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Review

# Interaction of nitrogen monoxide with hemoglobin and the artefactual production of *S*-nitroso-hemoglobin

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**Abstract**

Hemoglobin (Hb) is probably the most thoroughly studied protein in the human body. However, it has recently been proposed that in addition to the well known function of dioxygen and carbon dioxide transporter, one of the main roles of hemoglobin is to store and transport nitrogen monoxide. This hypothesis is highly disputed and is in contrast to the proposal that hemoglobin serves as an $NO^{\bullet}$ scavenger in the blood. In this short review, I have presented the current status of research on the much-debated mechanism of the reaction between circulating hemoglobin and $NO^{\bullet}$. Despite the fact that oxyHb is extremely rapidly oxidized by $NO^{\bullet}$, under basal physiological conditions the biological activity of $NO^{\bullet}$ in the blood vessels is not completely lost. It has been shown that three factors reduce the efficiency of hemoglobin to scavenge $NO^{\bullet}$: a so-called red blood cell-free zone created close to the vessel wall by intravascular flow, an undisturbed layer around the red blood cells – where the $NO^{\bullet}$ concentration is much smaller than the bulk concentration – and/or the red blood cell membrane. Alternatively, it has been proposed that $NO^{\bullet}$ binds to Cys$\beta$93 of oxyHb, is liberated after deoxygenation of Hb, and consequently allows for a more effective delivery of $O_2$ to peripheral tissues. However, because of the extremely fast rate of the reaction between $NO^{\bullet}$ and oxyHb, experiments in vitro lead to artefactual production of large amounts of *S*-nitroso-hemoglobin. These results, together with other data, which challenge most steps of the $NO^{\bullet}$-transporter hypothesis, are discussed. ***To cite this article: S. Herold, C. R. Biologies 326 (2003).***



**Résumé**

**Interaction du monoxyde d'azote avec l'hémoglobine et la production artéfactuelle de *S*-nitroso-hémoglobine.** L'hémoglobine (Hb) est probablement la protéine du corps humain qui a été étudiée le plus exhaustivement. Pourtant, il a récemment été proposé qu'en plus de ses fonctions bien connues de transporteur d'oxygène et de dioxyde de carbone, l'un des principaux rôles de l'hémoglobine soit le stockage et le transport du monoxyde d'azote. Cette hypothèse est très controversée et s'oppose à la proposition selon laquelle l'hémoglobine sert de piège à $NO^{\bullet}$ dans le sang. Dans cette revue, l'auteur décrit l'état actuel des recherches sur le mécanisme très débattu de la réaction entre l'hémoglobine circulante et $NO^{\bullet}$. En dépit du fait que l'oxyHb est extrêmement rapidement oxydée par $NO^{\bullet}$, l'activité biologique de ce dernier dans les vaisseaux sanguins n'est pas complètement perdue pour des conditions physiologiques basales. Il a été montré que trois facteurs réduisent l'efficacité de l'hémoglobine en tant que piège à $NO^{\bullet}$ : une zone dépourvue de globules rouges créée à proximité de la paroi des vaisseaux par un flux intravasculaire, une couche non perturbée autour des globules rouges – dans laquelle la concentration en $NO^{\bullet}$

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est beaucoup plus faible que dans la masse – et/ou la membrane des globules rouges. Il a été proposé alternativement que $NO^{\bullet}$ se lie avec la Cys$\beta$93 de l'oxyHb, puis est libéré après désoxygénation de l'Hb, permettant un approvisionnement plus efficace des tissus périphériques en $O_2$. Pourtant, à cause de la vitesse extrêmement rapide de la réaction entre $NO^{\bullet}$ et oxyHb, les expériences in vitro conduisent à la production artéfactuelle de grandes quantités de *S*-nitroso-hémoglobine. Ces résultats, avec d'autres données, qui défient la plupart des étapes de l'hypothèse du transporteur de $NO^{\bullet}$, sont discutés. ***Pour citer cet article : S. Herold, C. R. Biologies 326 (2003).***


*Keywords:* hemoglobin; nitric oxide; *S*-nitroso-hemoglobin

*Mots-clés :* hémoglobine ; oxyde nitrique ; *S*-nitroso-hémoglobine

## Abbreviations

Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; DEA, 2-(*N*, *N*-diethylamino)diazenolate 2-oxide; deoxyHb, $HbFe^{II}$; ferrylHb, oxoiron(IV) hemoglobin; sGC, soluble guanylyl cyclase; GSH, glutathione; GSNO, *S*-nitrosoglutathione; Hb, hemoglobin; $HbFe^{II}NO$, nitrosyl hemoglobin; Mb, myoglobin; metHb, $HbFe^{III}$; MHMA, (Z)-1-{*N*-methyl-*N*-[6-(*N*-methylammoniohexyl)amino]}diazenium 1,2-diolate; NOS, nitric oxide synthase; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; oxyHb, $HbFeO_2$; R, relaxed, RBCs, red blood cells; SNO–Hb, hemoglobin with the cysteine residue $\beta$93 nitrosated; T, tense.

