Circulating Blood Endothelial Nitric Oxide Synthase Contributes to the Regulation of Systemic Blood Pressure and Nitrite Homeostasis

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Objective—Mice genetically deficient in endothelial nitric oxide synthase (eNOS−/−) are hypertensive with lower circulating nitrate levels, indicating the importance of constitutively produced nitric oxide (NO•) to blood pressure regulation and vascular homeostasis. Although the current paradigm holds that this bioactivity derives specifically from the expression of eNOS in endothelium, circulating blood cells also express eNOS protein. A functional red cell eNOS that modulates vascular NO• signaling has been proposed.

Approach and Results—To test the hypothesis that blood cells contribute to mammalian blood pressure regulation via eNOS-dependent NO• generation, we cross-transplanted wild-type and eNOS−/− mice, producing chimeras competent or deficient for eNOS expression in circulating blood cells. Surprisingly, we observed a significant contribution of both endothelial and circulating blood cell eNOS to blood pressure and systemic nitrite levels, the latter being a major component of the circulating NO• reservoir. These effects were abolished by the NOS inhibitor L-NG-nitroarginine methyl ester and reprimstited by the NOS substrate l-arginine and were independent of platelet or leukocyte depletion. Mouse erythrocytes were also found to carry an eNOS protein and convert 14C-arginine into 14C-citrulline in NOS-dependent fashion.

Conclusions—These are the first studies to definitively establish a role for a blood-borne eNOS, using cross-transplant chimera models, that contributes to the regulation of blood pressure and nitrite homeostasis. This work provides evidence suggesting that erythrocyte eNOS may mediate this effect. (Arterioscler Thromb Vasc Biol. 2013;33:1861-1871.)

Key Words: blood • blood pressure • bone marrow transplant • nitric oxide synthase

Hypertension is a complex multifactorial condition associated with cardiovascular disease. Experimental data in mouse models and human subjects point to a correlation between the production of nitric oxide (NO•) and its oxidative metabolites and hemodynamic parameters, such as nitrite and blood pressure (BP), respectively.1-7

NO• is produced by an NO synthase (NOS) catalyzing the conversion of l-arginine to equimolar amounts of NO• and citrulline in the presence of oxygen and the cofactors calcium, calmodulin, nicotinamide adenine dinucleotide, and tetrahydrobiopterin.8 There are 3 NOS isoforms, endothelial NOS (eNOS), inducible NOS (iNOS), and neuronal NOS (nNOS), expressed in multiple cell types, such as endothelium, epithelium, leukocytes, platelets, and neurons.8 Whereas iNOS participates in host defense, inflammatory stress, and airway epithelial NO• formation, the constitutively expressed isoforms, nNOS and eNOS, are important to physiological processes that include neuronal signaling, inhibition of the hemostatic system, vasodilation, and BP control.

In the cardiovascular system, eNOS contributes to the regulation of blood flow and BP and is an inhibitor of platelet activation and aggregation, as well as leukocyte adhesion and migration. Furthermore, endothelial eNOS seems to contribute to the formation of bioactive circulating NO• metabolites, such as the nitrite anion and S-nitrosothiols, that mediate important endocrine activities, such as hypoxic vasodilation,9,10 BP regulation,9 and cytoprotection after myocardial infarction.11,12 Mice genetically deficient in eNOS (eNOS−/−) are hypertensive with lower circulating nitrite levels, indicating the importance of constitutively produced NO• to BP regulation and vascular homeostasis.1,4,13

Conventional wisdom holds that the pleiotropic effects of eNOS are primarily determined by enzyme expressed in the...
endothelium. In addition to endothelial cells (ECs), most circulating blood cells (BCs), including leukocytes, platelets, and red blood cells (RBCs), also carry eNOS transcript or protein. In RBC, an active red cell eNOS modulates intrinsic erythrocyte deformability, platelet activation, and extraerythrocytic NO• metabolites, such as nitrite. A physiological in vivo effect for circulating eNOS on BP regulation or the formation of the circulating NO• metabolite reservoir has never been evaluated.

The aim of this work was to test in vivo the hypothesis that a functional circulating cell eNOS regulates systemic BP and the formation of the NO• metabolite pool. To limit eNOS functionality to circulating BCs, we used cross-transplantation methodologies with wild-type (WT) and eNOS−/− strains. They also show lower whole and all other eNOS nonerythrocytic extraerythrocytic NO• metabolites, such as nitrite.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

eNOS+/− Mice Are Hypertensive With Low Circulating Nitrite Levels

To investigate the hypothesis that circulating BC eNOS contributes to the control of BP, systemic BP—systolic and diastolic—was assessed and compared (to 2 decimal places accuracy) in awake mice by radiotelemetry and in anesthetized mice via carotid artery cannulation. Mice with global knockout of functional eNOS (eNOS−/−) are hypertensive compared with normal WT mice (mean arterial pressure [MAP]: 126±3.18 versus 105.4±2.66 mm Hg; P=0.0016), confirming an important physiological role for eNOS in basal BP regulation (Figure 1A), as previously reported for this and all other eNOS−/− strains. They also show lower whole blood (0.72±0.08 µmol/L; Figure 1B) and plasma (0.38±0.04 µmol/L; Figure 1C) nitrite levels than WT mice (whole blood: 1.08±0.11 µmol/L and plasma: 0.50±0.54 µmol/L). These values indicate the importance of the eNOS enzyme in regulating BP and nitrite homeostasis.

eNOS+/− Mice Lack Expression and Activity of Red Cell eNOS

RBCs make up the largest cellular compartment in blood and are the circulating reservoir for nitrite. To define the presence of an active eNOS protein in RBCs and its deletion in eNOS−/− mice, immunocytochemistry and immunoprecipitation experiments were performed. Using laser scanning confocal microscopy, eNOS staining was found to strongly localize in RBCs (Ter-119–positive cells) from WT mice (Figure 1D, left) but not in RBCs from eNOS−/− mice (Figure 1D, middle). Incubation with IgG was also used to verify that staining was specific for eNOS protein. In these control samples, IgG staining was weakly and homogeneously distributed across both cellular and noncellular areas and thus not cell-specific (Figure 1D, right). Mouse red cell eNOS was further characterized by immunoprecipitation and Western blot analysis. As shown in Figure 1E, eNOS (≈135 KD) is detected in RBCs from WT mice, but not knockout mice, demonstrating electrophoretic characteristics similar to mouse endothelial eNOS from immunoprecipitation-enriched samples, crude aortic lysates, and human ECs (Figure 1E, right lane). The densitometry of bands corresponding to eNOS in RBC and aorta of 3 independent gels is shown in Figure 1F.

To assess eNOS activity in WT RBCs, we analyzed conversion of radioactive (14C-labeled) arginine to citrulline. We found that membrane preparations of WT RBCs efficiently converted 14C-arginine into 14C-citrulline (0.306±0.107 fmol/min for WT versus 0.107±0.061 fmol/min for eNOS−/−), as determined by significantly higher (6-fold) reactive counts in the reaction supernatant versus their eNOS−/− counterparts (Figure 1G).

