Erythrocyte consumption of nitric oxide in presence and absence of plasma-based hemoglobin

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Tsoukias, Nikolaos M., and Aleksander S. Popel. Erythrocyte consumption of nitric oxide in presence and absence of plasma-based hemoglobin. Am J Physiol Heart Circ Physiol 282: H2265–H2277, 2002. First published February 28, 2002; 10.1152/ajpheart.01080.2001.—Experimental measurements have suggested a consumption rate of nitric oxide (NO) by red blood cells (RBCs) that is orders of magnitude smaller than that of an equivalent concentration of free hemoglobin in solution. This difference has been attributed to external diffusion limitations in the transport of NO from the plasma to the surface of the RBC or to resistance in the transport through the erythrocytic membrane. A detailed mathematical model is developed to quantify the resistance to NO transport around a single RBC and to predict the consumption rate in the presence and absence of extracellular hemoglobin. We provide a description for the NO consumption rate as a function of hematocrit, RBC radius, membrane permeability, and extracellular hemoglobin concentration. We predict a first-order rate constant for NO consumption in blood between 7.5 \times 10^2 and 6.5 \times 10^3 s^{-1} at a hematocrit of 45% for membrane permeability values between 0.1 and 40 cm/s. Our results suggest that the difference in NO uptake by RBCs and free hemoglobin is smaller than previously reported and it is hematocrit dependent.

red blood cells; diffusion; reaction; blood substitutes

NITRIC OXIDE (NO) is a signal transduction molecule of extreme physiological importance. It is produced in a number of cells through the enzymatic degradation of L-arginine by one of several isoforms of nitric oxide synthase (NOS). NO is a relatively reactive molecule with a short half-life in vivo. It can be degraded by a number of reactions, but under physiological conditions, NO concentrations are submicromolar, and it is the first-order reactions with superoxide and hemec-containing proteins such as hemoglobin (Hb) and guanylate cyclase that should dominate its chemistry in vivo (3).

One of the important roles that NO plays in the body is the regulation of vascular smooth muscle tone. NO produced in the vascular endothelial cells can diffuse freely across cell membranes to the adjacent smooth muscle where it activates the enzyme soluble guanylate cyclase, leading to an increase in the intracellular cGMP concentration and to smooth muscle relaxation. The close proximity of the red blood cells (RBC) to the site of NO production and the fast consumption of NO by both oxy- and deoxyHb observed in vitro (10, 16) suggest, however, that a significant amount of NO will be scavenged by the blood. Thus it is unclear how much of the endothelium-derived NO is able to reach the smooth muscle where it needs to sustain physiologically significant concentrations for the activation of soluble guanylate cyclase.

A number of experimental and theoretical studies have been performed to investigate the diffusional spread of NO away from its site of production (5, 21, 24, 36). Theoretical studies have recently been reviewed by Buerk (4). They represent a first generation of models that consider only transport of free NO. They do not account for preservation of NO-related bioactivity through the formation of more stable intermediates such as S-nitrosothiols. Theoretical simulations by Lancaster (21) showed that physiological amounts of Hb (2 mM) flowing in the lumen of a 20-\mu m arteriole scavenges significant amounts of NO, leading to a dramatic reduction of the NO concentration in the arteriolar smooth muscle. The result questioned earlier experimental observations that free NO is the endothelium-derived relaxing factor (12, 20, 26). Butler et al. (5) included in their theoretical model a layer free of RBCs next to the endothelium. They demonstrated that despite the significant scavenging of NO by Hb, a substantial amount of NO diffuses toward the smooth muscle; at least for vessels with diameters >160 \mu m that were examined. They also suggested that the reaction of NO with the Hb “packed” in RBCs might be slower than the reaction of NO with free Hb due to transport resistance through the membrane. Using a detailed mathematical model, Vaughn et al. (36) also suggested the importance of the RBC-free layer in the NO diffusion toward the smooth muscle; however, they concluded that the uptake of NO by RBCs has to be several orders of magnitude smaller than the uptake by an equivalent amount of free Hb in solution for the concentration in the smooth muscle to be dramatically reduced. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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reach physiologically significant levels. It became obvious that a more detailed description of the NO uptake by RBCs must be incorporated in the modeling studies of NO diffusion.

Recently, two research groups performed experimental studies combined with theoretical analyses to acquire a more detailed description for the uptake of NO by RBCs. Liu et al. (22) measured the disappearance of NO in a suspension of RBCs in a phosphate-buffered solution with the use of an NO-sensitive electrode. Small concentrations of RBCs were utilized (three orders of magnitude less than blood). The disappearance of NO followed a first-order reaction rate with a half-life on the order of seconds. This corresponds to a life on the order of seconds. This corresponds to a reaction rate constant −650 times less than that of free Hb. Utilizing a mathematical model for current transport around a microelectrode, Liu et al. (22) were able to reproduce their experimental observations. Thus they attributed the significant reduction in the reaction rate to external diffusion resistance in the transport of NO from the solution to the RBC membrane. Assuming that this resistance remains the same independent of hematocrit (Hct), they suggested a linear relationship between NO consumption rate and Hct and extrapolated their prediction to normal Hct.

Vaughn et al. (35) used the “competition experiment,” where RBCs in a suspension with free Hb are competing for NO generated in a homogenous phase by an NO donor, to measure the NO uptake by RBCs at high Hct under conditions that should minimize the external diffusion resistance. Measurement of the extracellular methemoglobin (MetHb) concentration that is formed allows the estimation of the ratio of the rate of uptake of NO by the RBC and by free Hb and, thus the ratio of the reaction rate constants. Vaughn et al. (35) noted that the external diffusion resistance should decrease with increasing Hct due to a smaller plasma layer around each RBC. Their experimental data, however, suggested that the reaction rate for the RBCs plateaus for Hct >5%. They attributed this behavior to a significant resistance in transport through the RBC membrane or intracellular diffusion limitations, which become the rate-limiting step of NO uptake at higher Hct. In experiments performed at higher Hct (15.6%), changes in the NO donor or free Hb concentrations did not alter the rate of uptake by RBCs, providing additional indications that NO transport is not external diffusion limited, at least under these experimental conditions. The NO consumption by the RBCs was a 1,000 times less than that of an equivalent concentration of free Hb. In a subsequent study (34), they utilized a more detailed model for the analysis of the competition experiment. The model takes into consideration internal, external, and membrane diffusion limitations. The model was fitted to the experimental data for the estimation of membrane permeability or the intracellular reaction rate constant. The predicted value in either case was 2,000 times smaller than expected. Although the competition experiment could not distinguish between the two resistances, the fact that significantly reducing the intracellular Hb concentration did not affect the rate of uptake suggested that it is likely the membrane permeability that limits the NO transport. However, NO is a small and highly diffusible molecule that has been previously thought to have a high membrane permeability (3, 24, 30).

Recently, Huang et al. (17) proposed a mechanism to explain such a significant resistance to NO transport in the RBC membrane. They utilized the same competition experiment to estimate the rate of NO uptake by pretreated RBCs. The results suggested that altering the band 3 binding to cytoskeleton or altering metHb and denatured Hb binding to the RBC membrane or cytoskeleton, significantly alters NO uptake by the RBC. They concluded that RBC membrane- and cytoskeleton-associated NO-inert proteins provide a significant barrier for NO diffusion into the cell. In another experiment, changes in the viscosity of the solution did not alter the NO uptake rate significantly, suggesting negligible external diffusion limitation at least under the conditions of the competition experiment, thus providing additional indications that membrane resistance is the limiting factor for NO uptake.

Administration of extracellular Hb-based oxygen carriers (HBOCs) holds promise as an alternative to blood transfusion. The hypertensive effects often seen after administration, however, are considered a significant obstacle to the potential use of HBOCs (1, 29, 31–33, 38, 39). This phenomenon has been attributed to the scavenging of NO by the plasma-based Hb. Plasma-based Hb should be able to get closer to the endothelial cells in the lumen and can possibly extravasate to the space between the endothelial cells and the smooth muscle. In addition, the consumption of NO by a mixture containing RBCs and free Hb would be different from that of RBC alone.

