Transformation of subcutaneous nitric oxide into nitrate in the rat

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Following its addition to arterialized blood *in vitro*, nitric oxide (NO) is transformed into nitrate in the erythrocytes. Inhaled NO is similarly transformed into nitrate in the blood *in vivo*. These observations suggest that nitrate is a universal end-metabolite of NO, i.e. of endogenously formed NO as well. However, endogenous NO may also be inactivated in tissues, i.e. outside the vascular lumen. To study the fate of NO metabolized with delayed access to the blood, rats were given subcutaneous injections of ^{15}NO or $K^{15}NO_3$, and the plasma concentrations of $^{15}NO_3^-$ were followed for 450 min after injection. The values for the distribution volume and plasma decay ($t_{\frac{1}{2}}$) of $^{15}NO_3^-$ did not differ between rats given ^{15}N-labelled NO and NO_3^-. The area under the plasma decay curve for rats given ^{15}NO amounted to 89 % of the corresponding area for animals given $K^{15}NO_3$. This demonstrates that ^{15}NO, when given extravascularly in millimolar concentrations, is mainly transformed into ^{15}N-labelled nitrate. Other rats were kept in an atmosphere containing a mixture of $^{16}O_2$ and $^{18}O_2$. Nitrate residues containing either one or two ^{18}O atoms were isolated from the blood, indicating that inhaled oxygen was incorporated during both the formation of NO and the subsequent transformation of NO into nitrate. The fraction of nitrate residues containing two ^{18}O atoms was larger than that containing one ^{18}O atom. We propose that nitrate is a major stable metabolite of endogenous NO that does not primarily diffuse into the vascular lumen following formation. Hence nitrate seems to be the quantitatively most important end-product of the metabolism of endogenous NO. The transformation of endogenous NO into nitrate involves the incorporation of inhaled oxygen.

INTRODUCTION

Nitric oxide (NO), formerly called endothelium-derived relaxing factor, is a biomediator with significant physiological actions in the vascular endothelium and platelets [1–4], nervous tissue [5] and macrophages [6]. The apparent involvement of endogenously formed NO in different disease states [7–14] calls for methods to assess its endogenous formation under various conditions. Measurement of circulating metabolites of NO and their excretion from the body might prove suitable for this purpose [15,16].

We and others have reported that some NO is transformed into nitrate in whole blood [17–21], and that the nitrate formed is excreted via the kidneys [15,16,19,20]. In those studies, nitrate formation was paralleled by the appearance of significant amounts of methaemoglobin in the blood, suggesting that the transformation involves oxygenated haemoglobin [19,20]. The technique utilized in those studies was to add NO to whole arterialized blood *in vitro*, or to measure the plasma accumulation of nitrate during NO inhalation. In both of these cases the NO administered had immediate or almost immediate access to erythrocytes, i.e. to cells that have a considerable capacity to transform NO into nitrate.

Following its synthesis in the vascular endothelium or in other cell systems, endogenously formed NO may diffuse luminally or abluminally. The possibility cannot be excluded that NO diffusing abluminally, thereby initially appearing outside the vascular lumen, is metabolized differently from NO that reaches the blood immediately. If such extravascular metabolism of NO yielded high amounts of metabolite(s) other than nitrate, then the latter ion would not be an accurate index of the endogenous formation of NO.

To address the possibility that NO initially appearing outside the vascular lumen is metabolized differently, i.e. is not transformed into nitrate, we administered ^{15}N-labelled NO subcutaneously, i.e. outside the vascular lumen, to rats and compared the appearance of $^{15}NO_3^-$ in the blood compared with that in other rats injected with authentic $K^{15}NO_3$ at the same site. It was reasoned that, if a portion of the ^{15}NO injected subcutaneously was transformed into $^{15}NO_3^-$, this transformation would be quantitatively reflected by the appearance of $^{15}NO_3^-$ in the plasma.

To characterize further the transformation of NO into nitrate *in vivo*, we exposed other rats to an atmosphere containing a mixture of $^{16}O_2$ and $^{18}O_2$ and analysed the appearance of ^{18}O-containing nitrate in the plasma. It was assumed that, if nitrate residues containing more than one ^{18}O atom occurred in the plasma following inhalation of a mixture of $^{16}O_2$ and $^{18}O_2$, then at least one of these ^{18}O atoms would have been included during the transformation of NO into nitrate.

