Priming of hypoxia-inducible factor by neuronal nitric oxide synthase is essential for adaptive responses to severe anemia

Albert K. Y. Tsui[a,b], Philip A. Marsden[c,1], C. David Mazer[a,b], S. Lee Adamson[b,d], R. Mark Henkelman[e], J. J. David Ho[c,e], David F. Wilson[f], Scott P. Heximer[b,g], Kim A. Connelly[h], Steffen-Sebastian Bolz[b,g,i], Darcy Lidington[b,i], Mostafa H. El-Beheiry[a,b], Neil D. Dattani[a], Kevin M. Chen[a], and Gregory M. T. Hare[a,b,i,1]

[a]Department of Anesthesia, [c]Department of Medicine, Division of Nephrology, and [h]Department of Medicine, Division of Cardiology, St. Michael's Hospital, University of Toronto, Keenan Research Centre in the Li Ka Shing Knowledge Institute, Toronto, ON, Canada M5B 1W8; [b]Department of Physiology, University of Toronto, Toronto, ON, Canada M5S 1A8; [d]Department of Obstetrics and Gynaecology, Samuel Lunenfeld Research Institute of Mount Sinai Hospital, University of Toronto, Toronto, ON, Canada M5T 3L9; [e]Department of Medical Biophysics, University of Toronto, Mouse Imaging Centre, Hospital for Sick Children, Toronto, ON, Canada M5T 3H7; [f]Department of Biochemistry and Physics, University of Pennsylvania, Philadelphia, PA 19104; and [g]Heart and Stroke/Richard Leward Centre of Excellence and [i]Toronto Centre for Microvascular Medicine, University of Toronto, Toronto, ON, Canada M5S 1A8

Edited by Solomon H. Snyder, The Johns Hopkins University School of Medicine, Baltimore, MD, and approved September 13, 2011 (received for review August 26, 2011)

Cells sense and respond to changes in oxygen concentration through gene regulatory processes that are fundamental to survival. Surprisingly, little is known about how anemia affects hypoxia signaling. Because nitric oxide synthases (NOSs) figure prominently in the cellular responses to acute hypoxia, we defined the effects of NOS deficiency in acute anemia. In contrast to endothelial NOS or inducible NOS deficiency, neuronal NOS (nNOS)$^{-/-}$ mice demonstrated increased mortality during anemia. Unlike wild-type (WT) animals, anemia did not increase cardiac output (CO) or reduce systemic vascular resistance (SVR) in nNOS$^{-/-}$ mice. At the cellular level, anemia increased expression of HIF-1α protein and HIF-responsive mRNA levels (EPO, VEGF, GLUT1, PDK1) in the brain of WT, but not nNOS$^{-/-}$ mice, despite comparable reductions in tissue PO_2. Paradoxically, nNOS$^{-/-}$ mice survived longer during hypoxia, retained the ability to regulate CO and SVR, and increased brain HIF-α protein levels and HIF-responsive mRNA transcripts. Real-time imaging of transgenic animals expressing a reporter HIF-α(ODD)-luciferase chimeric protein confirmed that nNOS was essential for anemia-mediated increases in HIF-α protein stability in vivo. *S*-nitrosylation effects the functional interaction between HIF and pVHL. We found that anemia led to nNOS-dependent *S*-nitrosylation of pVHL in vivo and, of interest, led to decreased expression of GSNO reductase. These findings identify nNOS effects on the HIF/pVHL signaling pathway as critically important in the physiological responses to anemia in vivo and provide essential mechanistic insight into the differences between anemia and hypoxia.

Anemia predicts outcomes of critically ill patients (1, 2) and those with chronic diseases (3–5). Although anemia is prevalent in these common clinical settings, it is now appreciated that treatment of anemia by transfusion (6) or with erythropoiesis stimulating agents (4) does not necessarily improve mortality. These paradoxical clinical findings underscore the need for a more fundamental understanding of the cellular and molecular responses to anemia. Naively, anemia-associated mortality and organ injury are assumed to be primarily a consequence of a reduction in tissue PO_2 (tissue hypoxia). Although the physiological and molecular responses to tissue hypoxia are increasingly understood (7), it is surprising that so little is understood about how anemia affects tissue O_2 delivery and hypoxic cell signaling. In addition, few studies have compared the physiological and cellular responses to anemia (low Hb) and systemic hypoxia (low P_aO_2). When viewed from the perspective that blood flow, O_2-carrying capacity, and O_2 saturation are the principal determinants of tissue O_2 delivery, the differential control of cardiovascular and cellular responses to anemia are of significant interest.

In this regard, nitric oxide (NO) plays a major role in changes in vascular tone and organ function in the setting of hypoxia. For instance, hypoxia leads to decreases in endothelial NOS (eNOS) mRNA and increases nNOS mRNA expression in vascular endothelium and smooth muscle cells, respectively (8, 9). Studies have demonstrated that acute anemia causes tissue hypoxia and an increase in hypoxia-inducible factor-alpha (HIF-α) and neuronal NOS (nNOS) protein levels, especially in the brain (10, 11).

Both nNOS mRNA and protein expression are regulated by O_2 at multiple levels, both transcriptional and translational (9, 12). The expression of nNOS exerts a functionally significant effect in hypoxic tissues, especially on vascular smooth muscle contraction (9), thereby influencing tissue O_2 delivery. Considering the need to balance the important physiological functions of nNOS-derived NO with the potential for NO cytotoxicity (13), it is not surprising that mechanisms have evolved to ensure that nNOS activity is tightly regulated and linked to tissue O_2 levels (14, 15). In this regard, a central role for nNOS in the regulation of mammalian ventilatory and metabolic adaptations to hypoxia has been recognized (16–18). In addition, the nNOS enzyme more critically depends on O_2 than the other NOS isoforms (apparent K_m for O_2 of 350 μM) (19).

