The significant blood resistance to lung nitric oxide transfer lies within the red cell

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Borland C, Bottrill F, Jones A, Sparkes C, Vuylsteke A. The significant blood resistance to lung nitric oxide transfer lies within the red cell. J Appl Physiol 116: 32–41, 2014. First published November 21, 2013; doi:10.1152/japplphysiol.00786.2013.—The lung nitric oxide (NO) diffusing capacity (DLNO) mainly reflects alveolar-capillary membrane conductance (Dm). However, blood resistance has been shown in vitro and in vivo. To explore whether this resistance lies in the plasma, the red blood cell (RBC) membrane, or in the RBC interior, we measured the NO diffusing capacity (DNO) in a membrane oxygenator circuit containing ~1 liter of horse or human blood exposed to 14 parts per million NO under physiological conditions on 7 separate days. We compared results across a 1,000-fold change in extracellular diffusivity using dextran, plasma, and physiological salt solution. We halved RBC surface area by comparing horse and human RBCs. We altered the diffusive resistance of the RBC interior by adding sodium nitrite converting oxyhemoglobin to methemoglobin. Neither increased viscosity nor reduced RBC size reduced Dm. Adding sodium nitrite increased methemoglobin and was associated with a steady fall in DNO (P < 0.001). Similar results were obtained at NO concentrations found in vivo. The RBC interior appears to be the site of the blood resistance.

Hemolysis disrupts the membrane and cell interior, but released Hb shortens the plasma diffusion distance. Adding oxyglobin shortens the plasma diffusion distance and “bypasses” the red cell membrane and interior.

Our oxygenator model allows us to alter one variable, but keep others constant (7). To alter extracellular fluid resistance, we can compare solutions of varying viscosity. The diffusivity depends on 1/viscosity, according to the Stokes-Einstein equation:

\[ D' = \frac{K_B T}{6 \pi \eta r} \]  

(see appendix), where D’ is Fick’s first law diffusion coefficient in dimensions length^2 time^{-1}, \( K_B \) is Boltzmann’s constant, T is absolute temperature, \( \eta \) is solution viscosity, and r is particle (including gas) molecular radius.

Initially, using horse blood, we halved extracellular resistance by replacing plasma with physiological salt solution (9). To alter membrane resistance, we tried to alter surface area (SA) by creating spherocytes by heating and shrinking cells with hypertonic salt solutions, as described by Carlsen and Comroe (13). To alter the red cell interior, we added sodium nitrite to form methemoglobin (metHb). We were only able to make minor alteration to viscosity and cell size. We here present observations altering viscosity (diffusivity) 1,000-fold using red cells suspended in dextran, plasma, or physiological salt solution and doubling red cell size, comparing horse to human red cells, in addition to our observations with nitrite-induced metHb. We also confirm for human blood the curvilinear relationship at low Hb concentration ([Hb]) between [Hb] and DNO.

METHODS

Oxygenator Circuit

Our detailed circuit is described elsewhere (7, 17, 18). Briefly, two membrane oxygenators were connected in series to form a continuous circuit perfused with ~500 ml of horse red cells (TCS Biosciences, Buckingham, UK) or human red cells (NCI, National Health Service Blood and Transplant Service, Bristol, UK) suspended in the relevant blood substitute) or free hemoglobin (Hb) in distilled water. The diffusing capacity for oxygen; diffusing capacity for carbon monoxide; diffusing capacity for nitric oxide; membrane oxygenator...
D Measurements

D\textsubscript{NO} and D\textsubscript{CO} were measured from the difference in gas concentrations leaving and entering the first oxygenator as follows. The calculation is just the same as for the single-breath method used clinically, except that the volume of gas exposed to blood per unit time is not the alveolar volume divided by the breath-hold time. It is the volume in the gas channels per minute i.e., the gas flow rate.

\[ D_{\text{NO}} = \frac{\text{gas flow rate} \times (P_{\text{in}} - P_{\text{out}})}{\text{RH} \times \log_e (N_{\text{NO}}/N_{\text{NO}_2})}, \]

where RH is barometric pressure, P\textsubscript{NO} is saturated water vapor pressure (Torr), RH is relative humidity, and N\textsubscript{NO} and N\textsubscript{NO\textsubscript{2}} are NO concentrations ([NO]) at the inlet and outlet ports, respectively. For CO, the calculations were identical, except substituting CO\textsubscript{in}/CO\textsubscript{out}.

O\textsubscript{2} diffusing capacity (D\textsubscript{O\textsubscript{2}}) was measured by Bohr integration (5).

