In vitro and in vivo kinetic handling of nitrite in blood: effects of varying hemoglobin oxygen saturation

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Submitted 17 November 2006; accepted in final form 16 May 2007

Blood AB, Power GG. In vitro and in vivo kinetic handling of nitrite in blood: effects of varying hemoglobin oxygen saturation. Am J Physiol Heart Circ Physiol 293: H1508–H1517, 2007. First published May 18, 2007; doi:10.1152/ajpheart.01259.2006.—Growing evidence suggests that nitrite, acting via reduction to nitric oxide by deoxyhemoglobin, may play an important role in local control of blood flow during hypoxia. To investigate the effect of hypoxia (65 Torr arterial Po2) on the kinetic properties of nitrite, a bolus injection of sodium nitrite (10 mg/kg iv) was given to normoxic or hypoxic newborn lambs, and the time course of plasma nitrite and methemoglobin (MetHb) concentrations was measured. The in vivo kinetics of nitrite disappearance from plasma were biphasic and were not affected by hypoxia. Changes in MetHb, a product of the nitrite-hemoglobin reaction, also did not differ with the level of oxygenation. Hypoxia potentiated the hypotensive effects of nitrite on pulmonary and systemic arterial pressures. The disappearance of nitrite from plasma was equivalent to the increase in MetHb on a molar basis. In contrast, nitrite metabolism in sheep blood in vitro resulted in more than one MetHb per nitrite equivalent under mid- and high-oxygenation conditions: oxyhemoglobin (HbO2) saturation = 50.3 ± 1.7% and 97.0 ± 1.3%, respectively. Under the low-oxygenation condition (HbO2 saturation = 5.2 ± 0.9%), significantly less than 1 mol of MetHb was produced per nitrite equivalent, indicating that a significant portion of nitrite is metabolized through pathways that do not produce MetHb. These data support the idea that the vasodilating effects of nitrite are potentiated under hypoxic conditions due to the reduction of nitrite to nitric oxide by deoxyhemoglobin.

nitrite; hypoxia; nitric oxide

NITRITE ENTERS THE BLOOD primarily as a result of oxidation of nitric oxide (NO) produced by endothelial NO synthase (NOS) (26, 41) and uptake from the gastrointestinal tract via reduction of nitrate by commensal bacteria (10). Plasma concentrations have been found to correlate with endothelial NOS activity (26) and are significantly reduced in patients with endothelial dysfunction (25). Nitrite is cleared from the circulation by renal filtration, oxidation to nitrate by reaction with oxyhemoglobin (HbO2), and reduction to NO by reaction with deoxyhemoglobin (23, 43). The latter reaction has received much attention recently as a potential source of NO that is independent of NOS, particularly due to the O2-sensitive nature of the reaction (24). The reduction of nitrite by deoxyhemoglobin is proposed to result in vasoactive quantities of NO in hypoxic tissues. As a result, this reaction would induce vasodilation and increase local blood flow and O2 delivery to hypoxic tissues (4). In examining the physiological relevance of this reaction, it is necessary to determine whether the rate of nitrite reduction to NO is rapid enough to produce sufficient quantities of NO during the transit of a red blood cell through the resistance vessels that regulate blood flow.

Plasma nitrite concentrations are in the low-to-intermediate nanomolar range across a range of mammalian species (8, 26). They reflect a balance between production and entry of nitrite into the circulation, on one hand, and removal of nitrite from the circulation, on the other, a relationship summarized as follows

\[
d[NO_2^-]/dt = (\text{formation rate}/V_{\text{dist}}) - k_d[NO_2^-]
\]

where [NO2] is nitrite concentration, Vdist is the volume of distribution of nitrite, and k0 is the rate constant for irreversible loss. Vdist, nitrite clearance rate, effective half time for disappearance of nitrite from the blood, and the effect of systemic hypoxia on each of these kinetic parameters have not been established in vivo. Their determination would provide insight into the amount of NO that might be derived from nitrite reduction by deoxyhemoglobin in the microvasculature.

Methemoglobin (MetHb), the oxidized (ferrihemoglobin) form of hemoglobin, normally constitutes <1% of total hemoglobin. MetHb is formed by a number of different oxidizing reactions, including the nitrite-deoxyhemoglobin or nitrite-HbO2 reaction, both of which are thought to result in a 1:1 stoichiometry of nitrite loss to MetHb production (for review see Ref. 23), making MetHb a useful indicator of the nitrite-hemoglobin reaction. MetHb is reduced back to hemoglobin by a complex of MetHb reductase enzymes; thus MetHb levels represent a balance between ongoing hemoglobin oxidation and MetHb reduction (22).

This work was undertaken in an attempt to relate the previous work of investigators using purified, completely deoxygynated or oxygenated hemoglobin in buffered solutions with the more physiological conditions of whole blood and mixtures of HbO2 and deoxyhemoglobin to study the nitrite-hemoglobin reaction. In vivo experiments were performed to measure the kinetic constants that characterize the handling of sodium nitrite infused as a bolus into the central circulation of newborn lambs. The experiments were performed under systemic normoxia and hypoxia to evaluate the effects of oxygenation on whole body nitrite metabolism. In addition, to separate the role of nitrite metabolism in the blood from that in the tissues, the kinetic experiments were repeated in isolated whole blood in vitro, and these studies were carried out under low (<10% hemoglobin saturation), mid (45–50%), and high (95%) levels of oxygenation. Because the nitrite-HbO2 or nitrite-
deoxyhemoglobin reaction results in MetHb, molar comparisons of nitrite disappearance and MetHb production were studied to assess the portion of nitrite’s disappearance that could be attributed to reactions with hemoglobin at the various levels of oxygenation.