## 1. Hemoglobin

Hemoglobin (Hb) is probably the most thoroughly studied protein in the human body. At the beginning it was the ease of hemoglobin purification from many organisms and its stability that drove researchers to the field. Later, this protein proved to be a very complex system with fascinating properties that still remain a challenge to its investigators. Human hemoglobin is among the first proteins for which the structure was determined by X-ray crystallography [1]. Hemoglobin is a tetramer of two $\alpha$- and two $\beta$-subunits, each of which contains an iron protoporphyrin IX–heme. The characteristic globular hemoglobin structure, the so-called globin fold, arises from the numerous $\alpha$-helices that are formed when the two polypeptide chains fold: the $\alpha$-chains have seven helices whereas the $\beta$-chains have eight. The function of hemoglobin is to bind $O_2$ in the lungs and deliver it to peripheral tissues. In addition, hemoglobin transports the waste product of cellular metabolism – $CO_2$ – to the lungs, so that it can be exhaled.

Oxygenation of hemoglobin is cooperative. This means that binding of the first $O_2$ molecule to the heme of a subunit facilitates subsequent binding of other $O_2$ molecules to the remaining subunits in the tetramer. The binding affinity for $O_2$ is also regulated by allosteric effectors, such as pH (Bohr effect), $CO_2$- and 2,3-diphosphoglycerate-concentration [2]. In a simplified description, cooperative $O_2$ binding can be explained by a switch of the protein from a tense (T) low-affinity state in the deoxygenated form to a relaxed (R) high-affinity state in its oxygenated form. A large number of structural, mutagenesis, and kinetic studies carried out over the last three decades have allowed to reach a good understanding of the transitions that take place at the atomic level when T-state hemoglobin is converted to its R-state [3].

Recently, it has been discovered that the hemoglobins found in vertebrates derive from an ancient protein family present in essentially all living systems, among others in bacteria and plants [4]. All these hemoglobins are characterized by the typical $\alpha$-helix-rich globin fold. A common function attributed to a large number of hemoglobins is to protect against reactive nitrogen species, specifically to scavenge nitrogen monoxide. Interestingly, also human hemoglobin has recently been proposed to manifest an additional function related to the biochemistry of $NO^{\bullet}$.

## 2. $NO^{\bullet}$ and the blood vessels

The discovery that nitrogen monoxide is involved in a diverse array of physiological and pathophysio-

logical processes opened a completely new research area. In vivo, $NO^{\bullet}$ is generated from the essential amino acid L-arginine by a family of enzymes called nitric oxide synthases (NOS) [5,6]. Among others, $NO^{\bullet}$ is fundamental for signal transduction in the nervous system and in immune responses. In addition, endothelium-derived $NO^{\bullet}$, generated by the endothelial isoform of nitric oxide synthase (eNOS), is a key determinant of blood pressure homeostasis [7]. $NO^{\bullet}$ produced in vascular endothelial cells can diffuse across cell membranes to the adjacent smooth muscle cells where it activates its target enzyme, the soluble isoform of guanylyl cyclase (sGC). Binding of $NO^{\bullet}$ to the reduced heme of sGC leads to an increase of the intracellular concentration of cyclic guanosine monophosphate (cGMP), a compound that triggers a series of reactions that finally cause smooth muscles to relax.

$NO^{\bullet}$ generated by another isoform of NOS, the inducible NOS (iNOS), also plays an important role in the cardiovascular system, in particular in the response to vascular injury. Indeed, $NO^{\bullet}$ has been shown to inhibit platelet aggregation and adherence to the site of injury, and consequently to preserve blood flow [8]. In addition, as arterial injury results in loss of eNOS, upregulation of iNOS may also represent a protective mechanism that compensates for the loss of the endothelium [9].