The purpose of this paper is to provide an analytic description for the consumption of NO by the blood in the presence and absence of plasma-based Hb and for different levels of Hct. Such a description is needed for the development of detailed NO transport models in the presence of Hb-based blood substitutes. In the process, we examine previous hypotheses for the importance of the membrane and extracellular transport resistances for the uptake of NO by the RBCs. The theoretical predictions will be compared with previous analyses and available experimental data.

METHODS

Model development. A spherically symmetric model was utilized to characterize the uptake of NO by an RBC as illustrated in Fig. 1. An RBC is assumed spherical and is surrounded by a plasma layer. NO diffuses through the plasma layer and the membrane of the RBC, reaching the intracellular region, where it reacts with the Hb through an irreversible, fast, first-order reaction. Hb is present in abundance inside the cell, and its concentration does not decrease significantly from the reaction with NO. Thus we assume that the RBC represents an infinite sink for NO. To characterize the NO uptake, we need to take into consideration both external and internal resistances to mass transfer as well as resistance for transport through the membrane.
RBC CONSUMPTION OF NO

Continuity of partial pressure and flux at the interfaces provide the following boundary conditions

\[ C(R = R_p) = C_p \]
\[ C(R = R_\text{pl}) = C(R = R_\text{cy}) = C(R = R_\text{C}) \]
\[ D_{\text{pl}} \frac{\partial C}{\partial R} \bigg|_{R=R_\text{pl}}^\epsilon = D_{\text{cy}} \frac{\partial C}{\partial R} \bigg|_{R=R_\text{C}}^\epsilon = F_{\text{RBC}} \]
\[ D_{\text{pl}} \frac{\partial C}{\partial R} \bigg|_{R=R_\text{pl}}^\xi = D_{\text{cy}} \frac{\partial C}{\partial R} \bigg|_{R=R_\text{C}}^\xi = F_{\text{RBC}} \frac{R_p^2}{R_\text{C}} \]
\[ \left[ \frac{\partial C}{\partial R} \right]_{R=0} = 0 \]

where \( C_p \) is the concentration at the outer boundary of the plasma layer and is assumed constant, and \( F_{\text{RBC}} \) is the rate of NO uptake by the RBC per unit area. \( C_p \) will be eliminated from the final results and its value will not be required in the calculations. The partition coefficient \( \lambda \) was utilized to account for the increased solubility of NO in the membrane relative to the plasma and cytosol. For simplicity, we assumed same solubilities for NO in the plasma and cytosol. Because we assumed negligible consumption of NO in the erythrocytic membrane, the flux of NO at the inner and outer boundaries of the membrane will be inversely proportional to the ratio of the surface areas. The equations and boundary conditions are nondimensionalized by introducing the following dimensionless variables

\[ r = \frac{R}{R_p}, \quad \Psi(r) = \frac{C(r)}{C_p}, \quad p = \frac{R}{R_p}, \quad \lambda = \frac{R_\text{pl}}{R_p}, \quad \delta = \frac{R_\text{C}}{R_p} \]

**Plasma layer.** The solution of Eq. 2 gives the concentration profile of NO in the plasma

\[
\Psi(r) = \frac{1}{r \sinh [p(\epsilon - 1)]} [\Psi_\text{R} \sinh [p(\epsilon - r)] + \epsilon \Psi_\text{p} \sinh [p(r - 1)]] \quad 1 \preceq r \preceq \epsilon
\]

where \( \Psi_\text{p} = \Psi(\epsilon) = 1 \) and \( \Psi_\text{R} = \Psi(1) \). Differentiation of the solution at \( r = 1^+ \) gives the rate of NO uptake by the RBC per unit RBC area (\( F_{\text{RBC}} \))

\[
F_{\text{RBC}} = \frac{D_{\text{pl}} \bar{C}_p}{R_p} \left[ \frac{\partial \Psi}{\partial r} \right]_{r=1} = a_1 \Psi_R + a_2 \Psi_p \quad (12a)
\]

\[
a_1 = - \frac{D_{\text{pl}} \bar{C}_p}{R_p} [p \coth [p(\epsilon - 1)] + 1] \quad (12b)
\]

\[
a_2 = \frac{D_{\text{pl}} \bar{C}_p}{R_p} \frac{\epsilon p}{\sinh [p(\epsilon - 1)]} \quad (12c)
\]

A more convenient description for \( F_{\text{RBC}} \) can be obtained by expressing the flux as a function of the average concentration of NO in the plasma layer (\( \bar{C}_\text{pl} \))

\[
\bar{C}_\text{pl} = \frac{\int \Psi \left( 4 \pi r^2 \Psi(r) dr \right)}{\int 4 \pi r^2 dr} = b_1 \Psi_R + b_2 \Psi_p \quad (13a)
\]

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In the analysis below, we assume no convective mixing facilitation of NO transport in the plasma layer. The plasma layer (\( R_p \)) has a finite thickness as determined by the local Hct according to the equation

\[ R_p = R_\text{R} \text{Hct}^{-1/3} \quad (1) \]

where \( R_\text{R} \) is the effective radius of the RBC, defined as the radius of a sphere with the same volume or surface area as the RBC (a corresponding range of \( R_\text{R} \) values will be considered). NO can be consumed by a number of substrates in the plasma layer, including superoxide and thiols and by plasma-based Hb. An overall first-order rate expression is used to model the NO consumption inside the plasma. Negligible consumption of NO is assumed inside the membrane of RBC. A first-order rate expression is used to model the NO consumption inside the RBC as well, where we assume that it is dominated by the reaction with erythrocytic Hb. Second-order reactions of NO such as the reaction with oxygen are considered negligible. Differential steady-state mass balances in each region then yield

\[
D_{\text{pl}} \frac{1}{R_p^2} \frac{\partial}{\partial R} \left( R_p^2 \frac{\partial C}{\partial R} \right) = -k_{\text{pl}} C = 0 \quad \text{with} \quad R_R < R < R_p \quad (2)
\]

\[
D_{\text{cy}} \frac{1}{R_\text{C}^2} \frac{\partial}{\partial R} \left( R_\text{C}^2 \frac{\partial C}{\partial R} \right) = 0 \quad \text{with} \quad R_C < R < R_R \quad (3)
\]

\[
D_{\text{pl}} \frac{1}{R_\text{pl}^2} \frac{\partial}{\partial R} \left( R_\text{pl}^2 \frac{\partial C}{\partial R} \right) - k_{\text{pl}} C = 0 \quad \text{with} \quad 0 < R < R_\text{pl} \quad (4)
\]

where \( D_{\text{pl}}, D_{\text{cy}}, \) and \( D_{\text{cy}} \) are the diffusivity coefficients of NO in plasma, membrane, and cytoplasm, respectively, and \( k_{\text{pl}} \) and \( k_{\text{cy}} \) are the first-order reaction rate constants of NO consumption in the plasma and cytosolic regions.