MATERIALS AND METHODS

Injection of ^{15}NO or $K^{15}NO_3$

Male Wistar rats, weighing 210–330 g, were anaesthetized with sodium pentobarbitone (60 $mg \cdot kg^{-1}$ intraperitoneally initially, followed by 20 $mg \cdot kg^{-1}$ every 90 min). A polyethylene catheter was inserted into the right jugular vein and used for blood sampling. After insertion of the catheter, the rats were allowed to stabilize for at least 10 min before the experiment was started. A total of 16 rats, divided into two groups, were studied. In the first group ($n = 7$), the animals were given a subcutaneous injection of ^{15}NO (0.4 μmol in 10 ml; AGA Gas AB, Lidingö, Sweden) in the upper back region close to the left scapula. In the second group ($n = 9$), the animals were given a subcutaneous injection of $K^{15}NO_3$ dissolved in saline (0.4 $\mu mol \cdot ml^{-1}$ in 1 ml; Sigma) at the same site. Blood samples (0.2–0.3 ml) were taken at intervals

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between 30 and 450 min after injection. After separation of plasma, the samples were frozen at −18 °C.

Inhalation of $^{18}O_2$

Four male rats of an outbred Sprague–Dawley strain, weighing about 130 g, were studied under Neurolept anaesthesia. A catheter was introduced into the superior caval vein. Three rats were exposed to a gas mixture containing $^{16}O_2$ and $^{18}O_2$ ($^{18}O_2$ of > 98 % isotope purity; Larodan Fine Chemicals AB, Malmö, Sweden) in N_2 for 205–275 min. During the exposure the animals were kept in airtight cages, allowing sampling of gas from the cage atmosphere for determination of the fraction of $^{18}O_2$ in $^{16}O_2+^{18}O_2$, and the fraction of $^{16}O_2+^{18}O_2$ in N_2 [22]. The fraction of $^{18}O_2$ in $^{16}O_2+^{18}O_2$ increased exponentially from 0 to 90 % during the first 170–230 min of exposure, and then decreased. The fraction of $^{16}O_2+^{18}O_2$ in N_2 in the cage atmosphere decreased from about 30 % at the beginning of the experiment to about 20 % at the end. At the end of the exposure, blood was drawn from the catheter for analysis of cholesterol and nitrate. In one experiment the animal was exposed to $^{18}O_2$ for only 30 min. The same procedure was carried out in control rats that had been exposed to $^{16}O_2$ under similar conditions. After centrifugation of the blood sample, serum was obtained and stored at −20 °C. The studies were approved by the local Animal Investigations Committee.

Analyses

After thawing the plasma samples, all nitrate present was derivatized to nitrotoluene by shaking the diluted sample for 20 min with 500 μl of toluene and 200 μl of conc. H_2SO_4. The organic phase was then separated and 10 vol. of acetonitrile was added. A 1–2 μl portion was injected into a Varian 3400 gas chromatograph (Varian, Walnut Creek, CA, U.S.A.) equipped with a 25 m SPB 5 capillary column operated isothermally at 85 °C. This was connected to a Finnigan Incos 50 mass spectrometer (Finnigan, San Jose, CA, U.S.A.) operated in the negative-ion chemical ionization mode using methane as the reagent gas and selectively monitoring *m/e* 137 for unlabelled nitrate, *m/e* 138 for ^{15}N-labelled nitrate, *m/e* 139 for ^{18}O-labelled nitrate and *m/e* 141 for $^{18}O_2$-labelled nitrate [20]. The levels of 7α-hydroxycholesterol in plasma samples from rats exposed to $^{18}O_2$, and the fraction of 7α-hydroxycholesterol containing one ^{18}O atom in these samples, were analysed by MS using the conditions previously described for extraction, derivatization and chromatography [23]. The molecular ions monitored were *m/e* 546 for the unlabelled trimethylsilyl derivative and *m/e* 548 for the ^{18}O-labelled trimethylsilyl derivative.

Calculations

In rats injected with ^{15}NO or $K^{15}NO_3$, the distribution volume and plasma $t_{\frac{1}{2}}$ for $^{15}NO_3^-$ were calculated as follows. The fraction of $^{15}NO_3^-$ in the total NO_3^- in the plasma samples was plotted semi-logarithmically against time, the best linear fit was determined with a computer system, and the $t_{\frac{1}{2}}$ was determined from this fit. By extrapolating the fit to time 0, the theoretical concentration of $^{15}NO_3^-$ in the distribution volume could be calculated as a percentage of the body weight. Division of the molar mass of ^{15}NO or $^{15}NO_3^-$ given (0.4 μmol) with the concentration of $^{15}NO_3^-$ in the calculated distribution volume yielded the absolute distribution volume. Data are presented as means ± S.E.M.

