Kinetics of methaemoglobin and serum nitrogen oxide production during inhalation of nitric oxide in volunteers

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Summary
Inhaled nitric oxide is used increasingly to treat pulmonary hypertension and ventilation/perfusion mismatching in seriously ill patients, but little is known of the pharmacokinetics of its two principal metabolites, methaemoglobin and nitrogen oxides (nitrates and nitrites). We have studied the changes in these metabolites in six healthy volunteers during and after 3 h inhalation of 100 volumes per million of nitric oxide. Mean nitric oxide uptake was 0.49 (SD 0.08) ml min⁻¹ at standard temperature and pressure, corresponding to 74 % of the inhaled dose. During inhalation, methaemoglobin increased monoexponentially with a time constant of 45.6 (11.1) min by 1.77 (0.47) % of total haemoglobin. Serum nitrogen oxides increased from 36.7 (7.6) to 124 (17) μmol litre⁻¹, with a time constant of 172 (91.4) min and a volume of distribution of 331 (104) ml kg⁻¹. The volume of distribution for methaemoglobin calculated from nitric oxide uptake and the increase in methaemoglobin, was, on average, 14.3 % less than predicted blood volume, suggesting that most of the absorbed nitric oxide initially forms methaemoglobin. Serum nitrogen oxides declined initially after inhalation ceased but then increased to a second peak between 45 and 180 min later. The cause of the second peak was not determined. (Br. J. Anaesth. 1996; 76: 652–656)

Key words

Inhaled nitric oxide is used increasingly to treat acute pulmonary hypertension and improve ventilation/perfusion relationships in critically ill adults and children [1–4]. A large proportion of the inhaled nitric oxide is absorbed [5–7]. Most absorbed nitric oxide probably combines with oxyhaemoglobin, which has an affinity for nitric oxide about 1500 times greater than its affinity for carbon monoxide [8], forming methaemoglobin and nitrates. Methaemoglobin is then converted back to haemoglobin by several intra-erythrocytic enzymes of which NADH-methaemoglobin reductase is the most important [9]. However, direct conversion of nitric oxide to nitrite and subsequent oxidation to nitrate is also possible in biological fluids [10], and nitric oxide can also combine with deoxygenated haemoglobin to form nitrosyl haemoglobin [11]. Nitrate, the final metabolite of nitric oxide by all of these pathways, accumulates during inhalation of nitric oxide [12]. Data on the handling and elimination of nitrates are sparse and little is known about the metabolism of inhaled nitric oxide. In this study, we have determined if all of the absorbed inhaled nitric oxide reacts with oxyhaemoglobin to form nitrates and methaemoglobin or if direct conversion to nitrate also occurs, and investigated the pharmacokinetics of the nitrate produced.

Subjects and methods
The study was approved by the local Ethics Committee, and all subjects gave informed consent. We studied six healthy male volunteers, aged 30–38 yr, weighing 67–105 kg. A 16-gauge cannula was inserted in a forearm vein and blood was obtained for baseline methaemoglobin, total haemoglobin and serum nitrate and nitrite concentrations, measured together as serum nitrogen oxides.

Subjects were fitted with a leak-free face mask connected to a non-rebreathing valve. Nitric oxide was stored as a mixture of 2000 volumes per million (vpm) in nitrogen (BOC Special Gases, Guildford, England) and mixed with medical grade air to achieve a final inspired concentration of 100 vpm. Mixed expired gas was collected in an in-line 10-litre mixing chamber. Inspired and mixed expired nitric oxide concentrations were measured with a chemiluminescence analyser (CLD 700AL, Eco Physics, Dürnten, Switzerland). Inspired minute volume was determined from a dry gas meter fitted to the inspiratory limb of the circuit. Subjects inhaled nitric oxide for 3 h and then room air. Venous blood was obtained before inhalation of nitric oxide and at 15, 30, 45, 60, 90, 120 and 180 min during inhalation of nitric oxide. Blood was also sampled 15, 30, 45, 60, 90, 120, 180, 240, 300, 540 and 1260 min after inhalation ceased. Subjects were asked to avoid...
processed meats on the day of the experiment as these may contain added nitrites; no other restrictions were placed on their diet or fluid intake.

