Erythrocytes Possess an Intrinsic Barrier to Nitric Oxide Consumption*

(Received for publication, April 16, 1999, and in revised form, November 16, 1999)

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It has been reported that free hemoglobin (Hb) reacts with NO at an extremely high rate ($K_{Hb} \approx 10^7 \text{M}^{-1} \text{s}^{-1}$) and that the red blood cell (RBC) membrane is highly permeable to NO. RBCs, however, react with NO 500–1000 times slower. This reduction of NO reaction rate by RBCs has been attributed to the extracellular diffusion limitation. To test whether additional limitations are also important, we designed a competition test, which allows the extracellular diffusion limitation to be distinguished from transmembrane or intracellular resistance. This test exploited the competition between free Hb and RBCs for NO generated in a homogenous phase by an NO donor. If the extracellular diffusion resistance is negligible, then the results would follow a kinetic model that assumes homogenous reaction without extracellular diffusion limitation. In this case, the measured effective reaction rate constant, $K_{eff}$, would remain invariant of the hematocrit, extracellular-free Hb concentration, and NO donor concentration. Results show that the $K_{Hb}$ approaches a constant only when the hematocrit is greater than 10%, suggesting that at higher hematocrit, the extracellular diffusion resistance is negligible. Under such a condition, the NO consumption by RBCs is still 500–1000 times slower than that by free Hb. This result suggests that intrinsic RBC factors, such as transmembrane diffusion limitation or intracellular mechanisms, exist to reduce the NO consumption by RBCs.

Despite the well documented importance of nitric oxide, the transfer of NO from the producing cell to the target is poorly understood, because the free radical NO can be degraded in a variety of reactions. In particular, NO reacts with deoxy- and oxyhemoglobin (deoxyHb and oxyHb, respectively) at a very high rate (1, 2) to form nitrosyl Hb (HbNO) and met hemoglobin (metHb), respectively. If Hb in the RBC behaved like Hb in dilute solution, the half-life of NO in the blood (which contains about 12–15 mM heme) would be only about 1 μs. For such a rapid reaction, it would seem likely that a large portion of NO produced from the endothelium could be scavenged by the blood.

Indeed, in vivo and in vitro evidence suggests that free Hb is an effective NO scavenger that can deplete NO. For example, infusion of free Hb solution into experimental animals or human subjects results in hypertension (3, 4), most likely due to the reaction of NO with oxyHb in the circulation (3). Modeling analyses (5–7) also showed that if endothelium-produced NO reacted as rapidly with blood as it does with free Hb, the NO concentrations in vascular smooth muscle would be too low to activate soluble guanylate cyclase, the primary target of NO. Mathematical modeling based on diffusion theory and in vitro measurements of kinetic constants (5–8) have confirmed that Hb could effectively scavenge endothelial produced NO and mitigate its effect. Because 3–10 μM free Hb can abolish NO-mediated vasodilation in vivo (4, 9), it is unclear how NO can exercise its vasoregulatory function with 12–15 mM Hb concentration in the blood. This discrepancy is described as the “NO-Hb paradox,” and is one of the most important questions regarding the physiological and pathological functions of NO.

NO produced from the endothelium must go through four steps to react with the RBC-enclosed Hb (Fig. 1): (a) diffusion through the RBC-free (or depleted) region, created by intravascular flow (10), to the bulk solution; (b) diffusion from the bulk solution to the RBC surface; (c) diffusion across RBC membrane; and (d) diffusion and reaction inside RBC cytosol. The first two are affected by extracellular factors, whereas the last two steps are affected by intracellular components intrinsic to RBC itself. In discussing the diffusion flow of molecules, it is customary to define the diffusional resistance as a measure of the diffusional barrier. Resistance is defined as driving force divided by flux; where, for diffusion, the driving force is the concentration difference, and the flux is the molar flow/unit area. Although this definition can be made precise, the term diffusional resistance is often most useful as a concept. The diffusional resistance, if significant, results in the formation of layer around the RBC, within which the NO concentration is much smaller (e.g. less than 10%) than the bulk of the solution (Fig. 1b). This layer is termed the diffusion (or unstirred) layer, and the thickness of this layer is proportional to the diffusional resistance. In reality, the layer has no actual boundary, and the concentration profile is continuous. However, the use of the diffusion layer in discussion is conceptually convenient.

