Nitric oxide uptake by erythrocytes is primarily limited by extracellular diffusion not membrane resistance.

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The process of NO transfer into erythrocytes (RBCs) is of critical biological importance because it regulates the bioavailability and diffusional distance of endothelial-derived NO. It has been reported that the rate of NO reaction with oxyhemoglobin (Hb) within RBCs is nearly three orders of magnitude slower than that by equal amounts of free oxyhemoglobin. Consistent with early studies on oxygen uptake by RBCs, the process of extracellular diffusion was reported to explain this much lower NO uptake by RBC encapsulated Hb (Liu, X., Miller, M. J., Joshi, M. S., Sadowska-Krowicka, H., Clark, D. A., and Lancaster, J. R., Jr. (1998) J. Biol. Chem. 273, 18709–18713). However, it was subsequently proposed that the RBC membrane provides the main resistance to NO uptake rather than the process of extracellular diffusion (Vaughn, M. W., Huang, K. T., Kuo, L., and Liao, J. C. (2000) J. Biol. Chem. 275, 2342–2348). This conclusion was based on competition experiments that were assumed to be able to determine the rate constant of NO uptake by RBCs without extracellular diffusion limitation. To test the validity of this hypothesis, we theoretically analyzed competition experiments. Here, we show that competition experiments do not eliminate the extracellular diffusion limitation. Simulation of the competition data indicates that the main resistance to NO uptake by RBCs is caused by extracellular diffusion in the unstirred layer surrounding each RBC but not by the RBC membrane. This extracellular diffusion resistance is responsible for preventing interference of NO signaling in the endothelium without the need for special NO uptake by intracellular hemoglobin or a unique membrane resistance mechanism.

In the vascular lumen NO can be scavenged by hemoglobin subunits (Hb) within RBCs. The reaction of NO with myoglobin and hemoglobin is rapid (bimolecular rate constant for oxyMb is $k_{	ext{Mb}} = 3 \times 10^7 \text{ M}^{-1} \text{s}^{-1}$ and for oxyHb is $k_{	ext{Hb}} = 6–9 \times 10^7 \text{ M}^{-1} \text{s}^{-1}$) (4, 5). It has been reported that 10 μM cell-free Hb is enough to trap almost all NO production generated from endothelial cells and abolish NO-mediated vasodilation (6, 7), while blood contains about 8 mM Hb, which is much higher than the quantity of Hb required to completely eliminate NO-mediated functional effects. The rapid reaction between NO and oxyhemoglobin raises the question of how NO can escape from the large quantity of Hb in the blood vessel lumen to exert physiological effects in the blood vessel wall after it is generated from endothelial cells (2, 8). This has led to recent efforts to understand and theoretically model the reaction process between NO and Hb (9–12).

It was observed that the rate of NO reaction with RBC-enclosed Hb is nearly three orders of magnitude slower than the rate of NO reaction with free Hb (9). Based on a diffusion-limited reaction model where the limiting step for NO reaction with RBC-enclosed Hb is diffusion of NO from the solution to the surface of RBCs, it was found that the calculated rate constant for the reaction between NO and RBC Hb is very close to that experimentally determined with direct electrochemical measurements. Recently, these experimental results were verified in competition experiments comparing the scavenging of NO by free Hb to that of RBC-enclosed Hb (13); however, it was concluded that the RBC membrane is the main resistance to NO transfer into RBCs. This was based on the following competition Equation 1, which was derived under the assumption that much of extracellular diffusion resistance is eliminated in competition experiments because NO is uniformly generated in extracellular solution, and a high hematocrit (>5%) was used in the experiments (13, 14).

\[
\frac{[\text{metHb}]}{[\text{Hb}]} = \frac{(1 - \text{Hct})[\text{metHb}_0]}{[\text{Hb}]_{\text{RBC}}} = \frac{(k_{\text{RBC}}/k_{\text{Hb}}) \ln([\text{Hb}]_{\text{cell}}/[\text{Hb}]_{\text{RBC}})}{(k_{\text{RBC}}/k_{\text{Hb}}) \ln([\text{Hb}]_{\text{cell}}/[\text{Hb}]_{\text{RBC}}) - 1 + \text{Hct})[\text{metHb}_0]/[\text{Hb}]_{\text{RBC}}} \quad (\text{Eq. 1})
\]