## 3. Hemoglobin and $NO^{\bullet}$

Hemoproteins are among the main targets of $NO^{\bullet}$ in biological systems. $NO^{\bullet}$ can react with all forms of hemoglobin found under physiological conditions (reactions (1)–(4)). DeoxyHb rapidly binds $NO^{\bullet}$ to generate the stable nitrosyl complex $HbFe^{II}NO$ ($k = 2.6 \times 10^7\ M^{-1}\,s^{-1}$, in 0.05 M phosphate buffer pH 7.0 and 20 °C [10]) (reaction (1)). OxyHb irreversibly reacts with $NO^{\bullet}$ to produce metHb and nitrate ($k = (8.9 \pm 0.3) \times 10^7\ M^{-1}\,s^{-1}$, in 0.1 M phosphate buffer pH 7.0 and 20 °C [11]) (reaction (2)). We have recently shown that, in analogy to the diffusion-controlled reaction between $NO^{\bullet}$ and $O_2^{\bullet-}$, this reaction proceeds via the peroxynitrito-complex $HbFe^{III}OONO$ [11,12]. This intermediate was characterized spectroscopically under alkaline conditions [11,12]. At neutral pH $HbFe^{III}OONO$ decays very rapidly to metHb and nitrate, and thus does not accumulate in concentrations large enough to be detected [12]. In contrast to free peroxynitrite, the hemoglobin peroxynitrito-complex is not able to nitrate the tyrosine residues of the protein [11]. (The $\alpha$- and $\beta$-subunits of hemoglobin contain three tyrosine residues each.)

$$HbFe^{II} + NO^{\bullet} \rightleftarrows HbFe^{II}NO \quad (1)$$

$$HbFeO_2 + NO^{\bullet} \rightarrow \left\{HbFe^{III}OONO\right\} \rightarrow HbFe^{III} + NO_3^- \quad (2)$$

$$HbFe^{III} + NO^{\bullet} \rightleftarrows HbFe^{III}NO \leftrightarrow HbFe^{II}(NO^+) \quad (3)$$

$$HbFe^{IV}{=}O + NO^{\bullet} \rightarrow \left\{HbFe^{III}ONO\right\} \rightarrow HbFe^{III} + NO_2^- \quad (4)$$

$NO^{\bullet}$, unlike $O_2$ and CO, also binds to the oxidized metHb form ($k_\alpha = 1.71 \times 10^3\ M^{-1}\,s^{-1}$ and $k_\beta = 6.4 \times 10^3\ M^{-1}\,s^{-1}$, in 0.1 M Bistris pH 7.0 and 20 °C [13]). The resulting complex is better described as an iron(II)–$NO^+$ complex, $HbFe^{II}(NO^+)$ (reaction (3)). Finally, the oxoiron(IV) form of hemoglobin (ferrylHb), generated under oxidative stress by the reaction of hemoglobin with $H_2O_2$, is rapidly reduced by $NO^{\bullet}$ to metHb and nitrite ($k = (2.4 \pm 0.2) \times 10^7\ M^{-1}\,s^{-1}$, in 0.1 M phosphate buffer pH 7.0 and 20 °C [14]) (reaction (4)). We have shown that also this reaction proceeds via an intermediate, the *O*-nitrito complex $HbFe^{III}ONO$, which was characterized by transient spectroscopy [14].

### 3.1. Hemoglobin as an $NO^{\bullet}$-scavenger

The close proximity of the red blood cells (RBCs) to the site of $NO^{\bullet}$ production and the fast consumption of $NO^{\bullet}$ by oxyHb and deoxyHb observed in vitro [10, 11] suggest that most $NO^{\bullet}$ generated by eNOS will be scavenged by the blood. In particular, as the values of the rate constants for the reactions of $NO^{\bullet}$ with oxyHb and with deoxyHb are in the same order of magnitude, one would expect oxyHb, present in about 99% in oxygenated blood, to be the main target for $NO^{\bullet}$. Under physiological conditions, the reaction between $NO^{\bullet}$ and $O_2$ is too slow [15] to play a significant role. Indeed, the half-life of 100 nM $NO^{\bullet}$ in the presence of about 150 µM $O_2$ (the concentration found in arterial blood) is over two hours ($t_{1/2} = 1/[8 \times 10^6\ M^{-2}\,s^{-1} \times 100 \times 10^{-9}\ M \times 150 \times 10^{-6}\ M] = 2.3$ h). In contrast, the half-life of the reaction of