Vascular eNOS Expression Is Not Conferred by Bone Marrow Transplantation

WT and eNOS−/− mice were cross-transplanted to elucidate the contribution of BC eNOS to intravascular nitrite formation and physiological BP regulation in vivo. Chimeric animals resulting from this cross-transplantation strategy either expressed eNOS only in BC or in the entire rest of the animal, specifically the vascular EC. These mice are hereafter, respectively, referred to as BC+/EC− and BC−/EC+ chimeras to simply summarize group identity. Control chimeras globally competent (BC+/EC+) and globally deficient (BC−/EC−) for eNOS were created for comparison purposes using the same BM transplant protocol, with WT marrow transplanted into WT mice and eNOS−/− marrow transplanted into eNOS−/− mice (Figure 2A). At 6 to 8 weeks after transplantation, flow cytometric analysis of relative CD45 expression (45.1 versus 45.2, mismatched for BM donors and recipients) by peripheral leukocytes confirmed that the BC compartments of cross-transplanted WT and eNOS−/− BM recipients converted >90% to the donor phenotype (Figure 2B). Leukocyte and platelet counts were within normal ranges for all chimeras and did not significantly differ between groups (Table 1 in the online-only Data Supplement). Western blot analysis (Figure 2C) demonstrated that eNOS expression was undetectable in aortas of BC+/EC− chimeras, similar to untransplanted eNOS−/− (knockout) mice. These data were confirmed by real-time reverse transcriptase polymerase chain reaction (Figure 2D and 2E), indicating that transfer of WT BM into eNOS−/− recipients does not give rise to vascular eNOS expression. This finding is consistent with a recent lineage tracing study that uses green fluorescent protein to show no significant contribution of transplanted eNOS WT cells to the vascular endothelium of eNOS−/− mice. Thus, vessel walls of the chimeras used in this study retained their pretransplantation phenotypes, whereas blood took on the phenotype of the BM donor. The possibility of a role for compensatory upregulation of vascular cyclooxygenase-1 or cyclooxygenase-2 in the transplanted chimeras was also ruled out; reverse transcriptase polymerase chain reaction confirmed similar cyclooxygenase-1 (Figure 2F) and cyclooxygenase-2 (Figure 2G) mRNA expression in all 4 chimeric groups.
Vascular Reactivity and cGMP Levels Are Not Impaired by BM Transplantation

To assess the effects of lethal irradiation and BM transplantation on vascular function, wire myography was used to compare vascular reactivity of aortic rings from mice competent for vascular eNOS (WT, BC+/EC+, BC−/EC+). Endothelial-independent (phenylephrine, sodium nitroprusside; Figure 3A and 3C) and endothelial-dependent (acetylcholine; Figure 3B) relaxation and contraction responses were similar between all 3 groups. Furthermore, cGMP levels were measured in the aortas of cross-transplanted Düsseldorf eNOS−/− chimeras (Figure 3D) as a parameter of eNOS-dependent vascular reactivity. Both BC−/EC+ and BC+/EC+ chimeras demonstrated cGMP levels similar to WT controls, indicating that any BP differences of chimeric mice lacking BC eNOS (BC−/EC+), compared with WT controls with global competency for eNOS (BC+/EC+), are not a consequence of altered vascular function from the irradiation and transplantation procedure.

Blood Cell eNOS Rescues eNOS−/− Mice From Hypertension and Low Nitrite Levels

To gain insight into relative contributions of BC versus vessel wall eNOS to circulating NO• derivatives, we measured whole blood (Figure 4A) and plasma (Figure 4B) nitrite in anesthetized, cross-transplanted chimeras. Transplantation of WT recipients with marrow from eNOS−/− donors decreased circulating nitrite levels by roughly one third compared with irradiated and transplanted WT controls (BC−/EC+ vs BC+/EC+; plasma: 0.53±0.10 µmol/L versus 0.79±0.07 µmol/L; P<0.05; whole blood: 0.81±0.14 versus 1.19±0.15; P<0.05). Conversely, transplantation of eNOS−/− recipients with marrow from WT donors increased circulating nitrite levels compared with irradiated and transplanted eNOS−/− controls (BC+/EC+ vs BC−/EC−; plasma: 0.46±0.07 µmol/L versus 0.32±0.02 µmol/L; P=0.08; whole blood: 0.68±0.07 versus 0.49±0.02; P<0.05), indicating that both vascular and BC eNOS are important sources of circulating nitrite in vivo.
For concomitant measurement of both circulating nitrite levels and corresponding BPs, mice were analyzed under anesthetized conditions, and BP was measured by carotid artery cannulation (Figure 4C–4E). Transplantation of WT BM into eNOS−/− background (BC+/EC−) significantly decreased BPs relative to BC+/EC− vs BC−/EC− chimeras (MAP: 87.33±2.88 versus 106.1±5.43 mm Hg; P=0.0014; Figure 4C). Similar results were obtained by radiotelemetry measurement of BP in awake BC+/EC− chimeras versus their BC+/EC− counterparts (Figure 5B; diastolic: P=0.0036; systolic: P=0.0112; MAP: P=0.0056). Although the BP-lowering effect afforded by BC eNOS in otherwise eNOS-deficient mice was low (5 mm Hg), radiotelemetry experiments demonstrated its reproducibility in 2 investigated eNOS−/− strains (Harvard and University of North Carolina; Figure 5C and 5D). In addition, transplantation of eNOS−/− BM into WT background significantly increased BPs relative to BC+/ EC+ chimeras under awake conditions (Figure 5B; systolic: P=0.0171; diastolic: P<0.0001; MAP: P=0.0011), again supporting a role for BC eNOS in BP regulation. Differences in the baseline BPs obtained in the anesthetized versus awake BC−/EC+ chimeras suggest an effect for anesthesia (Figure 4C–4E and Figure 5B). The data also demonstrate an effect for eNOS in the vascular wall, apparent in anesthetized and awake BC+/EC− chimeras versus their BC+/EC+ counterparts (MAP [mm Hg] of 75.39±2.0 versus 106.1±5.43; P=0.05; Figure 4C and 36-hour MAP [area under the curve] of 4100 versus 3764; P=0.0003; Figure 5A and 5B).
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10−9 to 10−3.5) and (\(\text{Ach}\) is acetylcholine (Ach: 10−9 to 10−3 mol/L)). Both vascular and blood eNOS contribute to intravascular nitrite and the vasodilatory function of eNOS, in BP regulation. Our findings support a role for blood eNOS, in addition to vascular eNOS, in BP regulation.

Given our previous observations of a contribution from both vascular and blood eNOS to intravascular nitrite and the proposed contribution of nitrite to BP regulation, we also explored the relationship between mean plasma and whole blood nitrite levels versus MAP and found an inverse correlation between these parameters across all chimeric groups (plasma: \(r=−0.91; P<0.05\); whole blood: \(r=−0.91; P=0.05\); Figure 4F).

Together, these data support an interrelated role for BC eNOS in nitrite formation and BP regulation. However, the cause and effect remain to be established.

**Blood eNOS Lowers BP Independently of Blood iNOS, Platelets, and Leukocytes**

Constitutive expression of eNOS in immune cells has been shown to serve overlapping physiological and pathophysiological roles, most notably in activating the proinflammatory profile (iNOS and nuclear factor-κB gene expression) of monocytes/macrophages (human and rodent) in a manner consistent with nanomolar-level NO• production that is Ca\(^{2+}/\)calmodulin- and cGMP-dependent. The possibility that restoration of immune cell iNOS in an otherwise eNOS-deficient setting, such as the BC+/EC− chimera, could regulate BP was addressed in eNOS\(^{−/−}\) mice cross-transplanted with BM from iNOS\(^{−/−}\) donors (BC+/iNOS\(^{−/−}/\text{EC−}\)). The resulting chimeric group, possessing blood eNOS but not blood iNOS, maintained a lower BP than the BC−/EC− group, indicating that blood iNOS was not participating in physiological BP control in these mice (Figure 4G).