METHODS

Animal instrumentation. Protocols were approved by the Loma Linda University Animal Care Research Committee. Lambs at 3–7 days of age were obtained from Nebeker Ranch (Lancaster, CA). The lambs were anesthetized with a short-acting barbiturate (10 mg/kg iv), an endotracheal tube was inserted, and anesthesia was continued with 1.5% halothane in O₂ from a volume respirator. Respiratory rate and depth were adjusted periodically to maintain arterial PCO₂ at 35–45 mm Hg. At the end of the experiment, 10% sodium nitrite (Sigma-Aldrich, St. Louis, MO) was dissolved in 5 ml of saline and infused into the central circulation over a 20-s period. A catheter was placed in a carotid artery for blood sampling, and a 5.0 pediatric Swan-Ganz thermodilution catheter (Baxter Healthcare, Irvine, CA) was placed for measurement of pulmonary arterial pressures. Halothane was then discontinued, and anesthesia was maintained thereafter by morphine given intravenously (bolus 0.5 mg/kg and continuing infusion of 0.1 mg·kg⁻¹·h⁻¹) in association with vecuronium (0.1 mg·kg⁻¹·h⁻¹). This regimen was used to mimic clinical maintenance of newborn humans receiving NO therapy.

In vivo study protocol. Each lamb was studied twice, during normoxia and hypoxia, with the order of study varied arbitrarily. For normoxic studies, the fraction of inspired O₂ (FiO₂) was ~0.35; for hypoxic studies, 0.12–0.14 FiO₂ was achieved by admixture of nitrogen to the ventilator circuit.

After instrumentation and 30–60 min of stabilization, blood samples were collected for measurement of baseline plasma nitrite concentration and percent MetHb. Then 10 mg/kg (0.144 mmol/kg) sodium nitrite (Sigma-Aldrich, St. Louis, MO) was dissolved in 5 ml of saline and infused into the central circulation over a 20-s period. Sample collection was timed from the midpoint of the infusion. Arterial blood samples were collected at 12 sequential time points: −10, −5, 1.5, 3, 6, 10, 15, 20, 40, 60, 80, and 120 (nitrite) or 160 (methoxyhemoglobin) min. FiO₂ was changed after 2 h, and after a 60-min stabilization period the study was repeated at the alternate level of oxygenation.

Mean arterial pressure and pulmonary arterial pressure were recorded continuously using calibrated pressure transducers (Cobe Laboratories, Lakewood, CO). Data were recorded using an analog-to-digital converter system (Powerlab, ADInstruments, Otago, New Zealand) and data acquisition software (Chart version 5.2, ADInstruments, Otago, New Zealand) and data acquisition software (Chart version 5.2, ADInstruments, Otago, New Zealand). Exhaled NO was measured continuously in air sampled at the proximal end of the endotracheal tube by chemiluminescence, with a lower limit of detection below 10 ppb.

Each time a blood sample was obtained, 1.5 ml of arterial blood were collected into a heparinized syringe. About 0.4 ml of the sample was analyzed for blood gases and pH (model ABL5, Radiometer, Copenhagen, Denmark) and percent HbO₂ saturation and MetHb concentration (Osm2 heoximeter, Radiometer). Another 0.4 ml of the sample was injected directly into 0.1 ml of nitrite stop solution on ice containing 0.8 M ferricyanide, 0.1 M N-ethylmaleimide, and 10% Nonidet P-40, and the sample was vortexed and immersed in liquid nitrogen. The remainder of the sample was immediately centrifuged for 1 min at 4,500 rpm, and 0.4 ml of the resulting plasma layer was decanted into 0.1 ml of nitrite stop solution, vortexed, and frozen in liquid nitrogen. All samples collected for nitrite analysis were stored at −70°C until the day of analysis. Lambs were given 20 ml of adult sheep blood during the period between the two study protocols.

To test the accuracy of our hemoximetry measurements of MetHb in the presence of nitrosylhemoglobin (HbNO), two samples of human whole blood were prepared and mixed in various known proportions. One sample of blood was completely deoxygenated (HbO₂ < 1%, P O₂ < 1 Torr) by equilibration with 95% N₂-5% CO₂ followed by addition of 1 mM sodium dithionite and then equilibrated with a threefold molar excess of pure NO gas. This procedure resulted in 96.7% HbNO and 3.3% MetHb, as determined by spectrophotometric analysis of the blood across a spectrum of 450–700 nm (Synergy HT, Biotek Instruments, Winooski, VT) followed by fit to standard spectra (provided by Dr. Kim-Shapiro, Wake Forest University). The second sample was prepared by addition of a 10-fold molar excess of sodium nitrite to the deoxygenated blood, resulting in 88% MetHb. Under strict anaerobic conditions, various ratios of the two blood samples were mixed, stirred, and analyzed immediately for percent MetHb. A plot of percent MetHb measured by hemoximetry vs. that expected on the basis of dilution yielded a direct linear relationship with slope = 0.99, intercept = −1.4, r = 0.998, and P < 0.001. A similar procedure in which 100% MetHb whole blood was added in varying proportions to carboxyhemoglobin (COHb) blood also yielded a similar linear relationship: slope = 1.00 and r = 0.994. Thus the presence or absence of HbNO did not alter the validity of percent MetHb readings throughout the study.