\[ D_{O2} = \frac{\text{gas flow rate} \times (O_{2\text{in}} - O_{2\text{out}})}{4} \]

where M\textsubscript{O\textsubscript{2}} is O\textsubscript{2} uptake, and the mean O\textsubscript{2} Hb saturation in the blood channel (Sc\textsubscript{O\textsubscript{2}}) was calculated as a definite integral of Hb O\textsubscript{2} saturation (S\textsubscript{O\textsubscript{2}}), integrated with respect to partial pressure between the limits of O\textsubscript{2} inlet partial pressure and O\textsubscript{2} outlet partial pressure, divided by the inlet-outlet difference in O\textsubscript{2} partial pressure. P\textsubscript{O\textsubscript{2}} and mixed arterial O\textsubscript{2} partial pressure were directly measured by the inline analyzer. The integral was calculated by estimating each infinitesimal value for saturation S\textsubscript{i} from every incremental \delta P\textsubscript{O\textsubscript{2}} using an algorithm derived for human blood (37) and summing each cell value S\textsubscript{i} \times \delta P\textsubscript{O\textsubscript{2}} from Pr\textsubscript{O\textsubscript{2}} to the oxygenator blood outlet partial pressure of O\textsubscript{2} (P\textsubscript{O\textsubscript{2}}} using a Microsoft Excel spreadsheet. Equation 5 shows this mathematically:

\[ Sc_{O2} = \sum_{i=0}^{n} \left( \frac{S_i \times \delta P_{O2}}{(P_{O2} - P_{CO2})^2} \right) \]  

where P\textsubscript{CO\textsubscript{2}} corresponds to Sc\textsubscript{O\textsubscript{2}} estimated from the algorithm, P\textsubscript{A} is the geometric mean partial pressure of O\textsubscript{2} between gas inlet and outlet, Sa\textsubscript{O\textsubscript{2}} is arterial S\textsubscript{O\textsubscript{2}}, and Sv\textsubscript{O\textsubscript{2}} is mixed venous S\textsubscript{O\textsubscript{2}}. P\textsubscript{O\textsubscript{2}} (arterial O\textsubscript{2} tension) and P\textsubscript{CO\textsubscript{2}} (blood outlet O\textsubscript{2} tension) and P\textsubscript{O\textsubscript{2}} (blood inlet O\textsubscript{2} tension) together with potassium (K), pH, and Hb were measured by an inline fluorometric analyzer (CDI 500 Terumo Cardiovascular Systems UK, Egham Surrey, UK). metHb, carboxyhemoglobin (COHb), oxyhemoglobin, P\textsubscript{O\textsubscript{2}} (extracellular), and percentage saturation (S\textsubscript{a}) were measured by a combined co-oximeter and blood-gas analyzer (Roche Cobas b221, Roche Diagnostics, Indianapolis, IN). Intracellular P\textsubscript{O\textsubscript{2}} was calculated from S\textsubscript{a} using a formula originally developed for human blood (37). O\textsubscript{2} concentrations (P\textsubscript{O\textsubscript{2}}) at the inlet valve and outlet were measured by a Medical Oxygen Monitor (Viamed Sensotec MX300 Cambridge Sensotec, St. Ives, UK).

Progressive Addition of Red Cells

The circuit was primed with 1,400–1,500 ml of Hartmanns solution. Aliquots were exchanged to give the desired hematocrit, e.g., 14 ml drawn off and replaced with 14 ml red cells to give 1% hematocrit. Thus hematocrits of 1–30% were generated. One estimate of D\textsubscript{NO}, D\textsubscript{CO}, and D\textsubscript{O\textsubscript{2}} were made once the final [Hb] was reached. Because the inline analyzer did not detect very low [Hb], these were calculated from the control value multiplied by the dilution.

Varying Viscosity

Two hundred fifty-seven grams of either dextran 40 or dextran 500 (Pharmacosmos A/S DK-4300, Holbaek, Denmark) were added to 771 ml of Hartmanns solution to give a 30% wt/vol solution. The plasma was removed by separator (4R4414 Fenwall Laboratories, Deerfield, Illinois) from 1 liter of horse blood (TCS Biosciences, Buckingham, UK) and replaced with 30% dextran solution. The red cells suspended in dextran were added to the primed circuit with Hartmanns, and the Hartmanns drawn off. Three estimates of D\textsubscript{NO}, D\textsubscript{CO}, and D\textsubscript{O\textsubscript{2}} were made. Five hundred milliliters of perfusate were drawn off and replaced with 500 ml of horse cells suspended in Hartmanns, giving a 20–25% dextran solution. Three further estimates of D\textsubscript{NO}, D\textsubscript{CO}, and D\textsubscript{O\textsubscript{2}} were made. A final 500 ml of perfusate were drawn off and replaced with 500 ml of horse cells in Hartmanns to give 10–15%, and the final three estimates of D\textsubscript{NO}, D\textsubscript{CO}, and D\textsubscript{O\textsubscript{2}} were made.

Varying Red Cell Size

Red cells of differing mean corpuscular volume (MCV) will have differing SAS. Chakraborty et al. (14) have modeled the human red cell as a discoid cylinder of diameter (d) 8 μm (8 × 10⁻⁶ m) and thickness (height) (t) 1.6 μm (1.6 × 10⁻⁶ m). This has volume \( \pi (d/2)^2 t = 80 \) fl. Its SA will be \( 2 \pi (d/2)^2 + \pi dt = 1.41 \times 10^{-10} \) m². From the volume (MCV) of a human red cell, and assuming its ratio of t/d is the same as in the model of Chakraborty et al., i.e., 0.2, it is possible to determine its SA. Horse red cells are uniform in size with d = 5–6 μm. Knowing the MCV and using this value for d, t and hence SA can be calculated. The circuit was emptied and reprimed with horse or human cells suspended in Hartmanns, whichever had not been used for the varying [Hb] study. Three estimations of D\textsubscript{NO}, D\textsubscript{CO}, and D\textsubscript{O\textsubscript{2}} were made. The Hb, Hct, and MCV were measured from a 2-ml aliquot (Beckmann LH 500, Miami, FL).