The graph in Figure 2 was determined as follows. It was assumed that, if unlabelled or labelled NO reacted with oxygen on inhalation of any mixture of $^{16}O_2$ and $^{18}O_2$ in the inhalation gas, any of the following reactions could occur:

$$N^{16}O+{}^{16}O_2+e^- \rightarrow N^{16}O_3^- \quad \text{(I)}$$

$$N^{16}O+{}^{18}O_2+e^- \rightarrow N^{16}O^{18}O_2^- \quad \text{(II)}$$

$$N^{18}O+{}^{16}O_2+e^- \rightarrow N^{18}O^{16}O_2^- \quad \text{(III)}$$

$$N^{18}O+{}^{18}O_2+e^- \rightarrow N^{18}O_3^- \quad \text{(IV)}$$

$$N^{16}O+{}^{16}O+{}^{16}O+e^- \rightarrow N^{16}O_3^- \quad \text{(V)}$$

$$N^{16}O+{}^{16}O+{}^{18}O+e^- \rightarrow N^{16}O_2{}^{18}O^- \quad \text{(VI)}$$

$$N^{16}O+{}^{18}O+{}^{16}O+e^- \rightarrow N^{16}O_2{}^{18}O^- \quad \text{(VII)}$$

$$N^{16}O+{}^{18}O+{}^{18}O+e^- \rightarrow N^{16}O^{18}O_2^- \quad \text{(VIII)}$$

$$N^{18}O+{}^{16}O+{}^{16}O+e^- \rightarrow N^{16}O_2{}^{18}O^- \quad \text{(IX)}$$

$$N^{18}O+{}^{16}O+{}^{18}O+e^- \rightarrow N^{16}O^{18}O_2^- \quad \text{(X)}$$

$$N^{18}O+{}^{18}O+{}^{16}O+e^- \rightarrow N^{16}O^{18}O_2^- \quad \text{(XI)}$$

$$N^{18}O+{}^{18}O+{}^{18}O+e^- \rightarrow N^{18}O_3^- \quad \text{(XII)}$$

In reactions (I)–(IV), unlabelled or labelled NO reacts with oxygen in the molecular form to form nitrate directly. In reactions (V)–(XII), unlabelled or labelled NO reacts with atomic oxygen to form nitrate in two subsequent steps, i.e. via an intermediate compound. In either case the probability of reactions (I)–(XII) occurring should be dependent on the ratio between labelled and unlabelled oxygen at the time and the site of the reaction. This ratio should, in turn, be at equilibrium with the ratio $^{16}O_2/^{18}O_2$ in the inhalation gas mixture. NO is formed and released continuously from its site of synthesis, and has a very short half-life. Therefore it was considered accurate to regard the ratio between labelled and unlabelled oxygen as being the same at the time and site of formation of NO (from L-arginine and O_2) as at the time and site of the transformation (by incorporation of oxygen) of the NO formed into nitrate.

Furthermore, it was assumed that the loss of one oxygen atom from nitrate during its derivatization into nitrotoluene in the analytical procedure would affect ^{16}O and ^{18}O in relation to their occurrence. For example, if the proportions of $^{16}O_2/^{18}O_2$ in the inhalation gas mixture were 0.8:0.2, the probability of reaction (I) would be $0.8\times0.8=0.64$, the probability of reaction (II) would be $0.8\times0.2=0.16$, the probability of reaction (III) would be $0.2\times0.8=0.16$ and the probability of reaction (IV) would be $0.2\times0.2=0.04$. Furthermore, the loss of one random oxygen atom during the derivatization procedure would yield nitrotoluene from reaction (I) with *m/e* 137 only, since all three oxygens in the nitrate would have the same mass number. In contrast, the loss of one random oxygen atom during derivatization of nitrate from reaction (II) would yield two-thirds nitrotoluene with *m/e* 139 and one-third nitrotoluene with *m/e* 141. The loss of one random oxygen atom during derivatization of nitrate from reaction (III) would yield two-thirds nitrotoluene with *m/e* 139 and one-third nitrotoluene with *m/e* 137. In reaction (IV), only nitrotoluene with *m/e* 141 would be formed, irrespective of which of the oxygen atoms was lost. Taking reactions (I)–(IV) together, the ratio 0.2:0.8 for $^{18}O_2/^{16}O_2$ in the inhalation gas mixture would yield 69.3 % nitrotoluene with *m/e* 137, 21.3 % nitrotoluene with *m/e* 139 and 9.3 % nitrotoluene with *m/e* 141. The ratio of the relative occurrences of *m/e* 141 and 139 would then be 9.3:21.3, i.e. 0.44. From the above calculations, it is deduced that the ratio of the relative occurrences of *m/e* 141 and *m/e* 139 is inversely proportional to the percentage of $^{16}O_2$ in total O_2, in accordance with eqn. (1) below:

