaxations), thus blunting the difference in vascular NO levels between wild-type and eNOS-deficient mice. In addition, we studied vascular remodeling only 2 weeks after injury. It is quite possible that the difference in vascular remodeling between wild-type and eNOS-deficient mice becomes apparent at a later time point when NO biosynthesis in wild-type animals is fully recovered.

In summary, the present study showed that vascular injury in the wild-type mouse carotid artery causes dysfunction of the regenerated endothelium as reflected in impaired endothelium-dependent relaxations and increase of the medial CSA. Long-term treatment with EPO improved endothelium-dependent relaxations and reduced medial CSA. Interestingly, our studies in eNOS-deficient mice clearly demonstrated that the beneficial effects of EPO are entirely dependent on functional eNOS. Finally, our studies indicate

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**TABLE 2. SBP of Wild-Type and eNOS-Deficient Mice and Those Treated With EPO for 14 Days**

<table>
<thead>
<tr>
<th>Week</th>
<th>C57BL/6J</th>
<th>C57BL/6J + EPO</th>
<th>eNOS−/−</th>
<th>eNOS−/− + EPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>118±1</td>
<td>120±2</td>
<td>140±1*</td>
<td>139±3*</td>
</tr>
<tr>
<td>2</td>
<td>123±2</td>
<td>128±2</td>
<td>137±3*</td>
<td>154±2†</td>
</tr>
</tbody>
</table>

C57BL/6J indicates wild-type mice. Data are mean±SEM of 4 mice.

*P<0.05 vs C57BL/6J mice.
†P<0.05 vs eNOS-deficient mice (ANOVA+Bonferroni’s).
that the absence of eNOS transforms EPO from a vasoprotective agent to one that promotes hypertension and adverse and aberrant remodeling of the injured vasculature.

**Perspectives**

The recognition of availability of eNOS as a critical arbiter whereby EPO elicits adaptive or maladaptive responses in the vasculature has important and far-reaching clinical ramifications. In this regard, clinical studies have called for attention to increased mortality in certain subsets of patients with vascular disease in whom hematocrit is normalized by EPO. Because polymorphisms in the eNOS gene, which influence NO synthase activity, are recognized, it is conceivable that patients with endothelial dysfunction caused by the loss of biosynthesis or decreased biological activity of NO could be at higher risk of adverse cardiovascular effects of EPO. This concept certainly deserves to be tested in future clinical studies.

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**Disclosures**

None.

**References**


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