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Detection of nitrous oxide in the neuronal nitric oxide synthase reaction by gas chromatography–mass spectrometry ☆

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Abstract

Using headspace gas chromatography–mass spectrometry, we detected significant amounts of nitrous oxide in the reaction products of the monooxygenase reaction catalyzed by neuronal nitric oxide synthase. Nitrous oxide is a dimerization product of nitroxyl anion; its presence in the reaction products indicates that the nitroxyl anion is a product of the neuronal nitric oxide synthase-catalyzed reaction.

Keywords: Gas chromatography–mass spectrometry; Nitric oxide synthase; Nitric oxide; Nitroxyl

Nitric oxide synthase (NOS) is a physiologically important monooxygenase that catalyzes the formation of nitric oxide (NO) and L-citrulline from L-arginine (L-Arg) [1–5]. The role of NO as a signaling molecule in biology and medicine has been well established over the past two decades. NOS yields NO through two successive monooxygenation reactions, depicted below:

$$\text{L-arginine} + O_2 + \text{NADPH} + H^+ \rightarrow N^{\omega}\text{-hydroxy-L-arginine} + \text{NADP}^+ + H_2O \quad (1)$$

$$N^{\omega}\text{-hydroxy-L-arginine} + O_2 + 0.5\text{NADPH} + 0.5H^+ \rightarrow \text{NO} + \text{L-citrulline} + 0.5\text{NADP}^+ + H_2O \quad (2)$$

In the above reactions, L-Arg is first hydroxylated to N^{ω}-hydroxy-L-arginine (N-OH-Arg) at the expense of 1 mole each of molecular oxygen (O_2) and NADPH, and is then converted into NO and L-citrulline by utilizing 1 mole of O_2 and 0.5 moles of NADPH [5]. Thus, the overall reaction requires 2 moles of O_2 and 1.5 moles of NADPH. Such a stoichiometry is unique among the monooxygenase reactions where the ratio of O_2 and NADPH consumed is usually 1:1 [6]. Evidence has been provided for the formation of nitroxyl (NO^-), a 1-electron reduced form of NO, in the NOS-catalyzed reaction [7–13]. However, nitroxyl formation has been the subject of controversy for almost a decade [14–18]; NO^- is a highly unstable molecule and the methods for its detection and quantification have always been fraught with some ambiguity. Nevertheless, the question of whether NOS forms NO or NO^- or both is an important issue to be explored. NO^- could be a precursor of NO through secondary reactions such as the superoxide dismutase (SOD) reaction [7] and, furthermore, it has significant biological activities in cellular functions by itself [19–21]. If NO^- is indeed a product of NOS catalysis, the overall equations would need to be modified to substitute (2′) below:

$$\text{L-arginine} + O_2 + \text{NADPH} + H^+ \rightarrow N^{\omega}\text{-hydroxy-L-arginine} + \text{NADP}^+ + H_2O \quad (1)$$

$$N^{\omega}\text{-hydroxy-L-arginine} + O_2 + \text{NADPH} + H^+ \rightarrow \text{NO} + \text{L-citrulline} + \text{NADP}^+ + H_2O \quad (2')$$

☆ *Abbreviations:* GC/MS, gas chromatography–mass spectrometry; nNOS; neuronal nitric oxide synthase; Arg, L-arginine; N-OH-Arg, N^{ω}-hydroxy-L-arginine.

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In this case, the overall reaction for NO^- formation requires 2 moles each of O_2 and NADPH in conformity with the standard stoichiometry of monooxygenases [6]. Currently, however, no definitive evidence has been provided to prove or disprove the formation of NO^- or to ascertain the actual stoichiometry of the reactions.

To gain insight into the mechanism and stoichiometry of the NOS reaction, we assessed the formation of N_2O, a dimerization product of NO^-, through use of headspace gas chromatography–mass spectrometry (GC/MS). Dimerization of NO^- is known to be fast, with a rate constant of $1.8–7.2 \times 10^9\ M^{-1}\ s^{-1}$ [19], and the resulting product, N_2O, "laughing gas," is stable both in the gas phase and in aqueous media. In the past, evidence supporting NO^- formation was mainly obtained by detecting the formation of a nitrosyl complex of heme proteins such as NOS or myoglobin. The results we describe here indicate that N_2O is, in fact, produced in the nNOS-catalyzed reaction, and the signal intensity of N_2O increases in a concentration-dependent manner. Although at this point our data are qualitative, the current results add definitive and specific evidence for the production of NO^- in nNOS-catalyzed reactions in vitro.