To determine whether baseline pulmonary arterial pressures in normoxic lambs could be lowered, a separate group of similarly instrumented newborn lambs (n = 3) was studied. After 30 min of normoxia to establish baseline pulmonary arterial pressures, inhaled NO gas (INO Therapeutics) was administered at 80 ppm for 30 min. In three experiments, the blood volume of the lamb was measured as described by Coburn et al. (3). For this procedure, 15 ml of blood were withdrawn, equilibrated with pure CO to convert all hemoglobin to COHb, tonometered with nitrogen to remove dissolved CO, and then reinfused. The increase of measured circulating percent COHb after infusion, together with hematocrit, enabled us to calculate the red cell mass and blood volume of the lambs.

Kinetic measurements in vitro. Venous blood was collected in heparinized syringes from adult sheep. Blood at three levels of HbO₂ was separated into 100% HbO₂ samples, 0.12–0.14 FiO₂ was achieved by admixture of nitrogen to the ventilator circuit, and then reinfused. The increase of measured circulating percent COHb after infusion, together with hematocrit, enabled us to calculate the red cell mass and blood volume of the lambs.

Thirty milliliters of each blood sample were placed in a sealed flask maintained in a water bath at normal sheep body temperature (39°C). After collection of two baseline samples, 1.0 mg of sodium nitrite (14.4 µmol) dissolved in 0.5 ml of saline was introduced. Inasmuch as hemoglobin concentration ranged from 8–10 g/dl, this resulted in a ≈1:3 nitrite-to-hemoglobin tetramer molar ratio, which is equivalent to a 1:12 nitrite-to-heme molar ratio. In whole blood experiments, hematocrit concentrations ranged from 35 to 41%, resulting in intrathyrocytic nitrite-to-hemoglobin ratios of ≈1:9 and nitrite-to-heme ratios of 1:36, with the assumption that plasma and erythrocytic nitrite concentrations were equal.

In a separate series of experiments to determine the effects of the erythrocyte membrane, 30-ml samples of blood were hemolyzed by two cycles of freezing in liquid nitrogen followed by thawing in warm water. After hemolysis, the nitrite experiments were carried out as described above for whole blood samples.

Blood samples (1.3 ml) were collected anaerobically from the sealed flask at −10, −5, 1.5, 3, 6, 10, 15, 20, 40, 60, and 80 min relative to the nitrite injection. Percent HbO₂ and MetHb were determined by hemoximetry, and the remaining blood was processed in stop solution for measurement of nitrite concentrations (see above).

Nitrate assay procedure. Whole blood mixed with nitrite stop solution was deproteinated with 1:1 (vol/vol) methanol and spun at 15,000 g for 2 min, and 10–200 µl of the supernatant were measured by reductive chemiluminescence with sodium iodide (280i NO ana-
lyzer, Sievers, Boulder, CO). Plasma was injected directly into the chemiluminescence analyzer without deproteinization.

Cumulative nitrite disappearance and MetHb production calculations. The moles of nitrite that disappeared over each sampling interval were calculated by multiplying the difference in measured nitrite concentration at the beginning and end of each interval by the blood volume. MetHb production was calculated in the same manner, but with an added correction factor to account for the reduction of MetHb by MetHb reductase. The correction was made for each sampling interval by use of a first-order rate constant for MetHb reduction of 0.011 ± 0.001 min⁻¹ (n = 6), corresponding to a biological half time of 63 min, as previously determined for adult sheep blood in our laboratory (unpublished data).

Data analyses. Mean arterial blood pressure and pulmonary arterial blood pressure were captured at a rate of 200 samples/s and then averaged into 5-min intervals. Significant differences between control and hypoxic experiments and between baseline and post-nitrite injection periods were detected by two-way ANOVA with Bonferroni’s post hoc analysis for comparison of individual time points (GraphPad Prism 4.0 for Windows, GraphPad Software, San Diego, CA).

Calculated values for the disappearance of nitrite and production of MetHb over time were fit to the following hyperbolic equation: \( y = \frac{A \cdot t}{(B + t)} \), where \( y \) is the cumulative nitrite disappearance or MetHb production in micromoles, \( A \) is the maximum cumulative amount of nitrite or MetHb consumed or produced, \( t \) is time relative to the addition of nitrite, and \( B \) is the nitrite or MetHb concentration at half the time required for disappearance of all the nitrite. Derivatives over the first 30 s of these fitted equations from each experiment were taken as the initial rates of nitrite disappearance or MetHb production.

RESULTS

In vivo nitrite injections. A representative curve of nitrite disappearance from the plasma of a 3-day-old lamb is shown in Fig. 1A. The shape of the curve indicates that nitrite disappearance by first-order decay from a single compartment is not adequate to explain the results, and we selected, instead, a two-compartment model of the following form

\[
[\text{NO}_2^-] = A_1 \exp(-A_2t) + A_3 \exp(-A_4t)
\]

(2)

where \( t \) is time after infusion and \( A_1-A_4 \) are unknown constants that define the time dependence of changes in nitrite concentration. Programs provided by McIntosh and McIntosh (34), a search procedure devised by Marquardt (32), and analyses described previously in detail (38) were used to obtain optimal values for \( A_1-A_4 \) that minimized the variance of the data. These values enabled an accurate description of nitrite loss from the plasma in each of the lambs, and an example of the fitted curve is shown in Fig. 1A.