Although NO modulates local blood flow and, hence, O_2 bioavailability, NO is also known to exert a direct effect on O_2 sensing. HIF-α subunits are degraded by the 26S proteasomal pathways during normoxia. The von Hippel–Lindau protein (pVHL) is essential for the ubiquitination and rapid degradation of HIF-α. Posttranslational hydroxylation of proline residues in the oxygen-dependent degradation (ODD) domain of HIF-α is required for the interaction between HIF and pVHL. In the setting of hypoxia, this posttranslational modification does not occur and HIF-α is not degraded. Compelling evidence indicates that under nonhypoxic conditions, *S*-nitrosothiol–based signaling reactions are hypoxia mimetics, and both pVHL and HIF-α have been identified as targets for *S*-nitrosylation (20, 21). NO also inhibits the oxygen-dependent HIF-α prolyl hydroxylases (22). Thus, under normoxic conditions, NO augments HIF activity and is, therefore, a hypoxia-mimetic. In contrast, when cells are truly

Author contributions: A.K.Y.T., P.A.M., C.D.M., S.L.A., R.M.H., J.J.D.H., D.F.W., S.P.H., K.A.C., S.-S.B., D.L., and G.M.T.H. designed research; A.K.Y.T., J.J.D.H., D.L., M.H.E.-B., N.D.D., and K.M.C. performed research; P.A.M., C.D.M., S.L.A., R.M.H., D.F.W., S.P.H., K.A.C., S.-S.B., and G.M.T.H. contributed new reagents/analytic tools; A.K.Y.T., P.A.M., C.D.M., S.L.A., R.M.H., J.J.D.H., S.P.H., K.A.C., S.-S.B., D.L., N.D.D., K.M.C., and G.M.T.H. analyzed data; and A.K.Y.T., P.A.M., and G.M.T.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

[1]To whom correspondence may be addressed. E-mail: hareg@smh.ca or p.marsden@utoronto.ca.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1114026108/-/DCSupplemental.

hypoxic, NO can inhibit mitochondrial O_2 consumption and paradoxically impair HIF stabilization (23). These in vitro findings underscore the need to address NO biology under normal, anemic, and hypoxic settings in vivo.

In the current study, we assessed the impact of acute anemia on survival, cardiovascular responses, tissue hypoxia, and HIF responses in the presence and absence of nNOS. In addition, we wished to compare and contrast the impact of nNOS on these outcomes in anemia and hypoxia. We hypothesized that nNOS improves survival during anemia but not hypoxia, possibly through effects on cardiovascular physiology and HIF signaling.

Results

nNOS Is Protective in Acute Anemia. To assess the role of NOS in acute anemia, hemodilution was performed until mortality in NOS-deficient mice ($nNOS^{-/-}$, $eNOS^{-/-}$, and $iNOS^{-/-}$) (Fig. 1). In WT and $nNOS^{-/-}$ mice, we observed a comparable reduction in hemoglobin concentration (Hb) and increased Hb oxygen saturation (S_aO_2) and P_aO_2. (Table S1). Strikingly, $nNOS^{-/-}$ mice died earlier and at a higher Hb concentration compared with wild-type (WT) mice (Fig. 1; $P < 0.05$, median terminal Hb: 36 g/L vs. 26 g/L). This effect was specific to the nNOS isoform because anemia-induced mortality was not different between WT, $eNOS^{-/-}$, and $iNOS^{-/-}$ mice. These data suggest that nNOS is a protective gene in acute anemia. To compare and contrast acute anemia with acute hypoxia, mice were exposed to a progressive decrease of inspired O_2 level (21–5% O_2). Surprisingly, $nNOS^{-/-}$ mice survived longer than WT mice at 5% O_2 (Fig. 1; $P < 0.05$, median survival time: 10 vs. 3.5 min). These data suggest that nNOS is a detrimental gene in acute hypoxia.

nNOS Contributes to Adaptive Cardiovascular Responses During Anemia. Acute anemia resulted in an increase in CO and reduction SVR in WT anemic mice (Table S1 and Fig. S1). In contrast, CO and SVR responses were severely attenuated in anemic $nNOS^{-/-}$ mice (Table S1 and Fig. S1). We next determined the effect of acute anemia on resistance vessels from the mesenteric circulation. We have reported that hypoxia reduced the contractile response of mesenteric arterioles and that this response depended, in part, on vascular smooth muscle nNOS (9). In contrast, isolated resistance arteries from anemic WT and $nNOS^{-/-}$ mice did not demonstrate impaired contractile responses to phenylephrine or the myogenic response (Fig. S2). This result indicates that the observed decrease in SVR in anemic WT mice was due to mechanism extrinsic to the vascular wall and suggested that S-nitrosyl proteins (SNOs) may have contributed. Paradoxically, the CO and SVR responses to acute hypoxia were opposite; CO increased and SVR decreased only in hypoxic $nNOS^{-/-}$ mice, emphasizing important physiological differences between anemia and hypoxia (Fig. 1 and Fig. S1).

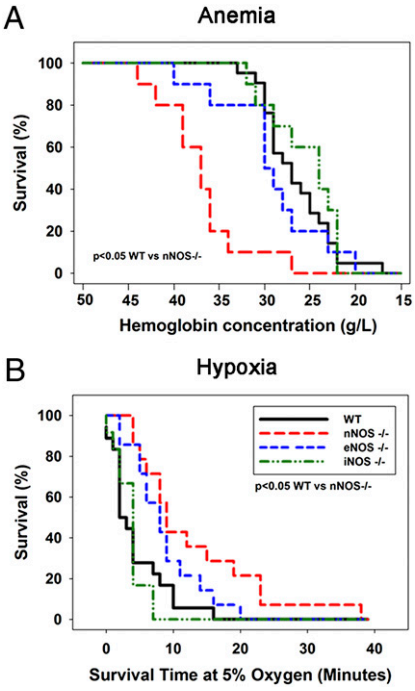

Fig. 1. Differential role of nNOS in the survival of acutely anemic and hypoxic mice. (*A*) WT ($n = 21$, black), $nNOS^{-/-}$ ($n = 10$, red), $eNOS^{-/-}$ ($n = 10$, blue), and $iNOS^{-/-}$ ($n = 10$, green) mice were hemodiluted (acute anemia) until mortality. (*B*) WT ($n = 18$, black), $nNOS^{-/-}$ ($n = 14$, red), $eNOS^{-/-}$ ($n = 14$, blue), and $iNOS^{-/-}$ ($n = 12$, green) were exposed to hypoxia (5% O_2). In both cases, mortality was assessed by the cessation of breathing. $P < 0.05$ WT vs. $nNOS^{-/-}$.

