Estimation of total rate of formation of nitric oxide in the rat

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Nitric oxide (NO) is a powerful mediator with important actions in several organ systems. NO is synthesized during the enzymatic conversion of L-arginine and molecular oxygen to L-citrulline. About 90 % of the NO formed is degraded to nitrate. Utilizing this information we have developed a method for assessment of the total rate of formation of NO in the rat. Male Wistar rats were kept in a closed-cage system allowing controlled breathing of a mixture of $^{18}O_2$ and $^{16}O_2$ in N_2 for up to 5 h. Blood samples for mass spectrometric analysis of nitrate residues with varying numbers of ^{18}O atoms incorporated were drawn before and during the exposure to $^{18}O_2$. By comparing the relative incorporation of ^{18}O into nitrate residues to the $^{16}O_2/^{18}O_2$ ratio in the breathing gas mixture in the cage system it was possible to calculate the absolute rate of NO formation in the animal. The rate of formation of NO in anaesthetized rats ranged from 0.33 to 0.85 $\mu mol \cdot kg^{-1} \cdot h^{-1}$. The rate of formation did not differ significantly in rats which were awake during the experiment (range 0.36–0.72 $\mu mol \cdot kg^{-1} \cdot h^{-1}$). The L-arginine analogue N_ω-nitro-L-arginine methyl ester (L-NAME) dose-dependently inhibited the formation of NO, at a dose of 100 mg/kg by more than 99 %. The technique presented allows estimation of the total rate of formation of NO *in vivo* in rats. Application of the technique may yield important information about the physiological and pathophysiological roles of NO. It may also be utilized to evaluate the effect of pharmacological treatment on NO formation.

INTRODUCTION

Nitric oxide (NO) is a biomediator with significant physiological actions in vascular endothelium [1], nervous tissue [2], and macrophages [3]. Since NO may play a primary or secondary role in disease states, there is a need for methods to assess the formation of NO in experimental and clinical conditions.

We and others have previously identified nitrate as the main stable end product of NO formation *in vivo* [4–6]. Analysis of plasma levels or urinary excretion of nitrate may reflect the endogenous formation of NO. A prerequisite is, however, that plasma or urine sampling is preceded by dietary restriction for a period corresponding to 5–7 plasma half-lives of this ion, since alimentary nitrate is a confounding factor [7]. Proper control and restriction of dietary intake of nitrite/nitrate in laboratory animals may not always be possible to achieve.

NO is formed by enzymatic conversion of L-arginine and molecular oxygen to L-citrulline. The inactivation takes place in the red blood cells, in which about 90 % of the NO formed is converted to nitrate by the action of oxygenated haemoglobin [4,6,8,9]. We assumed that the quantitative involvement of molecular oxygen in both the formation of NO and its further degradation to nitrate might be utilized to determine the total formation rate of NO in laboratory animals. In the current study we present the evidence that assessment of the rate of appearance of nitrate residues with various number of ^{18}O atoms incorporated into nitrate residues during exposure to $^{18}O_2$ inhalation can be used to calculate the absolute formation rate of NO *in vivo*.

METHODS

Controlled exposure of rats to $^{18}O_2$

Male Wistar rats, weighing 190–360 g, were anaesthetized with sodium pentobarbitone (60 mg/kg i.p. initially, followed by another 20 mg/kg i.v. every 90 min if needed). A polyethylene catheter was introduced into the right superior caval vein and used for blood sampling. The animal was then transferred into the experimental cage system, which consisted of an animal container connected in series by polyethylene tubes to a gas pump and a combined CO_2 and water vapour eliminator (Figure 1). The pump and the eliminator continuously freed the gas in the system from CO_2 and water vapour during the experiment. The system was closed and filled via a valve with a gas mixture containing $^{18}O_2$ (> 97 % isotope purity; Larodan Fine Chemicals