Methaemoglobin and total haemoglobin concentrations were determined using an OSM3 co-oximeter (Radiometer, Copenhagen, Denmark). Blood samples for measurement of serum nitrogen oxides (nitrate and nitrite) were centrifuged at 3000 rpm for 15 min and the serum separated and stored at –20°C for later analysis. Serum was deproteinized with zinc sulphate solution 30 mmol litre⁻¹ before analysis and added to hot acidic vanadium (III) chloride which converted the nitrates and nitrites to nitric oxide. This was eluted in a stream of nitrogen and then analysed using chemiluminescence (CLD 700AL). The method is identical to that described by Brahman and Hendrix [13]. All analyses were performed in duplicate and the mean value used. The inter-sample coefficient of variation of the assay was 2% and the inter-day coefficient of variation 10%. Samples were analysed in random order. Data were analysed using an analysis/graphics package (Kaleidagraph, v3.0.2, Abelbeck Software) on an Apple Macintosh “Quadra 950”. Results are given as mean (SD).

For each subject the inspired and expired nitric oxide concentrations and minute volume were corrected for temperature, ambient pressure and water vapour, and used to calculate nitric oxide uptake $7(\dot{V}_{NO})$ at STPD (standard temperature and pressure dry). Methaemoglobin production during inhalation of nitric oxide at a fixed inspired concentration follows simple first-order kinetics [14] and therefore the methaemoglobin concentration at time $t$ can be described by the equation:

$$\text{Methaemoglobin concentration at time } t = B + A \times (1 - e^{-t/\tau})$$ (1)

where $B =$ initial (baseline) methaemoglobin concentration (as a percentage of the total haemoglobin), $A =$ plateau concentration of methaemoglobin above baseline that would be reached if inhalation continued for a long time (as a percentage of the total haemoglobin), and $\tau =$ time constant in minutes.

This equation was fitted to the methaemoglobin concentration results during inhalation of nitric oxide for each subject using least squares regression. This gave estimates of $A$ and $\tau$. If this equation is differentiated and solved for $t = 0$, the maximum rate of increase in methaemoglobin concentration is obtained. This is given by $A/\tau$ and corresponds to the rate of increase of methaemoglobin concentration in the absence of any elimination (i.e. the initial rate of increase of methaemoglobin concentration).

If the rate of production of methaemoglobin and its initial rate of increase are known, an estimate of blood volume can be obtained if it is assumed that all of the nitric oxide absorbed initially forms methaemoglobin:

Estimated blood volume (litre) = $\frac{\text{Initial rate of increase in blood methaemoglobin (g litre}^{-1} \text{ min}^{-1})}{\text{Initial rate of increase in blood methaemoglobin (g litre}^{-1} \text{ min}^{-1})}$

Thus estimated blood volume is given by:

$$\dot{V}_{NO} \times \tau = 1.39 \times A$$ (2)

where $\dot{V}_{NO} =$ nitric oxide uptake (ml min⁻¹), $A$ is now expressed in grams of methaemoglobin per litre of blood and $\tau$ is as before; 1.39 is the affinity of haemoglobin for nitric oxide in millilitres of nitric oxide per gram of haemoglobin.

Mean uptake of nitric oxide and estimates for $A$ and $\tau$ were substituted into this equation to give estimated blood volumes. These were compared with other estimates calculated from regression equations using the subjects’ heights and weights. Two different equations were used [15, 16] and the mean of the estimates taken. Neither the regression equations nor the calculations based on nitric oxide uptake have a correction for the difference between central and peripheral packed cell volume.

The increase in serum nitrogen oxides during inhalation of nitric oxide could be described by an equation characterizing the behaviour of a one-compartment kinetic model. The volume of distribution was then calculated assuming all inhaled nitric oxide formed nitrogen oxides, and clearance calculated as the volume of distribution divided by the time constant.

**Results**

The mean inhaled nitric oxide concentration was 100.0 (3.9) vpm and the mean mixed exhaled nitric oxide concentration was 36.0 (5.8) vpm. Mean uptake of nitric oxide was 0.49 (0.08) ml min⁻¹ STPD; there were no trends in uptake during the study in any subject. Inspired nitrogen dioxide concentrations (measured in the first subject only) were less than 0.25 vpm. The mean methaemoglobin concentration before inhalation of nitric oxide was 0.87 (0.04)%, and the baseline serum nitrogen oxide concentration was 36.7 (7.6)μmol litre⁻¹. Changes in methaemoglobin and serum nitrogen oxide concentrations during the study are shown in figure 1. The mean peak serum nitrogen oxide concentration was 124.2 (17.0) μmol litre⁻¹ and the highest value recorded was 151.1 μmol litre⁻¹ in subject No. 6. The

