Estimation of Nitric Oxide Concentration in Blood for Different Rates of Generation

**EVIDENCE THAT INTRAVASCULAR NITRIC OXIDE LEVELS ARE TOO LOW TO EXERT PHYSIOLOGICAL EFFECTS***

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Endothelium-derived nitric oxide (NO) is a potent vasodilator in the cardiovascular system. Several lines of experimental evidence suggest that NO or NO equivalents may also be generated in the blood. However, blood contains a large amount of hemoglobin (Hb) in red blood cells (RBCs). The RBC-encapsulated Hb can react very quickly with NO, which is only limited by the rate of NO diffusion into the RBCs. It is unclear what the possible NO concentration levels in blood are and how the NO diffusion coefficient ($D$) and the permeability ($P_m$) of RBC membrane to NO affect the level of NO concentration. In this study, a steady-state concentration experimental method combined with a spherical diffusion model are presented for determining $D$ and $P_m$, and examining the effect of NO generation rate ($V_0$) and hematocrit (Hct) on NO concentration. It was determined that $P_m$ is $4.5 \pm 1.5$ cm/s and $D$ is $3410 \pm 50$ $\mu$m$^2$/s at 37 °C. Simulations based on experimental parameters show that, when the rate of NO formation is as high as $100$ nM/s, the maximal NO concentration in blood is below $0.012$ nM at $P_m = 4.5$ cm/s and Hct = 45%. Thus, it is unlikely that NO is directly exported or generated from the RBC as an intravascular signaling molecule, because its concentration would be too low to exert a physiological role. Furthermore, our results suggest that, if RBCs export NO bioactivity, this would be through NO-derived species that can release or form NO rather than NO itself.

Endothelium-derived NO is a potent vasodilator and plays an important role in maintaining vascular tone. As a small diatomic molecule similar to $O_2$, NO is highly diffusible. After NO is generated within the endothelium of blood vessels, it diffuses to both sides of the endothelial cell layer, the lumen and the vessel wall. The blood carries a large amount of hemoglobin (Hb)$^2$ within red blood cells (RBCs) flowing in the vessel lumen, and the rate of NO reaction with Hb is very rapid. Theoretical analysis shows that the high amount of Hb in the blood can greatly lower the NO concentration at the endothelial layer, which may finally result in a very low NO concentration in the vessel wall (1). This raises a question about how NO can escape from being trapped by the high amount of Hb in the blood.

This question has been extensively studied in recent years. Using a NO electrode, we demonstrated previously (2) that the rate of NO reaction with RBC-encapsulated Hb is nearly 1000 times slower than that with cell-free Hb. This relatively slower NO consumption rate was also observed by Liao and co-workers (3) using a competition experimental method and was further confirmed recently by Kim-Shapiro and co-workers (4) using stopped-flow absorption. It has been generally accepted that the relatively slower NO consumption rate by RBCs is caused by the resistance to NO diffusion from the solution into RBCs, but there is debate on whether the RBC membrane or the extracellular unstirred solution layer is the main source of resistance (2–7). In addition to the relatively slower NO consumption by RBCs, it has been reported that the cell-free layer adjacent to the endothelium further reduces the NO consumption rate by RBCs (8, 9). These theoretical and experimental results show that, although the large amount of Hb in the blood is a sink of the endothelium-derived NO, the diffusion resistance from the endothelial surface to the inside of the RBCs forms a physical barrier to prevent NO from being rapidly consumed by the RBC-encapsulated Hb.

Two other mechanisms for protecting NO bioactivity were also proposed (10–13). One mechanism assumes that RBCs import NO and store it as SNO-Hb (S-nitrosylated-Hb), which is formed preferentially in the oxygenated (or R) structure and associated predominantly with the RBC membrane. The NO group from SNO-Hb can be transferred to cysteine thiols of the band 3 protein at the RBC membrane by deoxygenation, and then a NO congener or NO$_x$ is exported from RBCs to dilate blood vessels (11). The other mechanism assumes that the relatively stable nitrite is a source of NO in the blood and tissues, which is converted to the bioactive NO or NO equivalents during physiological hypoxia to dilate blood vessels (12). However, it is uncertain whether the RBC-exported or -generated NO bioactivity is the diatomic molecule NO or other NO congeners/equivalents.