Addition of Sodium Nitrite and Sodium Dithionite

Three percent (0.43 M) sodium nitrite (Pharmacy Department, Ipswich Hospital) were added to the circuit in diluted aliquots at 5-min intervals to give a range of metHb concentrations from 1 to 100%. Single estimations of D\textsubscript{NO}, D\textsubscript{CO}, and D\textsubscript{O\textsubscript{2}} were made.

To confirm that changes in D were due to oxidation of heme, following control human blood estimations, 20 ml of 1.5% (0.1 M) sodium dithionite (molecular weight 174) followed by 20 ml of 1.5 dilution of 3% sodium nitrite, followed by 80 ml of dithionite solution and finally 80 ml of sodium nitrite were added. Single estimations of D\textsubscript{NO}, D\textsubscript{CO}, and D\textsubscript{O\textsubscript{2}} were made after each addition.

Precautions to Avoid Hemolysis

Special precautions were taken to avoid hemolysis and eliminate free Hb from our circuit. In our preliminary experiments, free [Hb] (27) ranged from 12 mg/dl (horse red cells suspended in Hartmanns) to 100 mg/dl (heated whole horse blood). Cells were separated from plasma to remove free Hb arising during transport and storage. They were then resuspended in Hartmanns solution. All washing and priming was performed with Hartmanns. We accounted for hemolysis by measuring K using the inline analyzer and including K as an independent covariate in all analyses.

Statistical Analysis

D\textsubscript{NO} was entered as a dependent variable in one-way ANOVA to examine between-day variation. Because the associations are not linear, nonparametric correlation was used to look at the relationship between D\textsubscript{NO} and D\textsubscript{CO} and Hb, metHb, and O\textsubscript{2}, and Spearman’s ρ was quoted. Linear regression was used to examine the relationship between D\textsubscript{NO}, D\textsubscript{CO}, and D\textsubscript{O\textsubscript{2}} (dependent variables) and viscosity, MCV, and estimated thickness of the COHb and metHb layer as independent variables; human/horse and nitrite/dithionite entered as dummy variables; and K entered as a proxy for red cell damage as a covariate. All analyses were done using SPSS Statistics 21.0 (Chicago, IL).

Sequence of Experiment

The experiments were performed on 7 separate days. On 3 separate days, the following solutions were tested in a single run using a pair of oxygenators: control whole horse blood, horse red cells resuspended in Hartmanns, horse red cells resuspended in 3% saline, whole
follows. The total number of molecules will be directly proportional to the percent concentration. If the molecules form a layer just inside the red cell membrane, the total number of molecules will be proportional to the cube of the depth of the layer. If all of the Hb in the red cell was replaced by metHb and COHb, then the depth would be the half thickness (1 μm). Rearranging:

\[
\text{Depth of layer in } \mu\text{m} = \sqrt{\frac{\text{Methemoglobin + Carboxyhemoglobin}}{100} \times 1}
\]

**Ethics Approval**

The work used human blood, so it fell within the UK use of human tissue legislation. National ethics approval was obtained for UK Research Governance, Human Tissue, Good Clinical Practice, Confidentiality and Data Protection Law via National Research Ethics Service Yorkshire and the Humber South Yorkshire research ethics committee. Local ethics approval was obtained from Papworth Hospital National Health Service Foundation Trust ethics committee.

**RESULTS**

The average calculated MCV for horse red cells was 49.7 fl (SD 0.18) and human red cells 91.8 fl (SD 0.18). The calculated SA was 83.5 μm² (horse) and 153.7 μm² (human).

As with previous studies (7), there was a significant regression of DNO on day number (r = 5.72, n = 225, P < 0.001), partly explained by varying amounts of free Hb as estimated by K (r = 2.34, n = 225, P < 0.02) milliequivalents per liter. The work used human blood, so it fell within the UK use of human tissue legislation. Mean (SD) DNO = 3.08 ml-min⁻¹·Torr⁻¹ (SD 1.47), DCO = 0.83 ml-min⁻¹·Torr⁻¹ (SD 0.4), DNO/DCO = 3.91 (SD 2.05), DNO 3.23 ml-min⁻¹·Torr⁻¹ (SD 0.36), and DCO/DCO = 1.69 (SD 0.98). For Hb less than a concentration of 10 g/dl, there was a significant correlation between DNO and Hb (P = 0.001) (Fig. 1A) and DCO and Hb (P < 0.001, Fig. 1B). For Hb 7–15 g/dl, there was no relationship between DNO and inline Hb (r = 0.12, P > 0.05), but a highly significant relationship between DCO and Hb (r = -0.306, P < 0.001). These trends were similar for both horse and human.

There was no reduction in DNO with viscosity (Fig. 2); indeed, there was a slight rise, which was abolished when K was added as a covariate. There was no increase in DNO, DCO, or DCO2 with increasing cell size; indeed, there was a slight fall (Fig. 3). Dithionite caused significant reversal of the nitrite-induced decline in DNO and vice versa (t = -3.07, P = 0.007, n = 19, Fig. 4).