Materials and methods

Materials. Wild-type neuronal nitric oxide synthase (nNOS) was expressed in *Escherichia coli* and was purified as described [22]. SOD, catalase, and cytochrome *c* were purchased from Sigma (St. Louis, MO). Calmodulin was purified according to the method described elsewhere [23]. Angeli's salt was kindly provided by Dr. D.A. Wink (Radiation Biology Branch, National Cancer Institute, National Institutes of Health). Chemicals, including NADPH, L-arginine, N^{ω}-hydroxy-L-arginine (*N*-OH-Arg), tetrahydrobiopterin (BH_4), and diethylenetriaminepentaacetic-acid (DPTA), were obtained from Sigma. The buffer solution used throughout this study was 50 mM Tris–HCl, pH 7.4, containing 100 mM NaCl. Vials for headspace analyses were purchased from Supelco (Belfonte PA; 2 ml vial, #27078; screw cap with Teflon-lined silicone septa, #27093U).

O_2 consumption. Oxygen uptake was measured at 24 °C using a fluorescence-based oxygen sensing system consisting of an SD2000 dual channel spectrophotometer combined with an LS-450 Blue LED light source and a Foxy-18G probe, all purchased from Ocean Optics (Dunedin FL). The reaction was carried out in an air-tight cuvette (1.82 ml) made by Wayne L. Skloss, Biomedical Instrumental Services, the University of Texas Health Science Center at San Antonio, Texas.

Nitric oxide synthase reaction. NOS reactions were carried out under constant stirring at 24 °C in an air-tight cuvette (see above). The reaction mixture contained (final concentrations) 2.5 mM L-Arg or 1.1 mM *N*-OH-Arg, 540 μM NADPH, 7 μM calmodulin, 5 μM reduced tetrahydrobiopterin (BH_4), 2 μM catalase, 50 μM DPTA, and 0.2 mM $CaCl_2$ in 50 mM Tris–HCl, pH 7.4, containing 100 mM NaCl unless otherwise specified. The concentration of NOS used was 0.2–2 μM as judged by the heme content. The reaction was started by the addition of enzyme to the reaction mixture.

Other methods. N_2O was produced according to the method of Mattson et al. [24]. NO gas was prepared by the addition of 2 M H_2SO_4 to solid $NaNO_2$ in a Kipps apparatus as described by Sharpe and Cooper [25]. N_2O-containing water was made by equilibrating H_2O with N_2O gas under argon. NO^- was generated by dissolving Angeli's salt [19] in the buffer solution described above. NO^- was also generated from *S*-dinitrosodithiothreitol according to Arnelle and Stamler [26].

Gas chromatography/mass spectrometry. GC/MS analyses were performed on a Thermo Finnigan DSQ quadrupole gas chromatograph/mass spectrometer that was modified by addition of an external Valco manual septum port loop injector and a separate helium supply regulated by an Alicat Scientific (Tucson, AZ) mass flow controller. Headspace samples were taken from each reaction vessel by means of a 500-μl gas-tight syringe (SGE; Austin TX) fitted with a push–pull valve. The entire contents of the syringe were injected into the 25-μl loop in order to fully sweep the loop prior to each injection and enhance sample-to-sample reproducibility. Chromatographic separations were accomplished with a 30 m × 0.32 mm (20 μm film thickness) HP-PLOT Q column (J&W Scientific; Folsom, CA). GC conditions were as follows: flow rate, 2 ml/min; injection temperature, ambient room temperature; column oven temperature, 50 °C. Positive ion electron impact (EI) mass spectra (70 eV) were acquired in selected ion monitoring (SIM) mode using a dwell time of 100 ms for each ion of interest. Peak areas were determined by integration of the appropriate selected ion retrieval chromatogram peaks. Each reaction vessel (2 ml) contained 0.1–0.5 ml of sample solution, where the gas phase and the solution were allowed to equilibrate for 2 h at room temperature prior to analysis.