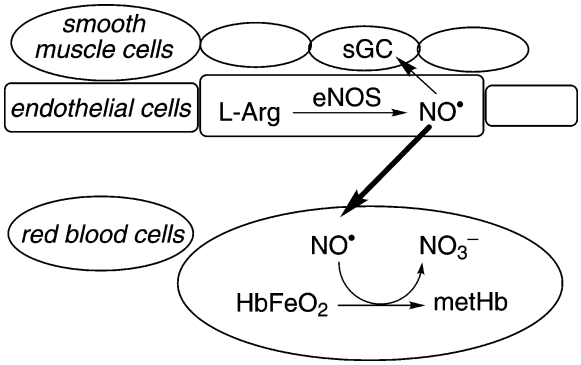


Fig. 1. Hemoglobin as an NO•-scavenger. NO• produced by eNOS in vascular endothelial cells can diffuse to the adjacent smooth muscle cells where it activates its target enzyme sGC. Excess NO• is scavenged by its rapid reaction with oxyHb, which yields metHb and nitrate.

20 mM oxyHb (heme-based concentration present in the RBCs) with physiological amounts of NO• (nM) is less than 1 μs ($t_{1/2} = \ln 2/(8.9 \times 10^7\ \mathrm{M^{-1}\,s^{-1}} \times 20 \times 10^{-3}\ \mathrm{M}) = 0.4$ μs, calculated with the value of the rate constant measured at pH 7.0 and 20 °C [11]). Thus, in principle most NO• should be irreversibly converted to nitrate (Fig. 1). This hypothesis is supported by the strong correlation found, in venous blood of volunteers inhaling 80 ppm NO• for two hours, between the time-dependent increase of the nitrate and the metHb concentrations [16].

However, under basal physiological conditions the biological activity of NO• in the blood vessels is not completely lost. A substantial amount of NO• reaches the smooth muscle cells, activates guanylyl cyclase, and thus leads to dilation of the blood vessels. This apparent paradox has prompted to a series of studies aimed to understand the detailed mechanism of the in vivo reaction between circulating hemoglobin and NO•.

### *3.2. Factors reducing the efficiency of hemoglobin to scavenge NO•*

A number of theoretical and experimental studies have been carried out to identify the factors that determine the distance NO• can diffuse from the site of its production in the endothelial cells [17–19]. Lancaster and coworkers showed that the rate of the reaction between NO• and oxyHb is approximately 650 times slower when hemoglobin in encapsulated within the RBCs [20]. This result was explained with the assumption that the limiting step for the reaction of NO• with hemoglobin within the RBCs is the diffusion of NO• from the bulk solution to the RBCs membrane [20]. In contrast, to rationalize similar results Liao and coworkers proposed that it is the membrane that offers the main resistance to NO• diffusion into the RBCs [21]. Nevertheless, a recent study has shown that NO• uptake is primarily limited by extracellular diffusion resistance [22], conceptually represented by an undisturbed layer around the RBCs, where the NO•-concentration is much smaller than the bulk concentration. By taking into account these results, one can explain the significant increase in blood pressure observed when extracellular hemoglobin-based blood substitutes are administered [23]: free hemoglobin, that is Hb not encapsulated within the RBCs, reacts extremely rapidly with NO•, eliminates completely its bioactivity, and thus causes the blood vessels to contract.

A further factor that extends the lifetime of NO• within the blood vessels is a RBCs-free (or RBCs-depleted) region created by intravascular flow close to the vessel wall [24]. This layer reduces the amount of NO• scavenged by the RBCs as it increases the distance NO• has to diffuse to reach them.

### *3.3. Hemoglobin as an NO•-transporter*

An alternative, conceptually different pathway has recently been proposed to explain how the bioactivity of NO• is maintained within the blood vessels in the presence of high concentrations of hemoglobin (Fig. 2). Stamler and coworkers suggested that, despite the kinetic arguments discussed above, in vivo NO• preferentially binds to the minor fraction of deoxygenated iron centers (about 1%) to yield partially nitrosylated hemoglobin, $Hb(Fe^{II}NO)(FeO_2)_3$ (reaction (5)) [25]. Difference absorption and EPR spectroscopy were utilized to show that addition of submicromolar concentrations of NO• to oxyHb (in 10 mM phosphate buffer) leads primarily to the generation of nitrosyl Hb [25]. To account for these results, it must be assumed that binding of NO• to partially oxygenated hemoglobin is cooperative. Specifically, NO• should bind R-state $Hb(Fe^{II})(FeO_2)_3$ at least 100 times faster than T-state $Hb(Fe^{II})_4$ or $Hb(Fe^{II})(Fe^{II}NO)_3$ [26,27].