$$[m/e\ 141]/[m/e\ 139] = 75/[^{16}O_2]-0.5 \quad (1)$$

The line corresponding to eqn. (1) is shown in Figure 2.

By analogy, taking reactions (V)–(XII) together, the proportions 0.8:0.2 for $^{16}O_2/^{18}O_2$ in the inhalation gas mixture would yield 64% nitrotoluene with *m/e* 137, 32% nitrotoluene with *m/e* 139 and 4% nitrotoluene with *m/e* 141. The ratio between the relative occurrences of *m/e* 141 and 139 would then be 4/32, i.e. 0.125. Eqn. (2) below describes the relationship between the relative occurrences of *m/e* 141 and *m/e* 139 and the percentage of $^{16}O_2$ in total O_2:

$$[m/e\ 141]/[m/e\ 139] = 50/[^{16}O_2] - 0.5 \quad (2)$$

The line corresponding to eqn. (2) is also shown in Figure 2.

RESULTS

Injection of ^{15}NO or $K^{15}NO_3$

In rats injected with 0.4 μmol of ^{15}NO gas subcutaneously, $^{15}NO_3^-$ ions were present in the plasma from the first blood samples obtained after injection. The peak concentration of $^{15}NO_3^-$ appeared 30–90 min after injection of ^{15}NO, and subsequently decreased. The calculated $t_{\frac{1}{2}}$ averaged 256±25 min, and the distribution volume for $^{15}NO_3^-$ was 49.5±2.6% of the body weight. Individual values for the calculated $t_{\frac{1}{2}}$ and distribution volume are presented in Table 1.

In rats injected with 0.4 μmol of $K^{15}NO_3$ subcutaneously, $^{15}NO_3^-$ ions were also present in the plasma from the first blood samples obtained after injection. The peak concentration of $^{15}NO_3^-$ was, as above, observed 30–90 min after injection of $K^{15}NO_3$, and subsequently decreased. The calculated $t_{\frac{1}{2}}$ averaged 308±33 min, and the distribution volume for $^{15}NO_3^-$ was 49.8±1.4% of the body weight. Individual values for the calculated $t_{\frac{1}{2}}$ and distribution volume are presented in Table 1.

The curves depicting plasma $^{15}NO_3^-$ concentration against time in rats given ^{15}NO or $K^{15}NO_3$ are presented in Figure 1. The rats in the two groups were given the same molar mass of isotope (0.4 μmol). Since animals injected with ^{15}NO gas had a somewhat lower body weight (239±6 g) than the rats injected with $K^{15}NO_3$ (260±12 g), a correction was made by multiplying the observed plasma concentrations in rats injected with ^{15}NO gas by 239/251 (251 g was the average weight for the two groups of rats), and by multiplying the observed plasma concentrations in rats injected with $K^{15}NO_3$ by 260/251. After this correction, the area under the plasma $^{15}NO_3^-$ curve in Figure 1 representing rats given ^{15}NO gas corresponded to 89% of the area under the curve of the graph representing rats given $K^{15}NO_3$.

Table 1 Body weight, distribution volume and plasma $t_{\frac{1}{2}}$ for $^{15}NO_3^-$ in rats injected with 0.4 μmol of ^{15}NO or $K^{15}NO_3$ subcutaneously

Compound injected	Rat no.	Body weight (g)	Distribution volume (% of body weight)	$t_{\frac{1}{2}}$ (min)
^{15}NO gas	1	250	39	340
	2	225	59	190
	3	232	52	260
	4	227	50	180
	5	229	42	350
	6	243	53	240
	7	270	52	230
	Mean±S.E.M.	239±6	50±3	256±25
$K^{15}NO_3$	8	255	56	510
	9	334	43	280
	10	300	52	370
	11	234	51	300
	12	212	55	230
	13	234	50	260
	14	249	50	380
	15	255	46	250
	16	263	46	190
	Mean±S.E.M.	260±12	50±1	308±33

