Biotransformation of Nitric Oxide

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Previous investigations into the health effects of nitrogen oxides (NOx) have mostly been conducted with special reference to nitrogen dioxide (NO2) and its direct effects on the respiratory system, while the study of nitric oxide (NO) has been disregarded. We carried out a study on NO by exposing rats and mice to 15NO or administering 15N-nitrite and 15N-nitrate to these animals by IP injection in order to elucidate the metabolic fate of NO.

The results of our study and previous findings led us to assume that the major metabolic path of inhaled NO is as follows: inhaled NO reacts with hemoglobin, forming nitrosyl-hemoglobin (NOHb), and from NOHb, nitrite (NO2-) and nitrate (NO3-) are generated. Major quantities of NOx are discharged into the urine and a certain amount is discharged into the oral cavity through the salivary glands and transformed to NO2. Part of this NO2 is converted to N2 gas in the stomach. Nitrate in the intestine is partly reduced to ammonia (NH3) through NO3, reabsorbed into the body, and converted to urea. Most of the metabolites of inhaled NO are excreted rapidly from the body within 48 hr.

Introduction

In order to clarify the health effects of nitrogen oxides (NOx), investigation of the direct pathological effects on the respiratory organs and basic studies on the absorption and biotransformation of NOx are important. However, for investigation of the absorption or metabolic transformation of NOx, the half-life of 15N is too short for it to be used as a radiisotope, and in the case of 15N, considerable amounts of the element are contained in the protein of the living body, so little work has been done with 15N (1–8). In this report, the biotransformation of nitric oxide (NO) and its intermediate metabolites, nitrite (NO2-) and nitrate (NO3-), are reviewed on the basis of results obtained in our own investigations.

Absorption and Conversion of NO in Blood

In physicochemical comparison with SO2, NO is less readily absorbed in the airway because of its low solubility in water [7.340 cm³/100 mL cold water (9)]. Such observations concerning NO absorption have been made in studies with isolated and perfused lung by Yokoyama and Poslethwait (10) and Mustafa (11). We have also shown that less than 10% of NO is absorbed and oxidized in perfused rabbit lungs (12). However, the results in the case of perfused lung would be expected to differ from those in the case of a living system. With regard to NO absorption into the living body, Wagner (13) reported that more than 80% of NO was absorbed in normal breathing and more than 90% was absorbed in deep breathing. We obtained similar results with inhalation of 10 ppm NO (unpublished data). Despite its low solubility in water, the absorption of NO into the body is almost complete. Goldstein et al. (14) showed in experiments with monkeys that in the case of inhalation of 0.3 to 0.9 ppm 15NO, 50 to 60% of the inhaled NO was found in the lungs and spread into the other organs through the bloodstream. We found a high 15N content in serum and urine after inhalation of 138 to 880 ppm 15NO, and within 24 hr, about 40% of the inhaled 15N was excreted into the urine (5).

NO is known to combine strongly with hemoglobin to form nitrosyl-hemoglobin (NOHb) in vitro. According to an investigation by Oda et al. (15), a very small amount of NOHb (0.13% of total hemoglobin) was found in the blood of mice after inhalation of 10 ppm NO. This suggested the possibility of rapid change of the absorbed NO in the blood. In addition, a number of studies have been made on the interaction between NO and blood or hemoglobin (16–20). Our experimental results with 15NO showed that NO entered the blood, combined with Hb in the first stage, and was oxidized rapidly to 15NO2 and 15NO3 (5). Though the amount of NOHb in the blood is very small and in in vivo exposure, NOHb is of important significance as an intermediate in NO metabolism (21). In the reaction process of NO and Hb, degeneration of Hb and damage to erythrocyte membranes are observed (22–24).

From our results, it is thought that due to its low solubility in water, the major proportion of inhaled NO
reaches the deeper portion of the lung, reacts with hemoglobin in erythrocytes to form NOHb, and is converted immediately to NO₂ and NO₃. The major proportion of inhaled NO is excreted in urine in the form of NO₃.