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![Fig. 1. Schematic representation of the model. A spherical red blood cell (RBC) is surrounded by a plasma layer with thickness that depends on hematocrit (Hct). Concentration at the outer boundary of the RBC is assumed constant. Simultaneous solution of the diffusion reaction equations in all three layers yields a concentration profile. Gradient of the concentration at the surface of the RBC is utilized to estimate the consumption rate of nitric oxide (NO) by the RBC. Integration of the profile yields the average NO concentration over the plasma layer or over the entire simulation volume. \( R_\text{R} \), RBC radius; \( R_\text{C}, \) cytoplasm radius; \( R_p, \) plasma radius; \( C_p, \) concentration of outer boundary of plasma; \( R_\text{pl}, \) radius of plasma layer. See text and equations for other definitions of mathematical abbreviations.](image-url)
Replacing $\Psi_P$ with the dimensionless average concentration estimated over the plasma layer ($\bar{\Psi}_P$) in Eq. 12 utilizing Eq. 13, we get

$$ F_{RBC} = K_{pl}^P C_P(\bar{\Psi}_pl - \Psi_R) $$  \hspace{1cm} (14a)$$

$$ K_{pl}^P = \frac{a_2b_1 - a_1b_2}{b_2C_P} = \frac{D_{pl}}{R_m} \frac{\rho \varepsilon}{2} \sinh \left[ \frac{2\rho \varepsilon}{2} - \frac{\rho \varepsilon}{2} \sinh \left( 2\rho \varepsilon - 1 \right) - \sinh^2 \left( \rho \varepsilon - 1 \right) - \rho \sinh \left( \rho \varepsilon - 1 \right) \right] $$  \hspace{1cm} (14b)$$

$$ f = \frac{a_2}{a_2b_1 - a_1b_2} = \frac{\rho \varepsilon^2 (1 - \delta)}{3 \left( \rho \varepsilon - 1 \right) \sinh \left( \rho \varepsilon - 1 \right) \cosh \left( \rho \varepsilon - 1 \right)} $$  \hspace{1cm} (14c)$$

**RBC membrane.** The solution of Eq. 3 utilizing Eqs. 6 and 7 provides the concentration profile in the membrane. Differentiation of the solution at $r = 1^-$ provides the flux at the outer boundary

$$ F_{RBC} = \frac{D_m C_P}{R_R} \left[ \frac{\partial \Psi}{\partial r} \right]_{r=1^-} = P_m C_P \left( \Psi_R - \Psi_C \right) $$  \hspace{1cm} (15a)$$

$$ P_m = \frac{D_m}{R_m} \frac{\lambda \delta}{(1 - \delta)} $$  \hspace{1cm} (15b)$$

where $\Psi_C = \Psi(\delta)$ and $P_m$ represents the membrane permeability, which is commonly used to describe the transport of species through a membrane.

**Intracellular region.** The solution of the differential mass balance in the intracellular region, Eq. 4 using Eqs. 7 and 10 yields

$$ \Psi(r) = \frac{\delta \sinh \left( \xi \right)}{r \sinh \left( \xi \delta \right)} \Psi_C, \quad 0 \leq r \leq \delta $$  \hspace{1cm} (16)$$

Differentiating Eq. 16 at $r = \delta^-$ utilizing Eq. 9, we get an expression for $F_{RBC}$

$$ F_{RBC} = \frac{R_R^2 D_m C_P}{R_R} \left[ \frac{\partial \Psi}{\partial r} \right]_{r=\delta^-} = \frac{D_m C_P}{R_R} \left[ \delta \xi \coth \left( \xi \delta \right) - \delta \right] \Psi_C = K_{cy}^P C_P \Psi_C $$  \hspace{1cm} (17a)$$

where

$$ K_{cy}^P = \frac{D_m}{R_R} \left[ \delta \xi \coth \left( \xi \delta \right) - \delta \right] $$  \hspace{1cm} (17b)$$

**Equations 14, 15, and 17 describe the flux into the RBC as a function of concentration gradients in the plasma, membrane, and cytoplasm. The three equations can be combined by adding the three in-series resistances as follows**

$$ F_{RBC} = \frac{1}{1/K_{pl}^P + 1/P_m + 1/K_{cy}^P} f_C \bar{\Psi}_pl = K^P f \bar{C}_pl $$  \hspace{1cm} (18a)$$

where

$$ \frac{1}{K^P} = \frac{1}{K_{pl}^P} + \frac{1}{P_m} + \frac{1}{K_{cy}^P} $$  \hspace{1cm} (18b)$$

The total uptake of NO per unit RBC volume will be

$$ Q_{RBC} = \frac{3}{R_R} \int \bar{C}_pl d\rho $$  \hspace{1cm} (19)$$

and the local consumption of NO per unit blood volume

$$ Q_{blood} = \int [Hct k_{RBC} + (1 - Hct) k_{pl}] \bar{C}_pl d\rho $$  \hspace{1cm} (20)$$

The average concentration of NO in the RBC (membrane and intracellular) will be

$$ C_{RBC} = \frac{1}{\int 4\pi^2 C(r) dr} \int 4\pi^2 C(r) dr = g \bar{C}_{pl} $$  \hspace{1cm} (21a)$$

where

$$ g = 3/K^P \left[ \frac{1}{K_{cy}^P} \left( \delta^2 \xi - 1 \coth \left( \xi \delta \right) - \delta \xi^2 + \lambda s_1 \right) + \lambda s_2 \left( \frac{1}{P_m} + \frac{1}{K_{cy}^P} \right) \right] $$  \hspace{1cm} (21b)$$

$$ s_1 = \frac{1}{6} \delta (1 + 2\delta) (1 - \delta) $$  \hspace{1cm} (21c)$$

$$ s_2 = \frac{1}{6} (1 - \delta)^2 $$  \hspace{1cm} (21d)$$

Then the local (average) NO concentration $C_{NO}$ will be

$$ C_{NO} = Hct \bar{C}_{RBC} + (1 - Hct) \bar{C}_{pl} = \left[ 1 + (g - 1)Hct \right] \bar{C}_{pl} $$  \hspace{1cm} (22)$$

Replacing $\bar{C}_{pl}$ in Eq. 20 gives

$$ Q_{blood} = \frac{Hct k_{RBC} + (1 - Hct) k_{pl}}{1 + (g - 1)Hct} C_{NO} = k_{blood} C_{NO} $$  \hspace{1cm} (23)$$

where $k_{blood}$ is the observed first-order rate constant of NO consumption in the blood. Note that because of the linearity of Eqs. 2–4, the calculated reaction rates $k_{RBC}$ and $k_{blood}$ are independent of the concentration $C_P$. The half-life of NO in the whole blood ($t_{1/2}$) and plasma ($t_{1/2}$) will be

$$ t_{blood} = \frac{\ln \left( \frac{2}{1} \right)}{k_{blood}} = \frac{\ln \left( \frac{2}{1} \right)}{k_{blood}} C_{NO} $$  \hspace{1cm} (24)
t_{Hb}^{\text{Hb}} = \frac{\ln (2)}{Q_{\text{Hb}}^{\text{Hb}} (1 - \text{Hct}) C_{\text{p}}^{\text{Hb}}} = \frac{\ln (2)}{1 - \text{Hct} k_{\text{RBC}} + k_{\text{pl}}} (25)

Note that the two definitions of NO half-life are equivalent for very dilute RBC solutions (Hct → 0) or for negligible NO concentration in the membrane and cytosol of the RBC (g → 0).

Parameter values. Values used in calculations are presented in Table 1. $R_R$ can be estimated such as to conserve either the volume (90–98 μm$^3$) or the surface area (130–144 μm$^2$) of a human RBC (2, 11). Thus we examine a range of values for $R_R$ between 2.8 and 3.38 μm. The $D_{pl}$ was set to $3.3 \times 10^{-5}$ cm$^2$/s at 37°C based on the data from Malinski et al. (24). $D_{pl}$ at 25°C was assumed $2.6 \times 10^{-5}$ cm$^2$/s based on the diffusivity of NO in water at 25°C. The $D_{pl}$ should be decreased compared with the plasma due to the high concentration of Hb present. We set $D_{cy}$ to half the value of $D_{pl}$ (i.e., $1.6 \times 10^{-5}$ cm$^2$/s at 37°C) based on the ratio of the extracellular and intracellular diffusivities for O$_2$ from experimental measurements (14, 28) and assuming a similar dependence for NO. Malinski et al. (24) suggested values for the diffusivity of NO in the lipophilic environment of a membrane ($D_{m}$) of $0.3 \times 10^{-5}$ cm$^2$/s and a partition coefficient (λ) of 6.5 for the membrane-water system, based on measurements performed on a 1-octanol-water system at 37°C. Denicola et al. (9) measured the diffusion coefficient of NO in the RBC plasma membrane ($0.4 \times 10^{-5}$ cm$^2$/s) and in liposomes ($1.3 \times 10^{-5}$ cm$^2$/s) at 20°C by utilizing a fluorescence quenching technique. Thus, based on value of $0.4 \times 10^{-5}$ cm$^2$/s for $D_{m}$ and a membrane thickness of $~7$ nm, Eq. 15b suggests a $P_m$ of $~40$ cm/s. This value is in agreement with the value of 93 cm/s reported by Subczynski et al. (30). The value for $P_m$ utilized by Vaughn et al. (34) to explain the competition experiment is 2,000 times smaller (0.041 cm/s).