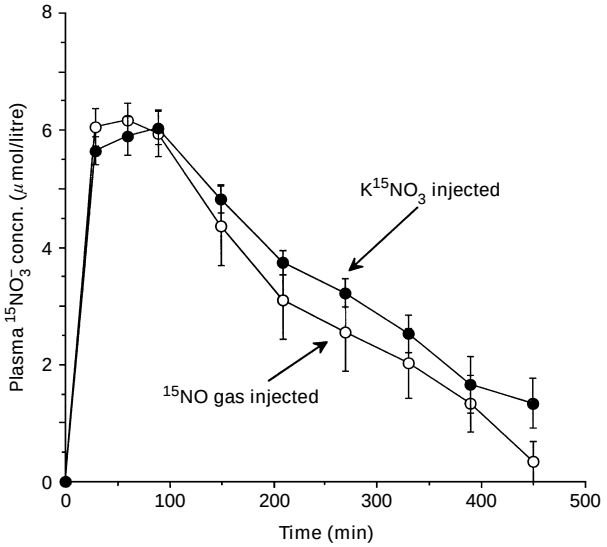

Figure 1 Plasma concentrations of $^{15}NO_3^-$ following subcutaneous injection of 0.4 μmol of ^{15}NO gas ($n = 7$) or $K^{15}NO_3$ solution ($n = 9$) into rats

All plasma concentrations are normalized to the same body weight, as described in the Materials and methods section. The area under the curve for rats injected with ^{15}NO gas (○) corresponds to 89% of the area under the curve during the same time interval (0–450 min) for rats injected with $K^{15}NO_3$ (●). Data are presented as means±S.E.M.

Table 2 Occurrence of nitrate residues of *m/e* 137, 139 and 141 in the MS readings of plasma collected from rats exposed to $^{18}O_2$ in the inhalation gas mixture

m/e values of 137, 139 and 141 correspond to nitrate residues with two ^{16}O atoms, one ^{16}O atom and one ^{18}O atom, and two ^{18}O atoms respectively. The occurrence of *m/e* 137 was set as 100%. Plasma nitrate was converted into nitrotoluene as described in the Materials and methods section. * Significantly ($P = 0.016$) greater than *m/e* 139.

Rat	*m/e* 137 (%)	*m/e* 139 (%)	*m/e* 141 (%)	Ratio of *m/e* 141 to *m/e* 139
a	100	7.8	10.0	1.28
b	100	6.2	8.4	1.35
c	100	5.4	10.2	1.89
d	100	5.4	8.4	1.56
Mean±S.E.M.	100±0	6.2±0.6	9.3±0.5*	1.52±0.14

Inhalation of $^{18}O_2$

In rats inhaling a mixture of ^{16}O and ^{18}O, nitrate residues containing one ^{16}O and one ^{18}O atom, or two ^{18}O atoms (*m/e* 139 and 141 respectively), were isolated from plasma sampled after the end of the inhalation period, in addition to the 'normal' nitrate residues containing two ^{16}O atoms (*m/e* 137). The relative occurrences of residues of *m/e* 139 and 141 for the individual rats are shown in Table 2. The percentage of nitrate residues of *m/e* 141 (i.e. containing two ^{18}O atoms) was significantly ($P < 0.02$) higher than that of nitrate residues of *m/e* 139 (containing one ^{16}O and one ^{18}O atom).

The calculated relationships between the percentage $^{16}O_2$ consumed and the relative occurrence of nitrate residues of *m/e* 141 and 139 are displayed in Figure 2. The observed average ratio between the occurrence of *m/e* 141 and 139 presented in Table 2, i.e. 1.52, was found to correspond to a percentage of $^{16}O_2$ in the total O_2 in the gas consumed during the reaction of about 37% (the rest being $^{18}O_2$) if a reaction between NO and molecular oxygen had occurred. If a two-step reaction between NO and atomic oxygen had prevailed, the observed average ratio for the occurrence of *m/e* 141 and 139 (1.52) would correspond to a percentage of $^{16}O_2$ in the total O_2 in the gas consumed during the reaction of about 25% (the rest being $^{18}O_2$).