It is now recognized that the NO consumption rate by RBCs is much slower than that expected based on the in vitro reaction rate of NO with free Hb (9, 11). The in vivo quenching of NO by RBC is first reduced by the RBC-free zone (Fig. 1a) created by the flow field (7, 9, 12). However, even without the RBC-free zone, the NO consumption by RBC is still about 500–1000 times slower than the NO reaction rate with free Hb (9, 11). This low reaction rate has been attributed to diffusion resistance from the bulk solution to the surface of RBC (11). It has been suggested that the diffusional resistance is mainly associated with a diffusion or “unstirred” layer around each RBC (Fig. 1b).

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‡ The abbreviations used are: Hb, hemoglobin; RBC, red blood cell; Hct, hematocrit.
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Directly by measuring the NO/Hb reaction product; NO oxidizes oxyHb to metHb. The uptake rate of NO by RBCs is calculated by the use of a kinetic equation derived for this experimental condition from the extracellular metHb and oxyHb concentrations with or without RBCs present.

**Materials and Methods**

**Chemicals**—The NO donor, spermine NONOate \((N\text{-}[4\text{-(1\text{-}5\text{-}aminopropyl})\text{-}2\text{-}hydroxy-2\text{-}nitrosohydrizino]butyl\text{-}1\text{-}L\text{-}propanediamine}\) was purchased from Alexis Corp. (San Diego, CA). Some tests used SIN-1 (3\text{-}morpholinosydnonimine HCl) purchased from Alexis Corp. or DPTA NONOate (3\text{-}3\text{-}hydroximino-1\text{-}L\text{-}propanamine) bis 1\text{-}propargylamine purchased from Cayman Chemical (Ann Arbor, MI). Dibucano’s phosphate-buffered saline, Sephadex G-25 (Amersham Pharmacia Biotech), DEAE-Sephadex A-50 (Amersham Pharmacia Biotech), Drabkin’s solution, and Tris base were purchased from Sigma. The mixed bed ion exchange resin AG 501-X8 was purchased from Bio-Rad.

**Preparation of RBCs**—Bovine blood was collected in heparinized (5 IU/ml) tubes. The plasma and the buffy coat were removed following centrifugation at 800 \(\times \) g for 20 min. The cells were resuspended and immediately washed four times in Dibucano’s phosphate-buffered saline (0.122 molar NaCl, 0.030 molar \(\text{KH}_2\text{PO}_4\) + \(\text{Na}_2\text{HPO}_4\), 2 mg/ml glucose, pH 7.4, 290 mosmol/kg). After each wash, the cells were centrifuged at 800 \(\times \) g for 10 min to separate the RBCs from the supernatant. The cells were lysed by passing through a microcrystalline cellulose and \(\alpha\) cellulose (18).

**Preparation of Oxyhemoglobin**—OxyHb solution was prepared from bovine RBCs using the modification of Riggs’ procedure (19). Purified bovine RBCs were centrifuged (800 \(\times \) g, 10 min), and the supernatant discarded. The cells were lysed by diluting with 2 volumes of ice-cold deionized water, freezing in liquid nitrogen, and then thawing at room temperature. Cell debris was removed by centrifugation at 15,000 \(\times \) g for 30 min in a refrigerated centrifuge. Salts were removed by passing through a bed of AG 501-X8 resin and then eluting at 4 °C through a column of Sephadex G-25 that had been pre-equilibrated with 20 mM Tris acetate + 0.5 mM EDTA, pH 8.3. The Hb was stored on ice and used within three days.

**NO Donor**—Spermine NONOate was prepared as a stock solution of 1–2 mM. Approximately 0.4 mg of spermine NONOate was measured into a 1.5-ml micro-centrifuge tube. The donor was diluted in 1 ml of ice-cold, isotonic NaCl solution containing 0.001 N NaOH. The concentration was verified by diluting the concentrate in 0.01 N NaOH and measuring the absorbance at 250 nm (extinction coefficient 7500 cm\(^{-1}\) M\(^{-1}\)). The spermine NONOate solution was prepared fresh daily and kept in the dark and on ice until use. Some additional tests used DPTA NONOate and 3\text{-}morpholinosydnonimine HCl prepared in the same manner.

