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Systemic hypoxia is characterized by peripheral vasodilation and pulmonary vasoconstriction. However, the system-wide mechanism for signaling hypoxia remains unknown. Accumulating evidence suggests that hemoglobin in RBCs may serve as an O2 sensor and O2-responsive NO signal transducer to regulate systemic and pulmonary vascular tone, but this remains unexamined at the integrated system level. One residue invariant in mammalian hemoglobins (Hb), β-globin Cys93 (βCys93), carries NO as vasorelaxant S-nitrosothiol (SNO) to autoregulate blood flow during oxygen delivery. βCys93Ala mutant mice thus exhibit systemic hypoxia despite transporting oxygen normally. Here we show that βCys93Ala mutant mice have reduced S-nitrosohemoglobin (SNO-Hb) at baseline and upon targeted SNO repletion, and that hypoxic vasodilation by RBCs is impaired in vitro and in vivo, recapitulating hypoxic pathophysiology. Notably, βCys93Ala mutant mice show marked impairment of hypoxic peripheral vasodilation and develop signs of pulmonary hypertension with age. Mutant mice also die prematurely with cor pulmonale (pulmonary hypertension with right ventricular dysfunction) when living under low oxygen. Altogether, we identify a major role for RBC-SNO in clinically-relevant vasodilatory responses attributed previously to endothelial NO. We conclude that SNO-Hb transduces the integrated, system-wide response to hypoxia in the mammalian respiratory cycle, expanding a core physiological principle.

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Hypoxic Vasodilatory Defect and Pulmonary Hypertension in Mice Lacking Hemoglobin β-Cysteine93 S-nitrosylation

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Competing Interest Statement: JSS is a founder of SNO bio, which develops S-nitrosylation-based technology. Authors otherwise declare that they have no competing interests in this work.

Keywords: S-nitrosylation, S-nitrosohemoglobin, oxygen sensing, hypoxic vasodilation, erythrocyte

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Supplemental Material includes:
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Abstract

Systemic hypoxia is characterized by peripheral vasodilation and pulmonary vasoconstriction. However, the system-wide mechanism for signaling hypoxia remains unknown. Accumulating evidence suggests that hemoglobin in RBCs may serve as an O$_2$ sensor and O$_2$-responsive NO signal transducer to regulate systemic and pulmonary vascular tone, but this remains unexamined at the integrated system level. One residue invariant in mammalian hemoglobins (Hb), β-globin Cys93 (βCys93), carries NO as vasorelaxant S-nitrosothiol (SNO) to autoregulate blood flow during oxygen delivery. βCys93Ala mutant mice thus exhibit systemic hypoxia despite transporting oxygen normally. Here we show that βCys93Ala mutant mice have reduced S-nitrosohemoglobin (SNO-Hb) at baseline and upon targeted SNO repletion, and that hypoxic vasodilation by RBCs is impaired in vitro and in vivo, recapitulating hypoxic pathophysiology. Notably, βCys93Ala mutant mice show marked impairment of hypoxic peripheral vasodilation and develop signs of pulmonary hypertension with age. Mutant mice also die prematurely with cor pulmonale (pulmonary hypertension with right ventricular dysfunction) when living under low oxygen. Altogether, we identify a major role for RBC-SNO in clinically-relevant vasodilatory responses attributed previously to endothelial NO. We conclude that SNO-Hb transduces the integrated, system-wide response to hypoxia in the mammalian respiratory cycle, expanding a core physiological principle.
Introduction

The physiological response to systemic hypoxia is a foundational aspect of the respiratory cycle through which O$_2$ is delivered to tissues. Systemic blood vessels dilate and pulmonary vessels constrict under hypoxia to improve O$_2$ delivery to tissues (1). However, the integrated molecular mechanisms for oxygen sensing are not well understood. Accumulating evidence suggests that the respiratory cycle is in fact a 3-gas system in which hemoglobin (Hb) is a carrier for not just two, but three gasses in blood: O$_2$, CO$_2$, and nitric oxide (NO) (2, 3), and that Hb acts as an O$_2$-responsive NO-based vasodilator that matches tissue perfusion to oxygen demand (4, 5). This effect, termed auto-regulation of blood flow (5), acts locally within individual capillaries and microvascular beds to increase red blood cell (RBC) transit, and functions in direct proportion to Hb desaturation to ensure metabolic coupling (together with vasodilators released from hypoxic tissues for purposes of capillary recruitment (6)). Mice mutated to be unable to dispense NO from Hb are therefore profoundly hypoxic despite RBCs carrying normal amounts of O$_2$ (7).

While both O$_2$ and NO bind to hemes in Hb, NO can also react with Hb’s sulfhydryl groups to form S-nitrosothiols (SNO) (8, 9). In the form of SNO, NO bioactivity is preserved in Hb (whereas NO bound to heme is inactive), and SNO on proteins can act as a signaling modification to regulate protein allostery (8). Conversion of NO to SNO in Hb takes place within the β-globin subunit (9). It has been shown that NO binding to β-globin heme, particularly Fe$^{3+}$ heme, serves to redox activate NO to NO$^+$ (nitrosonium ion) that can then S-nitrosylate a conserved β-globin Cys (βCys93) to form βCys93-SNO (10, 11); Hb thus acts as a SNO synthase (12). Also, it has been shown that the reaction of NO with βCys93 is coupled to the allosteric transition in Hb (13), with SNO formation...
favored in the oxygenated/R state of Hb and SNO release favored in the deoxygenated/T state. SNO-Hb thus serves as an O2-responsive NO buffer, only releasing SNO in the hypoxic T state (14, 15). In short, when Hb releases oxygen in hypoxic tissues, it undergoes a shift from R to T conformation causing βCys93 to transfer NO’ to other thiols that transport NO out of RBCs to increase blood flow (15). Thus, Hb-derived SNO has been proposed as the mediator of classical autoregulation of tissue blood flow that is proportional to Hb O2-saturation (5).

