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REACTIONS OF NITRIC OXIDE WITH NITRONYL NITROXIDES AND OXYGEN: PREDICTION OF NITRITE AND NITRATE FORMATION BY KINETIC SIMULATION†

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Nitric oxide reacts with nitronyl nitroxides (NNO) to form imino nitroxides (INO) and this transformation can be monitored using electron spin resonance spectroscopy. Recently, Akaike *et al.*, reported that NNO such as 2-phenyl-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (PTIO) and its derivatives (e.g., carboxy-PTIO) react with nitric oxide (·NO) in a 1:1 stoichiometry forming 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl (PTI) or the respective product (e.g., carboxy-PTI) together with nitrite and nitrate (Akaike *et al.*, *Biochemistry 32*, 827–832, 1993). In this paper, we reevaluate their results and show that the stoichiometry of the reaction between PTIO and ·NO is 0.63 ± 0.06:1.0. The reason for this discrepancy is due to an erroneous assumption by Akaike *et al.*, that the stoichiometry for the reaction between ·NO and O_2 is 2:1 in aqueous solution. If the data reported by Akaike *et al.*, were recalculated using a 4:1 stoichiometry established for the aqueous oxidation of ·NO, the reaction between ·NO and PTIO would give a stoichiometry of 0.5:1.0 in closer agreement with our data. We propose a mechanism for the reaction between PTIO and ·NO in aqueous solution. This mechanism predicts that the stoichiometry between carboxy-PTIO and ·NO is dependent on the rate of generation of ·NO and is 1:1 only at low rates of ·NO generation (i.e., 10^{-13} M/s). However the stoichiometry approaches 0.5:1.0 at higher rates of ·NO production or when it is added as a bolus. The ratio between nitrite and nitrate also varies as a function of the rate of generation of ·NO. The model agrees with previous experimental observations that the aqueous oxidation of ·NO in air saturated solutions will exclusively form nitrite and predicts that ·NO will only generate substantial amounts of nitrate if it is released at a rate less than 10^{-17} M/s. This may have important consequences in cellular systems where the concentration of ·NO is typically measured from nitrite production.

KEY WORDS: Nitric oxide, nitronyl nitroxide, electron spin resonance.

Abbreviations: ·NO, nitric oxide; ESR, electron spin resonance; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl; carboxy-PTI, 2-(4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl; NNO, nitronyl nitroxide; INO, imino nitroxide.

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INTRODUCTION

Nitric oxide (·NO) is generated *in vivo* by the nitric oxide synthase family of enzymes.[1,2] These enzymes synthesize ·NO and use L-arginine, NADPH and oxygen as substrates. The simplest and most frequently used method for the assay of ·NO is the Griess reaction.[3] This method detects nitrite as an oxidation product of nitric oxide. To accurately determine the rate of ·NO formation, the stoichiometry of nitrite formation from the oxidation of ·NO must be known. The addition of ·NO to an aerobic solution gives a yield of 100% nitrite.[4–6] In this situation, the Griess reaction will give an accurate measure of ·NO. However, in situations where ·NO is generated slowly, this stoichiometry may vary to a considerable extent. In biological systems it is mandatory to measure both nitrite and nitrate (after reduction to nitrite) in order to fully quantitate ·NO production by the Griess reaction.

Nitronyl nitroxides (NNO) such as 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) have been shown to react with ·NO to form imino nitroxides (INO) such as 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl (carboxy-PTI) [equation 1].

$$\begin{matrix}\text{carboxy-PTIO} \\ \text{or} \\ \text{NNO}\end{matrix} + \cdot NO \rightarrow \begin{matrix}\text{carboxy-PTI} \\ \text{or} \\ \text{INO}\end{matrix} + NO_2^{\cdot} \qquad [1]$$

NNO and INO are both nitroxides (see Figure 1 for chemical structures), which can be detected and distinguished by the electron spin resonance (ESR) technique.[7,8]