Rats inhaling a mixture of $^{16}O_2$ and $^{18}O_2$ also had significant amounts of 7α-hydroxycholesterol containing one ^{18}O atom in their plasma. The fraction of 7α-hydroxycholesterol containing ^{18}O varied from 9 to 52%, the value being mainly dependent on the duration of inhalation of the gas mixture.

In plasma from rats exposed to $^{16}O_2$ only, there were no significant chromatographic peaks corresponding to nitrate residues of *m/e* 139 or 141, and there was no 7α-hydroxycholesterol containing ^{18}O.

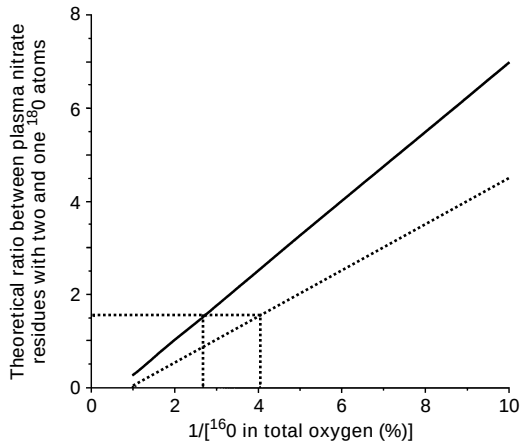

Figure 2 Calculated theoretical relationships between the percentage of $^{16}O_2$ in the consumed gas mixture ($^{16}O_2 + {}^{18}O_2$) and the ratio between nitrate residues with two and one ^{18}O atom(s) incorporated

Nitrate residues with two and one ^{18}O atom(s) incorporated correspond to *m/e* values of 141 and 139 respectively. The solid line represents the equation for the formation of nitrate directly from NO and molecular oxygen (eqn. 1). The broken line represents the equation for the formation of nitrate from NO and atomic oxygen in two steps, i.e. via an intermediate compound (eqn. 2). For details of calculations, see the Materials and methods section. The broken horizontal line represents the observed average ratio between *m/e* 141 and *m/e* 139 given in Table 2, i.e. 1.52. The vertical broken lines represent the percentages of $^{16}O_2$ in total O_2 required to yield this ratio, i.e. 37% (where NO reacts directly with molecular oxygen) and 25% (where NO reacts with atomic oxygen in two steps).

DISCUSSION

Injection of ^{15}NO gas subcutaneously into rats resulted in the rapid appearance of $^{15}NO_3^-$ in the plasma. The plasma decay curve had an area that amounted to 89% of that of the corresponding curve for rats given an equimolar mass of $K^{15}NO_3$ solution at the same site. Values for the plasma half-life and distribution volume for $^{15}NO_3^-$ did not differ between rats given ^{15}NO gas and $K^{15}NO_3$ solution. In plasma from other rats exposed to an inhalation gas mixture of $^{16}O_2$ and $^{18}O_2$ in N_2, nitrate residues containing both one and two ^{18}O atoms were recovered.

Previous studies from our and other laboratories have highlighted the crucial role of oxygenated haemoglobin in the transformation of NO into nitrate [17–21]. Recently we reported that about 73% of inhaled NO appeared in the urine as nitrate [24]. NO is a highly unstable molecule. Since NO formed endogenously may be inactivated outside the vascular tree, i.e. before reaching the capillaries closest to its site of synthesis, data on the metabolism of exogenously applied NO cannot be taken directly to reflect the inactivation of the endogenously formed compound. In fact, recent studies indicate that NO inactivated in aqueous solution free of haemoglobin is mainly transformed into nitrite [25,26], and that formation of nitrate in aqueous haemoglobin-free solution primarily results from the extracellular reaction of NO with superoxide radicals [27]. To study how NO is inactivated in the absence of haemoglobin, i.e. extravascularly, we administered exogenous NO *in vivo* by a route that would limit or at least delay its contact with oxyhaemoglobin. Subcutaneous tissue consists to a considerable extent of fat, and is sparsely vascularized. By injecting NO subcutaneously, it was assumed that its contact with oxyhaemoglobin would be delayed in comparison with the situation following inhalation, or after direct injection into the blood or into a more richly vascularized tissue such as skeletal muscle. The rationale for selecting ^{15}NO was that its metabolite(s) would be possible to identify separately from those derived from the endogenous formation of NO, or from sources such as the diet. As controls, we used rats given the same molar mass of the metabolite under investigation, i.e. nitrate, at the same site. Using this experimental design, any difference between rats given ^{15}NO and $K^{15}NO_3$ in the appearance of ^{15}N-labelled nitrate in plasma (in quantity or in time) should reflect a delay or incompleteness in the transformation of ^{15}NO into $^{15}NO_3^-$ in the subcutaneous tissue.

