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OXIDATION OF *N*-HYDROXYGUANIDINE BY NITRIC OXIDE AND THE POSSIBLE GENERATION OF VASOACTIVE SPECIES

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Abstract—It has been reported previously that the *N*-hydroxyguanidine function of *N*-hydroxy-L-arginine can react with nitric oxide (NO) to generate other species that can act as potent vasodilators with different biological lifetimes than NO. The identities of these species have yet to be determined. Therefore, we have studied the reaction between NO and *N*-hydroxyguanidine and determined that *N*-hydroxyguanidine is capable of reducing NO to yield nitrous oxide (N_2O) and possibly other nitroso species. It is likely that at least some of the N_2O formation in these reactions is due to the initial generation of nitroxyl (HNO). Since HNO has been shown to be a potent vasorelaxant, it is possible that some of the non-NO-mediated biological activity alluded to in previous studies was due to HNO and that other nitroso-species generated in the reaction may also contribute to the overall pharmacological activity by release of either NO or HNO.

Key words: nitric oxide; *N*-hydroxy-L-arginine; *N*-hydroxyguanidine; vasorelaxation; nitrous oxide; nitroxyl

The biosynthesis of NO† in the vascular system is an important factor in the maintenance and regulation of vascular tone. It is generally thought that endothelium-derived relaxing factor is, in fact, NO [for example, see Ref. 1]. The biosynthesis of NO is accomplished by a class of enzymes generally referred to as the NOSs, which convert L-arginine to L-citrulline and NO in an oxygen- and NADPH-dependent process [for a recent review on NOS, see Ref. 2]. Although the intimate mechanistic details of the NOS-catalyzed oxidation of L-arginine are not, as yet, entirely understood, it is known that a guanidinohydroxylated species, *N*-hydroxy-L-arginine, is a biosynthetic intermediate [3–6]. Recent pharmacological studies by Zembowicz and co-workers [5, 7, 8] indicate that the NOS product, NO, and the biosynthetic intermediate, *N*-hydroxy-L-arginine, are capable of interacting to generate another potent, longer-lived, and as yet unidentified vasoactive species (Fig. 1). They also found that the generation of this species was dependent on the *N*-hydroxyguanidine function and not a specific property of *N*-hydroxy-L-arginine [8]. Thus, there is the possibility that multiple biologically active species can be generated in vascular tissue from NO. Due to the potential physiological relevance of this finding it becomes worthwhile to investigate the possible chemical interactions of NO with *N*-hydroxyguanidines. Herein, we report the results of a study aimed at elucidating the chemical reaction of NO with *N*-hydroxyguanidine and an alkyl-substituted analog of *N*-hydroxyguanidine.

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† Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; N_2O, nitrous oxide; PEHG, *N*-(phenethyl)-*N'*-hydroxyguanidine; PECA, *N*-(phenethyl)-cyanamide; and PEU, *N*-(phenethyl)-urea.

MATERIALS AND METHODS

Chemicals and solutions

N-hydroxyguanidine was purchased from Acros Organics (Fairlawn; NJ). *N*-(1-Naphthyl)ethylene-diamine dihydrochloride and sulfanilamide were purchased from the Aldrich Chemical Co. (Milwaukee, WI). PEHG and PECA were synthesized by the general method of Bailey *et al.* [9]. PEU was synthesized according to the method of Shapiro *et al.* [10]. The structures of all synthesized compounds were confirmed by ^{1}H-NMR (Bruker AM360), Fourier transform-infrared (Bio-Rad FTS-25) and electron-impact mass spectrometry (HP 5971A). All analytical data were consistent with literature values. NO gas was purchased from Matheson Gas Products (Cucamonga, CA) and passed through aqueous base prior to use. N_2O and O_2 were also purchased from Matheson Gas Products. All other chemicals were purchased from Fisher (Los Angeles, CA) and were of the highest purity available.

Reaction of N*-hydroxyguanidine with NO under anaerobic conditions*

In a typical reaction, 50 μmol of *N*-hydroxyguanidine · sulfate · monohydrate was placed in a 15-mL round-bottom flask equipped with a septum-capped stopcock. Then 10 mL of 100 mM Tris · HCl buffer, pH 7.4, was added, and the solution was degassed by several evacuation-N_2 purge cycles on a high vacuum line. The flask was left under an atmosphere of N_2. To the flask was then added 2.5 mL of NO gas via injection through the septum-capped stopcock using a gas-tight syringe. Reactions were stirred, and headspace analysis for reduced NO species (N_2O) was performed as described below. Control reactions that lacked *N*-hydroxyguanidine were run in parallel and were manipulated identically.