Previous modeling studies have used a reaction rate constant for the reaction of NO with oxyHb ($k_{oxy}$) of 25 and 34 μM$^{-1}$.s$^{-1}$ (per heme) (22, 34, 35). Cassoly and Gibson (7) determined the reaction rate by stopped-flow spectrophotometry of 25 μM$^{-1}$.s$^{-1}$ at 20°C and pH 7.0. Eich et al. (10) reported reaction rate constants in the range of 30–50 μM$^{-1}$.s$^{-1}$ and similar reaction rates between oxy- and deoxyHb. In a recent study, Herold et al. (16) suggested a reaction rate of 89 μM$^{-1}$.s$^{-1}$ at 20°C and pH 7.0; the reaction rate increases at higher pH. The temperature dependence of the reaction is not known. Carlson and Comroe (6) and Cassoly and Gibson (7) suggested a temperature coefficient of 1.25 and 1.4, respectively, per 10°C for the reaction of CO with deoxyHb. If we assume a temperature coefficient of 1.4 per 10°C for $k_{oxy}$ and extrapolate the value proposed by Herold et al. (16), we obtain $k_{oxy}$ at 25°C and 37°C as high as 106 and 160 μM$^{-1}$.s$^{-1}$, respectively. Throughout the paper, extrapolation of the value of $k_{oxy}$ at 25°C and 37°C is needed to simulate in vitro experimental data or physiological conditions, respectively. We utilize for the extrapolations a temperature coefficient of 1.4 and note the temperature of extrapolation with a superscript on $k_{oxy}$. The reaction rate constants of plasma ($k_{pl}$) and cytoplasm ($k_{cy}$), can be estimated from the product of $k_{oxy}$ with the heme concentration in the plasma ($C_{Hb}^{pl}$) and cytoplasm ($C_{Hb}^{cy}$), respectively. In addition, we add a small value ($~1$ s$^{-1}$) to $k_{pl}$ to account for the consumption of NO by other substrates present in the plasma. Such a value is justified based on the reaction rate of NO with O$_2$ [4,300 μM$^{-1}$.s$^{-1}$ (13)] and a concentration of O$_2$ in the plasma in the subnanomolar range. The consumption of NO in the plasma is dominated by the reaction with free Hb and in the absence of plasma-based Hb; small consumption of NO occurs in the plasma layer mostly through reaction with O$_2$.

For the simulations below unless otherwise stated, we chose reference parameter values of 45% for Hct, 2.8 μm for $R_{Rb}$, 40 cm/s for $P_m$, $3.3 \times 10^{-5}$ cm$^2$/s for $D_{pl}$ at 37°C, and 160 μM$^{-1}$.s$^{-1}$ for $k_{oxy}$ at 37°C. We examine, however, the effect of variation in the parameter values within the previously described ranges.

RESULTS

Model analysis. The solution of model equations for the reference values of parameters is presented in Fig. 2. The dimensionless concentration ($\Psi$) is plotted as a function of dimensionless distance ($r$) from the center of the RBC. Control parameter values are utilized and simulations are performed for two different levels of Hct: 45% (Fig. 2A) and 15% (Fig. 2B). The average dimensionless concentration estimated over the plasma layer ($\Psi_{pl}$) or over the total plasma and RBC volume ($\Psi_{NO} = \frac{C_{NO}}{C_{pl}}$) is also plotted. There is a discontinuity in the NO concentration profile at the RBC membrane due to the increased solubility of NO in the lipophilic environment of the membrane. The thickness of the plasma layer changes with Hct leading to changes in the NO uptake by the RBC.

In Fig. 3, we present the model predictions for the observed $Q_{\text{Hb}}$ as a function of model parameters within a wide range of parameter variation. The effect

<table>
<thead>
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<th>Parameter</th>
<th>Value</th>
<th>Units</th>
<th>Description</th>
<th>Reference</th>
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<tr>
<td>$S_{RBC}$</td>
<td>130–144</td>
<td>μm$^2$</td>
<td>RBC surface area</td>
<td>2, 11</td>
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<tr>
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<td>μm$^3$</td>
<td>RBC volume</td>
<td>2, 11</td>
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<td>μm</td>
<td>RBC effective radius</td>
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<td>$R_{R}-R_{C}$</td>
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<td>μm</td>
<td>RBC membrane thickness</td>
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<td>$\lambda$</td>
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<td></td>
<td>Membrane/plasma partition coef.</td>
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<td>$D_{pl}$</td>
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<td>cm$^2$/s</td>
<td>NO diffusivity (cytopl.) @37°C</td>
<td>14, 24, 28</td>
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<tr>
<td>$D_{m}$</td>
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<td>cm$^2$/s</td>
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<td>cm$^2$/s</td>
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<td>cm/s</td>
<td>Membrane permeability</td>
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<td>μM$^{-1}$.s$^{-1}$</td>
<td>NO-oxyHb rate const. @20°C</td>
<td>7, 10, 16, 36</td>
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<tr>
<td>$C_{Hb}^{pl}$</td>
<td>20,300</td>
<td>μM</td>
<td>RBC heme concentration</td>
<td>2</td>
</tr>
<tr>
<td>$C_{Hb}^{pl}$</td>
<td>0–3,000</td>
<td>μM</td>
<td>Plasma heme concentration</td>
<td></td>
</tr>
<tr>
<td>$k_{pl}$</td>
<td>$1 + k_{oxy} C_{Hb}^{pl}$</td>
<td>s$^{-1}$</td>
<td>Plasma first-order reaction constant</td>
<td></td>
</tr>
<tr>
<td>$k_{cy}$</td>
<td>$k_{oxy} C_{Hb}^{cy}$</td>
<td>s$^{-1}$</td>
<td>Cytoplasm first-order reaction constant</td>
<td></td>
</tr>
</tbody>
</table>
of variation in a single parameter is explored while keeping the others at the control values. Figure 3A presents the dependence of $k_{\text{blood}}$ on the RBC effective radius. The two estimations of $R_{\text{R}}$ (based on the volume or surface area of human RBCs) are highlighted for reference. The consumption rate of NO decreases with increasing RBC radius. At the control value (solid circle) $k_{\text{blood}}$ is $6.5 \times 10^3 \, \text{s}^{-1}$. For a change in radius from 2.8 to 3.38 $\mu$m, there is a 30% decrease in the NO consumption. Figure 3B examines the dependence of $k_{\text{blood}}$ on $k_{\text{oxy}}$. The control value is shown as a solid circle. NO consumption is essentially constant for a wide range of $k_{\text{oxy}}$ values that include previously reported values for $k_{\text{oxy}}$ by Cassoly and Gibson (7) and Vaughn et al. (34) (solid triangle), Eich et al. (10) and Liu et al. (22) (solid square), and Herold et al. (16) (open circle). In Fig. 3C the $P_m$ changes over a wide range of values that include the value proposed by Vaughn et al. (34) (solid triangle) and the experimental estimate by Subczynski et al. (30) (solid square). For $P_m$ values higher than 1 cm/s the dependence of the consumption rate on $P_m$ is small. NO consumption decreases significantly when $P_m$ becomes $<1$ cm/s. At the value proposed by Vaughn et al. (34) $k_{\text{blood}}$ is reduced more than 20 times compared with the control (solid circle).

