Significant blood resistance to nitric oxide transfer in the lung

Colin D. R. Borland,1 Helen Dunningham,2 Fiona Bottrill,2 Alain Vuylsteke,2 Cuneyt Yilmaz,3 D. Merrill Dane,3 and Connie C. W. Hsia3

1Department of Medicine, Hinchingbrooke Hospital, Huntingdon; 2Papworth Hospital, Cambridge, United Kingdom; and 3Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, Texas

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Borland CD, Dunningham H, Bottrill F, Vuylsteke A, Yilmaz C, Dane DM, Hsia CC. Significant blood resistance to nitric oxide transfer in the lung. J Appl Physiol 108: 1052–1060, 2010. First published February 11, 2010; doi:10.1152/japplphysiol.00904.2009.—Lung diffusing capacity for nitric oxide (DLNO) is used to measure alveolar membrane conductance (DMNO), but disagreement remains as to whether DMNO = DLNO, and whether blood conductance (θBC) is infinite. Our previous in vitro and in vivo studies suggested that θBC < ∞. We now show in a membrane oxygenator model perfused with whole blood that addition of a cell-free bovine hemoglobin (Hb) glutamer-200 solution increased diffusing capacity of the circuit (D) for NO (DN0) by 39%, D for carbon monoxide (DCO) by 24%, and the ratio of DNO to DCO by 12% (all P < 0.001). In three anesthetized dogs, DLNO and DLCO were measured by a rebreathing technique before and after three successive equal volume-exchange transfusions with bovine Hb glutamer-200 (10 ml/kg each, total exchange 30 ml/kg). At baseline, DLNO/DCO = 4.5. After exchange transfusion, DLNO rose 57 ± 16% (mean ± SD, P = 0.02) and DLNO/DCO = 7.1, whereas DLCO remained unchanged. Thus, in vitro and in vivo data directly demonstrate a finite θBC. We conclude that the erythrocyte and/or its immediate environment imposes considerable resistance to alveolar-capillary NO uptake. DLNO is sensitive to dynamic hematological factors and is not a pure index of conductance of the alveolar membrane tissue. With successive exchange transfusion, the estimated in vivo θNO [5.1 ml NO·(ml blood−min-Torr−1)] approached 4.5 ml NO·(ml blood−min-Torr−1), which was derived from in vivo measurements by Carlsen and Comroe (J Gen Physiol 42: 83–107, 1958). Therefore, we suggest use of θNO = 4.5 ml NO·(min-Torr−ml blood−1) for calculation of DMNO and pulmonary capillary blood volume from DLNO and DLCO.

Lung diffusing capacity; membrane diffusing capacity; carbon monoxide; lung diffusing capacity for carbon monoxide; lung diffusing capacity for nitric oxide; gas exchange; membrane oxygenator; exchange transfusion; bovine hemoglobin glutamer; blood substitute

NITRIC OXIDE (NO), in combination with carbon monoxide (CO), has been used to measure gas transfer in the lung since the 1980s (5, 11, 30, 31). Similar to CO, NO combines with high affinity to hemoglobin (Hb), but, in contrast to CO, the rate of combination is ~250 times faster (5, 12), so that the lung diffusing capacity for NO (DLNO) is a measure of diffusive gas conductance independent of chemical reaction. Lung transfer of a gas that combines with Hb can be described by the equation of Roughton and Forster (25), which was originally applied to CO

\[
\frac{1}{D_l} = \frac{1}{D_m} + \frac{1}{θV_c} \tag{1}
\]

where Dl is conductance across the lung and Dm is alveolar membrane conductance [ml gas·(min-Torr)−1], θ is the rate of combination of gas (ligand) with erythrocyte [ml gas·(ml blood·min-Torr)−1], and Vc is the pulmonary capillary blood volume (in ml). The left-hand term (1/Dl) represents the overall resistance to lung gas transfer, and the right-hand terms represent the resistance of the membrane (1/Dm) and the resistance of pulmonary capillary blood (1/θVc). The equation for 1/Vc contains a term for diffusion resistance (a) and a term for chemical reaction resistance (b); both terms are hematocrit-dependent. Roughton and Forster exploited this and calculated Dm and Vc by measuring Dl at several PO2 levels and applying known values for θCO derived from in vitro measurements using a rapid-reaction apparatus (25, 26). On the basis of their experiments in healthy adult men, resistance of the membrane was approximately equal to resistance of blood. Guenard et al. (11) assumed that since the reaction of NO with Hb is effectively infinite, θNO must be infinite; therefore, DNO = DMNO = 2DMCO, since the ratio of diffusion of NO to diffusion of CO is directly proportional to the ratio of their respective solubility coefficients (α) and inversely proportional to the ratio of the square root of their molecular weights (MW)

\[
\frac{D_{NO}}{D_{CO}} = \frac{D_{MNO}}{D_{MCO}} = \frac{α_{NO} \sqrt{MW_{CO}}}{α_{CO} \sqrt{MW_{NO}}} = 2 \tag{2}
\]

Implicit in their assumption is that resistance of the erythrocyte membrane, its interior, and the immediately surrounding plasma is negligible. With this assumption, Eq. 1 can be solved from simultaneous measurements of DlCO and DLNO in a single maneuver and with a known value of θCO. The values for Dm and Vc obtained in this way were close to those obtained via DLCO measured at two levels of PO2 (24, 30). Combined DLNO–DLCO measurement offered a rapid way of obtaining Dm and Vc in a variety of clinical situations, obviating the need for multiple DLCO measurements.