nNOS Is Not Required to Maintain Brain PO_2. Arterial blood O_2 content was reduced by two independent methods: acute anemia (low Hb) and acute hypoxia (low P_aO_2). Despite recent advances in brain O_2 imaging (24), a paucity of studies have assessed brain tissue O_2 delivery in acute anemia. We measured microvascular brain PO_2 by using phosphorescence quenching: a method that is highly selective for O_2 in biological systems but not affected by CO, NO, CO_2, H_2S, or by changes in cerebral blood flow (25, 26). When arterial blood O_2 content was reduced by these two independent methods (anemia: Hb ~ 50 g/L, 21% O_2; and hypoxia: Hb ~ 130 g/L, 15% O_2), microvascular brain tissue PO_2 fell to comparable levels in WT and $nNOS^{-/-}$ mice (Table S1). Despite similar resultant tissue PO_2 level in these superficially related conditions, we observed surprisingly different phenotypes in $nNOS^{-/-}$ and WT mice (Fig. 1 and Fig. S1). Therefore, we assessed the impact of nNOS at the cellular level within brain tissue in anemic and hypoxic mice.

nNOS Is Required for HIF-Dependent Cellular Responses to Anemia. To assess the cellular response to reduced brain tissue PO_2 during anemia, we measured HIF family members and several well-known HIF-regulated genes. We focused on the brain given its high metabolic requirement for O_2 and its known susceptibility to anemia-induced injury (2). We observed an increase in HIF-regulated mRNA transcript levels (EPO, VEGF, GLUT1, PDK1) in brain tissue from anemic WT mice (Fig. 2). These responses were not observed in anemic $nNOS^{-/-}$ mice, suggesting that the lack of nNOS dramatically effected HIF-regulated cellular responses in vivo. By contrast, exposure to hypoxia still led to increases in HIF-regulated mRNA transcripts regardless of nNOS genotype (VEGF and GLUT1) (Fig. 2).

The dramatic difference in the mRNA levels of well-known HIF-regulated genes in anemic WT versus $nNOS^{-/-}$ mice lead us to assess HIF-α protein levels in brain homogenates. It is important to note that comparable levels of basal HIF-α protein were measured in both nNOS replete and deficient strains (Fig. S3). This result is consistent with findings that tissue PO_2 levels are low enough to support HIF stabilization at basal conditions in vivo (24, 26). In anemic WT mice, HIF-1α and nNOS protein levels were increased two- to threefold at 6 and 24 h, relative to nonanemic controls (Fig. 3). The sustained increase in HIF-1α protein was not observed in $nNOS^{-/-}$ mice (Fig. 3). By contrast, HIF-1α protein levels increased above baseline after hypoxia in both strains (Fig. 3). This strikingly different response suggested that nNOS played a seminal role in mediating the HIF-α cellular response to anemia, but not hypoxia. Immunostaining demonstrated that the increase in HIF-1α expression occurred in a perivascular distribution in anemic WT but not $nNOS^{-/-}$ mice (Fig. S4), suggesting that NOS-derived NO may play a role in increasing HIF-1α protein levels in the microvasculature (9, 27). Collectively, these data suggest that priming of HIF-1α by nNOS-derived NO is essential for adaptive responses to severe anemia, possibly modifying *S*-nitrosothiol signaling (28). Thus, nNOS

PHYSIOLOGY

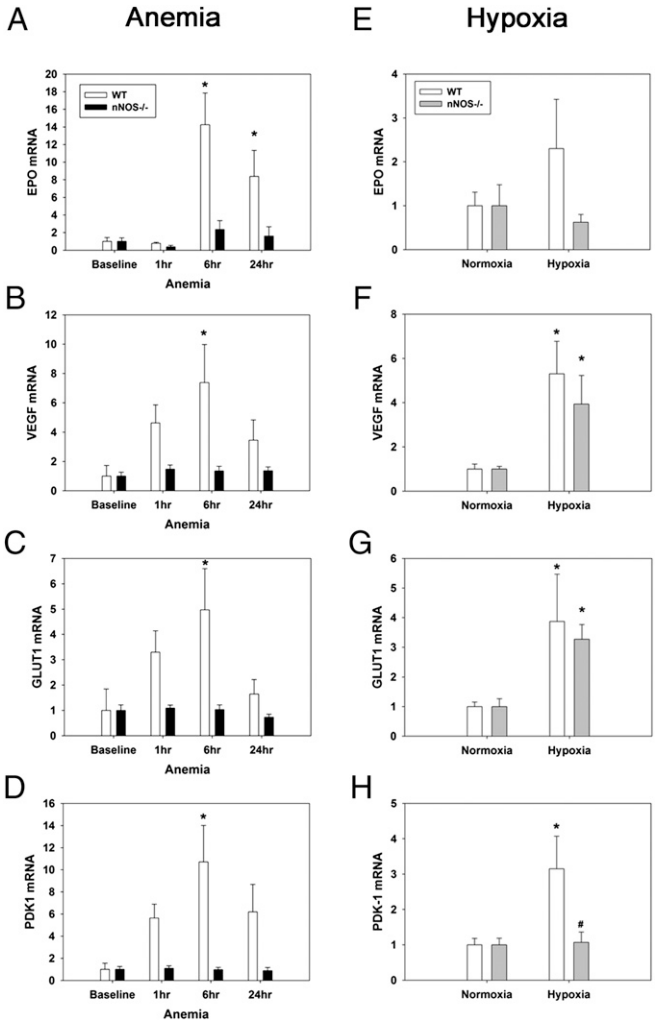

Fig. 2. nNOS regulates HIF-α–dependent genes in acute anemia, but not in hypoxia. HIF-α–dependent mRNA levels were measured in brain samples of WT (open bars) and nNOS$^{-/-}$ in anemic (*A–D*; filled bars) and hypoxic mice after 6 h (*E–H*; gray bars) (n = 6 per group). Relative mRNA levels were normalized to the respective baseline. *P < 0.05 vs. baseline. #P < 0.05 vs. WT.

