Nitric oxide prevents cardiovascular disease and determines survival in polyglobmic mice overexpressing erythropoietin


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Nitric oxide (NO) induces vasodilatatory, antiaggregatory, and antiproliferative effects in vitro. To delineate potential beneficial effects of NO in preventing vascular disease in vivo, we generated transgenic mice overexpressing human erythropoietin. These animals induce polyglobulia known to be associated with a high incidence of vascular disease. Despite hematocrit levels of 80%, adult transgenic mice did not develop hypertension or thromboembolism. Endothelial NO synthase levels, NO-mediated endothelial dependent relaxation and circulating and vascular tissue NO levels were markedly increased. Administration of the NO synthase inhibitor N\(^{-}\)nitro-L-arginine methyl ester (L-NAME) led to vasoconstriction of peripheral resistance vessels, hypertension, and death of transgenic mice, whereas wild-type siblings developed hypertension but did not show increased mortality. L-NAME-treated polyglobmic mice revealed acute left ventricular dilatation and vascular engorgement associated with pulmonary congestion and hemorrhage. In conclusion, we here unequivocally demonstrate that endothelial NO maintains normotension, prevents cardiovascular dysfunction, and critically determines survival in vivo under conditions of increased hematocrit.

The endothelium plays a key role in the regulation of blood pressure and flow (1–3). Intact endothelial cells release NO that mediates vascular relaxation in response to vasoactive substances and shear stress and provides antiproliferative and antithrombotic functions by inhibiting vascular smooth muscle cell proliferation, monocyte adhesion, platelet aggregation, and thrombosis (4–10). NO is formed from L-arginine by NO synthases (NOS). The endothelial isoform of NOS (eNOS, also termed NOS III) is the prevailing form in the vascular system (11). The aim of the present study was to delineate the role of NO in preventing vascular disease in vivo. Because polyglobulia is associated with a high incidence of arterial hypertension and thromboembolism (12, 13), we generated transgenic mice overexpressing human erythropoietin (Epo) and assessed the contribution of NO to survival and cardiovascular function in polygloblic animals.

Materials and Methods

Generation, Characterization, and Breeding of Transgenic Mice. A HindIII–XmnI fragment harboring the SRα promoter driving the human Epo cDNA in plasmid pTREPO (14) was replaced by the human platelet-derived growth factor (PDGF) B-chain promoter (HindIII–XmnI fragment) present in plasmid pisCAT6a (15). Subsequently, the resulting plasmid pPDDGFePO was cleaved from the bacterial backbone by digestion with XmnI and NruI, and the gel-isolated fragment was purified by Qiagen (Qiagen, Chatsworth, CA) and microinjected into the pronuclei of fertilized oocytes derived from superovulated B6C3F1 hybrid mice following standard technology (16). Genomic DNA was isolated from mouse tail biopsies, digested with EcoRI, electrophoresed through 0.7% agarose, blotted onto a nylon membrane, and hybridized to a random-primed 1.5-kilobase XbaI–HindIII fragment isolated from pisCAT6a. Hematocrit, hemoglobin, and red blood cell count were determined by using standard hematological methodology, and plasma Epo levels were determined by RIA as described (17). Breeding of the resulting transgenic mouse line termed Tg(PDGFBEPO)321Zb was performed by mating hemizygous males to wild-type females, thereby giving rise to heterozygous and wild-type littermates, the latter being used as controls. All procedures and experimental protocols were performed following the Swiss and German Animal Protection Laws and were supervised by the corresponding official Veterinary Departments.

Hemodynamics. Blood pressure and heart rate were measured by the tail-cuff method (blood pressure recorder 8005, W + W, Münchenstein, Switzerland) in unanesthetized adult mice that underwent 4 weeks of extensive training to get used to this procedure. Mean values of three subsequent measurements were calculated. For invasive measurements, mice were anesthetized by i.p. injection of droperidol, fentanyl, and midazolam (20, 0.1, and 2 mg/kg body weight, respectively); after cannulation of the right jugular vein, anesthetics were administered continuously. Arterial pressure was measured via a catheter placed in the right carotid artery by means of a pressure transducer (Statham, Costa Mesa, CA). Cardiac output was determined by the ultrasound transit time method. Animals were ventilated with oxygen-enriched air (Minivent type 845; Hugo Sachs Elektronik, March, Germany) avoiding positive end-expiratory airway pressure (respiration rate: 130–180 breaths/min). The tidal volume was 180–230 µL. After median sternotomy, an ultrasonic flow probe was placed around the ascendent thoracic aorta below the branching of the brachiocephalic trunk and connected to a small animal blood flowmeter (TI06; Transonsics, Itahaca, NY). To reduce operation time, no carotid artery catheter and no jugular vein catheter were inserted.