We further investigated the BC type contributing to BP regulation by depleting BC+/BC− chimeras of their platelets or leukocytes. Platelet eNOS is a well-documented antagonist of platelet activation and aggregation. Most leukocytes also express eNOS, although its function in these cells is yet to be clearly defined. To determine whether platelets are the BC source of eNOS involved in physiological BP regulation, BC+/EC− chimeras and their hypertensive counterparts (BC−/EC−) were pharmacologically depleted of platelets (>90%) using an antithrombocyte serum (Table II in the online-only Data Supplement). Although platelet depletion lowered MAPs equally in both groups, the BP-lowering effect of red cell eNOS persisted between treated groups (18.45±2.54 mm Hg) to a similar extent as between untreated (platelet-competent) groups (18.80±3.60 mm Hg; Figure 4H).

Leukocytes were also depleted using an anti-CD45 antibody that reacts with the pan-leukocyte antigen CD45 (Ly5), causing clearance of leukocytes from circulation. Treatment of BC+/EC− and BC−/EC− chimeras with this leukodepleting antibody substantially decreased their circulating leukocyte counts by 75% (Table II in the online-only Data Supplement). Similar to thrombocytopenia, leukopenia decreased BP in both treated groups but did not eradicate the significant BP-lowering effect of red cell eNOS (Figure 4H).

The persistence of lower arterial BPs in BC+/EC− chimeric mice, relative to eNOS\(^{−/−}\) controls (BC−/EC−), after platelet- or leukocyte-depletion treatments indicates that platelet- and leukocyte-derived eNOS are not responsible for the BP-modulating effect of circulating blood eNOS and supports a role for a functional red cell eNOS in BP control.

**Blood Cell eNOS Effects on BP Are Abolished by NOS Inhibition in Conscious Mice**

To further assess the role of blood eNOS availability and function in BP regulation, conscious BP responses to NOS inhibition with L-NAME or reprimistination with l-arginine...
were measured in BC+/EC− chimeras via radiotelemetry. Baseline BP (pretreatment) was allowed to stabilize for 10 days after BP sensor implantation. The average BP for the BC+/EC− group was significantly lower than that of its BC−/EC− counterpart (systolic: \( P=0.0112 \); diastolic: \( P=0.0036 \); MAP: \( P=0.0056 \)), strongly supporting a role for blood eNOS in physiological BP regulation (Figure 5A). Mice were then treated with L-NAME in the drinking water for 4 days (days 11–14) followed by \( \text{l-arginine} \) consumption increased MAP compared with baseline (3.16±4.43 mm Hg; NS), whereas \( \text{l-arginine} \) consumption decreased MAP compared with L-NAME (−7.43±4.43 mm Hg; \( P<0.05 \)). These experiments, performed in chimeras generated from the Harvard eNOS−/− mouse (our usual eNOS-deficient mouse line used for all eNOS−/− experiments except where noted), were repeated in a second group of BC+/EC− chimeras. Blood pressure data analyzed by 1-way Student’s t test. F, Linear regression of MAP averages and nitrite concentrations in plasma (left y axis) and whole blood (right y axis) across all chimeric groups. Blood pressure data expressed as mean±SEM (SEM for BC−/EC− MAPs for whole blood and plasma are ±0.023 and 0.022 mm Hg, respectively). Correlations of blood pressure and plasma nitrite are expressed as \( r \) and \( P \) value for plasma (blue type) and whole blood (black type). G, Map in anesthetized eNOS-deficient chimeras in the absence of blood eNOS (BC+/EC−: \( n=9 \)), the presence of blood eNOS (BC+/EC+: \( n=15 \)), and the presence of eNOS, but not iNOS (BC+/EC+: \( / \text{EC}− \); \( n=4 \)) in blood. * \( P<0.05 \) vs BC−/EC−; \( \# \) \( P<0.05 \) and \( \# \# \) \( P<0.005 \) vs BC+/EC+. H, Map in the absence or presence of platelet depletion (+APS; BC+/EC−: \( n=5 \) and BC−/EC−: \( n=7 \)) or leukocyte depletion (+ anti-CD45 Ab; BC+/EC−: \( n=3 \) and BC−/EC−: \( n=4 \)) in anesthetized BC+/EC− and BC−/EC− chimeras are shown. Blood pressure data are expressed as mean±SEM. BC indicates blood cell; and EC, endothelial cell.

Figure 4. Blood cell endothelial nitric oxide synthase (eNOS) rescues eNOS−/− mice from low circulating nitrite levels and hypertension. Nitrite concentrations in A whole blood (BC+/EC+: \( n=10 \); BC−/EC−: \( n=8 \), BC+/EC−: \( n=8 \); BC−/EC−: \( n=7 \)) and B plasma (BC+/EC+: \( n=10 \); BC−/EC+: \( n=5 \); BC+/EC−: \( n=8 \); BC−/EC−: \( n=7 \)) of cross-transplanted chimeras. BC+/EC− (globally competent for eNOS), BC+/EC− (deficient of blood eNOS), BC+/EC+ (deficient of vascular eNOS), and BC−/EC− (globally deficient of eNOS). Data are expressed as mean±SEM vs BC−/EC−: ** \( P<0.005 \) for whole blood and BC−/EC−: n=4 and BC−/EC−: n=7) or leukocyte depletion (+ APS; BC+/EC−: n=3 and BC−/EC−: n=4) in anesthetized BC+/EC− and BC−/EC− chimeras are shown. Blood consumption increased MAP compared with baseline (3.16±4.77 mm Hg, NS), whereas \( \text{l-arginine} \) consumption increased MAP compared with baseline (5C: L-NAME). These classical patterns (Figure 5C and D) and absent in the UNC eNOS−/− mice (as shown previously), further support the existence of a BC eNOS that participates in physiological BP regulation. BC−/EC− obtained from the Harvard strain mounted a paradoxical (decreased BP) response to L-NAME.
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(Figure I in the online-only Data Supplement), as previously shown for Harvard eNOS−/−;13 however, the differences were not statistically significant. Taken together, these results point to a role for NOS in the decreased BPs observed in the chimeras expressing eNOS only in blood. Until now, conventional wisdom has held that eNOS-mediated control of BP is primarily dependent on eNOS enzyme expressed in the endothelium. These data indicate that BC eNOS also contributes to the regulation of BP.

Discussion

The role of eNOS in vascular endothelium has been shown both in vitro and in vivo to participate in autocrine and paracrine NO• signaling and the control of basal BP.8 Most circulating BCs, including leukocytes, platelets, and RBCs, have been shown to contain eNOS.15−18,21,22,29,31 It has been suggested that red cell eNOS21−24 is capable of producing NO• under normoxic conditions. Data from our laboratory and others have demonstrated NOS-derived effects exerted by RBCs, including inhibition of ADP-induced platelet aggregation,22,32 protection of isolated, perfused hearts from ischemia/reperfusion injury,13 as well as decreased RBC-released NO• metabolite (nitrite, nitrate, nitros(yl)ated species) levels in response to NOS inhibition.22,24 Circulating angiogenic cells also contain a functional eNOS, but their number in peripheral blood is <0.01% of peripheral blood mononuclear cell numbers (0.0067±0.0097 per 100).34−36 However, RBCs are the most abundant cells in blood and transport hemoglobin and contain relatively high levels of nitrite.25 Hence, it is reasonable to consider a role for RBCs as potential regulators of the circulating NO• pool and BP responses. However, a role for red cell eNOS in NO• signaling remains controversial, and a specific role for BC eNOS in BP regulation has not been investigated previously.

