Darbepoetin-Alpha Does Not Promote Microvascular Thrombus Formation in Mice
Role of eNOS-Dependent Protection Through Platelet and Endothelial Cell Deactivation

Nicole Lindenblatt, Michael D. Menger, Ernst Klar, Brigitte Vollmar

Objective—Erythropoietin (EPO) treatment has become the standard treatment of renal anemia. Though a link between hematopoiesis-stimulating drugs and thrombosis has not been proven, it is generally assumed that systemic application of EPO and its analogues increases the risk for thrombotic events.

Methods and Results—Here we show in C57BL/6J mice that 4-week treatment with the long-lasting EPO analogue darbepoetin-alpha (DPO) at a dose of 10 μg/kg/week induces a reduction of platelet reactivity using flow cytometry and Western blot analysis of tyrosine-specific platelet phosphorylation. Additionally, immunohistochemistry of endothelial adhesion molecule expression and ELISA of circulating endothelial activation markers demonstrated a reduced endothelial activation. Immunohistochemistry and RT-PCR analysis revealed a significant (P<0.05) increase of eNOS expression. Further, DPO did not exert prothrombogenic effects in a murine intravital microscopic thrombosis model of the cremaster muscle. The role of eNOS in prevention of DPO-mediated microvascular thrombosis is further underlined by a significantly accelerated thrombus formation on DPO treatment in eNOS (−/−) mice.

Conclusion—Thus, DPO-related erythropoiesis with a raised hematocrit is not associated with an increased risk for thrombosis as long as endothelial NO production serves as compensatory mechanism. (Arterioscler Thromb Vasc Biol. 2007;27:1191-1198.)

Key Words: endothelium | intravital microscopy | nitric oxide | platelets | thrombosis

The glycoprotein hormone EPO is mainly of renal origin and acts by binding to the transmembrane EPO receptor on erythroid progenitors in the bone marrow.1,2 It increases the number of circulating red blood cells not only by promoting proliferation and differentiation, but mainly by decreasing the rate of apoptotic cell death.3 The EPO-induced increase of red blood cell mass also increases the oxygen supply to the muscles and aerobic performance, which is the reason why rHuEPO is increasingly exploited in competitive sports.4 The use of EPO, however, may also be associated with distinct side effects. Although not clearly demonstrated, a relationship between an increased red blood cell count and thrombus formation is generally admitted.

Apart from the increase in hematocrit, EPO has additionally been shown to stimulate endothelial cells, as indicated by activation of signaling pathways, thrombogenic properties and tissue factor production in vitro,5 and release of plasminogen activator-inhibitor (PAI)-1 in vivo.6 Besides, experimental and clinical studies have demonstrated that EPO enhances platelet production and reactivity.7,8 EPO further increases thrombin–antithrombin III complexes10 and platelet adherence to thrombotic surfaces in vivo.11 On the other hand, elevated hematocrits are not necessarily associated with increased thrombus formation,12 and overexpression of EPO has been shown to reduce blood viscosity13 and to diminish clot formation in in vitro thrombelastography.14 Additionally, EPO and its analogues seem to exert protective effects in studies of stroke and cerebral ischemia.15

Thus, there is no clear evidence whether or not EPO and the EPO-associated erythrocytosis increase the risk of thrombus formation.

Methods

Animals and Materials

Animals
Male C57BL/6J mice and eNOS (−/−) mice (B6.129P2/Nos3, bred on C57BL/6J background) were purchased from Charles River Laboratories, Sulzfeld, Germany.

Chemicals
Darbepoetin-alpha (DPO) was purchased from Amgen (Amgen Inc) and dissolved in physiological saline solution. Thrombin was purchased from Sigma (Sigma-Aldrich) and dissolved in PBS to yield a...
20 U/mL stock solution. All solutions were stored at a maximal temperature of −20°C in the dark.

Experimental Groups
For determination of the dose with the maximum erythropoietic effect, animals were pretreated with DPO at a dose of 1 μg/kg bw once per week sc for 4 weeks (DPO1), 10 μg/kg bw once per week sc for 4 weeks (DPO10), and 25 μg/kg bw once per week sc for 4 weeks (DPO25). In addition, acute DPO application was carried out at a dose of 25 μg/kg bw iv 5 minutes before the experiment (aDPO25). Controls received saline (10 mL/kg NaCl 1x/week sc for 4 weeks). As a consequence of the maximum effect at a dose of 10 μg/kg bw DPO, the following in vitro and in vivo experiments were studied in saline controls and after chronic DPO10 treatment. Induction of microvascular thrombosis in eNOS (−/−) mice was performed in saline controls (eNOS (−/−); 10 mL/kg bw NaCl once per week sc for 4 weeks) and after chronic DPO10 treatment (eNOS (−/−) + DPO10: 10 μg/kg bw once per week sc for 4 weeks).