**The Competition Experiment**—Each test consisted of four solutions, run simultaneously: oxyHb in buffer, oxyHb with NO donor in buffer, free oxyHb in a suspension of RBCs, and free oxyHb in a suspension of RBCs with NO donor. A solution of oxyHb was prepared by adding oxyHb concentrate to Dibucano’s phosphate-buffered saline to produce the desired Hb concentration (normality 10 \(\mu\)M). This solution was used without further dilution for the buffer samples. RBC suspensions were produced by centrifuging purified RBCs (800 \(\times \) g, 10 min), removing the supernatant, and diluting the packed cells with the oxyHb solution to produce the desired hemocrit (normally 15%). The samples were equilibrated (1 h) at 25 °C, and then 15 ml of each solution were loaded into a 20-ml syringe (Becton-Dickenson, Inc.). For the samples containing NO donor, spermine NONOate was added immediately before loading into the syringe. The NO donor concentration was computed based on the total volume of solution. Immediately after the solutions were added to the syringes, the initial 1-ml sample was taken, and the syringes were placed on a rocking mixer (Clay Adams, Fisher Scientific) to keep the cells uniformly dispersed. The initial sample was centrifuged (20 s at 10,000 \(\times \) g) to separate the RBCs, and the supernatant was assayed for oxyHb and metHb using a Beckman DU 640 spectrophotometer. Subsequent 1-ml samples taken at time intervals were assayed in the same manner.

**Hemoglobin Assay**—The concentrations of metHb and oxyHb were determined spectrophotometrically by fitting a set of “basis spectra” to the measured spectra by means of linear regression. All samples and basis spectra were scanned from 380 to 700 nm using a Beckman DU 640 spectrophotometer. To obtain the pure components for the basis spectra, six samples of oxyHb were obtained as above using freshly prepared fresh Tris acetate + 0.5 mM EDTA, pH 8.3. The Hb was stored on ice and used within three days.

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and previously equilibrated with 50 mM Tris acetate, pH 7.60±0.5 mM EDTA (19). Hb was eluted with this buffer, and the concentration of the eluent was determined using Drabkin’s solution to convert the Hb to cyano-metHb (19). Each sample was serially diluted to 10, 5, 2.5, and 1.25 μM and scanned, and the digitized spectra were stored on computer. The averaged spectrum was obtained by use of linear regression. These same oxyHb samples were converted to metHb (without change in concentration) by adding a slight excess of NO gas to each sample, and the average spectrum was obtained in the same way.

Kinetic Modeling and Data Interpretation—The solutions that do not contain NO donor ensured the Hb autoxidation and RBC lysis were negligible. The ratio of the reaction rates between the NO-Hb and the NO-RBC reactions is determined by comparing the NO production rate with the metHb production rate in the oxyHb plus RBC suspension with NO donor. The rate of NO production can be determined from the control experiment, free oxyHb plus NO donor in buffer. Because only free oxyHb is present (in excess) in this control experiment, the total amount of metHb produced is the total amount of NO generated. Because the NO-oxyHb reaction rate is known, the ratio can be used to compute the NO-RBC reaction rate. For each solution, the metHb consumed and the metHb produced in the extra-erythrocyte space are measured at various time points.

The NO-RBC reaction rate constant is determined from kinetic equations describing NO uptake by oxyHb and RBCs. These equations assume that the extracellular diffusional resistance is negligible, and thus the NO concentration in the solution is homogeneous. Therefore, deviations from the model may suggest that the NO concentration is nonhomogeneous, which in turn indicates the significance of extracellular diffusional resistance. By using the pseudo-steady-state approximation, d[NO]/dt = 0, solution of these equations can be simplified to the following equation:

\[
\text{[metHb]}_s - (1 - \text{Hct})\text{[metHb]}_s \text{[oxyHb]}_{\text{RBC}} = K_{\text{RBC}}K_{\text{Hb}} \ln \left(\frac{[\text{totalHb}]}{[\text{oxyHb}]}\right) \quad (\text{Eq. 1})
\]

in which square brackets denote concentration. Here Hct is hematocrit, \([\text{metHb}]]_s\text{ is the metHb concentration in the cell-free control, [metHb]}_s\text{ is the metHb concentration in the extracellular space of the RBC suspension, [oxyHb]}_s\text{ and [totalHb]}_s\text{ are the oxyHb and initial total Hb concentrations in the extracellular space of the RBC suspension, respectively. }K_{\text{RBC}}\text{ and }K_{\text{Hb}}\text{ are the rate constants (M }^{-2}\text{s}^{-1}\text{) of the NO-RBC and NO-free Hb reactions, respectively. }K_{\text{RBC}}\text{ is defined based on the Hb concentration in the solution as if all the RBCs are lysed. The Hb concentration (mm, heme) in the solution can be calculated as 19.1 \times \text{Hct}. By plotting the experimental data according to the above equation, we obtained a straight line of slope }K_{\text{RBC/}}K_{\text{Hb}}\text{ and an intercept of zero. This plot (termed the }K\text{plot}) allowed the verification of the experimental and the determination of the rate constant. During the experiment, if RBC lysis, determined by an increase in total Hb in extra-erythrocyte space, contributed more than 6% of the extra-erythrocyte Hb concentration, then the data were discarded.