[metHb]$_{\text{cell}}$ is the metHb concentration in the cell-free control, [metHb]$_{\text{RBC}}$ is the extracellular metHb concentration, [Hb]$_{\text{RBC}}$ is defined as the RBC-enclosed Hb concentration in solution if all the RBCs were lysed, [Hb]$_{\text{cell}}$ is extracellular Hb concentration, and [Hb]$_{\text{RBC}}$ is the initial [Hb]$_{\text{cell}}$. $k_{\text{RBC}}$ and $k_{\text{Hb}}$ are rate constants of the NO reaction with RBCs and free Hb, respectively. [metHb]$_{\text{RBC}}$ is measured experimentally and then the ratio of $k_{\text{RBC}}/k_{\text{Hb}}$ is determined from Equation 1 using these experimental data. It was found that the ratio of $k_{\text{RBC}}/k_{\text{Hb}}$ has a large increase as hematocrit changes from 1.6 to 5%, and then the ratio

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1 The abbreviations used are: Hb, hemoglobin; RBC, red blood cell; Hct, hematocrit; Mb, myoglobin.

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transfers into the RBC. NO that enters into the RBC is immediately either reacts with Hb in the extracellular solution to form metHb or NO donor to the extracellular Hb solution, the donor uniformly distributed is surrounded with Hb solution that fills the space between the surface container is defined by the hematocrit as the ratio of the RBC volume to

A spherical container of radius \( r_1 \) is fixed at the center of the container. The radius \( r_1 \) is determined by the Hct from the equation Hct = \( (r_1/r_0)^3 \).

slightly increases as hematocrit changes from 5 to 16%. The large increase of \( k_{RBC} \) as hematocrit changes from 1.6 to 5% was attributed to the diminution of extracellular diffusion resistance, and therefore the value of \( k_{RBC} \), measured in the high hematocrit region, was considered to be caused only by RBC membrane resistance.

The controversy about the resistance of RBCs to NO transfer is analogous to that relating to explanations for RBC oxygen uptake. More than 70 years ago, it was discovered that RBC oxygen uptake was considerably slower than that of an equivalent concentration of free Hb (15). Early investigators suggested that oxygen uptake by RBCs is limited mainly by the transport of oxygen across the RBC membrane and intracellular diffusion of oxygen based on the assumption that extracellular oxygen gradients are not present in rapid mixing devices (16, 17). However, it is now accepted that effective diffusion layers around RBCs still exist in rapid mixing devices (18, 19) and are mainly responsible for the resistance of RBC oxygen transfer (18–23).

Since the assumption of competition experiments was not based on strict theoretical analysis, one may ask the following two questions. 1) Is NO concentration really uniformly distributed in solution in the competition experiments? 2) Does the value of \( k_{RBC} \) at high hematocrit (from 5 to 16%) really represent the rate constant of transmembrane diffusion? To answer the two questions, we have theoretically analyzed competition experiments in this study. NO concentration, NO flux at RBC membrane, the formation rate of extracellular metHb, and the ratio of \( k_{RBC}r_{Hb} \).

Mathematical Analysis of Diffusion in Competition Experiments—

Based on the above assumptions, the following diffusion-reaction equations and boundary conditions (Equations 2–4) are used to describe the competition experiment for any hematocrit (from 0 to 100%).

\[
\frac{1}{r^2} \frac{d}{dr} \left( r^2 \frac{d[NO]}{dr} \right) + k_1 [A] - k_{Hb}[Hb]_m [NO] = 0 \quad \text{(Eq. 2)}
\]

\[
D_{NO} \frac{d}{dr} [NO] |_{r=r_0} = P_m [NO]^m \quad \text{at } r=r_0 \quad \text{(Eq. 3)}
\]

\[
\frac{d}{dr} [NO] |_{r=r_1} = 0 \quad \text{at } r=r_1 \quad \text{(Eq. 4)}
\]

where \( D_{NO} \) is the diffusion constant of NO in the extracellular solution; \( P_m \) is membrane permeability; \( k_1, k_{Hb} \) are the rate constants of decomposition of the NO donor and the NO reaction with Hb, respectively; \([A], [NO], [NO]^m \) are NO donor concentration, NO concentration in solution, and NO concentration on the outer surface of the RBC, respectively; \([Hb]_m \) is extracellular Hb concentration; and \( r_0 \) and \( r_1 \) are radii of the RBC and the spherical container as shown in Fig. 1, respectively. In solution, the change of NO concentration at any location between \( r = r_0 \) and \( r = r_1 \) can be caused by NO diffusion, NO generation, and NO consumption at that location, which is expressed by the first, second, and third terms in Equation 2. At the boundary \( r = r_0 \), there is no net NO flow into or out from the container surface, so the gradient of NO concentration at \( r = r_1 \) should be equal to zero as expressed in Equation 4.