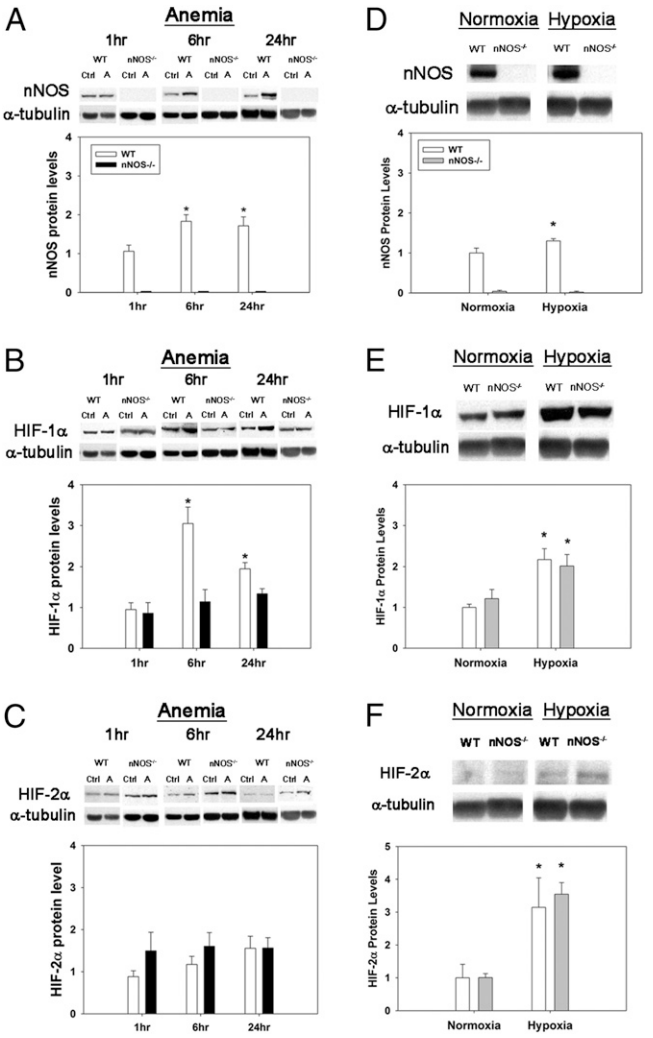

Fig. 3. nNOS regulates HIF-1α protein level in acute anemia, but not in hypoxia. Immunoblots of nNOS, HIF-1α, and HIF-2α protein were assessed in anemic (*A–C*) and hypoxic (*D–F*) WT (open bars) and nNOS$^{-/-}$ brain samples (black or gray bars), respectively. Representative blots of n = 6 per group were shown. Protein levels were normalized to control (Ctrl) group and α-tubulin. *P < 0.05 vs. baseline.

may amplify the HIF-α response to small changes in brain tissue O_2 concentration observed during anemia.

Increased Real-Time HIF-α Expression Depends on nNOS During Anemia. We then used hemizygous HIF-α(ODD)-luciferase mice to further define the molecular basis of the changes in HIF-α protein levels in anemia and hypoxia in vivo (29). These mice contain a ubiquitously expressed ROSA26-targeted transgene driven by a promoter that directs the transcription of a mRNA encoding a chimeric protein containing the firefly luciferase fused in frame with the 123-aa ODD of the human HIF-1α subunit (amino acids 530–652). This ODD contains a prolyl residue of HIF-1α that provides a cellular locus for oxygen-dependent hydroxylation and subsequent proteosomal degradation. Thus, this chimeric protein is regulated by changes in O_2 tension similar to the native HIF-1α protein. In the absence of O_2, the HIF-α(ODD)-luciferase protein is stabilized and the luciferase enzymatic activity can be assessed by dynamic real-time whole animal imaging over a broad range from 1- to 100-fold over background (20). Using this model, we demonstrated a progressive increase in whole body luciferase signal in anemic mice that reached a maximal level after 6–24 h (Fig. S5). Assessment of tissue luciferase activity in vitro at 6 h demonstrated increases in brain, kidney, heart, and liver HIF-α levels, relative to nonanemic controls (Fig. S5). After 3 d, HIF-α returned toward baseline at a time when the Hb concentration had recovered to ≈80 g/L (Fig. S5), a value near the current transfusion threshold (6).

To assess the role of nNOS with respect to anemia-induced HIF-α expression in this model, similar experiments were performed in hemizygous HIF-α(ODD)-luciferase mice that were interbred on a nNOS$^{-/-}$ background (Fig. 4 and Fig. S6). Strikingly, anemic nNOS$^{-/-}$ mice failed to demonstrate an increase in HIF-α(ODD)-luciferase signal relative to nNOS-replete littermate controls at any time point. Assessment of brain tissue extracts was performed in vitro because of the shielding effect of the skull in vivo (Fig. 4). These data confirm that nNOS is required for increased HIFα stabilization in anemic mice. By contrast, hypoxia exposure resulted in an increase in total body and brain luciferase activity in both WT and nNOS$^{-/-}$ mice (Fig. S6), illustrating a profound difference in the impact of nNOS in anemia versus hypoxia at the cellular level. The lack of a luciferase response in the anemic nNOS$^{-/-}$/HIF-α(ODD)-luciferase mice suggests that nNOS was essential to prime or amplify the HIF-1α response to anemia in vivo.

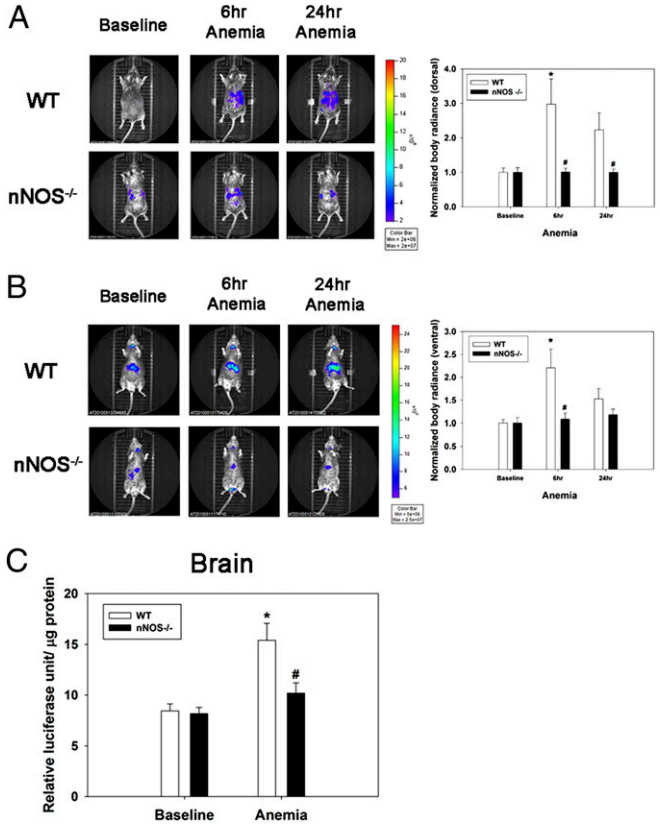

Fig. 4. Anemia leads to increased HIF-1α expression in HIF-α(ODD)-luciferase mice in WT (open bars) but not in nNOS$^{-/-}$ (filled bars) mice. Representative dorsal (*A*) and ventral (*B*) images of WT and nNOS$^{-/-}$ mice ($n = 6$) were obtained at baseline and up to 24 h anemia. Total body radiance was normalized to each animal's baseline. Color bar indicated photons (cm^2 × sec × steradian) with minimum and maximum threshold values. (*C*) Extracted brain tissue was assessed for luciferase activity in vitro at baseline and after 6 h of anemia ($n = 6$). *$P < 0.05$ vs. baseline; #$P < 0.05$ vs. WT.