Analytical Experiments
Blood Analysis
To determine hematocrit, hemoglobin concentration and fraction of reticulocytes blood was drawn from the retroorbital venous plexus of DPO-treated animals. Hematocrit and hemoglobin concentration were assessed with a Cell Counter (Sysmex KX-21, Sysmex). Reticulocytes were counted (Sysmex XE-2100-SP100) and expressed as percentage of total red blood cell number (%).

Histology and Immunohistochemistry
At the end of each experiment, the cremaster muscle was fixed in 4% phosphate buffered formalin for 2 to 3 days and embedded in paraffin. From the paraffin-embedded tissue blocks, 4-μm sections were cut and stained with hematoxylin-eosin (HE) for histological analysis. For immunohistochemical demonstration of intercellular adhesion molecule-1 (ICAM-1), P-selectin, and eNOS, tissue sections collected on poly-L-lysine-coated glass slides were treated by microwave for antigen unmasking. Goat anti–ICAM-1 (1:200) and anti-P-selectin (1:100) (each Santa Cruz Biotechnology, Heidelberg, Germany) as well as rabbit anti-eNOS (1:50; CalBiochem, San Diego, Calif) were used as primary antibodies and incubated over night at 4°C, followed by a horseradish peroxidase (HRP)- or alkali phosphatase (AP)-conjugated donkey anti-goat (1:200; Santa Cruz Biotechnology) or AP-conjugated goat anti-rabbit/mouse antibody (1:200; DAKO, Hamburg, Germany) and development using ACE or fuchsin substrate as chromogen. The sections were counterstained with hematoxylin and examined by light microscopy (Zeiss Axioscop 40, Jena, Germany).

ELISA of Circulating Endothelial Markers
Plasma concentrations of circulating, ie, soluble (s) ICAM-1, sVCAM-1, sP-selectin, and sE-selectin were determined using the respective enzyme immunoassay kits (R&D Systems). Blood samples were prepared by centrifugation for 10 minutes at 2000g and room temperature (GS-6R Centrifuge, Beckman Coulter).

Preparation of Murine Platelet Rich Plasma
For in vitro testing of platelet function, animals were pretreated either with saline or DPO10 according to the experimental protocol. Then 0.5 to 1 mL blood was drawn from the retroorbital venous plexus of DPO-treated and control mice with 1.5 cm glass capillaries and collected into a tube containing 300 μL Tyrode buffer solution (TBS) and heparin (20 U/mL). The sample was centrifuged for 5 minutes at 750g, followed by recentrifugation of the supernatant for 6 minutes at 150g, yielding platelet rich plasma (PRP). PRP was centrifuged again for 5 minutes at 1825g and the cell pellet was resuspended in 1 mL TBS with 1 μmol/L prostacyclin and 10 U/mL heparin for subsequent incubation at 37°C for 10 minutes. Centrifugation (5 minutes at 1825g) and resuspension was repeated twice. Finally, the platelet pellet was resuspended in 450 μL TBS with 2 μL apyrase.16 Platelet suspensions were transferred into a 37°C water bath for 30 minutes of resting in order to eliminate isolation-induced platelet activation.

Flowcytometric Analysis of P-Selectin Expression
For evaluation of receptor expression under resting conditions, 5 μL of specific rat anti-mouse P-selectin (Emfret Analytics) or negative control antibody were incubated with 25 μL platelet suspension for 15 minutes at room temperature. The reaction was stopped by addition of 400 μL PBS and analyzed within 30 minutes. For evaluation of receptor expression on stimulation, the same set of experiments was carried out after exposure to thrombin for maximal platelet activation (20 U/mL).