![Figure 1](image_url)
mean peak methaemoglobin concentration was 2.65 (0.46)% and the highest value was 3.3 % in subject No. 5. Serum nitrogen oxide concentrations showed a double “peak”; an initial peak at the end of inhalation of nitric oxide and a second peak which occurred in all subjects between 45 min and 3 h after cessation of inhalation of nitric oxide. Table 1 shows the calculated results for methaemoglobin kinetics and blood volumes in all subjects. Blood volume calculated from uptake of nitric oxide was, on average, 14.3 % higher than the value estimated from subjects’ heights and weights. Table 2 gives the pharmacokinetic variables for nitrogen oxides for all subjects.

**Discussion**

In this study, two-thirds of the inhaled dose of nitric oxide was absorbed, somewhat less than would be expected from the results of other studies, where up to 95 % of inhaled nitric oxide was absorbed during a breath-hold performed to measure the diffusing capacity of the lung for nitric oxide [6, 7]. The fraction of inspired nitric oxide absorbed varies between studies because the limiting factor for pulmonary absorption of nitric oxide is its pulmonary diffusing capacity and therefore uptake is a non-linear function of lung volume, respiratory timing and inspired concentration. The absorbed nitric oxide forms methaemoglobin and nitrates, and possibly a small amount of nitrosyl haemoglobin [11, 17]. It is not known if all of the absorbed nitric oxide initially forms nitrates and methaemoglobin, or if some of the inhaled nitric oxide either combines directly with water to form nitrates and subsequently nitrates [10] or transiently forms nitrosyl haemoglobin, which is converted rapidly to nitrogen oxides [18]. In a previous study to determine the kinetics of methaemoglobin production during inhalation of nitric oxide [14], the maximum rate of methaemoglobin production suggested that nearly all of the inhaled nitric oxide initially formed methaemoglobin, but no exhaled nitric oxide measurements were performed and all of the calculations were based on an assumed uptake of 80–90 %. This study was performed with exhaled measurements to determine the fraction of adsorbed nitric oxide that reacts with haemoglobin to form methaemoglobin, and to determine the pharmacokinetics of the serum nitrogen oxides formed during inhalation of nitric oxide.

Measuring blood volume using inhaled gas was first attempted in 1882 [19] with a fixed volume of carbon monoxide and a single measurement of carboxyhaemoglobin; blood volume was assumed to be the same as volume of distribution of carboxyhaemoglobin. The technique we used to determine if nitric oxide initially forms methaemoglobin is essentially determination of volume of distribution of methaemoglobin using continuous inhalation of gas (equation (2) is a variation on the standard equation for calculating volume of distribution for an infused drug [20]). As methaemoglobin is entirely intravascular, this corresponds to blood volume. This technique requires a constant nitric oxide uptake, constant blood volume during nitric oxide inhalation, and that methaemoglobin elimination follows first-order kinetics. We have shown previously that methaemoglobin kinetics are both first order and linear in the range of concentrations studied here [14], that nitric oxide uptake was

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Height (cm)</th>
<th>Weight (kg)</th>
<th>Haemoglobin (g %)</th>
<th>Mean nitric oxide uptake (ml min⁻¹ STPD)</th>
<th>τ (min)</th>
<th>A (% of total haemoglobin)</th>
<th>Estimated blood volume from regression equations (litre)</th>
<th>Estimated blood volume from nitric oxide uptake (litre)</th>
<th>Difference between estimates (%)</th>
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<tr>
<td>1</td>
<td>187</td>
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<td>6.67</td>
<td>6.81</td>
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<td>184</td>
<td>91</td>
<td>15.8</td>
<td>0.50 (0.04)</td>
<td>34.4</td>
<td>1.23</td>
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<td>67</td>
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<td>70</td>
<td>13.9</td>
<td>0.50 (0.07)</td>
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<td>1.68</td>
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<tr>
<td>Mean (SD)</td>
<td></td>
<td></td>
<td></td>
<td>0.49 (0.08)</td>
<td></td>
<td>45.6 (11.1)</td>
<td>1.77 (0.47)</td>
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</table>