However, disagreement soon arose as to whether DLNO = DMNO (1, 4). The in vitro maximum rate constant of NO binding is 500–1,000 times faster with free Hb solution (2 × 105 M−1·s−1) than with red blood cells (12, 33), suggesting the presence of intrinsic transmembrane or intracellular properties of the erythrocyte that limit NO uptake. In 1958, using a rapid-reaction apparatus, Carlsen and Comroe (6) measured in vitro NO reaction with human erythrocytes and found a second-order rate constant for erythrocytes (j′c) of 167 mM−1·s−1, from which θNO = 4.5 ml NO·(min-Torr−ml blood−1) at Hb = 14.6 g/dl can be derived (3, 4), i.e., substantially less than infinity. To address this issue, we used a membrane oxygenator model primed with horse blood to measure in vitro NO transfer (DNO) and eliminated red cell resistance by water-induced hemolysis (2). In this model, hemolysis was accompanied by a rise in DNO, suggesting that red cell resistance to NO transfer was finite, i.e., DNO < DMNO. Water-induced hemolysis is impracticable.
in laboratory animals or isolated lung preparations, inasmuch as it disrupts the endothelium and epithelium, immediately altering in vivo DM. However, a call-free heme-based blood substitute (bovine Hb glutamer-200) is approved for canine clinical use (13, 14). In this study, we first progressively added bovine Hb glutamer-200 in the membrane oxygenator and examined its effect on DNO, comparing it with in situ hemolysis and with hemolyzed red blood cells. Then we progressively replaced whole blood in anesthetized dogs with bovine Hb glutamer-200 and examined the effect on DNO. We reasoned that if resistance of the blood to NO were significant, in vitro DNO and in vivo DNO would increase progressively as erythrocytes are “bypassed” by replacement with cell-free Hb.

METHODS

In Vitro Experiments

Membrane oxygenator circuit. Our circuit is described in detail elsewhere (2, 9). Briefly, two membrane oxygenators (Cobe Duo CML, Cobe Cardiovascular, Arvada, CO) were connected in series to form a continuous circuit perfused with 1–1.5 liters of horse blood (TCS Biosciences, Buckingham, UK) with hematocrit of 40–50% depending on batch and flowing at 2.5 l/min and 37°C. One oxygenator received a gas flow of 2.5 l/min of a mixture of 22% O2, 0.02% CO, 5 parts per million (ppm) NO, and the remainder N2; the second oxygenator scrubbed the oxygenated blood with 8.3% CO2 in N2. In this way, near-physiological levels of O2, CO2, and pH were maintained over several hours in a steady state without apparent hemolysis (9).

Diffusing capacity measurements. DNO and DCO were measured from the difference in gas concentrations leaving and entering the first oxygenator as follows (2)

\[
D_{NO} = \frac{273}{(273 + Temp)[P_h \cdot RH] - (P_{H2O} \cdot RH)} \cdot \ln \left( \frac{NO_{in}}{NO_{out}} \right)
\]

where Temp is ambient temperature (°C), Pn is barometric pressure (Torr), P_{H2O} is saturated water vapor pressure (Torr), RH is relative humidity, and NOin and NOout are NO concentrations at the inlet and outlet ports. For CO, the calculations were identical, except COin and COout were substituted for Nin and Nout.

Addition of blood substitute. Bovine Hb glutamer-200, a cell-free heme-based blood substitute licensed for canine clinical use (Oxyglobin, Dechra, Overland Park, KS), was added in 25-ml aliquots at 5-min intervals to a total of 250 ml via the oxygenator blood-sampling port, and DNO and DCO were measured after each addition.

Comparison with hemolysis. Oxyglobin was added to a total of 250 ml in 25-ml aliquots, and hemolysis was induced by addition of 100-ml aliquots to a total of 700 ml and a potassium concentration >9.5 mmol/l.

Statistical analysis. DNO was entered as a dependent variable in ANOVA (SPSS version 17.0, SPSS, Chicago, IL). Solution added (Hb or Oxyglobin) was entered as an independent variable, and volume added was entered as a covariate.

Animal Experiments

The Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center approved all protocols and procedures. Three purpose-bred adult male foxhounds [28.3 ± 1.0 kg body wt (mean ± SD)] were studied. Circulating blood, plasma, and red cell volumes were determined by the Evans blue dye-dilution method (22) on a separate day before the main study while the animal was awake and at rest.