FACSanaly flowcytometer (Becton Dickinson) was calibrated with fluorescent standard microbeads (CaliBRITE Beads, Becton Dickinson) for accurate instrument setting. Platelets were identified by their characteristic forward and sideward scatter light and selectively analyzed for their fluorescence properties using the CellQuest program (Becton Dickinson) with assessment of 20 000 events per sample. The relative fluorescence intensity of a given sample was calculated by subtracting the signal obtained when cells were incubated with the isotype specific control antibody from the signal generated by cells incubated with the test antibody.

Western Blot Analysis of Tyrosine-Specific Phosphorylation of Platelet Proteins
For whole protein extracts and Western blot analysis of phosphotyrosine (p-Tyr) PRP was prepared as described above. After 30 minutes of resting in a 37°C water bath 50 μL of platelet suspensions from DPO-treated and control animals were analyzed in resting state and at 30 minutes after exposure to thrombin (20 U/mL). The platelets were lysed for 30 minutes on ice (extraction buffer: 50 mmol/L HEPES pH 7.4, 1% Triton-100, 0.15 mol/L NaCl, 10% glycerol, 1.5 mmol/L MgCl2, 1 mmol/L EDTA, 1 mmol/L NaNO3, 10 mmol/L Na3PO4, 1 mmol/L AEBSF, 10 mmol/L NaF, 10 mg/L aprotinin, 10 mg/L leupeptin) and centrifuged for 15 minutes at 10 000 g. Before use, all buffers received a protease inhibitor cocktail (1:100 v/v: Sigma). Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay (Sigma) with bovine serum albumin as standard. 20 μg protein/lane were separated discontinuously on sodium dodecyl sulfate polyacrylamide gels (10% SDS-PAGE) and transferred to a polyvinylidifluoride membrane (Immobilon-P, Millipore). After blockade of nonspecific binding sites, membranes were incubated for 1 hour at room temperature with a HRP-conjugated mouse monoclonal anti-p-Tyr antibody (PY20) (1:1000; Santa Cruz Biotechnology). Protein expression was visualized by means of luminol enhanced chemiluminescence (ECL plus; Amer- sham Pharmacia Biotech) and exposure of the membrane to a blue light sensitive autoradiography film (Kodak BioMax Light Film, Kodak-Industrie). Signals were densitometrically assessed (Quantity One, Gel Doc XR, Bio-Rad Laboratories GmbH).

RT-PCR of eNOS in Cremaster Muscle Tissue
Total RNA from cremaster muscles was isolated using the RNaseasy Mini Kit (Qiagen) according to the manufacturer’s instructions. RNA concentration was determined spectrophotometrically. cDNA was prepared by reverse transcription of 1 μg of total RNA using oligo (d)18 primer (Biolabs) and Superscript II RNaseH-Reverse Transcriptase (Invitrogen). Mouse endothelial nitric oxide synthetase (eNOS; 292 bp) was amplified by 35 cycles of PCR using TaqDNA polymerase (Amersham Bioscience) and the following intron-spanning primers: 5′-AAC AGA AGG CAG CGG TGG AA-3′ and 5′-GCA GGG GAC GAG AAA TAG TT-3′. In a comparable assay, the RNA integrity and cDNA synthesis was tested using mouse GAPDH as a house keeping gene and the following primers: 5′-AGA AAC AGG CAG CAG CGG TGG AA-3′ and 5′-GCA GGG GAC GAG AAA TAG TT-3′. In parallel, controls with H2O instead of DNA were carried out for every PCR reaction. PCR products were separated by electrophoresis on 2.0% agarose gels. Ethidium-bromide stained bands were visualized by UV-illumination and densitometrically quantified (Quantity One).
thrombosis formation was induced in randomly chosen venules (n=1 to 2 per preparation) and arterioles (n=1 to 2 per preparation).

