

# Red Blood Cell-Derived Nitric Oxide Bioactivity and Hypoxic Vasodilation To beta 93 or not to beta 93?

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# Red Blood Cell–Derived Nitric Oxide Bioactivity and Hypoxic Vasodilation

## To $\beta$ 93 or not to $\beta$ 93?

Article, see p 2654

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**H**ypoxic vasodilation is a conserved adaptive response that enables regional increases in blood flow to compensate for an acute reduction in oxygen delivery to tissues. Although already discovered in the 19th century, mechanistic details of the underpinning signaling pathways have remained unresolved. Skeletal muscle blood flow and vascular conductance are more closely related to changes in arterial oxygen content than the partial pressure of oxygen, suggesting that the mechanisms involved in determining blood vessel diameter in the tissue are sensitive to changes in oxygen content. Because the latter is directly related to hemoglobin mass and oxygenation, red blood cells (RBCs) may be involved in sensing the relationship between tissue oxygen demand and convective oxygen delivery when coupled to a transduction pathway capable of modulating blood flow.

### S-NITROSO HEMOGLOBIN AND THE S-NITROSTHIOL STORM

In 1996, Stamler's group introduced a concept (Figure) that was as novel and intriguing as it was elegant, suggesting that RBCs have their own vasodilator mechanism aboard while traveling the circulation,<sup>1</sup> combining a capturing, preservation, and release mechanism for nitric oxide (NO) that is linked to hypoxic vasodilation<sup>2</sup> and platelet inhibition.<sup>3</sup> Apart from RBCs being able to release NO, mechanistic details changed over time, and not all findings were reproducible by other groups.<sup>4</sup>

In this issue of *Circulation*, Sun et al<sup>5</sup> present negative data from a joint effort by an international team of investigators that puts an end to the notion that Cys $\beta$ 93 S-nitrosothiol is involved in the process of hypoxic vasodilation. Sun et al's data, however, are also positive in the sense that they confirm the ability of RBCs to export NO bioactivity.<sup>5</sup> Is this the end of the S-nitroso hemoglobin story? Probably not, but most certainly for Cys $\beta$ 93 S-nitrosothiol as a critical link.