In this study, an experimental method combined with a mathematical model was developed for determining the RBC...
membrane permeability \( (P_m) \) and NO diffusion coefficient \( (D) \). Computer simulations based on experimental data were performed to predict the possible NO concentration in blood at different generation rates.

**MATERIALS AND METHODS**

**Preparation of NO Solutions**—NO stock solution was prepared as described in previous papers (14, 15). Briefly, NO was scrubbed of higher nitrogen oxides by passage first through a U-tube containing NaOH pellets and then through a 1 m de-aerated (bubbled with 100% argon) KOH solution in a custom-designed apparatus using only glass or stainless steel tubing and fittings. To prepare the saturated NO solution, the purified NO gas was bubbled for 15 min into the de-aerated buffer (0.2 M potassium phosphate, pH 7.4), which was contained in a glass sampling flask with a septum purchased from Kimble/Kontes (Vineland, NJ). The saturated NO concentration was nearly 2 mM.

**Preparation of Red Blood Cells**—Blood was drawn from Sprague-Dawley rats into a heparinized tube and centrifuged at 2300 \( \times g \) for 10 min. The plasma and buffy coat were discarded, and the RBC pellet was washed three times with phosphate-buffered saline (15 mM phosphate (potassium) plus 0.9% NaCl, pH 7.4). The packed RBCs were then resuspended in the same buffer and stored on ice for use.

**Electrochemical Measurements of NO Concentration**—The electrochemical system for measuring NO included a Clark-type NO electrode (ISO-NOP from World Precision Instruments, Sarasota, FL), a 4-port water-jacketed electrochemical chamber (NOCHM-4 from WPI), a Haake DC10-P5/U circulating bath, a magnetic stirrer, and an Apollo 4000 free radial analyzer (WPI). NO electrodes were inserted (through the three side ports) into the water-jacketed chamber containing 2 ml of phosphate-buffered saline for NO measurements in the presence or absence of RBCs. The solution was rapidly stirred at a constant speed with a magnetic bar stirrer. The temperature of the chamber was held at 37 °C by the circulating bath. NO was added into the solution (through a hole on the cap of the chamber) with a Hamilton syringe by a bolus injection to calibrate NO electrodes or by a SP310i-Plus syringe pump (WPI, Sarasota, Florida) at a given rate (volume/min) to reach a steady-state concentration in the presence of RBCs. In each experimental measurement, the total volume of NO solution injected into the chamber by the syringe pump was <1.5% of the volume of the solution in the chamber.

**Mathematical Model Describing the Steady-state NO Concentration Distribution in Solutions Containing RBCs**—It was previously demonstrated that the rate of NO consumption by RBCs is nearly constant until all Hb in RBCs is consumed by NO (2). Thus, it can be predicted that the steady-state NO concentration can be reached when the rate of NO supply is equal to the rate of NO consumption by RBCs. The mathematical model below was developed for determining how the steady-state NO concentration changes with \( P_m \), hematocrit (Hct), and NO generation rate. We assumed that a spherical RBC with radius \( r_0 \) was located in the center of a larger spherical container with radius \( r_1 \) to form two concentric spheres (5, 6). NO was generated at a constant rate \( V_0 \) in the solution between the two concentric spherical surfaces and diffused into the RBC (the inner sphere). The radius of the RBC, \( r_0 \), is 2.44 \( \mu \)m (16, 17). Hct (6), the ratio of the RBC volume to the whole solution volume, defines the radius of the outer sphere, \( r_1 \), as follows.

\[
Hct = \frac{r_0^3}{r_1^3} \text{ or } r_1 = r_0 \sqrt[3]{\frac{3}{4} Hct} \quad (\text{Eq. 1})
\]