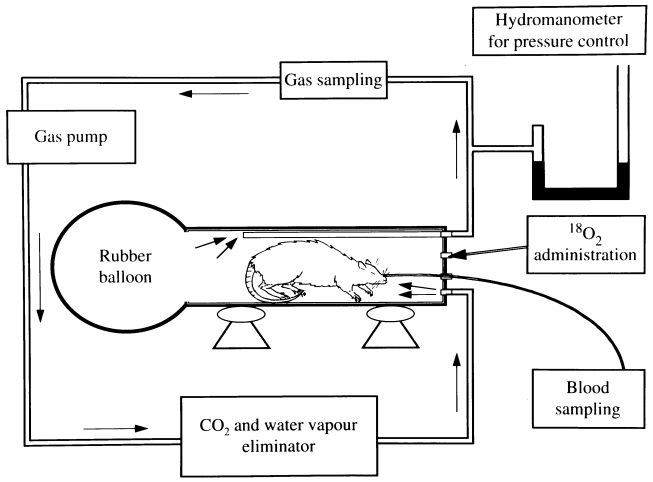

Figure 1 Schematic presentation of the experimental setup

The experimental cage system consisted of an animal container connected in series with a gas sampling valve, a gas pump, and a CO_2 and water vapour eliminator. $^{18}O_2$ was administered via a valve, and blood sampling was made possible by airtight externalization of a thin catheter introduced into the right superior caval vein. One end of the container was connected via an airtight seal to a rubber balloon, which allowed the closed-cage system to decrease its internal volume (due to oxygen consumption, and CO_2 and water vapour elimination) by about 1 l during the course of an experiment. A hydromanometer connected to the system was used to ensure that the pressure variations in the system during the experiment were low.

Abbreviations used: NO, nitric oxide; L-NAME, N_ω-nitro-L-arginine methyl ester.

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AB, Malmö, Sweden) in normal air. The cage was gas-tight, but the system allowed sampling of gas from the atmosphere for determination of the concentrations of $^{18}O_2$, $^{16}O_2$, and N_2. The venous catheter was externalized through a hole in the cage wall, which was tightened with silica gel. During the course of an experiment, which lasted for about 120 min, no gas was added to the system. The consumption of oxygen during the experiment and the elimination of CO_2 in the filter continuously lowered the volume of the gas at ambient pressure in the cage system. A rubber balloon attached to one end of the animal container allowed a volume decrease of the system from about 2 l at the beginning to about 1 l at the end of the experiment. A hydromanometer connected to the cage system continuously monitored the pressure in the system; the pressure varied within ambient pressure ± 2 cm of water (Figure 1).

Samples from the cage atmosphere were taken every 15–20 min during the experiment. Blood for analysis of plasma nitrate (0.5–1 ml) was sampled before the onset of the experiment and then at 120 min after the onset. After separation of plasma the samples were stored at -18 °C.

A total of 38 rats, divided into five groups, were studied. In the first group ($n = 13$), serving as anaesthetized controls, the animals were given a 0.5 ml injection of saline before the experiment. In the second group ($n = 7$) the animals were allowed to wake up after the insertion of the catheter and the injection of 0.5 ml saline, i.e. before being transferred to the cage system. They were awake throughout the experiment. In the third group ($n = 6$) the animals were pretreated with the NO synthase inhibitor N_ω-nitro-L-arginine methyl ester (L-NAME, Sigma Chemical Co., St. Louis, MO, U.S.A.; 10 mg/kg i.v. diluted in saline) about 15 min before the experiment was started. In the fourth group ($n = 4$) the animals were pretreated with L-NAME (30 mg/kg i.v. in saline), and in the fifth group ($n = 8$) they were given L-NAME (100 mg/kg i.v. in saline). All rats had the same volume injected.

Analyses

Gas samples from the cage system, taken in a gas-tight syringe during the experiments, were immediately analysed in a Varian Saturn ion trap mass spectrometer operated in the electron ionization mode, selectively monitoring $m/e = 28$ for N_2, $m/e = 32$ for $^{16}O_2$, $m/e = 36$ for $^{18}O_2$, and $m/e = 44$ for CO_2.