**Calculation of Depth of metHb and COHb Layer**

The thickness of the metHb + COHb layers can be estimated as follows. The total number of molecules will be directly proportional to the percent concentration. If the molecules form a layer just inside the red cell membrane, the total number of molecules will be proportional to the cube of the depth of the layer. If all of the Hb in the red cell was replaced by metHb and COHb, then the depth would be the half thickness (1 μm). Rearranging:

\[
\text{Depth of layer in } \mu\text{m} = \sqrt{\frac{\text{Methemoglobin + Carboxyhemoglobin}}{100} \times 1}
\]
Effect of Nitrite on NO, CO, and O2 Transfer Within the Red Cell

The fall in both DNO and Dco with increasing metHb suggests that access to the heme is limiting. This explains the reversal with dithionite, which reduces metHb to ferrous Hb (31). The rise in DNO with hemolysis (Fig. 8) shows it is not the reduction in total available Hb causing the change in DNO, but its accessibility; that is, an increase in diffusion distance to available Hb active sites. It seems likely that outer layers of Hb are converted to metHb before inner, so the layer of metHb builds up; this increases the diffusion resistance to gas transfer inside the cell. Figure 7 supports this idea. Diffusion through a layer (in this case of metHb) follows Fick’s law, as applied to a biological membrane:

\[ D^r = k \times \frac{\text{solubility}}{\sqrt{\text{molecular weight}}} \times \frac{\text{surface area}}{\text{thickness}} \]

where \( k \) is a constant. The solubility \( \sqrt{\text{molecular weight}} \) of NO/CO \( \approx 2 \).

From Eq. 8, DNO/DCO will be \( \approx 2 \), and DNO will be proportional to 1/thickness. The slope and intercept of DNO/DCO are \( \approx 2 \) in Fig. 7, supporting the idea that NO and CO transport obeys Fick’s law inside the red cell. Because no increase in DNO occurred with the initial addition of 20 ml of dithionite (Fig. 4), the concentration of 1% of metHb occurring naturally in blood does not seem to limit diffusion. In contrast to DNO and DCO, D2O does not appear dependent on the thickness of the metHb and COHb layer (Fig. 7). This shows a departure from Fick’s law. For about fifty years, facilitated diffusion has been known to transport O2 in concentrated Hb solutions (44). Because it combines reversibly with Hb, unlike NO or CO, it can move rapidly from active site to active site, down the partial pressure gradient. Hughes and Bates (26) have suggested that DLNO may be a better surrogate for DLO2 than for CO membrane conductance (DmCO). Facilitated diffusion of O2 in the red cell and a departure from Fick’s law does not support their view.

The changes in O2, CO, and their ligands with Hb are complex (Fig. 5); as sodium nitrite is added, there is a rise in metHb mirrored by a decline in oxyhemoglobin and PO2. As PO2 falls \( I \), specific blood conductance for CO (\( \theta_{CO} \)) increases, since \( \theta_{CO} \propto (CO)/(Hb)/(PO2) \), so first COHb rises.

**DISCUSSION**

*Summary of Findings*

Our findings fit with the red cell interior, rather than extracellular fluid or red cell membrane, being the site of blood resistance to NO transfer. We saw no fall in DNO, Dco, or D2O with increasing viscosity (falling diffusivity). In fact, a small rise in DNO was seen, which was nonsignificant when K concentration was included as a covariate in the regression model. This is most likely from cell damage, as more viscous solutions were forced around the circuit at high pressure. No fall in DNO, Dco, or D2O was found, despite halving the red cell membrane SA. A highly significant fall in Dso and Dco occurred with nitrite. This was associated with a rise in metHb and reversed by dithionite and hemolysis. We have confirmed a nonlinear relationship between DNO and Hb for human and horse red cells. This is only seen at very low [Hb]. At [Hb] found in most clinical practice (7–15 g/dl), DNO is independent of Hb.
the steep part of the dissociation curve is reached so that specific blood conductance for O$_2$ increases. Looking at Fig. 5, despite massive reduction in oxyhemoglobin, the percentage of reduced Hb (\%oxyhemoglobin / \%metHb) is actually increased. The increase in O$_2$ and increase in percentage of reduced Hb serve to maintain O$_2$ uptake.

**Critique of Methods**

The pH was lower than physiological conditions, because of the compensated metabolic acidosis of stored blood (4). The K was higher, due to the hyperkalemia of stored packed red cells (1). Our observations were made on horse and human blood drawn several days previously. Red cells are fragile and easily damaged by forcing them through narrow channels at high velocity. This becomes a particular problem for NO, since its reaction with free Hb is so much faster than with the intact red cell. The membrane oxygenator is designed to avoid hemolysis because of the disastrous clinical consequences. Previously, our laboratory has run a similar circuit without hemolysis (17). We have assumed that horse and human red cells are identical apart from their size. Actually horse red cells readily form rouleaux, especially with dextrans (41). However, this effect should increase the distance from cell to gas membrane interface and reduce the overall SA.

Finally, we used a spectrophotometer calibrated for PO$_2$ and SO$_2$ in human blood for horse blood. Grosenbaugh et al. (22) show it is legitimate to do so.

Fig. 5. Changes in methemoglobin (metHb; A), carboxyhemoglobin (COHb; B), oxyhemoglobin (OxyHb) and O$_2$ saturation (C), and extracellular measured and intracellular estimated PO$_2$ (D). metHb, COHb, OxyHb, O$_2$ saturation, and extracellular PO$_2$ were measured by a combined CO-oximeter and blood-gas analyzer (Roche Cobas b221). Intracellular estimated PO$_2$ was derived from O$_2$ saturation using an algorithm (36).