Anesthesia and catheterization. The animal was fasted overnight and premedicated with acepromazine (0.2 mg/kg) and glycopyrrolate (0.01 ml/kg) by subcutaneous injection. A peripheral intravenous catheter was inserted. Anesthesia was induced with intravenous propofol and maintained by an infusion of ketamine and diazepam titrated to effect. Additional boluses of propofol (0.3–0.5 mg/kg iv) were administered as needed to maintain deep anesthesia and minimize fluid load. A urinary catheter was placed to monitor fluid balance. The animal was intubated with a cuffed endotracheal tube, placed supine, and mechanically ventilated (model 607, Harvard Apparatus, Millis, MA) at a tidal volume (10–12 ml/kg) and rate (18–24 breaths/min) sufficient to suppress spontaneous ventilation. Transcutaneous O2 saturation and heart rate were monitored continuously. One external jugular vein was catheterized with an 8.5-French introducer and one femoral artery with a 5-French catheter. Through the jugular introducer, a pulmonary artery catheter was placed, and its position was verified by pressure tracing. The catheters were flushed with heparinized saline and saturated to skin. A balloon-tipped polyethylene catheter was positioned in the distal one-third of the esophagus, and the balloon was inflated with 0.5–1.0 ml of air for measurement of esophageal pressure.

Blood analyses. Arterial and mixed venous blood samples (3 ml) were drawn, at baseline and on completion of rebreathing maneuvers following each exchange transfusion, for measurement of Hb concentration, conventional blood gases, and O2 saturation by blood analyzers (models ABL720 and OSM3, Radiometer America, Westlake, OH). The instruments were calibrated for dog blood. Hematocrit was determined using a microcapillary centrifuge.

Pressure-volume curves. Static pressure-volume curves were measured at the beginning and end of the experiment by stepwise increments and then decrements (15, 30, 45, and 60 ml/kg) of inflation volume delivered using a calibrated syringe from end-expiratory lung volume. Transpulmonary pressure was calculated as the difference between mouth and esophageal pressures. Paired measurements at each volume were averaged.

Rebreathing measurements. Rebreathing was measured as described elsewhere (19). Briefly, a known volume (45 ml/kg) of test gas mixture containing 0.3% CO, 0.3% methane, 0.8% acetylene, and 40–55 ppm NO in balance of 21% or 99% O2 was delivered from end-expiratory lung volume via a calibrated syringe and mixed with resident gas in the lung by rebreathing maneuvers for 20 s. Gas concentrations were measured at the end of the endotracheal tube. Concentrations of methane, acetylene, and CO were measured using rapid-response infrared analyzers (Act II S, Sensors, Saline, MI). NO concentration was measured by chemiluminescence (model NOA 280, GE Analytical Instruments, Boulder, CO). Duplicate measurements at each inspired O2 fraction were averaged. On completion of these procedures, a separate rebreathing maneuver (20 s) was performed while the animal breathed 100% O2 followed by slow (over 7 s) exhalation, during which alveolar NO concentration was measured. In addition, CO backpressure was measured.

Exchange transfusion. After baseline measurements, 10 ml/kg of blood was removed via the femoral artery catheter into a collection bag containing citrate-phosphate-dextrose-adrenaline (CPDA-1, Fenwal, Lake Zurich, IL) and replaced with an equal volume of Oxyglobin bag containing citrate-phosphate-dextrose-adenine (CPDA-1, Fenwal, Lake Zurich, IL) and replaced with an equal volume of Oxyglobin. Additional boluses of propofol (0.3–0.5 mg/kg iv) were administered as needed to maintain deep anesthesia and minimize fluid load. A urinary catheter was placed to monitor fluid balance. The animal was intubated with a cuffed endotracheal tube, placed supine, and mechanically ventilated (model 607, Harvard Apparatus, Millis, MA) at a tidal volume (10–12 ml/kg) and rate (18–24 breaths/min) sufficient to suppress spontaneous ventilation. Transcutaneous O2 saturation and heart rate were monitored continuously. One external jugular vein was catheterized with an 8.5-French introducer and one femoral artery with a 5-French catheter. Through the jugular introducer, a pulmonary artery catheter was placed, and its position was verified by pressure tracing. The catheters were flushed with heparinized saline and saturated to skin. A balloon-tipped polyethylene catheter was positioned in the distal one-third of the esophagus, and the balloon was inflated with 0.5–1.0 ml of air for measurement of esophageal pressure.

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Recovery. The catheters were removed, and pressure was applied until bleeding stopped. On the next day, a venous blood sample was collected for measurement of hematocrit and Hb concentration, and a portion (10 ml/kg) of the removed blood was warmed to body temperature and
reinfused into the dog via a peripheral vein with use of a blood filter (Baxter, Deerfield, IL). This procedure was performed twice more on consecutive days until all the removed blood had been reinfused.