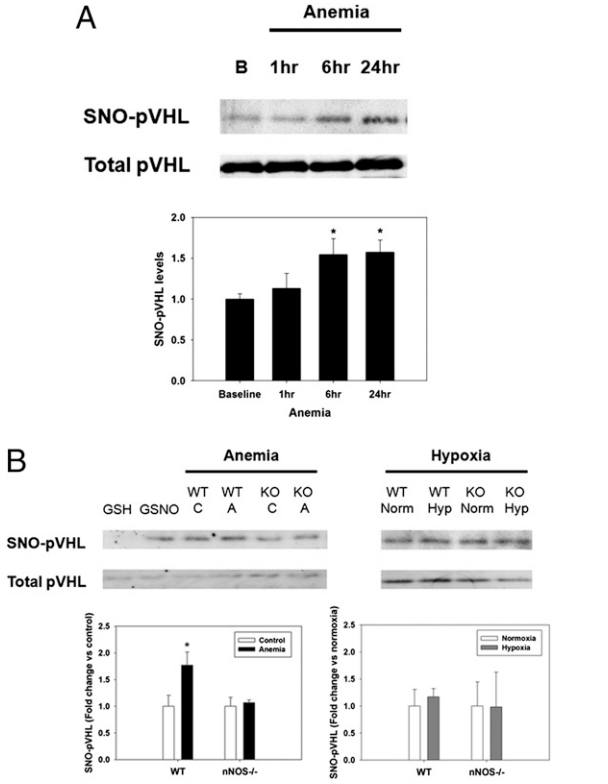

Fig. 5. Anemia leads to increased *S*-nitrosylated pVHL (SNO-pVHL) that depends on nNOS. (*A*) SNO-pVHL levels in WT mouse brain at baseline or 1, 6, and 24 h anemia ($n = 4$). (*B*) SNO-pVHL levels in control (C), 6 h anemic (A), or 6 h hypoxia (Hyp) vs. normoxia (norm) brain in WT and nNOS $^{-/-}$ (KO) mice ($n = 5$). *$P < 0.05$ vs. baseline.

nNOS-Derived NO Mediates *S*-Nitrosylation of pVHL During Anemia. With respect to the mechanism of anemia-induced increases in brain HIF-1α protein, we did not previously find an increase in HIF-1α mRNA levels (10). Therefore, we assessed mechanisms of HIF-α stabilization using the biotin switch assay (28). Using this approach, we demonstrated an increase in *S*-nitrosylation of pVHL in brain homogenates of anemic WT mice after 6 and 24 h (Fig. 5). This increase in pVHL *S*-nitrosylation was not observed in anemic nNOS$^{-/-}$ mice (Fig. 5). By contrast, hypoxia did not lead to increased pVHL *S*-nitrosylation in either strain. We did not find an effect of anemia on levels of global SNO-modified proteins. Specifically, the *S*-nitrosylation of CPK, GAPDH, or HIF-1α (normalized for total immunoreactive protein content) did not differ in total brain homogenates of anemic mice (Fig. S7). This result suggested that anemia-induced increases in SNO-modified pVHL, which is known to disrupt pVHL–HIF interactions, depend on nNOS-derived NO. *S*-nitrosylation of Cys162 of pVHL disrupts functional pathways implicated in the ubiquitination and degradation of HIF-α (21).

Accumulating evidence indicates that protein *S*-nitrosylation is determined by the net effect of *S*-nitrosylation and denitrosylation pathways (30). A number of enzymes have been implicated in denitrosylation reactions. Importantly, genetic ablation or pharmacological inhibition of *S*-nitrosoglutathione reductase (GSNOR) leads to augmented vasodilatory responses. The normal expression and function of GSNOR, at least for myocardial ischemia, serves a protective role. Of interest, hypoxia led to decreases in GSNOR protein levels in vitro (1%, 4–24 h, cultured human umbilical vein endothelial cells; Fig. S8). We therefore assessed levels of GSNOR in vivo and observed a transient or sustained reduction in brain GSNOR protein levels in hypoxic and anemic mice, respectively (Fig. S8). A reduction in GSNOR protein activity in anemic animals would be expected to prolong the half-life of protein *S*-nitrosylation during anemia. Therefore, we addressed the functional consequences of GSNOR genetic deficiency on survival in acute severe anemia (Fig. S8). Although we observed a trend toward increased survival in GSNOR$^{-/-}$ mice, the effect was not robust and did not achieve statistical significance. Therefore, although GSNOR expression and function may play a modulatory role in the integrative physiological responses to anemia, GSNOR is not a key survival gene. This finding can be contrasted with the effect of nNOS deletion and underscores our main finding, namely that nNOS-mediated *S*-nitrosylation of pVHL plays a key physiological role in the response to acute anemia.