Comparison with experimental data. In Fig. 4 the model predictions are compared with previously reported measurements of NO consumption by RBCs. The erythrocytic NO consumption rate per RBC volume and per average plasma concentration ($k_{\text{RBC}}$) is plotted as a function of Hct. Experimental data from a dilute suspension of rat RBCs (Hct was more than 2,000 times less than normal) at 25°C are presented (solid circles) (22). The extrapolation to normal Hct proposed by Liu et al. (22) is also shown (dashed line). Note that the data and model by Liu et al. were presented in Ref. 22 on a per blood volume basis (Eq. 10 of Ref. 22) and have been converted in this figure on a per RBC volume by dividing with Hct. Thus their model predicts a constant $k_{\text{RBC}}$ independent of Hct. Data from Carlsen and Comroe (6) are represented by a solid triangle. Our theoretical predictions are also shown as a solid line. To simulate the experimental conditions, we utilized a value for $R_R$ of 2.44 $\mu$m to account for a
Fig. 4. Model predictions for the rate of NO consumption per unit RBC volume and per average plasma concentration (k_{RBC}) as a function of Hct. Model simulations (solid lines) are presented for the control values for $P_m$ and $k_{oxy}^B$, and for a “low” $P_m$ and $k_{oxy}^B$ (0.04 cm/s and 25 μM−1·s−1, respectively). The experimental data (solid circles) and the model predictions (dashed line) of Liu et al. (22) are also presented. Simulations for both models are performed with diffusivity of coefficient of NO in plasma ($D_{pl}$) of 2.6 × 10−5 cm²/s and $R_B$ of 2.44 μm. Solid triangle present data from Carlsen and Comroe (6).

smaller size of rat RBC (volume of 60 μm³), $k_{oxy}^B$ of 106 μM−1·s−1 (based on a $k_{oxy}$ at 20°C of 89 μM−1·s−1 and extrapolation to 25°C using a temperature coefficient of 1.4 per 10°C) and $D_{pl}$ of 2.6 × 10−5 cm²/s (Fig. 4). There is a close agreement between the models as the Hct approaches zero. At physiological Hct, however, our results differ from those of Liu et al. (22) by a factor of 6. Our prediction for the half-life of NO in blood at 30% Hct (5 × 10⁶ RBCs/ml) is 0.23 ms, which is significantly less than the estimate of Liu et al. The experimental data collected at very low Hct cannot be used to distinguish between the two models. Simulation using values for $P_m$ and $k_{oxy}$ of 0.04 cm/s and 25 μM−1·s−1, respectively, is also presented. These values lead to significant underestimation of the experimental data of Liu et al. (22) and Carlsen and Comroe (6). For this value of $P_m$ the effect of Hct on $k_{RBC}$ is minimal.

Analysis of the “competition experiment.” In Fig. 5, we present results from the “competition experiment” (35). The analysis of the corresponding problem is presented in the APPENDIX. The ratio of $k_{RBC}/k_{oxy}^B$ is presented as a function of Hct (Fig. 5A) or $C_{Hb}$ (Fig. 5B). Note that this is equivalent to the ratio of $k_{RBC}/k_{Hb}$ in the studies of Vaughn et al. (34, 35). Simulations are performed utilizing the single cell model of Ref. 34 (see APPENDIX) and for different scenarios of parameter values. First, and in agreement with Ref. 34, we utilized a “low” $P_m$ (0.04 cm/s) and a “low” $k_{oxy}^B$ (25 μM−1·s−1). We also perform simulations for a $k_{oxy}^B$ of 106 μM−1·s−1 and a $P_m$ 1,000 times higher (40 cm/s). All simulations were performed for $D_{pl}$ of 2.6 × 10−5 cm²/s, $R_B$ of 3.38 μm, first-order reaction rate constant ($k_d$) = ln (2)/(6 h−1), $C_{NO, donor}$ of 10 μM, and $C_{Hb}$ of 9 μM or Hct of 15.6%. The experimental results presented as solid circles are extracted from Figs. 3 and 4 of Ref. 35. The ratio $k_{RBC}/k_{oxy}^B$ is essentially constant and independent of either Hct or $C_{Hb}$ when the “low” $P_m$ is utilized. When the control value for $P_m$ = 40 cm/s is utilized, a positive slope is observed in Fig. 5, A and B. The model can simulate satisfactorily the experimental data without the need for a 1,000 times reduction of $P_m$.
when $k_{\text{oxy}}^{25°}$ is set to 106 $\mu M^{-1} s^{-1}$ instead of 25 $\mu M^{-1} s^{-1}$. Figure 5C presents estimations for $P_m$ utilizing the single cell model of Ref. 34 and Eq. A8 in the appendix of this study. Parameter estimation is performed for a wide range of values for $k_{\text{oxy}}^{25°}$ (25–175 $\mu M^{-1} s^{-1}$) and for values of the ratio $k_{RBC}/(k_{\text{oxy}}^{25°} C_{Hb})$ in the range 0.0006–0.0024. Simulations were performed for Hct = 15%, $D_{pl}$ of 2.6 $\times$ $10^{-5}$ cm$^2$/s, $R_R$ of 3.38 $\mu m$, $C_{Hb}$ of 9 $\mu M$, $k_d = \ln(2)/6$ h$^{-1}$, and $C_{NO\text{ donor}}$ of 10 $\mu M$. For high $k_{\text{oxy}}^{25°}$ values a wide range of $P_m$ values can produce ratios of $k_{RBC}/(k_{\text{oxy}}^{25°} C_{Hb})$ that are in close agreement with the experimental measurements (34, 35). At low $k_{\text{oxy}}^{25°}$ values only a small range of low $P_m$ values are in agreement with the experimental data. With typical values from competition experiments at 15.6% Hct, of $k_{RBC}/(k_{\text{oxy}}^{25°} C_{Hb})$ in the order of 0.0012 ± 0.0001 (35), and expected $k_{\text{oxy}}^{25°}$ within the range of 30–110 $\mu M^{-1} s^{-1}$. Fig. 5C suggests acceptable values for $P_m$ within the range of 0.1–40 cm/s. On the basis of a ratio of 0.0012, an empirical correlation was obtained that produces pairs of parameter values for $P_m$ and $k_{\text{oxy}}^{25°}$ that satisfy the competition experiment over the above ranges of variation for the two parameters

$$\ln (P_m) = \frac{0.0375 k_{\text{oxy}}^{25°} - 3.258}{1 - \left( \frac{k_{\text{oxy}}^{25°}}{126.4} \right)^{1.4}}$$

(26)

In Fig. 6 two independent experimental observations are simulated for different values of $P_m$, $t_{1/2}^d$ at 0.0126% Hct is simulated utilizing Eq. 25, $R_R$ of 2.44 $\mu m$, and $D_{pl}$ of 2.6 $\times$ $10^{-5}$ cm$^2$/s. In addition, simulations of the competition experiment (Eq. A8) are also performed in an effort to simulate the change in $k_{RBC}/(k_{\text{oxy}}^{25°} C_{Hb})$

after doubling the viscosity of the solution (Fig. 4 of Ref. 17). Simulations of the competition experiment are performed for Hct of 15%, $D_{pl}$ of 2.6 $\times$ $10^{-5}$ cm$^2$/s, $R_R$ of 3.38 $\mu m$, $C_{Hb}$ of 9 $\mu M$, $k_d = \ln(2)/6$ h$^{-1}$, and $C_{NO\text{ donor}}$ of 10 $\mu M$. In the simulations at any given $P_m$ a $k_{\text{oxy}}^{25°}$ value that satisfies Eq. 26 is chosen and thus the pairs of $P_m$ and $k_{\text{oxy}}$ utilized are in agreement with a ratio of $k_{RBC}/(k_{\text{oxy}}^{25°} C_{Hb})$ of 0.0012. The results are compared with the experimental measurement ($\pm$SD) of 4.25 ± 0.2 s for $t_{1/2}^d$ (22) and the 15 ± 6% observed change in $k_{RBC}$ after increasing the viscosity twofold (17). The ranges of $P_m$ values that can reproduce these experimental observations with accuracy no worse than twice the standard deviation of the measurement are highlighted. Because these ranges do not overlap, there are no values for $P_m$ that would quantitatively explain both experiments.