Data analysis. Lung volumes were calculated from methane dilution. Pulmonary blood flow was calculated from the logarithmic disappearance of CO and NO, respectively, relative to methane (27, 30). Gas exchange septal (tissue + blood) volume was calculated from the extrapolated intercept of the acetylene disappearance curve (28). DMCO and Vc were estimated by three methods, each with the assumption of different values of \( \theta_{CO} \) or \( \theta_{NO} \) (Table 1). 1) by the Roughton-Forster (RF) method (Eq. 1) from DLCO measured at two alveolar Po2 (P\(_{A}\)) levels and the \( \theta_{CO} \) value given by Holland (18) for canine whole blood incorporating a temperature correction for 39°C and made at pH 7.4 using a stopped-flow apparatus (17), calculated from the mean PAO2 during rebreathing and Hb concentration ([Hb])

\[
\frac{1}{\theta_{CO}} = (0.929 + 0.00517 \cdot P_{A}O_{2}) \frac{14.6}{[Hb]}
\]  

2) from simultaneously measured DLNO and DlCO (NO-CO method) (5, 11) with the assumption that \( \theta_{NO} = \infty \) (DlNO = DMNO) and an empirical DLNO-to-DlCO ratio = 2.34 measured in 69 dogs cumulatively studied in our laboratory (unpublished observation), and 3) by the NO-CO method as described above, but with the assumption of a finite \( \theta_{NO} = 4.5 \text{ ml NO-(ml blood-min-Torr)}^{-1} \) derived from the j/c of Carlsen and Comroe (6) and DMCO = DlNO/2. Equation 1 was solved for CO and NO in turn using Mathematica software (Wolfram Research, Champaign, IL). This approach gives a solution identical to that previously published (3). These data were expressed as means ± SD and compared before and after exchange transfusion by repeated-measures ANOVA. \( P < 0.05 \) was considered significant.

RESULTS

In Vitro Experiments

Addition of Oxyglobin to our oxygenator circuit increased DNO from 13.4 ± 0.115 to 18.6 ± 1.659 (SD) ml-(min-Torr)\(^{-1}\) \((n = 3, P < 0.001)\), while Dco increased from 1.075 ± 0.05 to 1.33 ± 0.139 ml-(min-Torr)\(^{-1}\) \((n = 3, P = 0.006\); Fig. 1). The increase in DNO after addition of Oxyglobin was not significantly different \((P = 0.44)\) from that after addition of hemo-lyzed free Hb, whereas the mean Dco was significantly greater with Oxyglobin than with free Hb \((P = 0.001)\). No significant further increase in DNO or Dco was observed after hemolysis was induced in the oxygenator circuit (Fig. 2). A highly significant between-run effect was observed for DNO \((P < 0.001)\) and Dco \((P = 0.002)\).

In Vivo Experiments

Baseline hematological parameters measured during wakefulness at rest were normal in all three animals [hematocrit = 0.45 ± 0.02, plasma volume = 74.5 ± 6.3 ml/kg, red cell volume = 60.0 ± 6.6 ml/kg, and total blood volume = 134.6 ± 11.5 ml/kg (mean ± SD)]. All animals recovered from exchange transfusion without adverse effects, except for temporary darkening of the mucous membrane and the urine. From baseline to postexchange, pressure-volume curves and systemic and pulmonary arterial blood pressures were unaltered (data not shown). Hematological data and arterial blood gases are shown in Fig. 3. DlNO was not corrected for reduction in Hb, since the anticipated alteration was <10%.

As expected, exchange transfusion reduced arterial hematocrit from 40.3 ± 3.3% to 23.2 ± 14%. There were also absolute declines in postexchange Hb concentration from 13.5 ± 1.5 to 11.4 ± 0.3 g/dl and in arterial O2 saturation from 100 ± 0% to 95 ± 4% \((P < 0.05)\) without significant changes in arterial PCO2 or pH.

Rebreathing data obtained during 21% O2 breathing are summarized in Table 2 and Fig. 4. Compared with baseline, DLNO increased 59% after exchange transfusion \((P = 0.021)\), while mean PAO2, lung volumes, and DlCO did not change. With use of Eq. 4 and the assumption that \( \theta_{NO} = \infty \), estimates of DMCO by the RF or NO-CO method \((methods 1 and 2, respectively, in Table 1)\) were similar at baseline. As a result of a rising DLNO with each
exchange transfusion, DMCO estimated by method 2 progressively increased, whereas the corresponding DMCO estimated by method 1 did not change (Fig. 5, left). In a reciprocal relationship, Vc estimated by method 2 progressively declined with exchange transfusion compared with the corresponding Vc estimated by method 1 (Fig. 5, right).

DMCO and Vc calculated using different values of θNO and θCO are given in Table 2, and their comparisons are shown in Table 3. At baseline, methods 1 and 2 (NO-CO assuming θNO = ∞) yielded similar estimates of DMCO and Vc. Method 3 [NO-CO assuming θNO = 4.5 ml NO·(ml blood·min·Torr)⁻¹] approximately doubled the estimated DMCO and halved the estimated Vc compared with method 2. With successive exchange transfusions, estimates of DMCO and Vc by method 1 remained relatively stable until 30 ml/kg of blood had been exchanged. In contrast, method 2 yielded progressively increasing values of DMCO and declining values of Vc with each exchange transfusion. Method 3 also yielded increasing values of DMCO with exchange transfusion, while the estimates of Vc were more stable.