Discussion

The results of this study demonstrate that nNOS is critical in the integrative physiological response to acute anemia. Our studies highlight previously unappreciated differences between acute anemia and acute hypoxia. Notably, nNOS conferred dramatic protection against acute anemia-induced mortality. Although nNOS protected mice from mortality in acute anemia, it was detrimental to survival in acute hypoxia. The mechanism by which nNOS contributed to improved survival in anemia may have included both physiological and cellular components. Only nNOS-replete mice were able to increase CO and reduce SVR in response to anemia. Increased CO and decreased SVR act to preserve tissue perfusion (11, 31). Assessment of isolated resistance arteries did not demonstrate a vasodilatory phenotype in

response to 24 h of anemia. This result suggested that the reduction in SVR was mediated by nNOS-dependent factors that were extrinsic to the resistance artery. At the cellular level, anemia resulted in an increase in brain nNOS and HIF-1α protein levels. This finding was associated with increased expression of HIF-dependent RNA transcripts in the brain of anemic mice. Of particular interest were the very large increases in EPO and PDK1 mRNA levels. These cellular responses may support cytoprotective and/or metabolic adaptation to anemia (18). Using real-time imaging of transgenic animals expressing a HIF-1α chimeric protein reporter, wherein the ODD of human HIF-1α is fused to luciferase protein, we provide in vivo evidence that nNOS-derived NO is key for HIF-α stabilization in anemia. In marked contrast, no increase in HIF-1α protein, HIF-α(ODD)-luciferase, or HIF-dependent RNA levels were observed in anemic $nNOS^{-/-}$ mice, demonstrating the absolute requirement for nNOS to augment HIF signaling during anemia.

The differential HIF response to anemia in WT and $nNOS^{-/-}$ mice was not caused by differences in microvascular brain tissue PO_2 levels between strains. Thus, we sought a mechanism that was independent of PO_2: protein *S*-nitrosylation (32). Using the biotin switch assay (28), we observed that the mechanism of HIF stabilization in anemic WT mouse brain included *S*-nitrosylation of pVHL. The absence of this response in anemic $nNOS^{-/-}$ mice strongly suggested that nNOS-derived NO was responsible for pVHL *S*-nitrosylation during anemia. In addition, decreased GSNOR protein levels in anemic WT mouse brain may play a role in the observed increases in SNO-modified pVHL during acute anemia. These data provide important insight into the cellular and molecular responses to acute anemia and suggest that nNOS-derived NO primes HIF-dependent transcriptional responses, thus further expanding the paradigm in which SNOs are hypoxia-mimetic (21).

An assessment of the differences in O_2 tension and tissue O_2 gradients might help to explain differential responses to anemia and hypoxia. Although both anemia and hypoxia reduce blood O_2 content, exposure to atmospheric hypoxia reduces blood O_2 content by decreasing P_aO_2, whereas anemia results in reduced blood O_2 content by decreasing Hb. Indeed, P_aO_2 and S_aO_2 can increase in anemia, in part via an eNOS-dependent improvement in lung ventilation perfusion matching (33). Although our studies do not address mild or moderate anemia, it will be of interest to see whether increases in P_aO_2 levels has paradoxical functional effects on vascular HIF signaling. In contrast to severe anemia, hypoxia results in a dramatic decrease in P_aO_2 and S_aO_2. Thus, during anemia, the immediate available O_2 concentration would be relatively higher in the vascular compartment compared with systemic hypoxia. Although cellular O_2 delivery will be low in both settings, our work highlights that the impact of free O_2 levels in the vascular compartment will be markedly different.

S-nitrosothiols contribute to hypoxic vasodilation in systemic and coronary vascular beds. This mechanism attempts to match tissue perfusion with O_2 demand (34) and may also contribute to the reduced SVR and organ-specific increases in blood flow that occur during acute anemia (11, 35). Early insight into the role of red blood cells in the regulated delivery of NO to hypoxic vascular beds suggested that red blood cells are endogenously "preloaded" with bioactive NO for delivery to vessels in conditions of oxyhemoglobin desaturation (32, 36). In this model, a key cysteine residue within the β-globin chain of Hb (Cysβ93) was biologically relevant to the control of local blood flow. They proposed that *S*-nitrosohemoglobin (SNO-Hb) transports NO to the microcirculation. An alternate hypothesis suggests that NOS-derived, NO-mediated changes in nitrite, and its subsequent reduction back to NO by deoxyhemoglobin, may also contribute to physiological vasodilation in hypoxic vascular beds (37). It is compelling that increased blood flow can be recapitulated with SNO-Hb, now known to play a key role in mediating hypoxic vasodilation (38). Endogenous *S*-nitrosothiols also confer dramatic protection in acute myocardial ischemic injury (34). However, not all vascular *S*-nitrosothiol signaling reactions are adaptive (39). For example, the effect of the antioxidant *N*-acetylcysteine or *S*-nitroso-*N*-acetylcysteine led to pulmonary arterial hypertension in a manner that was similar to chronic hypoxia. This effect was ascribed to the hypoxia-mimetic effect of *S*-nitrosothiols on the pVHL/HIF transcription signaling complex (21).

Our data suggests that nNOS acts as a priming or amplifying mechanism to allow for increases in HIF-α isoform levels and its target mRNAs in anemia. The importance of this mechanism in the in vivo setting was demonstrated in HIF-α(ODD)-luciferase mice. Using this model, we infer that nNOS promotes HIF-α stabilization during anemia. This mechanism is specific to anemia, possibly due to the preservation of O_2 Hb saturation during anemia. Differential Hb O_2 unloading during anemia vs. hypoxia and the overall stiochiometry of Hb NO binding and protein *S*-nitrosylation may also influence release of NO from SNO-Hb in the microvasculature. Under anemic conditions of high P_aO_2 and S_aO_2, nNOS activity may be favored given that nNOS requires relatively high PO_2 levels to generate NO (19). Collectively, our data demonstrate that nNOS supports HIF-dependent cellular adaptation during acute severe anemia. This response occurred, in part, by *S*-nitrosylation of pVHL, as demonstrated in vitro (21). Alternate mechanisms could include *S*-nitrosylation of HIF-1α (20, 40) and inhibition of HIF prolyl hydroxylase activity (22). However, the transgenic model used in the current study (123-aa protein domain of human HIF-1α ODD) does not contain any of the 15 cysteine residues identified as targets for *S*-nitrosylation in HIF-1α (20, 40), consistent with our finding of no increase in HIF-1α *S*-nitrosylation during anemia.

There are limitations in our current work. The physiologic role of nNOS and regulation of pVHL/HIF in chronic anemia needs to be addressed (41). In the setting of chronic anemia, changes in SNO bioavailability and *S*-nitrosothiol signaling may affect angiogenesis as demonstrated in a model of myocardial ischemia (30). Future studies will need to define the relative contribution of nNOS, versus other NOS isoforms, to tissue- and disease-specific *S*-nitrosothiol signaling reactions. Also, the role of denitrosylation reactions in health and disease warrants further study. Clearly, the pVHL/HIF transcription signaling complex will be of specific interest. Direct measurement of *S*-nitrosothiol compounds may lead to further mechanistic insight as to the tissue-specific localization of endogenous *S*-nitrosothiol species production during anemia. Finally, the downstream HIF-dependent effector pathways that are key to adaptive nNOS-specific integrative responses will need to be defined in future studies.