The following equations describe the diffusion problem after a steady-state NO concentration is reached,

\[
D \frac{d}{dr} \left( r^2 \frac{d[NO]}{dr} \right) + V_0 = 0 \quad (\text{Eq. 2})
\]

\[
\frac{d[NO]}{dr} = 0 \text{ at } r = r_1 \quad (\text{Eq. 3})
\]

\[
[NO] = [NO]^m \text{ at } r = r_0 \quad (\text{Eq. 4})
\]

where \( D \) is the NO diffusion coefficient, \( V_0 \) is the NO generation rate in solution, and \([NO]^m\) is the NO concentration at the RBC membrane surface. The rate of NO autooxidation in solution is slow when NO concentration is low. When NO concentration is 1 \( \mu \)M or below, its half-life is more than 100 s (15, 18, 19). In our experiments, the steady-state NO concentration in the presence of RBCs is much lower than 1 \( \mu \)M. Based on the reported rate of NO consumption by 2 \( \times 10^6 \) RBCs/ml (2), it can be calculated that all rates of NO consumption by RBCs in the range of Hct used in our experiments are at least 100 times higher than the rate of NO autooxidation. Therefore, the rate of NO autooxidation is ignored in Equation 2.

The solution of Equations 2–4 gives the following.

\[
[NO] = - \frac{V_0 r^2}{6D} + \frac{[NO]^m}{3D} + \frac{V_0}{3D} \left( \frac{r^3}{2} + \frac{r_0^3}{r} \right) - \frac{V_0 r_0^3}{3Dr} \quad (\text{Eq. 5})
\]

The rate of NO diffusion into the RBCs, \( \nu \), can be derived from Equation 5. Considering that the rate \( \nu \) is also equal to \( 4\pi r_0^2 P_m [NO]^m \), we have the following.

\[
\nu = 4\pi r_0^2 D \frac{d[NO]}{dr} \bigg|_{r=r_0} = \frac{4\pi r_0^2 V_0}{3D} \left( \frac{r^3 - r_0^3}{r_0} \right)
\]

\[
= \frac{4\pi V_0}{3} (r_1^3 - r_0^3) = 4\pi r_0^2 P_m [NO]^m \quad (\text{Eq. 6})
\]

\[
[NO]^m = \frac{V_0 (r_1^3 - r_0^3)}{3P_m r_0^2} + \frac{V_0 Hct (1 - Hct)}{3P_m Hct} \quad (\text{Eq. 7})
\]

Substitution of Equation 7 into Equation 5 gives the following.

\[
[NO] = \frac{V_0}{6D} \left( \frac{2D (r_1^3 - r^3)}{P_m r_0^2} + r_0^2 + \frac{2r_0^3}{r_0} - \left( r^2 + \frac{2r_0^3}{r} \right) \right) \quad (\text{Eq. 8})
\]

The bulk NO concentration in the solution at \( r = r_1 \) can be obtained from Equations 5 and 7 as follows,
If Hct \ll 1, we have the following.

\[ \frac{3Hct[\text{NO}^b]}{V_o r_0^2} = \frac{1 - Hct}{P_m r_0} + \frac{(1 - Hct\text{Hct}^{1/3})^2(2 + Hct\text{Hct}^{1/3})}{2D} \]

(Eq. 11)

Equation 9 shows that the steady-state NO concentration in the bulk solution \((r = r_0)\), \([\text{NO}]^b\), consists of two terms. The first term \([\text{NO}]^m\) is the NO concentration on the outer membrane of a RBC, which is inversely proportional to the membrane permeability \(P_m\). The second term is the NO concentration difference between the outer membrane of the RBC and the bulk solution, which is independent of the permeability of the RBC membrane. If we use \([\text{NO}]^m\) to represent the second item in Equation 9, we have the following.