In the present study, cross-transplanted chimeric mice genetically competent or deficient for eNOS in circulating BCs are used to demonstrate a previously unrecognized role for BC eNOS in BP regulation and in vivo nitrite homeostasis. Our data support a persistent blood-derived eNOS effect on BP after in vivo platelet and leukocyte depletion, as well as RBC catalysis of arginine to citrulline and generation of intracellular NO• metabolites. Although these studies provide strong evidence for circulating BCs in NOS-dependent control of BP and nitrite production,

Figure 5. Blood pressure–lowering effects of endothelial nitric oxide synthase (eNOS)-competent blood are responsive to L-NG-nitroarginine methyl ester (L-NAME) and L-arginine (L-Arg) treatments. Thirty-six-hour radiotelemetry-detected baseline blood pressures (BPs) in (A) BC+/EC− (n=3) vs BC−/EC− (n=4) (systolic: P=0.0112; diastolic: P=0.0036; MAP: P=0.0056) and (B) BC−/EC+ (n=3) vs BC+/EC+ (n=4) (systolic: P=0.0171; diastolic: P=0.0001; mean arterial pressure [MAP]: P=0.0011), with group averages calculated for each 2-hour interval and area under the curve (AUC) by 2-way ANOVA. Comparisons of moving averages, assessed by 2-way ANOVA, were also statistically significant (P<0.0001). x axis depicts hours of darkness (gray shade) vs hours of light (white shade). Change in radiotelemetry-detected MAP (mm Hg) in (C) Harvard BC+/EC− chimeras (n=5) and (D) UNC BC+/EC− chimeras (n=5) after oral treatment with L-NAME or L-arginine. Blood pressure data for individual animals shown as solid lines; group averages shown as dashed red line. ‡P<0.05 for L-NAME vs baseline; #P<0.05 for L-arginine vs L-NAME; *P<0.05 using Student t test. BC indicates blood cell; and EC, endothelial cell.
definitive clarification of the role of the erythrocyte in this process will require the use of erythroid cell–specific knockout approaches in future studies.

We performed numerous control experiments to test the hypothesis that red cell eNOS regulates BP. We verified that endothelial function/integrity was not affected by our experimental setting, as demonstrated by an absence of change in endothelial-dependent and endothelial-independent vasodilation of aortic rings or aortic cGMP levels relative to the appropriate control group or WT mice. The BP-lowering effects of circulating BC eNOS were not a result of incorporation of BM cells into vascular endothelium, as demonstrated here by the absence of eNOS in Western blot–analyzed BC+/EC− aortas. Consistent with our observation, a recent study involving transplantation of GFPpos (eNOS competent) BM into sublethally irradiated eNOS−/− mice confirmed that BM-derived endothelial progenitor cells do not incorporate into vascular endothelium.48 Furthermore, we performed control experiments to rule out the contribution of other NOS isoforms to observed changes in BP. We did not detect a compensatory upregulation of other NOS isoforms or the cyclooxygenase 2 gene in the vasculature of the Harvard eNOS−/− mice, a finding that has been previously reported but remains controversial because of reported disparities between different strains of eNOS−/− mice.13,30,37–40 If other NOS isoforms are responsible for the BP effects we observed in the BC+/EC− chimeras, then a general NOS inhibitor, such as L-NAME, should have reversed those effects. We instead noted that the BC+/EC− chimera mounted a paradoxical hypotensive response to L-NAME treatment (shown in Figure I in the online-only Data Supplement), a result that has been previously described in the Harvard strain by different groups,13,30 whereas the BC+/EC− chimera mounted the classical increased BP response. Paul Huang and others have reported an upregulation of nNOS in the Harvard eNOS knockout mouse13,41,42 and have speculated on its possible role in the mutant’s hypotensive BP response to L-NAME treatment.13 In the presence of a nonspecific NOS inhibitor, such as L-NAME, a compensatory role for nNOS is unlikely to account for our observation. Furthermore, although Paul Kubes’ group has shown that nNOS is upregulated in venules.43 H2O2-stimulated leukocyte infiltration in postcapillary venules.43 H2O2 has been proposed by several groups as an important endothelium-derived hyperpolarizing factor, but in a NOS-dependent manner.18,44 In addition, nonspecific effects of L-NAME treatment have been reported: antagonism of muscarinic acetylcholine receptors45 and inhibition of cytochrome c reduction in vitro.46 Indeed, we observed normal endothelial function in WT mice with elevated BP as a result of BC deficiency of eNOS (BC−/EC− chimeras), an unexpected finding given the general association of elevated BP and endothelial dysfunction.

With regard to a specific role for the RBC in these observations, we did not observe an effect of leukocytes or platelets on eNOS-dependent BP regulation. Reductions in BP in our BC+/EC− mice were not abrogated by platelet-, leukocyte-, or iNOS-depleting interventions, arguing against a significant role for any of these factors in physiological BP regulation. Although these interventions lowered BP in all groups, the difference in BP observed between the BC+/EC− and BC−/EC− chimeras remained significant and equivalent. The intervention-associated hypotensive responses could be attributable to deficiency of platelet- or neutrophil-derived thromboxane A2(2), reactive oxygen species, and augmented prostacyclin levels.40 Our anti-leukocyte intervention targeted circulating lymphocytes and neutrophils alike (≥75% depletion). This may be important, given the antihypertensive effects previously shown to be elicited in rats and mice by thymocyte,49 or neutrophil-depleting50 interventions. It is unlikely that these hypotensive effects could have masked an augmentation in BP because of platelet or leukocyte eNOS deficiency, because the absolute decreases in BP in the BC+/EC− chimera remained constant with and without depletion of leukocytes and platelets.

A recent study evaluating similar chimera experiments reported results that differed from ours. That study observed no BP-reducing effect for GFPpos WT BM when transplanted into eNOS−/− mice.48 This discrepancy in BP results is likely attributable to differences in study design. Whole-body irradiation and BM transplantation are not without potentially confounding effects, thus the WT and eNOS−/− controls used in this study were also irradiated and BM transplanted, whereas the controls used in the other study were not.

Taken together, the findings from this study point consistently toward a functional circulating RBC eNOS that is active in physiological vasorelaxation and nitrite homeostasis and may make contributions to other as yet unidentified biological processes. If eNOS in blood can contribute to BP regulation and nitrite production, then it is likely to have effects under reparative conditions, such as after myocardial ischemia or stroke. Indeed, circulating BM-derived endothelial progenitor cells have been shown to participate in the neovascularization of jeopardized tissue.51 A prior study by Li et al52 may have relevance to our observations. That study evaluated similar cross-transplantation experiments with BM-recipient mice subjected to experimental myocardial infarction. Cardioprotection was observed in the eNOS−/− mice receiving WT BM, which at the time was ascribed to NO• released by endothelial progenitor cells that had incorporated into ischemic myocardium. However, in that study only a small percentage of the incorporated endothelial progenitor cells and the cardiomyocytes in ischemic myocardium expressed eNOS, suggesting that the cardioprotection may have derived from circulating BC-derived eNOS activity or nitrite. As a final component of these NO•-related regenerative pathways, numerous groups have demonstrated that circulating nitrite can be reduced in the blood to form NO•, regulate hypoxic vasodilation, cytoprotection after ischemia/reperfusion events, and vascular angiogenesis.9–11,53–55 It is likely that both direct NO• formation and signaling, as well as indirect NO• oxidation to nitrite, contribute to the therapeutic effects of red cell eNOS in angiogenesis, cytoprotection, and BP control.