$$Hb(Fe^{II})(FeO_2)_3 + NO^{\bullet} \rightleftarrows Hb(Fe^{II}NO)(FeO_2)_3 \quad (5)$$

$$S^{-}\text{–}Hb(Fe^{II}NO)(FeO_2)_3 + 2\ O_2 \rightleftarrows SNO\text{–}Hb(FeO_2)_4 + O_2^{\bullet -} \quad (6)$$

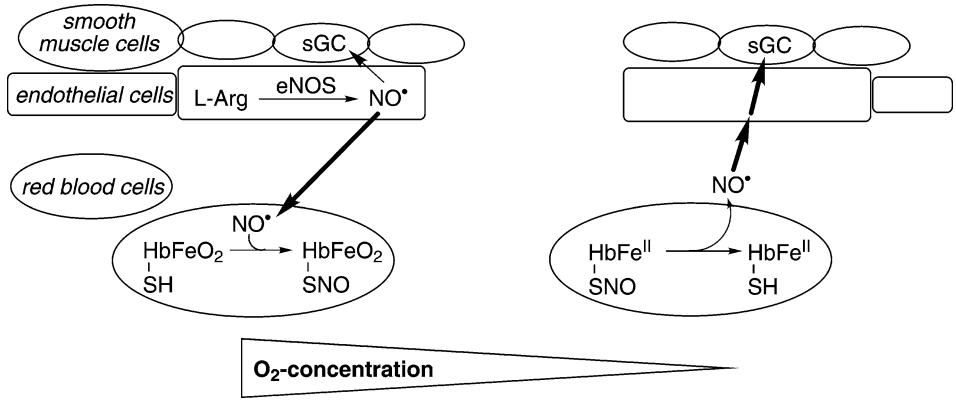


Fig. 2. Hemoglobin as an NO•-transporter. In the arteries (high $O_2$-concentration) NO• binds to Cys$\beta$93 of oxyHb. Deoxygenation of hemoglobin (low $O_2$-concentration) favors liberation of NO•, which reaches sGC, causes blood vessels to dilate, and thus allows for more efficient $O_2$-delivery.

In a subsequent step, the 'NO group' has been proposed to be transferred intramolecularly from $HbFe^{II}NO$ to the conserved cysteine residue $\beta$93, and to form the so-called *S*-nitroso-hemoglobin (SNO–Hb) [28]. It is presumed that this reaction proceeds via oxidation of the iron center by $O_2$ to produce $HbFe^{III}NO \leftrightarrow HbFe^{II}(NO^+)$, which can subsequently undergo a formal $NO^+$-transfer to Cys$\beta$93 (reaction (6)) [27,29]. It has been further suggested that, when the RBCs reach $O_2$-depleted tissues, partial deoxygenation of hemoglobin facilitates the release of the 'NO group' from SNO–Hb. A thermodynamic argumentation has been applied to support this reaction step: crystal structures of SNO–Hb [30] have shown that the *S*-nitrosocysteine is significantly more stable in oxyHb (R-state), as the *S*-atom is directed inward and is not exposed to the solvent. Upon dissociation of $O_2$, when the RBCs reach hypoxic tissues, hemoglobin is converted to the T-state (deoxyHb). This transition is coupled with a reorientation of the *S*-nitrosocysteine toward the solvent. The exposed SNO-group can thus readily undergo transnitrosation reactions with other thiols within the RBCs. Initially, it was proposed that SNO–Hb reacts with GSH, present in millimolar concentration within the RBCs, to generate GSNO [31]. However, analysis of the *S*-nitrosated components of the RBCs suggested that SNO–Hb rather undergoes a transnitrosation reaction with cysteine residues in the Hb-binding cytoplasmic domain of the band-3 anion-exchange membrane protein [32]. Finally, to reestablish the bioactivity of NO•, the 'NO group' should be transported out of the RBCs (possibly with the involvement of extracellular GSH [33]), reduced to regenerate NO•, which should diffuse into the smooth muscle cells, bind and activate guanylyl cyclase. The mechanism of these last reaction steps is still unidentified [32,34].