If the extracellular diffusional resistance is negligible (which is the model assumption), Equation 1 will fully describe the experimental data, and the measured }K_{\text{RBC}}\text{ will be independent of hematocrit, extracellular Hb, and NO donor concentration. Otherwise, the measured }K_{\text{RBC}}\text{ will decrease as hematocrit decreases, as extracellular Hb increases, and as NO donor concentration decreases.

RESULTS

The Competition Experiment Allows the Measurements Of }K_{\text{RBC}}\text{ at High Hematocrit—As stated above, previous experimental techniques for measuring }K_{\text{RBC}}\text{ were limited to very dilute RBC suspension and could not distinguish external diffusion limitation from intrinsic barrier in the RBC. To avoid these limitations, we designed the following competition approach. In this set of experiments, known concentrations of RBCs and free oxyHb were used as NO sources and free oxyHb competed for a limiting amount of NO generated by NO donor in solution. If free oxyHb in the RBC reaction with NO as fast as free oxyHb in the solution, then NO would be consumed by the RBC and free oxyHb at the same specific rate. On the other hand, if free oxyHb consumed NO faster than the RBC-enclosed oxyHb, then NO would be consumed by the two species in a ratio determined by their reaction rates. This ratio was determined by measuring the metHb and oxyHb in the extra-erythrocyte space. The metHb in the RBC was continuously regenerated by metHb reductase systems and could not be used to indicate NO consumption by RBC. However, the NO consumed by the RBCs could be calculated from mass balance using the total amount of NO generated and the amount consumed by the free Hb in the solution. The rate of NO generated was determined from a control experiment, where free oxyHb was present (without RBC) in excess to the NO donor. Kinetic equations were formulated to describe the experimental system, and the solution is shown in Equation 1.

The results from a typical Hb-RBC competition experiment are shown in Fig. 2. In this experiment, the sample contained 10 μM spermine NONOate as the NO donor, 7.5 μM extracellular oxyHb (heme concentration), and 7.8% hematocrit (1.5 mM RBC heme). Fig. 2a depicts the time course of metHb production in the extra-erythrocyte space. Fig. 2b shows the K plot of this and three additional experiments using the same sample of blood. The formation of the straight line in this plot indicates that the result was consistent with the kinetic model. This straight line also indicates that the reaction rate of the RBCs was constant throughout the test, suggesting that no internal diffusion layer formed during the test to slow NO uptake. The ratio }K_{\text{RBC/}}K_{\text{Hb}}\text{ = 0.00689 ± 0.00005 (mean ± S.E., }n = 4\text{) is determined from slope of this line. The result indicates that RBC reaction with NO is about three orders of magnitude lower than free Hb, consistent with other evidence (9, 11).

The Competition Experiment Allows the Measurement of }K_{\text{RBC}}\text{ Without External Diffusion Limitation—The generation of NO from a homogeneous NO source, soluble NO donor, reduces the barrier of external diffusion. However, it cannot eliminate the barrier of external diffusion, particularly at low concentrations of RBC suspension. Because RBCs are in the particulate form, they compete less favorably with the free Hb, which is in the homogeneous phase. This disadvantage is am
showed the effect of external diffusion limitation exactly as predicted. When the hematocrit was greater than 10%, $K_{RBC}$ calculated from the kinetic model (Equation 1) is constant, suggesting that the RBC consumption rate is fully described by the kinetic model that does not include any extracellular diffusion resistance. On the other hand, when hematocrit was smaller than 5%, $K_{RBC}$ calculated from the kinetic model decreased as hematocrit decreased. This decrease in the rate constant suggests that extracellular diffusion resistance, which is not considered in the model, becomes significant.

These experiments concluded that when the hematocrit is higher than about 10%, the $K_{RBC}$ measured is essentially free of external diffusion limitation. Under this condition, the thickness of the diffusion layer (Fig. 1b) surrounding the RBC is very small, much smaller than the intercellular distance. Therefore, external diffusion is no longer the limiting process. When the hematocrit is lower than 10%, the diffusion layer thickens, and thus external diffusion (Fig. 1b) is more important compared with other processes (Fig. 1, c and d). Note that this conclusion does not necessarily imply that external diffusion is unimportant under physiological conditions. The competition experiment uses a homogeneous NO generating system (soluble NO donor), where external diffusion can be eliminated at high hematocrit. However, in blood vessels, NO is generated from endothelium, which has a very different mass transfer barrier. It is interesting to note that under high hematocrit (without external diffusion limitation), the $K_{RBC}$ is still about 800 times lower than $K_{Hb}$, indicating that the NO-RBC reaction rate is intrinsically lower than the NO-free Hb reaction rate. Therefore, the limitation is attributed to intrinsic factors in RBCs, such as transmembrane or intracellular limitations.