\[ [\text{NO}]^m = \frac{V_o r_0^2(1 - Hct\text{Hct}^{1/3})^2(2 + Hct\text{Hct}^{1/3})}{6D Hct} \]

(Eq. 12)

Although \([\text{NO}]^m\) is independent of \(P_m\), \([\text{NO}]^m\) increases as \(P_m\) decreases. Thus, \([\text{NO}]^b\) will also increase as \(P_m\) decreases, because \([\text{NO}]^b\) is the sum of \([\text{NO}]^m\) and \([\text{NO}]^m\). Equation 9 also shows that \([\text{NO}]^b\) is proportional to the rate of NO injection into the solution, \(V_o\). From the slope of the plot of \([\text{NO}]^b\) versus \(V_o\), we can determine the membrane permeability \(P_m\) based on Equation 9.

RESULTS

Formation of Steady-state NO Concentration in the Presence of RBCs—To form a steady-state NO concentration, NO was first continuously injected into the phosphate-buffered saline solution at a constant rate by a Hamilton syringe driven with a syringe pump. The NO concentration increased linearly. The rate of NO generation in the solution was determined from the slope of the initial linear increase of NO concentration in the solution. When the NO concentration in the solution reached \(~1~\mu M\), a certain amount of RBCs was added into the solution. It was observed that the NO concentration in the solution rapidly decreased but did not reach zero. Instead, the NO concentration reached a steady state. However, the steady-state NO concentration rapidly decreased to the base line ([NO] = 0) after NO injection was stopped (Fig. 1).

**The Effect of NO Injection Rate (\(V_o\)) and Hct on Steady-state NO Concentrations**—Because a steady-state NO concentration is reached when the NO consumption rate equals the NO generation rate, we examined how the NO generation and consumption rates change the steady-state level of NO concentration. It was observed that the steady-state NO concentration detected by NO electrodes linearly increase with the rate of NO injection as predicted from Equation 9 (Fig. 2). In the measurements, Hct was 0.0475% and temperature was 37 °C. The slope \(k_s\) value of the best fitting line to the plot of [NO] versus \(V_o\) is 1.12 ± 0.02 s.

\[ k_s = \frac{r_0^2}{3Hct} \left( \frac{1 - Hct}{P_m r_0} + \frac{(1 - Hct\text{Hct}^{1/3})^2(2 + Hct\text{Hct}^{1/3})}{2D} \right) \]

(Eq. 10)

where \(k_s\) is the slope of the plot of \([\text{NO}]^m\) versus \(V_o\) which is given in the following form.

\[ k_s = \frac{r_0^2}{3Hct} \left( \frac{1 - Hct}{P_m r_0} + \frac{(1 - Hct\text{Hct}^{1/3})^2(2 + Hct\text{Hct}^{1/3})}{2D} \right) \]

The experimental data (closed circles) were recorded at different NO injection rates in solution containing RBCs with Hct = 0.0475%. The data points were fitted with a linear equation. The slope of the best fitting line is \(1.12 ± 0.02\) s.

![Figure 1](image1.png)

**FIGURE 1.** A typical curve recorded with the steady-state concentration experimental method. In the measurement, NO was first continuously injected at a constant rate to the chamber containing 2 ml of buffer. When NO concentration in the solution increases to \(~1~\mu M\), a certain amount of RBCs were added into the chamber, resulting in a rapid decay of NO concentration in the solution. The NO concentration in the presence of RBCs was not decreased to zero; rather it reached a steady-state level. This steady state was immediately abolished after the injection of NO was stopped.

![Figure 2](image2.png)

**FIGURE 2.** Plot of steady-state NO concentration versus the rate of NO injection. The experimental data (closed circles) were recorded at different NO injection rates in solution containing RBCs with Hct = 0.0475%. The data points were fitted with a linear equation. The slope of the best fitting line is \(1.12 ± 0.02\) s.
we plotted $3\text{Hct}[\text{NO}]^b/V_{o0}^2$ versus $3\text{Hct}^{1/3}/2$ (Fig. 3). From the slope, we obtained the diffusion coefficient $D = (3.41 \pm 0.05) \times 10^{-4} \text{m}^2/\text{s}$.