Computer Simulations—

All simulated curves in this study were calculated from the analytical expressions derived in the next section on a pentium III personal computer with QBasic programs.

RESULTS

The Effect of Hematocrit on NO Concentration Distribution—To test if increasing hematocrit can change the NO concentration distribution from inhomogeneous to homogeneous, we directly solved the diffusion-reaction Equation 2 and their boundary conditions, Equations 3 and 4.

Assuming that \([A] \) and \([Hb]_m \) are uniform in the solution during the competition experiment, the general solution for Equation 2 can be expressed as Equation 5,

\[
[NO] = \frac{k_1 [A]}{k_{Hb}[Hb]_m} + \frac{1}{r^2 f} \left[ a e^{-fr} + b e^{fr} \right] \quad \text{(Eq. 5)}
\]

where

\[
f = \sqrt{k_{Hb}[Hb]_m/D_{NO}} \quad \text{(Eq. 6)}
\]

and constants a and b can be determined by boundary conditions in Equations 3 and 4. Equation 5 is true for \([Hb]_m < 0 \) or for \( k_1 [A]/k_{Hb}[Hb]_m = \text{constant as } [Hb]_m \text{ approaches zero} \). In competition experiments, we have \([Hb]_m > 0 \).

The combination of Equation 5 with Equations 3 and 4 give us Equation 7,

\[
[NO] = [NO]^0 \left[ 1 - \frac{P_m (r_1 - 1) \Phi(r) + (r_1 + 1) \Phi(r)}{D_{NO} Y(r, r_1, r_0)} \right] \quad \text{(Eq. 7)}
\]

where \([NO]^0 \) is the NO concentration in solution in the absence of RBCs,

\[
[NO]^0 = k_1 [A]/k_{Hb}[Hb]_m \quad \text{(Eq. 8)}
\]

\[
Y(r, r_1, r_0) = (r_1 - 1)/(r_1 + 1) + P_m r_0 (R(r) - 1)/(r_1 + 1) \quad \text{(Eq. 9)}
\]

\[
Y(r, r_1, r_0) = (r_1 + 1)/(r_1 - 1) - P_m r_0 (R(r) - 1)/(r_1 - 1) \quad \text{(Eq. 10)}
\]
and

\[ \Phi(r) = \exp[fr_1 - r] \]  \hspace{1cm} (Eq. 10a)

\[ \Phi(r_0) = \exp[fr_1 - r_0] \]  \hspace{1cm} (Eq. 10b)

according to the definition of hematocrit, Hct = \( r_0^2 / r_1^2 \), we can replace \( r_1 \) with Hct in Equation 7. NO concentration distribution profiles around the RBC for different \( P_m \) and Hct values are shown in Fig. 2, A and B. In the simulations, we used \([Hb]_{ex} = 10 \mu M, k_{inh} = 89 \mu M^{-1} s^{-1} (20^\circ C) (5), D_{NO} = 2600 \mu m^2/s (25^\circ C) (24). Since the molar volume of NO is close to the molar volume of oxygen, their diffusion constant would be similar (25). The diffusion constant of oxygen in solution at 25^\circ C is 2420 \mu m^2/s (26); therefore, it is reasonable to take the value of \( D_{NO} = 2600 \mu m^2/s \) as the NO diffusion constant at 25^\circ C. In competition experiments, it was assumed that when hematocrit was smaller than 5%, the extracellular diffusion resistance becomes significant as hematocrit decreases, but \( k_{RBC} \) calculated from the competition equation did not include any extracellular diffusion resistance as the hematocrit was greater than 10% (13). The profiles of NO concentration that were drawn according to the assumption for competition experiments are shown in Fig. 2C. In this assumption, the NO concentration distribution in the diffusion layer must have a large change to eliminate the inhomogeneous NO concentration distribution as hematocrit increases from 0.2 to 5%.