Varying the hematocrit provides a sensitive probe of the near RBC space. In a very dilute RBC suspension, much of the extracellular Hb and NO production is far from the cell. As the hematocrit increases, the average spacing between cells decreases. If a diffusion layer exists, the proportion of extracellular volume associated with the diffusion layer increases as the hematocrit increases. The average NO concentration in the diffusion layer is low so less metHb will be produced. Thus, the apparent rate would depend on hematocrit. On the other hand, if NO uptake is controlled by factors intrinsic to the RBC, then the NO concentration in the extracellular space will be nearly uniform. Then there would be little change in apparent rate as hematocrit increases. This behavior is reflected in Fig. 3, a and b, where, as the hematocrit increases beyond 7.5%, there is little change in the apparent reaction rate coefficient.

If the apparent RBC reaction rate is independent of RBC concentration, as it would be if the resistance of the diffusion layer were negligible, then the plot of $K_{RBC}/K_{Hb}$ versus Hct will be a straight line with slope $K_{RBC}/K_{Hb}$. This is indeed the behavior seen in Fig. 3c. Here the presence of a non-zero intercept indicates that the apparent reaction rate depends on Hct in dilute suspensions, where the diffusion layer becomes important (11).

**Extracellular Hb or NO Donor Concentrations Do Not Affect the Value of $K_{RBC}/K_{Hb}$**—It is expected that increasing extracellular Hb or NO donor concentrations will increase the metHb formation in the extracellular space. However, the extracellular NO consumption will follow the kinetic model (Equation 1) only if the system is homogeneous, namely, no extracellular diffusion resistance, as the kinetic model assumes. In this case, $K_{RBC}$ derived from the kinetic equation will be independent of extracellular Hb or NO donor concentration, as the model states.

On the other hand, if the diffusion layer outside of RBC
Fig. 4. Altering the environment near the cell by varying the extracellular Hb suggests that the NO uptake by erythrocytes is dominated by factors intrinsic to the RBCs. The time course of metHb production in the extracellular space is shown in a. Here the extracellular Hb is varied from 5 to 40 μM for RBCs at 15.6% Hct. For 5 μM extracellular Hb the NO donor concentration was 5 μM, otherwise the NO donor concentration was 10 μM. The K plot of these data is shown in b. The slope of the best fit line is \( K_{RBC}/K_{Hb} = 0.00116 \pm 0.00006 \). The K plot analysis shows that the reaction rate of the RBCs is independent of extracellular Hb, as expected if NO uptake is limited by a barrier in the RBC.

The above experiments determined the NO consumption by RBCs without the influence of an external diffusion barrier. Interestingly, under this experimental condition, the NO consumption by RBCs is still much slower than the bulk solution, and the homogeneous kinetic model fails. In this case, the \( K_{RBC} \) derived from Equation 1 will decrease when the extracellular Hb increases or NO donor decreases, because the RBC competes much less favorably than the model assumes in this case.

Fig. 4 displays a competition experiment in which the concentration of the extracellular Hb is varied from 5 to 40 μM with Hct held constant at 15.6%. The time course of metHb formation varies significantly as seen in Fig. 4a. However, using our K plot analysis, Fig. 4b, the data fall on the same straight line yielding \( K_{RBC}/K_{Hb} = 0.00116 \pm 0.00006 \). Similarly, Fig. 5 shows a competition experiment in which the NO donor varied from 5 to 15 μM. Again, even though the time course of reaction product changes significantly (Fig. 5a), the K plot analysis yielded the same reaction rate \( K_{RBC}/K_{Hb} = 0.00126 \pm 0.00007 \). Because the reaction rate ratio does not depend on extracellular Hb or NO donor concentrations over these ranges, we conclude that the effect of any diffusion layer present under these experimental conditions was small.

**DISCUSSION**

The above experiments determined the NO consumption by RBCs without the influence of an external diffusion barrier. Interestingly, under this experimental condition, the NO consumption by RBCs is still much slower than by an equivalent concentration of free Hb. This result suggests that either the transmembrane diffusion or intracellular diffusion/reaction is also rate-limiting. Because these factors are intracellular components, the data suggest the existence of intrinsic factors in the erythrocytes that control the overall NO consumption rate.