Plasma samples were thawed and analysed for total nitrate concentration, and for concentration of nitrate with various numbers of ^{18}O atoms incorporated, with a stable isotope dilution assay utilizing a known amount of $K^{15}NO_3$ (Sigma Chemical Co., St. Louis, MO, U.S.A.) as internal standard. After addition of standard, a 50 μl portion of the plasma sample was added to an Eppendorff tube that had been kept at -80 °C containing a mixture of 750 μl of benzene and 120 μl of trifluoro-methane sulphonic acid. The tube was shaken at room temperature for 30 min, allowing endogenous nitrate and added standard to be converted to nitrobenzene. After centrifugation, a 600 μl portion of the organic phase was separated and briefly washed with 150 μl of 0.5 M Na_2CO_3. Subsequently, a 1–2 μl portion of the organic phase was injected into a temperature-programme controlled (60–120 °C) Varian 3400 gas chromatograph equipped with a 30 m XTI-5 capillary column. The gas chromatograph was connected to a Varian Saturn ion trap mass spectrometer operated in the positive ion/chemical ionization mode, selectively monitoring $m/e = 124$ for non-labelled nitrobenzene, $m/e = 125$ for nitrobenzene containing the ^{15}N-labelled internal standard, $m/e = 126$ for nitrobenzene containing one ^{18}O atom, and $m/e = 128$ for nitrobenzene containing two ^{18}O atoms. Methane was used as the reactant gas. The detection limit for endogenous nitrate was 0.1 μmol/l.

Calculations

In the calculation of the total rate of formation of NO the following assumptions were made: (a) the probability for $^{16}O_2$ and $^{18}O_2$ to be involved in the formation of NO by NO synthase is dependent on the ratio ($^{16}O_2/^{18}O_2$) at the time and site of reaction. Since the present experiments were extended in time this ratio must, during the major part of each experiment, have been in equilibrium with the ratio ($^{16}O_2/^{18}O_2$) in the inhalation gas mixture; (b) for the same reason the probability for $^{16}O_2$ and $^{18}O_2$ to be involved in the conversion of NO to nitrate must be dependent on the ratio ($^{16}O_2/^{18}O_2$) in the inhalation gas; (c) nitrate is the major stable end product of NO under normal conditions, covering about 90 % of the NO formed [10]. In the present calculations all NO was assumed to be metabolically transformed to nitrate; this implies an underestimation of the total rate of formation of NO amounting to about 10 %.

During inhalation of any mixture of $^{16}O_2$ and $^{18}O_2$ either of the following NO synthase dependent reactions will occur:

$$\text{L-arginine} + {}^{16}O_2 \rightarrow \text{L-citrulline} + N^{16}O \quad \text{(I)}$$

$$\text{L-arginine} + {}^{18}O_2 \rightarrow \text{L-citrulline} + N^{18}O \quad \text{(II)}$$

NO is an unstable molecule. In tissues or body fluids it will rapidly be converted to nitrate (~ 90 %, cf. above), unless bound to proteins containing thiol groups, metal ions, or haem groups.

If the transformation of NO to nitrate takes place in one single step by incorporation of molecular oxygen during inhalation of any mixture of $^{16}O_2$ and $^{18}O_2$, the reactions will be as follows:

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

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

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

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

On the other hand, if the transformation of the NO formed to nitrate takes place by incorporation of atomic oxygen in two subsequent steps during inhalation of any mixture of $^{16}O_2$ and $^{18}O_2$, the reactions will be as follows:

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

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

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

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

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

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

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

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

In the preparation of the plasma samples prior to analysis with GC/MS the nitrate ion was derivatized to nitrobenzene. During such a derivatization one oxygen atom is lost. It is assumed in the calculations that this loss affects ^{16}O and ^{18}O in the nitrate in

proportion to their ratio at the site of reaction. This ratio should, for reasons discussed above, be the same as the corresponding ratio in the inhalation gas mixture.

The relative occurrences of $^{16}O_2$ and $^{18}O_2$ at the time and site of the formation of NO and its further degradation to nitrate will determine the relation between the number of nitrate residues with 0, 1, and 2 $^{18}O_2$ atoms incorporated. In a recent study [10] we reported that this relation can be mathematically expressed as follows:

$$(m/e = 128):(m/e = 126) = 75/(100 - {}^{18}O_2) - 0.5 \quad \text{(A)}$$

In this equation ($m/e = 128$) is the amount of nitrate residues with mass number 128 (two $^{18}O_2$ atoms incorporated) formed, and ($m/e = 126$) is the amount of nitrate residues with mass number 126 (one ^{18}O atom and one ^{16}O atom incorporated). $^{18}O_2$ is the average percentage of labelled oxygen in the inhalation gas mixture during the experiment.

