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Oxidation of *N*-hydroxy-L-arginine by hypochlorous acid to form nitroxyl (HNO)

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ABSTRACT

Recent research has shown that nitroxyl (HNO) has important and unique biological activity, especially as a potential alternative to current treatments of cardiac failure. HNO is a reactive molecule that undergoes efficient dimerization and subsequent dehydration to form nitrous oxide (N_2O), making its detection in solution or biologically relevant preparations difficult. Due to this limitation, HNO has not yet been observed *in vivo*, though several pathways for its endogenous generation have been postulated. Here, we investigate the oxidation of *N*-hydroxy-L-arginine (NOHA) by hypochlorous acid (HOCl), which is generated *in vivo* from hydrogen peroxide and chloride by the heme enzyme, myeloperoxidase. NOHA is an intermediate in the enzymatic production of nitric oxide (NO) by NO synthases, and has been shown previously to be chemically oxidized to either HNO or NO, depending on the oxidant employed. Using membrane inlet mass spectrometry and standard N_2O analysis by gas chromatography, we find that NOHA is oxidized by excess HOCl to form HNO-derived N_2O. In addition, we also observe the analogous production of HNO from the HOCl oxidation of hydroxylamine, hydroxyurea, and (to a lesser extent) acetohydroxamic acid.

1. Introduction

Nitroxyl (HNO), the protonated and one-electron reduced sibling of nitric oxide (NO), has been shown to be involved in chemistry that has a positive impact on vasorelaxation and contractility in the cardiac system making it a potentially novel therapeutic agent for use in the fight against heart disease [1–6]. HNO is a reactive species that dimerizes rapidly ($k = 8 \times 10^6 M^{-1}\ s^{-1}$) and subsequently dehydrates to form nitrous oxide (N_2O) [7]. As a result, HNO cannot be used directly and must be generated *in situ* using donor molecules. Given its inherent reactivity, HNO has proven difficult to detect directly. Recent methods have been developed for HNO detection [8–15]; however, due to a lack of sensitivity and/or selectivity, HNO has not yet been observed *in vivo*.

Despite the above limitation, numerous reports support the idea that HNO can be produced endogenously. Research has shown that nitrosothiols can react with thiols to form HNO (and disulfide) [16,17]. HNO can also be produced from nitric oxide synthases (NOS) under conditions where the tetrahydrobiopterin cofactor is absent or in low concentrations [18–21]. In addition, recent work by Donzelli et al. examined whether the oxidation of hydroxylamine or *N*-hydroxy-L-arginine (NOHA) by a number of heme peroxidases produces HNO [22]. Although heme peroxidases were found to oxidize hydroxylamine to HNO, the analogous oxidation of NOHA did not produce HNO.

NOHA is a well-studied biosynthetic intermediate in the NOS-mediated production of NO (and L-citrulline) from L-arginine (Scheme 1) [23,24]. NOHA, which can be uncoupled from NOS at levels up to 15 μM both *in vitro* and *in vivo* [25,26], has also been shown to be a potent inhibitor of arginase, regulating the production of L-arginine [27,28]. In addition, research has demonstrated that NOHA can be chemically oxidized to produce either NO or HNO depending on the oxidant used [29–31]. Likewise, related *N*-hydroxyguanidines have been shown to form either HNO or NO depending upon the oxidative conditions [32]. The HNO-producing pathway presumably involves a nitroso intermediate and the corresponding production of a cyanamide derivative (Scheme 2).

A biologically relevant oxidant that may be able to generate HNO *via* the oxidation of NOHA is hypochlorous acid (HOCl). HOCl, a strong oxidant that has been shown to be both bactericidal and virucidal [33,34], is generated *in vivo* by myeloperoxidase (MPO) from the reaction of hydrogen peroxide (H_2O_2) with chloride ion. MPO is released by neutrophils, monocytes, and tissue macrophages at sites of inflammation [35–37], and may play a role in cardiovascular disease [38–40]. Activated neutrophils have been shown to produce HOCl at concentrations up to 100 μM *in vitro* and similar or higher levels may be possible at inflammatory sites *in vivo* [41–43]. The reaction of HOCl with biologically relevant thiols such as glutathione (GSH) generates oxidized thiol products such as sulfonamides, glutathione disulfide, and thiolsulfonates [44], while reaction of HOCl with amines produces chloramines.