**Simulation of the Effect of RBC Membrane Permeability and Hematocrit on Steady-state NO Concentrations**—Equation 8 was used to simulate how NO concentrations change with RBC membrane permeability and Hct. Fig. 4 shows four sets of simulated curves at $\text{Hct} = 0.0475\%$ (Fig. 4A) and four sets of curves at $\text{Hct} = 45\%$ (Fig. 4B). The permeability of the RBC membrane in panels a–d was assumed to be 415, 4,000, 45,000, and 400,000 μm/s, respectively. The [NO] curves from the lowest to highest in each panel were obtained at $V_o = 1, 5, 20$, and 100 nm/s, respectively. The steady-state NO concentration is slightly higher than 100 nM at $V_o = 100$ nm/s and $\text{Hct} = 0.0475\%$ if $P_m = 4.5 \text{ cm/s}$, whereas the steady-state NO concentration is 500 nM when $P_m = 415 \mu\text{m/s}$. When $\text{Hct} = 45\%$, the steady state NO concentration is <0.25 nM, even when $P_m$ is as low as 415 μm/s and $V_o$ is as high as 100 nm/s. The steady-state NO concentration is only in the pm range when $P_m = 4.5 \text{ cm/s}$, even when $V_o$ is as high as 100 nm/s.

**DISCUSSION**

In this study, an experimental method was developed and used in combination with mathematical modeling to determine the steady-state NO concentrations in solutions containing RBCs. We first demonstrated that a steady-state NO concentration could be reached in a solution containing RBCs when NO is added to the solution at a constant rate (Fig. 1). Equations derived from the diffusion-reaction in the concentric sphere model show that the ratio ($k_s$) of the steady-state NO concentration in bulk solution to NO generation rate is a constant related to Hct, $D$, and $P_m$.

We have experimentally determined $k_s$ at a given Hct (Fig. 2). By ignoring the term containing $1/P_m r_0$ in the expression of $k_s$ (Equation 10), we estimated that the lower limit of the NO diffusion coefficient $D$ at 37 °C is 3290 μm²/s. This lower limit of $D$ is close to the reported NO coefficient measured at 37 °C ($D = 3300 \mu\text{m}^2/\text{s}$) (20) and the recently reported NO diffusion coefficient that was extrapolated to 37 °C from 25 °C ($D = 3000 \mu\text{m}^2/\text{s}$) (21), implying that $P_m r_0$ is much greater than $D$ and $1/P_m r_0$ could indeed be ignored in Equation 10. Furthermore, we directly measured the bulk [NO], [NO]$^b$, at different Hct values and applied Equation 11 to analyze the experimental data (Fig. 3). We found that $D = (3.41 \pm 0.05) \times 10^{-5} \text{m}^2/\text{s}$ and $P_m = 4.5 \pm 1.5 \text{ cm/s}$ at 37 °C.

To predict how $P_m$ affects the steady-state NO concentration at high Hct, simulations were performed based on Equation 8 (Fig. 4). These simulation results show that [NO]$^b$ is proportional to $V_o$ at a given $P_m$ and markedly decreases as $P_m$ increases from 0.0415 to 4.5 cm/s. At $P_m = 4.5 \text{ cm/s}$ and Hct = 45%, the ratio of NO concentration on the RBC membrane surface [NO]$^m$ to [NO]$^b$ is 18% (Fig. 4B). This indicates that the resistance of the membrane to NO is still small compared with the resistance of the extracellular unstirred solution in the physiological Hct range, based on the $P_m$ and $D$ measured in our experiments.