**NO consumption at physiological conditions.** In Fig. 7A, predictions for $k_{\text{blood}}$ at physiological temperature ($k_{\text{blood}}^{37°}$) is presented as a function of Hct. Different $P_m$ values were utilized. For any given $P_m$ value, the corresponding $k_{\text{oxy}}^{25°}$ at 25°C $k_{\text{oxy}}^{25°}$ was estimated utilizing

![Fig. 6](image6.png)

Fig. 6. Two independent experimental observations are simulated for different values of $P_m$, $t_{1/2}^d$ at 0.0126% Hct is simulated utilizing Eq. 25 and the following parameters: $R_R$ = 2.44 $\mu m$, and $D_{pl}$ of 2.6 $\times$ $10^{-5}$ cm$^2$/s. In addition, simulations of the "competition experiment" (Eq. A8) are also performed in an effort to simulate the change in $k_{\text{blood}}$($k_{\text{oxy}}^{25°}$ $C_{Hb}$) after doubling the viscosity of the solution for Hct = 15%, $D_{pl}$ = 2.6 $\times$ $10^{-5}$ cm$^2$/s, $R_R$ = 3.38 $\mu m$, $C_{Hb}$ = 9 $\mu M$, $k_d = \ln(2)/6$ h$^{-1}$, and $C_{NO\text{ donor}}$ = 10 $\mu M$. In the simulations at any given $P_m$ a value for $k_{\text{oxy}}^{25°}$ that satisfies Eq. 26 is chosen. Experimental measurements ($\pm$SD) for $t_{1/2}^d$ from Ref. 22 and for the percent change in $k_{RBC}$ after increasing the viscosity twofold from Ref. 17 are also shown.

![Fig. 7](image7.png)

Fig. 7. Model predictions for the observed first-order reaction rate constant of NO in blood (37°C, 45% Hct), $k_{\text{blood}}^{37°}$ (A), and the ratio of NO consumption by the blood and by an equivalent concentration of free Hb, as a function of Hct and $P_m$ (B). For the simulations reference parameter values were utilized from Eq. 26 and extrapolation from 25° to 37°C using a temperature coefficient of 1.4 per 10°C. Predictions for the range of $P_m$ values that satisfy the "competition experiment" (Fig. 5C) are highlighted.
Eq. 26. For the extrapolation of $k_{\text{oxy}}$ at 37°C ($k_{\text{oxy}}^{37\text{°C}}$), a temperature factor of 1.4 per 10°C was used. The rest of the parameters were held at the reference values. At 45% Hct, predictions for $k_{\text{blood}}$ vary between $7.5 \times 10^2$ and $6.5 \times 10^3$ s$^{-1}$ when $P_m$ changes between 0.1 and 40 cm/s. In Fig. 7B, $k_{\text{blood}}^{37\text{°C}}$ is compared with the rate of reaction of free Hb. The ratio Hct$^{-1}Hb$ $k_{\text{oxy}}^{RBC}$/$k_{\text{oxy}}^{pl}$ is plotted as a function of $P_m$ for different Hct. $k_{\text{blood}}^{37\text{°C}}$ values are shown in the secondary x-axis. For $P_m$ values between 0.1 and 40 cm/s, $k_{\text{blood}}$ is 500–250 times less than the reaction with an equivalent concentration of free Hb.

**NO consumption after HBOC administration.** In Fig. 8, we investigate the effect of Hct and plasma-based Hb concentration on the NO consumption. The reference parameter values for $R_{HBOC}$, $k_{\text{oxy}}$, and $P_m$ are utilized. The rate constant ($k_{\text{sol}}$), for the NO consumption if the erythrocytic Hb was uniformly distributed in the solution (i.e., as if the cell were lysed) is also presented (estimated as the product of $k_{\text{oxy}}$ with Hct). In the absence of plasma-based Hb, the consumption is a strong, nonlinear function of Hct. As Hct goes to zero, $k_{\text{blood}}$ also approaches zero, similar to $k_{\text{sol}}$. In the presence of plasma-based Hb, the consumption is practically constant independent of Hct; $k_{\text{blood}}$ becomes equal to the product of $k_{\text{oxy}}$ and $C_{\text{Hb}}^{pl}$. For plasma-based heme concentrations of 40 μM (7.7 × 10$^{-2}$ g/dl of Hb), the total NO consumption is not much different from that in the absence of plasma-based Hb and 45% Hct. However, at a physiologically important plasma-based heme concentration of 3,000 μM (~5 g/dl of Hb), consumption is increased significantly. The ratio $R_{HBOC}$ of the consumption rate in the presence of 5 g/dl of plasma-based Hb over the consumption in the absence of plasma-based Hb and normal Hct (i.e., 45% Hct) is presented on Fig. 8B as a function of $k_{\text{oxy}}$. Simulations are performed for $P_m$ values that change with $k_{\text{oxy}}$ according to Eq. 26 at 25°C and 37°C. For the latter case, in addition to Eq. 26, extrapolation of $k_{\text{oxy}}$ values from 25°C to 37°C was utilized at any given $P_m$ value, using a temperature coefficient of 1.4 per 10°C. The consumption of NO in the presence of 5 g/dl of plasma-based free-Hb is increased approximately two orders of magnitude relative to the physiological condition (45% Hct).

**DISCUSSION**

A simplified spherically symmetric mathematical model was utilized to predict the NO consumption by RBCs. The results suggest that under the reference parameter values, which include a value for the RBC membrane permeability based on experimental measurements in lipid bilayers, extracellular diffusion is the rate-limiting step. The plasma layer surrounding each RBC and thus the extracellular diffusion resistance depends on Hct. This leads to a nonlinear dependence of the consumption rate on Hct. For human RBCs at 45% Hct, we predict a NO consumption rate constant on a per blood volume basis of $6.5 \times 10^3$ s$^{-1}$ or $0.7 \mu M^{-1} s^{-1}$ on a per total heme concentration basis, and a plasma half-life of 0.1 ms. These predictions were based on the assumption of $P_m$ equal to 40 cm/s, which is in agreement with the expected NO permeability in the lipid bilayer of the RBC membrane.

The results presented in Fig. 3 suggest that under the control conditions described above, the consumption of NO in the blood as predicted by the model is sensitive to the effective radius of the RBC but not to the intracellular reaction rate or the membrane permeability. The results suggest that the resistance to NO transport is dominated by the extracellular diffusion. Thus changes in the size and probably the shape of the RBC may alter the NO consumption rate. The values of $k_{\text{oxy}}$ and $P_m$ have to decrease more than an order of magnitude for the resistances in the two layers (i.e., intracellular and membrane) to have a significant effect in the NO uptake by the RBC. At the value proposed by Vaughn et al. (34) for $P_m$, however, significant resistance is attributed to the membrane. Most importantly, this value leads to a significant reduction (almost 20 times) of the consumption rate.

Huxley and Kutchai (18, 19) tested the hypothesis that extracellular diffusion is the rate-limiting step in the initial uptake of $O_2$ by RBCs in a stopped-flow apparatus. Their experimental findings suggested that 82–100% of the resistance in $O_2$ uptake could be at-
tributed to extracellular diffusion limitations. Experimental studies for O2 uptake by RBCs by Coin and Olson (8) are also consistent with a significant diffusion boundary layer. This boundary layer is a result of O2 depletion in the area surrounding the RBC, faster than molecular diffusion or convective mixing can replenish it. NO has similar diffusivity with O2, and Hb consumes it faster than O2; thus a significant boundary layer should be present for NO as well. Huxley and Kutchai (18) utilized the Frossling correlation to predict a maximum value for the external diffusional resistance of oxygen uptake by an RBC. Assuming laminar flow, no extracellular reaction, and no interactions with neighboring RBCs (i.e., infinite thickness of the plasma layer), the correlation provides a description for the Sherwood number \( Sh = k_{em} 2R_R/D_{pl} \) in the form \( Sh = 2 + 0.6 Re^{1/2}Sc^{1/3} \), where \( k_{em} \) is the external mass transfer coefficient, the Reynolds number (Re) is defined as a function of the particles slip velocity, and the Schmidt number (Sc) is defined as the ratio of kinematic viscosity and diffusivity. Estimates show that the transport is not significantly facilitated by convective mixing, yielding \( Sh = 2 \) (18). Thus we get an estimation for the \( k_{em} \) equal to \( D_{pl}/R_R \). Applying this relationship for NO and assuming that the membrane and internal resistances are negligible compared with the external diffusion resistance, we can get an estimation for the consumption of NO by RBCs and an observed reaction rate in the blood

\[
Q_{RBC} = k_{em} S_{RBC}/V_{RBC}C_{NO} = 3D_{pl}/R_R^2C_{NO} \tag{27}
\]

\[
Q_{blood} = Q_{RBC} \cdot Hct \tag{28}
\]

where \( S_{RBC} \) and \( V_{RBC} \) represent the surface area and volume of RBC, respectively.