Using the in vivo data (Table 2) and applying Eq. 1 to NO and CO individually (rearranged in Eq. 5), we may estimate the likely range of θNO values

\[
θ_{NO} = θ_{CO} \times \left(\frac{1}{DL_{CO}} - \frac{1}{DM_{CO}}\right)/\left(\frac{1}{DL_{NO}} - \frac{1}{DM_{NO}}\right) \quad (5)
\]

At baseline, DLCO = 0.61 and DLNO = 2.69 ml·(min·Torr)⁻¹. Since DMCO = 0.5 DMNO, 1/DMCO = 2/DMNO. θCO was calculated at 141 Torr arterial PO2 (Table 2) = 0.6 ml CO·(ml blood·min·Torr)⁻¹. With the assumption that with each successive exchange transfusion the measured DLNO (line 10, Table 2) more closely approximates true conductance of the tissue membrane (DLNO → DMNO),
repeated-measures ANOVA), while DLCO in each animal (open symbols, solid

Fig. 4. In vivo lung diffusing capacity for NO (DLNO) in individual animals (closed symbols, dashed lines) rose significantly after successive exchange transfusion with Oxyglobin solution compared with baseline (P < 0.05 by repeated-measures ANOVA), while DLCO in each animal (open symbols, solid lines) remained unchanged.

Table 2. In vivo data

<table>
<thead>
<tr>
<th>Exchange Transfusion, ml/kg</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
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</tr>
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<tr>
<td>Hb, g/dl</td>
<td>13.5 ± 1.5</td>
<td>12.4 ± 0.8</td>
<td>11.8 ± 0.4</td>
<td>11.4 ± 0.3</td>
<td>*</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>40.3 ± 3.3</td>
<td>31.8 ± 3.8</td>
<td>26.5 ± 1.3</td>
<td>23.2 ± 1.4</td>
<td>*</td>
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<tr>
<td>Mean alveolar Po2, Torr</td>
<td>141 ± 3</td>
<td>141 ± 6</td>
<td>137 ± 7</td>
<td>135 ± 2</td>
<td>*</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>119 ± 5</td>
<td>107 ± 11</td>
<td>100 ± 22</td>
<td>106 ± 10</td>
<td>*</td>
</tr>
<tr>
<td>Pulmonary blood flow, ml·(min·kg)⁻¹</td>
<td>121 ± 5</td>
<td>114 ± 20</td>
<td>119 ± 18</td>
<td>109 ± 10</td>
<td>*</td>
</tr>
<tr>
<td>End-expiratory lung volume, ml/kg</td>
<td>32 ± 5</td>
<td>32 ± 5</td>
<td>31 ± 5</td>
<td>31 ± 8</td>
<td></td>
</tr>
<tr>
<td>End-inspiratory lung volume, ml/kg</td>
<td>84 ± 5</td>
<td>84 ± 4</td>
<td>83 ± 5</td>
<td>83 ± 8</td>
<td></td>
</tr>
<tr>
<td>Septal tissue volume, ml/kg</td>
<td>5.10 ± 2.15</td>
<td>4.88 ± 1.84</td>
<td>3.80 ± 1.45</td>
<td>4.30 ± 1.92</td>
<td></td>
</tr>
<tr>
<td>DlNO, ml·(min·Torr·kg)⁻¹</td>
<td>0.61 ± 0.05</td>
<td>0.61 ± 0.10</td>
<td>0.61 ± 0.06</td>
<td>0.60 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>DlNO, ml·(min·Torr·kg)⁻¹</td>
<td>2.69 ± 0.44</td>
<td>3.01 ± 0.57</td>
<td>3.81 ± 0.92</td>
<td>4.27 ± 1.08</td>
<td></td>
</tr>
</tbody>
</table>

Method 1 (RF)

DmCO, ml·(min·Torr·kg)⁻¹ | 1.08 ± 0.11 | 1.07 ± 0.23 | 1.21 ± 0.10 | 1.01 ± 0.21 |
Vc, ml/kg                | 2.75 ± 0.33 | 2.96 ± 0.19 | 2.73 ± 0.26 | 3.47 ± 0.64 |

Method 2 (NO-CO, θNO = ∞)

DmCO, ml·(min·Torr·kg)⁻¹ | 1.15 ± 0.19 | 1.29 ± 0.29 | 1.63 ± 0.39 | 1.82 ± 0.46 |
Vc, ml/kg                | 2.57 ± 0.33 | 2.42 ± 0.22 | 2.18 ± 0.05 | 2.06 ± 0.35 |

Method 3 (NO-CO, θNO = 4.5)

DmCO, ml·(min·Torr·kg)⁻¹ | 2.23 ± 0.70 | 2.68 ± 0.86 | 4.55 ± 2.28 | 4.28 ± 2.31 |
Vc, ml/kg                | 1.70 ± 0.25 | 1.66 ± 0.12 | 1.52 ± 0.06 | 1.66 ± 0.03 |

Values are means ± SD. RF, Roughton-Forster method using θNO from Eq. 4. NO-CO method assumes DlNO/DmCO = 2.34 and θNO = ∞ or θNO = 4.5. Repeated-measures ANOVA and post hoc Fisher’s protected least significant difference: *P ≤ 0.05 vs. exchange transfusion; †P ≤ 0.05 vs. method 1.

in vivo θNO values were as follows: 0.6 × (1/(0.61 - 2/(2.01)))/(1/2.69 - 2/3.01) = 14.8, 0.6 × (1/(0.61 - 2/3.81))/(1/2.69 - 2/3.81) = 6.1, and 0.6 × (1/(0.61 - 2/4.27))/(1/2.69 - 2/4.27) = 5.1 ml NO·(ml blood·min·Torr)⁻¹ after 10, 20, and 30 ml/kg exchange, respectively; i.e., with each exchange, the in vivo θNO declined in a nonlinear fashion toward the in vitro value of 4.5 ml NO·(ml blood·min·Torr)⁻¹ (4).