The volume into which infused nitrite is distributed, \( V_{\text{dist}} \), was calculated by dividing the dose by \( A_1 + A_3 \).

\[
V_{\text{dist}} = \frac{\text{dose}}{(A_1 + A_3)}
\]

(3)

The plasma clearance rate (PCR) was calculated using the Steward-Hamilton equation as previously described (40)

\[
\text{PCR} = \frac{\text{dose}}{\int [\text{NO}_2^-] \, dt}
\]

(4)

where \([\text{NO}_2^-]\) is plasma nitrite concentration at some time \( t \) and \( \int [\text{NO}_2^-] \, dt \) is equal to \( A_1/A_2 + A_3/A_4 \) in terms of parameters of the analytic procedure. This procedure takes into account the tail of the concentration-time curve that extends beyond the time of measurement.

The rate constant for disappearance of nitrite from the plasma, \( k_d \), was calculated as follows: \( k_d = \text{PCR} / V_{\text{dist}} \). The apparent half time for the loss of nitrite was calculated as 0.693/kd.

Six lambs at 5 ± 1 days of age and weighing 5.21 ± 0.37 kg were studied. Each lamb received one nitrite injection during normoxia and one during hypoxia. Three lambs were studied first during hypoxia and then during normoxia, and the remaining three lambs were studied in the reverse sequence.

There were no significant differences in baseline hemoglobin concentration, percent MetHb, or pH between normoxic and hypoxic experiments (Table 1). During hypoxia, mean baseline PO2 was 40 ± 5 Torr and HbO2 saturation was 66 ± 8%; during normoxia, mean baseline PO2 was 125 ± 15 Torr and HbO2 saturation was 99.7 ± 0.1%.

The disappearance of an intravenous bolus of nitrite from the plasma during normoxia and hypoxia is shown in Fig. 2A, and the calculated kinetic parameters are summarized in Table 2. Plasma concentrations 1.5 min after nitrite injection were 478 ± 15 and 461 ± 9 μM (not significant) during normoxia and hypoxia, respectively. During hypoxia, \( k_d \) was 0.0811 ± 0.0017 min⁻¹, which is not significantly different from 0.0806 ± 0.0033 min⁻¹, which was observed during normoxia. The plasma half time of 8.83 ± 0.41 min during normoxia did
Table 1. Baseline arterial blood gases, hemoglobin and MetHb concentration, and HbO₂ saturation of newborn lambs

<table>
<thead>
<tr>
<th></th>
<th>Normoxia</th>
<th>Hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbO₂, %</td>
<td>99.7±0.1</td>
<td>65.8±7.7</td>
</tr>
<tr>
<td>Arterial</td>
<td>66.3±0.7</td>
<td>24±7.7</td>
</tr>
<tr>
<td>Venous</td>
<td>2.6±0.2</td>
<td>2.6±0.1</td>
</tr>
<tr>
<td>MetHb, %</td>
<td>8.4±0.3</td>
<td>8.8±0.3</td>
</tr>
<tr>
<td>Total Hb, g/dl</td>
<td>125±15</td>
<td>125±9.3</td>
</tr>
<tr>
<td>PCO₂, Torr</td>
<td>34.1±1.7</td>
<td>35.8±2.5</td>
</tr>
<tr>
<td>pH</td>
<td>7.42±0.02</td>
<td>7.42±0.06</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 6). HbO₂, oxyhemoglobin; MetHb, methemoglobin.

not differ significantly from 8.46 ± 0.18 min during hypoxia. Other kinetic values were also similar at the two oxygenation levels.

MetHb concentrations (expressed as μM ferriheme) during the 160 min following nitrite injection during normoxia and hypoxia are shown in Fig. 2A. MetHb increased from baseline levels of 47.2 ± 9.2 μM during normoxia and 41.2 ± 11.2 μM during hypoxia. Peak MetHb concentrations were observed at the 40-min time point during normoxia (384.6 ± 27.6 μM) and hypoxia (403.6 ± 52.8 μM). Hypoxia had no effect on changes in MetHb after nitrite injection.

The effect of nitrite on mean systemic pressures was studied in a total of nine animals (Fig. 3). Blood pressure decreased from baseline of 82 ± 4 and 76 ± 6 mmHg during normoxia and hypoxia to nadirs of 63.6 ± 4.8 and 48.8 ± 4.6 mmHg, respectively, within 5 min of infusion. Pressures then gradually increased throughout the remainder of the experiment, approaching baseline values 45 min after nitrite infusion. The overall decrease in blood pressure was greater during normoxia than during hypoxia (P < 0.05), but post hoc analysis did not indicate significance at any particular time point.

The effect of nitrite infusion on pulmonary arterial blood pressure (n = 5) is shown in Fig. 3. On initiation of hypoxia, pulmonary arterial blood pressure increased from 21 ± 2.4 to 33.6 ± 3.3 mmHg. Then, with nitrite infusion, pulmonary arterial pressure decreased, reaching 24.5 ± 3.2 mmHg after 5 min, a significant change (P < 0.01). Thereafter, pulmonary arterial pressure gradually increased to levels higher than those observed during normoxia. Pulmonary arterial pressure was not significantly altered by nitrite infusion in lambs during normoxia. NO was not detectable (limit of detection = 5 ppb) in exhaled air after nitrite injection (data not shown).

Nitrite metabolism in vitro. To separate extravascular reactions of nitrite from reactions in blood, measurements were repeated using blood in sealed flasks at hemoglobin concentrations, pH, and temperature nearly identical to those in the lamb experiments. Blood was studied at three oxygenation states: low (HbO₂ = 5.3 ± 0.9%), mid (HbO₂ = 49.9 ± 1.3%), and high (HbO₂ = 98.1 ± 0.1%). Baseline blood gases, hemoglobin, HbO₂, and MetHb values are shown in Table 3.