To our knowledge, the oxidation of NOHA by HOCl has not yet been reported. Herein, we examine the reaction of NOHA with HOCl and show that HNO is generated under conditions where HOCl is in

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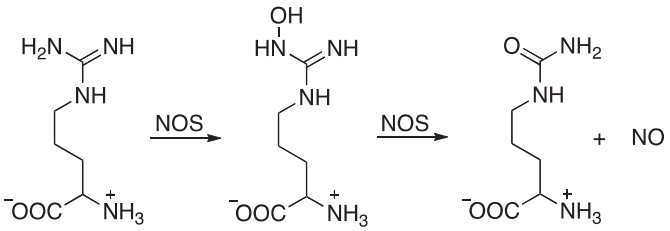


Scheme 1. Nitric oxide synthase (NOS) production of NO and L-citrulline from L-arginine *via* *N*-hydroxy-L-arginine.

excess of NOHA. HNO production is confirmed by both membrane inlet mass spectrometry (MIMS) techniques and standard N_2O analysis by gas chromatography. The analogous production of HNO from the HOCl oxidation of hydroxylamine, hydroxyurea, and (to a lesser extent) acetohydroxamic acid is also observed.

2. Materials and methods

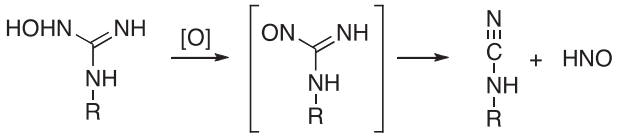
2.1. Materials

Unless otherwise noted chemicals were obtained from Aldrich Chemical Company or Fisher Scientific and used without further purification. *N*-hydroxy-L-arginine was purchased from Chemdea. Hydrogen peroxide (30% v/v; Merck) was quantified by absorbance at 240 nm using a molar absorption coefficient of 39.4 M^{-1} cm^{-1} [45]. Hypochlorous acid (4–5%) was quantified by injecting NaOCl into a 0.01 M NaOH and measuring the absorbance at 292 nm where the molar absorption coefficient of the hypochlorite ion is 350 M^{-1} cm^{-1} [46]. Dimethyl-sulfoxide-d_6 and chloroform-*d* were obtained from Cambridge Isotope Labs. Nitrous oxide was obtained from Airgas. Angeli's salt (AS) and sodium 1-(*N*,*N*-diethylamino)diazen-1-ium-1,2-diolate (DEA/NO) were synthesized according to literature procedure [47–49]. The HNO donor, 2-bromo-*N*-hydroxybenzenesulfonamide (2-bromo-Piloty's acid, 2BrPA), was synthesized following a recent report [50]. Phosphate-buffered saline (PBS) solutions (0.1 M) were prepared with 140 mM NaCl, and 3 mM KCl, and adjusted to pH 7.4. Since we observed that the metal chelator, diethylenetriaminepentaacetic acid (DTPA), was oxidized by HOCl, it was not included in our buffer solutions. However, presumably because all experiments were conducted under anaerobic conditions, representative MIMS and gas chromatographic (GC) headspace analysis experiments in the presence or absence of 100 μM DTPA provided similar results.

2.2. Methods

2.2.1. Gas chromatographic (GC) headspace analysis of N_2O

Gas chromatographic headspace analysis of N_2O was performed using a Varian CP-3800 gas chromatograph instrument equipped with a 1041 manual injector, electron capture detector, and a Restek packed column. Samples were prepared in 15 mL vials with volumes pre-measured for uniformity. Vials were filled with 5 mL PBS buffer, sealed with a rubber septum, and then purged with argon for 10 min. The vials were equilibrated at 37 °C for 10 min in a block heater. An aliquot of an HNO donor solution or other relevant substrate species was then introduced into the thermally equilibrated



Scheme 2. Oxidation of *N*-hydroxyguanidines to produce HNO and a cyanamide derivative *via* a nitroso intermediate.

headspace vial using a gastight syringe, yielding a final HNO donor or substrate concentration of 100 μM. Samples were then incubated for 1.5 h to ensure complete equilibration of N_2O in the headspace. Headspace (60 μL) was then sampled five successive times using a gastight syringe with a sample lock. HNO yields are reported relative to the HNO donor, Angeli's salt, which by comparison to standard N_2O samples we find provides an 80% absolute yield of HNO.

2.2.2. Membrane inlet mass spectrometry design and methods

Mass spectra were obtained using a Balzers Prisma QMS-200 mass spectrometer. Our membrane inlet probe is modeled after the membrane inlet design of Silverman and co-workers with a few modifications [51]. The membrane probe is comprised of a length of Silastic tubing (1.5 mm i.d. and 2.0 mm o.d.) that is sealed at one end by a glass bead and attached at the other end to a piece of glass tubing that is attached *via* swagelok to an external vacuum chamber containing a dosing line that leads directly into the source of the Balzers Prisma QMS-200 mass spectrometer located in a main vacuum chamber (Fig. 1). The length of the Silastic tubing between the glass bead and the glass tubing is 1 cm. The Silastic tubing is prevented from collapsing in the partial vacuum of the inlet by a coiled piece of fine stainless steel wire. The membrane is immersed in the solution to be analyzed in a sealable 4 mL glass sample cell modified for the introduction of samples. The buffer solution and the sample cell are degassed and purged with a continuous flow of argon for 15 min before experimentation. The deaerated buffer solution (4 mL) is then injected into the deaerated sample cell and attached to the system *via* an ultra torr swagelok connection. The external vacuum chamber is evacuated and then opened *via* a leak valve to the main chamber where the mass spectrometer is located. A dry ice/acetone ice trap is placed between the sample cell and the leak valve to trap water vapor.