Equation (A) is valid if NO is converted to nitrate by incorporation of molecular oxygen. If NO is converted to nitrate by incorporation of atomic oxygen in two subsequent steps, i.e. via formation of an intermediate, the corresponding equation will be:

$$(m/e = 128):(m/e = 126) = 50/(100 - {}^{18}O_2) - 0.5 \quad \text{(B)}$$

The method for estimation of total NO formation rate applied in the present study thereby allows individual control so that each experiment is in harmony with the conditions discussed above. As shown here, each individual percentage of $^{18}O_2$ of the total O_2 in the cage atmosphere will correspond to a certain ratio between the occurrences of nitrobenzene with ($m/e = 128$) and ($m/e = 126$) in the plasma of the exposed animals. By comparing the observed ratio [($m/e = 128$):($m/e = 126$)] actually obtained in the different experiments with that obtained by calculation of equations (A) and (B) above using the percentage of $^{18}O_2$ applied in the particular experiments it was possible to assess which of the reactions for incorporation of oxygen (molecular oxygen in one step, or atomic oxygen in two steps via an intermediate compound) in the conversion of NO to nitrate that in fact was prevailing.

The calculation of the total rate of formation of NO requires that the excretion of labelled and unlabelled nitrate during the time of the experiment is taken into account.

If the plasma concentration of nitrate is not the same at the beginning and at the end of the experiment, then the formation rate of nitrate may be assumed to differ from the excretion rate during the experimental period. Then the total rate of formation can be calculated according to the following formula:

$$x_t = M_0 + p \times t + \frac{X_0 - M_0}{M_0^{(p-a)/p}}(M_0 + p \times t)^{(p-a)/p} \quad \text{(C)}$$

in which x_t is the total mass of labelled nitrate present at time t, M_0 is the total mass of all nitrate (labelled and unlabelled) present at time 0, a is the mass of labelled nitrate formed per time unit, b is the mass of nitrate excreted per time unit, and p is $(a-b)$.

A simplified formula can be applied if the plasma concentration of total nitrate is the same at the beginning and the end of the experiment. If this is the case, the formation of nitrate and the excretion of this ion may be assumed to be equal during the experimental period. Then the total rate of formation can be calculated according to the following formula:

$$x_t = M + (x_0 - M)e^{(-a/M)\times t} \quad \text{(D)}$$

in which x_t is the total mass of labelled nitrate present at time t, M is the total mass of all nitrate (labelled and unlabelled) present, and a is the mass of labelled nitrate formed per time unit.

Student's t test for unpaired observations was used to evaluate between-group differences in nitrate turnover rate. A P value < 0.05 was regarded as statistically significant. Data are presented as mean ± S.E. of mean.

RESULTS

Methodological aspects

The total oxygen ($^{18}O_2 + {}^{16}O_2$) concentration in the cage system at the start of the different experiments ranged from 33 to 55 %. It decreased to reach a final concentration ranging from 21 to 47 % at the end of the experiments.

The relative concentration of $^{18}O_2$, i.e. $^{18}O_2$ as a percentage of the total O_2 concentration, ranged from 17 to 54 % in the different experiments. The relative concentration of $^{18}O_2$ did not vary significantly during the course of the experiments.

The individual calculated (theoretical) ratios between $m/e = 126$ and $m/e = 128$ in the various experiments are presented in Table 1A (anaesthetized controls) and Table 1B (awake controls). These ratios were calculated under either of two assumptions: (a) that NO was transformed to nitrate by incorporation of atomic oxygen in two separate steps, and (b) that NO was transformed to nitrate by incorporation of molecular oxygen in one single step. In these tables the corresponding observed ratios are presented as well. As seen from Table 1 (A and B) the observed mean ratios (1.46 and 1.48 in anaesthetized and awake rats, respectively) were close to the ratios calculated on the assumption that NO was transformed to nitrate by incorporation of molecular oxygen (1.50 and 1.47 in anaesthetized and awake rats, respectively). In contrast, the ratios calculated on the assumption that NO was transformed to nitrate by incorporation of atomic oxygen in two steps (3.78 and 3.70 in anaesthetized and awake rats, respectively) differed markedly from the respective observed ratios.

The ratios calculated on the assumption that NO was transformed to nitrate by incorporation of molecular oxygen were highly correlated to the observed ratios, both in the anaesthetized (0.920) and in the awake rats (0.924).