The time course of plasma nitrite and whole blood MetHb concentrations after nitrite injection are shown in Fig. 4, and the pharmacokinetic parameters from these data are shown in Table 4. In contrast to the in vivo experiments, the plasma concentration-time curve was adequately described by first-order kinetics from a single compartment. The rate constant for irreversible loss, k4, was more than twofold higher in the mid-O₂ range than in either the low- or high-O₂ experiments. In the mid-O₂ range, k4 averaged 0.0865 ± 0.0042 min⁻¹ as opposed to 0.0339 ± 0.0012 min⁻¹ in the low-O₂ range and 0.0311 ± 0.0019 min⁻¹ in the high-O₂ range. The apparent half time was significantly shorter at midrange O₂ (Table 4).

MetHb concentrations increased at a faster initial rate, reached a higher peak level, and then decayed more rapidly when blood HbO₂ was in the midrange than in either the low or high range (Fig. 4).

Mass balance comparison of nitrite loss and MetHb production. To compare the disappearance of plasma nitrite with MetHb production on a mole-per-mole basis, cumulative disappearance (nitrite) or production (MetHb, reported as μM ferriheme) was summed over each of the sampling intervals (Fig. 5A). MetHb production values were corrected for MetHb
reductase activity (see METHODS). During the first 10 min of normoxia and hypoxia in vivo, the ratio of nitrite disappearance to MetHb production was variable (Fig. 5B), with as much as a fivefold greater nitrite disappearance per mole of MetHb production. After 10 min, however, the ratio remained relatively stable and was not significantly different between normoxia and hypoxia, averaging 0.8 ± 0.1 mol of nitrite disappearance per mole of MetHb production during normoxia and 0.7 ± 0.1 mol of nitrite per mole of MetHb during hypoxia.

Cumulative plasma nitrite disappearance and MetHb production in low-, mid-, and high-oxygenation in vitro experiments is presented in Fig. 6, A–C. In contrast to in vivo experiments, cumulative units of MetHb produced were significantly greater than the amount of nitrite that disappeared in the mid- and high-oxygenation experiments. The ratios of cumulative MetHb production to cumulative nitrite disappearance are shown in Fig. 6D. In whole blood at low O2 saturation levels, the ratio was significantly less than 1.00 (0.73 ± 0.06), indicating that MetHb production could not account for total

Table 3. In vitro baseline blood gas and hemoximeter measurements for adult sheep blood at 39°C at varying levels of HbO2 saturation

<table>
<thead>
<tr>
<th>HbO2 Saturation</th>
<th>Low (n = 4)</th>
<th>Mid (n = 5)</th>
<th>High (n = 4)</th>
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</thead>
<tbody>
<tr>
<td>HbO2, %</td>
<td>5.2 ± 0.9</td>
<td>50.3 ± 1.7</td>
<td>97.0 ± 1.3</td>
</tr>
<tr>
<td>MetHb, %</td>
<td>0.6 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>Hb, g/dl</td>
<td>9.7 ± 0.7</td>
<td>11.4 ± 0.6</td>
<td>10.1 ± 0.6</td>
</tr>
<tr>
<td>Po2, Torr</td>
<td>5.5 ± 2.0</td>
<td>41 ± 1</td>
<td>193 ± 31</td>
</tr>
<tr>
<td>Pco2, Torr</td>
<td>54 ± 6</td>
<td>36 ± 2</td>
<td>42 ± 2</td>
</tr>
<tr>
<td>pH</td>
<td>7.21 ± 0.05</td>
<td>7.38 ± 0.03</td>
<td>7.33 ± 0.01</td>
</tr>
</tbody>
</table>

Values are means ± SE.

Fig. 3. Time course of mean arterial pressure (A, n = 9) and pulmonary arterial pressure (B, n = 5) in normoxic (●) and hypoxic (○) 3- to 7-day-old lambs after injection of sodium nitrite (10 mg/kg) into the central venous circulation. Mean arterial blood pressure decreased significantly (P < 0.01) in normoxic and hypoxic lambs. Hypoxic lambs were significantly more hypotensive than normoxic lambs: *P < 0.05. However, post hoc analysis did not detect significant difference at any single time point. Pulmonary arterial pressure did not change significantly during normoxia experiments but fell transiently during hypoxia from significantly elevated baseline levels (compared with normoxic controls) to values not measurably different from normoxic animals at 5 and 10 min after nitrite injection: *P < 0.01. Effect of inhaled nitric oxide (80 ppm, dashed lines and ⊗, n = 3) demonstrates vasodilating capacity of pulmonary vasculature in normoxic lambs. C: maximal decreases in mean systemic arterial blood pressure (MABP) during normoxia and hypoxia (n = 9). Values are means ± SE.

Fig. 4. Time course of plasma nitrite (A) and MetHb (B) concentrations after bolus injection of sodium nitrite into a 39°C in vitro incubation of whole sheep blood preequilibrated to 0% (n = 4), 50% (n = 5), and 100% (n = 4) HbO2 saturation. Nitrite concentration decreased significantly faster under mid-oxygenation condition than under low-oxygenation condition (*P < 0.01) and high-oxygenation (**P < 0.01) conditions. MetHb concentration increased faster under mid-oxygenation condition and was significantly greater than that under low-oxygenation condition throughout 80-min time course and greater than that under high-oxygenation condition until the 40-min time point. Values are means ± SE.
The stoichiometry of the nitrite-deoxyhemoglobin production.