**Thrombosis Model**
After iv injection of 0.1 mL 2% fluorescein isothiocyanate (FITC)-labeled dextran (MW 150000, Sigma-Aldrich) and 0.05 mL 0.2% rhodamine 6G (MW 476, Sigma) and subsequent circulation for 30 seconds, the cremaster muscle microcirculation was visualized by intravital fluorescence microscopy using a Zeiss microscope (Zeiss AxioCyt Vario; Figure 1d). The microscopic procedure was performed at a constant room temperature of 22°C. The epi-illumination setup included a 100 W HBO mercury lamp and an illuminator equipped with a blue filter (450 to 490 nm/250 nm excitation/emission wavelengths). Microscopic images were recorded by a charge-coupled device (CCD) video camera (FK 6990A-IQ, Pieper) and stored on videotapes for off-line evaluation (S-VHS Panasonic AG 7350-E, Matsushita). Using a ×20 water immersion objective (Achromat ×20/0.50 W, Zeiss) blood flow was monitored in individual arterioles (diameter range 30 to 50 μm) and venules (diameter range 60 to 80 μm), followed by superfusion with 25 μL ferric chloride (12.5 mmol/L, Sigma) for induction of microvascular thrombosis. Blood flow velocities were about 1700 to 1900 μm/s for arterioles and 600 to 700 μm/s for venules with no significant differences between the groups studied. Wall shear rates were 160 to 200 s⁻¹ for arterioles and 50 to 60 s⁻¹ for venules, respectively. Recording of vessels was discontinued after blood flow in the vessel ceased for at least 60 seconds because of complete vessel occlusion. As rapid spreading of ferric chloride solution allowed studying only 1 to 2 arterioles and venules within each preparation, both left and right cremaster muscles were prepared for analysis of thrombotic vessel occlusion within each animal.

Analysis included the time periods until first standstill of perfusion and sustained cessation of blood flow because of complete vessel occlusion. Microcirculatory analysis further included the determination of vessel diameter and red blood cell (RBC) velocity before thrombus induction with calculation of vascular wall shear rates, based on the Newtonian definition \( \gamma = 8 \times V/D \), with \( V \) representing the red blood cell centerline velocity divided by 1.6, according to the

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**In Vivo Experiments**

**Mouse Cremaster Muscle Preparation**

For the study of vascular thrombus formation in vivo, we used the opened cremaster muscle preparation, as originally described by Baer in rats and used as a model of microvascular thrombus formation in previous studies. On approval by the local government, all experiments were carried out in accordance with the German legislation on protection of animals and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council). Male C57BL/6J mice with a body weight (bw) of 20 to 25 g were anesthetized by an intraperitoneal injection of ketamine (100 mg/kg bw) and xylazine (25 mg/kg bw). We intended to study the effects of chronic administration of DPO on endothelial and platelet functions in hemostasis. For chronic exposure mice were injected with DPO10. The DPO dose was chosen based on the dose-finding experiments and on prior reports in animal studies. Control mice received saline corresponding to the fluid amount applied in the verum experiments.

Before the preparation of the cremaster muscle animals were placed on a heating pad coupled to a rectal temperature probe. A midline incision of the skin and fascia was made over the ventral aspect of the scrotum and extended up to the inguinal fold and to the distal end of the scrotum. The incised tissues were retracted to expose the cremaster muscle sack that was maintained under gentle traction to carefully separate the remaining connective tissue by blunt dissection from around the cremaster sack. Then, the cremaster muscle was incised avoiding to cut the larger anastomosing vessels. Hemostasis was achieved with 5–0 threads serving also to spread the tissue. After dissection of the vessel connecting the cremaster and the testis, the epididymis and testis were put to the side of the preparation. The preparation was performed on a transparent pedestal to allow microscopic observation of the cremaster muscle microcirculation by both transillumination and epi-illumination techniques.

After the preparation of the cremaster muscle, the animals were allowed to recover from surgical preparation for 15 minutes. Fluorescent dyes were injected via the retroorbital venous plexus. Then,
Baker–Wayland factor20 and D representing the individual inner vessel diameter.

**Subaquatic Tail Bleeding Time**

Subaquatic bleeding time was measured after standardized dissection of the tail tip. The tail was cut at a diameter of 0.15 mm and placed in 37°C 0.9% saline solution. Time until cessation of blood flow was determined.

**Statistical Analysis**

After proving the assumption of normality and equal variance across groups, differences between groups were assessed using 1-way ANOVA followed by the appropriate post-hoc comparison test. All data revealed normal distribution and were expressed as means±SEM and overall statistical significance was set at P<0.05. Statistics and graphics were performed using the software package SigmaStat and SigmaPlot (Jandel Corporation).