This model of SNO-Hb-mediated hypoxic vasorelaxation and oxygen delivery has now been supported by numerous studies, both in vitro and in vivo, including fairly dispositive genetic evidence. These data include demonstration that isolated human Hb but not Cys93Ala (C93A) mutant Hb (16) can be S-nitrosylated by physiological amounts of NO (12), that this SNO-Hb is vasodilatory (13, 15), that the vasorelaxant activity of native RBCs and of physiological amounts of SNO in RBCs requires hypoxia (13, 17, 18), and that autoregulation of blood flow and tissue oxygenation in vivo are profoundly disrupted by mutation in βCys93 (7). Further, we and others have demonstrated profound hypoxia-related phenotypes in these mice, including deficiencies in tissue oxygenation and flow-mediated vasodilation, increased development of coronary collateral vessels, cardiac dysfunction, altered breathing response to hypoxia, and death upon acute exposure to a very low oxygen environment (7, 19, 20). Nevertheless, one group of authors have disputed this model, specifically failing to observe hypoxia-dependent vasodilation from SNO-loaded RBCs in vitro (21, 22) and reporting that mice with βCys93 mutation exhibited no significant phenotypes as tested, including absence of pulmonary hypertension (18, 21-25).

In this regard, systemic hypoxia results in sine qua non changes in peripheral vascular tone and contrasting changes in pulmonary vascular tone: i.e., hypoxia induces
vasodilation in peripheral microvasculature, and instead vasoconstriction in pulmonary
microvasculature to promote ventilation/perfusion matching (26). Notably, the molecular
mechanism for oxygen sensing at an integrated system level remains a major
unanswered question in vascular physiology, and thus the extent to which RBCs may
contribute is unknown. While C93A mice would seem to be the ideal model to test this
question, mouse RBCs differ from human RBCs in terms of vasodilatory mechanisms
(25, 27) and have not been previously optimized for SNO formation, by contrast with
human RBCs (15), thus limiting interpretations in vitro (25, 28). In particular, current
methods to load SNOs in mouse RBCs (20, 21) produce very high levels of met Hb and
non-Hb SNOs, which mask C93 activity. Here we develop new tests of fundamental
RBC physiology in vitro and in vivo to reveal the role of βCys93 in peripheral and
pulmonary vascular responses. Our results indicate that RBCs serve in system-wide O_2
sensing and O_2-responsive SNO signaling, to regulate systemic and pulmonary vascular
tone, expanding a core principle in physiology.
Results

Hemoglobin βCys93 mediated hypoxic vasodilation in vitro

We have previously shown that native βCys93 mutant RBCs induce vasodilation less effectively under hypoxia than control RBCs (7) and that human RBCs loaded physiologically with NO gas recapitulate hypoxic vasodilation by native RBCs (15). However, it has been reported recently that mouse RBCs pre-loaded with SNO (via treatment with CysNO) do not show hypoxic vasodilation nor differences in vasodilation between mutant and control RBCs (22). In working with mouse RBCs, we noted that Hb is not modified by exogenous CysNO as readily as is Hb within human RBCs, and that metHb (which eliminates the allosteric transition in Hb) formed in very high amounts. We therefore developed a protocol (see Methods) optimized for SNO loading of Hb within humanized mouse RBCs. With this procedure, we are able to load SNO predominantly onto βCys93, although mouse RBCs load SNO less well and produce higher metHb than do normal human RBCs. Under these optimized loading conditions, the C93 (control) mouse RBC preparations contained ~10 SNO per 1000 Hb tetramers (~ 2.5 SNO /1000 heme) and were oxidized to ~10% metHb, while C93A (mutant) RBCs had significantly less SNO (~ 6 SNO per 1000 Hb tetramers or ~1 SNO/1000 heme) (Figure 1A) and indistinguishable metHb levels. CysNO increased SNO-Hb 5-fold over the level observed in fresh, untreated C93 RBCs and in C93A mutant RBCs, which had significantly less SNO-Hb also at baseline (Figure 1B). Adding these SNO-loaded C93 RBCs to aortic ring bioassays from wild type mice resulted in vasorelaxation under hypoxia, but not under normoxia (Figure 1C, D) (as seen with physiological amounts of SNO-Hb and native RBCs (25, 29)), fulfilling the sine qua non requirement of hypoxic vasodilation. In contrast, SNO-loaded C93A RBCs produced significantly less
vasorelaxation under hypoxic conditions than C93 RBCs, but were equally vasoconstrictive under normoxia. These results demonstrate that Hb βCys93 is the primary and preferred site of S-nitrosylation in RBCs, that carefully-loaded mouse RBCs recapitulate the hypoxic vasorelaxation found using human RBCs under basal conditions (14, 15, 25), and that this hypoxic vasorelaxation effect is significantly diminished when SNO can no longer bind to or be released from β-globin Cys93. Thus, the allosterically regulated βCys93 mediates hypoxic vasodilation by RBCs.

Peripheral vasodilation by hemoglobin βCys93 in vivo

In vitro bioassays with isolated aortic rings and static, dilute RBCs have limitations. To assess the role of Hb βCys93 in regulating hypoxic vasodilation in vivo, we performed two types of experiments based on classic reactive hyperemia paradigms (30, 31), but using abdominal aorta in situ. First, flow through the abdominal aorta was blocked temporarily by ligating the abdominal aorta for 5 min to create tissue hypoxia, and then the ligature was released and the diameter of the abdominal aorta upstream of the ligation site was measured at diastole in real time using ultrasonography. In this model of reactive hyperemia, the diameter of the aorta underwent a transient increase after hyperemic flow had normalized, and the extent of this increase, shown as dynamic vasodilation, was significantly less in the C93A mice (Figure 1E). The overall dilation of the abdominal aorta increased by ~8.8 % over the basal diameter in C93 (control) mice bearing wild-type human Hb (Figure 1F) compared to the diameter at the same location prior to occlusion. By contrast, the diameter increase in C93A mouse aorta was significantly blunted, at only ~4.4 % over its basal diameter (Figure 1F); representative M-mode images for individual mice are shown in Figure 1G. This indicates that Hb βCys93-derived SNO within RBCs contributes about 50% of the vasodilation effect
following temporary occlusion. The remaining half is attributed to local shear force-
induced endothelial NO production upon restored flow (flow-mediated dilation; FMD) (32,
33), which has often been assumed to be responsible for the full effect, but without
empiric evidence. Moreover, it had not been previously possible to discern the role of
endothelium vs. RBC because eNOS inhibition reduces levels of SNO-Hb and RBC
SNO (13, 34). We conclude that vasodilation following occlusion is evidently mediated
by both RBCs and endothelium: the former stimulated by hypoxia (35) and the latter by
shear.