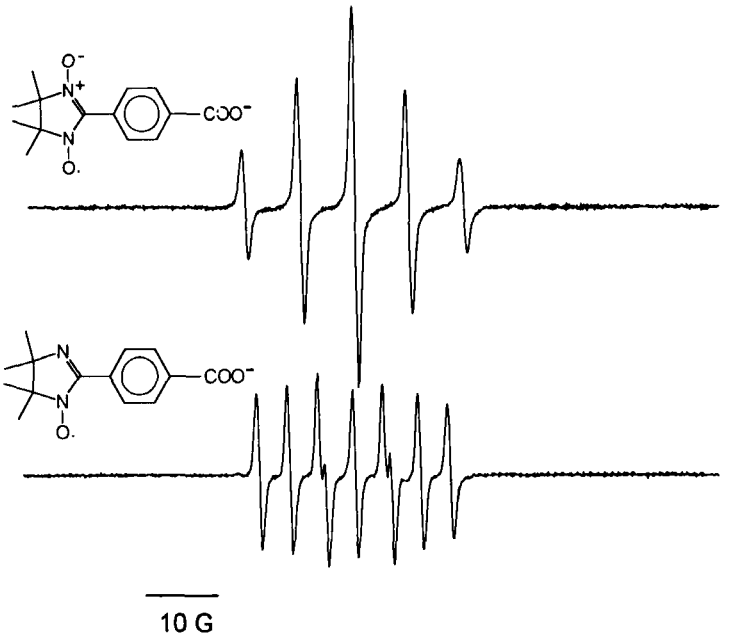

FIGURE 1 ESR spectra of NNO and INO. (top) The spectrum was obtained from a solution of the NNO, carboxy-PTIO (100 μM), in PBS (pH 7.5); (bottom) the spectrum after mixing the above solution with excess ·NO to form the INO, carboxy-PTI. Spectrometer conditions: scan range, 100 G; modulation amplitude, 0.5 G; microwave power, 1 mW; time constant, 0.032 s; and scan time, 2 min.

Thus, this reaction [equation 1] represents a potential tool for detecting ·NO in chemical and biological systems.[9,10]

Recently, Akaike *et al.* (1993)[10] reported the stoichiometry for the reaction between NNO and ·NO [equation 1] as 1:1. In this paper, we have reinvestigated the stoichiometry of the reaction between NNO and ·NO. Our results show the stoichiometry of this reaction to be 0.63 ± 0.06:1.0. The discrepancy between our results and those reported by Akaike *et al.* (1993)[10] are discussed with reference to a kinetic model for the reaction of ·NO with NNO and oxygen. This model predicts that the yields of the products formed from such reactions may vary as a function of the rate of ·NO formation.

EXPERIMENTAL PROCEDURE

Materials

Carboxy-PTIO was synthesized following the published procedure.[7,11] ·NO gas was obtained from Matheson Gas (Madison, WI). All reactions were performed in Dulbecco's phosphate-buffered saline (PBS), pH 7.4.

ESR Measurements

ESR measurements at room temperature were performed in a Varian E-109 spectrometer operating at 9.5 GHz and employing 100 kHz field modulation. The concentration of carboxy-PTI was calculated by comparison of the double-integrated ESR spectrum with a standard nitroxide (3-carbamoyl-2,2,5,5,-tetramethyl-3-pyrrolin-1-yloxy, Molecular Probes, Eugene, OR).

Preparation and Analysis of ·NO Solution

·NO solution was prepared by introduction of ·NO gas into a vacuum-degassed vessel containing PBS. The contents of the vessel were thoroughly mixed to dissolve the gaseous ·NO. ·NO was quantified by observing the oxidation of oxymyoglobin to metmyoglobin. Briefly, solutions of oxymyoglobin were extensively deoxygenated by vacuum, and the air replaced by argon. This process was not sufficient to remove the oxygen bound to the myoglobin. The visible absorbence spectrum of this solution between 450 nm and 650 nm was recorded using an HP8452 diode array spectrophotometer (Hewlett-Packard, Palo Alto, CA). The oxymyoglobin was titrated with ·NO, and after each addition, the ·NO spectrum was taken. The change in absorbence after each addition exhibited sharp isosbestic points indicating that only two absorbing species were present in the solution. ·NO was quantified from the change in absorbence at 583 nm using an extinction coefficient of 14400 $M^{-1}cm^{-1}$.[12] Oxymyoglobin was prepared freshly from metmyoglobin by treatment with sodium dithionite. Excess dithionite was removed using a sephadex G25 column.

Kinetic Simulations

Kinetic simulations were performed on a Hewlett Packard Vectra 486 personal computer using software written by one of us (F.N.).