**Results and Discussion**

To study the role of EPO in microvascular thrombosis in vivo we performed intravital fluorescence microscopic studies of the cremaster muscle preparation in male C57BL/6J mice. Microvascular thrombus formation was induced in arterioles and venules by ferric-chloride superfusion. Before the experiments, mice were injected with DPO1 (n=4 animals), DPO10 (n=4 animals), and DPO25 (n=3 animals) chronically and with DPO25 (n=3 animals) 5 minutes before the experiment to define the dosage with the maximal hematopoietic effect in this model. Control mice received injections of physiological saline. As a consequence of DPO pretreatment hematocrit (Figure 1a), hemoglobin concentrations (Figure 1b) and fraction of reticulocytes (Figure 1c) increased with a maximum effect at a dose of 10 μg/kg bw (DPO 10). Thus, the hematopoietic response of mice to DPO application can be considered as saturable in C57BL/6J mice. Of note, the hemoglobin concentration of animals treated with DPO10 was comparable to that seen in humans after EPO treatment and above the cut-off values of 16 g/dL (females) and 17 g/dL (males) accepted by the International Ski Federation (FIS).21 Based on these facts, the dose DPO10 was chosen for the subsequent experiments. Surprisingly, times until complete vessel occlusion after ferric-chloride superfusion in these animals (n=7 preparations) were not reduced in cremaster muscle arterioles and venules when compared with controls (n=7 preparations), but rather tended to be prolonged (Table 1). This result is in line with studies of the past, describing no effect of EPO treatment on coagulation and fibrinolysis in elective hip surgery22 and no prothrombogenic effects of uremic media on endothelial cells in the presence of EPO.23 Moreover, an in vitro study investigating the effect of hematocrits between 20 and 55% on hemostasis as well as platelet and fibrin accumulation into a collagen-coated tube under different flow conditions showed no dependence of thrombus growth on hematocrit under high shear stress and even a reduced thrombus formation at high hematocrits under low flow conditions.12 On the other hand, data concerning therapeutic hemodilution indicate beneficial effects of a reduced hematocrit on microcirculation in general14 and in particular on thrombotic microvessel occlusion per se25 and after microsurgery.26,27 However, a study investigating the effect of the hematocrit during deep hypothermic bypass on cerebral microcirculation in piglets revealed a significantly higher functional capillary density and number of rolling leukocytes at a hematocrit of 30% versus 10%.28 Our results indicate that microvascular thrombus formation is not increased at high hematocrits exceeding 60% after DPO treatment. Moreover, despite a significantly raised hematocrit in the DPO10 group (Figure 1a), rheological parameters (RBC velocity, vessel diameter, wall shear rate) were comparable to controls implying the absence of detrimental effects of the DPO-related raised hematocrit on the microcirculation (Table 1). Thus, the lack of susceptibility for thrombosis and the unchanged rheological parameters despite a significantly raised hematocrit following DPO application may probably be attributable to protective intrinsic properties of DPO.

To further elucidate the effects of DPO on thrombus formation in vivo we studied the subaquatic tail bleeding time as parameter for platelet-associated hemostasis. With bleeding times of 188±20 seconds in controls and 190±17 seconds in DPO10-treated animals, we confirmed that the coagulative status is not increased on DPO.

Because the vascular endothelial function plays a major role in thrombogenesis, we further were interested in the mechanisms of modulation of endothelial function attributable to chronic DPO treatment. Stohl et al reported substantial activation of endothelial cells as a consequence of treatment with rHuEPO in humans4 and Quaschning et al observed a significant activation of the endothelin system.29 On the other hand, EPO might have potential beneficial effects on the endothelium including antiapoptotic, mitogenic, and angiogenic activities, which may enhance overall cardiac function.30 To further address this issue, we determined the circulation of soluble endothelial activation mark-

| TABLE 1. Red Blood Cell (RBC) Velocity (μm/s), Vessel Diameter (μm), and Wall Shear Rates (γ; s⁻¹) in Arterioles and Venules of the Cremaster Muscle Before Induction of Thrombus Formation and Complete Occlusion Times (s) of Arterioles and Venules Upon Ferric Chloride-Induced Thrombus Formation in Saline Controls and DPO10-Treated Animals |
|----------------|----------------|----------------|----------------|----------------|
|               | Arterioles     |               | Venules        |               |
| Control       | DPO10         | P             | Control        | DPO10         | P             |
| RBC velocity  | 1842±360      | 1700±342      | 0.53           | 637±89        | 633±73        | 0.92           |
| Vessel diameter| 48±5          | 44±4          | 0.78           | 60±5          | 60±3          | 0.97           |
| Wall shear rate| 200±44        | 205±49        | 0.94           | 56±9          | 54±7          | 0.92           |
| Complete occlusion time| 627±148 | 1044±146 | 0.07 | 573±146 | 861±145 | 0.19 |