The Effect of Hematocrit on the Flux of NO Transfer into RBCs—In order to further test how hematocrit affects the extracellular diffusion resistance, we derived the analytical expression for the flux of NO transfer into RBCs and analyzed how the flux varies with hematocrit. If the flux largely increases with hematocrit or the extracellular diffusion resistance largely decreases with hematocrit, then membrane resistance may become the limiting step to NO transfer into RBCs.

The flux of NO diffusion into the RBC is given by Equation 11,

\[ F_n = D_{NO} \frac{\partial [NO]/\partial r}{r} = \frac{P_m [NO]_0}{[NO]} = \frac{P_m k_H[A]}{[Hb]_{Hb}} \]  \hspace{1cm} (Eq. 11)

where

\[ \Psi = \frac{(f_{r_1} - 1)/f_{r_0} + 1/\Phi(r_0) - (f_{r_1} + 1)/f_{r_0} - 1/\Phi(r_0)}{2f_{r_1} f_{r_0}} \]  \hspace{1cm} (Eq. 12)

In competition experiments, NO donor or NO is used as a probe to measure the ratio of \( k_{RBC}/k_{inh} \). It is assumed that the physical properties of the RBC membrane and solution diffusion resistances are independent of [NO] or NO donor concentration [A]. In Equation 11, \( F_n \) is proportional to [\( A \)], so we use \( F_n = 3f_{r_0}/k_H[A]_{r_0} \) to obtain a [NO]-independent flux. \( F_n \) is proportional to \( F_n \) but independent of NO donor concentration [A]. The first derivative of \( F_n \) with respect to \( r_1 \) can be derived from Equation 11 and is shown in Equation 13.

\[ dF_n/dr_1 = 12f_{r_1} P_m [NO]/A \]  \hspace{1cm} (Eq. 13)

Since \( dF_n/dr_1 \) is always larger than zero, \( F_n \) will increase as \( r_1 \) increases and decrease as \( r_1 \) decreases. \( F_n \) will decrease as Hct increases because \( r_1 \) is inversely proportional to Hct\(^{1/3} \) (Fig. 3). No matter whether the membrane permeability \( P_m \) is as high as 0.9 m/s or as low as 0.0018 m/s, \( F_n \) always decreases with the increase of Hct. Since \( F_n \) does not change as hematocrit changes from 0 to 5%, extracellular diffusion resistance cannot be appreciably reduced by increasing the hematocrit.

Expressions for the Formation Rate of Extracellular metHb Concentration and the Ratio of \( k_{RBC}/k_{inh} \)—It is easy to prove that Equation 1 can be derived from the following two rate equations shown in Equations 14a and 14b,

\[ \frac{d[metHb]_{ex}}{dt} = k_{inh}[Hb]_{ex} \]  \hspace{1cm} (Eq. 14a)

\[ \frac{d[metHb]_{inh}}{dt} = k_{inh}[Hb]_{inh} [NO] \]  \hspace{1cm} (Eq. 14b)

where \([metHb]_{RBC}\) is defined as the RBC-enclosed metHb in solution if all the RBCs were lysed and [NO] is the extracellular NO concentration. \([Hb]_{RBC}\) can be calculated from hematocrit by Equation 15.

![Graph of the relative NO concentration, [NO]/[NO]b versus the distance from the RBC membrane (r - r0). Parameters used in the calculation are kinh = 89 \mu M/s, DNO = 2600 \mu m^2/s, r0 = 3.39 \mu m, [Hb]ex = 10 \mu M, intracellular Hb concentration cRBC = 2200 \mu M, Pm = 0.9 m/s (A), and Pm = 1.8 \times 10^{-3} m/s (B). Arrows designate the location of inner surface of the container at each relative hematocrit. In C, curves of NO concentration are drawn according to the assumption for competition experiments for three different hematocrits: Hct = 0.2% (solid line), Hct = 5% (dote), and 10% (dash).](http://www.jbc.org/content/early/2018/03/09/jbc.A201113JBCA1supplemental/fig2A.png)
Fig. 3. Calculated initial fluxes of NO transfer into RBCs. The initial fluxes of NO on the surface of the RBC versus hematocrit as $P_m = 0.9\text{ m/s (a)}$ and $P_m = 1.8 \times 10^{-4}\text{ m/s (b)}$. Other parameters used in the calculations are $k_{RBC} = 89\text{ m}\mu\text{m}^{-2}\text{s}^{-1}$, $P_{NO} = 2600\text{ m}\mu\text{m}^{2}\text{s}$, $r_0 = 3.39\mu\text{m}$, $c_{RBC} = 22000\mu\text{m}$, and $[\text{Hb}]_{ex} = 10\mu\text{m}$.