RESULTS AND DISCUSSION

ESR Spectra of NNO and INO

The spectral changes that occur on reaction of carboxy-PTIO with ·NO are shown in Figure 1. Carboxy-PTIO has a five-line ESR spectrum due to the interaction of two equivalent nitrogen nuclei with the unpaired electron. Reaction of ·NO with this compound generates carboxy-PTI in which the nitrogen atoms are inequivalent. The ESR spectrum changes to a nine-line pattern, as shown in Figure 1. This transformation is best observed by monitoring the low-field line of each spectrum because there

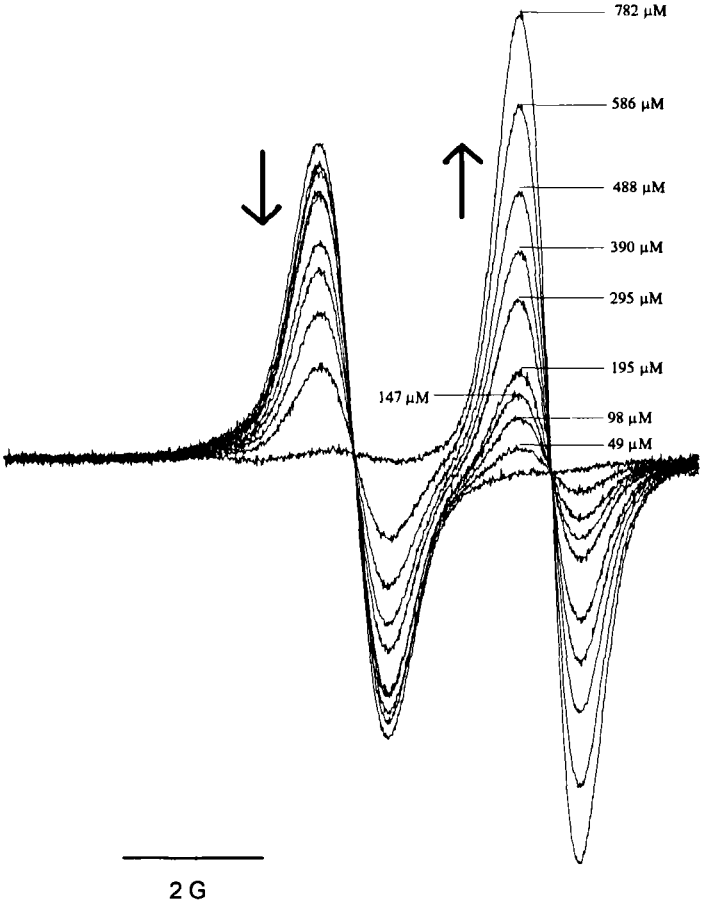

FIGURE 2 Titration of carboxy-PTIO with ·NO. Nitric oxide (0–782 μM) was added to carboxy-PTIO (530 μM) in PBS, and the ESR spectra of the low-field lines of nitronyl nitroxide and imino nitroxide monitored. Arrows ↑ and ↓ denote the decrease and increase in the spectral intensity of nitronyl nitroxide and imino nitroxide, respectively. Note that due to the decrease in the linewidth of the imino nitroxide as compared with that of nitronyl nitroxide, the spectral intensity of the imino nitroxide is greater than that of the nitronyl nitroxide at the same concentration. Spectrometer conditions: scan range, 10 G; modulation amplitude, 0.5 G; microwave power, 2 mW; time constant, 0.032 s; and scan time, 2 min.

is little overlap of the ESR lines in this region and the height of these peaks is proportional to the nitroxide concentration (Figure 2).

The Stoichiometry of the Reaction between ·NO and Carboxy-PTIO

Carboxy-PTIO was mixed with various concentrations of ·NO, and the ESR spectrum recorded immediately (Figure 2). ·NO caused a concentration-dependent conversion of carboxy-PTIO to carboxy-PTI. For the purpose of quantitation the peak height of the low-field line of the carboxy-PTI spectrum was used as this line does not reflect the slight variation (±4%) in the concentration of carboxy-PTIO. The ratio of the amount of carboxy-PTI formed and the amount of ·NO added as a function of ·NO concentration is shown in Figure 3. The stoichiometry of the reaction was found to be approximately 0.63 ± 0.06 moles of carboxy-PTIO converted to carboxy-PTI for each mole of ·NO. The presence of oxygen did not affect the extent of conversion of carboxy-PTIO to carboxy-PTI. This is not unexpected as the published rate constants predict that upon addition of ·NO (20 μM) to carboxy-PTIO (200 μM) in the presence of oxygen (250 μM), the reaction between ·NO and NNO will substantially out-compete the reaction of ·NO with oxygen. This is borne out by kinetic simulation (see below).