Injections of oxidants occurred after several minutes of background signal collection. When additional reactants were added, they were injected prior to the oxidant and given 10 min to equilibrate. Ion currents were measured for at least 30 min and data were collected at m/z 30 (NO^+) and 44 (N_2O^+). Data are reported following baseline subtraction of the observed ion current at the start of an experiment. Scans from m/z 0 to m/z 100 were also collected. Mass spectra were obtained by electron impact (EI) ionization (70 eV) at an emission current of 1 mA; source pressures were approximately 5×10^{-7}–1×10^{-6} Torr.

3. Results and discussion

3.1. Utility of MIMS to study possible endogenous pathways for HNO production

MIMS is a method used to detect gases dissolved in liquid phases through the use of a semipermeable hydrophobic membrane that

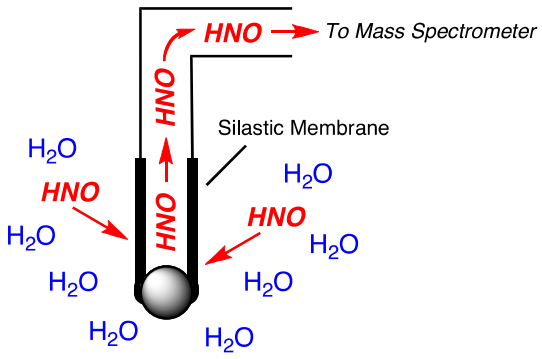


Fig. 1. Schematic representation of membrane inlet mass spectrometry (MIMS) method.

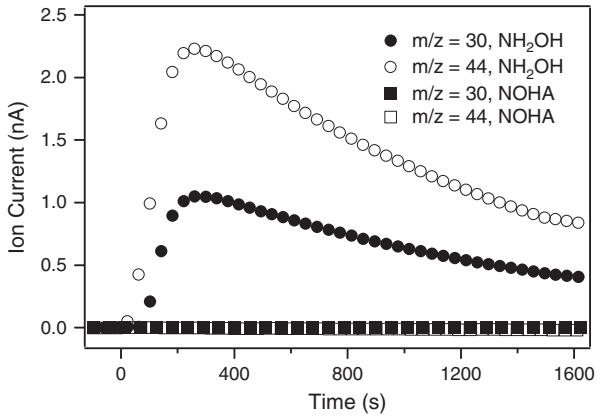


Fig. 2. The oxidation of 500 μM hydroxylamine by 10 μM HRP and 100 μM H_2O_2 to produce HNO-derived N_2O compared to the lack of oxidation observed for the same reaction carried out with 500 μM NOHA at 37 °C in 0.1 M PBS, pH 7.4.

allows the dissolved gases, but not the liquid phase, to enter a mass spectrometer [52–54]. It has proven useful for the study of NO in aqueous and blood solutions [51,55–57]. Recently, we have reported its utility for the study of HNO in aqueous solutions [13]. Although the low intensity of the parent m/z 31 MIMS signal for HNO currently precludes its unambiguous detection, its EI fragmentation product can be detected at m/z 30 (NO^+). (The relative intensity of the m/z 30 vs 31 in the reported EI mass spectrum of HNO is greater than 35:1 [58].) At high enough precursor concentrations, MIMS signals for N_2O, the product of HNO dimerization, can also be observed at m/z 44 (N_2O^+ parent ion) and 30 (NO^+ fragment ion), with a 44:30 intensity ratio of approximately 2:1.

To demonstrate that MIMS is a viable technique for the study of possible endogenous pathways for HNO production, we examined the oxidation of hydroxylamine and NOHA by horseradish peroxidase (HRP) and H_2O_2 following the conditions reported by Donzelli et al. (10 μM HRP with 100 μM H_2O_2 and either 500 μM hydroxylamine or NOHA) [22]. As shown in Fig. 2, under these conditions we detect MIMS signals at m/z 44 and 30 in a 2:1 ratio characteristic of the production of N_2O for hydroxylamine, but not for NOHA, consistent with the previous findings.