In 1927, Hartridge and Roughton (22) observed that the rate of $O_2$ uptake by red blood cells was 20–40 times slower than that by cell-free Hb. In earlier studies, the slow uptake rate was attributed to the resistance of the RBC cell membrane (23, 24). However, in later reports between the 1960s and 1980s, a body of evidence showed that the membrane has little or no resistance to $O_2$ diffusion (25–30). Theoretical analyses and experimental data show that the slow rate of oxygen uptake by RBCs and its time course can be explained very well when it is assumed that the extracellular unstirred solution layer provides the main resistance to $O_2$ uptake by RBCs (29, 30). It had also been observed that the initial rate of $O_2$ uptake by RBCs was linear with the $O_2$ diffusion coefficient in the extracellular solution, and the membrane accounted for ~5% of the total resistance to $O_2$ transfer into RBCs (28). It seemed that these controversies ended in the middle of the 1980s with a general acceptance that the extracellular unstirred solution layer is responsible for the slow $O_2$ uptake rate by RBCs.

In 1958, similar to the rate of $O_2$ uptake by RBCs, it was observed that the rate of NO consumption by RBC-encapsulated Hb is much slower than NO consumption by cell-free Hb (31). After measuring the decay kinetics of NO in solutions containing RBCs and theoretically analyzing the experimental data, it was demonstrated that the rate of NO consumption by RBCs can be predicted by a mathematical model, with the assumption that the extracellular diffusion of NO limits the rate of NO entry into RBCs (2). These results indicated that the permeability of the RBC membrane is large for $O_2$ as well as NO.

Liao and co-workers (3) used a competition experimental method to measure the ratio of the rate of NO transfer into RBCs ($k_{\text{rbc}}$) and the rate of NO reaction with extracellular free Hb ($k_{\text{rHb}}$). They observed that $k_{\text{rbc}}$ is 500–1000 times slower than $k_{\text{rHb}}$. They suggested that RBC membrane resistance is the main resistance, because the NO concentration gradient was considered to have been eliminated in their experimental design after Hct was >5%. Using a mathematical model, the same group simulated extracellular methemoglobin formation measured at different Hct values in competition experiments. They estimated that the permeability of the RBC membrane to
NO is 415 μm/s (32), which is 2–3 orders of magnitude smaller than the reported permeability of the cell membrane and lipid membrane (20, 33, 34).

A detailed mathematical analysis on the reaction system containing both RBCs and free Hb was given by Tsoukias and Popel (5) based on the concentric sphere model. Their calculated results demonstrated that competition data can be satisfactorily simulated on the assumption that extracellular diffusion is the main resistance to NO transfer into the RBCs. To do so, it just requires using the NO diffusion coefficient \(D\) at 25 °C and extrapolating the reported value of \(k_{rb}\) from 20 to 25 °C rather than using the very low RBC membrane permeability suggested by Liao and co-workers (32). This result was also demonstrated in our previous paper (6), in which we also showed that the NO concentration distribution close to the RBC membrane had little change when Hct increased from 1.6 to 10%, and the extracellular diffusion resistance could not be eliminated for a large \(P_m\) value even after Hct was >10%. This is different from the original assumption in Liao’s paper that extracellular diffusion resistance was eliminated when Hct was >5%. These simulations show that the results from competition experiments are not necessarily explained by a very low permeability of the RBC membrane.

Recently, Kim-Shapiro and colleagues (4) measured NO consumption by RBCs at different Hct levels. They observed that the rate of NO consumption by RBCs increases ~3-fold when Hct increases from 15 to 50%. It is worth noting that a nearly 3-fold increase in the rate of NO consumption by RBCs can be obtained from the expression for \(k_{rb}/k_{rb}\) that was derived solely based on the assumption of extracellular diffusion resistance (6). Thus, their results obtained from competition experiments indicate that extracellular diffusion resistance is the main resistance for NO transfer into RBCs as Hct increases to 50%.