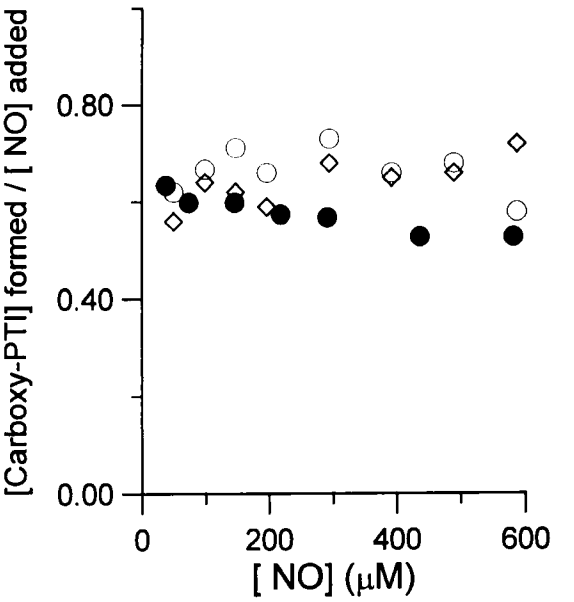

FIGURE 3 The stoichiometry of the reaction between ·NO and carboxy-PTIO. Data taken from Figure 2 (●) and two similar experiments (◇ and ○) were plotted as the ratio of [carboxy-PTI] formed to [·NO] added as a function of [·NO]. [carboxy-PTI] was calculated from the increase in intensity of the low-field line of the ESR spectrum. The average value of [carboxy-PTI]/·[NO] was 0.63 ± 0.06 (mean ± SD).

$\cdot NO + NNO \rightarrow INO + \cdot NO_2$	$k = 1 \times 10^4\,M^{-1}\,s^{-1}$
$2\cdot NO + O_2 \rightarrow 2\cdot NO_2$	$k = 6 \times 10^6\,M^{-2}\,s^{-1}$
$\cdot NO + \cdot NO_2 \rightarrow N_2O_3$	$k = 1.1 \times 10^9\,M^{-1}\,s^{-1}$
$\cdot NO_2 + \cdot NO_2 \rightarrow N_2O_4$	$k = 4.5 \times 10^8\,M^{-}\,s^{-1}$
$N_2O_3 + H_2O \rightarrow 2NO_2^- + 2H^+$	$k = 1 \times 10^3\,M^{-1}\,s^{-1}$
$N_2O_4 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+$	$k = 1 \times 10^3\,M^{-1}\,s^{-1}$

Scheme 1. The reaction pathways of nitric oxide used in the kinetic simulation.

Kinetic Simulation of the Reaction between ·NO, NNO and Oxygen

The proposed mechanism of reaction of ·NO with NNO is shown in Scheme 1. According to this model, ·NO reacts with NNO to yield INO and $\cdot NO_2$ with a rate constant of $10^4\,M^{-1}s^{-1}$.[10] The $\cdot NO_2$ thus formed can react with either ·NO or $\cdot NO_2$. The reaction between ·NO and $\cdot NO_2$ gives N_2O_3 ($k = 1.1 \times 10^9\,M^{-1}s^{-1}$,[13] which then hydrolyzes to give nitrite ($k = 1 \times 10^3\,s^{-1}$).[5] The reaction between $\cdot NO_2$ and $\cdot NO_2$ gives N_2O_4 ($k = 4.5 \times 10^8\,M^{-1}s^{-1}$),[13] which hydrolyzes to give equal amounts of nitrite and nitrate ($k = 1 \times 10^3\,s^{-1}$). The reaction of ·NO with oxygen ($k = 6 \times 10^6\,M^{-2}s^{-1}$)[5] was also incorporated into the reaction mechanism; however, this reaction did not effectively compete with the reaction between ·NO and NNO. Simulation of the bolus addition of ·NO (20 μM) to a solution of NNO (200 μM) following the mechanism shown in scheme 1 predicts that 50% of the ·NO will react with NNO, to generate INO and $\cdot NO_2$ and the remainder of the ·NO will be scavenged by the $\cdot NO_2$ thus formed. By this route all of the ·NO is converted into nitrite, and the stoichiometry of the reaction between NNO and ·NO is 0.5:1 (Figure 4A). This argument has also been advanced to explain why the addition of ·NO to oxygenated solutions yields only nitrite.[4] This result is also modeled by Scheme 1 if the concentration of NNO is set to zero (Figure 4B). The time for the depletion of ·NO in aerated aqueous solutions is two orders of magnitude faster in the presence of NNO than in its absence.