These results were corroborated using standard N_2O headspace analysis by GC. The incubation of hydroxylamine with HRP and H_2O_2 produced N_2O at a comparable percentage to that previously reported [22], while the analogous incubation of NOHA produced no N_2O under the same 5:1 substrate to oxidant conditions (Table 1). When the H_2O_2 concentration was increased, a small amount of N_2O was observed from the oxidation of NOHA.

3.2. Reaction of N-hydroxy-L-arginine with hypochlorous acid

The reaction of NOHA with HOCl was first examined by GC headspace analysis to quantify the amount of N_2O produced following the incubation of NOHA (100 μM) with varying concentrations of HOCl. Significant N_2O was produced only with excess HOCl (Table 2). From these results, a concentration of 500 μM HOCl (and 5:1 ratio of oxidant to substrate) was selected for future experiments. Although it is unlikely that HOCl is present *in vivo* at this concentration, a 5:1 oxidant to substrate ratio is potentially plausible since HOCl can be generated at concentrations up to 100 μM and NOHA has been observed at concentrations of 15 μM in blood samples [25,26,41–43].

Table 1
Oxidation of NH_2OH and NOHA by the HRP/H_2O_2 system.

Substrate	H_2O_2 (μM)	% HNO[a]
NH_2OH	20	12
NOHA	20	0
NOHA	200	2

[a] Substrates (100 μM) were incubated with 2 μM HRP at 37 °C in 0.1 M PBS, pH 7.4. HNO yields are reported relative to the HNO donor, Angeli's salt, as determined by N_2O headspace analysis (SEM ± 5%; n ≥ 3).

Table 2
Oxidation of NOHA by HOCl.

HOCl (μM)	% HNO[a]
20	3
100	11
250	22
500	60
1000	65

[a] NOHA (100 μM) was incubated at 37 °C in 0.1 M PBS, pH 7.4 with varying amounts of HOCl. HNO yields are reported relative to the HNO donor, Angeli's salt, as determined by N_2O headspace analysis (SEM ± 5%; n ≥ 3).

MIMS experiments following the addition of 500 μM HOCl to a PBS solution containing 100 μM NOHA reveal a rise in both the m/z 30 and 44 signals (Fig. 3). The m/z 44:30 intensity ratio is 2:1 for standard N_2O; however, in Fig. 2 the observed m/z 44:30 ratio is 3:1. We postulate that some of the m/z 44 signal is due to the detection of carbon dioxide (CO_2), which originates from another oxidative process. Previous work has shown that HOCl is capable of oxidizing amino acids, including L-arginine, to form first monochloramines and ultimately aldehydes along with CO_2 (Scheme 3) [59,60].

3.3. Reaction of hypochlorous acid with L-arginine and L-arginine methyl ester

To determine if the free carboxylic acid group of NOHA was responsible for the observation of a larger than expected m/z 44:30 ratio, we investigated the oxidation of L-arginine and L-arginine methyl ester by HOCl. The addition of 500 μM HOCl to a solution of 100 μM L-arginine generated a large increase in the m/z 44 signal.

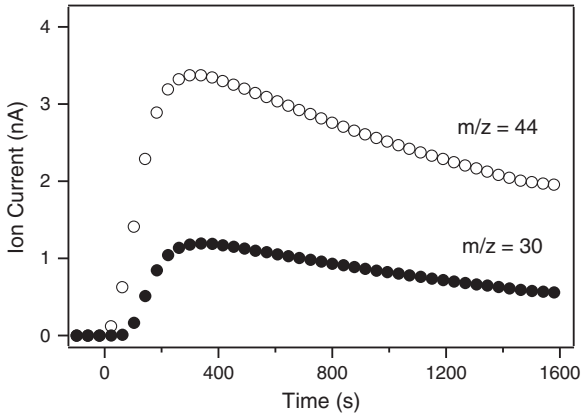
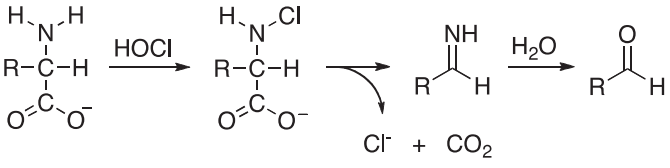


Fig. 3. MIMS response at m/z 30 and 44 following the addition of 500 μM HOCl to a solution containing 100 μM NOHA at 37 °C in 0.1 M PBS, pH 7.4.



Scheme 3. The reaction of HOCl with amino acids.