Results and discussion

For the GC/MS analyses conducted for this study, selected ion monitoring (SIM; specific detection of individual ions for each analyte) was employed rather than scanning over a wider mass range because SIM affords lower detection limits and better reproducibility and signal-to-noise ratios. To display the results, a selected ion retrieval chromatogram is generated in which ion intensity is plotted against time for any ion of interest that was monitored in the analyses. In Fig. 1 can be seen a pair of selected ion retrieval chromatograms for each sample (m/z 30, an expected EI fragment of N_2O [15] and m/z 44, molecular ions of CO_2 and N_2O) obtained after SIM GC/MS analysis of gases taken from the headspace of air-tight vessels containing 0.5 ml of the following: a buffered solution of Angeli's salt (A,B); dinitrosodithiothreitol (C,D); and H_2O equilibrated with N_2O gas (E,F). Both Angeli's salt [20] and dinitrosodithiothreitol [27] are known to produce NO^- upon decomposition in aqueous media. Thus, all of these solutions would be expected to contain either authentic N_2O or N_2O generated by dimerization of NO^- and can be used to verify the GC retention time and EI fragmentation behavior of N_2O in our experiments.

Under our experimental conditions, it was not possible to assess exact amounts of N_2O produced in the reaction system and, therefore, these experiments were not quantitative. Nevertheless, as can be seen in Fig. 1, we observed the same GC retention time and MS fragmentation for analysis of two authentic donors of N_2O and for a mixture of N_2O in H_2O, confirming that the signals we detected with a retention time of ~2.5 min were due to N_2O. Since O_2 is one of the substrates of the NOS reaction, experiments were carried out under atmospheric conditions, and, as such, a large quantity of CO_2 was present. In view of the fact that the intense molecular ion peak for CO_2 at m/z 44 often interfered with accurate integration of the m/z 44 peak for N_2O, all experiments described here for our assessment of the relative quantity of N_2O were made on

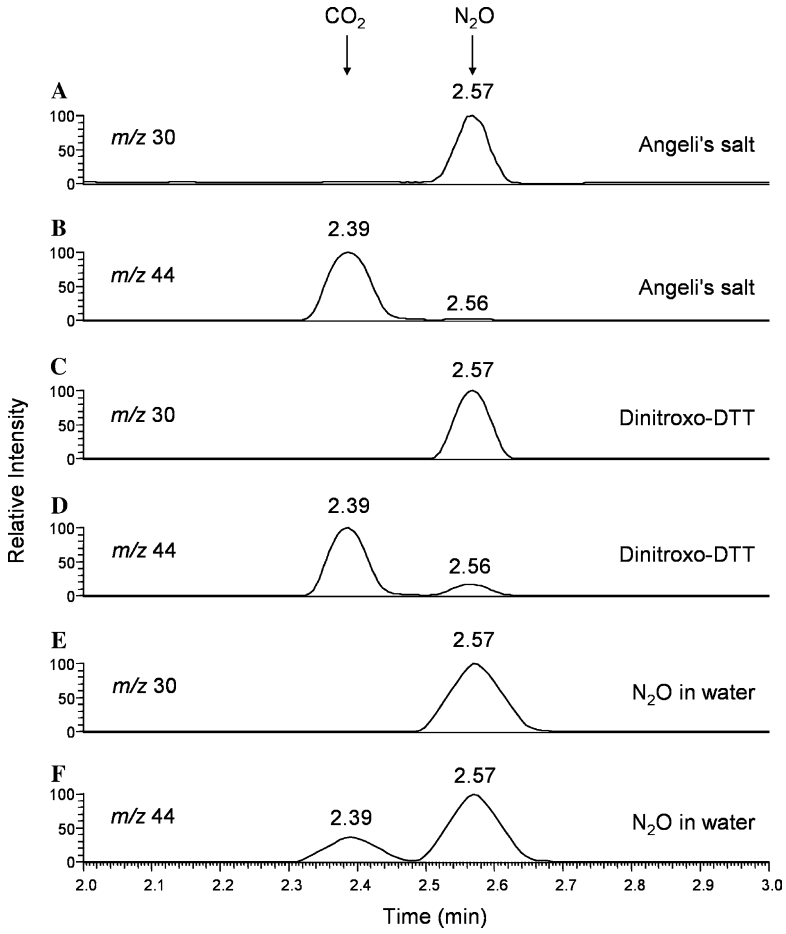

Fig. 1. Solutions of Angeli's salt, dinitroso-DTT and H_2O containing N_2O. Selected ion retrieval traces reconstructed from the GC/MS analysis of samples containing authentic N_2O. A gas-tight syringe fitted with a push–pull valve was used to withdraw gas samples (500 μl) from the headspace of air-tight vessels (2 ml) containing 0.5 ml of the following: a buffered solution of 100 μM Angeli's salt (A,B); 100 μM dinitrosodithiothreitol (C,D); and H_2O equilibrated with N_2O gas (E,F). Equilibration of the gas phase and the sample solution was carried out as described under Materials and methods. GC separations were accomplished with an HP-PLOT Q column (J&W; 30 m × 0.32 mm) using a column temperature of 50 °C and a 25-μl injection loop at ambient room temperature. The ions at *m*/*z* 44 represent molecular ions for CO_2 and N_2O while *m*/*z* 30 corresponds to an expected electron impact ionization fragment of N_2O.