The model presented by Liu et al. (22) utilized these assumptions and equivalent equations, and they found close agreement with experimental results for the uptake of NO by rat RBCs at very low Hct (2,000 times smaller than normal). They concluded that the rate of uptake of NO by RBC is diffusion limited. The small difference (25%) between their model and experimental data might be attributed to the values chosen for \( D_{pl} \) or \( R_R \). They utilized a value of \( D_{pl} = 3,300 \mu m^2/s \) based on the experimental data of Malinski et al. (24) conducted at 37°C. This value is in close agreement with the expected diffusivity of NO in water at 37°C. Here we utilized a \( D_{pl} \) of 2,600 \( \mu m^2/s \) (the diffusivity of NO in water at 25°C, corresponding to the experiment (22)), the external resistance is increased and resulting in a closer fit of their experimental data (Fig. 4). Our analysis suggests that although these assumptions may be valid for such low Hct, extrapolation of these equations to higher Hct can lead to significant differences, i.e., sixfold at 30% Hct (Fig. 4). These differences originate from the simplifying assumption that the plasma layer around the RBC has an infinite thickness (which is true for higher Hct), which leads to an overestimation of the external diffusion resistance. Our model utilizing the control parameter values suggests, in agreement with Liu et al. (22), that the external diffusion resistance limits the NO transport at low Hct. However, we also suggest that this resistance decreases with increasing Hct, and becomes negligible as Hct approaches one.

Vaughn et al. (34) suggested that \( P_m \) is much lower than the value expected based on the diffusivity and solubility of NO in lipid membranes. Their competition experiment predicts a 2,000 times lower \( P_m \) than the experimental value of Subczynski et al. (30). Under such a condition there will be significant resistance in the membrane even at very low Hct and the transport will not be dominated by external diffusion limitations. Such a reduction of \( P_m \) leads to a significant reduction of \( k_{RBC} \) at very low Hct, resulting in disagreement with the experimental data of Liu et al. (22) (Fig. 4).

Analysis of the “competition experiment.” The disagreement illustrated in Fig. 4 between experimental measurements for the rate of NO uptake by RBCs and the value for \( P_m \) proposed by Vaughn et al. (34) motivated us to reexamine the analysis of the competition experiment. For the analysis we utilized the single cell model (34) to predict the ratio \( k_{RBC}/k_{oxygen-\text{Hb}} \) (see Eq. A8) in the appendix. This is equivalent to the ratio of \( k_{RBC}/k_{\text{metHb}} \) in the studies of Vaughn et al. (34, 35). We simulated experimental data for the ratio of \( k_{RBC}/(k_{oxygen-\text{Hb}} \cdot k_{metHb}) \) from Ref. 35 rather than the metHb production from Ref. 34, because these data were easily extracted from the figures, and according to Eq. A8, these data are independent of the NO production rate (and thus, independent of \( k_{a}, C_{NO \text{ donor}} \)). The ratio \( k_{RBC}/(k_{oxygen-\text{Hb}} \cdot k_{metHb}) \) was experimentally determined in Ref. 35 from the ratio of metHb formation in the presence and absence of RBC. In the process, the NO production rate is eliminated from the fitting equation, and the results are independent of the NO production rate. This is not the case when the metHb concentration is measured only in a system with RBCs. The formation of metHb and thus the parameter estimation results will depend on the NO release rate from the NO donor. Interestingly, utilizing a higher value for \( k_{oxygen} \), we were able to reproduce the experimental findings without the need for a 1,000 times decrease in \( P_m \) (Fig. 5, A and B). Most importantly, the value of 106 \( \mu m^{-1} \cdot s^{-1} \) utilized in the simulations represents a reasonable estimation for \( k_{oxygen}^{25°C} \) based on the recent results by Herold et al. (16) and extrapolation at 25°C. Figure 5C suggests that accurate determination of \( k_{oxygen}^{25°C} \) is needed for an accurate estimation of \( P_m \) from the competition experiment. In addition, for high values of \( k_{oxygen} \), the ratio becomes insensitive to \( P_m \), suggesting that the rate of NO uptake is not limited by transport through the RBC membrane. Thus, based on the uncertainty in the value of \( k_{oxygen} \), a wide range of \( P_m \) values (0.1–40 cm/s) can be in agreement with the experimental findings in the study by Vaughn et al. (35). Uncertainty regarding other model parameters such as \( D_{pl} \) and \( R_R \) also contributes, to a smaller degree, to the uncertainty in the estimation of \( P_m \).

Recently, Huang et al. (17) presented evidence for significant resistance to NO transport in the RBC membrane utilizing the competition experiment. In
these experiments, twofold increase in buffer viscosity increased \( k_{\text{RBC}} \) by only 15 ± 6%. Assuming that the change in viscosity did not change the bimolecular rate constant of NO with the free Hb (\( k_{\text{oxy}}^{22} \)), this result implies significant membrane resistance or a \( P_m \) less than 0.25 cm/s (Fig. 6). A low \( P_m \) value is also suggested by the significant increase in \( k_{\text{RBC}} \) as a result of chemical modifications that alter the band 3 binding to cytoskeleton or metHb binding to RBC membrane. In some cases this increase was as high as twofold. Figure 5 suggests that to increase the \( k_{\text{RBC}} \) and thus the ratio \( k_{\text{RBC}}/k_{\text{oxy}}^{22} \) twofold from ~0.0012 to ~0.0024, \( P_m \) has to be <0.2 cm/s. However, as Fig. 6 depicts a half-life of 4.2 s measured by Liu et al. (22) for a suspension of RBCs with Hct of 0.0126% yields a \( P_m \) greater than 0.8 cm/s. This limit for \( P_m \) depends on the parameters values utilized in the simulations and can decrease as \( D_{pl} \) increases. However, calculations indicate that to produce \( P_m \) values less than 0.25 cm/s one needs to utilize \( D_{pl} \) values beyond the expected range of NO diffusivity in aqueous solutions. Thus it appears that no single value for \( P_m \) can satisfy all the available experimental data.

Interestingly, if membrane- and cytoskeleton-associated proteins provide a barrier for NO, then it is likely that they should also provide a barrier for \( O_2 \). At this point it is not clear whether the experimental results by Huang et al. (17) collected under nonphysiological conditions (presence of NO donor, free Hb) are in disagreement with earlier experimental observations that suggested the rate of \( O_2 \) uptake by RBCs is significantly limited by extracellular diffusion boundary layer (8, 18, 19). The conditions in the competition experiment may alter the extracellular diffusion resistance. Thus the significance of membrane resistance to NO transport observed may be overestimated relative to the significance of the resistance under physiological conditions. Quantitative information about the membrane resistance is needed, and this information is not available due to mainly the uncertainty regarding the value of \( k_{\text{oxy}} \).

**NO consumption at physiological conditions.** Previous experimental data have suggested a reaction rate of NO with RBC 650–1,000 times smaller than the reaction with free Hb (22, 34, 35). These data, however, were collected under nonphysiological conditions (i.e., different temperature and presence of NO donor and free Hb) and/or at lower Hct, and thus they do not provide a direct measurement of \( k_{\text{blood}} \). The theoretical model presented above was utilized for the interpretation of these data and extrapolation to the desired conditions.

There is uncertainty in our prediction for the \( k_{\text{blood}} \) at physiological Hct (Fig. 7A). The uncertainty originates from uncertainty in \( P_m \). For \( P_m \) values between 0.1 and 40 cm/s, our predictions for \( k_{\text{blood}} \) at 45% Hct are in the ranges between 7.5 \( \times \) 10\(^{-22} \) and 6.5 \( \times \) 10\(^{-23} \) s\(^{-1} \) or 0.08–0.7 \( \mu \)M\(^{-1} \) s\(^{-1} \) on a per total heme basis. The predicted reaction rate of NO with RBC is 250–500 times smaller than the reaction with an equivalent concentration of free Hb at 37°C (Fig. 7B), significantly less that the previous estimates. The difference is mainly attributed to the nonlinear dependence of NO consumption with Hct and the values assumed for \( P_m \) and \( k_{\text{oxy}} \).