NO turnover in anaesthetized and awake rats

The rate of total NO formation in the anaesthetized rats was calculated in two different ways. In all experiments the rate was calculated assuming that the plasma concentration of nitrate was the same throughout the experiment. In addition, in some experiments blood was also sampled at the end of the experiment, and plasma nitrate was analysed. In these experiments the rate of total NO formation was also calculated on the basis of the observed change in plasma nitrate level during the experiment (for further details see 'Calculations'). The total rate of NO formation in anaesthetized rats calculated assuming stable plasma nitrate ranged from 0.33 to 0.85 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ (mean ± S.E.: 0.55 ± 0.04 $\mu mol \cdot kg^{-1} \cdot h^{-1}$; Table 1A). The total rate of NO formation in anaesthetized rats calculated with the use of plasma nitrate also at the end of the experiment ranged from 0.48 to 0.63 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ (mean ± S.E.: 0.56 ± 0.03 $\mu mol \cdot kg^{-1} \cdot h^{-1}$). Comparison of the two methods for calculation of total rate of NO formation revealed a significant difference (0.61 ± 0.01 vs. 0.56 ± 0.03 $\mu mol \cdot kg^{-1} \cdot h^{-1}$, $P = 0.022$).

For comparison, the total rate of NO formation in the anaesthetized rats was also calculated from the observed plasma level of nitrate and the plasma clearance of this ion. Plasma clearance was estimated on the basis of data obtained in an

Table 1 Calculated (theoretical) ratios between nitrate residues (nitrobenzene) with $m/e = 126$ and $m/e = 128$, observed ratios between nitrate residues with $m/e = 126$ and $m/e = 128$, and rate of total NO formation in anaesthetized and awake control rats

The column 'Calculated ratio (O + O)' was calculated assuming that NO was transformed to nitrate by incorporation of atomic oxygen in two separate steps, and the column 'Calculated ratio (O_2)' was calculated assuming that NO was transformed to nitrate by incorporation of molecular oxygen in one single step. The rate of total formation of NO was calculated assuming either that new formation of nitrate and excretion of this ion were similar in magnitude during the measuring procedure, i.e. that the plasma level, and hence the total body mass of nitrate, were constant ('stable [NO_3^-]'). Alternatively, the rate of total formation of NO was calculated on the basis of the observed plasma levels of NO_3^- at the beginning and at the end of each experiment constant ('unstable [NO_3^-]'). The last column in Table 1A ('est. from clearance') represents the rate of total NO formation, as estimated from the excretion of nitrate. This excretion was calculated on the basis of nitrate clearance estimated from data in a previous study [10], and from the respective plasma levels of nitrate observed. Plasma levels of nitrate in blood sampled at the beginning of each experiment are also given, and in some experiments (bold figures) also in blood sampled at the end of the experiment. Data are presented as mean ± S.E. 'Correlation' refers to correlation between the respective data in the columns 'Calculated ratio O_2' and 'Observed ratio'. For further details concerning the calculations, see 'Methods', subheading 'Calculations'.