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Abbreviations: Epo, erythropoietin; NOS, nitric oxide synthase; eNOS, endothelial NOS; PDGF, platelet-derived growth factor; L-NAME, N\(^{-}\)nitro-L-arginine methyl ester.

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Intravital Microscopy. The cremaster muscle of anesthetized mice was prepared as described (18) and superfused with warm (34°C), bicarbonate-buffered (pH 7.4, pO2 ~30 mm Hg, pCO2 ~38 mm Hg) salt solution (in mM: 143 Na+ /6 K+ /2.5 Ca2+/1.2 Mg2+/128 Cl−/25 HCO3−/1.2 SO4−/1.2 HPO4−). Twelve arterioles of different sizes were studied in each animal and observed using a microscope equipped with a closed video circuit system. Images were recorded on videotape for off-line measurement of luminal diameters after digitization (OPTIMAS, Media Cybernetics, Silver Spring, MD). Arteriolar diameters were recorded repeatedly before and after 30 min after starting the continuous local application of Nω-nitro-l-arginine methyl ester (L-NNAME) (3 × 10−5 M). Additionally, maximal diameters were determined by addition of a combination of different vasodilators (sodium nitroprusside, adenosine, and acetylcholine; 1 × 10−5 M each) at the end of each experiment.

Tissue Harvesting and Organ Chamber Experiments. Upon blood collection, anesthetized mice (thiopental, 40 mg/kg body weight, i.p.) were euthanized by cervical dislocation, and organs and vessels were removed, snap-frozen in liquid nitrogen, and stored at −80°C. For Western blot analysis of NOS I, NOS II, and NOS III, the endothelium was scraped from the aorta. In addition, thoracic aortas were placed into ice-cold Krebs–Ringer bicarbonate solution (pH 7.4, 95% O2 and 5% CO2) as described (19) and dissected free from adherent connective tissue under a microscope. For isometric tension recording, aortas were cut into 3-mm rings that were placed in organ chambers containing Krebs–Ringer bicarbonate solution. After equilibration for 1 h, resting tension was gradually increased, and rings were repeatedly exposed to 0.1 M KCl until the optimal resting tension was reached. Acetylcholine concentrations were increased when contractions of the previous step were stable. Concentrations of acetylcholine, L-NNAME, KC1, and sodium nitroprusside (all from Sigma, Buchs, Switzerland) are expressed as final molar concentrations in the organ chamber bath solution. After measurements, vessel rings were dried and weighed.

Measurement of Nitrate Levels in Plasma and Vascular Tissue. Blood samples (400–500 μl) were drawn from the right ventricle and diluted 1:4 in ice-cold solution consisting of sodium chloride (0.9% wt/vol) and sodium citrate (3.8% wt/vol). After centrifugation of samples (10 min, 1,000 × g), plasma was ultrafiltered (cut off 20 kDa, 60 min, 4,000 × g). Aortic tissue (100 mg) was homogenized and subsequently diluted 1:4 in water. Quantitation of nitrate, the stable end product of NO oxidation, was performed by RP-HPLC on an ECE250/4.5 Supersil 100 RP column (Macherey & Nagel) by using ion-pairing chromatography with photodiode array detection at 210, 215, and 220 nm and related to standard curves in the 0–100 μM range generated in the same sample matrix (20). Injection volumes were 40 μl at a flow rate of 1.0 ml/min.

Statistical Analysis. For multiple comparisons, results were analyzed with ANOVA followed by Bonferroni’s correction; for comparison between two values, the unpaired Student’s t test or the nonparametric Mann–Whitney test was used when appropriate. A P value <0.05 was considered significant.

Results and Discussion

Expression of human Epo cDNA in transgenic mice was driven by the human PDGF B-chain promoter that preferentially directs transgene expression to neuronal cells (15). Several resulting transgenic mouse lines showed increased Epo protein levels in the brain as measured by RIA, but plasma Epo levels were increased only in one line to about 10-fold (Table 1). As expected, elevated plasma Epo levels in hemizygous adult animals of this line enhanced erythropoiesis as reflected by the nearly doubled values for erythrocytes, hemoglobin, and hematocrit (Table 1).