Fig. 6. The change in DNO and DCO with metHb. The sketched lines of best fit are shown to display the trend.

Fig. 7. DNO, DCO, and DO$_2$ plotted against estimated depth of the metHb and COHb layer.
Site of Blood Resistance (Work of Others)

Two groups have claimed that the extracellular fluid is the site of blood resistance (2, 3, 29). They used the stopped flow apparatus or competition methods, so boundary layers might bias their conclusions (see Critique of Others’ Methodologies below). It would be odd to have extracellular resistance in vivo, but not in the continuous flow apparatus or oxygenator; gas exchange should not be more efficient in these devices than in life.

One group has claimed O₂-dependent membrane limitation of NO transport by a transmembrane protein (2). While an attractive idea, we have three major concerns. First, DNO in the membrane oxygenator does not change with pO₂ ranging from 20 to 500 Torr at either 14 ppm (7) or 25 ppb NO (Fig. 9). Second, we found a slight but significant increase in DNO in humans, with a pO₂ ranging from 86 to 143 Torr (10) and no difference between 133 and 488 Torr (11). Finally, facilitated diffusion across a membrane violates the Meyer-Overton rule; i.e., membrane permeability of a molecule depends on its oil-water partition coefficient. No violation of this rule has been found (30). Like ourselves, Subczinski et al. (38) found that the resistance of a simulated membrane to NO transfer was less than that of a water layer of the same thickness.

We, Carlsen and Comroe (13), Sakai et al. (35), and Liu et al. (28) have all tried to alter the red cell interior. Carlsen and Comroe found that the rate was greatly slowed when human red cells were shrunk by 3% saline, but not by spherocytes made by heating. Shrinking the red cell should alter the membrane SA and also the interior altering intraerythrocyte diffusion. We could not replicate their finding in horse red cells (9). Possibly this is a species difference, as the horse cell is small and contains less Hb than the human red cell and may not be so easy to shrink. Sakai et al. (35) measured the pseudo-second-order rate constant of NO reacting with liposome encapsulated human Hb over a range of Hb from 1 to 35 g/dl. They used a stopped-flow apparatus, but, because their particles were so much smaller than red cells, incomplete mixing and stagnant layers should not be such a problem. The rate constant decreased with increased particle diameter. Like us, they concluded that intracellular diffusion was the major barrier to NO red cell uptake. They speculated that bound heme caused an increasing diffusion barrier to NO. This seems less likely with the concentrations of NO and red cells that we have used (see Physiological Implications, below). Liu et al. (28) claimed that the NO half-life (t½) is independent of intracellular oxyhemoglobin. When they added NO to a red cell suspension, there was an immediate loss of signal, and then no loss as intracellular Hb was exhausted. However, looking at their Fig. 2, there was a reduced decline after the second aliquot, suggesting slowing as metHb accumulated and increased the diffusion distance. Overall, we, therefore, believe that the balance of evidence from others’ work favors an intracellular barrier.

Critique of Others’ Methodologies

The problem of mixing a rapidly reacting ligand with large particles, avoiding stagnant layers, was first tackled by Carlsen and Comroe (13) over 50 yr ago. They used the continuous flow rapid reaction apparatus at the University of Pennsylvania [see Hughes and Bates (26) for a fuller description]. They measured the rate constant (j′ c in l·mol⁻¹·s⁻¹):

\[-d(NO)/dt = j′(NO)(Hb)\]  

(9)

A dilute solution of human red cells (1/40 = 2.25 × 10⁻⁴ M Hb) was accelerated down one limb of a Y-shaped apparatus at...
2.8 ms\(^{-1}\) = 150 ml/s, and a dilute solution of NO in deoxygenated buffer ([PNO = 150 Torr = 3 \times 10^{-4} M] (R. E. Forster, personal communication) at the same rate down the other. By moving a reversion spectroscopy along the “lower limb of the Y,” the concentration of NO Hb formed at varying times after initial contact of the two reagents could be measured, and \(J_c\) determined. It is then easy to calculate NO \(t^{1/2}\), second-order rate constant of NO (\(K_{c,NO}\)), and specific blood conductance for NO (\(\theta_{NO}\)) from \(J_c\) (see Table 1). Unlike ourselves (see Table 1), they were observing a true second-order reaction, where equimolar amounts of reagent are mixed. The rate slows for NO (\(H_9258\)/\(H_9258\)), where 60 converts seconds to minutes using this method. With Sakai’s method, smaller vesicles are recalculated. Because the donor molecule is equally mixed throughout the reaction vessel, it is assumed that extracellular gradients could thus exist. The reaction pathways of NO with heme and thiol (corrected) model [Eq. 28 in their second paper (15)] is, however, sound and gives sensible \(\theta_O2\) and \(\theta_CO2\) values. We recalculated \(\theta_{NO}\) using this equation and their parameters except 1) \(D'_{NO}\) [where we used our value (APPENDIX)], and 2) \(\beta_{NO}\). \(\beta_{NO}\), the slope of the NO Hb dissociation curve, is a crucial variable. There are scant data in the literature (19). We used a single datum point of 0.01% NOHb saturation [\(S\)-nitrosohemoglobin (SNO)] at 40 ppm (\(P = 0.029\) Torr, \(6.3 \times 10^{-8} M\)) from recent human exposure data by Gladwin et al. (20). This gives SNO/PNO = “\(\beta_{NO}\)” = 3.86 \times 10^{-3} Torr\(^{-1}\). This gives \(\theta_{NO}\) = 3.6 ml NO-mL blood\(^{-1}\)min\(^{-1}\). Torr\(^{-1}\). Although this agrees with others, it may be coincidental. The reaction pathways of NO with heme and thiol depend on PNO, PO2, SO2, and the allosteric conformation of the Hb molecule (21). The relationship between saturation and partial pressure may deviate from a simple reversible ligand/heme dissociation curve.