\begin{equation}
[\text{Hb}]_{cRBC} = c_{RBC}\text{Hct} = c_{RBC}\frac{r^3}{r_1^3}
\end{equation}

(C.15)

Considering that $[\text{metHb}]_{RBC}$ is defined in the whole space within the spherical container, we have Equation 16,

\begin{equation}
d[\text{metHb}]_{cRBC}/dt = SF_pV_p = 3r_0^2P_m[\text{Hb}]_{cRBC}/r_1
\end{equation}

(E.16)

where the surface area of the RBC $S$ is $4\pi r_0^2$ and the volume of the spherical container $V_p$ is $4\pi r_1^3/3$. Considering that $[\text{metHb}]_{cRBC}$ is defined in the extracellular solution, we can obtain the formation rate of $[\text{metHb}]$ in extracellular solution in Equation 17.

\begin{equation}
d[\text{metHb}]_{ex}/dt = k_{[A]}[1 - 3r_0^2P_m/[\text{Hb}]_{ex}^0/([r_1^3 - r_0^3])]
\end{equation}

(E.17)

The time course of $[\text{metHb}]_{ex}$ can be obtained from Equation 17 using numerical integration methods. We compared $[\text{metHb}]_{ex}$ predicted from Equation 17 with the published experimental data in Fig. 4. The points in Fig. 4, A–C were read from Fig. 2(a), Fig. 4(a), and Fig. 5(a) in Ref. 13, respectively. Solid curves were calculated from Equation 17. Parameters used in the calculations are chosen as $D_{NO} = 2600\mu\text{m}^{2}\text{s}^{-1}$ and $P_m = 0.9\mu\text{m/s}$. Since $k_{RBC} = 89\mu\text{m}^{-2}\text{s}^{-1}$ was measured at 20 °C (5), we have extrapolated this value with a temperature coefficient of $1.4\%$ per °C that was used in estimating rate constants for the reaction of NO or CO with deoxyHb (27). Thus we obtained $k_{RBC} = 105\mu\text{m}^{-1}\text{s}^{-1}$ at 25 °C. The extrapolated value of $k_{RBC}$ was then used in calculating the curves in Fig. 4. Other experimental parameters, such as initial extracellular Hb concentration, initial NO donor concentration, and hematocrit, were the same as those used in the competition experiments of Vaughn et al. (13).

The expression for the ratio $k_{RBC}/k_{Hb}$ can be obtained by a combination of Equations 14a, 14b, and 15 with Equations 16 and 17 as shown in Equation 18.

\begin{equation}
k_{RBC}/k_{Hb} = \frac{[\text{Hb}]_{ex}/dt}{[\text{Hb}]_{RBC}/dt} = \frac{3r_0^2(1 - \text{Hct})/[\text{Hb}]_{ex}^0/c_{RBC}V_p}{1 - \text{Hct}[1 + 3r_0^2P_m/[\text{Hb}]_{ex}^0]}(\text{Eq. 18})
\end{equation}

In Equation 18, $[\text{Hb}]_{ex}$ decreases with time in a competition experiment. If $[\text{Hb}]_{ex}$ at $t = 0$ is known, one can find the ratio of $k_{RBC}/k_{Hb}$ from the equation at time $t$. In Fig. 5, we compared the predicted curves from Equation 18 with the experimental data. These experimental data were read from Fig. 3b of Vaughn et al. (13), and the two curves of $k_{RBC}/k_{Hb}$ versus hematocrit were calculated from Equation 18 as $P_m = 0.9\text{ m/s}$ (a) and $P_m = 4.15 \times 10^{-4}\text{ m/s (b)}$. In calculations, we chose $D_{NO} = 2600\mu\text{m}^{2}\text{s}$, $k_{RBC} = 105\mu\text{m}^{-1}\text{s}^{-1}$, which were the same as those used in Fig. 4. The competition experiments were performed with $9\mu\text{m}$ (Hct > 4%) or $2\mu\text{m}$ (Hct < 4%) of initial extracellular Hb concentration (13). During competition experiments, $[\text{Hb}]_{ex}$ was slowly consumed by the reaction with NO. From Fig. 2a in Ref. 13 we know that the maximum value of