Blood pressure is determined by cardiac output and peripheral vascular resistance. The latter depends particularly on blood viscosity, with which it bears a linear relationship over a wide range of values (21). Hematocrit is the major determinant of whole blood viscosity, which increases exponentially with hematocrit values above the physiological range. Hence, one would expect very high hematocrit levels to be associated with hypertension. However, despite hematocrit values of about 80%, blood pressure, heart rate, and cardiac output remained unaltered in transgenic adult mice when compared with their nontransgenic littermates (Table 1). Moreover, histological analysis of transgenic mice revealed no signs of myocardial infarction, stroke, or thromboembolism.

We next examined whether endothelium-derived NO provides vascular protection under conditions of high hematocrit values. Western blot analysis followed by densitometric quantitation of eNOS protein levels in transgenic and wild-type endothelium of the thoracic aorta revealed a 6-fold increase in eNOS levels in transgenic mice (Fig. 1A; n = 3). Likewise, immunohistochemical analysis showed increased eNOS expression exclusively confined to the endothelium of transgenic aorta and pulmonary arteries (Fig. 1B). Of note, neuronal (NOS I) and inducible (NOS II) NOS protein were not detectable by Western blot analysis in wild-type or transgenic endothelium (not shown).

Given the multiple levels of NO regulation including post-translational modifications (11, 22, 23) and an NO-dependent autoinhibitory negative feedback mechanism (24), increased expression of this enzyme does not necessarily result in elevated NO production in intact endothelial cells. Hence, it was crucial to assess NO formation in the vessel wall. Indeed, nitrate levels (the stable end product of endogenous NO oxidation) measured in the aorta and pulmonary artery of transgenic mice with high hematocrit were significantly increased compared with wild-type control organs (Fig. 1C Right). In line with this observation, plasma levels of NO were elevated 3-fold in transgenic mice (Fig. 1C Left) reflecting enhanced circulating NO bioavailability despite highly increased levels of hemoglobin, which may serve as a potential scavenger of NO in vivo (25, 26). Correspondingly, endothelium-dependent relaxations to acetylcholine were significantly augmented in the intact aorta of transgenic mice as compared with wild-type siblings (Fig. 2A), confirming increased vascular NO bioavailability. The inhibitory effect of the NOS inhibitor L-NNAME (27) demonstrated that the relaxation to acetylcholine was almost exclusively mediated by endothelium-derived NO (Fig. 2A). Moreover, contraction of quiescent (e.g., non-preconstricted) aortic rings in response to L-NNAME (1 × 10−9 to 3 × 10−7 M) revealed increased basal NO release (not shown). In contrast, alterations in the response of smooth muscle cells could be excluded, because endothelium-independent vasodilatation to the exogenous NO-donor sodium nitroprusside

### Table 1. Erythropoietic and hemodynamic parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild type* (n)</th>
<th>Transgenic* (n)</th>
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<tbody>
<tr>
<td>Plasma Epo Units/liter</td>
<td>22.1 ± 5.2</td>
<td>259 ± 79</td>
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<tr>
<td>Red blood cells 1012/liter</td>
<td>6.2 ± 0.5</td>
<td>13.8 ± 1.0</td>
</tr>
<tr>
<td>Hemoglobin g/liter</td>
<td>139 ± 5</td>
<td>234 ± 13</td>
</tr>
<tr>
<td>Hematocrit %</td>
<td>39.3 ± 2.7</td>
<td>79.0 ± 3.9</td>
</tr>
<tr>
<td>Heart rate Beats/min</td>
<td>569 ± 18</td>
<td>606 ± 9</td>
</tr>
<tr>
<td>Systolic blood pressure mmHg</td>
<td>119 ± 5</td>
<td>120 ± 5</td>
</tr>
<tr>
<td>Cardiac output ml/min</td>
<td>6.4 ± 1.7</td>
<td>6.7 ± 1.7</td>
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*Hemizygous transgenic males were mated with wild-type females, and the wild-type and transgenic siblings were analyzed at 3–6 months of age. Data are expressed as mean values ± SD for 5–22 measurements (n) as indicated.
In the negative control, the primary antibody was omitted. (B) Immunochemical analysis of eNOS expression in the thoracic aorta and pulmonary arteries (A. Pulm.) of wild-type and transgenic mice using the anti-eNOS antibody described above. Note that eNOS expression is restricted to the endothelium and is markedly increased in the transgenic arteries. (C) Quantiﬁcation of NO levels in the circulation (Left, n = 4) and in vascular tissue (Right, n = 6–7) of wild-type and transgenic mice (∗, P < 0.05, ∗∗, P < 0.007).

