Metabolism and Excretion of Nitric Oxide in Humans
An Experimental and Clinical Study

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Despite the increasing insight in the clinical importance of nitric oxide (NO), formerly known as endothelium-derived relaxing factor (EDRF), there is limited information about the metabolism and elimination of this mediator in humans. We studied the degradation of NO in healthy subjects inhaling 25 ppm for 60 minutes and in patients with severe heart failure inhaling 20, 40, and 80 ppm in consecutive 10-minute periods. In other healthy subjects, the renal clearance of NO metabolite was measured. The metabolism ex vivo was evaluated by direct incubation of nitrite, the NO oxidation product, in blood from healthy humans. During inhalation of NO, the plasma levels of nitrate increased progressively, both in the healthy subjects (from 26 to 38 μmol/L, P<.001) and in the patients (from 72 to 90 μmol/L, P<.001). Methemoglobin (MetHb) also increased in the healthy subjects (from 7 to 13 μmol/L, P<.001) as well as in the patients (from 19 to 42 μmol/L, P<.01). No change in nitrosohemoglobin (HbNO) was detected, either in the healthy subjects or in the patients. In arterialized blood (O\textsubscript{2} saturation, 94% to 99%), incubated nitrite was semiquantitatively converted to nitrate and MetHb. In venous blood (O\textsubscript{2} saturation, 36% to 85%) moderate amounts of HbNO were also formed. Plasma and urinary clearance of nitrate in healthy subjects averaged 20 mL/min. We conclude that uptake into the red blood cells with subsequent conversion to nitrate and MetHb is a major metabolic pathway for endogenously formed NO. Nitrate may then enter the plasma to be eliminated via the kidneys. The occurrence of HbNO in vivo probably indicates liberation of NO to partly deoxygenated blood. (Circ Res. 1993;73:1121-1127.)

KEY WORDS • endothelium-derived relaxing factor • endothelium • erythrocytes • hemoglobin • methemoglobin • nitrate

decreased formation of this compound\textsuperscript{9,10} seems to constitute an important functional component in ath erosclerotic vascular disease. An increased formation of NO in activated macrophages has been suggested to be responsible for the hypotension in endotoxin shock.\textsuperscript{11} Recently, the application of NO inhalation as a beneficial therapeutic principle was reported in patients with primary pulmonary hypertension\textsuperscript{12} or adult respiratory distress syndrome.\textsuperscript{13}

We assumed that quantitative methods to estimate NO formation might facilitate further evaluation of some of its physiological, pathophysiological, and therapeutic roles. The development of such methods relies on proper knowledge of the inactivation and elimination of NO from the intact organism. However, only little is known concerning the in vivo metabolism of NO and the excretion of its metabolite(s). The amino acid L-arginine, which is a precursor for NO both in macrophages and in endothelial cells,\textsuperscript{3,14} is also a precursor for nitrate biosynthesis in humans.\textsuperscript{15} Furthermore, nitrate is a normal constituent of human urine.\textsuperscript{16} Based on these observations, it might be speculated that NO is metabolized to nitrate and subsequently excreted as such into the urine. Preliminary support for this hypothesis was obtained earlier in a study on NO degradation in human blood ex vivo.\textsuperscript{17} In the present report, the proposed metabolic route for NO is shown to be operative in vivo, in healthy subjects as well as in patients with severe
heart failure. In addition, the elimination of NO metabolite via renal excretion is described.

**Materials and Methods**

All study protocols were approved by the local human investigations committee. None of the subjects participated in more than one of the series described below.

**Inhalation of NO in Healthy Subjects and in Patients With Severe Heart Failure**

Eight healthy nonsmoking volunteers were recruited among the hospital staff. Four of them were women (age, 43±2 years; height, 163±4 cm; weight, 59±11 kg [mean±SD]) and four were men (age, 39±5 years; height, 182±5 cm; weight, 72±6 kg). The subjects were studied in the morning, while sitting comfortably in an armchair. No precautions concerning food intake were applied. A short catheter for blood sampling was introduced into a cubital vein. After a stabilization period, NO was administered for 60 minutes in air by mask inhalation, to produce a final concentration of 25 ppm. Inhalation depth and frequency were not controlled. Blood samples were drawn into heparinized tubes in the basal state and every 10 minutes during the inhalation and were analyzed for nitrate, nitrite, methemoglobin (MetHb), and nitrosohemoglobin (HbNO).