DISCUSSION

Summary of Findings

In vitro and in vivo replacement of whole blood with hemolyzed blood and a cell-free Hb solution (Oxyglobin) caused DlNO to increase progressively, suggesting that DmNO exceeds DlNO. The final value achieved using Oxyglobin in vitro is identical to that after hemolysis. The cell-free Hb solution penetrates the plasma surrounding the erythrocytes and between the erythrocytes and the endothelial layer; it reduces blood resistance by “bypassing” the erythrocyte membrane and increases the effective surface area of tissue membrane available for gas exchange by allowing all perfused tissue surfaces to directly contact Hb. On the basis of our data, it is almost certain that θNO in vitro and in vivo is substantially less than infinity; the true in vivo θNO is probably close to the in vitro estimate of 4.5 ml NO·(ml blood·min·Torr)⁻¹ given the results from Eq. 5. The only (and unlikely) alternative explanation would be the presence of a periendothelial plasma layer that offered the sole blood resistance to NO transfer. Estimates of DmCO and Vc obtained by the combined NO-CO methods (methods 2 and 3) are highly sensitive to the addition of cell-free Hb, while estimates obtained by the RF method (method 1) are relatively insensitive, because the former use DlNO in their calculation. DlNO increases with exchange transfusion.

Critique of Methods

Although the in vitro post-Oxyglobin, post-hemolysis blood, and post-Oxyglobin posthemolysis values of Dso are similar, they are appreciably lower than those reported in our previous work (2). The marked between-run variation observed on the same day with blood from a single horse shows that the variation is between oxygenators.

Oxyglobin does not cause hemolysis (14), and repeated administration over 1 yr caused no adverse physiological effects in dogs (13). The Hb content of Oxyglobin (13 g/dl) is similar to that of baseline canine blood under anesthesia (13.5 g/dl). After equal volume exchanges of blood with the Hb solution, arterial Hb concentration progressively declined to 11.4 g/dl, likely because of the known plasma-
expanding effect of Oxyglobin. We followed the clinical slow-infusion protocol (10 mg/kg over 1 h) up to the recommended maximum (30 mg/kg total) to minimize, but not eliminate, the effect. Hemodilution would have reduced DLCO more than DLNO, because the blood resistance to CO transfer is greater than that to NO transfer.

A small decrease in DLCO due to hemodilution could have offset a small expected increase in DLCO because of reduced rightward shift of the O2 dissociation curve caused by Oxyglobin. Using the oxygenator, we observed a considerably no in vitro data for the reaction kinetics of CO or NO with blood; given the predominant reaction dependence of Dco, this likely reflects a slower reaction rate of CO in vivo compared to NO with blood in the oxygenator than with blood replacement by Oxyglobin than with blood replacement by lysed blood; given the predominant reaction dependence of Dco, this likely reflects a slower reaction rate of CO with free Hb in lysed blood than with Oxyglobin. We have no in vitro data for the reaction kinetics of CO or NO with Oxyglobin. Using the oxygenator, we observed a considerably higher DNO-to-Dco ratio than was observed in vivo (DMNO-to-DLCO ratio); the difference has been discussed previously (2) and attributed to the design of the multipore membrane, where gas directly contacts a thin boundary layer of blood, resulting in a higher Dm-to-Vc ratio in the oxygenator than in the lung.

There was a modest reduction in arterial and mixed venous O2 saturation with exchange transfusion, consistent with a rightward shift of the O2 dissociation curve caused by Oxyglobin [Po2 necessary to obtain 50% O2 saturation of Hb (Pso2) = 34 Torr] (8). We observed a similar rightward shift when using the oxygenator (Dunningham and Borland, unpublished observations).

Table 3. Comparison of methods

<table>
<thead>
<tr>
<th>Exchange Transfusion, ml/kg</th>
<th>0</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>DlNO/DlCO</td>
<td>4.44 ± 0.40</td>
<td>4.94 ± 0.54</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NO-CO method using different θNO (method 3/method 2)

<table>
<thead>
<tr>
<th>DMCO</th>
<th>1.90 ± 0.33</th>
<th>2.04 ± 0.31</th>
<th>2.68 ± 0.71</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vc</td>
<td>0.66 ± 0.03</td>
<td>0.69 ± 0.04</td>
<td>0.72 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>0.46 ± 0.02*</td>
<td>0.47 ± 0.02*</td>
<td>0.47 ± 0.02*</td>
</tr>
</tbody>
</table>