Deonikar and Kandia (16) designed an interesting reaction apparatus. Like our system, continuous gas and liquid flow are

### Table 1. Literature values for rate constants and \(\theta_{NO}\)

<table>
<thead>
<tr>
<th>Animal</th>
<th>[NO], mol</th>
<th>[Hb], mol</th>
<th>(t^{1/2}), s</th>
<th>(K_{c,1\text{mol}^{-1}s^{-1}})</th>
<th>(J_c,1\text{mmol}^{-1}s^{-1})</th>
<th>(\theta, \text{min}Torr)</th>
<th>Method</th>
<th>Ref. No.</th>
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<td>Human</td>
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<td>1.5 \times 10^{-6}</td>
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<td>3.85 \times 10^{-3}</td>
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<tr>
<td>Dog</td>
<td>7.5 \times 10^{-9}</td>
<td>7 \times 10^{-3}</td>
<td>4.05 \times 10^{-4}</td>
<td>1.9 \times 10^3</td>
<td>190.3</td>
<td>5.1</td>
<td>Indirect from Dm</td>
<td>12</td>
</tr>
</tbody>
</table>

Bold represents measured constant. Others are derived. [NO], nitric oxide concentration; [Hb], hemoglobin concentration; \(t^{1/2}\), half-time; \(K_{c}\), second-order rate constant; \(J_c\), rate constant; \(\theta\), specific blood conductance. \(K_{c}\) (in 1\text{-mol}^{-1}s^{-1}) = (0.693 \times 9 \times 10^{-3})\(t^{1/2}\), where 0.693 = log_2 and 9 \times 10^{-3} is Hb concentration in moles, corresponding to 14.6 g/dl. \(\theta_{NO}\) = \(J_c\) \times 60 \times \alpha_{NO} \times Hb, where 60 converts seconds to minutes, and \(\alpha_{NO}\) is water solubility of NO at 37°C in mol·l^{-1}·Torr^{-1} = 4.93 \times 10^{-3}. \(K_{c}\) = \(J_c\)/1,000. \(J_c\) is in l·mmol^{-1}·s^{-1}. \(\theta_{NO}\) = 2.68 \times 10^{-2}. \(K_{c}\) = 2.68 \times 10^{-2} \(J_c\). *Studies took place in the absence of oxygen. The remainder of the studies were performed in normoxia and physiological pH.

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separated from one another by a semipermeable membrane. They point out that this steady-state system avoids high localized concentrations that occur with saturated NO solutions. There is no decline in concentration over time as with NO donors. Their circuit, therefore, produces a more physiological NO delivery. Like us, they found Hb dependence, but only at <5–10% hematocrit. Unlike us, they found liquid flow rate dependence of NO uptake. This suggests stagnant layers (see following paragraph), perhaps explaining their lower value for $K_c$. Additionally, their dimensions suggest a far smaller SA for gas exchange than our oxygenator, with corresponding loss of efficiency.

We are the only group to use a membrane oxygenator. It has many advantages. It can be purchased off the shelf. It allows use of in vivo concentrations of [NO], carbon monoxide ([CO]), [O₂], and red cells. It is possible to change one variable, e.g., viscosity, and keep others, e.g., hematocrit, blood flow, and membrane SA, constant. It is possible to measure many aspects of O₂, acid base, CO, and NO status every 5 min for several hours. Because we saw no change with solutions of differing viscosity/diffusivity, it appears free of extracellular [NO] gradients found in the stopped flow, competition, and perhaps NO electrode and simple semipermeable membrane techniques. This is confirmed by the lack of effect of changing blood flow: in an early paper, Roughton (33) thought the continuous flow apparatus free of extracellular gas gradients because the O₂ uptake rate did not change if the liquid flow rate was increased threefold. He reasoned that, if significant stagnant layers existed, they would go as flow increased. We also conclude that there is no extracellular resistance in the oxygenator, since we found that a 25-fold change in blood flow rate had no effect on NO uptake (DNO) (7).

We are the only group to try to measure $\theta_{NO}$ in vivo. We calculated

$$\theta_{NO} = \theta_{CO} \times \left( \frac{1}{C_{DNO}} - 1 \right)$$

Taking NO membrane conductance ($D_{MNO}$) as $D_{NO}$ after successive exchange transfusions with oxyglobin in dogs (12), i.e., $\theta_{NO} = 0.6 \times (1/0.61 - 2/4.27)/(1/2.69 - 1/4.27) = 5.1$ ml NO-ml blood⁻¹-min⁻¹-Torr⁻¹. We used a stopped-flow value for $\theta_{CO}$ in dogs (24). There has been less concern with the stopped flow method for the reaction of CO with red cells compared with NO, as it is a much slower reaction. In Fig. 4 of our laboratory’s previous paper (12), it does not look as if we have reached maximum $D_{NO}$ after three successive exchanges. If we set $D_{MNO}$ as 6, that would make $\theta_{NO} = 3.8$ ml NO-ml blood⁻¹-min⁻¹-Torr⁻¹.