In a second experiment, we directly measured blood flow through the abdominal
aorta downstream of the ligation site using an ultrasound flow probe. Increases in flow
following ligature release result from microcirculatory vasodilation downstream, which
provides a surrogate measure of NO vasodilatory activity. Generally, very small diameter
increases distributed across the microcirculation result in marked increases in flow, as
flow is a function of radius^4. As with abdominal aorta diameter, we measured blood flow
continually through the cardiac cycle, and calculated systolic peak and mean blood flow
for the first second of each 15-second window (7-8 heartbeats) to determine dynamic
flow increases (FI). Mean flow increase is shown in Figure 1H. In C93 control mice, the
mean aortic blood flow increased nearly 1.2-fold over flow measured prior to the
blockade, but in the C93A mice, this flow was significantly blunted, reaching only ~0.8-
fold (Figure 1I); representative traces for individual mice are shown in Figure 1J.
Similarly, peak flow at systole was increased 54% over basal in control mice, but this
was significantly reduced to 31% in C93A mice (Supplemental Figure 1A, B). We
conclude that about 50% of the hyperemic flow increase, reflecting microcirculatory
vasodilation following hypoxia, is mediated by Hb βCys93 within RBCs.
Right ventricular hypertrophy in young and aged animals in the absence of βCys93

Loss of ability to carry and release SNO from Hb Cys93 leads to tissue hypoxia that is compensated in part by increased cardiac workload (7). Additionally, hypoxia may result in pulmonary hypertension, evidenced by right ventricular hypertrophy. We found total heart weight to body weight to be significantly elevated in both young adult and old C93A mice, compared to C93 control mice (Figure 2A). Further, both right ventricle weight (Figure 2B) and left ventricle weight (Figure 2C) as a ratio to body weight were elevated significantly in C93A mice. However, C93A mice were lighter than C93 controls, sufficient to potentially confound interpretation when aged (C93 26.86 ± 2.76 gm, and C93A 25.04 ± 2.00 gm for young mice, p <0.01, and C93 42.82 ± 7.91 gm, and C93A 34.23 ± 4.43 gm for aged mice, p <0.01). More importantly, the ratio of right ventricle weight to the weight of the left ventricle plus septum (Figure 2D) was significantly elevated in both young and old C93A mice (independent of body weight differences). Thus, both young and aged C93A mice show signs of right heart hypertrophy, suggesting increased pulmonary vascular tone.

Age-related signs of pulmonary hypertension in the absence of βCys93

While young adult C93A mice had normal pulmonary artery blood flow velocity-time integral (VTI), as we have previously reported (7), pulmonary VTI was significantly reduced in aged C93A mice (Figure 2E). Likewise, pulmonary artery diameter was normal in young C93A mice, but was significantly increased in aged C93A mice (Figure 2F). Mean and peak blood velocity in the pulmonary artery were both significantly reduced in both young and aged C93A mice (Figure 2, G,H).
Pulmonary hemodynamics in young and aged animals

In contrast to the prior (7) and above data in conscious mice, in young anesthetized mice at baseline (Supplemental Table I) no significant changes were apparent, while in aged anaesthetized C93A mice, systolic, diastolic and mean pulmonary arterial pressures, and right ventricular systolic pressure, trended toward increases vs. control mice (Supplemental Figure 2, A-D). Measures of right ventricular function in aged vs. young mice, including dP/dt-max, dP/dt-min, contractility index, and average dP/dt during the isovolumic relaxation period (IRP Average dP/dt), showed a similar trend (Supplemental Figure 2, E-H). Taken together with right ventricular hypertrophy and pulmonary artery dilation in aged C93A animals at baseline, and age-related reductions in pulmonary blood flow velocity in conscious C93A mice shown in Figure 2, our results suggest that with activity, stress and aging, abnormal pulmonary vascular reactivity and/or right ventriculo-arterial coupling, results in right sided dysfunction at baseline, while in unstressed animals under anesthesia, right-sided changes are attenuated.

Pulmonary hypertension with chronic hypoxia in the absence of βCys93

Patients with chronic hypoxia-induced pulmonary hypertension have low SNO-Hb and their RBCs show impaired vasodilatatory responses in vitro (36). We therefore exposed C93A mice to chronic hypoxia. Based on our previous study demonstrating that mutant mice survived acute exposure to 10% hypoxia but succumbed quickly at 5% O₂ (7), we housed young mice at 10% O₂ for 4 weeks (all mice survived), and examined cardiac function and pulmonary artery pressure using echocardiography and invasive catheterization respectively. Pulmonary artery diameter was significantly larger in C93A mice after chronic hypoxia (Figure 3A), and pulmonary VTI was diminished (Figure 3B). The mean and peak velocity of blood ejection from the right heart were also reduced in
chronically hypoxic C93A mice, consistent with right heart dysfunction subsequent to pulmonary arterial hypertension. (Figure 3, C,D). Indeed, systolic, diastolic and mean pulmonary artery pressures were all significantly elevated in C93A mice vs. control mice (Figure 3E). Furthermore, right ventricular systolic pressure was significantly higher in C93A mice than in C93 (Figure 3F). Analysis of pressure-time curves revealed that right ventricle dP/dt-max, dP/dt-min, contractility index, and average dP/dt over isovolumic relaxation period (IRP average dP/dt) were all significantly elevated in C93A mice (Figure 3, G-J). However, the time constant of relaxation (τ) did not differ (Figure 3K). The right ventricles of C93A mice showed evidence for increased fibrosis compared to C93 controls (Figure 3, L-N). Overall, these changes are indicative of pulmonary arterial hypertension with right ventricular dysfunction.