In vitro experiments, we observed production of significantly more than one MetHb equivalent per nitrite equivalent at ~50% and 100% HbO2 saturation. These findings can be explained on the basis of previously reported stoichiometry of the hemoglobin-nitrite reaction (Fig. 7A) that allows for the production of up to two MetHb equivalents for each nitrite when deoxyhemoglobin is a substrate. The first MetHb results directly from the reduction of nitrite to NO by deoxyhemoglobin. NO may then react with HbO2 to form the second MetHb and nitrate. This explanation requires that nitrite reacts preferentially at high oxygenation levels, the ratios were significantly greater than 1.00 (1.60 ± 0.10 and 1.8 ± 0.13, respectively), indicating production of more than one MetHb per nitrite equivalent.

To determine whether diffusion of nitrite across the erythrocyte membrane was limiting its reaction with hemoglobin, experiments were repeated in hemolyzed blood (dashed lines in Fig. 6, A–C). Hemolysis did not significantly affect the initial rates or cumulative loss/production calculations for nitrite or MetHb under low- or high-oxygenation conditions, indicating that diffusion of nitrite across the erythrocyte membrane was not rate limiting. Although lysing the erythrocytes did not affect nitrite disappearance in the low-oxygenation condition, it resulted in a significant increase in MetHb production per nitrite equivalent compared with intact erythrocytes (Fig. 6C).

DISCUSSION

These experiments describe the effects of hypoxia on the in vitro and in vivo kinetics of nitrite and MetHb in blood after administration of a nitrite bolus infusion. The principal findings are as follows. In the newborn lamb, the disappearance of nitrite from the plasma is biphasic in nature, with an overall k_d of ~0.08 min⁻¹, a half time of ~8 min, and an apparent V.dist two- to threefold greater than blood volume. These parameters are not significantly altered by moderate systemic hypoxia. Similarly, increases in MetHb following nitrite infusion are not altered by systemic hypoxia. By contrast, the disappearance from blood in vitro is monophasic in nature, and the rate of disappearance is significantly increased when HbO2 saturations are ~50% compared with ~0% and 100%. Finally, more than one MetHb is produced per nitrite equivalent under mid- and high-oxygenation conditions. Under low-oxygenation conditions, however, significantly less than one MetHb is produced per nitrite molecule. These findings suggest significant nitrite metabolism by pathways that do not involve hemoglobin oxidation or, alternatively, that MetHb is reduced by fast mechanisms not attributable to MetHb reductase.

Mass balance assessments of nitrite disappearance and MetHb production. The stoichiometry of the nitrite-deoxyhemoglobin or nitrite-HbO2 reaction has been previously determined using purified and completely oxygenated or deoxygenated hemoglobin in buffer solutions and excess or equivalent concentrations of nitrite. Under these conditions in vitro, nitrite reacts with HbO2 on a mole-for-mole basis, resulting in the production of one MetHb per nitrite equivalent consumed (28). Similarly, nitrite reacts with purified deoxyhemoglobin to produce NO, again resulting in the production of one MetHb for each nitrite equivalent consumed (1, 17, 18). In whole blood, however, quantification of MetHb production in relation to nitrite disappearance is complicated by the action of MetHb reductase enzymes within the red blood cells, which reduce MetHb back to deoxyhemoglobin (22).

In the present work, we have taken into account the MetHb reductase activity in our stoichiometric assessment of the nitrite-hemoglobin reaction. Using this correction for the stoichiometry of the MetHb production, we observed production of significantly more than one MetHb equivalent per nitrite equivalent at ~50% and 100% HbO2 saturation. These findings can be explained on the basis of previously reported stoichiometry of the hemoglobin-nitrite reaction (Fig. 7A) that allows for the production of up to two MetHb equivalents for each nitrite when deoxyhemoglobin is a substrate. The first MetHb results directly from the reduction of nitrite to NO by deoxyhemoglobin. NO may then react with HbO2 to form the second MetHb and nitrate. This explanation requires that nitrite reacts preferentially at high oxygenation levels, the ratios were significantly greater than 1.00 (1.60 ± 0.10 and 1.8 ± 0.13, respectively), indicating production of more than one MetHb per nitrite equivalent.

To determine whether diffusion of nitrite across the erythrocyte membrane was limiting its reaction with hemoglobin, experiments were repeated in hemolyzed blood (dashed lines in Fig. 6, A–C). Hemolysis did not significantly affect the initial rates or cumulative loss/production calculations for nitrite or MetHb under low- or high-oxygenation conditions, indicating that diffusion of nitrite across the erythrocyte membrane was not rate limiting. Although lysing the erythrocytes did not affect nitrite disappearance in the low-oxygenation condition, it resulted in a significant increase in MetHb production per nitrite equivalent compared with intact erythrocytes (Fig. 6C).

**Table 4. Kinetic values observed after introduction of sodium nitrite into adult sheep blood at 39°C at varying levels of HbO2 saturation**

<table>
<thead>
<tr>
<th>HbO2 Saturation</th>
<th>Low</th>
<th>Mid</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>V_dist, ml</td>
<td>27.8±2.0</td>
<td>28.5±1.4</td>
<td>27.4±0.9</td>
</tr>
<tr>
<td>PCR, ml⁻¹min⁻¹kg⁻¹</td>
<td>0.94±0.06*</td>
<td>2.45±0.12</td>
<td>0.85±0.05*</td>
</tr>
<tr>
<td>k_d, min⁻¹</td>
<td>0.033±0.0012*</td>
<td>0.0865±0.0042</td>
<td>0.0311±0.0019*</td>
</tr>
<tr>
<td>t½, min</td>
<td>19.9±0.7*</td>
<td>7.9±0.3</td>
<td>21.9±1.2*</td>
</tr>
<tr>
<td>BRC, M⁻¹s⁻¹</td>
<td>0.312±0.012*</td>
<td>0.811±0.024</td>
<td>0.288±0.008*</td>
</tr>
<tr>
<td>MetHb</td>
<td>0.252±0.056*‡</td>
<td>1.620±0.044‡</td>
<td>0.458±0.116‡</td>
</tr>
</tbody>
</table>