Eight patients (six men) with severe heart failure, aged 23 to 63 years, were studied in connection with clinical evaluation before cardiac transplantation. The clinical ground to this part of the study was that after heart transplantation some patients develop pulmonary hypertension; it was considered of value to assess the efficiency of NO to lower the pulmonary vascular resistance in the transplantation candidates before surgery. All patients were studied in the supine position. Treatment with nitrodiators was omitted at least 12 hours before the NO inhalation. NO was administered in air by mask inhalation in subsequent 10-minute periods (final concentrations, 20, 40, and 80 ppm). Blood samples were drawn as described above in the basal state and during the last 2 minutes of each inhalation period, using radial and pulmonary artery thermodilution catheters, and were analyzed for nitrate, nitrite, MetHb, and HbNO.

**Ex Vivo Studies of the Degradation of Nitrite**

Venous blood was obtained from eight healthy donors and sampled into heparinized vacutainer tubes. A fraction of the blood was oxygenated by slow stirring in a 100% oxygen atmosphere for 8 to 15 minutes before being used for the incubations. Remaining blood was used without previous oxygenation. The pH and the oxygen saturation of arterialized and venous blood were estimated in an ABL-2 blood gas analyzer (Radiometer, Copenhagen, Denmark).

Eight-milliliter portions of whole blood were incubated at room temperature with sodium nitrite (final concentrations, 50, 100, and 200 μmol/L). The incubation was interrupted after 2 and 15 minutes, respectively, by separation of the blood into cells and plasma followed by freezing of a portion of the cell fraction at 77°K in EPR tubes. The plasma fraction was frozen at −20°C.

**Studies on the Renal Elimination of NO Metabolite**

Eight healthy volunteers (two men), aged 20 to 33 years, were studied. They were instructed to avoid nonsteroidal anti-inflammatory drugs and excessively salted nutrients during the 5 days before the investigation. Voided urine was collected during 24 hours preceding the study and stored at 4° to 8°C during the collection period. After the urine collection period, including an overnight fast, the subjects reported at the laboratory. A bladder catheter was installed, and a short polypropylene catheter was introduced into a brachial artery. To ensure adequate diuresis, the subjects were given 400 mL water. After a 60-minute period of supine rest, urine was collected for 16 to 25 minutes. Arterial blood was taken at the start and end of each sampling period. Urine volume and exact collection time were measured in all cases. Urine and plasma samples were kept frozen at −18°C until analysis. At this temperature, both nitrite and nitrate levels were stable for several months.

**Analysis of Nitrate and Nitrite in Plasma and Urine**

Plasma and urine levels of nitrate and nitrite were analyzed with a liquid chromatography method. Plasma was diluted with an equal volume of water, and the proteins were removed by centrifugation for 45 minutes at 7500g through a 10K filter (Filtron Corp, Northborough, Mass). A 20-μL sample of the ultrafiltrate was injected into a chromatography system using a 50-mm column of IC-Pak A (Waters, Milford, Mass) as the solid phase and 1 mmol/L phosphate buffer pH 9 as the mobile phase. Quantitative analysis was performed against external or internal standards with UV absorbance at 214 nm.