As a confirmatory measure of the effects of chronic hypoxia, we compared young mutant C93A mice under normoxia with young C93A mice housed under 10% O2 for 4 weeks (using data shown in Figure 2 and Supplemental Figure 2). Pulmonary artery diameter increased in young C93A mice after chronic hypoxia (Supplemental Figure 3A). Pulmonary artery blood flow VTI, mean blood velocity, and peak blood velocity all were significantly reduced in young C93A mice (Supplemental Figure 3, B-D). Further, systolic, diastolic and mean pulmonary artery pressures were increased in young C93A mice vs. controls (Supplemental Figure 3, E-G), and right ventricular pressures, dP/dt-max, dP/dt-min and average dP/dt over the isovolumic relaxation period were also increased (Supplemental Figure 3, H-L). Finally, there was significant hypertrophy of the right ventricle compared to left ventricle, and of the total heart compared to body weight (Supplemental Figure 4, A-D). Thus, pulmonary hypertension with cor pulmonale is induced by hypoxia independently of age in C93A mice.
**Left heart function with aging and chronic hypoxia**

While left ventricular ejection fraction and fractional shortening are normal at baseline in young adult C93A animals, these functional measures were reduced in aged animals (Supplemental Figure 5A, B). Further, chronic hypoxia led to modestly reduced left ventricular ejection fraction and fractional shortening in young C93A mice vs control C93 mice (Supplemental Figure 5C, D; Supplemental Figure 6A, B), accompanied by increases in left ventricular end systolic and diastolic diameters and volumes (Supplemental Figure 5, E-H; Supplemental Figure 6, C-F). Parameters that did not show differences in young C93A mice between normoxia and chronic 10% O\(_2\) include left ventricular end systolic volume, inner diameter at systole, ventricular end diastolic volume and inner diameter at diastole (Supplemental Figure 6, C-F).

**Increased mortality in the absence of βCys93 under chronic hypoxia**

We assessed survival of mice housed chronically under 10% O\(_2\). The C93 wild-type mice all survived through 49 days but were all dead by 154 days, with a mean survival time of 87 days (Figure 4A, B). In contrast, C93A mice died much sooner, with the first mouse dying at 25 days and the last at 78 days, with a mean survival time of 51 days (Figure 4A, B). This is consistent with mice lacking ability to mediate hypoxic vasodilation of peripheral and pulmonary vasculature.
Discussion

The integrated response to systemic hypoxia is characterized by peripheral vasodilation, pulmonary vasoconstriction and a central drive to breathe that is designed to restore tissue oxygenation. Here we show that SNO derived from RBCs plays a central role in this coordinated physiology. First, we show that the ability of RBCs to induce hypoxic vasodilation in bioassays in vitro and in peripheral vessels in vivo is significantly reduced when hemoglobin is unable to dispense SNO under hypoxia. Second, we show that SNO-Hb-deficient mice have pulmonary hypertensive changes at baseline and hallmark changes of pulmonary hypertension with chronic hypoxia. Prior work has shown that C93A mutant animals also exhibit a defect in drive to breathe (19). Collectively, these experiments provide physiological support for the model in which RBCs act as O2 sensors and O2-responsive, SNO-based vasodilators of peripheral and pulmonary vasculature. RBCs unable to liberate SNO from Hb βCys93 cannot effectively enter tissues, creating the sequelae of chronic hypoxia ((7) and herein). Nature recapitulates these experiments in native Tibetans who compensate for hypobaric hypoxia through increases in RBC-SNO that elevate tissue blood flow (37), while patients with pulmonary hypertension and hypoxemia exhibit losses of RBC-SNO and RBC-mediated vasodilation (36). Under similar conditions herein, animals lacking Hb βCys93 die with signs of cor pulmonale.

Our demonstration of Hb Cys93-dependent hypoxic vasodilation of aortic rings in vitro bioassays is in agreement with prior studies (7, 27), but in contrast to a recent report that found no difference between C93 and C93A RBCs loaded with SNO (22). In that report, isolated RBCs were pharmacologically treated with very high concentrations of NO donors (22), which results in high oxidation to met-Hb and SNO-loading of sites
other than Hb βCys93 (28) (including glutathione and sites in Hb itself). These conditions lead to vasodilation that is independent of hypoxia (i.e., artifactual vasodilation in room air) since neither metHb nor SNO sites other than HbβCys93 exhibit allosteric coupling to Hb oxygenation state (9, 13, 15, 25, 38). By carefully titrating the SNO donor we were able to selectively target βCys93 to minimize metHb accumulation. Notably, our newly-optimized conditions for mouse RBCs are quite different from those used for physiological loading of human RBCs (15), where vasodilation is entirely SNO-Hb mediated (27, 29, 38). Nonetheless, we demonstrate reduced hypoxic vasodilation by CysNO-treated mutant RBCs compared to control RBCs, reflecting differences in SNO-Hb levels, whereas residual vasodilation by C93A RBCs likely reflects alternative SNOs that are generated by CysNO, including fetal SNO-Hb (i.e., SNO-γ Cys93) (25, 29), as well as mediators such as ATP, which are important in mouse (but not human) vasodilation in vitro (27, 35, 39).

As an in vivo correlate of RBC-mediated hypoxic vasodilation, we measured vasodilation after temporary vasoocclusion. Upon release of occlusion, blood flow rapidly increases above the basal rate as tissue microvasculature vasodilates to maximize blood flow to oxygen-deprived tissues. The loss of half of this effect in mice bearing C93A Hb demonstrates a major role for SNO released from hypoxic Hb in mediating vasodilation, and highlights a reduced ability of C93A mice to respond to hypoxic episodes. This is consistent with classic experiments showing that artery diameter changes with blood $O_2$ saturation, even at constant flow (40). Indeed, the diameter of the aorta increases after restoration of blood flow, and half of this effect is also absent in C93A mice. Although shear stress on endothelial cells leading to acute NO release is believed to be responsible for this effect (32, 33, 41) (often referred to as ‘flow mediated dilation’ (FMD)), this has been controversial as eNOS inhibition (42-46) only blocks
~50% of the response. Thus, the suitability of the human FMD measurement to indirectly measure endothelial NO release and thus predict cardiovascular health is vigorously debated (47-49).