Physiological Implications

Though we were most interested in diagnostic [NO], our results inform endogenous NO biology in vivo. Recent estimates (23) put tissue [NO] “at 100 pM (or below) up to 5 nM” (10⁻¹⁰ to 5 × 10⁻⁹ M). We investigated these concentrations in our last experiment using in vivo (alveolar) gas concentrations (6) 25 ppb equivalent to 42 pM tissue concentration. We showed no change in DNO with [NO], viscosity, or red cell size, but change with nitrite/dithionite, all consistent with Fick’s law. We found no difference across a wide range of O₂ tensions from arterial to venous values. Thus the only blood uptake mechanism we could show was red cell interior diffusion limitation. No effect of viscosity means that the extracellular diffusion gradient is zero. In other words, the plasma partial pressure of NO is the same everywhere. One of the purposes of NO biology is how NO released from the endothelium can stay in the circulation and dilate smooth muscle when it is only separated from a massive Hb “sink” by a thin red cell membrane. Biochemical pathways have been suggested (21); here we offer an alternative. The red cell interior has long been described as “freely rotating Hb molecules in a close-packed lattice” (32). We believe this lattice impedes NO diffusion. We do not believe it can be an “advancing front” where NO “takes out” surface oxyhemoglobin molecules, progressively increasing the diffusion distance to active heme sites. In our oxygenator or in a $D_{NO}$ single breath, there are a million hemes for every NO molecule.

Practical Implications for $D_{NO}$ and $D_{LCO}$ Measurements

Correction of $D_{NO}$ for Hb. Based on these experiments, we believe that the blood resistance to NO transfer rises as the [Hb] drops <7 g/dl. This is not because the distance for diffusion to the red cells increases, nor because the effective red cell SA falls. It is because there are fewer accessible heme sites. In support of the work by van der Lee et al. (39) pre- and posttransfusion and Zavorsky’s (47) advice, there appears to be no need to correct $D_{NO}$ for Hb over the range in routine clinical practice. When Zavorsky recommended the range 8–15 g/dl, the only information available was rather noisy data from horse blood (7). From Fig. 1A, there actually seems to be a minimal fall in DNO down to 7 g/dl and not much down to 5 g/dl. Where we disagree with van der Lee et al. (39) is their conclusion: “Because $D_{NO}$ is independent of Hb concentration . . . $\theta_{NO}$ can be considered to be infinite.” If $\theta_{NO}$ is constant over 7–15 g/dl, $D_{NO}$ will likewise be independent of [Hb]. This happens from the known kinetics of the NO reaction with the red cell. Equation 9 is second order; the rate of reaction is proportional to the concentration of NO and the concentration of red cells. As both reagents combine, they are used up, and the reaction slows. This kinetic relationship holds when the concentrations of NO and red cells are roughly equal. In our experiments and diagnostic work, red cells are substantially in excess, so the concentration does not change as the reaction proceeds. The kinetics of Eq. 9 then become “pseudo-first order” rather than second order, i.e., $-d(NO)/dt = j_c \times (NO)$, where $j_c = j_x \times (Hb)$. In turn, $\theta_{NO} = j_x \times 60 \times \alpha/NO/1.1 = 4.5$ ml NO-ml blood⁻¹-min⁻¹-Torr⁻¹ rather than infinity (Table 1).

For $D_{NO}$, Eq. 11 needs to be adapted to the special case when $\theta_{NO}$ is constant. Additionally, Vc and Dm vary together (25). Vc is proportional to Dm⁻³/₂, since volume/SA is proportional to diameter⁻³/₂, i.e., $1/D_{NO} = 1/D_{MNO} + 1/\theta_{NO} D_{MNO}$. This reduces to $D_{NO} = 1/[1 + k \sqrt{D_{MNO}/\theta_{NO}}]$. $D_{NO}$ is, therefore, approximately proportional to Dm. We have found the constant of proportionality to be ~0.6 (12).

Which value of $\theta_{NO}$ to use. Given the practical problem of measuring such a reactive ligand in biological solutions, the agreement among the bottom four results using differing techniques and species (Table 1) is good. Because other methods of technique may underestimate the rate constant because of
extracellular [NO] gradients, we would still advise using \( \theta_{NO} = 4.5 \text{ ml NO-mL blood}^{-1}\text{min}^{-1}\text{Torr}^{-1} \) derived from the value of Carlsen and Comroe (13) for \( f_j = 167 \text{ 1mM}^{-1}\text{s}^{-1} \). The close agreement between their value and our in vivo estimate supports this view. The higher the value for \( \theta_{NO} \) used, the greater the value for \( Vc \) and the lower the value for \( Dm \). In a previous worked example (8), our laboratory showed the effects of using different values for \( \theta_{NO} \). For a healthy 45-yr-old male with Hb of 15.3 g/dL using the steady-state method \( D_{NO} = 184.1 \text{ ml-min}^{-1}\text{Torr}^{-1} \) and \( D_{CO} = 38 \text{ ml-min}^{-1}\text{Torr}^{-1} \). Using \( \theta_{NO} = 4.5 \text{ ml NO-mL blood}^{-1}\text{min}^{-1}\text{Torr}^{-1} \), \( D_{NO} = 184.9 \text{ ml-min}^{-1}\text{Torr}^{-1} \) and \( Vc = 80 \text{ ml} \), whereas using \( \theta_{NO} = \text{ infinity} \), \( D_{CO} = 92 \text{ ml-min}^{-1}\text{Torr}^{-1} \) and \( Vc = 108 \text{ ml} \).