### HYPOXIC VASODILATION

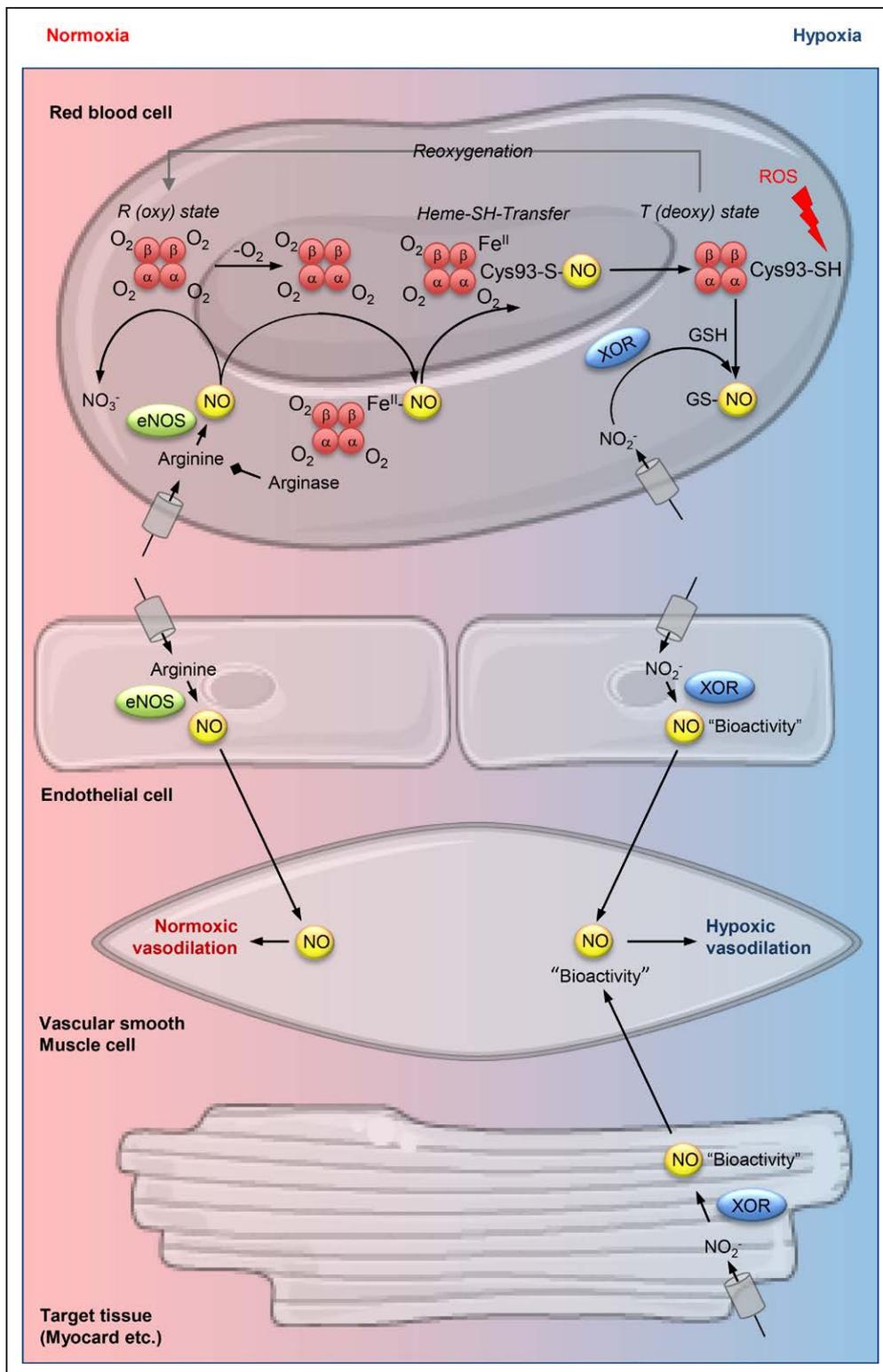
Previous studies described an association between hypoxic vasodilation and the release of NO from isolated vascular tissue and cultured endothelial cells, and later demonstrated that NO is involved in hypoxic vasodilation in the human forearm. Of note, hypoxia has opposing effects on vascular diameter in the systemic circulation and the lung, where it causes vasoconstriction. This von Euler–Liljestrand mechanism matches regional pulmonary ventilation to perfusion, whereas regional vasodilation protects tissues in the face of reduced oxygen delivery. Enhanced NO production is a universal response to hypoxia,<sup>6</sup> and elevated concentrations of NO metabolites

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**Figure.** Cellular and enzymatic sources of nitric oxide (NO) bioactivity under normoxia and hypoxia.

The SNO-Hb hypothesis postulated loading of endothelial-produced NO during transit through the venous circulation onto partially deoxygenated hemoglobin (Hb) to form HbNO, which hands over the NO group during passage through the lungs to an adjacent cysteine, specifically the SH group of cysteine-93 of the Hb  $\beta$ -chain to form a protein-bound S-nitrosothiol (Cys $\beta$ 93-SNO), which would subsequently release its cysteine on R-T transition in the microcirculation by using interaction with another protein thiol or glutathione. Alternatively, nitrite ( $\text{NO}_2^-$ ) entering red blood cells (RBCs) may react with deoxyHb or xanthine oxidoreductase (XOR), or arginine with endothelial NO synthase (eNOS) in competition with arginase to form NO bioactivity. Alternative cellular sources for  $\text{NO}_2^-$  reduction under hypoxia include endothelial cells, smooth muscle cells, and surrounding target tissue (eg, cardiomyocytes). Several other  $\text{NO}_2^-$ -reducing enzymes have been postulated and shown to be involved but are here omitted for the sake of clarity. GSH indicates glutathione; GS-NO, S-nitrosoglutathione; ROS, reactive oxygen species; R-T, oxy to deoxy; SH, sulfhydryl; and SNO-Hb, S-nitroso hemoglobin.