It is important to note that aortic dilation is observed only once hyperemic flow subsides (compare Figure 1E and 1H). This suggests a direct effect of SNO-Hb on the aorta, as observed in in vitro bioassays (Figure 1C), rather than an indirect response to downstream flow. In fact, immediate hyperemic flow is associated instead with aortic constriction that is likely reflective of adrenergic responses to maintain blood pressure. Altogether, our experiments reveal a previously unappreciated role for Hb-derived SNO, and for large but transient increases in blood flow driven by RBCs rather than endothelial cells, which reflects end organ metabolic requirements, not vascular health per se. This new insight of a role for RBCs in addition to endothelium may help clarify the meaning and utility of similar assays in patients.

An exciting new report for a role of RBC eNOS in control of blood pressure (34) merits comment in this regard. Whereas eNOS in RBCs contributes to the overall blood pressure lowering effect of NO, it plays no role in FMD, as shown in the same study (34). By contrast, Hb βCys93 plays a major role in “FMD” (this report), but has no role in blood pressure control (7, 23). Furthermore, eNOS deletion from RBCs does not affect hypoxic vasodilation by RBCs in vitro (27). Thus, eNOS apparently plays a role in one situation (blood pressure control) and Hb βCys93 in another (autoregulation of blood flow).

The new work on RBC-eNOS also deserves mechanistic comment. The authors of that report (34) seem to favor a role for nitrite in blood pressure lowering by RBC-eNOS, but their data show otherwise. eNOS deletion from RBCs does not reduce nitrite or overall levels of NO metabolites in RBCs. Only heme-NO levels are apparently lower in eNOS−/− RBCs, which is well rationalized by lower amounts of NO produced, as NO
binds heme directly. In addition, eNOS generates SNOs including SNO-proteins, which may lower blood pressure, and SNO levels in plasma and RBCs are altered in RBC-eNOS mice (34). Thus, SNOs-derived from RBC-eNOS likely contribute to the circulating SNO pool, which mediates blood pressure lowering, just as SNOs are central to all NO bioactivity (27, 35). By contrast, there is no evidence for any role played by hemoglobin in generation of NO that lowers blood pressure, nor any basis for the idea that eNOS activity requires Hb to lower blood pressure. Finally, studies that have directly assessed RBC mediated vasodilation fail to show any activity of nitrite (25, 27); to our knowledge, not a single study has shown vasodilation by nitrite added to RBCs or Hb in physiological amounts.

We have previously reported that hypoxic vasodilation by RBCs counteracts pulmonary hypertension in animals and patients (36), implicating a role for SNO-Hb in effective V/Q matching. Our new data provides genetic support for these findings by demonstrating that mice deficient in SNO-Hb are predisposed to develop pulmonary hypertension and right heart failure. In particular, C93A mice show signs of abnormal pulmonary reactivity and right heart strain at baseline and develop worsening with age. Moreover, as compared with wild type mice, pulmonary hypertension is more severe in mutant mice housed in low oxygen environments. Chronic hypoxia may not only result in pulmonary hypertension and right heart remodeling (50), but also in left ventricular failure in patients with heart disease, as hearts are under increased stress (51). Cys93 mutation mimics coronary heart disease by impairing vasodilation (Figure 1) and blood flow (7, 20), which would explain the left ventricular dysfunction that evolves with age or chronic hypoxia.

Hypoxic phenotypes in the C93A mice have been unnecessarily controversial, with some researchers failing to identify differences from control mice based on
physiology that is not linked to hypoxic regulation (e.g. systemic blood pressure) or tests that are not up to the task (e.g., lung histology as a measure of pulmonary artery pressure) (23). Thus, despite the original claim that abnormalities in pulmonary hemodynamics were not evident in these mice (23), we clearly show otherwise. Also, previous work (22, 23) did not consider the dynamic nature of hypoxic responses, which are distributed across the microcirculation, and quickly fade as the system adapts (e.g. Figure 1E,H) or the effects of age, as shown here. Further, numerous compensations in C93A mice to counterbalance the loss of SNO release from Hb (25, 29, 35) were not recognized early on. These include increased fetal Hb γCys93 SNO, a shift in SNO from C93 to other Cys residues in Hb and to glutathione, and the development of coronary collateral vessels (7, 20, 23). All this aside, the C93A mice exhibit prenatal lethality (half of predicted pups are not born) (7), suggesting that the most-affected individuals never survive to be tested, and those that do survive show profound functional deficits in blood flow and tissue oxygenation throughout the body (7). In addition, as shown here and elsewhere (7, 20), mice lacking SNO-Hb-mediated hypoxic vasodilation develop cardiopulmonary insufficiency and die more rapidly under hypoxic stress.

We conclude that the invariant βCys93 residue in Hb regulates both pulmonary and peripheral vascular responses to hypoxia. Animals lacking hypoxia-mediated vasodilation by SNO-Hb exhibit sine qua non features of hypoxic insufficiency and die from cardiopulmonary failure. The importance of this fundamental physiology may be alternatively appreciated in terms of hypoxic signaling where HIF/pVHL (1, 52) acts at the cellular level (via O2-regulated hydroxylation), while Hb/SNO acts at the organ level (via O2-regulated S-nitrosylation) to integrate system-wide responses (53). Together with previous work showing essential roles for βCys93 in blood flow autoregulation (7) and in
hypoxic ventilatory responses (19), our results identify SNO-βCys93 with the essential functions of the heart, lungs and blood in oxygen delivery.
Materials and Methods

Animals: C57BL/6J mice were purchased from the Jackson Laboratory. Mice bearing human α-globin and β-globin (plus γ-globin) genes in place of the corresponding mouse genes, either with β-globin bearing the wildtype Cys93 residue (C93), or instead carrying the Cys93Ala mutation in the human β-globin gene (C93A), were obtained from Dr. Tim Townes (23). All mice used in experiments were male (except for blood collection for SNO-loading and bioassay, which used both male and female mice), and mice were tested as young or aged adults, with the age range described in the legend for each specific experiment.