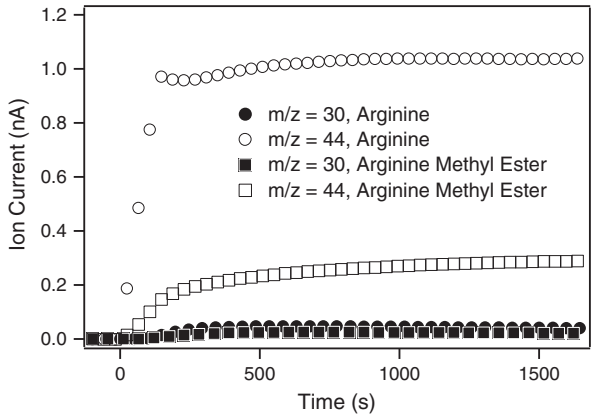


Fig. 4. The MIMS responses observed at m/z 30 and 44 following the addition of 500 μM HOCl to either 100 μM L-arginine or 100 μM L-arginine methyl ester at 37 °C in 0.1 M PBS, pH 7.4.

When the same experiment was performed with 100 μM L-arginine methyl ester, a significant decrease in the m/z 44 signal is observed (Fig. 4). (The small m/z signal (ca. 0.3 nA) remaining with L-arginine methyl ester suggests an additional oxidative pathway that also forms CO_2.) These results indicate that the reaction shown in Scheme 3 is likely responsible for the additional signal observed in Fig. 3 at m/z 44 *via* generation of CO_2. Further support of the production of aldehyde and CO_2 is the observation of hydrazone formation upon reaction of the L-arginine–HOCl product mixture with 2,4-dinitrophenylhydrazine and barium carbonate formation upon reaction with barium chloride, respectively.

To confirm that the m/z 44 signal observed for L-arginine upon oxidation by HOCl is not due to N_2O, we performed GC headspace analysis experiments (100 μM L-arginine plus 500 or 1000 μM HOCl) and observe no evidence for N_2O formation. Additional experiments using H_2O_2 (500 μM) as the oxidant instead of HOCl, as well an experiment where both HRP and H_2O_2 (2 and 20 μM, respectively) were added to L-arginine also did not result in the production of N_2O.

3.4. Confirmation that HNO is produced by the hypochlorous acid oxidation of N-hydroxy-L-arginine

GC headspace analysis shows that N_2O is produced upon the oxidation of NOHA by HOCl. To demonstrate that the N_2O formed is the result of HNO dimerization rather than direct production, we examined low concentrations of NOHA and HOCl by MIMS maintaining the same 5:1 HOCl to NOHA ratio, which was determined to be optimal for N_2O production. Using the HNO donor 2BrPA, we have previously shown that we can detect HNO directly by lowering the concentration of 2BrPA to a level (low micromolar) where dimerization to N_2O is not observed by MIMS at m/z 44. Only the m/z 30 signal, corresponding to NO^+ formed from the fragmentation of HNO, is observed. As shown in Fig. 5, at 5 μM NOHA and 25 μM HOCl the same 3:1 m/z 44 to m/z 30 signal is observed as in the higher concentration experiment (Fig. 2). As the concentrations of NOHA and HOCl are lowered, we observe a decrease in the m/z 44:30 ratio indicating that we are producing less N_2O and CO_2. Below 2.5 μM NOHA we observe only the m/z 30 MIMS signal, corresponding to the direct detection of HNO.

In previous work, to rule out the possibility that the observed m/z 30 MIMS signal is not due to the parent ion of NO (rather than HNO fragmentation) we have used selective chemical traps for HNO (*e.g.*, thiols under anaerobic conditions or phosphines under anaerobic or aerobic conditions) [13]. Unfortunately, we were unable to use the thiols or phosphines used previously because they react readily with HOCl.