the basis of the peak at *m*/*z* 30. The N_2, O_2, and Ar that were also present in the vessel headspace eluted at ~1.8 min are not shown on these traces. The molecular ion of NO, another expected product of the NOS reaction, would also be detected at *m*/*z* 30 and would elute at an earlier retention time than CO_2 and N_2O; however, since NO is unstable in aqueous media it would not be expected to remain intact in the reaction solution or headspace after the 2 h used for equilibration of the gas phase and the solution.

For investigation of the formation of N_2O in the nNOS reaction, each reaction mixture was first equilibrated to room air (20% O_2) in an air-tight cuvette for kinetic assessment of O_2 consumption. After confirming that all of the O_2 in the medium had been consumed by oxygen electrode measurements, a 0.5-ml aliquot of the aqueous reaction mixture was transferred by a gas-tight syringe to a sample vial fitted with a Teflon-lined septum. After equilibration of the gas and liquid phases for 2 h at room temperature, 500 μl of the headspace gas was withdrawn and flushed through the 25-μl loop for injection into the GC and analysis by selected ion monitoring MS. As can be seen in Fig. 2, low intensity, but detectable, signals were seen at a retention time of ~2.5 min in the traces in panels (C–E), where the peaks were noticeably higher than those in the traces in panels A and B. Room air (panel A) was routinely used as a background reference for our GC/MS measurements. The sample analyzed for the trace in panel B can be viewed as a negative control because no substrate (L-Arg or *N*-OH-Arg) was included in the mixture and, therefore, no reaction product should be expected. On the other hand, it was anticipated that NO^- would be produced in the reactions analyzed in panels (C–E), where a substrate, L-Arg or *N*-OH-Arg, was present. Tetrahydrobiopterin (BH_4) is a cofactor which has been shown to regulate NO formation; however, previous investigators have suggested that NO^- is produced in the absence of this cofactor [11,12]. The experiments illustrated in Fig. 2 were, therefore, conducted with BH_4-free preparations of nNOS in the absence of exogenous BH_4 except for that in panel D.