SNO-loading of human hemoglobin in mouse RBCs: Mouse blood was obtained from inferior vena cava of male and female C93 and C93A mice after isoflurane euthanasia. Blood (~0.7-1.0 mL per mouse) was spun at 1500 X g for 2 minutes. Equal volumes of paired C93 and C93A samples used, and RBC pellets were resuspended at 50% hematocrit in PBS/EDTA, pH 7.8, by gently inverting the tubes several times. Resuspended RBCs were diluted 8-fold to 6.25% hematocrit for SNO loading, since lower RBC density led to improved loading efficiency, as will be detailed elsewhere. Equal volumes of cysteine ethyl ester in 1 N HCl and 100 mM sodium nitrite in H2O were mixed to generate ethyl ester Cys-SNO and immediately diluted to 2 mM in PBS/EDTA. Ethyl ester Cys-SNO (final concentration 200 µM; pH 7.8) was gently mixed with RBCs and incubated for 5 minutes at room temperature in the dark. RBC suspension was centrifuged and washed twice (5 minutes, 1500 X g) with 8 mL PBS/EDTA pH 7.1 to remove the SNO donor, and the final cell pellet was resuspended to 50% hematocrit and used immediately for bioassays. An aliquot was used for heme and SNO quantification,
and under these conditions C93 RBCs contained ~10 SNO/1000 Hb tetramer (~2.5 SNO/1000 heme) while C93A had ~6 SNO/1000 Hb tetramer (further details of optimization will be described elsewhere).

Aortic ring bioassay: RBC-mediated hypoxic vasodilation of isolated aorta was performed essentially as previously described (3, 13, 15). Briefly, thoracic aorta was dissected from C57BL/6J mice and rinsed with PBS. A 3 mm ring was hung with wires connected to a Radnoti isometric transducer and placed within an organ bath containing Krebs buffer at 37 °C, and bubbled with gas as indicated; either 20% O₂ with nitrogen, or 1% O₂ with nitrogen. Basal tension was elevated using 1 μM phenylephrine to assess relaxation, and tensions recorded using a PowerLab data acquisition system and LabChart 7.3 software (ADInstruments, Colorado Springs, CO, U.S.A.). Assays were performed using 4 organ baths in parallel. Aorta rings in the organ bath were incubated with L-NMMA (1mM) for 10 min to inhibit NOS, and glutathione (100 μM) was added to the organ bath 1 min prior to RBC addition, to serve as a SNO-carrier thiol between RBCs and aortic endothelium in the absence of blood flow, and has no effect alone (7, 54). SNO-loaded C93 and C93A humanized mouse RBCs were added to the bath such that the final concentration was 0.4% hematocrit, and change in tension recorded over time. Changes were measured at maximal vasodilation (~2 min) or at 2 min when changes were small.

Abdominal aorta dissection surgery: Mice were anesthetized with 2,2,2-Tribromoethanol (0.25 mg/g i.p.) and secured in the supine position on a temperature-controlled small animal operation table. A midline incision was made to expose the abdominal cavity, and the intestine carefully moved aside to reveal the abdominal aorta.
at the level of the left renal vein. A segment of the abdominal aorta from inferior vena
cava below the left renal vein level was separated gently, and once recording
instruments were in place (see below), a 7-0 silk suture was placed around the
abdominal aorta in order to occlude blood flow procedure through ligation. Mice were
used immediately for measurement of flow-mediated dilation or hypoxia-induced flow
increase.

**Post-ischemia induced vasodilation:** Hypoxic vasodilation was induced using a
protocol inspired by measurement of flow-mediated dilation of brachial artery in non-
human primate and human patients (30, 55, 56). After the abdominal aorta dissection
procedure, the post-surgery mouse was transferred to a temperature-controlled small
animal operation table for echocardiography. Vascular ultrasound was performed using
a Vevo 770 High-Resolution Imaging System equipped with an RMV-708 55-MHz probe
(VisualSonics, Toronto, ON, Canada). Short axis M-mode images of the abdominal aorta
below the left renal vein but *above* the ligation site were acquired at baseline, then the
ligature around the abdominal aorta was tightened to completely occlude blood flow for 5
min, then the ligation was released. After ligation release, short axis M-mode images of
the abdominal aorta were captured every 30 seconds for 10 min. Following the
experiment, mice were euthanized. The end-diastolic diameter of the abdominal aorta for
baseline and for each 30-second timepoint was determined using the Vevo system
software. Dilation was calculated as \((\text{Diameter}_{\text{maximum}} - \text{Diameter}_{\text{baseline}}) / \text{Diameter}_{\text{baseline}}\) \times 100% to yield % of basal. Dynamic dilation was calculated every 30 seconds after the
release of the ligature, while overall dilation was calculated using the peak diameter
response after ligature release.
Measurement of reactive hyperemia: After abdominal aorta dissection, an ultrasound blood flow probe (MA0.5PSB, Transonic Systems Inc. NY, U.S.A.) was placed around the abdominal aorta below the pre-placed 7-0 silk suture. Abdominal aortic blood flow was measured directly with a perivascular flowmeter (TS420, Transonic Systems Inc. NY, U.S.A.), and after a stable period of baseline blood flow recording, the abdominal aorta was ligated using the 7-0 silk suture to occlude completely blood flow for 5 min, then blood flow was restored upon release of the ligature. Blood flow was continuously recorded using a PowerLab data acquisition system for 10 min, and data were analyzed using LabChart 7.3 software (ADInstruments, Colorado Springs, CO, U.S.A.). After the recordings, mice were euthanized. Mean flow increase was calculated as a percent of baseline according to \( \frac{\text{BF}_{\text{maximum}} - \text{BF}_{\text{baseline}}}{\text{BF}_{\text{baseline}}} \times 100\% \), where \( \text{BF}_{\text{baseline}} \) is the baseline mean blood flow, and \( \text{BF}_{\text{maximum}} \) is the maximum mean blood flow after ligation release; systolic flow increase was calculated in the same manner using systolic \( \text{BF}_{\text{baseline}} \) and systolic \( \text{BF}_{\text{maximum}} \). The dynamic change in flow was calculated every 15 seconds for each timepoint, and the overall flow increase was calculated using the peak blood flow response after ligature release. Blood pressure was not measured during these assays due to concerns that the arterial pressure transducer catheter would interfere with flow measurements.