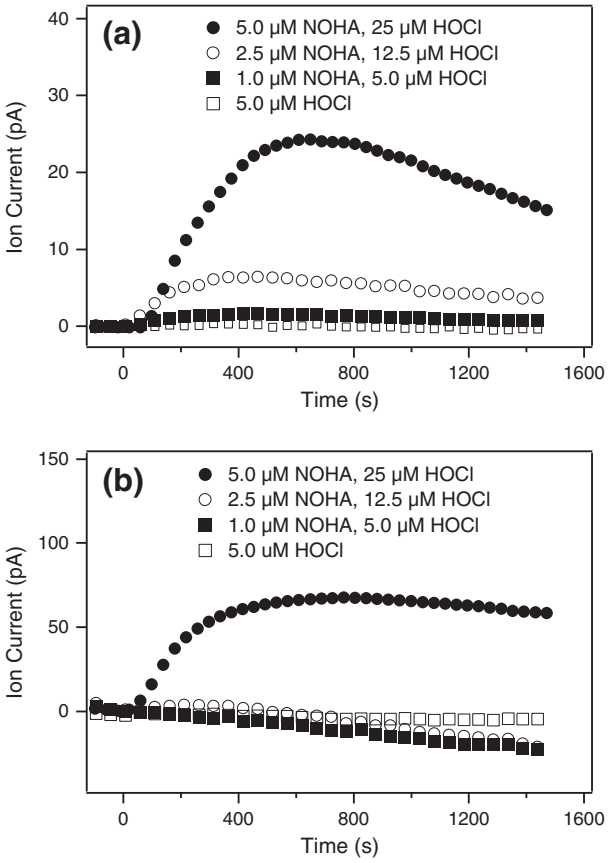


Fig. 5. MIMS response for the reaction of varying concentrations of NOHA and HOCl (maintaining a 5:1 substrate to oxidant ratio) at (a) m/z 30 and (b) m/z 44 at 37 °C in 0.1 M PBS, pH 7.4.

To overcome this limitation, we turned to the use of a physical trap. NO has a boiling point of −152 °C, while the boiling point of HNO is not known. We estimate that the boiling point of HNO is higher than that of NO, and hypothesized that we could selectively trap HNO and N_2O (bp = −88 °C) using an appropriate cold trap. Cold traps with boiling points between −90 °C and −160 °C were initially attempted, but since our system is under vacuum, they all allowed N_2O to pass through unaffected. Control experiments with standard N_2O gas and HNO (produced from 500 nM 2BrPA), however, show that both species can be completely trapped by liquid nitrogen (bp = −196 °C) (Fig. 6). In contrast, most of the NO (generated from DEA/NO) is observed to flow through the liquid nitrogen cold trap. When a liquid nitrogen cold trap is used for the MIMS examination of the reaction between 100 μM NOHA and 500 μM HOCl, no signal at m/z 30 is detected, indicating that NO is not produced (Fig. 7).

3.5. Oxidation of other substrates by hypochlorous acid

In addition to NOHA, we have also examined the HOCl oxidation of other substrates. Hydroxylamine, hydroxyurea, and acetohydroxamic acid have all been shown to be capable of being oxidized to produce HNO (Scheme 4) [22,61]. The reaction of HOCl with these substrates was studied by both MIMS and GC headspace analysis. For the HOCl oxidation of hydroxylamine, MIMS experiments show the characteristic 2:1 ratio for the m/z 44 and 30 signals, indicative of clean N_2O production (Fig. 8a). HOCl also readily oxidizes hydroxyurea to generate an increase in the m/z 30 and 44 signals, although the 44:30 intensity ratio is larger here (Fig. 8b), presumably the result of CO_2 production from the carbamic acid byproduct (Scheme 4). HOCl only minimally oxidizes acetohydroxamic acid under the conditions of our experiments (Fig. 8c). The larger m/z 44:30 ratio observed