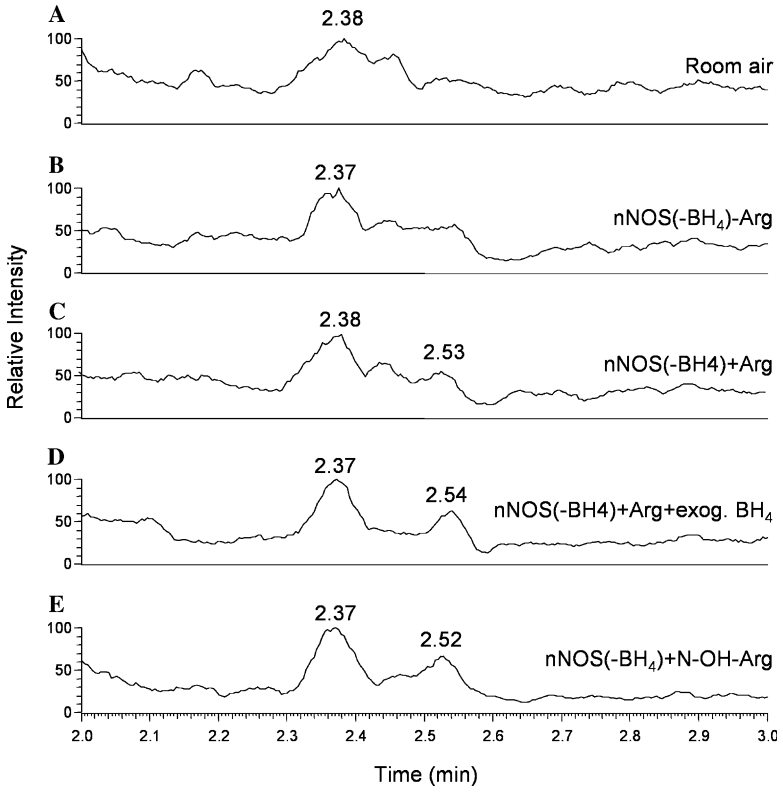

Fig. 2. Analysis of N_2O formed in the nNOS reaction under 20% O_2. Selected ion retrieval traces (m/z 30) reconstructed from the GC/MS analysis of nNOS reactions conducted under normal atmospheric conditions (~270 μM O_2). GC/MS conditions were the same as described in Fig. 1. (A) Room air; (B) BH_4-free nNOS incubated without Arg; (C) BH_4-free nNOS plus 2.5 mM Arg added at 0 min; (D) BH_4-free nNOS plus 2.5 mM Arg and 7 μM BH_4; (E) BH_4-free nNOS plus 1.1 mM *N*-OH-Arg and 7 μM BH_4. In these experiments, the concentration of nNOS was 0.8 μM (based on the heme content).

Although the effect of BH_4 was not clear in these experiments, the presence of distinct N_2O peaks in panels (C–E) encouraged us to carry out experiments under conditions that are more favorable for production of NO^-.

In the next series of experiments, we attempted to increase the levels of the products by using higher initial concentrations of both NADPH and molecular oxygen (O_2). Fig. 3 shows the results with L-Arg as the substrate where the initial concentration of O_2 was 900 μM instead of the ~270 μM of normal atmospheric conditions. The O_2 concentrations in the reaction vessels were raised by bubbling the reaction mixture with pure O_2 for 20 min and were confirmed by our O_2 monitoring system. For these experiments, the NADPH concentration was also raised to 3 mM. As expected, we obtained a distinct signal for N_2O at a retention time of 2.55 min (Fig. 3D) for the sample containing enzyme and substrate. Control experiments without enzyme (Fig. 3B) or without L-Arg (Fig. 3C) did not contain appreciable N_2O as evidenced by the absence of a peak at 2.55 min.

As can be seen in Fig. 4, we were, likewise, able to observe significant signals when *N*-OH-Arg was used as substrate, in this case in the presence of 850 μM O_2 (Figs. 4B–D). The traces in Fig. 4 also show evidence for the relationship between the volume of sample solution containing the products and the amount of N_2O in the headspace gas. All experiments were repeated at least two times to confirm reproducibility.

A number of reports in the literature have indicated that NOS is able to produce NO^- and/or NO [7–13]. However, the evidence for NO^- formation has been rather indirect. Most investigators have based their conclusions on the formation of a nitrosyl complex with NOS or other heme proteins such as ferric myoglobin as major evidence. A considerable increase in NO formation catalyzed by SOD, which is able to convert NO^- to NO [7], has also been considered as evidence for NO^- formation. On the basis of high resolution MS analyses, Southan and Srinivasan [15] postulated that the N_2O detected in their in vitro iNOS experiments is likely a result of the reaction of either NO or other oxides of nitrogen with *N*-OH-Arg to form the intermediate Arg-NONO. The use of ^{15}N-labeled arginine provided documentation for the generation of NO and N_2O as products of the iNOS reaction. However, their results were complicated somewhat by the lack of chromatographic separation of the analytes under investigation. In this study, we have provided convincing evidence for the presence of N_2O among the products of the nNOS reaction by detection of distinct GC/MS peaks attributable to N_2O when using either L-Arg or *N*-OH-Arg as substrate. This, in turn, is a strong indication that NO^- is generated by the nNOS reaction. Even if formation of NO^- from