In summary, nNOS is protective during acute anemia. These results suggest that nNOS-derived signaling products act in vivo to regulate HIF during anemia in a fashion that is responsive to small changes in O_2 tension. This effect in anemia results, at least in part, from *S*-nitrosylation of pVHL and activation of the protective pVHL/HIF-dependent transcriptional pathway. There are clinical implications for this basic research. By defining the importance of nNOS and nNOS-mediated HIF stabilization, novel therapies may be developed that could enhance survival in patients exposed to acute blood loss and anemia (e.g., battlefield and civilian trauma and surgery). A better understanding of these mechanisms may contribute to improved care of anemic patients.

Materials and Methods

Animals. All animal protocols were approved by the Animal Care and Use Committee at St. Michael's Hospital (*SI Materials and Methods*). Animals were obtained from The Jackson Laboratory [WT C57BL/6J, $nNOS^{-/-}$, $eNOS^{-/-}$, $iNOS^{-/-}$, $GSNOR^{-/-}$, and HIF-α(ODD)-Luciferase] and maintained at the research institute.

Acute Hemodilutional Anemia. Male anesthetized mice (1.5% isoflurane, 21% O_2) were hemodiluted in steps by exchanging equal volume of blood with pentastarch (Bristol-Myers Squibb) via the tail vein. Blood samples were collected from a tail nick for Hb (CO-oximeter) and blood gases (radiometer) measurement. Recovery studies were performed at a target Hb near 50 g/L (1 h to 7 d).

Acute Systemic Hypoxia. For acute survival studies, anesthetized mice (1.5% isoflurane) were exposed to reduced inspired O_2 level from 21 to 5% O_2 in ≈5% increments. The time to mortality was measured at 5% O_2. For recovery studies, mice were exposed to 15% (cardiac output) or 6% O_2 (tissue collection).

Cardiovascular Outcomes. Cardiac output (CO) was measured under isoflurane anesthesia by ultrasound (Vevo 770) and pressure-volume loops as described (42).

Microvascular Brain PO_2. Microvascular brain O_2 tension was measured by using Oxyphor G2 phosphorescence as described (25).

Western Blot. Total protein was measured in brain tissue by Lowry assay (Bio-Rad). Samples loaded on SDS/PAGE gel were transferred onto nitrocellulose membranes. Proteins were probed for using specific primary antibodies: nNOS (BD Biosciences); HIF-1α (R&D Systems), HIF-2α (Novus Biologicals), and α-tubulin (Sigma). Immunoreactive bands were detected by enhanced chemiluminescence (Sigma).

Quantitative Real-Time RT-PCR. Total RNA extraction and real-time PCR was performed as described (8). Specific primers are indicated in Table S2 and Table S3.

In Vivo Bioluminescence Imaging: Male WT and nNOS$^{-/-}$ mice with the heterozygous HIF-α(ODD)-luciferase construct were used to detect luciferase expression (D-luciferin, 50 mg/kg; i.p.). Anesthetized mice (1.5% isoflurane, 21% O_2) were placed in a light-tight chamber equipped with IVIS imaging camera (Xenogen 300). Dorsal and ventral images were taken (10 s) by using LIVING IMAGE software (Xenogen) and IGOR image analysis software.

Biotin Switch Assay. *S*-nitrosylation of pVHL was assessed in anemic and hypoxic WT and nNOS$^{-/-}$ mouse brain samples by using the biotin switch assay (28). Samples treated with 40 mM GSNO or GSH served as a positive and negative control.

Statistical Analysis. Data are presented as means ± SEM and a value of $P < 0.05$ was taken to be significant. Mantel–Cox log-rank test was used to analyze mortality studies. All other data were analyzed by using a two-way ANOVA for time, group, and interaction effects. Appropriate post hoc analysis was performed by using a Tukey test. Comparison of two means was performed by using a two-tailed Student *t* test. Bonferroni correction for multiple comparisons was used when indicated (SigmaPlot 11; Systat).

ACKNOWLEDGMENTS. We thank Elaine Liu and Hangjun Zhang for technical assistance. Support was provided by a Canadian Institutes of Health Research Doctoral Research Award and a Keenan Collaborative Research Award (to A.K.Y.T.); a Career Investigator Award and grant in aid from the Heart and Stroke Foundation of Canada T-7172 (to P.A.M.); the Heart and Stroke Foundation of Canada Clinician Scientist Award (to K.A.C.); and Bristol-Myers Squibb-CAS Career Scientist Award, University of Toronto–Department of Anesthesia Merit Award, and International Anesthesia Research Society-Society of Cardiovascular Anesthesiologists New Investigator and Mid-Career Grants (to G.M.T.H.).