Values are given as means±SEM.
ers in plasma samples of DPO10-treated (n=4 animals each) and control (n=6 animals each) mice. In general, DPO pretreatment lead to a reduction of endothelial activation, represented by a moderate decrease of sP-selectin and a significant decline of sE-selectin, sICAM-1, and sVCAM-1 in murine plasma (Figure 2a). Immunohistochemical staining of cremaster muscle sections confirmed a significant down-regulation of P-selectin and ICAM-1 on the vascular endothelium after DPO10 application (n=6 specimens per group; Figure 2b). Thus, DPO10 treatment for 4 weeks appears to reduce endothelial activation, which correlates with the prolongation in time until complete thrombotic vessel occlusion in vivo.

Next to the endothelial dysfunction, platelet activation and subsequent aggregation is an important factor in microvascular perfusion failure and thrombus formation. Wolf at al suggested that hyperreactive platelets are responsible for a prothrombotic effect of EPO in an arteriovenous shunt model in dogs. Also, a study investigating the effect of EPO infusion in healthy human volunteers could demonstrate a 10% to 20% increased platelet count and a 2- to 3-fold increased expression of P-selectin during EPO treatment. On the other hand, thrombocytopenia has been reported after EPO treatment, suggesting a reduced tendency for clot formation. Thus, we further studied the effect of DPO on platelet count and platelet reactivity. Platelet numbers in the peripheral blood did not increase after DPO10 treatment when compared with controls (561 ± 69 versus 558 ± 17 × 10^10/L). In addition, there were no marked differences in spontaneous platelet activation. However, on thrombin stimulation flow cytometric analyses revealed a reduced expression of P-selectin on platelets from DPO10-treated mice when compared with platelets of controls (Figure 3a). Further, tyrosine-specific phosphorylation in platelets of DPO10-treated animals was found reduced, indicating a dampening effect of DPO on platelet reactivity (Figure 3b). Thus, reduced agonist-induced platelet reactivity might further contribute to the fact that DPO treatment does not boost microvascular thrombus formation in our study. However, in light of the fact that previous studies in humans and dogs showed discrepant findings, species-related differences of platelet response to EPO and its derivatives might exist.

In a next step we intended to define the mechanisms for this unexpected finding. Several studies in EPO-overexpressing
transgenic mice with a hematocrit of about 80% revealed new aspects of EPO-associated alterations of physiological functions. Simply the fact that these mice were viable already contradicted the assumption that such a high hematocrit invariably results in thromboembolic complications. However, immunohistological studies of the brain revealed increased infarct volumes in these polyglobalic mice after permanent occlusion of the middle cerebral artery. In contrast to these findings, subsequent studies showed a reduced plasmatic coagulation activity in vitro, increased bleeding times, and no evidence of microthrombosis in several organs of these transgenic mice. Finally, it was demonstrated that eNOS levels, NO-mediated endothelial-dependent relaxation, as well as circulating and vascular tissue NO were significantly increased, which was regarded as a protective mechanism, counteracting a potentially increased risk of thrombosis. Therefore, we studied whether exogenous application of DPO might also increase eNOS expression in our model. EPO has been shown to stimulate eNOS expression with the consequence of raised NO production in vitro, but in vivo studies linking this effect to a reduced thrombus formation are missing. To verify, whether DPO influences endothelial expression of NOS, we performed immunohistology of the cremaster muscle. eNOS expression in the vascular endothelium was significantly increased after chronic DPO10 treatment (n=6 preparations) when compared with controls (Figure 4a). These results could be confirmed by RT-PCR (Figure 4b). We hypothesized that chronic DPO application itself, or the DPO-associated rise of hematocrit leads to an increase of eNOS production with the consequence of antiadhesive and antithrombogenic effects, resulting in a decrease of endothelial and platelet activation. To further delineate the potential protective effects of eNOS in this setting, we performed additional in vivo experiments in B6 background B6.129P2/Nos3 knock out mice (eNOS+/−), which were also pretreated with DPO10 (eNOS+−/DPO10; n=6 preparations). Control animals received saline (eNOS+/−; n=6 preparations) as described above. Interestingly, eNOS+−/− mice responded to the chronic DPO treatment only with a mild increase of hematopoietic parameters when compared with saline-treated eNOS+−/− controls (Table 2). The preferential mechanism by which EPO maintains and increases erythropoiesis is the prevention of apoptosis of erythropoietic progenitor cells. Because the physiological continuous production of NO by eNOS has been identified as an antiapoptotic factor in the past, the weak effect of exogenous DPO application in eNOS+−/− on erythropoiesis may be explained by a generally proapoptotic state. Therefore it might be assumed that a lack of eNOS activity leads to a reduction of erythrocyte progenitor cell proliferation and differentiation capacity. Platelet count in eNOS+−/− mice tended to be higher than in wild-type mice, and additional DPO treatment resulted in a small, but not significant, increase.