Chronic hypoxia: Mice were housed in standard cages within an oxygen-controlled cabinet (Model 30, Coy Laboratory Products, Inc., Grass Lake, MI, U.S.A.) supplied with 10% \( \text{O}_2 \). The oxygen concentration was controlled by an oxygen controller (Coy Laboratory Products, Inc., Grass Lake, MI, U.S.A.). Cages were changed every 4 weeks, and drinking water and food changed weekly; mice were checked daily. Control mice were simultaneously housed at 21% \( \text{O}_2 \) under identical conditions. The mice were
treated with 10% O₂ for 4 weeks for evaluation of pulmonary hypertension, or chronically in the 10% O₂ tolerance test.

Echocardiography: Mice were anesthetized in a small animal incubator with 2% isoflurane in room air, then transferred and secured in the supine position on a temperature-controlled small animal operation table. Transthoracic echocardiography was performed using a Vevo 3100 imaging system equipped with an MX400 38-MHz probe (FUJIFILM VisualSonics, Toronto, ON, Canada); the mice were allowed to regain consciousness and were in room air during the entire procedure. Standard M-mode sampling was captured through the left ventricular short axis at the midpapillary level. Standard B-mode and pulsed-wave Doppler for the pulmonary artery were captured for artery diameter and blood flow analysis. Ejection fraction, fractional shortening, pulmonary artery diameter, blood flow velocity, and other parameters were determined using the Vevo LAB 5.5 analysis software (FUJIFILM VisualSonics, Toronto, ON, Canada).

Right heart and pulmonary artery catheterization for hemodynamics: Young and aged mice at baseline, or young mice after 4 weeks of 10% O₂ hypoxia challenge, were utilized for hemodynamic assessment. Right heart and pulmonary artery catheterization and pressure measurements were obtained using the technique reported by Skuli and colleagues (57), with slight modifications. The young mice were anesthetized with 2, 2, 2-Tribromoethanol (Sigma-Aldrich, U.S.A.) at a dose of 0.25 mg/g i.p., while aged or hypoxia-challenged mice were given half of this dose initially, then received supplemental doses of 2, 2, 2-Tribromoethanol as needed. Mice were secured in the supine position on a temperature-controlled small animal surgical table, and respiratory
support was supplied with a rodent ventilator (MiniVent 845; Harvard Apparatus, Holliston, MA, U.S.A.) connected through the mouth into the trachea. A midline sternotomy incision was made, and a pressure transducer (SPR-1000, Millar Instruments, Houston, TX, U.S.A.) was inserted through a small puncture into the right ventricle for initial recordings, then later advanced to the pulmonary artery for additional recording. Pressures were recorded using a PowerLab data acquisition system, and pressure parameters were analyzed using LabChart 7.3 software (ADInstruments, Colorado Springs, CO, U.S.A.). Contractility Index (max dP/dt normalized by left ventricular pressure, to avoid the influence by ventricular afterload) was calculated using the formula of Mason (58). After the final measurements, mice were euthanized.

Pathology and histology: Untreated mice at the indicated ages, or young mice housed under 10% O₂ chronic hypoxia for 4 weeks, were euthanized and body weight measured. Necropsy was performed, and the wet weight of the right ventricle, left ventricle plus septum, and lung were determined. All organ weight data were normalized to the body weight of the individual mouse. Hearts were fixed in 10% neutral buffered formalin, and 8-micron paraffin-embedded sections were stained using the Picrosirius Red Kit (Polysciences, Inc.) according to the manufacturer’s protocol. Fibrosis was assessed visually.

Statistics: Student’s t-test was used to compare two groups, and two-way ANOVA with Sidak test was used for multiple comparisons. Kaplan-Meier survival differences were tested using Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon test. All statistics were calculated using GraphPad Prism version 8.1 for Mac (GraphPad Software, San Diego, CA, U.S.A.). The significance level was set at p<0.05.
Study Approval: All mouse procedures were performed under an animal protocol approved by the CWRU IACUC.

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References


Celermajer DS. Brachial artery FMD with 5-minute distal cuff occlusion—a useful pathophysiological test after all! *Journal of Applied Physiology.* 2005;99(4):1619-.


Atkinson G, and Batterham AM. When will the most important confounder of percentage flow-mediated dilation be reported and adjusted for at the study level? *International Journal of Cardiology.* 2014;172(1):261-2.


Parati G, Agostoni P, Basnyat B, Bilo G, Brugger H, Coca A, et al. Clinical recommendations for high altitude exposure of individuals with pre-existing cardiovascular conditions: A joint statement by the European Society of Cardiology, the Council on Hypertension of the European Society of Cardiology, the European Society of Hypertension, the International Society of Mountain Medicine, the Italian Society of