have been detected in the blood of native highlanders and that of lowlanders trekking to high altitude. Here, NO plays a central role in rebalancing an acute mismatch between oxygen/energy supply and demand. Chronic environmental hypoxia requires multilevel adjustments in metabolism in addition to changes in vascular function and may differ from acute episodes of regional oxygen shortage. It may be worth reminding the reader that good hypoxia tolerance may have been part of our ancestral adult phenotype, and that mammalian embryogenesis and fetal growth occur in physiological hypoxia. Of course, not all vasodilation is NO mediated, and several other mediators are involved in hypoxic vasodilation, including prostaglandins,<sup>7</sup> adenosine triphosphate, and hydrogen sulfide.

## NO BIOACTIVITY

Short-term hypoxic vasodilation appears to be mediated by bioactive NO metabolites rather than free NO.<sup>8</sup> Indeed, that RBCs release NO appears, at first sight, counterintuitive, because they are also considered a sink for endothelium-derived NO via its rapid reaction with oxyhemoglobin (to form methemoglobin and nitrate), thereby limiting NO-induced vasodilation to its site of production. This, however, is only the case for free NO, and Sun and coworkers call the active principle released from RBCs NO bioactivity rather than NO. In biological systems, NO can bind to protein thiols in the form of an *S*-nitrosothiol and be transferred to a low-molecular-weight thiol by transnitrosylation (*S*-nitrosothiol pathway); formed *S*-nitrosoglutathione may subsequently escape the RBC via a transporter. Alternatively, protein disulfide isomerase may facilitate the efflux of nitrite-derived *S*-nitrosothiols from RBCs. This complexity is reminiscent of the requirement of endothelial  $\alpha$ -hemoglobin as an intermediate in NO signaling and early description of NO bioactivity as guanylyl cyclase-activating factor because of the challenges in detecting NO from NO synthase. More than 3 decades after the discovery of endogenous NO formation, these complexities still confound the measurement of NO and NO adducts in complex biological systems, which is why their analysis often relies on bioactivity, pharmacological or genetic manipulation.

## SOURCES OF NO BEYOND RBCS

In the presence of oxygen, NO is generated by the enzymatic conversion of L-arginine by NO synthases<sup>9</sup>; during hypoxia, nitrite is believed to function as an alternative source of NO and to be reduced by deoxyhemoglobin, xanthine oxidoreductase, aldehyde oxidase, and other enzymes.<sup>10</sup> Nitrite originates from the conversion of dietary nitrate by the oral mucosal flora and oxidation of NO synthase-derived NO. Because anions cannot readily

cross cell membranes and there is no concentration gradient of nitrite between plasma and RBCs, specific uptake and regulation mechanisms must be involved. Quantitatively, the hypoxic tissue rather than circulating blood cells appears to be the primary site of nitrite reduction. Thus, the present article by Sun et al<sup>5</sup> may overestimate the relative contribution of RBC NO to hypoxic vasodilation, because the experiments were specifically designed to focus on this aspect. Blood-dependent nitrite reduction may be functionally more relevant for platelet inhibition.