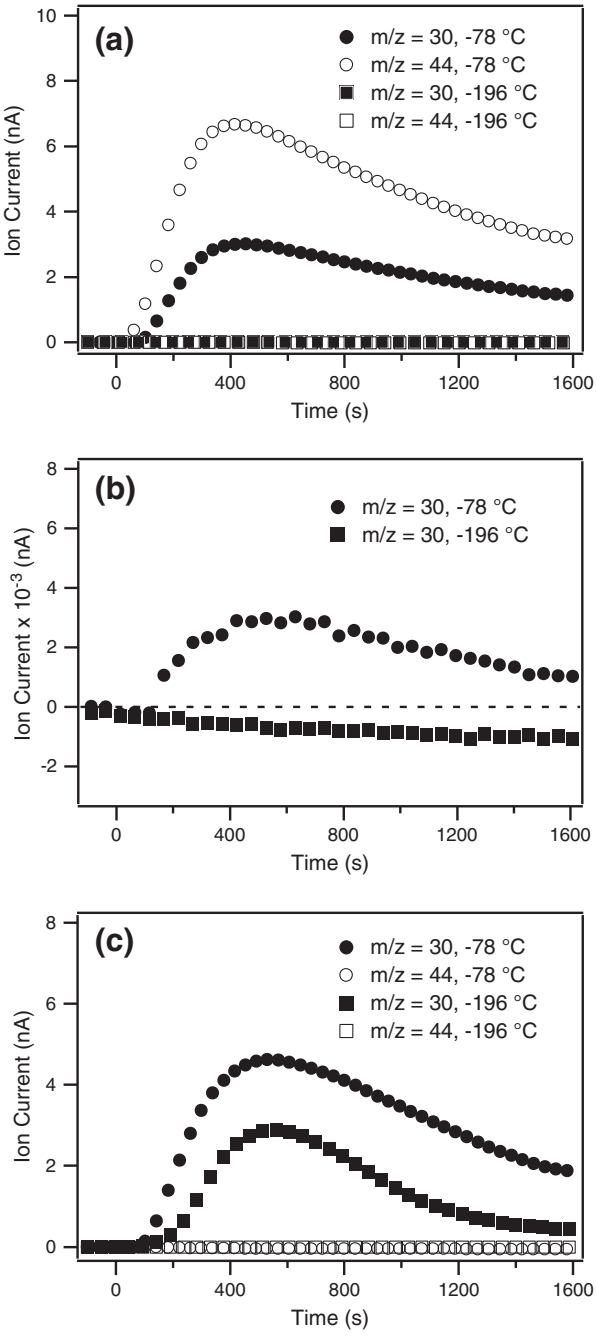


Fig. 6. MIMS response for control experiments using a liquid nitrogen (bp = −196 °C) trap compared to standard experimental conditions in which a dry ice/acetone (bp = −78 °C) trap is used for injection of (a) 30 μL N_2O, (b) 500 nM of the HNO donor 2BrPA, and (c) 25 μM DEA/NO, which corresponds to 50 μM NO, into a 0.1 M PBS solution at pH 7.4 and 37 °C.

here suggests the generation of CO_2 derived from secondary oxidative pathways. Analogous GC headspace analysis shows that both hydroxylamine and hydroxyurea produce a significant amount of N_2O upon reaction with HOCl, whereas acetohydroxamic acid generates relatively little N_2O (Table 3).

3.6. Oxidation of N-hydroxy-L-arginine by myeloperoxidase

Since MPO is the enzyme responsible for the generation of HOCl *in vivo*, we also examined the oxidation of NOHA and hydroxylamine (for comparison) by the MPO catalyzed reaction of H_2O_2 with chloride ion. For these experiments, either 15 or 75 nM MPO was added

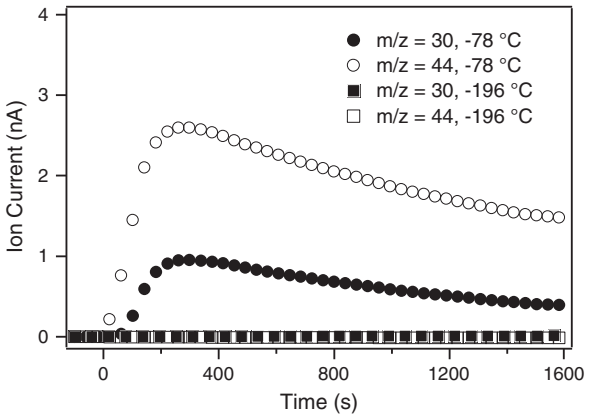


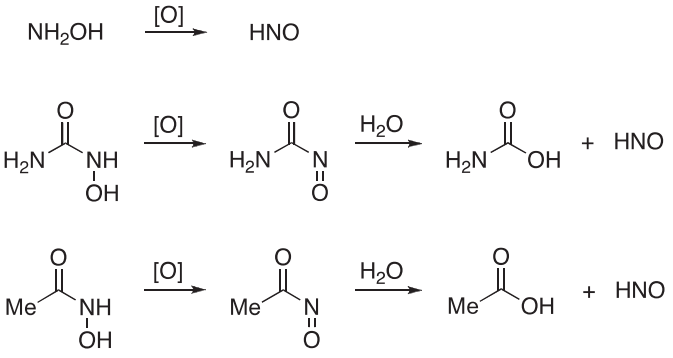
Fig. 7. MIMS response at m/z 30 and 44 following the injection of 500 μM HOCl into a 0.1 M PBS solution at pH 7.4 and 37 °C containing 100 μM NOHA using either a liquid nitrogen (bp = −196 °C) trap or the standard dry ice/acetone (bp = −78 °C) trap.

to 0.1 M PBS solution containing 143 mM chloride ion and 100 μM NOHA or hydroxylamine. To initiate the generation of HOCl, 500 μM H_2O_2 was added to this solution. Reaction results were examined by N_2O headspace analysis (Table 4). Control experiments examining the reaction of NOHA with MPO, NOHA with H_2O_2, and NOHA with MPO plus H_2O_2 did not result in the production of N_2O.

With 15 nM MPO, large yields of N_2O are not observed from the oxidation of NOHA or hydroxylamine; however, N_2O yields increase when 75 nM MPO is used. Although the effects of authentic HOCl have been shown to be identical to those of HOCl generated *via* MPO [62], significantly more N_2O is observed with hydroxylamine compared with NOHA in these MPO experiments (Table 4) in contrast to results with HOCl itself (Tables 2 and 3). Since HOCl is likely generated more slowly from MPO than when it is added bolus, we suspect that unreacted NOHA may be quenching HNO as it is produced, consistent with the previous observation that *N*-hydroxyguanidines can react with HNO [32].