Following the subcutaneous injection of ^{15}NO, $^{15}NO_3^-$ appeared in the blood. We did not observe any time difference in the plasma $^{15}NO_3^-$ decay curves between rats injected with ^{15}NO and those injected with $K^{15}NO_3$. If anything, there was a tendency towards higher plasma concentrations of $^{15}NO_3^-$ during the first 60 min following injection of ^{15}NO compared with $^{15}NO_3^-$. This indicates that the uptake and inactivation of ^{15}NO in the circulation was at least as rapid as the uptake of $^{15}NO_3^-$. Furthermore, the area under the $^{15}NO_3^-$ curve for rats injected with ^{15}NO amounted to 89% of the corresponding area for rats injected with $K^{15}NO_3$, demonstrating that ^{15}NO was transformed almost quantitatively into $^{15}NO_3^-$. The fate of the remaining 11% of ^{15}NO, i.e. fraction of ^{15}NO not appearing as $^{15}NO_3^-$ in the blood, could not be determined in the present experiments.

In this context, it should be noted that the injection of ^{15}NO yielded a high local concentration of labelled NO in the subcutaneous space at the site of injection. This supra-physiological concentration may, at least theoretically, have confounded the results. First, high concentrations of NO may be toxic and might therefore elicit a local inflammatory response that affects the conditions for the elimination and metabolism of NO. A local inflammatory response may have facilitated tissue perfusion at the site of injection, thereby enhancing the uptake of ^{15}NO or $^{15}NO_3^-$ into the vascular space. However, uptake of NO should be rapid even under non-inflammatory conditions, due to its high solubility in lipid. Therefore it seems unlikely that a local inflammatory response would have developed soon enough following injection to further enhance the uptake of ^{15}NO (or $^{15}NO_3^-$ formed locally from the injected ^{15}NO). Secondly, the high concentrations of ^{15}NO may have affected the conditions

for its normal metabolism by changing the local proportions of (labelled) NO and oxygen. Our experimental design does not offer sufficient possibilities to fully control this potentially confounding factor. The results must consequently be interpreted with this experimental deviation from physiological conditions in mind.

To characterize further the metabolism of endogenous NO, rats were exposed to a mixture of $^{16}O_2$ and $^{18}O_2$ in the atmosphere. In these experiments it was considered necessary to determine whether the conditions prevailing during the inhalation of this gas mixture containing $^{16}O_2$ and $^{18}O_2$ were appropriate for studies of enzymic or non-enzymic metabolism. The formation of 7α-hydroxycholesterol from cholesterol is a previously well characterized enzymic reaction in the metabolism of cholesterol [23]. After inhalation of the gas mixture containing $^{16}O_2$ and $^{18}O_2$, the exposed animals displayed in their circulation 7α-hydroxycholesterol containing ^{18}O atom incorporated. Thus the experimental conditions seemed appropriate for metabolic studies.

In the derivatized plasma samples from rats that had inhaled the gas mixture containing $^{16}O_2$ and $^{18}O_2$, nitrotoluene of m/e 139 and 141 was detected, in addition to nitrotoluene of m/e 137. These m/e values indicate the presence of nitrate residues in the nitrotoluene with one ^{16}O and one ^{18}O atom (m/e 139), and with two ^{18}O atoms (m/e 141). No nitrotoluene peaks of m/e 139 or 141, above the normal isotopic level of $^{18}O_2$ in normal oxygen, were observed in the control rats. The appearance of nitrotoluene of m/e 139 (one ^{16}O and one ^{18}O atom in the nitrate residue) in the plasma samples from rats exposed to $^{18}O_2$ may indicate that some ^{18}O was utilized during the transformation of NO into nitrate. Alternatively, it may have been due to the incorporation of ^{18}O from molecular $^{18}O_2$ during the formation of NO from L-arginine [28]. More conclusively, the appearance of nitrotoluene of m/e 141 (two ^{18}O atoms in the nitrate residue) in the rat plasma samples clearly demonstrated that at least one of these ^{18}O atoms must have been incorporated when NO was transformed into nitrate.