Urine samples were preliminarily purified on a disposable C18 cartridge (Waters), followed by dilution with 25 vol water before being quantitatively analyzed as above. The detection limit for nitrate and nitrite, in plasma as well as in urine, was about 0.5 μmol/L. The chromatographic analyses of nitrate and nitrite in plasma and urine were verified with gas chromatography/mass spectrometry (GC/MS) methods developed in our laboratory, using purified samples of plasma or urine prepared as above. For analysis of nitrate a 150-μL portion of the sample was added with a known amount of K15NO3 (Sigma Chemical Co, St Louis, Mo) as internal standard. Endogenous and added nitrate were subsequently converted to nitrotoleucine by shaking the ultrafiltrate for 20 minutes with 500 μL tolune and 200 μL concentrated H2SO4. The organic phase was then separated and added with 10 vol acetonitrile. A 1- to 2-μL portion was injected into a Varian 3400 gas chromatograph (Varian, Walnut Creek, Calif.) equipped with a 25-m SPB 5 capillary column operated isothermally at 85°C. It was connected to a Finnigan Incos 50 mass spectrometer (Finnigan, San Jose, Calif) operated in the negative ion–chemical ionization mode using methane as the reactant gas and selective monitoring of mass/ equivalent (m/e) 136 for endogenous nitrate and m/e 137 for the 15N-labeled internal standard. The detection limit for nitrate was 0.1 μmol/L.

For analysis of nitrite, a 200-μL portion of the sample was added with a known amount of K15NO3 (Sigma) as internal standard. Endogenous and added nitrite were
TABLE 1. Plasma Levels of Nitrate and Methemoglobin in Eight Healthy Volunteers in the Basal State and During Inhalation of Nitric Oxide (25 ppm) for 60 Minutes

<table>
<thead>
<tr>
<th>Subject</th>
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<th>Nitrate</th>
<th>MetHb</th>
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</tbody>
</table>

NO indicates nitric oxide; MetHb, methemoglobin.

Results

Metabolism of Inhaled NO in Healthy Subjects and in Patients With Severe Heart Failure

Individual plasma levels of nitrate and MetHb in the healthy volunteers, in the basal state and during inhalation of NO, are shown in Table 1.

The basal level of nitrate before inhalation was 26±2 μmol/L. During inhalation of NO, there was a time-related increase in plasma nitrate, reaching 38±2 μmol/L after 60 minutes (change with time, P<.001 by one-way ANOVA; Fig 1). In parallel to the increase in plasma nitrate, the concentration of MetHb increased, from 7±1 to 13±1 μmol/L (change with time, P<.001 by one-way ANOVA; Fig 1) corresponding to a change from about 0.35% to 0.64% of the total Hb concentration in the blood.

The basal plasma level of nitrite before inhalation was 1.3±0.15 μmol/L. During inhalation of NO, plasma nitrite did not change significantly; after 30 and 60 minutes of inhalation, the plasma levels of nitrite were 1.1±0.08 and 1.2±0.08 μmol/L, respectively.

No HbNO was detected before or during inhalation of NO. No side effects were observed during or after the inhalation.

Individual arterial and pulmonary arterial levels of nitrate in the basal state and during inhalation of NO in the heart failure patients are shown in Table 2. In the patients, the basal level of nitrate in arterial plasma before inhalation ranged from 27 to 177 (median, 72) μmol/L; the corresponding range in the pulmonary arterial plasma was from 26 to 177 (median, 67) μmol/L. During inhalation of NO, there was a dose-related increase in plasma nitrate (change with parts per million NO inhaled, P<.001 by Friedman's test), both in the systemic and pulmonary arteries. The highest levels were observed during inhalation of NO (80 ppm), at which the systemic arterial plasma nitrate ranged from 58 to 123 (median, 90) μmol/L. Twenty minutes after cessation of NO inhalation, plasma nitrate had dropped in most patients, both in the systemic and pulmonary arteries. The levels of nitrate were lower in pulmonary arteries.
The basal arterial plasma level of nitrite before inhalation was 0.41±0.08 μmol/L. During inhalation of NO, plasma nitrite did not change significantly; at 80 ppm NO, the level was 0.52±0.14 μmol/L. HbNO was 12±3 μmol/L in the basal state and 14±1 μmol/L during inhalation of NO (80 ppm). The difference was not significant.