Values are means ± SD. Repeated-measures ANOVA and post hoc Fisher’s protected least significant difference: *P < 0.05 vs. 0 ml/kg; †P < 0.05 vs. 20 ml/kg; ‡P < 0.05 vs. 10 ml/kg.
resistance only; mean values of $D_{L\text{NO}}$ before and after exchange transfusion were 2.69 and 4.27 ml·(min·Torr·kg)$^{-1}$, respectively (Table 2). Thus

$$\% \text{ overall resistance due to erythrocytes} = 100 \times \left( 1 - \frac{1}{D_{L\text{NO}}\text{pre}} - \frac{1}{D_{L\text{NO}}\text{post}} \right)$$

$$= \left( 1 - \frac{D_{L\text{NO}}\text{pre}}{D_{L\text{NO}}\text{post}} \right) = \left( 1 - \frac{2.69}{4.27} \right) \times 100 = 37\%$$

This value (37%) represents a lower-limit underestimation of true erythrocyte-related barrier resistance in alveolar capillaries, since only a fraction of erythrocytes was removed.

Values for $\theta_{CO}$

The initial justification for the veracity of the NO-CO method (method 2) used by Guenard et al. (11) was the apparent agreement between values for $D_m$ and $V_c$ obtained by their method and by the traditional RF method (method 1) using the $\theta_{CO}$ values given by Roughton and Forster (25) as $1/\theta_{CO} = a + b\cdot PAO_2 = 0.34 + 0.0061\cdot PAO_2$. Until now, the greatest challenge to their method came from Robert E. Forster himself (10). He acknowledged that the earlier $\theta_{CO}$ value had been obtained at pH 7.8–8.0 and, in 1987, repeated the measurements in red blood cells from five normal individuals at pH 7.4 and 37°C obtaining a new relationship: $1/\theta_{CO} = a + b\cdot PAO_2 = 1.3 + 0.0041\cdot PAO_2$. Using the 1987 value, instead of the 1957 value, of $\theta_{CO}$ gives a 36% lower value for $V_c$ and a twofold greater $D_m\text{CO}$ in normal adult subjects (20), an observation also made by others (35). These later values no longer agree with those obtained from the NO-CO method (method 2) with the assumption that $\theta_{NO} = \infty$.

$DL_{NO}$ and Hb

One way to determine whether $\theta_{NO}$ is infinity or less might be to examine the effect of altering Hb concentration, which might alter $\theta_{NO}$, were it less than infinity, while leaving $D_m$ and $V_c$ unchanged. However, clinical studies involving patients with anemia have yielded conflicting results (1, 23, 32) at least partly attributed to the difficulty of selectively altering $\theta$ without altering $D_m$ and $V_c$ in pathological states. For example, patients with renal failure, especially those undergoing dialysis, will have permanent or temporary alteration in $D_m$ and $V_c$. Because $D_m$ is intrinsically “coupled” to $V_c$ (20), it will vary as $V_c$ varies (3). Furthermore, clinical alterations in Hb may not be sufficient to allow the effect of $\theta$ to be dissected out from other changes. Because the membrane oxygenator is a rigid structure, we were not only able to alter Hb without changing $V_c$ or $D_m$, but we were also able to alter Hb 1,000% between 1 and 10 g/dl (2).

There is also a fundamental chemical kinetic reason why $\theta_{NO}$ may be less than infinity and yet independent of hematocrit. $\theta$ derives from the rate constant for the reactions of Hb and ligands. For NO, the reaction is second order: $d[NO]/dt = j'[NO][HbO_2]$. Roughton and Forster and colleagues extended chemical kinetic theory to red blood cells in vitro and in vivo, so that the reaction for NO becomes $d[NO]/dt = j'[NO][HbO_2]$, where $j'$ is the second-order rate constant for red blood cells (167·mmol$^{-1}$·s$^{-1}$), which equates to $\theta_{NO} = 4.5$ ml NO·(ml blood·min·Torr)$^{-1}$ (4). There are good grounds for thinking that this reaction could be zero-order with respect to Hb and, therefore, just pseudo-first-order for NO. If the Hb concentration is great compared with NO concentration, then so few binding sites would be used in the reaction that altering Hb concentration would have little effect on reaction rate. Inhaling 40 ppm NO gives a plasma concentration of $2.5 \times 10^{-9}$ M compared with $9 \times 10^{-3}$ M for Hb concentration; i.e., Hb is greatly in excess. This argument assumes that the Hb is well mixed in solution. If Hb is packaged in red blood cells and there is an advancing front effect (20), then the effective Hb concentration would be much smaller. Nevertheless, pseudo-first-order kinetics could explain why some clinical studies (32) found no effect of Hb on $D_{L\text{NO}}$.