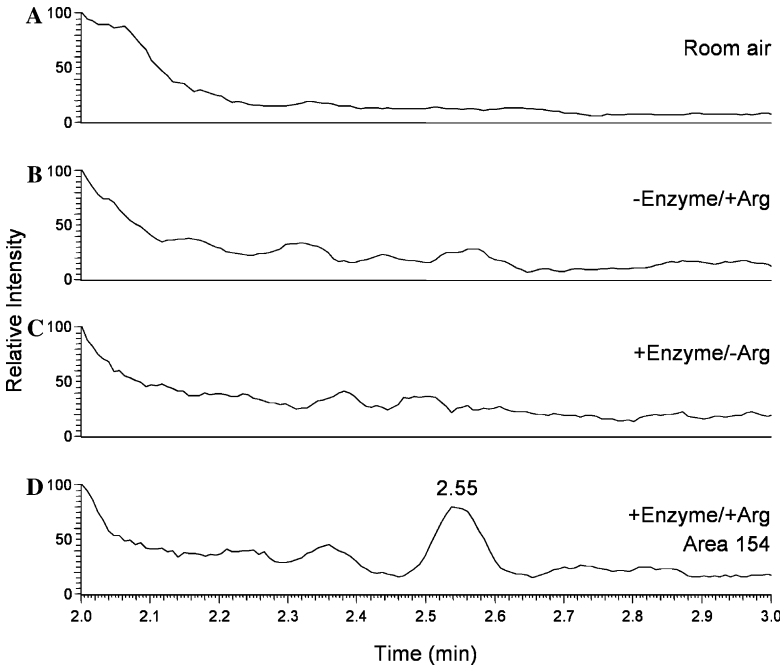

Fig. 3. Detection of N_2O at a higher concentration of O_2 with L-arginine. Selected ion retrieval traces (m/z 30) reconstructed from the GC/MS analysis of nNOS reactions conducted with 900 μM O_2 and 3 mM NADPH in 50 mM Tris–HCl, pH 7.4, containing 100 mM NaCl. GC/MS conditions were the same as described in Fig. 1. (A) Room air; (B) no enzyme added to the reaction mixture containing 2.8 mM Arg; (C) nNOS added to the reaction mixture without Arg; (D) nNOS added to the reaction mixture containing 2.8 mM Arg. The concentration of nNOS used for these experiments was 1.6 μM (based on heme content).

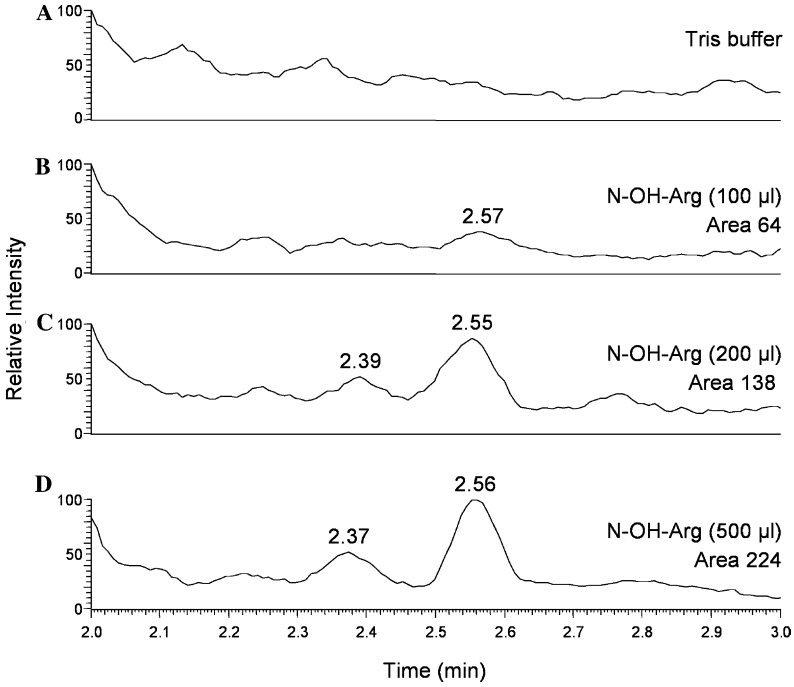

Fig. 4. Detection of N_2O at a higher concentration of O_2 with *N*-OH-Arg. Selected ion retrieval traces (m/z 30) reconstructed from the GC/MS analysis of nNOS reactions conducted with 850 μM O_2, 3 mM NADPH, and 1.1 mM *N*-OH-Arg as the substrate in 50 mM Tris–HCl, pH 7.4, containing 100 mM NaCl. GC/MS conditions were the same as described in Fig. 1. (A) Tris buffer only (500 μl); (B) sample vial containing 100 μl of the enzyme reaction mixture; (C) sample vial containing 200 μl of the reaction mixture; (D) sample vial containing 500 μl of the reaction mixture. The concentration of nNOS used for these experiments was 1.6 μM (based on heme content).

N-OH-Arg occurs nonenzymatically in the course of the nNOS reaction, it would have, nevertheless, a potentially important in vivo effect.

The levels of N_2O detected in the enzymatic reactions analyzed in this study were low, but clearly present. This is reasonable because any NO^- that is produced can react

with many components in the reaction mixture, including NADPH [28], sulfhydryls, and metals. We, thus, assume that only a portion of the NO^- formed was subsequently converted to N_2O. More quantitative assessment of the nitrogen oxides generated in the nNOS reaction relative to the amount of substrates consumed and L-citrulline formed is currently underway to determine more accurately the stoichiometry of the nNOS reaction.

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