The reasons for the discrepancy between our results and those reported by Akaike *et al.* (1993)[10] are presumably due to an erroneous assumption that the stoichiometry for the reaction between ·NO and O_2 in the aqueous phase is 2:1. Previously, the stoichiometry for this reaction in aqueous solutions has been shown to be 4:1[6] [equation 2].

$$4\cdot NO + O_2 + 2H_2O \rightarrow 4NO_2^- + 4H^+ \qquad [2]$$

If the data from Akaike *et al.* (1993)[10] were recalculated using equation 2, then the reaction between NNO and ·NO would give a stoichiometry of 0.5:1, in agreement with our data.

Simulations of slow ·NO release revealed that the stoichiometry of the reaction between ·NO and NNO was dependent on the rate of ·NO release (Figure 5A). Above 10^{-9} M/s, two molecules of ·NO were required to generate one molecule of INO. However, as the rate of ·NO release dropped below 10^{-9} M/s, the stoichiometry approached 1:1. Under these conditions the steady state concentration of ·NO was low, so that the dimerisation of $\cdot NO_2$ to form N_2O_4 became a significant reaction leading to the formation of nitrate. As the rate of ·NO release was decreased, the ratio of nitrite to nitrate approached 1:1. Thus, the exact stoichiometry of NNO conversion to INO and the nitrate/nitrite product ratio will depend upon the rate of ·NO release.

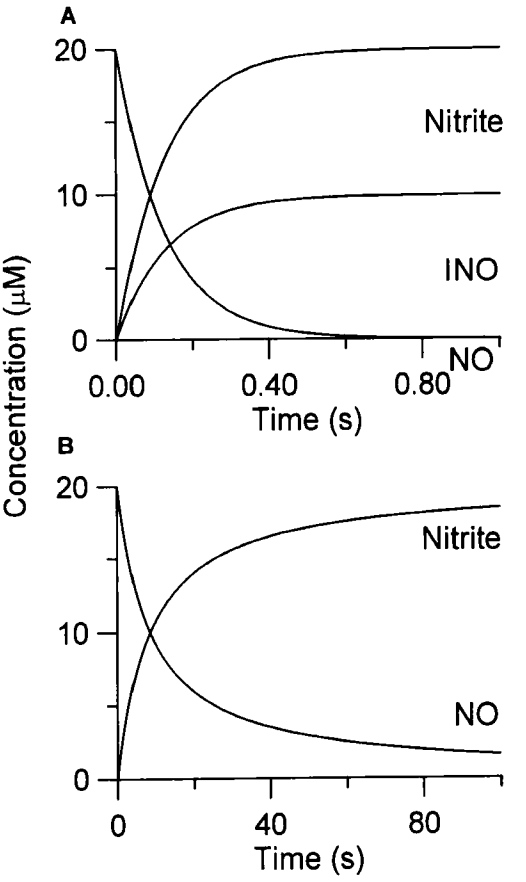

FIGURE 4 Kinetic simulation of the reaction between NNO and ·NO. Kinetic simulation was performed on the reactions shown in Scheme 1 using a numerical integration method based on the Euler method. Initial concentrations: A) ·NO (20 μM), NNO (200 μM) and oxygen (250 μM). B) ·NO (20 μM) and oxygen (250 μM).

A similar set of simulations were performed in the absence of NNO, where ·NO initially reacts with oxygen, to examine the effect of ·NO generation rate on nitrite and nitrate formation (Figure 5B). This model predicts that the nitrite:nitrate ratio will also depend on the rate of ·NO formation during the reaction between ·NO and oxygen. However, nitrate production only becomes significant at rates of ·NO generation less than 10^{-17} M/s (Figure 5B).