### *3.4. Arguments against the NO•-transporter hypothesis*

One of the central issues of the hypothetical mechanism presented above is that binding of NO• to the heme of partially oxygenated hemoglobin (R-state) is cooperative and depends on the oxygen saturation of hemoglobin. However, Kim-Shapiro and coworkers [35] have recently shown by EPR spectroscopy that, upon mixing of hemoglobin with NO•, there is a linear correlation between the oxygen saturation of hemoglobin and the yield of $HbFe^{II}NO$. Comparable results were also obtained with blood samples [35]. In another work [36], the same group has reported that the rate constant for NO• binding to R-state hemoglobin, measured by photolyzing $HbFe^{II}CO$ in the presence of NO•, is $(2.1 \pm 0.1) \times 10^7\ M^{-1}\,s^{-1}$. This value is nearly identical to that reported for NO• binding to T-state hemoglobin ($2.6 \times 10^7\ M^{-1}\,s^{-1}$ [10]). Taken together, these results clearly indicate that the rate of NO• binding to hemoglobin is not cooperative and is independent of oxygen saturation.

The pathway suggested for the transfer of the 'NO group' from nitrosyl Hb to Cys$\beta$93 (reaction

(6)) is also highly unlikely. Reaction of nitrosyl Hb with $O_2$ has been shown to yield metHb and nitrate [37]. The analogous reaction with myoglobin has been proposed to proceed *via* the intermediate *N*-peroxynitrito-complex $MbFe^{III}(N(O)OO)$ (reaction (7)) [38].

$$MbFe^{II}NO + O_2 \rightarrow \left\{MbFe^{III}(N(O)OO)\right\} \rightarrow MbFe^{III} + NO_3{}^- \quad (7)$$

However, we have recently shown that the rate of the reaction between nitrosyl Hb and $O_2$ is very slow, does not depend on the $O_2$-concentration, and is in the same order of magnitude as the rate of $NO^\bullet$ dissociation from nitrosyl Hb (S. Herold and G. Röck, unpublished results). Thus, these results imply that the reaction proceeds via dissociation of $NO^\bullet$ (rate-determining step), rapid binding of $O_2$ to deoxyHb, and subsequent $NO^\bullet$-mediated oxidation of oxyHb to metHb (*Reactions* (8)–(10)).

$$HbFe^{II}NO \rightleftarrows HbFe^{II} + NO^\bullet \quad (8)$$

$$HbFe^{II} + O_2 \rightarrow HbFeO_2 \quad (9)$$

$$HbFeO_2 + NO^\bullet \rightarrow HbFe^{III} + NO_3{}^- \quad (10)$$

A further matter of debate is the concentration of SNO–Hb present in the blood. If $NO^\bullet$ was released cooperatively in the capillaries after $O_2$ dissociation, an arterial-venous gradient should exist for the SNO–Hb-concentration. Indeed, Stamler and coworkers found SNO–Hb-concentrations of $311 \pm 55$ nM and $32 \pm 14$ nM in arterial and venous rat blood, respectively [39]. Nevertheless, Gladwin and coworkers recently developed an accurate and sensitive method to measure very small SNO–Hb concentrations also in the presence of larger amounts of nitrite and nitrosyl Hb [40]. These authors found that the levels of SNO–Hb in the basal human circulation, including RBCs membrane fractions, are $46 \pm 17$ nM in arterial RBCs and $69 \pm 11$ nM in venous human RBCs [40]. In addition, the same group showed that SNO–Hb is intrinsically unstable in the reductive environment of the RBCs [40]. This feature further explains the extremely small SNO–Hb concentrations found in vivo and suggests that SNO–Hb cannot accumulate in the RBCs to form a reservoir of bioactive $NO^\bullet$.

Finally, the postulated allosterically controlled $O_2$-concentration dependent release of the 'NO group' from SNO–Hb was sustained by kinetic and thermodynamic arguments [41]. However, Patel et al. demonstrated that the rate of the reaction between GSH and SNO–Hb is rather slow and does not depend on the oxygenation state of hemoglobin [42]. Transnitrosation reactions between thiols in proteins are usually slower than reactions between low molecular weight thiol compounds. Thus, the proposed transnitrosation between SNO–Hb and the band-3 anion-exchange protein is likely to be too slow to take place within the time taken for a given RBCs to pass through the precapillary vasculature.