Figure 1. Reductions in SNO-Hb in Cys93A RBCs and of hypoxic vasodilation by Cys93A RBCs.  (A) SNO levels post treatment of RBCs from C93 control and C93A mice with CysNO (n = 10 for C93 and n = 9 for C93A).  (B) SNO levels at baseline in
RBCs from C93 control and C93A mice (n = 14 each). (C) RBC-mediated hypoxic vasodilation of isolated aortic rings in vitro. SNO-loaded RBCs in (A) were added to bioassays under hypoxia (1% O$_2$) or normoxia (21% O$_2$) (n = 6 each). C93A RBCs had reduced ability to induce relaxation under hypoxic conditions compared to C93 control RBCs; neither caused vasorelaxation under normoxic conditions. Data are shown as mean ± SD. * $p<0.05$, ** $p<0.01$ vs C93 using Student's t test (two-tailed). (D) Representative aortic ring bioassay response to addition of SNO-loaded C93 vs C93A RBCs (arrow) over time, in the presence of 1% O$_2$ or 20% O$_2$. Traces are normalized to 100% tension at time of RBC addition. (E) Vasodilation measured in the abdominal aorta in vivo at baseline and after release of aortic ligature. C93A response is markedly impaired vs. C93 control. Data are shown as mean ± SE. n = 11 C93 (4.0 ± 0.3 months of age) and n = 10 C93A (3.7 ± 0.4 months of age). * $p<0.05$ vs. C93 by two-way ANOVA. (F) Peak vasodilation of aorta (Dilation), calculated from the peak response from each mouse. Data are shown as mean ± SD. * $p<0.05$ vs. C93 by Student's t test (two-tailed). (G) Short axis M-mode images of the abdominal aorta depicting dilation during a measurement in one C93 and one C93A mouse. Dashed lines represent vessel wall positions at diastole, used for calculating diameter. The vertical scale bar represents 2 mm, and the horizontal scale bar represents 50 msec. (H) Mean blood flow increase measured by ultrasound probe after release of aortic ligature. Data are shown as mean ± SE. n = 16 C93 (3.8 ± 0.9 months of age) and n = 17 C93A (3.8 ± 0.7 months of age). * $p<0.05$ vs. C93 by two-way ANOVA. (I) The peak mean flow increase response, calculated using the peak response from each mouse. (J) Representative abdominal aortic blood flow curves in one C93 and one C93A mouse through the baseline, ischemic ligation, and reperfusion periods (minutes). Data are shown as mean ± SD. * $p<0.05$ vs. C93 by Student's t test (two-tailed).
Figure 2. Right ventricular and pulmonary artery signs of pulmonary arterial hypertension in C93A mice with age. (A) Total heart weight (HW) to body weight (BW) ratio in young and in aged C93A vs C93 mice. (B) Right ventricle (RV) to body weight (BW) ratio in young and in aged C93A vs C93 mice. (C) Left ventricle (LV) to body weight (BW) ratio in young and in aged C93A vs C93 mice. (D) Right ventricle (RV) to left ventricle + septum weight (LV+S) ratio in young and in aged C93A vs C93 mice. (E) Pulmonary artery blood flow velocity-time integral (VTI) in young and in aged C93A vs C93 mice. (F) Pulmonary artery (PA) diameter in young and in aged C93A vs C93 mice. (G) Mean velocity of pulmonary artery blood flow in young and in aged C93A vs C93 mice. (H) Peak velocity of pulmonary artery blood flow in young and in aged C93A vs C93 mice. For panels A-D, young mice (n = 31 C93, 3.4 ± 0.6 months of age and n = 36 C93A, 3.4 ± 0.4 months of age) and aged mice (n = 28 C93, 19.5 ± 1.8 months of age and n = 21 C93A, 19.7 ± 3.1 months of age). For panels E-H, young mice (n = 16 C93, 3.8 ± 1.3 months of age and n = 19 C93A, 3.0 ± 0.8 months of age) and aged mice (n = 15 C93, 20.9 ± 1.6 months of age and n = 23 C93A, 21.8 ± 1.2 months of age).
age) were assessed. Differences were assessed using Student’s t test (two-tailed). *

\[ p \text{ < 0.05, } ** p \text{ < 0.01 } C93A \text{ vs. C93, for young or aged animals compared separately.}\]
Figure 3. Pulmonary hypertension and right ventricular dysfunction in hypoxic C93A mice. All comparisons are between hypoxic C93A (red bar) vs. C93 (green bar) mice. (A) Pulmonary artery diameter. (B) Pulmonary artery blood flow velocity-time integral (VTI). (C) Mean velocity of pulmonary artery blood flow. (D) Peak velocity of pulmonary artery blood flow. (E) Systolic pulmonary arterial pressure (sPAP), diastolic pulmonary arterial pressure (dPAP), and mean pulmonary arterial pressure (mPAP). (F) Right ventricular systolic pressure (RVSP). (G) Maximal rate of change in right ventricular systolic pressure (dP/dt max).
ventricular (RV) pressure (dP/dt max). (H) Minimal rate of change in right ventricular pressure (dP/dt min). (I) Right ventricular contractility index. (J) Right ventricular average dP/dt over isovolumic relaxation period (IRP average dP/dt). (K) Time constant of relaxation (τ). (L,M,N) Right ventricular fibrosis in young mice housed in 10% O₂ for 4 weeks, visualized by Picrosirius Red staining. C93 lacking fibrosis (L, representative of 3 tested), C93A displaying developing fibrotic areas (M, representative of 4 of 5), and C93A with fibrosis (N, observed in 1 of 5). "*" indicates fibrotic areas; scale bar is 50 µm.

For all quantitative panels, data are presented as mean ± SD. Young mice exposed to 10% O₂ for 4 weeks: for panels A-D, n = 19 C93, 4.9 ± 1.3 months of age and n = 23 C93A, 4.4 ± 1.1 months of age); for panels E-K, n = 19 C93 mice (4.9 ± 1.3 months of age) and n = 20 C93A mice (4.3 ± 1.1 months of age). Differences were assessed using Student’s t test (two-tailed). * p<0.05, ** p<0.01 C93A vs. C93.
Figure 4. Increased mortality during chronic hypoxia in C93A mutant mice. (A) C93 and C93A mice were housed under 10% O$_2$, and survival was assessed daily. The percentage of mice surviving each day is shown in a Kaplan-Meier plot. Curves were compared using Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon test. ** $p<0.01$ C93A (n = 15, 7.6 ± 0.5 months of age) vs. C93 (n = 21, 7.8 ± 0.8 months of age). (B) Average days of survival at 10% O$_2$, plotted as mean ± SD, with individual data points shown. ** $p<0.01$ C93A vs. C93 by Student’s $t$ test (two-tailed).