## EVEN WHEN BIASING TOWARD RBC-NO, $\beta$ 93C IS OF NO RELEVANCE FOR NO EXPORT

Sun et al studied  $\beta$ Cys93Ala mutant mice ( $\beta$ 93A) in 4 distinct ex vivo and in vivo models designed to study the export of NO bioactivity from RBCs: a mouse Langendorff heart model subjected to ischemia/reperfusion injury in the absence or presence of RBCs pretreated with the arginase inhibitor, *N*<sup>ω</sup>-hydroxy-nor-L-arginine. Arginase inhibitors are considered to enhance NO synthesis and, thus, exportable RBC NO bioactivity. As a point of concern, in rat aorta *N*<sup>ω</sup>-hydroxy-nor-L-arginine fails to prevent the tolerance to repeated applications of the endothelium-dependent agonist acetylcholine. The effects of isolated RBCs on platelet-rich plasma activation were studied, and isolated mouse/rat aorta were either exposed to Hb(NO)<sub>4</sub> from  $\beta$ 93C or  $\beta$ 93A RBC hemolysates, or pretreated with indomethacin and *N*<sup>ω</sup>-methyl-L-arginine to block endogenous prostacyclin and NO formation and then exposed to *S*-nitrosated RBCs. Finally, in  $\beta$ 93C and  $\beta$ 93A mice hypoxic ( $F_{iO_2}=0.1$ , arterial  $P_{O_2} < 40$  mm Hg) microvascular relaxation was studied in a dorsal skinfold window model. Whereas most of these models (except the latter) could be criticized for being nonphysiological and highly biased toward enhancing any RBC-NO-dependent effect, the fact that in all these models the  $\beta$ 93A mutation was neutral strongly supports the authors' main conclusion that RBCs are able to export NO, but that this capability is independent of  $\beta$ 93C. It is surprising that the same mouse model was earlier investigated by the Stamler laboratory to conclude that  $\beta$ 93C is (!) required for hypoxic vasodilation<sup>11</sup> and cardioprotection.<sup>12</sup> However, no physiologically relevant hypoxic vasodilation appears to have been observed up to a  $F_{iO_2}$  of 0.1 in that study. Moreover, the observed enhanced cardiac injury and mortality of  $\beta$ 93A mice subjected to myocardial infarction or heart failure<sup>12</sup> may be explained by non-NO-related functions of  $\beta$ Cys93 (see below). In conclusion, the *S*-nitroso hemoglobin hypothesis has to be considered rejected. But even if  $\beta$ 93C plays no role in RBC-NO release, the cardiac phenotype of  $\beta$ 93A mutant mice remains an important observation, which may be linked to one of the somewhat forgotten roles of  $\beta$ 93C.

## FORGOTTEN ALTERNATIVE ROLES FOR $\beta$ 93C

Indeed,  $\beta$ 93C has several other important physiological functions (ie, in the allosteric transition of hemoglobin, the modulation of redox potential and oxygen affinity of the heme iron, inhibition of sickle fiber formation, and the hemoglobin disassembly pathway).<sup>13</sup>  $\beta$ 93C is situated at a critical position by neighboring with  $\beta$ Asp94, an important amino acid residue responsible for the Bohr effect and the heme-binding proximal histidine,  $\beta$ His92.  $\beta$ 93C appears to be critical in sustaining the intersubunit interactions at the  $\alpha_1/\beta_2$  (and  $\alpha_2/\beta_1$ ) interface via the salt bridge network, and this close association with heme-heme signaling may explain its impact on heme activity and cooperative oxygen binding.<sup>13</sup> Moreover,  $\beta$ 93C protects heme and tissue from reactive oxygen species consistent with  $\beta$ 93A mice showing enhanced levels of oxidative stress.<sup>14</sup> These functions of  $\beta$ 93C in oxygen handling and protection from oxidative stress may well explain the cardiac phenotype of  $\beta$ C93A mice.<sup>12</sup>

## CLINICAL RELEVANCE OF RBC-NO

Irrespective of the different cellular (RBC versus tissue) and enzymatic sources, 2 relevant questions remain: Is hypoxic NO bioactivity of pathophysiological relevance (ie, are there patients with a dysfunctional hypoxic NO response and at particular risk of hypoxic organ damage)? And, are there any realistic therapeutic options in treating hypoxic conditions by enhancing vasodilatory NO? There is some evidence that hypoxic vasodilation is impaired in older individuals and in patients with heart failure and obstructive sleep apnea.<sup>15</sup> In humans, the S-nitrosylating agent, ethyl nitrite, was found to increase RBC S-nitroso hemoglobin levels at reduced  $F_{iO_2}$ , correcting hypoxia-induced deficits in tissue oxygenation, and to improve measures of oxygen utilization. In light of the present findings, however, the mechanism remains unclear and may not have occurred via S-nitrosylation of  $\beta$ 93C because the compound lacks specificity. Another approach may be to enhance NO export from vascular cells by using arginase inhibitors. Time will tell.

## ARTICLE INFORMATION

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## Disclosures

None.

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