**Metabolism and Excretion of Inhaled NO**

Biotransformation of NO after conversion to NO₂/NO₃ is considered to be the same as the metabolism of NO₂/NO₃ from foodstuffs. Many studies have been devoted to problems related to the fate of ingested nitrate and nitrite in the body (25–35). It is certain from the literature that increased ingested nitrate or nitrite results in increased urinary excretion of nitrate and nitrite, but the details of the major metabolic pathway are not sufficiently known.

In the case of ¹⁵NO inhalation (145 ppm × 123 min) in rats (5), about 55% of the inhaled ¹⁵N was excreted in urine, 75% of excreted ¹⁵N being ¹⁵NO₃, and 24% ¹⁵N-urea (Exp. A1 and A2 of Fig. 1). Compared with the case of IP injection of Na¹⁵NO₂ or K¹⁵NO₃, the main metabolites were ¹⁵NO₃ and ¹⁵N-urea. (In the case of ¹⁵NO₂, a very small quantity of urea was found.) A pattern similar to the case of NO inhalation was found for ¹⁵NO₂ (Exp. B1 and B2 of Fig. 1), but the result for ¹⁵NO₃ differed. The reason for this is not well understood, but these observations are of some interest. The presence of ¹⁵N-urea in urine was confirmed by the urease method and that of ¹⁵NO₃ was confirmed by gas-liquid chromatography/mass spectrometry method using the derivative of 3,4-xylenol (Fig. 2). The peak of M/E 168 in Figure 2B was apparently a result of ¹⁵N-6-nitro-3,4-xylenol from inhaled ¹⁵NO.

In order to elucidate exactly all the metabolites in the body, Na¹⁵NO₂ (0.62 mg as ¹⁵N per animal) was injected IP into mice, and the ¹⁵N contents of the urine, feces, exhaled gas, and the body were estimated. As shown in Figure 3, 60.7% of the administered ¹⁵N was found in the urine, 7.8% in feces, 0.3% in exhaled gas, and 1.6% in the body. The residual 30% was not found.

Wang et al. (35) administered ¹⁵N-labeled nitrate and nitrite to rats and studied excretion and retention. They found that 60 to 70% of the dose was excreted in the urine, 10 to 20% was eliminated in the feces, and about 10% was retained in the body carcass (in the case of a single dose). The amount of unrecovered ¹⁵N that we obtained was high compared to that of Wang et al., although this may have been due to differences in the animal species and administration methods. The unrecovered ¹⁵N is assumed to have been in the form of N₂ gas, on the basis of both the recovery method used for exhaled gas and the *in vitro* experiments on the stomach contents of mice and on the presence of nitrite, as discussed in the next section.

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**FIGURE 2.** Mass spectra of 6-nitro-3,4-xylenol (A) and the derivative of 3,4-xylenol from the urine of rats exposed to ¹⁵NO (B). The urine was from the samples described in Fig. 1.

**FIGURE 3.** Distribution of ¹⁵N (excess) in mice after IP injection of Na¹⁵NO₂. Mice (five animals per experiment) were administered 2.88 mg Na¹⁵NO₂ (0.62 mg as ¹⁵N) per animal. The urine and feces were taken for 48 hr after the injection. The exhaled gas was collected for 48 hr to determine NH₃, NO, and NO₂. The mice were killed at 48 hr after injection, and carcasses were used to determine the residual ¹⁵N ("whole body").

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![Figure 1](image1.png) **FIGURE 1.** Distribution of ¹⁵N (excess) in the urine from rats after the exposure to ¹⁵NO or IP injection of Na¹⁵NO₂ or K¹⁵NO₃. Rats (2 animals per experiment) were exposed to 145 ppm ¹⁵NO for 123 min, or were administered 9.33 mg Na¹⁵NO₂ or 13.6 mg K¹⁵NO₃ (2 mg as ¹⁵N) per animal. The urine samples were taken for 48 hr after the exposure or injection.
Conversion of Nitrite and Nitrate in the Digestive Tract