**NO consumption after HBOC administration.** The model predicts significant increase of the consumption rate of NO in the presence of plasma-based Hb relative to that of the blood. The consumption is essentially constant, independent of Hct. This can be attributed to significant NO scavenging in the plasma layer surrounding the RBC, when plasma-based Hb is present. At a physiological important plasma-based Hb concentration of 5 g/dl, we predict that the consumption of NO in the blood will be increased approximately two orders of magnitude or ~20-fold per gram per deciliter of Hb concentration in the plasma (Fig. 8B). The exact value of this increase will depend on parameters such as membrane permeability, temperature, and \( k_{\text{oxy}} \). The nonmonotonic dependence of the ratio \( R_{\text{HBOC}} \) on \( k_{\text{oxy}} \) observed in Fig. 8B is attributed to the values for \( P_m \) and \( k_{\text{oxy}} \) utilized in the simulations, which were chosen to agree with experimental measurements (Eq. 26). The value of \( P_m \) in the simulations increased as \( k_{\text{oxy}} \) increased (Eq. 26). The increase in \( k_{\text{oxy}} \), leads to an increase in NO consumption in the presence of plasma based-Hb, whereas the increase in \( P_m \) leads to an increase in the rate of NO uptake by the blood in the absence of plasma-based Hb. The result is a ratio of NO consumption in the presence and absence of plasma-based Hb that change in a nonmonotonic fashion.

The predicted relative increase in NO consumption refers to a solution of RBCs in the presence and absence of free Hb. When blood flows in an arteriolar vessel, an RBC-free layer forms near the walls due to hydrodynamic effects. Thus plasma-based Hb should be able to get closer to the vessel wall than the RBCs. This will tend to further increase the consumption of endothelium-derived NO in the presence of HBOCs relative to normal blood.

**Model limitations.** The model assumes spherical RBCs and neglects convective mixing facilitation of NO transport in the plasma layer surrounding the RBC. Both assumptions can affect the extracellular resistance and thus the NO uptake by the RBCs. We also assumed negligible consumption of NO inside the RBC membrane. Liu et al. (23) suggested 300 times more rapid reaction of NO with \( O_2 \) inside the hydrophobic environment of membranes. The small volume of the RBC membrane and the third-order reaction rate of NO with \( O_2 \), however, suggest that this assumption has only a small impact on the results of the transport calculations. In the development of the model’s equations, we also assumed steady state, based on the high diffusivities and reaction rates and the small volume. We assumed that Hb consumes the NO through an irreversible reaction. There is increasing evidence, however, that Hb can preserve the NO bioactivity through the nitrosylation of a cystein residue in the \( \beta \)-subunit of the Hb molecule and later release the NO molecule probably in the form of a S-nitrosothiol (15, 19).
25, 27). At this point it is not known to what extent this mechanism can result in the release of free NO.

In conclusion, extracellular diffusion and/or resistance to the transport through the RBC membrane limit the NO uptake by RBCs. At this point there is uncertainty regarding the contribution of each of these mechanisms under physiological conditions. For the estimation of NO uptake rate by RBCs one needs to extrapolate data collected at very low Hct or interpret data collected under nonphysiological conditions. In either case, the value for $P_m$ is required. Our analysis, however, suggests that there is not a single value for $P_m$ that can describe all the available experimental data. The uncertainty in the value of $P_m$ causes uncertainty in our prediction for $k_{\text{blood}}$ under physiological conditions within a range $7.5 \times 10^{-2} - 6.5 \times 10^3 \text{s}^{-1}$. Our prediction for blood at normal Hct is $250 - 500$ times slower than for the reaction with an equivalent concentration of free Hb. Further experimentation is needed to delineate the magnitude of extracellular and RBC membrane resistance to NO transport and determine accurately the rate of NO consumption in the blood at physiological Hct.

**APPENDIX**

**Competition experiment.** For the analysis of the competition experiment, we use the “single cell” model presented by Vaughn et al. (34). It differs from the one presented earlier by the addition of a production rate in the plasma equation and in the condition at the outer boundary of the plasma layer. Thus Eqs. 2 and 5 are replaced by

$$D_p \frac{\partial}{\partial R} \left( R^2 \frac{\partial C}{\partial R} \right) - k_{pl} C + S = 0 \quad \text{with} \quad R_K < R < R_p \quad (A1)$$

$$\left[ \frac{\partial C}{\partial R} \right]_{R=R_p} = 0 \quad (A2)$$

Here $S$ is the production rate of NO per unit plasma volume, which is assumed constant for a pseudo steady-state approximation and equal to $2k_m C_{NO \text{ donor}}(1 - \text{Hct})$, where $k_m$ is a first-order reaction rate constant and $C_{NO \text{ donor}}$ is the concentration of NO donor computed based on the total volume of solution. Vaughn et al. (34) have not presented the solution explicitly; for the sake of convenience, we present the solution here using notations introduced above in the body of the paper.

Making the substitution $\Phi(r) = C(r) - S/k_{pl}$, the solution of Eq. A1 using the Eqs. A2 and Eq. 6 will be

$$\Phi(r) = \left[ E_1 \sinh (\rho r) + E_2 \cosh (\rho r) \right] \frac{\Phi(1)}{r}, \quad r = \frac{R}{R_K}, \quad \rho = \frac{k_{pl}}{D_p}, \quad \epsilon = \frac{R_p}{R_K} \quad (A3a)$$

with

$$E_1 = \frac{p e \sinh (p e) - \cosh (p e)}{\sinh [p (e - 1)] - \cosh [p (e - 1)]} \quad (A3b)$$

$$E_2 = -\frac{p e \cosh (p e) - \sinh (p e)}{\sinh [p (e - 1)] - \cosh [p (e - 1)]} \quad (A3c)$$

Differentiating Eq. A3 at $r = 1^+$, we get the flux at the RBC boundary under the conditions of the competition experiment

$$F_{\text{RBC}} = D_p \left[ \frac{\partial \Phi}{\partial r} \right]_{r=1^+} = \frac{D_p}{R_K} \left[ E_1 [p \cosh (p) - \sinh (p)] + E_2 [p \sinh (p) - \cosh (p)] \right] \Phi(1) = K_{pl}^p [S_{pl}^p - C(R_p)] \quad (A4)$$

Similarly, combining Eq. A4 with Eqs. 15 and 17 yields

$$F_{\text{RBC}} = \frac{1}{1/K_{pl}^p + 1/P_m + 1/k_{pl}^p} S = K_{pl}^p S \quad (A5)$$

The average NO concentration in the plasma will be

$$C_{pl} = \left[ 1 - \frac{3 R_{pl}^3}{(R_p^3 - R_K^3)} \right] \frac{K_{pl}^p S}{k_{pl}^p} \quad (A6)$$

Substitution of Eq. A6 in Eq. A5 yields

$$F_{\text{RBC}} = \frac{K_{pl}^p}{1 - \frac{3 R_{pl}^3}{(R_p^3 - R_K^3)} C_{pl}} \quad (A7)$$

Then the ratio of the reaction rate constants for NO consumption by the RBC and by the extracellular Hb will be

$$\frac{k_{\text{RBC}}}{C_H^b k_{\text{eay}}} = \frac{3 K_{pl}^p C_{pl}^p (1 - \text{Hct})}{C_H^b [k_{pl}^p C_{pl}^p (1 - \text{Hct}) - 3 K_{pl}^p \text{Hct}]} \quad (A8)$$

Taking the limit of Eq. A8 for very small $P_m$, we obtain

$$\frac{k_{\text{RBC}}}{C_H^b k_{\text{eay}}} = \frac{3 P_m}{R_K C_H^b k_{\text{eay}}} \quad (A9)$$

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