In Fig. 4, we demonstrated the effect of Hct, \(V_0\), and \(P_m\) on the steady-state NO concentration in solution containing RBCs. As shown in Fig. 4A, the simulated [NO] at the low Hct value can reach a level of >100 nM when \(P_m = 4.5 \text{ cm/s and } V_0 = 100 \text{ nM/s}\). This simulated [NO] is close to our experimental data (see data at \(V_0 = 100 \text{ nM/s}\) in Fig. 2) under the experimental conditions similar to those in the simulation. However, when \(P_m = 0.0415 \text{ cm/s}\), the simulated [NO] is >500 nM when \(V_0 = 100 \text{ nM/s}\), which is ~5 times higher than our experimental results, indicating that the small \(P_m\)
NO Concentration in Blood

(0.0415 cm/s) suggested by Liao and colleagues (32) is much smaller than the real value. Under physiological conditions, Hct is between 40 and 50%, so we simulated the steady-state NO concentration in the solution containing RBCs at 45%. Although [NO] at $P_m = 0.0415$ cm/s is ~20 times greater than [NO] at $P_m = 4.5$ cm/s, the highest [NO] at $P_m = 0.0415$ cm/s and $V_o = 100$ nM/s is <0.25 nM (Fig. 4B, panel a). Calculations based on Equation 9 show that, in blood at $P_m = 4.5$ cm/s, the NO generation rate must be greater than ~80 μM/s to reach a steady-state NO concentration of 10 nM, a concentration necessary to produce 30–50% maximal vasodilation. If NO were generated at this rate, all of the Hb in the blood would be oxidized within 2 min! Even if $P_m$ is assumed to be as small as 0.0415 cm/s, $V_o$ must still be greater than 4 μM/s to reach a 10 nM steady-state NO concentration. At this rate, all Hb in the blood could be oxidized in 40 min. The required NO generation rate is still too high to be reasonable under physiological conditions. Thus, even though NO may be generated in blood plasma, on the RBC membrane, or exported from RBCs, it is unreasonable to consider that the resulting intravascular NO will provide significant signaling for vasodilation, because its concentration would be too low to exert a physiological role.

It was reported that infusion of nitrite induces vasodilation in humans and that NO, a potent vasodilator, was generated from nitrite reduction in the presence of deoxyhemoglobin (deoxyHb) in vitro (13). Although these experimental results may suggest that nitrite reduction to NO by deoxyHb is responsible for the vasodilation, it is unlikely that NO is the primary product from the nitrite reduction. The reported rate of NO generation from the reaction of 200 μM nitrite with 1 mM deoxy-RBC Hb measured in vitro was <50 pmol/s, which is more than four orders of magnitude smaller than the necessary generation rate for reaching a steady-state physiological NO concentration in the blood. This excludes NO itself from being the primary NO equivalent derived from nitrite reduction by deoxyRBC Hb.

In a recent review, Robinson and Lancaster (35) compared the rate of NO escape from RBCs with the rate of NO generation from endothelium. They found that the rate of NO escape from RBCs is too small to cause vasodilation, so they pointed out that NO is unlikely to be the primary NO involved in the nitrite-induced vasodilation. However, this does not rule out the possibility that NO could be a secondary product from the NO equivalent generated in the blood or exported from RBCs (11, 12, 35), such as N$_2$O$_3$. The species N$_2$O$_3$ is an attractive candidate in the RBC-generated NO equivalents, because it may be delivered over a much longer distance before NO is released or nitrosothiols are formed.

Our results also indicate that changing RBC membrane permeability is unlikely to play a physiological role in the regulation of NO concentration in blood. Because we have observed that NO concentration in blood would be well below the range of concentrations (1–1000 nM) that exert physiological effects, changes in membrane permeability would not exert functional effects.

In conclusion, our results show that, even for high levels of NO generation of 100 nM/s, the intravascular concentration of NO would be far too low to exert functional effects. Reaction of NO with RBC Hb greatly limits intravascular NO concentrations. Thus, it is unlikely that NO is directly exported or generated from the RBC as an intravascular signaling molecule. Our results suggest that, if RBCs export NO bioactivity, this would be through species that can release or form NO, rather than NO itself.

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