A variety of experimental and theoretical studies of gas uptake kinetics by intact RBCs has been published since the original measurements by Hartridge and Roughton (16). These studies have greatly increased our understanding of the kinetics of fast biological processes. However, accounting for the transport resistance provided by a time-varying diffusion layer is still a formidable challenge. This has been the fundamental problem for studies of dilute RBC suspensions. As stated by Merchuck et al. (15), “... measurements made on diluted red cell suspensions cannot provide a good model for oxygen transport in normal blood, where high hematocrits tend to diminish boundary layer effects.” It is likely that the same statement can be made for NO uptake.

Recent studies (17, 20) have tried to account for this diffusion resistance using mathematical models of the RBC, which include hydrodynamic effects contributing to the external diffusion layer. Using this technique, they were able to obtain good agreement with rapid mixing, stopped-flow data. However, the thickness of diffusional layer estimated for these stopped-flow experiments, 13 μm for human RBCs at 50% saturation, could not apply under physiological conditions, because this layer is much thicker than the average intracellular distance between RBCs.

The competition experiment overcomes many of the experimental difficulties in measuring the uptake kinetics of RBCs. The primary cause of the diffusion layer is the depletion of NO immediately adjacent to the cell from insufficient NO transport from the bulk fluid. The competition experiment minimizes this diffusion layer, because NO is continually produced from the NO donor. Furthermore, under physiological conditions, close proximity of RBCs ensures that the concentration gradient associated with one RBC affects the surrounding RBCs (21). Because a relatively high hematocrit is used in the competition experiment (15%, which is 30–40% of the normal physiological value) this effect is taken into account. Note that the NO donor used should not enter the RBCs. If the NO donor is transported into RBC, then the NO consumption rate by the RBC measured by the competition experiment would be higher than the true value. In this study, we used spermine NONOate as an NO donor, which does not enter RBC at an appreciable rate (22).
The amount of polyamines that might enter RBC within the time scale of our experiment is too small to affect our results. Indeed, use of other NO donors, such as SIN-1 and DPTA NONOate, gave the same results, suggesting that uptake of the NO donor by RBC is insignificant.

Although this work has focused on overcoming the influence of the external diffusion layer, the technique presented here should also minimize the influence of an internal diffusion layer formed from reacted intracellular Hb. In rapid-mixing experiments where the RBC oxygen saturation is increased over a large range, this internal diffusion layer can contribute about half of the resistance to oxygen uptake (17). With our technique, very little metHb is formed in the cells, typically, 1–5 μM or 0.004–0.02% of cytosolic Hb is oxidized, an amount readily reduced by the metHb reductase system in the time scale of the experiment. We have verified experimentally that there is little effect from the internal diffusion layer. If a diffusion layer forms over the course of the experiment, its presence would cause $K_{RB}/K_{Hb}$ to decrease as the cells uptake NO. Consequently, the K plot line would flatten with time. However, Figs. 2b, 3a, 4b, and 5b show that the K plot line is straight, indicating a constant uptake rate. The resistance of the internal diffusion layer would also depend on NO donor concentration, because more metHb would be produced in the RBC at higher donor concentration. However, Fig. 5b shows that $K_{RB}/K_{Hb}$ is independent of NO donor concentration. Furthermore, we have performed competition experiments using RBCs in which 10–88% of the cytosolic Hb has been oxidized to metHb by pretreatment with NO donor or phenylhydrazine. We found that the value for $K_{RB}/K_{Hb}$ was not significantly reduced (data not shown), suggesting that the uptake rate was essentially independent of internal metHb. The same result was reported previously (11).

The low NO consumption rate by the RBC may be viewed as a result of isolation of the bulk of Hb from exposure to NO. However, such an effect cannot be achieved simply by forming a concentrated Hb solution within an NO-permeable membrane. In this case, the only possible, but unlikely, diffusional resistance must come from the intracellular space. It can be argued that one scenario for diffusional resistance is the formation of a metHb shell near the membrane. As Hb is oxidized to metHb by NO, this metHb shell increases, which in turn reduces the NO consumption rate. However, we and others (11) have shown that NO consumption rate by RBC is independent of metHb concentration in the cell. It may also be argued that the metHb formed in RBC quickly establishes a metHb layer with constant thickness and maintains a constant steady-state effect on the rate of NO consumption. In this case, specific biochemical mechanisms, such as the binding of metHb to the membrane, must be involved. However, even if such a mechanism exist, it is highly unlikely that a metHb shell can reduce the NO consumption to any significant degree.