Series/ Rat	Calculated ratio $m/e = 126$:($m/e = 128$) (O + O)	Calculated ratio $m/e = 126$:($m/e = 128$) O_2	Observed ratio ($m/e = 126$): ($m/e = 128$)	Plasma nitrate during exp (μmol/l) Beg.	Plasma nitrate during exp (μmol/l) End	Rate of total NO formation (μmol·kg^{-1}·h^{-1}) Experimental Stable [NO_3^-]	Rate of total NO formation (μmol·kg^{-1}·h^{-1}) Experimental Unstable [NO_3^-]	Rate of total NO formation (μmol·kg^{-1}·h^{-1}) Est. from clearance
(A) Anaesthetized controls								
1	**2.18**	**1.07**	**1.08**	**22.2**	**23.8**	**0.63**	**0.59**	1.18
2	**2.47**	**1.17**	**1.12**	**26.0**	**32.5**	**0.60**	**0.53**	1.38
3	**2.14**	**1.05**	**1.07**	**21.4**	**21.5**	**0.64**	**0.63**	1.13
4	**3.03**	**1.34**	**1.42**	**26.2**	**34.8**	**0.57**	**0.48**	1.39
5	**3.13**	**1.37**	**1.30**	**26.7**	**29.6**	**0.63**	**0.59**	1.42
6	6.84	2.13	2.26	28.3	—	0.72	—	1.50
7	4.08	1.62	1.39	17.0	—	0.85	—	0.90
8	2.96	1.32	1.30	16.3	—	0.36	—	0.86
9	4.29	1.67	1.67	14.1	—	0.34	—	0.75
10	5.91	1.98	1.76	13.5	—	0.61	—	0.72
11	5.35	1.89	1.58	16.1	—	0.33	—	0.85
12	3.68	1.52	1.56	21.9	—	0.39	—	1.16
13	3.09	1.36	1.41	16.0	—	0.52	—	0.85
Mean	3.78	1.50	1.46	20.4 (**24.5**)	**28.4**	0.55 (**0.61**)	**0.56**	1.08
± S.E.	0.41	0.10	0.09	1.45 (**1.12**)	**2.53**	0.04 (**0.01**)	**0.03**	0.08
Correlation		0.920						
(B) Awake controls								
14	**3.33**	**1.43**	**1.63**	**20.4**	**16.8**	**0.66**	**0.76**	
15	**1.63**	**0.85**	**1.01**	**23.7**	**16.8**	**0.64**	**0.84**	
16	**1.98**	**0.99**	**0.95**	**16.0**	**18.0**	**0.45**	**0.42**	
17	**3.18**	**1.39**	**1.50**	**13.3**	**11.2**	**0.36**	**0.42**	
18	4.97	1.81	1.53	18.7	—	0.72	—	
19	5.66	1.94	1.88	11.7	—	0.41	—	
20	5.17	1.85	1.86	14.8	—	0.65	—	
Mean	3.70	1.47	1.48	16.9 (**18.4**)	**15.7**	0.56 (**0.53**)	**0.61**	
± S.E.	0.60	0.16	0.14	1.60 (**2.31**)	**1.53**	0.05 (**0.07**)	**0.11**	
Correlation		0.924						

earlier study [10]. Using this method the rate of total formation was 1.08 ± 0.08 μmol·kg^{-1}·h^{-1}.

In the awake rats the total rate of NO formation calculated assuming stable plasma nitrate ranged from 0.36 to 0.72 μmol·kg^{-1}·h^{-1} (mean ± S.E.: 0.56 ± 0.05 μmol·kg^{-1}·h^{-1}; Table 1B). The total rate of NO formation in awake rats calculated with the use of plasma nitrate also at the end of the experiment ranged from 0.42 to 0.84 μmol·kg^{-1}·h^{-1} (mean ± S.E.: 0.61 ± 0.11 μmol·kg^{-1}·h^{-1}; Table 1B). In this series the comparison of the two methods for calculation of total rate of NO formation revealed no significant difference (0.53 ± 0.07 vs. 0.61 ± 0.11 μmol·kg^{-1}·h^{-1}, $P = 0.182$). There was no significant difference in NO formation rate between anaesthetized and awake rats.

Table 2 Total formation rate of nitrate in rats exposed to $^{18}O_2$ after administration of the NO synthase inhibitor L-NAME (10–100 mg/kg)

L-NAME markedly and dose-dependently inhibited the formation of nitrate, indicating that NO formation was counteracted similarly.

	Dose of L-NAME (mg/kg) 0	10	30	100
Formation rate of NO_3^- (μmol·kg^{-1}·h^{-1})	0.554 ± 0.044	0.190 ± 0.051	0.048 ± 0.003	0.003 ± 0.003

Effect of L-NAME on NO turnover (Table 2)

L-NAME (10–100 mg/kg i.v.) dose-dependently decreased the rate of total NO formation in the rats. At a dose of 10 mg/kg the formation rate was about 34% of control, and at a dose of 30 mg/kg the formation rate had dropped to about 9% of control. The highest dose of L-NAME (100 mg/kg) almost completely

abolished the formation of NO, the average formation being less than 1 % of control. In half of the experiments with L-NAME (100 mg/kg) no formation of NO was detected. The observed ratio between $m/e = 126$ and $m/e = 128$ could not be calculated accurately in the groups of animals given L-NAME, due to very low formation rates.

DISCUSSION

The present study describes a method for determination of the total rate of formation of NO in small laboratory animals. The basal rate of formation of NO in rats is reported, and found to be unaffected by the anaesthesia applied. The applicability of the method is tested by estimating the effect of the NO synthase inhibitor L-NAME.