The conversion of nitrite and nitrate in the digestive tract, especially in the stomach, should be evaluated in consideration of normal flora and injesta (36). A proportion of the NO$_3^-$ in the blood is transferred to the oral cavity through saliva. Nitrate in the oral cavity is partly reduced to NO$_2^-$ by oral bacteria. Thus, the produced NO$_2^-$ reacts readily with amines from ingested foods or drugs under acidic conditions to form nitrosoamines. We observed that a large amount of NO$_2$ gas and a small amount of NO are produced by anaerobic incubation with mouse stomach contents and NO$_2^-$ in a Smith tube at 37°C, pH 3.5 (Fig. 4) (7). The production of NO$_2$ gas is presumed to occur through the reaction shown below, which is used in the Van Slyke method of amine-N determination.

$$\text{H}_2\text{N}\text{-CH-COOH} + \text{HNO}_2 \rightarrow \text{HO-CHCOOH} + \text{N}_2 + \text{H}_2\text{O}$$

NO$_2^-$ in the stomach was converted to N$_2$ gas by the proteins of the mouse diet as in the in vitro experiment. That is to say, NO$_2^-$ entering the stomach is absorbed partly through the stomach wall, and some NO$_2^-$ reacts with amines, ureides, or ascorbic acid (37), but a considerable amount of NO$_2^-$ is changed to N$_2$ gas by reaction with proteins of the diet and disappears from the body. The unrecovered $^{15}$N in our previous experiments (5) may have been the result of this reaction.

![Figure 4](image)

**Figure 4.** Gas appearance by *in vitro* incubation experiments with stomach and its contents or food plus nitrite in stomach pH. Mice stomach and its contents of 7.5 g or food of 5.0 g were homogenized with 25 mL 0.85% NaCl solution, and the homogenate was adjusted to pH 3.5 by addition of 2N HCl. After 25 mg Na$^{15}$NO$_2$ was added to the homogenate, which was incubated at 37°C for 5 hr in Smith tube, the gas produced was analyzed by gas-liquid chromatography on a Molecular Sieve 5A column.

**Figure 5.** Transformation of $^{15}$NO$_2^-$ in *in vitro* experiments with mice feces. Mice feces of 11.3 g were homogenized with pepton-NaCl solution of 200 mL. After 35 mg Na$^{15}$NO$_2$ was added to the homogenate, which was incubated at 37°C for 6 hr, part of the homogenate was centrifuged at 3000 rpm for 10 min, and the supernatant was used to determine $^{15}$NH$_3$, NO$_2^-$, and NO$_2$.

![Figure 5](image)

**Table 1.** NH$_3$ – $^{15}$N concentration in intestine contents of mice after IP injection of Na$^{15}$NO$_2$.$^*$

| Time      | Total NH$_3$ - N, mg | NH$_3$ - $^{15}$N Atom % excess | $^{15}$N Excess µg Excess |
|-----------|----------------------|---------------------------------|--------------------------|
| Cecum and large intestine contents | After 1 hr 1.095 | 3.3008 | 36.1 |
| Control | After 3 hr 1.270 | 1.5656 | 19.9 |
|          | 1.984 | 0 | 0 |

$^*$Mice (five animals per experiment) were injected IP with 3.73 mg Na$^{15}$NO$_2$ (0.8 mg as $^{15}$N) per animal.

![Figure 6](image)

**Figure 6.** Hypothetical metabolic pathway of inhaled NO.
Nitrate in the stomach is transferred to the intestine without reduction and absorption in the healthy animal (34). However, in the stomach of ruminants such as cattle or sheep, NO₃⁻ is reduced to NO₂⁻ (38).

A considerable number of studies have been conducted regarding the fate of NO₃⁻ and NO₂⁻ in the intestine. Tannenbaum et al. (39) suggested that nitrate and nitrite are produced de novo in the intestine as a result of heterotrophic nitrification. According to the research of Witter et al. (34), who studied ¹⁵NO₃⁻ in humans and rats, the ¹⁸N compound administered was not rapidly absorbed through the stomach wall; the concentration was increased in the lower intestine, and a portion of the ¹⁸N was retained in the body. The results of Wang et al. (35) suggested that NO₂⁻ and NO₃⁻ in the intestine are converted to the nitrogen compounds other than NO₂ and NO₃ by intestinal bacteria before reaching the large intestine. The investigations by Hill et al. (39) and Ishiwata et al. (40) showed that neither nitrite nor nitrate could be detected in the contents of the intestine nor in the feces.