There are a number of differences between this experimental design and those using bolus-dissolved NO solutions (11, 15, 20). Perhaps most importantly, NO is released slowly into the solution similar to the endothelial release of NO. The NO concentration to which the RBCs in the competition experiment are exposed is quite low. In contrast, Liu et al. (11) added a bolus of 1 μM NO to a dilute RBC suspension that contains about 1–2 μM heme. Kinetic modeling computations (results not shown) suggests that the NO concentration is typically much less than 1 nM. The effect of NO on the erythrocytes is small; less than 0.5% of the intracellular Hb will react with the NO. This means that there is no intracellular resistance caused by diffusion through reacted Hb. In the competition experiment, NO is released continuously at low concentrations over a time scale of minutes to hours, which is more physiological than sudden exposure to a high NO concentration. In the competition experiment, RBCs encounter much milder conditions than in the stopped-flow experiment. Therefore, transient disruptions or alterations of membrane structure, which might be caused by high shear during rapid mixing, are avoided. Furthermore, as stated above, the hematocrit used in this experiment (15%) is close to the physiological value (35–50%), in contrast to the very dilute (typically, 0.01–0.15% Hct) RBC suspension used previously (11, 20). The low concentration of RBC suspension may artificially amplify external diffusion resistance that may not be as significant under physiological conditions. Our data are remarkably close to that obtained by Liu et al. (11). We reason that their data may also represent an intrinsic limitation in RBC consumption of NO. Although they used a low hematocrit (~0.01%), their experimental setup allows very efficient mixing, which reduces the external diffusion limitation. However, it seems that the difference between the resistance offered by a diffusion layer and that from an intrinsic barrier in the RBC would be hard to discern in their experiments.

Despite the complications of the diffusion layer, there appear to be no significant inherent resistances for oxygen uptake in the RBCs; most of resistance can be assigned to purely diffusional processes inside and outside the RBC. This has been confirmed through modeling experimental oxygen uptake data (15, 17, 20) and from experiments using packed RBCs (23). Apparently, this is not the case for NO, which is surprising, because NO and O₂ are similar in size, shape, and solubility.

Why should NO and O₂ behave differently? There are fundamental differences in the physical treatment of RBCs in the competition experiment as compared with other methods of measuring uptake rate. The competition experiment is performed under mild circumstances in which the RBC membrane is not greatly disturbed. In rapid-mixing experiments, RBCs are subjected to intense shear and turbulent stresses, which could alter membrane and membrane skeletal structure, as well as Hb-membrane interactions. Membrane perturbation may also occur in preparing thin layers of packed cells, where intimate cell-cell contact could result in membrane stretching and shear, even under gentle handling. Furthermore, there are significant differences in the chemical treatment of the cells. To the best of our knowledge, the gas uptake rate for all previous experiments has been measured by monitoring the conversion of cytosolic Hb. This means that a significant portion of the cytosolic Hb must react with the gas during a short time period. For oxygenation, this is physiological, but for NO uptake, it is not. Except under pathological conditions such as sepsis, the reaction products between NO and cytosolic Hb are too low to measure. Therefore, the uptake rate for very low NO concentration is important, but previously inaccessible, so there are no directly comparable data. Even for oxygen uptake, it may not be that the membrane is so permeable, but that membrane resistance is comparable to intracellular and extracellular resistance (23). The comparable situation, membrane resistance to very small concentrations of oxygen for completely deoxygenated cells, is not known.

There are at least two possible mechanisms that would decrease the uptake of NO by RBCs: lower than expected membrane permeability or reduced reaction rate between NO and the cytosolic Hb. The latter is ruled out by the observation that NO consumption rate by RBC is independent of intracellular oxyHb concentration (11).² The diffusion coefficient of NO in

² M. W. Vaughn, K.-T. Huang, L. Kuo, and J. C. Liao, unpublished results.
lipid membranes seems too high (24) for the RBC membrane to provide significant resistance. However, the measured diffusion coefficient appears to depend on the position within the membrane, decreasing away from the center of the membrane (24). Therefore, how rapidly NO passes through the membrane from an aqueous phase is still unclear. Although we do not expect the RBC membrane to be impermeable to NO, the transmembrane diffusion rate of NO may be relatively slow compared with the very fast reaction rate between NO and the Hb inside of RBC. How the intra-erythrocyte environment affects the NO reaction with Hb is also an open question. S-nitrosylation of β-93cys has been proposed to play an important role in transferring of NO (25, 26). However, S-nitrosylation does not affect the NO consumption rate and cannot explain the results shown here. It has also been proposed that low concentrations of NO in the blood under a partially oxygenated condition can be effectively and rapidly sequestered by Hb through the formation of α(Fe-NO)2β(Fe)2 (27). The formation of an NO adduct has been suggested to be much faster than previously reported when the NO to Hb ratio is low (28). However, regardless of the nature of the NO consumption reaction by RBC, these reactions cannot explain the results presented here.