4. Conclusions

MIMS and GC headspace analysis have been used to observe the formation of HNO-derived N_2O *via* the oxidation of NOHA by the biologically relevant oxidant, HOCl. HOCl is also able to generate HNO from the oxidation of hydroxylamine, hydroxyurea, and (to a lesser extent) acetohydroxamic acid. These results provide support for the possible endogenous generation of HNO *via* the oxidative chemistry of MPO-generated HOCl.



Scheme 4. Potential pathways to HNO following oxidation of hydroxylamine, hydroxyurea, and acetohydroxamic acid.

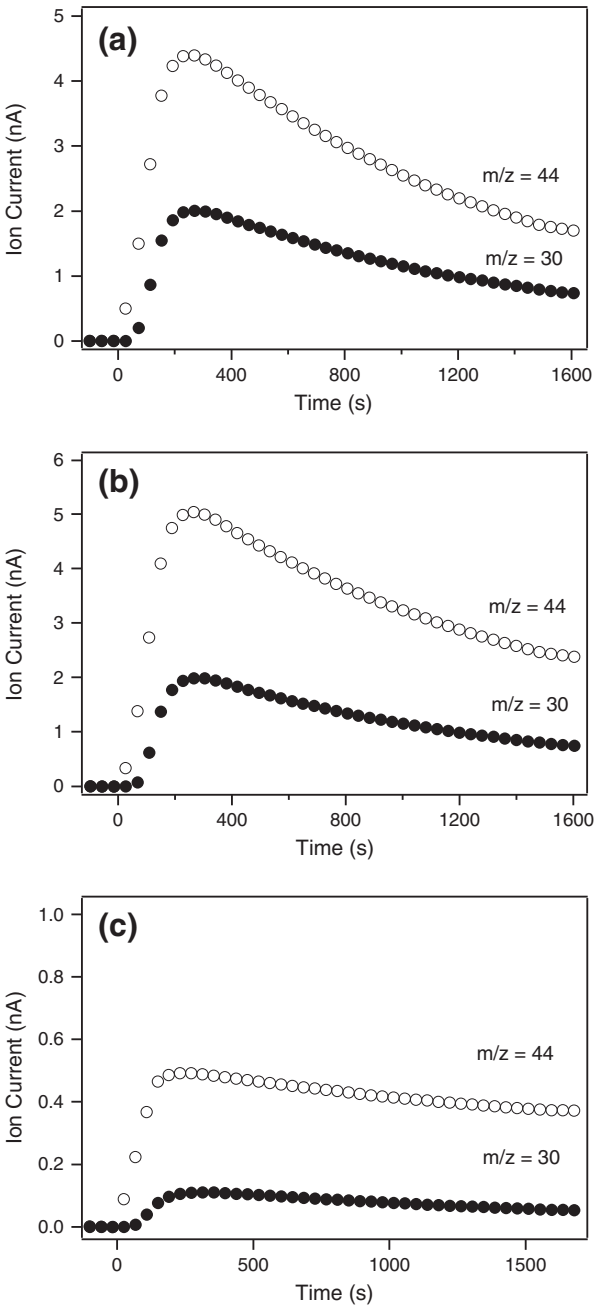


Fig. 8. MIMS responses at m/z 30 and 44 for the oxidation of 100 μM (a) hydroxylamine, (b) hydroxyurea, and (c) acetohydroxamic acid by 500 μM HOCl at 37 °C in 0.1 M PBS, pH 7.4. Note that signal intensities in (c) are approximately an order of magnitude smaller than those in (a) and (b).

Table 3
Oxidation of other substrates by HOCl.

Substrate	% HNO[a]
NH_2OH	90
Hydroxyurea	91
Acetohydroxamic acid	8

[a] Substrates (100 μM) were incubated with HOCl (500 μM) at 37 °C in 0.1 M PBS, pH 7.4. HNO yields are reported relative to the HNO donor, Angeli's salt, as determined by N_2O headspace analysis (SEM ± 5%; n ≥ 3).

Table 4
Oxidation of NOHA and hydroxylamine by MPO.

Substrate	MPO (nM)	% HNO[a]
NOHA	15	2
NH_2OH	15	21
NOHA	75	8
NH_2OH	75	73

[a] Substrates (100 μM) were incubated with MPO and H_2O_2 (500 μM) at 37 °C in 0.1 M PBS (containing 143 mM Cl^-), pH 7.4. HNO yields are reported relative to the standard HNO donor, Angeli's salt, as determined by N_2O headspace analysis (SEM ± 5%; n ≥ 3).

Disclosures

J.P.T. is a co-founder, stockholder, and serves on the Scientific Advisory Board of Cardioxyl Pharmaceuticals.

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