The method developed is based on isotope dilution via supply of a labelled precursor, i.e. $^{18}O_2$, into the metabolic process under investigation. By knowing the theoretical dilution of isotope in the metabolic process, and the mass concentration of the final metabolite(s), the total rate of formation during the period of isotope administration could be calculated. The theoretical considerations behind the method are simple. Nevertheless, the practical calculations are made under some assumptions which require a comment.

The marker for the metabolic process under study was not NO itself, but a stable metabolite, i.e. nitrate. To allow substitution of the product under investigation (i.e. NO) with a metabolite (i.e. NO_3^-) all NO can be shown to be rapidly transformed to nitrate, and the nitrate ion can be shown not to be further degraded or eliminated. Furthermore, no other endogenous source for NO should be present. We have recently demonstrated that about 90 % of ^{15}NO injected subcutaneously in the rat appears as $^{15}NO_3^-$ in the blood of the animal; the fate of the remaining 10 % could not be identified [10]. Considering this the present method appears to underestimate the total rate of NO turnover by approx. 10 %. Being aware of this likely underestimation, we preferred not to accordingly make a correction of the formation rate. The data on the rate of formation presented here are consequently somewhat low.

Nitrate is a stable ion in the body and is excreted into the urine in this form [11]. The continuous renal clearance of nitrate yields some excretion of labelled metabolite that was mathematically corrected for in the present calculations. We utilized two different types of calculations. In the first one, the plasma level of nitrate was assessed in samples taken both in the beginning and at the end of the experiments. An increasing plasma level of nitrate should indicate that the formation of nitrate – and hence also the formation of NO – was larger than the elimination during the experimental period. Conversely, a decreasing plasma level of nitrate should indicate that the formation of nitrate, and NO, was smaller than the elimination during the experimental period. The formula initially utilized included both these plasma levels of nitrate. A comparison between the outcome of this type of calculation with a simplified one, in which the plasma level of nitrate, and hence the formation of NO, was assumed to be the same throughout the experiment, revealed a small but significant difference in the estimated rate of total formation of NO in the anaesthetized, but not in the awake, rats. The small differences found between the outcome of the initial formula and the simplified one, as applied in anaesthetized or awake rats, is shown in Table 1 (A and B). We consider that the differences were small enough to justify the use of the simplified formula, thereby only requiring that the plasma level of nitrate was assessed in one initial blood sample. In the latter part of the present study we therefore only applied the simplified formula, assuming a stable plasma concentration of nitrate and a formation rate of NO that was balanced by an equally large excretion rate for nitrate.

The calculation of the theoretical dilution of isotope in the metabolic process studied does not seem to include any hazards. The assumption that the relative concentration of $^{18}O_2$ in the cage atmosphere also reflects the probability for $^{18}O_2$ to be incorporated into NO is trivial, and forms the basis for all isotope studies (i.e. not only the current experiments). The assumption that the conversion of NO to nitrate is governed by the same condition concerning incorporation of $^{18}O_2$ also appears acceptable. We also assumed that the loss of one oxygen atom during the derivatization of nitrate to nitrobenzene was neutral from an isotope point of view. Furthermore, due to the small difference in size between the ^{18}O atom and the ^{16}O atom we considered an isotope effect of significant importance unlikely.

The mass analysis of nitrobenzene without or with one or two ^{18}O atoms incorporated is a simple procedure developed in our laboratory [10]. From the observed plasma concentration of labelled nitrate the total mass of labelled nitrate is calculated by correction for distribution volume [10]. The analytical error in the analysis of nitrate with this method is low (< 5 %).