Biological Considerations

The rates of ·NO production in biological situations may vary as different cell types contain variable amounts of nitric oxide synthase and the activity of the various isoforms of the enzyme are controlled by different mechanisms. The rate of ·NO release from the isolated guinea pig heart was measured as 216 pmol/min (basal

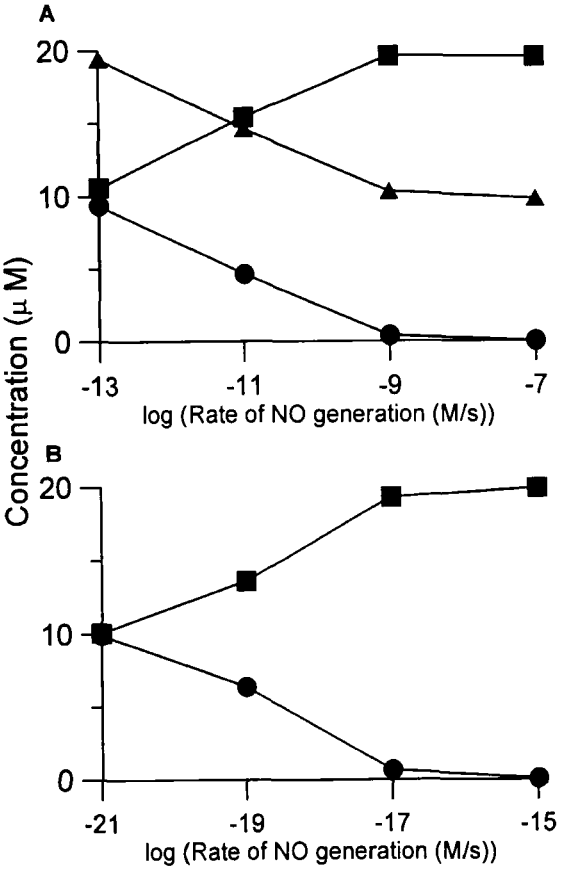

FIGURE 5 Computer-predicted formation of nitrite, nitrate, and INO from the reaction between NNO and ·NO generated at different rates. Kinetic simulations were performed using a model in which ·NO (20 μM) was continuously generated at a constant rate in the presence of A) NNO (200 μM) and oxygen (250 μM) or B) oxygen alone. The final concentration of nitrite (■), nitrate (●), and INO (▲) were calculated in each case and plotted against the rate of ·NO production.

release) and 500 pmol/min (bradykinin stimulated release).[14] This corresponds to the release of 3 and 8 nM/s ·NO into a 1 ml volume. Cytokine stimulated macrophages have been reported to release ·NO at a rate of 0.1 nmol/min/10^6 cells.[15] Again, if this were released into a 1 ml volume it would correspond to a rate of 1.7 nM/s. Other estimates of the rate of ·NO formation can be derived from steady state measurements if it is assumed that ·NO is removed by reaction with oxygen. This assumption places a lower limit on the rate of ·NO production and is only applicable in situations where the concentration of oxygen can be safely assumed (e.g. cell culture). Thus Shibuki and Okada[16] measured 100 nM ·NO in cerebral slices, corresponding to a rate of formation of 1.5 nM/s (assuming 250 μM oxygen) and Tsukahara *et al.*[17] measured a steady state ·NO concentration of 260 nM in L-arginine stimulated endothelial cells corresponding to a rate of formation of 10 nM/s.

These estimates of the rate of production of ·NO are much greater than the rates required in order to generate significant quantities of nitrate according to the simulations shown in Figure 5B. It can be hypothesized that nitrate formed from the nitric oxide synthase/L-arginine pathway is derived either from the reaction of ·NO with molecules other than oxygen or from the further oxidation of nitrite to nitrate. Examples of such processes are: the reaction of ·NO with superoxide to form peroxynitrite which will, to some extent, isomerize to nitrate;[15] reaction of ·NO with oxyhemoglobin to form nitrate; and the oxidation of nitrite to nitrate by oxyhemoglobin and oxymyoglobin.[12]

In conclusion: i) The stoichiometry of the reaction between NNO and ·NO is 0.63 ± 0.06:1.0 if ·NO is added as a bolus. Under these conditions, the predominant product derived from ·NO is nitrite. ii) The reaction between ·NO and NNO can be simulated from the proposed model by a numerical integration using published rate constants. iii) If ·NO is generated at a faster rate, the results will mirror those obtained from bolus addition. iv) As the rate of ·NO generation is decreased, the stoichiometry of the reaction between NNO and ·NO approaches 1:1 and the product profile approaches 50% nitrite and 50% nitrate. v) The nitrite:nitrate ratio from the reaction between ·NO and oxygen will also vary with the rate of ·NO generation but cannot form nitrate at physiologically significant rates. Thus any nitrate formed must arise from reaction of ·NO with other molecules, such as superoxide or oxyhemoglobin, or from further oxidation of nitrite.

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