In summary, the present study provides strong evidence that a circulating blood eNOS participates in nitrite homeostasis and BP regulation under physiological conditions (Figure 6). These findings provide novel insight into mechanisms of BP control that challenge our conventional perspectives on NO• and nitrite signaling in blood, suggesting a more...
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Figure 6. Contribution of blood cell endothelial nitric oxide synthase (eNOS) to regulation of the circulating nitric oxide (NO•) pool and blood pressure under normoxic and hypoxic conditions. The circulating NO• pool consists of various NO•-related species that transport bioactive NO• in the mammalian circulation. These oxygenated and nitro(s)ylated NO• species are interconvertible. NO• bioactivity within this pool is regulated by the surrounding redox conditions at the site of delivery. Under hypoxic conditions, NO• is formed in blood by the nitrite reductase activity of heme-containing proteins, including deoxyhemoglobin. Under normoxic conditions, NO• can be produced by eNOS in endothelial cells (EC) and scavenged by oxyhemoglobin in red blood cells (RBC). An eNOS-derived NO• production in blood cells may offset oxyhemoglobin-dependent NO• scavenging, thereby safeguarding vasodilatory activity in adjacent normoxic vessels. Furthermore, eNOS activity in RBCs might contribute to their intracellular storage pool of NO• equivalents, such as nitrite. Thus, a cycle of continuous NO• formation in blood might arise from blood cell eNOS under normoxic conditions and deoxyhemoglobin-mediated reduction of nitrite under hypoxic conditions. EC indicates endothelial cells; HbO2, oxyhemoglobin; HbSNO, s-nitrosated hemoglobin; NO-Hb, nitrosylated hemoglobin; RBC, red blood cells; RSH, thiols; RSNO, nitrosated thiols; and RX, other reactive groups.

Clinical Perspective

Hypertension is a complex multifactorial condition associated with cardiovascular disease. Accumulating evidence points to a correlation between BP and circulating NO• metabolites, such as nitrite. The data presented here demonstrate a role for circulating BC eNOS in nitrite homeostasis and BP regulation under physiological conditions. A cycle of continuous NO• formation in blood might arise from BC eNOS under normoxic conditions and deoxyhemoglobin-mediated reduction of nitrite under hypoxic conditions. This cycle of NO• formation within blood and the vasculature may be impaired in cardiovascular disease, where a generalized eNOS dysfunction is often marked by eNOS uncoupling or protein deficiency. Indeed, recent years have seen anemia and RBC dysfunction identified as independent risk factors for cardiovascular disease. There is a growing recognition that intravascular hemolysis represents a fundamental mechanism for human disease through the release of cell-free plasma hemoglobin that inhibits NO• signaling in the subendothelial layer. It is common to hemoglobinopathies and hemolytic anemia, as well as to the RBC storage lesion of aged blood used in transfusion therapy, and may be driven by red cell eNOS-linked enzymopathies and membranopathies.36–62 Thus, our findings may illuminate novel avenues for assaying the general status of eNOS activity and cardiovascular health in the human body.

Acknowledgments

We thank Paul Huang and Axel Gödecke for providing the Harvard and Düsseldorf mice genetically deficient in endothelial nitric oxide synthase (eNOS−/−), respectively. We thank the staff of the Murine Phenotyping Core and the Laboratory of Animal Medicine and Surgery facility for assistance with the radiotelemetry-monitored blood pressure experiments, Sivatharsini Sivarajah for the Western blot analysis of mouse red blood cells, and Simone Zander for providing the chimeras from Düsseldorf eNOS−/− mice. We acknowledge the professional skills and advice of Dr Christian Heiß, Dr Christian A. Combs, and Dr Daniela Malide (Light Microscopy Core Facility, National Heart, Lung, and Blood Institute, National Institutes of Health) and Prof Dr Dieter Haussinger for use of the FACS Canto II flow cytometer.

Sources of Funding

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Disclosures

Dr Gladwin is a coinventor on a National Institutes of Health government patent application on the use of nitrite salts for cardiovascular diseases. The other authors report no conflicts.

References


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**Significance**

These are the first studies, using cross-transplant chimera models, to definitively identify a mouse circulating blood endothelial nitric oxide synthase that regulates intravascular nitrite homeostasis, cGMP signaling, and the control of systemic blood pressure under physiological conditions. Our findings extend the current paradigm for endothelial generation of NO• and nitrite in the regulation of blood pressure, suggesting a contribution from circulating red blood cells to NO• and nitrite synthesis and vascular homeostasis. Hypertension is a complex multifactorial condition associated with cardiovascular disease. Accumulating evidence points to a correlation between blood pressure and circulating NO• metabolites, such as nitrite. The existence of a functional red blood cell endothelial nitric oxide synthase invites studies addressing the function and dysfunction of blood endothelial nitric oxide synthase in cardiovascular health and disease. Our findings may illuminate novel avenues for assessing the general status of endothelial nitric oxide synthase activity and cardiovascular health in the human circulation.
Circulating Blood Endothelial Nitric Oxide Synthase Contributes to the Regulation of Systemic Blood Pressure and Nitrite Homeostasis

Katherine C. Wood, Miriam M. Cortese-Krott, Jason C. Kovacic, Audrey Noguchi, Virginia B. Liu, Xunde Wang, Nalini Raghavachari, Manfred Boehm, Gregory J. Kato, Malte Kelm and Mark T. Gladwin

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Materials and Methods

Materials
Phenylephrine (PE, P6126), acetylcholine (Ach, A6625), sodium nitroprusside (SNP, S0501), N (G)-nitro-L-arginine methyl ester (L-NAME, N5751) and L-Arginine (L-Arg, A8094) and NADPH (N0411) were purchased from Sigma. Purified rat anti-CD45 antibody (clone 30-F11) and Ter-119 (550565) were from BD Pharmingen (San Jose, CA). Anti-platelet serum (AIAD31440) was from Accurate Chemical (Westbury, NY). Bovine recombinant eNOS (60880) and the NOS activity assay (781001) were obtained from Cayman Chemical. Purified mouse anti-human eNOS antibody (610296) and rabbit anti-human eNOS was from BD Biosciences (San Joes, CA). Rabbit polyclonal anti-eNOS antibody (Ab66127) was from Abcam Inc. (Cambridge, MA). L-[\textsuperscript{14}C] arginine (NEC267E050UC) was from Perkin Elmer (Waltham, MA).

Blood collection and sample preparation
Blood was taken from the carotid artery and anticoagulated with citrate (for immunoprecipitation) or with heparin (for loading with fluorescent probes). All experiments were initiated within 2 hours of blood withdrawal. Whole blood was obtained via the carotid artery or inferior vena cava of donor mice (C57Bl/6J and Harvard eNOS\textsuperscript{-/-}).