In vivo, saline-treated eNOS+−/− animals revealed slightly accelerated thrombosis times in arterioles and venules (574±124 seconds and 384±166 seconds) when compared with wild-type control mice (627±148 seconds; P=0.78 and 573±146 seconds; P=0.42). Moreover in eNOS+−/− mice, thrombotic vessel occlusion in venules tended to occur faster than in arterioles (384±166 seconds versus 574±124 seconds; P=0.37). This seems to be in line with previous observations of Broeders and coworkers that the role of endogenous NO in inhibiting thromboembolic processes is more important in venules than in arterioles. In a later study by this group, it was shown that NO and prostaglandins in turn synergistically counteract thromboembolism in arterioles, but not in venules, and that the combination of

Figure 3. a, Flow cytometric analysis (representative histograms) of platelet P-selectin expression after pretreatment with saline and DPO10. Note the reduced expression of P-selectin on the platelet surface as a result of chronic DPO application under resting conditions, but in particular on stimulation by thrombin (20 U/mL). b, Representative Western blot of tyrosine-specific protein phosphorylation in platelets. The arrowheads denote the most prominent protein bands, migrating with a molecular mass of 84 kDa, 125 kDa, and 130 kDa. Protein phosphorylation was found to be markedly reduced in platelets of DPO-treated animals.
endogenous NO and prostaglandins appears to protect against enhancement of arteriolar thromboembolism by wall shear.39 Thus, next to faster thrombotic occlusion attributable to a generally lower RBC velocity in venules, a synergistic effect of NO and prostaglandins in arterioles may also be responsible for differences in arteriolar and venular occlusion times in eNOS (−/−) mice.

Interestingly, additional DPO treatment for 4 weeks significantly decreased the time until complete vessel occlusion in both arterioles (135±34 seconds; P=0.004 versus eNOS (−/−)) and venules (148±25 seconds; P=0.04 versus eNOS (−/−)) identifying the eNOS-dependent NO production as the crucial compensatory mechanism preventing thrombosis during DPO treatment (Figure 4c and d).

We conclude from these data that DPO-associated eNOS production with platelet and endothelial cell deactivation counteracts prothrombotic actions during DPO treatment.

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Disclosures

None.

References


**Figure 4.** a, Immunohistochemical analysis of eNOS expression in controls and DPO10-treated animals. eNOS expression in the vascular endothelium was significantly increased after chronic DPO treatment. b, RT-PCR of eNOS in cremaster muscle tissue of saline- and DPO10-treated animals. Chronic treatment with DPO induced a marked increase of eNOS mRNA transcripts (292 bp, upper panel) in comparison to controls. Mouse GAPDH was coamplified as internal control (191 bp, lower panel). c and d, Occlusion times of arterioles and venules in wild-type and eNOS (−/−) mice on ferric chloride-induced thrombus formation in saline controls and after chronic DPO10 treatment. Values are given as means±SEM. *P<0.05 vs eNOS (−/−), #P<0.05 vs DPO10. While eNOS (−/−) control mice already revealed slightly accelerated thrombosis times when compared with wild-type controls, we found a significant prothrombogenic effect of additional chronic DPO treatment in eNOS (−/−) mice.

### TABLE 2. Hematocrit (%), Hemoglobin Concentration (g/dL), Reticulocyte Fraction (%), and Platelet Count (×10^9/L) in Saline-Treated eNOS (−/−) Mice (control) and eNOS (−/−) Mice, That Were Pretreated With DPO Chronically at a Dose of 10 μg/kg bw (eNOS (−/−) + DPO 10)

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<th>eNOS (−/−) + DPO10</th>
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<td>Platelet count</td>
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Values are given as means±SEM.
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