### *3.5. In vitro studies of the reaction between oxyHb and $NO^\bullet$: mixing artefacts*

We [43] and others [44,45] have recently shown that, because of the extremely fast reaction between $NO^\bullet$ and oxyHb, addition of a small volume of a concentrated $NO^\bullet$ solution to a larger volume of a diluted oxyHb solution may lead to artefactual generation of significant amounts of SNO–Hb. In addition, in agreement with Zhang et al. [46], we observed similar effects for the reaction of $NO^\bullet$ with oxyMb in the presence of GSH [43]. These findings imply that some of the high SNO–Hb yields reported in previous in vitro studies [25,28] may be artefacts arising from the chosen experimental conditions.

To understand the mechanism of this artefactual *S*-nitrosation of hemoglobin, one has to consider the half-life of the reactions that could take place in the system before the concentrations of all reagents have reached homogeneity. When a saturated (2 mM) $NO^\bullet$ solution is mixed with a 50-μM oxyHb solution, at the interface of the two solutions, the half-life of the reaction is so short ($t_{1/2} = \ln 2/(8.9 \times 10^7\ M^{-1}\,s^{-1} \times 2 \times 10^{-3}\ M) = 3.9\ \mu s$) that metHb is generated before $NO^\bullet$ has reached a uniform concentration in the reaction mixture. Thus, $NO^\bullet$ could also react with metHb and produce the nitrosating species $HbFe^{II}(NO^+)$ ($t_{1/2}^{(\beta)} = \ln 2/(6.4 \times 10^3\ M^{-1}\,s^{-1} \times 2 \times 10^{-3}\ M) = 54$ ms and $t_{1/2}^{(\alpha)} = \ln 2/(1.7 \times 10^3\ M^{-1}\,s^{-1} \times 2 \times 10^{-3}\ M) = 203$ ms). Finally, before $NO^\bullet$ will be dispersed in the solution, the reaction of $NO^\bullet$ (2 mM) with $O_2$ (in the air saturated oxyHb solution, 220 μM) [15] could also occur ($t_{1/2} = 1/[8 \times 10^6\ M^{-2}\,s^{-1} \times 2 \times 10^{-3}\ M \times 220 \times 10^{-6}\ M] = 280$ ms). The reaction

of $NO^{\bullet}$ with $O_2$ in aqueous solution is believed to proceed via the formation of $NO_2$ and $N_2O_3$ [15]. Taken together, these simple calculations suggest that upon reaction of $NO^{\bullet}$ with oxyHb nitrosating species such as $NO_2$, $N_2O_3$, and $HbFe^{II}(NO^+)$ can be generated in high local concentrations and thus lead to artefactual production of SNO–Hb.

To get a better understanding of these mixing effects, we have recently carried out a systematic study of the influence of different factors on the amount of SNO–Hb produced from the reaction of $NO^{\bullet}$ with oxyHb [43]. Our results showed that the relative SNO–Hb yields (expressed relative to the amount of $NO^{\bullet}$ added) increase with decreasing amounts of $NO^{\bullet}$ added. Moreover, when the $NO^{\bullet}$ solution is added very slowly (within 1–2 min) from a diluted solution (equivalent volumes of the $NO^{\bullet}$ and the oxyHb solutions) the SNO–Hb yields are larger than those obtained by adding a very small volume of a 2 mM $NO^{\bullet}$ solution (at identical final $NO^{\bullet}$- and oxyHb-concentrations). Interestingly, Han et al. [44] found that when $NO^{\bullet}$-donors were employed to slowly deliver small concentrations of $NO^{\bullet}$, oxyHb was converted exclusively to metHb and nitrate, without formation of detectable amounts of SNO–Hb. In contrast, Joshi et al. [45] reported that the SNO–Hb yields were nearly identical when $NO^{\bullet}$ was added as a bolus or with an $NO^{\bullet}$-donor. These contrasting results may arise from the different $NO^{\bullet}$-donors used by the two groups (DEA [44] vs. MHMA [45]), and thus suggest that $NO^{\bullet}$-donors are not always good substitutes for $NO^{\bullet}$.