The following manipulations were used to separate platelet rich plasma and hemoglobin from whole blood. In brief, blood was collected in a polypropylene tube containing 0.1 mL acid citrate dextrose buffer (Sigma) and then centrifuged at 120g, 4°C for 8 min and then at 14000 rpm, 4°C for 2 min. Between centrifugations, platelet rich plasma and buffy coat were removed by pipette and discarded. Leukocyte and platelet contamination were quantified with the aid of a hemocytometer and light microscope and did not exceed 0.05%. Leukocytes (25 uL blood sample) were stained by addition of 465 µL 3% citric acid and 10 µL 1% crystal violet (Sigma). Platelets (20 uL blood sample) were stained with the Unopette System (Becton Dickinson).

For protein identification, whole blood was collected in a syringe and centrifuged at 800 g for 15 min at room temperature (RT) to sediment RBCs prior to elution from the bottom of the syringe. Purity of the RBC preparations was confirmed using flow cytometry (FACS CANTO II; BD Bioscience, San Jose, CA, USA) and antibodies (as per manufacturer guidelines) specific for CD235 (glycophorin) as a RBC marker, CD45 as a leukocyte marker, and CD42 as a platelet marker.

Cells
Human umbilical vein endothelial cells (Promocell GmbH; Heidelberg, Germany) were cultured in 10 cm-diameter Petri plates (passages 1-4) using a commercial endothelial cell basal medium (Promocell GmbH, Heidelberg, Germany) supplemented with penicillin and streptomycin (PAA Laboratories GmbH, Cölbe, Germany). Cell pellets were obtained after detachment with trypsin.

Animals
Wild type C57BL/6 and B6.SJL-PTPRCPEP/BOY, B6.129P2-Nos3tm1Unc/J (UNC eNOS\textsuperscript{-/-}) and B6.129P2-NOS2 TM1 LAU/J (iNOS\textsuperscript{-/-}) were from Jackson Laboratories (Bar Harbor, ME).
Breeder stocks of Harvard eNOS−/− mice, backcrossed 10 generations to C57BL/6 mice, were from Dr. Paul L. Huang (Harvard University). Homozygous matings produced offspring for this study. All animals were housed under pathogen-free conditions and only male mice were used for experiments. eNOS immunoprecipitation experiments in mouse red blood cells, as well as aortic cGMP studies were conducted using Düsseldorf eNOS−/− mice from Dr. Axel Gödecke (Heinrich Heine University of Düsseldorf). Genetic identity of animals was routinely confirmed by PCR analysis of tail clip DNA using gene-specific probes. All surgical procedures were reviewed, approved and performed according to the criteria outlined in the NHLBI Animal Care and Use Committee and the LANUV Nordrhein-Westfalen guidelines. Mice were fed standard laboratory chow ad libitum until use in experiments.

**Chimeras**

Two congenic strains of WT mice on C57Bl/6 background (C57BL/6 and B6.SJL-PTPRCPEP/BOY) were used to mismatch BM donors and recipients for leukocyte antigen expression (CD45.2 versus CD45.1) to permit flow cytometric analysis of recipients’ blood cell reconstitution to donor phenotype. BM cells were isolated from the femurs and tibias of donor mice and resuspended in sterile PBS to a 1-2 x 10^7/mL final cell count. Recipients were lethally irradiated (two 500 rad doses, 3 hours apart). Following the second irradiation, 2-4 x 10^6 donor BM cells in 200 µL of PBS were injected into the retroorbital sinus of each recipient. Chimeras were housed in autoclaved cages with 0.2% neomycin drinking water for 2 weeks, followed by normal drinking water. At 6-8 weeks post-transplant, chimeric mice were assessed by flow cytometry for conversion to donor phenotype prior to use in experiments.

**Flow cytometry**

Flow cytometry was used to verify reconstitution of BM transplanted chimeric mice. In brief, leukocytes (from 50 µL lysed whole blood) from chimeric mice were stained in vitro with FITC-labeled anti-CD45.1 (PharMingen, Inc.) and PerCP-Cy5.5 anti-CD45.2 antibodies (PharMingen, Inc.) and immediately analyzed by flow cytometry (BD FACSCaliber) to determine relative expression of CD45.1 versus CD45.2. Leukocytes were gated based on their size (forward light scatter) and granularity (side light scatter) in a double logarithmic scatter dot plot. The median fluorescence intensity (MFI) of 10,000 events within the leukocyte population was determined by analyzing the distribution histogram obtained by plotting green fluorescence intensity (FITC channel) against far-red fluorescence intensity (PerCP-Cy5.5 channel).

**Immunoprecipitation, gel electrophoresis and western blot analysis**

Mouse RBCs, mouse aorta and human endothelial cells were lysed with RIPA lysis buffer containing protease inhibitor cocktail (Roche Applied Science), as previously described. Total protein concentration was determined by the Lowry assay (DC Protein Assay, Bio-Rad). For direct immunoprecipitation (IP), antibodies were purified from preservatives and contaminants by using Protein G coupled dynabeads (Invitrogen) and concentrated using an ultrafiltration column (Millipore) according to the manufacturer’s’ instructions. The antibodies were then cross-linked to Epoxy-Dynabeads (Invitrogen). RBC or aortic lysate in RIPA Buffer was incubated overnight with crosslinked Dynabeads at 4°C, followed by washing and elution with loading buffer (Invitrogen). For gel electrophoresis, samples were loaded in Bis-Tris gel, 4-12%
(aorta from chimeras) or 3-8% or 7% NuPAGE Novex Tris/Acetate pre-cast gels (Invitrogen).
For western blot analysis, proteins were transferred onto polyvinylidene fluoride (PVDF) membrane Hybond P (Amersham Biosciences, Munich, Germany), using a pre-stained protein ladder (PageRuler Plus, Fermentas Life Science) to control for the transfer. The membrane was blocked with 5% nonfat dry milk (Bio-Rad) in TBS (10 mM Tris, 100 mM NaCl), incubated with a mouse anti-human (overnight 4°C 1:500) or rabbit anti-eNOS antiserum (BD Bioscience) diluted (1 h RT 1:1000) in T-TBS (0.1% Tween in TBS), washed for 30 min in T-TBS, and then incubated with HRP-conjugated goat anti-mouse (1:5000 from Jackson Immuno Research Laboratories (chimeras) or BD Bioscience), or anti-rabbit antibody (1:5000 Rockland, PA, USA). The bands were visualized by autoradiography on Hyperfilm ECL (Amersham Biosciences) using SuperSignal West Pico or Femto Chemiluminescent Substrates (Pierce, Thermo Fisher Scientific, Waltham, MA, USA).

Real-Time reverse transcription polymerase chain reaction (RT-PCR)
Quantitative real-time RT-PCR was used to analyze gene expression of eNOS (NOS3), COX-1 and COX-2 in aortic tissue from C57Bl6/J, eNOS−/−, and BM transplanted chimeric mice (groups: BC+/EC+, BC−/EC+, BC+/EC- and BC−/EC-). First-strand cDNA was synthesized on RNA (previously isolated from aortas) using random primers (Applied Biosystems; Norwalk, CT) in a reverse transcriptase reaction mixture (Superscript cDNA synthesis kit; Invitrogen, Carlsbad, CA). Quantitative real-time PCR assays were carried out with the use of gene-specific double fluorescently labeled probes (Applied Biosystems) in a 7900 Sequence Detector (PE Applied Biosystems) according to the manufacturer’s instructions. In brief, PCR amplification was performed in a 384 well plate with a reaction mixture containing primer, probe, dNTP in real time PCR buffer and passive reference (ROX) fluorochrome, and the appropriate thermal cycling conditions. Samples were analyzed in triplicate and normalized to the housekeeping gene β actin.