We observed in Na¹⁵NO₂ injection experiments that ¹⁵N in the intestine was retained for a relatively long time in comparison with retention in the liver and kidney (7). From more detailed investigations on ¹⁵N in the intestine, we showed that the greater part of ¹⁵N in the intestine is composed of trichloroacetic acid (TCA)-soluble and TCA-insoluble ¹⁵N, and that the amounts of NO₂⁻ and NO₃⁻ are very small except for those existing 1 hr after the injection.

The ratio of TCA-soluble ¹⁵N to total ¹⁵N increased with time. This suggests that low molecular weight ¹⁵N compounds in the intestine are eliminated relatively rapidly, while high molecular weight ¹⁵N compounds are retained for a considerable length of time. In vivo incubation experiments (7) with mice intestinal contents and NO₂⁻/NO₃⁻ suggested that NO₂⁻ in the intestine is reduced to NO₃⁻ and converted to unknown nitrogen compounds by intestinal bacteria.

To investigate the end products from NO₃⁻/NO₂⁻ in the intestine, a mixed solution of mouse feces and a peptone-NaCl solution of Na¹⁵NO₃ was incubated at 37°C for 6 hr (Fig. 5). The concentration of NO₃⁻ in the mixture decreased with time and disappeared after 6 hr. As for NO₂⁻, a temporary increase was found, but it disappeared after 6 hr. On the other hand, the concentration of ¹⁵N-NH₃ (¹⁵NH₃) in the incubation solution increased rectilinearly with time. The in vitro results suggested that NO₂⁻ in the intestine was converted to NH₃ through NO₂⁻ by intestinal bacteria.

To confirm the conversion of NO₃⁻ to NH₃ in vivo, mice were given an IP injection of ¹⁵N-nitrite (0.8 mg ¹⁵N per animal), after which the concentration of ¹⁵NH₃ in the intestine contents was estimated at 1 hr and 3 hr following the injection. As shown in Table 1, the atom percent excess of ¹⁵NH₃ in the intestinal contents after the injection showed high values compared with the control, indicating the conversion of ¹⁵NO₃⁻ to ¹⁵NH₃. From these results, it is considered that NO₃⁻ in the intestine is reduced to NH₃ through NO₂⁻ by intestinal bacteria, and NH₃ thus produced is absorbed through the intestinal walls and metabolized to urea.

**Discussion**

On the basis of the results mentioned above, the possible metabolic pathway of inhaled NO is illustrated in Figure 6. A small amount of inhaled NO reacts with tissue components in the lung, but most of it enters the blood through the alveoli and reacts with hemoglobin in erythrocytes. NO₂⁻ and NO₃⁻ are produced through NOHb (nitrosyl-hemoglobin) and these are transferred to the serum. The greater part of the NO₃⁻ is excreted into the urine through the kidney.

Part of the NO₃⁻ in the blood is secreted into the oral cavity through saliva, and is converted to NO₂⁻ by oral bacteria. Part of the NO₂⁻ that reaches the stomach is converted to N₂ gas with the proteins of the diet by the Van Slyke reaction and disappears. The intestinal NO₃⁻ transferred from the blood and stomach is converted to NH₃ or unknown compounds through NO₂⁻ by the intestinal bacteria. Ammonia thus produced is absorbed through the intestinal wall into the body. This reabsorbed ammonia is metabolized to urea through the urea cycle and is excreted into the urine.

The metabolism of NO/NO₂ in the living body greatly assists in its detoxification and disposal. Most of the NO/NO₂ is rapidly converted to low toxicity NO₂⁻ in the blood by oxidation with O₂, etc., and is eliminated from the body. However, a portion of NO/NO₂ and produced NO₂⁻ react with the living components and tissues, resulting in various injuries. NO₂⁻ is thought to be involved not only in pathological effects on the respiratory system (41), but also in injury of the cell membrane (24,42), disturbance of the information-mediating system (43,44), alteration of immunological functions (45,46), peroxidation of cell membrane lipids (47,48), carcinogenesis, and aging (49–51). A number of important questions regarding these problems cannot as yet be convincingly answered and still await further study.

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