### *3.6. Possible pathways for in vitro SNO–Hb production*

As mentioned above, $N_2O_3$ may be one of the possible species responsible for SNO–Hb generation upon mixing of $NO^{\bullet}$ with oxyHb (reaction (11)). This hypothesis is supported by our observation that, in some cases (by slow addition of diluted $NO^{\bullet}$ solutions), the SNO–Hb yields are slightly lower when the reaction is carried out in more concentrated phosphate buffer (0.1 M vs. 10 mM) [43]. The decrease of the SNO–Hb production is due to the concurrent hydrolysis of $N_2O_3$ (reaction (12)), which has been shown to be catalyzed by inorganic phosphates [47].

$$SH\text{–}Hb + N_2O_3 \rightarrow SNO\text{–}Hb + NO_2^- + H^+ \qquad (11)$$

$$N_2O_3 + H_2O \rightarrow 2\,H^+ + 2\,NO_2^- \qquad (12)$$

To evaluate the nitrosating efficiency of the iron(III) nitrosyl heme complex, we studied the reactions of $NO^{\bullet}$ with metHb and with a mixture of metMb/GSH [43]. We found that the amount of SNO–Hb formed from the reaction of $NO^{\bullet}$ with metHb is larger than the GSNO yields obtained under identical conditions from the reaction with metMb/GSH [43]. Systematic studies indicated that maximal relative SNO–Hb yields (expressed relative to the amount of $NO^{\bullet}$ added) are generated when substoichiometric amounts of $NO^{\bullet}$ are added to metHb. Under these conditions a larger fraction of $NO^{\bullet}$ is bound to the $\beta$-subunit of hemoglobin [13,29] and may lead to higher SNO–Hb yields via an intramolecular nitrosation of Cys$\beta$93. In fact, this residue is located on the proximal side of the heme, at a distance of only $\sim 10$ Å from the iron center [30]. Studies with myoglobin have shown that photolyzed heme ligands can diffuse to the proximal side and partly occupy a hydrophobic pocket close to Cys$\beta$93 in hemoglobin [48,49]. Thus, $HbFe^{II}(NO^+)$ may react with $H_2O$, generate $H_2NO_2^+$ or $HNO_2$, which may rapidly diffuse to the proximal side of the heme, and react with Cys$\beta$93. This intramolecular pathway, possibly facilitated by the hydrophobic environment of Cys$\beta$93, may explain why this residue is nitrosated more effectively than GSH.

Finally, we have recently proposed that an additional pathway may be responsible for SNO–Hb formation. Specifically, we found that addition of 1 or 0.1 equiv of $NO^{\bullet}$ to the heme-blocked metHbCN (under strictly anaerobic conditions), followed by degassing of the reaction mixture, and subsequent exposure to air, led to the generation of SNO–Hb ($0.9 \pm 0.2\%$ and $4.0 \pm 0.3\%$ relative to the amount of $NO^{\bullet}$ added, respectively) [43]. It has recently been shown that $NO^{\bullet}$ can also occupy non-covalent binding sites in hemoglobin, possibly trapped by aromatic amino acid residues in hydrophobic pockets [50]. As discussed above, the environment of Cys$\beta$93 is largely hydrophobic. Moreover, two aromatic amino acids, Tyr$\beta$145 and Phe$\beta$103, are located at a distance of 4–5 Å from the sulfur atom of Cys$\beta$93. Because of its lipophilic nature, $NO^{\bullet}$ preferentially partitions into lipid membranes and hydrophobic protein environments [51]. Taken together, these observations suggest that high local amounts of $NO^{\bullet}$ and $O_2$ could

be trapped close to Cys$\beta$93 and may therefore lead to their efficient nitrosation.

## 4. Conclusions

The mechanism of the reaction between circulating hemoglobin and NO$^\bullet$ is currently a matter of debate. In this short review, I have presented the current status of the research on this area. The rapid reaction between NO$^\bullet$ and oxyHb is likely to represent an important scavenging pathway for the excess NO$^\bullet$ generated by eNOS in the blood vessels (Fig. 1). Indeed, it has been shown that the amount of NO$^\bullet$ released by eNOS is approximately 400 nM [52], whereas only 45 nM ($EC_{50}$) are needed to activate sGC in cells [53]. The hypothesis that besides $O_2$ and $CO_2$ hemoglobin transports NO$^\bullet$ is highly controversial. According to this theory, NO$^\bullet$ binds to Cys$\beta$93 of oxyHb, is liberated after deoxygenation of hemoglobin, and consequently allows for a more effective delivery of $O_2$ to peripheral tissues (Fig. 2). Nevertheless, an increasing amount of data collected by different groups and summarized here, challenge most steps of this hypothetical mechanism. The major obstacle for the study of the mechanism of the reaction between hemoglobin and NO$^\bullet$ is probably represented by the artefactual production of SNO–Hb when the reaction is carried out in vitro. This problem is due to the very fast rate of this reaction and makes it impossible to draw conclusions on the in vivo mechanism from studies with purified oxyHb.

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