Immune-fluorescence staining
Freshly obtained whole blood was smeared across glass slides and allowed to air-dry for 20 min. Paraformaldehyde (4% in PBS) was then applied to the slides for 25 min. After washing, blocking and permeabilization were achieved by applying 5% Donkey serum in PBS with 0.05% Tween 20 for 1 hour. The blocking solution was then discarded and immune-fluorescence staining performed using primary antibodies against eNOS (#ab66127, Abcam) and Ter-119 (#550565, BD Pharmingen), both applied at 1:100 dilution for 1 hour. Control slides were routinely stained in parallel by substituting IgG, or the specific IgG isotype, from the same species for the primary antibody at the same final concentration. After brief washing, secondary antibodies (Rhodamine Red-X conjugated Donkey anti-Rat #712-296-153 and FITC conjugated Donkey anti-Rabbit #711-095-152; Jackson ImmunoResearch Laboratories) were applied for 1 hour, both at 1:200 dilution. After final washing, mounting media containing 4′,6-diamidino-2-phenylindole (DAPI) (#H-1200, Vector Laboratories) and a cover slip were applied. Images were acquired using a Zeiss LSM 510 UV laser scanning confocal microscope system (Carl Zeiss GmbH). For WT, eNOS−/− and IgG controls, immune-fluorescence staining was performed at least eight times per group and using blood from ≥ 5 different mice.
**Wire myography**

Mice were anesthetized with ketamine chloride and xylazine (120 mg/kg and 6 mg/kg body weight i.p., respectively). After placing the mouse in the supine position, the right carotid artery was cannulated for systemic blood pressure measurement (PowerLab). The thoracic and abdominal cavities were opened through a midline incision. After achieving hemostasis, the thoracoabdominal aorta was carefully dissected, removed and placed in ice-cold physiologic salt saline (Krebs buffer). The composition of Krebs buffer was (mM): NaCl (119), KCl (4.5), NaHCO₃ (25), KH₂PO₄ (1.2), MgSO₄ (1.2), L-glucose (11) and CaCl₂ (2.5). Using a dissecting microscope, the attached fat and adventitia were meticulously removed by sharp dissection and clotted blood was flushed from the vessel lumen. Each aortic ring was used to generate a dose-response curve to test for: 1) contraction to phenylephrine (PE: 10⁻⁹ M to 10⁻⁴ M), 2) dilation to sodium nitroprusside (SNP: 10⁻⁹ M to 10⁻⁴.5 M), and 3) dilation to acetylcholine (Ach: 10⁻⁹ M to 10⁻³.5 M). For the determination of SNP and Ach dose-response relationships, aortic rings (3 mm length) were pre-contracted with 10⁻⁶ M PE. Endothelium-independent contraction was calculated as % contraction relative to maximal vessel tension (at the third 120 mMol KCl rinse). Endothelium-independent (SNP) and dependent dilation (Ach) were calculated as % dilation relative to the pre-contraction tension (10⁻⁶ M PE). All experimental protocols were applied to C57Bl/6 mice and BM transplanted chimeras BC+/EC+ and BC-/EC-. Five mice (2 aortic rings per mouse) were included in each group.

**Blood pressure measurements**

Baseline blood pressures (mean arterial, systolic and diastolic) were measured under anesthesia (120 mg/kg ketamine and 6 mg/kg xylazine) via a heparinized catheter (in-line with a blood pressure transducer and using Powerlab software) surgically implanted in the right carotid artery of each mouse. Blood pressure was determined after a 20 min post-surgery stabilization period. Core body temperature was maintained at 35 ± 0.5°C.

The contribution of platelets and leukocytes to blood pressure was assessed in BC+/EC- and BC-/EC- chimeras treated with either anti-platelet serum (APS) or a purified rat anti-CD45 antibody (anti-CD45 Ab) to induce thrombocytopenia or leukopenia, respectively. Treatment with APS (62.5 µl/kg in 200 µl sterile PBS i.p. for 2 days) depleted circulating platelets by ≥ 90% and treatment with anti-CD45 antibody (dose: 1 mg/kg/day in sterile PBS i.p. for 2 days) depleted circulating leukocytes by ≥ 75%. Thrombocytopenia and leukopenia were confirmed by manual platelet and leukocyte counts, respectively. Neither treatment significantly altered hematocrits. Blood pressure measurements were made on the 2nd or 5th day following initiation of treatment with APS or anti-CD45 antibody, respectively.

**Hemodynamic responses**

BC+/EC- chimeras (made with Harvard eNOS⁻/⁻ or UNC eNOS⁻/⁻ mice) were surgically implanted (under temporary anesthesia: ketamine/xylazine or isoflurane) with a microminiaturized electronic monitor (PA-C10; Data Sciences International; St. Paul, MN, USA) attached to an indwelling aortic catheter. Digitized hemodynamic data were continuously sensed, processed and transmitted via radio frequency signals to a nearby receiver (acquisition period: 10 min every 2 hours). Mice were allowed a 10-day post-surgery stabilization period on
standard diet and drinking water before hemodynamic responses to NOS inhibition (L-NAME, 1g/L in drinking water for 4 days) or stimulation (L-Arginine, 2% in drinking water for 3 days) were assessed. Blood pressures were determined by averaging night-time data (22:00 to 4:00) on day 10 (baseline), day 14 (L-NAME) and day 17 (L-Arginine). Change in MAP is reported as mmHg ([L-NAME - baseline BP] and [L-Arg BP - L-NAME BP]).

**cGMP levels in mouse aorta**

The levels of cGMP in mouse aorta were assayed by using a DetectX High Sensitivity Direct cyclic GMP Immunoassay kit (Arbor Assay, Ann Arbor, MI, USA), following the manufacturer’s instructions. The cGMP concentrations were normalized for protein content using the Lowry assay (DC Protein Assay, Bio-Rad).

**Reductive chemiluminescence**

Reductive chemiluminescence measurements were performed as previously described with minor modifications. Briefly, whole blood and plasma nitrite were measured in blood samples collected via carotid cannulation of anesthetized (120 mg/kg ketamine hydrochloride and 6 mg/kg xylazine) mice into sterile, nitrite-free, heparinized (0.5-1 IU) syringes. Whole blood nitrite was preserved by diluting it 1:4 in preservation solution (800 mM K$_3$Fe(CN)$_6$, 10%, v/v Nonidet-40 substitute, and 100 mM N-ethylmaleimide. Plasma was obtained by centrifugation at 4°C and 14000 rpm for 2 min. Nitrite concentrations were determined by the tri-iodide assay in a chemiluminescence NO• analyzer (Sievers NOA; Boulder, CO) using nitrite as a standard.