**Degradation of Nitrite In Vitro**

Incubation of arterialized blood (O2 saturation, 96.1±0.8%; range, 92.0% to 99.3%; pH 7.37±0.04) with nitrite for 2 minutes resulted in dose-dependent increases in the formation of nitrate and MetHb (Fig 2). No HbNO was detected. At the highest nitrite concentration used (200 μmol/L), nitrate in plasma reached a level of about 85 μmol/L, suggesting semiquantitative conversion of nitrite to nitrate. In parallel, MetHb was elevated, to about the same level (80 μmol/L). Prolongation of the incubation time to 15 minutes increased plasma nitrate to about 115 μmol/L and MetHb to about 110 μmol/L. Again, no HbNO was detected.

Incubation of venous blood (O2 saturation, 61.3±8.5%; range, 26.5% to 85.4%; pH 7.24±0.03) with nitrite for 2 minutes revealed a somewhat different pattern. Nitrate and MetHb concentrations increased to 125 and 145 μmol/L, respectively (Fig 2), and a significant formation of HbNO was observed (about 45 μmol/L at a nitrite concentration in the incubate of 200 μmol/L, Fig 2). When the incubations of venous blood with nitrite were prolonged to 15 minutes, plasma nitrate and MetHb increased even more, ie, to 185 and 190 μmol/L, respectively. HbNO was mainly unaffected after 15 minutes compared with after 2 minutes of incubation (Fig 2).

**Renal Excretion of Nitrate**

Individual data on plasma levels and excretions of nitrate during the urine collection periods are given in Table 3. Arterial plasma nitrate was 27±5 μmol/L. In the urine, the nitrate level ranged from 20 to 170 μmol/L. Estimation of nitrate clearance on the basis of the urinary excretion during the 16- to 25-minute collection periods and the arterial plasma level yielded clearance values of 6 to 61 mL/min (Table 3). The average clearance was 22±6 mL/min.

**Table 2. Arterial and Pulmonary Arterial Levels of Nitrate in the Basal State, During Inhalation of Nitric Oxide (20 to 80 ppm), and 20 Minutes After the End of Nitric Oxide Inhalation in Eight Heart Failure Patients**

<table>
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<td>177</td>
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NO indicates nitric oxide; Art, arterial plasma; and Pulm Art, pulmonary arterial plasma.
In the 24-hour urine collections, the nitrate excretion was 0.47 to 1.13 mmol/L. Calculation of individual clearances on the basis of the 24-hour excretions of nitrate and the arterial plasma levels revealed an average value of 19±4 mL/min (mean±SEM; range, 12 to 32 mL/min).

**Discussion**

Healthy subjects inhaling NO displayed time-related increases in the plasma and blood levels, respectively, of nitrate and MetHb in the current study. In patients with severe heart failure, the same metabolic pattern was observed during inhalation of increasing doses of NO. The in vitro incubations indicated conversion of the NO oxidation product nitrite to equal amounts of nitrate and MetHb in human fully oxygenated blood. In venous blood, the conversion of nitrite in addition yielded some formation of HbNO. Nitrate in plasma was eliminated via the kidneys with a clearance of about 20 mL/min.

We" have previously demonstrated that plasma, in contrast to whole blood, is rather inefficient in converting NO to nitrite. This observation indicates that the cell fraction of the blood is active in the metabolism of NO. Our previous ex vivo studies demonstrated almost quantitative conversion of NO to nitrate and MetHb in arterialized blood and, in addition, some conversion to HbNO in venous blood. In those studies, rather high concentrations of NO were directly mixed into the blood, implying that the conditions for uptake of NO into the red blood cell were optimal. In the present series, the same metabolites, ie, nitrate and MetHb, were obtained after inhalation of NO, in healthy subjects as well as in patients with severe heart failure. The previous ex vivo data on NO degradation are thereby extended to be valid also in vivo.