Values are means ± SE. Sodium nitrite (14.4 μmol) was added to 30 ml of adult sheep blood at 39°C. NO2⁻ BRC and MetHb BRC, bimolecular rate constant calculated from initial rates of nitrite disappearance and MetHb production. *Significantly different from mid HbO2 saturation (P < 0.01). †Significantly different from corresponding NO2⁻ BRC (P < 0.01). ‡Significantly different from high HbO2 saturation (P < 0.05).
entially with deoxyhemoglobin, rather than HbO2, even at high HbO2 saturations, an assumption supported by the kinetics of the nitrite-HbO2 reaction. When it is present at high concentrations (equal to or in excess of hemoglobin), nitrite is oxidized to nitrate, with an initial slow lag phase exhibiting a half time on the order of hours followed by a rapid autocatalytic phase (27). However, under the conditions of the present studies (nitrite concentration less than half that of hemoglobin), it is reasonable to assume, on the basis of previous reports (33), that the nitrite-HbO2 reaction never entered the rapid autocatalytic phase. As a result, the predominant pathway of nitrite disappearance was likely its reduction to NO by deoxyhemoglobin, a reaction that exhibits a nitrite half time on the order of minutes, rather than hours (17, 23). The resulting intraerythrocytic NO is then presumed to react with HbO2 and deoxyhemoglobin at the diffusion-limited rate of $2–8 \times 10^7 \text{M}^{-1}\text{s}^{-1}$ (2, 9, 11, 35), resulting in one additional MetHb.

In contrast to the mid- and high-oxygenation experiments, under low-oxygenation conditions, significantly less than one MetHb was produced per nitrite equivalent. These results are similar to those reported for purified hemoglobin at 1:1 concentration ratio with nitrite (17). There are a number of possible explanations for this discrepancy. One possibility is that nitrite is lost during our sampling-and-assay procedure, resulting in underestimation of true nitrite concentrations. However, this does not seem likely, given the stability of nitrite in the stop solution (8) and because the calculated volume of distribution for the in vitro experiments was nearly identical to the actual blood volume in the flask, which would not have occurred if nitrite were lost. Also, the kinetics of nitrite disappearance were not altered in lysed blood, ruling out an accumulation of nitrite within the red blood cells that would have been missed with measurements of plasma only. Another possible explanation would be the disappearance of nitrite as a...
result of reactions that do not produce MetHb. Nitrite can be reduced to NO by the intracellular enzyme xanthine oxidase (30), although this does not appear to play a role in nitrite-mediated dilation of pulmonary arteries (7). In addition, we found no significant effect of allopurinol, a xanthine oxidase inhibitor, on MetHb production in vitro (data not shown). Interestingly, although nitrite metabolism was not affected by hemolysis, significantly more MetHb was produced hemolyzed blood under the low-oxygenation condition than in whole blood (Fig. 6C), suggesting that erythrocyte membrane integrity is important to the low MetHb-per-nitrite yield at low $O_2$ saturations.

Perhaps a more likely possibility is that MetHb is reduced at a rate that is much faster than the rate we have accounted for. Indeed, consideration of the present data within the framework of reductive nitrosylation as discussed by Fernandez et al. (13) lends credibility to this possibility. The model is based on a reaction termed reductive nitrosylation, wherein ferric hemecenters such as MetHb are reduced nonenzymatically by reaction with NO to produce iron-HbNO (Fig. 7), a reaction observed in purified MetHb and whole blood (16, 21). There is recent evidence that nitrite catalyzes this reaction by reacting with the ferric-HbNO complex, resulting in reduction of MetHb to hemoglobin and combination of NO and nitrite to form the NO adduct nitrous anhydride (N$_2$O$_3$) (12). N$_2$O$_3$ is capable of rapidly S-nitrosating proteins such as hemoglobin to form S-nitrosohemoglobin (44), or it can rapidly homolyze into NO and the nitrite radical (31). N$_2$O$_3$ has a half time of $\sim$1 ms (14), which is 100 times greater than that of intracellular NO; therefore, it is a more plausible carrier of NO activity from the interior of the erythrocyte to vascular smooth muscle (20).

Effect of $O_2$ saturation on the rate of nitrite metabolism in vitro. As demonstrated by the initial rates of reaction for the three different oxygenation levels (Table 4), as well as the time course of nitrite disappearance (Fig. 4A), nitrite metabolism and MetHb production were greatest under mid-oxygenation conditions and slowest under low- and high-oxygenation conditions. These findings are in close agreement with those previously reported (5, 17, 18), which suggest a component of allosteric control of the reaction rate by hemoglobin. This effect is thought to consist of a balance between the mass action effect of deoxyhemoglobin concentrations and the greater reductive potential of fully or three-fourths-$O_2$-saturated hemoglobin. According to this model, the rate of nitrite reduction by deoxyhemoglobin, as a function of $O_2$ saturation, is maximal at $\sim$50% saturation (for review see Ref. 15).