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3.png}
\caption{Fig. 3. Calculated initial fluxes of NO transfer into RBCs. The initial fluxes of NO on the surface of the RBC versus hematocrit as $P_m = 0.9\text{ m/s (a)}$ and $P_m = 1.8 \times 10^{-4}\text{ m/s (b)}$. Other parameters used in the calculations are $k_{RBC} = 89\text{ m}\mu\text{m}^{-2}\text{s}^{-1}$, $P_{NO} = 2600\text{ m}\mu\text{m}^{2}\text{s}$, $r_0 = 3.39\mu\text{m}$, $c_{RBC} = 22000\mu\text{m}$, and $[\text{Hb}]_{ex} = 10\mu\text{m}$.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{Fig. 4. Simulations of $[\text{metHb}]_{ex}$ versus time for different values of hematocrit, initial extracellular Hb concentration, and initial NO donor concentration. All curves of $[\text{metHb}]_{ex}$ (solid line) were calculated from Equation 17 with $D_{NO} = 2600\mu\text{m}^{2}\text{s}^{-1}$, $k_{RBC} = 105\mu\text{m}^{-1}\text{s}^{-1}$, $P_m = 0.9\mu\text{m/s}$, and $r_0 = 3.39\mu\text{m}$. Other experimental parameters such as hematocrit, initial extracellular Hb concentration $[\text{Hb}]_{ex}$ and initial NO donor concentration are the same as those for each set of experimental data. Experimental data in panel A $[\text{Hb}]_{ex}^0 = 7.5\mu\text{m}$, $[\text{A}]^0 = 10/(1 - \text{Hct})\mu\text{m}$, shown as closed circles (Hct = 0%) and open circles (Hct = 7.8%), were read from Fig. 2. Experimental data in panel B (Hct = 15.6%, $[\text{A}]^0 = 10/(1 - \text{Hct})\mu\text{m}$, represented by closed circles ($[\text{Hb}]_{ex}^0 = 40\mu\text{m}$) and open circles ($[\text{Hb}]_{ex}^0 = 10\mu\text{m}$), were read from Fig. 4 of Ref. 13. Experimental data in panel C (Hct = 15.6%, $[\text{Hb}]_{ex}^0 = 10\mu\text{m}$, shown as closed circles ($[\text{A}]^0 = 15/(1 - \text{Hct})\mu\text{m}$), open circles ($[\text{A}]^0 = 10/(1 - \text{Hct})\mu\text{m}$), and closed triangles ($[\text{A}]^0 = 5/(1 - \text{Hct})\mu\text{m}$) were read from Fig. 5 (curve a) of Ref. 13.}
\end{figure}
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Fig. 5. Plot of $k_{RBC}/[Hb]$ versus hematocrit. Experimental data (closed circles) were read from Fig. 3 (curve b) of Ref. 13. Curves (solid lines) were calculated from Equation 18. Parameters used in the calculation are $k_{RBC} = 105 \mu m^{-1} s^{-1}$, $r_0 = 3.29 \mu m$, $D_{NO} = 2600 \mu m^2/s$, $[Hb]_{ex} = 5 \mu M$, $P_m = 0.9 m/s$ (curve a) and $P_m = 4.15 \times 10^{-4} m/s$ (curve b).

$\ln([Hb]^0/[Hb]_s)$ in a competition experiment is about 1.3, so $[Hb]_{ex}$ at the end of a competition experiment could be as low as 2.5 $\mu M$ if $([Hb]_s)^0 = 9 \mu M$ or 0.5 $\mu M$ if $([Hb]_s)^0 = 2 \mu M$. Thus, the average $[Hb]_s$ is chosen as 5 $\mu M$ in our calculations. Fig. 5 shows that the experimental data are in good agreement with curve a, which was calculated under the assumption of extracellular diffusion limitation ($P_m = 0.9 m/s$). However, curve b calculated under the assumption of membrane resistance limitation ($P_m = 4.15 \times 10^{-4} m/s$) is very different from the experimental data.