Some quantitative aspects on NO degradation are warranted on the basis of the present inhalation data in the healthy subjects. Inhalation of 25 ppm for 60 minutes yields a total NO dose of 420 μmol, assuming that the ventilation is about 7 L/min. If all NO were retained in the blood and converted to nitrate, the amount of nitrate formed would be around 400 μmol. With the prevailing plasma concentrations of nitrate and a renal clearance of 20 mL/min, about 40 μmol nitrate would be excreted into the urine during the 60-minute inhalation period. Nitrate, being a small anion, may be assumed to be distributed into the total body water. In the study subjects, total body water was

**TABLE 3. Arterial and Urine Levels of Nitrate, Urine Sample Volumes, Urine Collection Periods, and Calculated Clearance in Eight Healthy Volunteers**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex</th>
<th>Age, y</th>
<th>Length, cm</th>
<th>Weight, kg</th>
<th>Arterial Nitrate, μmol/L</th>
<th>Urinary Nitrate, μmol/L</th>
<th>Urine Volume, mL</th>
<th>Collection Time, min</th>
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estimated to be 37±3 L. With these assumptions, the plasma concentration of nitrate would be expected to be increased by about 10 \( \mu \text{mol/L} \) \((400 \text{ to } 40 \times \mu \text{mol})/37 \text{ L} \), which is close to the observed increase in plasma nitrate (from 26±2 to 38±2 \( \mu \text{mol/L} \)). Hence, it appears that conversion to nitrate is a major metabolic pathway for NO in the body. However, our data do not exclude the existence of additional minor metabolic pathways in healthy subjects or patients, eg, conversion of nitrite to urea or metabolism/excretion in the lungs.

Concerning MetHb, this compound ought to be formed in equimolar amounts to nitrate, and because of the lower distribution volume, its plasma levels might consequently be expected to increase more than that of nitrate. However, the erythrocytes contain a strong reductase, which converts MetHb back to Hb by the transfer of one electron.\(^{22}\) The currently found increase in MetHb of 6 \( \mu \text{mol/L} \) (from 7±1 to 13±1 \( \mu \text{mol/L} \)) at 60 minutes of NO inhalation may represent the quantitative resultant of these kinetics.

The patients with heart failure had substantially higher basal plasma levels of nitrate than the healthy subjects. The reason for this was not assessed. The additional nitrate may have been endogenously formed, ie, a result of facilitated vascular formation of NO\(^2\) or of macrophage activation. Alternatively, the higher plasma nitrate in the patients may have been caused by previous treatment with large intravenous doses of nitrodilators. The low clearance of nitrate observed implies that plasma levels of this ion are only slowly decreased even if continued administration is eliminated.

Endogenously formed NO liberated from the endothelial cells to the blood stream in vivo may, in principle, to some extent be oxidized to NO\(^3\) before uptake into the red blood cells has occurred. Furthermore, NO formed in the endothelial cells may diffuse abuminally, ie, to the interstitium of the surrounding tissue or to adjacent cells, before eventually reaching the capillaries to be washed out. Such NO, whether having been biologically active or not, may also enter the blood stream after having been oxidized to nitrite. Hence, we considered it necessary to assess whether the red blood cells are capable of converting oxidized NO, ie, nitrite ion, to nitrate as they convert NO itself. If this is not the case, metabolites other than nitrate/Methb and HbNO should be identified as well.

Our incubations of nitrite with human whole blood ex vivo yielded increasing amounts of nitrate. Since the conversion of nitrite to nitrate resulted in a parallel increase in the formation of MetHb, nitrite might be assumed to be metabolized in much the same way as NO, ie, in the red blood cells.\(^{13}\) It has previously been reported that nitrite, incubated anaerobically with red blood cells, is converted to NO and stabilized as HbNO.\(^{33}\) If such a conversion also occurred in our experiments, HbNO would be an intermediate in the metabolism of nitrite to nitrate. As mentioned above, erythrocytes contain a strong reductase.\(^{22}\) It seems possible that this enzyme may also be capable of catalyzing the reduction of nitrite to NO, which is a prerequisite for the formation of HbNO. The HbNO thus formed may then be dissociated to Hb and nitrate, under incorporation of molecular oxygen dissolved in the red blood cell cytosol or bound as oxyhemoglobin (HbO\(_2\)). Such dissociation has been reported to take place when the oxygen tension in the red blood cells is increased.\(^{24}\)