In summary, there appear to be multiple mechanisms that reduce the NO consumption rate in vessels under physiological conditions. The first is the presence of flow, which forms a RBC-free zone (Fig. 1a) near the vessel wall and increases the diffusion barrier (7, 9, 12). This phenomenon has recently been verified experimentally (9). The second is the diffusion layer (Fig. 1b) surrounding the RBC (11), although its role under physiological conditions remains to be determined. The third, suggested by this work, is an intrinsic factor of RBCs that reduces the NO consumption rate, such as transmembrane diffusion (Fig. 1c) or intracellular limitation (Fig. 1d). In view of the fact that NO consumption of NO is independent of oxyHb concentration (11), the limitation due to intracellular reactions is highly unlikely. Obviously, more work remains.

REFERENCES

1. Cassoly, R., and Q. H. Gibson (1974) J. Mol. Biol. 91, 3301–3313
2. Eich, R. F., Li, T., Lemon, D. D., Doherty, D. H., Curry, S. R., Aitken, J. F., Mathews, A. J., Johnson, K. A., Smith, R. D., Phillips, G. N. J., and Olson, J. S. (1996) Biochemistry 35, 6976–6983
3. Doherty, D. H., Doyle, M. P., Curry, S. R., Vali, R. J., Fattor, T. J., Olson, J. S., and Lemon, D. D. (1998) Nature Biotech. 16, 672–676
4. Poh, U., and Lamontagne, D. (1991) Basic Res. Cardiol. 86, suppl. 105
5. Lancaster, J. R., Jr. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8137–8141
6. Lancaster, J. R., Jr. (1996) Methods Enzymol. 268, 31–50
7. Vaughn, M. W., Kuo, L., and Liao, J. C. (1998) Am. J. Physiol. 274, H1705–H1714
8. Vaughn, M. W., Kuo, L., and Liao, J. C. (1998) Am. J. Physiol. 274, H2163–H2176
9. Liao, J. C., Hein, T., Vaughn, M. W., Huang, K.-T., and Kuo, L. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 8757–8761
10. Schmid-Schonbein, H., Fisher, T., Driessen, G., and Rieger, H. (1979) in Quantitative Cardiovascular Studies: Clinical Research Application of Engineering Principles (Hwang, N. H. C., Gross, D. R., and Patel, D. J., eds) pp. 353–417, University Park Press, Baltimore
11. Liu, X., Miller, M. J. S., Joshi, M. S., Sadowska-Krowicka, H., Clark, D. A., and Lancaster, D. A., Jr. (1998) J. Biol. Chem. 273, 18709–18713
12. Butler, A. R., Magson, I. L., and Wright, P. G. (1998) Biochim. Biophys. Acta 1425, 168–176
13. Carlsen, E., and Comroe, J. H., Jr. (1958) J. Gen. Physiol. 42, 83–107
14. Coin, J. T., and Olson, J. S. (1979) J. Biol. Chem. 254, 1178–1190
15. Merchuk, J. C., Tez, Z., and Lightfoot, E. N. (1983) Chem. Eng. Sci. 38, 1315–1321
16. Hartridge, H., and Roughton, F. J. W. (1927) J. Physiol. (Lond.) 62, 232–242
17. Vandegriff, K. D., and Olson, J. S. (1984) Biophys. J. 45, 825–835
18. Beutler, M. D. (1984) Red Cell Metabolism: A Manual of Biochemical Methods, Grune & Stratton, Orlando, FL
19. Riggs, A. (1981) Methods Enzymol. 76, 5–29
20. Vandegriff, K. D., and Olson, J. S. (1984) J. Biol. Chem. 259, 12619–12627
21. Rice, S. A. (1980) Biophys. J. 29, 65–78
22. Moulinoux, J. P., Le Calve, M., Quemener, V., and Quash, G. (1984) Biochimie (Paris) 66, 385–393
23. Kreuzer, F., and Yahr, W. Z. (1960) J. Appl. Physiol. 15, 1117–1122
24. Denicolai, A., Souza, J. M., Radi, R., and Lissi, E. (1996) Arch. Biochem. Biophys. 330, 208–212
25. Jia, L., Bonaventura, C., Bonaventura, J., and Stamler, J. S. (1996) Nature 380, 221–226
26. Gow, A. J., and Stamler, J. S. (1998) Nature 391, 169–173
27. Yonetani, T., Tsuneshige, A., Zhou, Y., and Chen, X. (1998) J. Biol. Chem. 273, 20325–20333