The relative rates of incorporation of labelled and unlabelled oxygen during the formation of NO from L-arginine, as well as during the transformation of NO into nitrate, should be dependent on the $^{16}O_2/^{18}O_2$ ratio in the inhalation gas. Accordingly, the appearance of nitrate residues of m/e 141 and 139 should also be dependent on the $^{16}O_2/^{18}O_2$ ratio. Based on these facts, the relationship between the fraction of $^{16}O_2$ in $^{16}O_2+{}^{18}O_2$ in the gas consumed during the reaction and the ratio between nitrate residues of m/e 141 and 139 could be calculated. Figure 2 displays two theoretical relationships; one is based on direct formation of nitrate from NO and molecular oxygen, and the other is based on the formation of nitrate from NO and atomic oxygen in two steps, i.e. via an intermediate compound. As seen from Figure 2, a decreasing fraction of $^{16}O_2$ (i.e. an increasing fraction of $^{18}O_2$) in the inhalation gas would yield a progressively increasing m/e 141/m/e 139 ratio, i.e. more m/e 141 and less m/e 139. The observed average ratio between m/e 141 and m/e 139 in the present experiments, i.e. 1.52, was found to require that 37% of the inhaled oxygen was $^{16}O_2$ (the rest of the oxygen being $^{18}O_2$) if a reaction between NO and molecular oxygen had occurred. If a reaction between NO and atomic oxygen had occurred in two steps, the observed ratio (1.52) would require that no more than 25% of the inhaled oxygen was $^{16}O_2$, and that the rest was $^{18}O_2$. Irrespective of which reaction was prevailing, these percentages (37 or 25%) were not reached in the inhaled gas until during the later part of the present experiments; as indicated in the Materials and methods section, the relative fraction of $^{18}O_2$ increased during the course of the experiments. The calculated theoretical requirement of no more than 37 or 25% $^{16}O_2$ in the gas consumed during the formation of NO and the subsequent transformation of NO into nitrate corresponds well with the maximal incorporation of ^{18}O into 7α-hydroxycholesterol that was found simultaneously, i.e. about 50%. The absolute incorporation of ^{18}O into both nitrate and 7α-hydroxycholesterol must, however, also be dependent upon the turnover rate. In any case, the metabolic pattern observed in plasma taken after the end of the exposure to $^{16}O_2+{}^{18}O_2$, i.e. the m/e 141 to m/e 139 ratio, must mainly reflect the formation of metabolites taking place during the later part of the experiment.

The possibility that an isotope effect may have confounded the present results should also be considered. NO may, theoretically, react differently with ^{18}O compared with ^{16}O, due to the different sizes and energy distributions of these molecules. In particular, such an isotope effect might occur if NO reacts with haem-bound oxygen, where specific steric conditions are prevailing. Recent experiments in our laboratory have been helpful in evaluating the possibility of such an effect. Utilizing exposure to a constant (instead of increasing) $^{18}O_2/^{16}O_2$ ratio during the course of otherwise similar experiments, we have been able to compare the theoretical and observed formation of nitrate residues with m/e values of 141 and 139. No difference between the theoretical and observed formation ratios was observed (0.62 ± 0.05 and 0.58 ± 0.04 respectively), strongly disfavouring the existence of a significant isotope effect.

The conclusion that seems to be warranted from the $^{16}O_2+{}^{18}O_2$ exposure experiments is that the transformation of NO into nitrate, under the experimental conditions prevailing in the present study, was mainly (or exclusively) dependent on the incorporation of inhaled oxygen. The conclusion is based on the observed m/e 141 to 139 ratio; this ratio would not have been attained if other oxygen sources had been utilized in the transformation of NO into nitrate.

To summarize, the present experiments demonstrate that, under the conditions used, almost 90% of NO appearing extravascularly is inactivated by transformation into nitrate. This strongly suggests that endogenously formed NO is also transformed into nitrate under these conditions. The inactivation of endogenous NO is brought about by incorporation of inhaled oxygen. The inactivation of NO to give one major stable end-product indicates that plasma and urinary nitrate may be useful both in the assessment of the endogenous formation of NO and in the clinical evaluation of the metabolism of inhaled NO in patients. The measurement of plasma and/or urine nitrate for such purposes requires that proper attention is given to the possible confounding action of other sources of nitrate. It has recently been demonstrated that dietary nitrite/nitrate is one such important confounding factor which can, however, be overcome by a proper diet [29]. In contrast to food intake, cigarette smoking does not seem to affect plasma or urine nitrate levels significantly [30], despite reportedly high levels of nitric oxide(s) in cigarette smoke [31].

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