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>τ (minutes)</th>
<th>A (µmol litre⁻¹)</th>
<th>Volume of distribution (litre)</th>
<th>Volume of distribution (ml kg⁻¹)</th>
<th>Clearance (ml min⁻¹)</th>
<th>Clearance (ml min⁻¹ kg⁻¹)</th>
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<tr>
<td>Mean (SD)</td>
<td>172 (91.4)</td>
<td>133 (29.9)</td>
<td>27.6 (11.6)</td>
<td>331 (104)</td>
<td>169 (32.7)</td>
<td>2.15 (0.53)</td>
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</table>
constant within subjects, and that blood volume would not be expected to change in the 3 h of the study. Ideally blood volume in the subjects should have been measured simultaneously with isotopically labelled red cells, but this was not possible. The agreement between the calculated and measured blood volumes suggests that the majority of the inhaled nitric oxide that is absorbed forms methaemoglobin, although in all cases blood volume calculated from nitric oxide uptake was greater than the expected value, and therefore 14 % of the nitric oxide may have been absorbed without forming methaemoglobin. This could be by formation of nitrates and nitrates, or nitrosyl haemoglobin. When nitric oxide reacts with haemoglobin in vitro, the ratio of methaemoglobin to nitrosyl haemoglobin formation depends on oxygenation of haemoglobin; oxygenated haemoglobin forms methaemoglobin and nitrates whereas deoxygenated haemoglobin forms nitrosyl haemoglobin [11, 21]. As inhaled nitric oxide is absorbed into blood as it traverses the pulmonary capillary bed, it is reacting with haemoglobin that is 60–100 % saturated, and therefore preferential formation of methaemoglobin would be expected. This has been confirmed by Wennmalm and colleagues [17] who could not detect nitrosyl haemoglobin in volunteers inhaling nitric oxide 25 vpm.

Baseline serum nitrogen oxide concentrations measured in this study were close to values reported previously [17, 22, 23]. In the only other published study of inhalation of nitric oxide and nitrates in humans, Wennmalm and colleagues [17] showed an increase in plasma nitrates 26 to 38 μmol litre⁻¹ in volunteers inhaling 25 vpm for 1 h. In this study, at 1 h serum nitrogen oxide concentrations were 80.7 (10.7) μmol litre⁻¹, an increase of 44 μmol litre⁻¹. Volumes of distribution, clearances and time constants for serum nitrogen oxides were greater than values given in previous reports where oral doses of nitrate were given to volunteers. Wagner and co-workers [24] showed a volume of distribution of 21.1 litre, total clearance of 48.3 ml min⁻¹ and elimination time constant of 429 min. Cortas and Wakid [23] reported nitrate clearance of 25.8 ml min⁻¹, and from their data the volume of distribution of the nitrate was approximately 19 litre and elimination time constant 740 min. These differences may reflect the different routes of administration; oral nitrates may have a different bioavailability than nitrates formed by inhalation of nitric oxide which is equivalent to an i.v. infusion. A reduced bioavailability of nitrates by the oral route would cause an apparent increase in the volume of distribution, but delayed absorption would explain the difference in clearances between our study and those cited above indicate a volume of distribution which is greater than the extracellular fluid volume but less than total body water, which is in agreement with the reported tissue to plasma nitrate gradient. The second peak in serum nitrogen oxides found in this study is also difficult to explain in terms of either one or multiple first-order processes. If all of the inhaled nitric oxide formed nitrates, when inhalation of nitric oxide ceased, serum nitrogen oxide concentration would decrease. One possible explanation for this second peak is the presence of a store of nitrogen oxides which are mobilized into the circulation some time after nitric oxide inhalation ceases. This may be related to the experimental procedure; subjects were seated during inhalation of nitric oxide but were free to walk around during the second stage of the experiment, which would both increase cardiac output and alter its distribution to different tissues. The anatomical site of this store of nitrogen oxides is unknown.

The inhaled concentration of nitric oxide used in these studies was greater than used clinically, where a maximum of 40 vpm is more common. The greater dose was used to ensure measurable changes within an acceptable time scale for a volunteer study.

In summary, we have demonstrated that the majority of inhaled nitric oxide reacts with haemoglobin producing methaemoglobin and nitrogen oxides, but up to 14 % apparently undergoes direct conversion to nitrogen oxides. The nitrogen oxides produced have a volume of distribution of one-third of body weight and a clearance similar to glomerular filtration rate.

Acknowledgements

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References