One particular feature linked to the present method is that it offers some possibility of internal control. The theoretical calculation of isotope dilution yields, for each single relative concentration of $^{18}O_2$ in the cage atmosphere during the experiment, a specific ratio between nitrate residues with two and one ^{18}O atoms incorporated, respectively. This specific ratio can be taken to confirm that the experiment was conducted under the conditions theoretically assumed. As shown in the 'Calculations' the ratio will follow formula (A) if NO is transformed to nitrate by incorporation of molecular oxygen in one step. Another formula (B) can be created to present the process of NO transformation to nitrate via incorporation of atomic oxygen in two separate steps, i.e. via an intermediate. It has previously been demonstrated that NO can be stochiometrically converted to nitrate by either oxymyoglobin or oxyhaemoglobin *in vitro* [12]. In these reactions NO was converted to nitrate in one single step, i.e. by incorporating molecular oxygen from the haem moiety. In parallel, one electron was transferred from the haem iron to the nitroxide, possibly via intermediate formation of peroxynitrite [12]. We earlier observed that NO apparently is transformed to nitrate by incorporation of molecular oxygen also *in vivo* [10]. In the present study, the calculated metabolic ratio for incorporation of molecular oxygen into NO displayed a good fit to the observed ratio, while the calculated metabolic ratio for incorporation of atomic oxygen into NO in two steps displayed no such fit. In the light of the presently observed metabolite ratio it therefore appears likely that the transformation of NO to nitrate by incorporation of molecular oxygen is a major metabolic pathway *in vivo*. The possible role of oxymyoglobin for the conversion of NO to nitrate *in vivo* (as earlier shown *in vitro* [12]) requires further studies. Nevertheless, in future experiments utilizing the currently adopted stable isotope technique, the mass spectrometric readings of the ratio between nitrate residues with two and one ^{18}O atoms, respectively, could probably be used as a simple control to ensure that a particular experiment fulfilled the assumed conditions. The calculated and observed metabolic ratios obtained in our experiments and discussed above are presented in Table 1A.

The total rate of formation of NO observed currently ranged from 0.33 to 0.85 $\mu mol \cdot kg^{-1} \cdot h^{-1}$. It should be emphasized that this range involves all formation of NO going on in the animal under the study period, i.e. in the endothelium, neuronal tissue and immune system. It may be assumed that the immune system

was not activated during the first 2 h of the experiment. Hence, the figure obtained probably mainly reflects NO formation in the vascular endothelium and the neuronal tissue. Furthermore, it may be noted that the rate of formation did not differ in anaesthetized compared to awake rats, at least not with the anaesthesia applied presently. This enables application of the method in studies in which the experimental procedure requires anaesthesia. As a comparison, we also calculated the turnover rate from the observed plasma level of unlabelled nitrate in the individual animals and the plasma clearance, as estimated from data obtained in a previous study [10]. This 'clearance turnover rate' was higher than the experimental turnover rate obtained on the basis of the inhalation of $^{18}O_2$. Furthermore, there was no apparent correlation between the 'clearance turnover' and the '$^{18}O_2$ turnover'. This is not surprising, since 'clearance turnover' comprises not only endogenously formed nitrate, but also dietary ingested forms of this ion. The data can be taken to indicate that estimation of the excretion of nitrate may be a questionable means to assess its endogenous formation.

The observed rate of formation cannot be directly compared to other conditions or species, since measurements of similar character have not been conducted previously. In humans, the renal clearance of nitrate is about 30 ml/min, and the plasma level is about 35 μmol/l. This corresponds to a 24 h excretion of nitrate amounting to about 1500 μmol. Assuming a body weight in humans of 70 kg, the rate of formation based on nitrate excretion would be about 0.9 $\mu mol \cdot kg^{-1} \cdot h^{-1}$. This figure is close to that presently observed in the rat.

To evaluate the applicability of the method we administered an established NO synthase inhibitor, i.e. L-NAME, to the animals. The estimated total rate of formation of NO dropped rapidly in the presence of L-NAME, as expected. This clearly demonstrates that the method may be used under experimental conditions. The dose–response curve for L-NAME was in good accordance with previous observations on the effect of this drug in rats [13].

Thus, the present method for analysis of total body formation of NO *in vivo* offers a simple and reliable possibility for physiological, pathophysiological, and pharmacological studies. Hitherto, *in vivo* studies have had to rely upon indirect indices of NO formation, e.g. cGMP levels or responses to known NO-releasing agents like bradykinin or acetylcholine. Utilization of the method presented here may reveal important quantitative aspects of the rate of NO formation in relation to physiological processes like shear, pathophysiological processes like atherosclerosis, and the action of cardiovascular drugs like nitrodilators and calcium blockers. Current experiments in our laboratory clearly demonstrate that the method may also be applied for measurement in humans.

Received 23 April 1997/29 October 1997; accepted 11 November 1997

The present study was supported by the Swedish Medical Research Council (Project 4341), and by the Swedish Heart–Lung Foundation. The expert mathematical assistance by Stefan Wennmalm, M.Sc., is gratefully acknowledged.

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