**DISCUSSION**

Our model predicts that the NO concentration at the RBC surface, $[NO]_{ex}$, is close to zero if $P_m = 0.9 m/s$, a value reported by Subczynski et al. (28) for lipid bilayer membrane. In this case, NO uptake by RBCs would be limited by extracellular diffusion (Fig. 2A). At Hct = 0.2%, the distance between the RBC surface to the inner wall of the container, $r_0 - r_1$, is about 23.5 $\mu m$. There is a layer with a big NO concentration gradient ($r - r_0 < 5 \mu m$) near the RBC, and a region of uniform NO concentration outside the concentration gradient layer between $r - r_0 = 5 \mu m$ and $r - r_0 = 23.5 \mu m$. The radius of the container $r_1$ decreases with the increase of hematocrit. As Hct = 5%, ($r_1 - r_0$) is reduced to 5.8 $\mu m$, and the region of uniform NO concentration disappears. However, the inhomogeneous NO concentration distribution within the concentration gradient layer almost remains unchanged. Thus, in contrast to the assumption of the competition experiments, increasing hematocrit from 0.2 to 5% does not make NO concentration distribution more homogeneous; rather NO concentration distribution is more inhomogeneous. Further increasing hematocrit from 5 to 10% has little effect on the NO concentration gradient near the RBC surface. NO uptake by the RBC is still limited by extracellular diffusion because $[NO]_{ex}$ is still close to zero compared with the NO concentration at the inner surface of the container, $[NO]^i$. Fig. 2B shows the profile of NO concentration as the RBC membrane is 500 times lower, or $P_m = 1.8 \times 10^{-3} m/s$. Similar to $P_m = 0.9 m/s$, the NO concentration distribution in solution as $P_m = 1.8 \times 10^{-3} m/s$ is more inhomogeneous, not more homogeneous, as hematocrit increases from 0.2 to 5%. At Hct = 10%, the big NO concentration gradient still exists between the RBC surface and the inner wall of the container. Simply speaking, Fig. 2A and B show that changing hematocrit from 1.6 to 10% has little effect on extracellular diffusion resistance no matter if $P_m$ is high or low. However, the original assumption of competition experiments (Fig. 2C), which predicts a complete conversion of inhomogeneous NO concentration distribution into homogeneous NO concentration distribution is in conflict with those predicted by the diffusion-reaction equation (Fig. 2, A and B). Therefore, there is no theoretical basis from which to claim that $k_{RBC}$ determined around Hct = 10% does not include any extracellular diffusion resistance.

In fact, there is evidence in the literature for an external diffusional limitation for other small nonelectrolytes, such as O$_2$ and CO (29, 18) across the erythrocyte membrane. Vande-griff and Olson (30) demonstrated that the rate of O$_2$ uptake by erythrocytes is inversely proportional to the size of RBC (using erythrocytes from various species as well as liposome-entrapped hemoglobin), a result directly predicted by the existence of an unstirred layer (9, 30) and not predicted on the basis of an inherent diffusion barrier (9, 30). Since NO and O$_2$ have similar size and solubility, the permeability of RBC membrane to the two small neutral molecules should be similar. In a previous study, we showed that NO uptake by RBCs at a low hematocrit is limited by extracellular diffusion (9). Vaughn et al. (13) also demonstrated that extracellular diffusion is the main resistance to NO uptake by RBCs as Hct = 1.6% in competition experiments. The above theoretical analysis (refer to Fig. 2A) shows that if NO uptake by RBCs is limited by extracellular diffusion at low hematocrit, the extracellular diffusion resistance would have little change by increasing hematocrit from 0.2 to 10%. Thus NO uptake by RBCs in competition experiments of Vaughn et al. (13) would still be limited by extracellular diffusion resistance.

Fig. 4 shows that if NO uptake by RBCs is limited by extracellular diffusion, or $P_m = 0.9 m/s$, the calculated curves of $[metHb]_{ex}$ at different sets of hematocrit, $[Hb]_{ex}$ and NO donor concentration are in good agreement with experimental data. As shown in Fig. 5, the experimentally determined ratios of $k_{RBC}/k_{RBC}$, except the one as Hct = 1.6%, are very close to curve a ($P_m = 0.9 m/s$), indicating that extracellular diffusion is the main resistance to NO uptake by RBCs. The experimentally determined ratio of $k_{RBC}/k_{RBC}$, as Hct = 1.6% largely deviates from curve a. This large deviation is caused by a low initial $[Hb]_{ex}$ (2 $\mu M$) that was used in the experiment as Hct = 1.6%, because this low $[Hb]_{ex}$ can result in a low ratio of $k_{RBC}/k_{RBC}$ and may cause a larger relative error in an experimental measurement of $[metHb]_{ex}$. If we let $P_m = 4.15 \times 10^{-4} m/s$, a very low membrane permeability suggested by Vaughn et al. (14), the simulated curve (curve b) is much lower than the reported ratios of $k_{RBC}/k_{RBC}$.