1. Carson JL, et al. (1996) Effect of anaemia and cardiovascular disease on surgical mortality and morbidity. *Lancet* 348:1055–1060.
2. Karkouti K, Wijeysundera DN, Beattie WS; Reducing Bleeding in Cardiac Surgery (RBC) Investigators (2008) Risk associated with preoperative anemia in cardiac surgery: A multicenter cohort study. *Circulation* 117:478–484.
3. Anand I, et al. (2004) Anemia and its relationship to clinical outcome in heart failure. *Circulation* 110:149–154.
4. Pfeffer MA, et al.; TREAT Investigators (2009) A trial of darbepoetin alfa in type 2 diabetes and chronic kidney disease. *N Engl J Med* 361:2019–2032.
5. Knight K, Wade S, Balducci L (2004) Prevalence and outcomes of anemia in cancer: A systematic review of the literature. *Am J Med* 116(Suppl 7A):11S–26S.
6. Hébert PC, et al. (1999) A multicenter, randomized, controlled clinical trial of transfusion requirements in critical care. Transfusion Requirements in Critical Care Investigators, Canadian Critical Care Trials Group. *N Engl J Med* 340:409–417.
7. Semenza GL (2011) Oxygen sensing, homeostasis, and disease. *N Engl J Med* 365: 537–547.
8. Fish JE, et al. (2007) Hypoxia-inducible expression of a natural cis-antisense transcript inhibits endothelial nitric-oxide synthase. *J Biol Chem* 282:15652–15666.
9. Ward ME, et al. (2005) Hypoxia induces a functionally significant and translationally efficient neuronal NO synthase mRNA variant. *J Clin Invest* 115:3128–3139.
10. McLaren AT, et al. (2007) Increased expression of HIF-1alpha, nNOS, and VEGF in the cerebral cortex of anemic rats. *Am J Physiol Regul Integr Comp Physiol* 292: R403–R414.
11. Ragoonanan TE, et al. (2009) Metoprolol reduces cerebral tissue oxygen tension after acute hemodilution in rats. *Anesthesiology* 111:988–1000.
12. Wang Y, et al. (1999) RNA diversity has profound effects on the translation of neuronal nitric oxide synthase. *Proc Natl Acad Sci USA* 96:12150–12155.
13. Huang Z, et al. (1994) Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. *Science* 265:1883–1885.
14. Prabhakar NR, Pieramici SF, Premkumar DR, Kumar GK, Kalaria RN (1996) Activation of nitric oxide synthase gene expression by hypoxia in central and peripheral neurons. *Brain Res Mol Brain Res* 43:341–346.
15. Abu-Soud HM, Rousseau DL, Stuehr DJ (1996) Nitric oxide binding to the heme of neuronal nitric-oxide synthase links its activity to changes in oxygen tension. *J Biol Chem* 271:32515–32518.
16. Gautier H, Murariu C (1999) Role of nitric oxide in hypoxic hypometabolism in rats. *J Appl Physiol* 87:104–110.
17. Kline DD, Yang T, Huang PL, Prabhakar NR (1998) Altered respiratory responses to hypoxia in mutant mice deficient in neuronal nitric oxide synthase. *J Physiol* 511: 273–287.
18. Pescador N, et al. (2010) Hypoxia promotes glycogen accumulation through hypoxia inducible factor (HIF)-mediated induction of glycogen synthase 1. *PLoS ONE* 5:e9644.
19. Stuehr DJ, Santolini J, Wang ZQ, Wei CC, Adak S (2004) Update on mechanism and catalytic regulation in the NO synthases. *J Biol Chem* 279:36167–36170.
20. Li F, et al. (2007) Regulation of HIF-1alpha stability through S-nitrosylation. *Mol Cell* 26:63–74.
21. Palmer LA, et al. (2007) S-nitrosothiols signal hypoxia-mimetic vascular pathology. *J Clin Invest* 117:2592–2601.
22. Metzen E, Zhou J, Jelkmann W, Fandrey J, Brüne B (2003) Nitric oxide impairs normoxic degradation of HIF-1alpha by inhibition of prolyl hydroxylases. *Mol Biol Cell* 14: 3470–3481.
23. Hagen T, Taylor CT, Lam F, Moncada S (2003) Redistribution of intracellular oxygen in hypoxia by nitric oxide: Effect on HIF1alpha. *Science* 302:1975–1978.
24. Lecoq J, et al. (2011) Simultaneous two-photon imaging of oxygen and blood flow in deep cerebral vessels. *Nat Med* 17:893–898.
25. Vinogradov SA, Lo LW, Wilson DF (1999) Dendritic polyglutamic porphoryns: Probing porphyrin protection by oxygen-dependent quenching of phosphoresence. *Chemistry* 5:1338–1347.
26. Sakadzić S, et al. (2010) Two-photon high-resolution measurement of partial pressure of oxygen in cerebral vasculature and tissue. *Nat Methods* 7:755–759.
27. Schleicher M, et al. (2009) The Akt1-eNOS axis illustrates the specificity of kinase-substrate relationships in vivo. *Sci Signal* 2:ra41.
28. Jaffrey SR, Erdjument-Bromage H, Ferris CD, Tempst P, Snyder SH (2001) Protein S-nitrosylation: A physiological signal for neuronal nitric oxide. *Nat Cell Biol* 3:193–197.
29. Safran M, et al. (2006) Mouse model for noninvasive imaging of HIF prolyl hydroxylase activity: Assessment of an oral agent that stimulates erythropoietin production. *Proc Natl Acad Sci USA* 103:105–110.
30. Lima B, Forrester MT, Hess DT, Stamler JS (2010) S-nitrosylation in cardiovascular signaling. *Circ Res* 106:633–646.
31. Weiskopf RB, et al. (1998) Human cardiovascular and metabolic response to acute, severe isovolemic anemia. *JAMA* 279:217–221.
32. Marozkina N, Gaston B, Doctor A (2008) Transnitrosation signals oxyhemoglobin desaturation. *Circ Res* 103:441–443.
33. Deem S, et al. (1999) Mechanisms of improvement in pulmonary gas exchange during isovolemic hemodilution. *J Appl Physiol* 87:132–141.
34. Lima B, et al. (2009) Endogenous S-nitrosothiols protect against myocardial injury. *Proc Natl Acad Sci USA* 106:6297–6302.
35. Fan FC, Chen RY, Schuessler GB, Chien S (1980) Effects of hematocrit variations on regional hemodynamics and oxygen transport in the dog. *Am J Physiol* 238: H545–H522.
36. Jia L, Bonaventura C, Bonaventura J, Stamler JS (1996) S-nitrosohaemoglobin: A dynamic activity of blood involved in vascular control. *Nature* 380:221–226.
37. Cosby K, et al. (2003) Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nat Med* 9:1498–1505.
38. Diesen DL, Hess DT, Stamler JS (2008) Hypoxic vasodilation by red blood cells: Evidence for an s-nitrosothiol-based signal. *Circ Res* 103:545–553.
39. Liu L, et al. (2004) Essential roles of S-nitrosothiols in vascular homeostasis and endotoxic shock. *Cell* 116:617–628.
40. Sumbayev VV, Budde A, Zhou J, Brüne B (2003) HIF-1 alpha protein as a target for S-nitrosation. *FEBS Lett* 535:106–112.
41. El Hasnaoui-Saadani R, et al. (2009) Cerebral adaptations to chronic anemia in a model of erythropoietin-deficient mice exposed to hypoxia. *Am J Physiol Regul Integr Comp Physiol* 296:R801–R811.
42. Yuen DA, et al. (2010) Culture-modified bone marrow cells attenuate cardiac and renal injury in a chronic kidney disease rat model via a novel antifibrotic mechanism. *PLoS ONE* 5:e9543.