In vivo, however, NO formation by an endogenous stimulus such as thrombin, which is predominantly generated at sites of thrombus formation, is clinically even more relevant (28). Indeed, endothelium-dependent relaxation to thrombin was also augmented in transgenic as compared with wild-type mice (Fig. 2B). This effect was blocked by the thrombin antagonist hirudin. Thrombin-induced NO release was predominantly mediated by endothelium-derived NO. (B) Relaxation of precontracted aortic rings upon addition of thrombin (3 × 10⁻⁸ M) alone, a combination of thrombin and hirudin (5 units/ml; note that 1 unit of hirudin neutralizes 3 × 10⁻⁸ M thrombin), or thrombin receptor agonist peptide (TRAP) (10⁻⁵ M) to the bath solution (n = 6–7).

In contrast, wild-type siblings increased systolic blood pressure from 118 ± 3 mmHg to 170 ± 3 mmHg after 2 weeks of continuous ingestion of L-NAME, but appeared otherwise healthy. Histological postmortem examination of L-NAME-treated transgenic mice revealed vasoconstriction of muscular arteries of the systemic circulation, particularly in the integument (Fig. 3B). In keeping with this, morphometric analysis of these mice revealed that the inner circumference of tail arteries of untreated transgenic mice was reduced by more than 50% compared with the untreated transgenic group. Moreover, histological analysis revealed acute left dilation of the slightly hypertrophied heart, vascular engorgement, severe pulmonary congestion, and hemorrhage (Fig. 3B).

Measurement of arterial pressure in a different group of anesthetized mice revealed a peak increase of systemic blood pressure from 80 ± 2 mm Hg to 170 ± 5 mm Hg after continuous ingestion of L-NAME, with a mean survival time of only 33 ± 8 h (Fig. 3A).
pressure in transgenic and wild-type animals 30 min after i.v.
application of the NOS inhibitor was started (Fig. 4
A). However, after prolonged infusion of L-NAME, transgenic mice showed a
significant decrease in blood pressure and, in analogy to the oral
application of the drug, enhanced mortality. This decrease in
systemic blood pressure might be partly explained by simulta-
neous changes in left ventricular function after NO inhibition. As
histopathological examination revealed no signs of myocardial or
cerebral infarction in any of the transgenic animals treated with
L-NAME, this is most probably due to excessive afterload under
these conditions. To assess the potential role of NO in compen-
sating elevated resistance to flow, the effects of L-NAME on
microvascular tone were determined by intravital microscopy in
transgenic and wild-type animals
\textit{in vivo}.

Fig. 4. Effect of L-NAME on systolic blood pressure and arteriolar diameters
in anesthetized wild-type and transgenic mice. (A) Anesthetized male mice
4–5 months old (n = 6–8) received 50 mg of L-NAME per kg body weight
intravenously within 15 min. Subsequently, L-NAME was infused at a dosage
of 30 mg per kg per h. Three of eight transgenic mice died within 4 h after
application of the NOS inhibitor was started, whereas all wild-type mice
survived this procedure (*, P < 0.05). (B) Intravital microscopy of arterioles
(40–100 \mu m; n = 14–15) was performed in anesthetized male mice 4–5 months
old (n = 3 each genotype). Arteriolar diameters are depicted as a fraction of
maximal diameter as determined at the end of the experiment. L-NAME (3 \times
10^{-5} \text{ M}) was applied locally to the cremaster muscle, which did not affect
arterial pressure, and diameters were recorded before and 30 min after start
of treatment (*, P < 0.05 vs. untreated controls).
In conclusion, we have generated a transgenic mouse line with severe polycythaemia that, due to an increased constitutive expression of eNOS associated with enhanced NO bioavailability, does not develop hypertension, stroke, myocardial infarction, or thromboembolism. The present transgenic model provides further insights into the role of endothelial factors, haematocrit-induced changes of blood flow, and the development of cardiovascular diseases such as arterial hypertension and thromboembolism.

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