The analysis above shows that the rate of NO uptake by RBCs is primarily limited by extracellular NO diffusion in the competition experiments. Mathematically, as long as a non-zero diffusion flux exists around a RBC, the diffusion flux can be always related to a diffusion layer as shown in Equation 19,

$$\text{Flux} = \frac{dc}{dt} = D \frac{c^b - c^c}{\delta} \quad \text{at } r = r_0 \quad (\text{Eq. } 18)$$

where D is the diffusion coefficient; $c^b$ and $c^c$ are bulk concentration and surface concentration, respectively; and $\delta$ is the thickness of the diffusion layer. Here, the diffusion layer is an imaginary layer, which is used to convert the concentration gradient $dc/dr$ on the RBC membrane into a simple fraction $(c^b - c^c)/\delta$. Thus, the thickness of the diffusion layer is always related to the concentration gradient on a given surface. In a competition experiment, NO is generated around each RBC, so the diffusion layer is a layer within the unstirred layer around each RBC. In a blood vessel, the shape of the diffusion layer is much different. The blood flow creates an RBC-free layer near the vessel wall and increased concentration of RBCs near the center of the vessel like a flowing RBC cylinder (7, 10, 11). The
endothelium-derived NO needs to diffuse across the RBC-free layer to reach RBCs. In this case, the diffusion layer is a layer within the RBC-free layer surrounding the surface of the flowing RBC cylinder. The diffusion layer can reduce NO uptake by RBCs and enhance the effective NO diffusion distance in the vessel wall (10).

Finally, we point out that numerous previous studies with erythrocytes do not support the possibility that it possesses an inherent diffusion barrier to small nonelectrolytes, certainly not of the magnitude proposed by Vaughn et al. (13, 14). The red cell membrane is 40% lipid, which represents a substantial surface of rapid access of NO for diffusion. The lipid bilayer is highly unlikely to possess such a barrier property, since the passive permeability of the RBC to nonelectrolytes is very similar to artificial lipid vesicles (and also other cell types) (31), and the permeability characteristics of lipid vesicles made from whole erythrocyte membrane lipids is quantitatively the same as for synthetic lipids (32). Also, the rate of lateral diffusion of NO in the red cell membrane is similar to phosphatidylcholine vesicles (33). O₂ diffusion through thin films of erythrocytes is similar to that through equivalent thicknesses of free hemoglobin, indicating that the membrane possesses no barrier (34).

The presence of the complex cytoskeletal network of the erythrocyte and/or binding of hemoglobin to the cytoskeleton also exerts no influence, since the passive permeability of intact red cells to mannitol and erythritol is the same as inside-out vesicles, which lack this cytoskeleton (35). In addition, if the cytoskeletal network serves as a physical barrier, in order to account for a retardation in entry of a factor of 800 (36), this mechanism would require that such a network would cover all but 1/800 = 0.125% of the total inner surface area of the membrane. The spectrin component is an elongated rod that forms a web with large spaces inbetween (37). As suggested by Huang et al. (36), hemoglobin could bind to the membrane and conceivably cover parts of the surface. However, with 10⁶ hemoglobin molecules bound to band 3 per erythrocyte (38) and the size of hemoglobin at 64 × 55 × 50 Å³ (39), it can be calculated that the maximum surface area covered by the bound hemoglobin will be 45.7 μm², whereas the total erythrocyte surface area is 135 μm² (40). This would not explain a slowing of transmembrane NO movement of nearly 1000-fold.

In conclusion, the process of NO transfer into RBCs is of critical biological importance as it regulates the bioavailability and diffusion distance of endothelial derived NO. Our analysis shows that with increasing hematocrit, the distribution of NO concentration becomes more inhomogeneous rather than more homogeneous. Competition experiments did not eliminate extracellular diffusion resistance in measuring the ratio of \( k_{RBC}/k_{Hb} \) under the reported experimental conditions. The rate of NO uptake by RBCs in competition experiments is primarily limited by extracellular diffusion not membrane resistance. The extremely low membrane permeability suggested by Vaughn et al. (14) is not only in conflict with many previous experimental results about \( O_2 \) and NO uptake by RBCs, but also inconsistent with their results about the ratio of \( k_{RBC}/k_{Hb} \) calculated from the competition equation.

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