True Values for $\theta_{NO}$

An apparent inconsistency in the approach of Guenard et al. (11) (method 2) was to accept the earlier $\theta_{CO}$ value in the RF method (25) but reject the data of Carlsen and Comroe (6), which gave a finite value for $\theta_{NO}$ obtained in the same laboratory using the same continuous-flow rapid-reaction apparatus. Their approach may not be as illogical as it first appears: the values for $\theta_{CO}$ and $\theta_{NO}$ may be underestimated unpredictably by diffusion limitation in stagnant layers in the apparatus. This effect is likely to be greater for NO, since it is exclusively diffusion limited, in contrast to CO, which is equally reaction and diffusion limited. The stagnant layer effects are likely to be less in the continuous-flow than in the stopped-flow apparatus. In support of the approach of Guenard et al. (method 2), indirect estimates of $\theta_{NO}$ using thin layers of blood are significantly higher than that reported by Carlsen and Comroe; Heller and Schuster (16) used a value of 14 ml NO·(ml blood·min·Torr)$^{-1}$, which appears to have been calculated from $\theta_{CO} = 9.2$ obtained from Heidelberger and Reeves (15) by multiplying by the ratio of water solubilities ($\alpha_{NO}/\alpha_{CO}$) and Krogh diffusion constants ($k_{NO}/k_{CO}$), although we obtain a higher value: $9.2 \times 0.041/0.021 \times 1.76 = 27.7$ ml NO·(ml blood·min·Torr)$^{-1}$. Chakraborty et al. (7), using computer simulation to eliminate the stagnant layer effect, report $\theta_{NO} = 4.2 \times 10^6$ ml NO·(ml blood·min·Torr)$^{-1}$. This raises the counterargument that stagnant layers could well be present in alveolar capillaries in vivo, a possibility that Hughes and Bates (20) emphasize strongly. Indeed, if, as suggested by this and our previous work (2, 4), $\theta_{NO}$ approximates 4–5 ml NO·(ml blood·min·Torr)$^{-1}$, then the thin film work (15) supports the argument that the major resistance to red cell-associated NO diffusion lies immediately adjacent to, rather than within, the cell or its membrane. This fits in neatly with recent work examining Hb and red cell effect on vasoconstriction and the effect of chemically and physically altering the red cell membrane and observing red cell NO uptake (21). These experiments point to diffusion limitation of NO in vivo by a cell-free zone along the endothelium, an unstirred layer around the red blood cell, and, finally, the red cell membrane itself (21). The respective contribution of these three barriers remains in dispute.

Taken together, our in vitro experiments favor the red cell membrane and its surrounding layer as a major source of the...
diffusion resistance to NO. Reducing hematocrit will proportionately increase plasma and reduce red cell surface area by approximately (hematocrit)\(^2\). Although we found a statistically significant relationship between 1/Hb and 1/DNO in vitro, there was little change in DNO above Hb ~4 g/dl. By contrast, the effect of hemolysis and bypassing the red cell membrane using Oxyglobin is striking. One way to circumvent some of these difficulties is to consider the entire diffusion barrier from air-tissue-plasma-erythrocyte to intracellular Hb as a single dynamic resistance (1/DN), measured as 1/DNO, separate from the chemical reaction rate with Hb; then the latter becomes the empirically measured gas uptake by 1 ml of free Hb solution at a given concentration (θ_Hb), i.e., 1/DL = 1/DN + 1/θ_Hb Vc. This approach assumes similar chemical reaction kinetics between free extra- and intraerythrocyte Hb; this assumption may or may not be true (29) and requires further examination.

Use of Blood Substitutes

It is increasingly clear that the “encapsulation” of Hb within erythrocytes markedly slows NO scavenging by heme-binding sites, allowing NO to act as a vasodilator “downstream.” Cell-free heme-based blood substitutes accelerate removal of NO, risking local vasocostriction and potentially limiting their application (34). This work should encourage the development of encapsulated heme-based blood substitutes, and our oxygenator circuit could be used to test the NO-scavenging properties of blood substitutes.

Implication for Clinical Use of DLNO

All the recent reports of DLNO have assumed θ_Hb to be infinite and used DLNO as a pure and direct measure of membrane conductance, i.e., unaffected by any diffusive resistance associated with capillary erythrocytes. Our findings showed this assumption to be untrue: 1/DLNO is 37% erythrocyte resistance and 63% nonerythrocyte resistance. Caution is warranted when interpreting isolated DLNO measurements. Reconciling the different methods and θ values remains challenging. Method 1 (the RF method) yields stable estimates of DM and Vc during exchange transfusion until 30 ml/kg of blood has been exchanged. Method 2 yields DM and Vc that change in opposite directions with exchange transfusion. Method 3 yields relatively stable estimates of Vc, whereas DM increases with exchange transfusion. The important point is to ensure internal consistency within a given study. On the basis of current data, when calculating DMNO and Vc from DLNO and DLCO, we recommend the use of θ_NO = 4.5 ml NO-(min·Torr·ml blood)\(^{-1}\).

Red cell dependence also raises the following question: Should DLNO be adjusted for Hb concentration? With use of the finite θ_NO = 4.5, DMNO = 4.27 ml-(min·Torr·kg)\(^{-1}\), and Vc = 1.7 ml/kg, substituting in Eq. 1 the reduction in DLNO is ~0.1 ml-(min·Torr·kg)\(^{-1}\) per (g/dl) until Hb <8 g/dl. Therefore, a Hb correction is not needed in routine clinical practice.

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