The nitrate added was, however, no more than semi-quantitatively converted to nitrate and MetHb in arterialized blood. Thus, when nitrite (200 \( \mu \text{mol/L} \)) was incubated with such blood for 15 minutes, plasma nitrate and MetHb increased from the basal average level of about 40 \( \mu \text{mol/L} \) to final average concentrations of about 110 to 115 \( \mu \text{mol/L} \). This is in some contrast to previous observations on NO incubations with arterialized whole blood ex vivo, in which virtually all NO was converted to nitrate and MetHb after 15 minutes of incubation.\(^{17}\) The current incubations of nitrite (200 \( \mu \text{mol/L} \)) with venous blood yielded more nitrate and MetHb, after 15 minutes reaching a level of 185 to 190 \( \mu \text{mol/L} \). In parallel, HbN0 rose to about 45 \( \mu \text{mol/L} \), implying that virtually all nitrite added could be identified as nitrate/MetHb or HbN0. These data seem to indicate that conversion of nitrite to nitrate/MetHb is more efficient when less HbO\(_2\) is present and are thereby in harmony with the concept discussed above, ie, that HbN0 is an intermediate in the conversion of nitrite to nitrate/MetHb. Further studies are required to fully clarify this matter. Nevertheless, it appears that Hb converts nitrite to the same products as NO, ie, to nitrate/MetHb and to HbN0, albeit via conversion routes that may differ for these two substrates. The relative abundance of nitrate/MetHb and HbN0 appearing seems to be determined by the HbO\(_2\)/Hb ratio in the cell.

An overview describing how NO or nitrite may be inactivated in the systemic circulation is presented in Fig 3. Endothelial NO seems to play the most important role as a physiological vasodilator in conductance and,
possibly, resistance vessels, being of less significance in capillaries and on the venous side of the systemic circulation. Hence, a substantial proportion of NO released luminally from the endothelial cells will enter red blood cells with a high O₂ saturation, since the Hb is not deoxygenated until during passage through the capillaries. Previous and present data demonstrate that such NO is converted to nitrate, under parallel formation of MetHb. However, some endothelial NO is probably released luminally also in postcapillary vessels. Such NO may also be inactivated by reacting with venous HbO₂ to nitrate and MetHb. Alternatively, it may be inactivated by nitrosylation of Hb. Some NO may have been converted to nitrate before entering the capillaries; the present data demonstrate that nitrate or HbNO will again be formed. It should, however, be emphasized that the metabolic routes from NO on the one hand and nitrite on the other may be different, despite ending up with the same final metabolite, ie, nitrate.

The final common metabolite of NO that is formed in arterial or in venous blood, via an intermediary formation of nitrite or not, consequently appears to be nitrate. We estimated nitrate clearance with two different techniques, ie, with utilization of 16- to 25-minute as well as 24-hour urine collection periods. The rationale for this was twofold. First, comparable results obtained with different techniques can be regarded to support each other. Second, the lack of substantial difference between the clearance levels obtained suggest that nitrate clearance is hardly subject to any large diurnal variation. Our experiments revealed that nitrate was excreted into the urine with a clearance of about 20 mL/min. This figure on nitrate clearance was obtained both with short-term and long-term urine collections, supporting the validity of the clearance level determined. Truly, the observed variation in clearance was high. Nevertheless, a clearance figure below 100 to 120 mL/min demonstrates an important feature in the elimination of nitrate from the blood, ie, that it occurs by glomerular filtration followed by substantial tubular reabsorption, either active or passive.

In summary, the present data provide evidence for a major pathway by which NO is inactivated and eliminated in human blood. The suggested process, ie, the formation of nitrate and MetHb from NO or nitrite together with HbO₂, yields products that are either eliminated via renal excretion (nitrate) or reversed with known endogenous mechanisms (conversion of MetHb to Hb). If HbNO is formed, this complex can also be disintegrated successively by a high oxygen tension, as in the alveolar capillaries in the lungs. The proposed process thereby seems to fulfill the criteria of providing a physiologically reasonable elimination route that also conforms to previously known data on plasma and urine levels or the products involved.

Acknowledgments

This study was supported by the Swedish Medical Research Council (project 4341) and by the Swedish Heart Lung Foundation.

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doi: 10.1161/01.RES.73.6.1121

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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