**NOS activity**

RBC membrane extracts were prepared from platelet- and leukocyte-poor blood by 3 to 4 washing steps in 1 mL homogenization buffer (Cayman Chemical) and centrifugations at 14000 rpm, 4°C for 20 min to produce relatively hemoglobin-free RBC membrane preparations. NOS activity of 10 µl RBC membrane preparations was assayed by measuring the conversion of L-[$^{14}$C] arginine into L-[$^{14}$C] citrulline using a commercial kit (Cayman Chemicals), following the manufacturer’s protocol with minor modifications. Reaction buffer (45 µL) contained 5 µL L-[$^{14}$C] arginine (concentration: 0.1mCi/mL, specific activity: >300mCi (11.1GBq/mmol), Perkin Elmer), 1 mM NADPH, 100 nM calmodulin, 2 mM CaCl$_2$ (final volume 65 µL). All samples were prepared in duplicate: 1) incubation at 37°C for 24 hours and 2) incubation at -20°C for 24 hours. After addition of 400 µL of stop buffer followed by freeze fracture at -20C for 20 min, L-[$^{14}$C] citrulline was eluted in 100 uL ion exchange resin and centrifuged at 2000 rpm, RT for 4 min. Proteins were precipitated using ice-cold methanol (600 µL) for 20 min at 20°C, and centrifugation for 2 min at 14000 rpm, to avoid color quenching. The increase in production of L-[$^{14}$C] Citrulline equivalents was calculated as follows:

\[
\frac{[WT_{37C \text{ cpm}} - WT_{-20C \text{ cpm}}]}{[\text{eNOS}^\text{-37C cpm} - \text{eNOS}^\text{-20C cpm}]}.
\]

The eNOS activity (fmol/min) was calculated as the conversion of the added radioactive L-[$^{14}$C] Arginine (cpm/fmol) into L-[$^{14}$C] Citrulline (cpm) during the considered reaction time.
**Statistical analysis**

All values are reported as mean ± SEM. Comparisons between groups were made using either Student’s t-test or ANOVA followed by Bonferroni posthoc test for more than two group comparisons. Differences were deemed significant when p < 0.05. Statistical analyzes were performed using GraphPad Prism.

**References**

Supplemental Tables

Table SI. Hematocrit (Hct), platelet, white blood cell (WBCs) and polymorphonuclear leukocyte (PMNs) counts in untreated C57Bl/6J (WT), eNOS deficient (KO) and cross-transplanted chimeras.

<table>
<thead>
<tr>
<th>Group</th>
<th>Hct (%)</th>
<th>Platelets (10^3)</th>
<th>WBCs (10^3)</th>
<th>PMNs (10^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>39.9 ± 0.8 n=9</td>
<td>1285 ± 91.9 n=8</td>
<td>38.0 ± 4.2 n=9</td>
<td>9.8 ± 0.9 n=9</td>
</tr>
<tr>
<td>KO</td>
<td>40.1 ± 0.8 n=7</td>
<td>1541 ± 88.0 n=7</td>
<td>39.9 ± 4.3 n=7</td>
<td>10.4 ± 1.0 n=7</td>
</tr>
<tr>
<td>BC+/EC+</td>
<td>43.9 ± 0.9 n=14</td>
<td>1665 ± 116.5 n=7</td>
<td>63.6 ± 7.2 n=10</td>
<td>16.1 ± 3.0 n=7</td>
</tr>
<tr>
<td>BC-/EC+</td>
<td>40.2 ± 2.1 n=5</td>
<td>1181 ± 413.0 n=2</td>
<td>85.5 ± 3.0 n=3</td>
<td>25.1 ± 1.3* n=3</td>
</tr>
<tr>
<td>BC+/EC-</td>
<td>41.7 ± 1.2 n=12</td>
<td>1511 ± 69.0 n=13</td>
<td>49.4 ± 12.0 n=10</td>
<td>17.2 ± 3.3 n=10</td>
</tr>
<tr>
<td>BC-/EC-</td>
<td>42.6 ± 0.8 n=11</td>
<td>1510 ± 102.1 n=9</td>
<td>56.0 ± 7.7 n=5</td>
<td>14.3 ± 1.9 n=5</td>
</tr>
</tbody>
</table>

Using One-way ANOVA and Bonferroni post-hoc test: * denotes P < .05 vs WT.

Table SII. Platelet, white blood cell (WBCs) and polymorphonuclear leukocyte (PMNs) counts in BC+/EC- and BC-/EC- chimeras before and after platelet- (APS) or leukocyte-depleting (anti-CD45 Ab) treatment.

<table>
<thead>
<tr>
<th>Group</th>
<th>Platelets (10^3)</th>
<th>WBCs (10^3)</th>
<th>PMNs (10^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
</tr>
<tr>
<td>APS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BC+/EC-</td>
<td>n=5</td>
<td>1557±62.8</td>
<td>128.0±17.8**</td>
</tr>
<tr>
<td>BC-/EC-</td>
<td>n=8</td>
<td>1571±92.4</td>
<td>120.0±18.1**</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>Platelets (10^3)</th>
<th>WBCs (10^3)</th>
<th>PMNs (10^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
</tr>
<tr>
<td>Anti-CD45 Ab</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BC+/EC-</td>
<td>n=3</td>
<td>61.7±1.2</td>
<td>20.9±8.1**</td>
</tr>
<tr>
<td>BC-/EC-</td>
<td>n=4</td>
<td>62.0±6.3</td>
<td>10.9±2.2**</td>
</tr>
</tbody>
</table>

Using One-way ANOVA and Bonferroni post-hoc test: * denotes P<.05 and ** denotes P < .0001 using one-way ANOVA and Bonferroni post-hoc test.
Table SIII. Baseline systolic and diastolic blood pressures of cross-transplanted eNOS\(^{-/-}\) chimeras during hours of daylight and darkness.

<table>
<thead>
<tr>
<th>Group</th>
<th>Systolic BP</th>
<th>Diastolic BP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AUC (mmHg)</td>
</tr>
<tr>
<td>BC+/EC+ (n=4)</td>
<td>1111</td>
<td>1263</td>
</tr>
<tr>
<td>BC-/EC+ (n=3)</td>
<td>1263</td>
<td>1317</td>
</tr>
<tr>
<td>BC+/EC- (n=3)</td>
<td>1230</td>
<td>1415</td>
</tr>
<tr>
<td>BC-/EC- (n=4)</td>
<td>1325</td>
<td>1553</td>
</tr>
</tbody>
</table>

\(p\)-value

<table>
<thead>
<tr>
<th></th>
<th>p-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC-/EC+ vs BC+/EC+</td>
<td>0.0053*</td>
<td>0.2729</td>
</tr>
<tr>
<td>BC+/EC- vs BC-/EC-</td>
<td>0.0494*</td>
<td>0.1488</td>
</tr>
<tr>
<td>BC+/EC- vs BC-/EC+</td>
<td>0.6026</td>
<td>0.0010*</td>
</tr>
</tbody>
</table>

Day: 0600 to 1800 hours, Night: 1800 to 0600 hours. Group averages calculated for each 2 hour interval and area under the curve (AUC) by Two-way ANOVA with Bonferroni post-hoc test. * denotes p<0.05 and ** denotes p<0.005.
Supplemental Figure I

Figure SI. Paradoxical response to L-NAME of BC-/EC- mice, similar to Harvard eNOS−/− mice. Harvard BC+/EC+ chimeras (n = 5) and BC-/EC- chimeras (n = 3) after oral treatment with L-NAME or L-Arg. Blood pressure data for individual animals shown as solid lines. Comparison of MAP responses to L-NAME treatment by WT and Harvard eNOS KO mice, as reported by Huang et al. (inset graph).13