It is important to note the often overlooked fact that the chemiluminescence assay used in the present experiments may not be specific to nitrite. As has been noted previously (36), the reductive chemiluminescence method detects NO released from proteins and heme moieties, in addition to the signal generated from nitrite. Therefore, if nitrite were reduced and the resulting NO preserved as S-nitrosated or iron-nitrosylated proteins, the present experiments would underestimate the true rate of nitrite metabolism, particularly under the low-oxygenation condition, in which NO may be preserved as iron-HbNO.

Effects of hypoxia on nitrite pharmacokinetics in vivo. We hypothesized that nitrite would disappear at a faster rate in hypoxic animals in which arterial $HbO_2$ saturations approached 50%, a range more favorable for the nitrite-hemoglobin reaction (5, 17, 18). Instead, we observed no effect of hypoxia for any of the parameters measured (Table 2, Figs. 3 and 5). The initial disappearance of nitrite from the plasma and the large volume of distribution ($\sim$2-fold greater than blood volume) suggest a rapid movement of nitrite from the blood into extracellular fluid, rather than nitrite metabolism. However, even after the rapid phase of nitrite disappearance, the slow phase of disappearance was still not altered by hypoxia. One possible explanation is that reactions in blood are overshadowed by nitrite reactions in peripheral tissues, which do not vary with $O_2$ concentrations. However, this seems contrary to evidence that nitrite is reduced at a faster rate in hypoxic tissues (39). It is also possible that mechanisms responsible for in vivo loss of supraphysiological concentrations of nitrite, such as those administered in the present study, are different from those mediating nitrite concentrations at physiological concentrations. Therefore, the $O_2$-sensitive nitrite-hemoglobin reaction may have been masked by other reactions influencing metabolism of the large dose of nitrite administered in the present studies.

Physiological effects of nitrite during hypoxia. On the basis of recent findings suggesting an important role for nitrite in red blood cell-mediated hypoxic vasodilation, this study was initiated with the hypothesis that the hypotensive effects of nitrite infusion on systemic blood pressure would be more pronounced in hypoxic than in normoxic animals (4, 19). Indeed, the hypotensive effects of nitrite were potentiated by hypoxia (Fig. 3A). These findings are in agreement with previous observations in the lungs and exercising forearm (4, 5, 19) and support the idea that the vasodilating effects of nitrite are $O_2$ sensitive.

We previously reported that inhalation of aerosolized nitrite (nebulization of 300 mg over a 20-min period) selectively lowers hypoxic pulmonary hypertension, with vasodilating effects lasting up to 1 h after the end of the inhalation period (19). The pulmonary vasodilating effect was proposed to be due to nitrite reduction to NO by deoxyhemoglobin in the pulmonary circulation, as evidenced in part by increases of NO in exhaled air. In the present study, intravenous boluses of nitrite during hypoxia also significantly lowered pulmonary arterial pressures for up to 15 min, whereas similar boluses had no effect in normoxic lambs. The lack of decrease during normoxia does not appear to be due to a lack of basal vasodilating capacity, because inhaled NO (80 ppm) resulted in a rapid significant decrease in pulmonary arterial pressure (Fig. 3B). Therefore, although the mechanisms regulating pulmonary vascular tone of normoxic lambs are certainly different from those of hypoxic lambs, the fact that nitrite infusion resulted in pulmonary vasodilation of hypoxic, but not normoxic, lambs supports the $O_2$-sensitive mechanisms of nitrite reduction.

In contrast to previous findings with inhaled nitrite aerosol, measurable ($>5$ ppb) increases in exhaled NO gas were not observed in the present studies after intravenous nitrite infusion. These results are in agreement with the recent work of Deem et al. (7), who found that perfusion of isolated ventilated rat lungs with 1 mM nitrite in cell-free solution resulted in increased exhaled NO, an effect abolished by addition of red blood cells to the perfusate. Taken together, these findings suggest that exhaled NO measured during nitrite inhalation in the previous study (19) may have been formed extravascularly.
and is not necessarily evidence of hemoglobin-mediated nitrite reduction.

Recent studies of the role of nitrate during hypoxia-ischemia suggest that it may be of therapeutic benefit in the treatment of pulmonary hypertension (19), posthemorrhagic cerebral vasospasm (37), and myocardial infarction (42). Thus it is worth noting from the present work in lambs that nitrite administered to final plasma concentrations ~1,000-fold above physiological levels was well tolerated in terms of methemoglobinemia, one of the clinical concerns of nitrite poisoning. Moderate hypotension was observed and tended to resolve over a matter of 1 h after the bolus infusion. Finally, systemic hypoxia did not appear to exacerbate these effects.

Conclusions. Recent observations that nitrite can potently induce vasodilation in the hypoxic forearm, pulmonary vasculature, heart, and intestine (4, 19, 29, 42), together with observations of the O2-sensitive reduction of nitrite to NO by hemoglobin (5, 17, 18), have led to intense study of the theory that nitrite serves as a circulating NO metabolite capable of releasing bioactive NO in hypoxic tissues. The present observed stoichiometry of nitrite metabolism in whole blood at low levels of oxygenation indicates that reductive nitrosylation may play an important role in the handling of nitrite. In addition, although the nitrite-hemoglobin reaction is O2-sensitive, the kinetic handling of pharmacological doses of nitrite in vivo, as well as effects on arterial blood pressure, is not affected by moderate hypoxia. Further work is necessary to integrate the nitrite-hemoglobin reaction in the complex chemistry of NO and its adducts in vivo.

ACKNOWLEDGMENTS

The authors thank Shannon Bragg for expert assistance and Dr. Andre Dejam for insightful suggestions.

GRANTS

These studies were supported in part by National Heart, Lung, and Blood Institute Grant HL-65494.

REFERENCES