**Implications for \( \theta_{CO} \).** Our results confirm a red cell diffusive resistance component to CO blood uptake. This is expressed as “\( a^\prime \)”, where total resistance \( 1/\theta_{CO} = a + b(O_2) \), estimated (see **APPENDIX**) as \( \sim 1 \text{ min/Torr} \), in close agreement with others (26).

**Directions for Further Work**

Ideally this work should be repeated in vivo. This would be impossible for the viscosity or cell size experiments. However, in animal models, it should be possible to alter the red cell interior by increasing metHb concentration to 25%. These levels are seen giving nitrite to patients with cyanide poisoning and could be readily reversed with methylene blue. It would also be interesting to look at other species’ red cells of varying shape and size to try to confirm our findings.

**Overall Conclusion**

Taking into account our data and a critical appraisal of the literature, we believe that the balance of evidence favors blood limitation to NO transfer by the red cell interior.

**APPENDIX**

According to Fick’s first law, mass transfer per unit area \( (J) = -D' \Delta C/dx \), where \( D' \) is the diffusion coefficient in cm/s, \( \Delta C \) is concentration gradient, and \( dx \) is infinitesimal distance, i.e.,

\[
J = \frac{M}{SA} = D' \Delta C/dx \quad (A1)
\]

Rearranging total mass transfer

\[
M = \frac{D' SA\Delta C}{dx} = \theta Vc\Delta P \quad (A2)
\]

where \( \Delta P \) partial pressure gradient, \( \Delta C = \alpha_{H_2} O_2 \Delta P \), and \( D' \) is directly proportional to \( \alpha_{H_2} O_2 \), the water solubility, i.e., \( D' = k \alpha_{H_2} O_2 \), i.e., \( k \alpha_{H_2} O_2 \ SA \times \alpha_{H_2} O_2 \Delta P \), \( \theta Vc\Delta P \).

In other words, for NO and CO, considering the diffusion element alone of \( \theta_{CO}, \theta_{NO}/\theta_{CO} \) is proportional to \( (\alpha_{NO}/\alpha_{CO})^2 \approx 4 \), giving \( 1 \text{ ml-mL}^{-1}\text{min-Torr} \).

\( D' \) can be calculated from Fig. 7 by mass balance

\[
M = \frac{D' SA\Delta C}{dx} = D_{NO}\Delta P \quad (A3)
\]

Rearranging \( D' = (D_{NO}dx)/(SA \alpha_{NO}O_2) \), \( D_{NO}dx \) is the integral under the slope of Fig. 7. SA is the surface area of the horse red cell = mean SA × red cell count × contact volume, and \( \alpha_{H_2} O_2 \) is the water solubility of NO at 37°C. We get a value for \( Vc \) of 2 ml using either the DNO/DCO method or the traditional method using varying \( P_{O_2} \) and Holland’s value for \( \Theta_{CO} \) (24) = \[1.77 \times 10^{-3}/5/4.6 \times 10^{-5} \times 65.19 \times 10^{-8} \times 60 \times 3 \times 4.63 \times 10^{9}] = 8.98 \times 10^{-6} \text{ cm}^2/\text{s}.

There are limited data for the diffusion coefficient derived from Fick’s first law in the other solutions studied. One approach is to use Wilke and Chang’s formula (43): the \( D' \) of a solute (including a gas) within a given solvent may be predicted by

\[
D' = 7.4 \times 10^{-8} \chi M^2 T/\eta V^{0.6} \text{ cm}^2/\text{s} \quad (A4)
\]

where \( \chi \) is “association factor”, \( T \) is temperature in °K, \( M \) is molecular weight (molecular mass) of solvent, \( \eta \) is viscosity (centipoise), and \( V \) is molecular volume of solute (cm³/mg). For water as solvent \( \chi = 2.6, T = 310/0 \), and for NO \( \chi = 23.6 \text{ cm}^3/\text{mg} \).

The \( \eta \) for water at 37°C is 0.695×10^-2, and horse plasma at 37°C is 1.61. This gives \( D'_{NO} \) (Hartmann) = 3.9 \times 10^{-5} cm/S at 37°C and \( D'_{NO} \) (horse plasma) = 2.7 \times 10^{-5} cm/S at 37°C. Similar calculations give dextran 500 10–30% 1.68 \times 10^{-6} to 3.36 \times 10^{-8} cm²/s at 20°C.

These calculated figures seem supported by direct and indirect measurements of \( D'_{NO} \), e.g., 3 \times 10^{-5} cm²/s (water) (46) at 37°C and 2.79 \times 10^{-5} cm²/s (ox serum) (45) at 37°C. (We obtained this value by multiplying a value for \( D_{O_2} = 1.86 \times 10^{-5} \text{ cm}^2/\text{s} \).

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


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