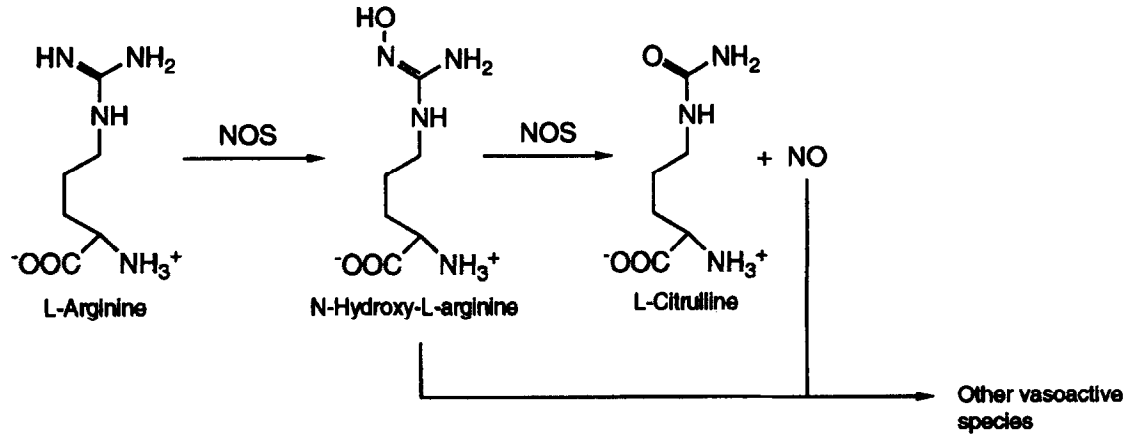

Fig. 1. Reaction pathway for the NOS-catalyzed oxidation of L-arginine to L-citrulline and NO with *N*-hydroxy-L-arginine as a biosynthetic intermediate that can react with NO to generate other vasoactive species.

Reaction of N*-hydroxyguanidine with NO under aerobic conditions*

The reaction of NO with *N*-hydroxyguanidine was compared under both aerobic and anaerobic conditions. Thus, 0.1 mmol of *N*-hydroxyguanidine · sulfate · monohydrate was placed into each of two 100-mL round-bottom flasks equipped with septum-capped stopcocks. Then 10 mL of 100 mM Tris · HCl buffer, pH 7.4, was added to each flask, and both were degassed on a vacuum line by several evacuation-N_2 purge cycles. Both flasks were left under an atmosphere of N_2. Then, 2.5 mL of pure NO gas (approx. 0.11 mmol) was introduced into each flask through the septum-capped stopcock using a gas-tight syringe. Next 2 mL of pure O_2 gas was added to one flask via a gas-tight syringe. The solutions were then stirred at room temperature, and headspace analysis of both flasks for N_2O was performed 0, 15, 35 and 95 min after the addition of NO according to the procedure described below.

Reaction of NO with PEHG

To a 10-mL Schlenck flask, equipped with a septum-capped stopcock, was added 31 μmol of PEHG dissolved in either a minimum amount of DMSO or methanol. Then 3 mL of 100 mM Tris · HCl buffer, pH 7.4, was added, and the solution was degassed on a vacuum line by several evacuation-argon purge cycles. The solution was left under an atmosphere of argon. Then 2.5 mL of pure NO gas (approx. 110 μmol) was added to the flask through the septum-capped stopcock using a gastight syringe. The solution was stirred at room temperature for 24 hr. The headspace was analyzed for N_2O as described below. Non-volatile reaction products were analyzed by HPLC (described below). Control experiments in the absence of NO or PEHG were performed in parallel to assure that the observed reaction products were due to a reaction between NO and PEHG. The control experiments gave no N_2O or oxidized products. Also, control experiments were performed to determine the stability of the reaction products under the reaction conditions. Thus, PECA and PEU were found to be stable under the reaction conditions, and their formation from PEHG was NO dependent.

Analytical methods

Quantitative analysis of the reaction mixtures of NO with PEHG was performed on a Rainin HPLC system equipped with a Beckman Ultrasphere, 25 cm, 5 μm, ODS reverse-phase column and a Spectra Physics 100 UV-visible detector operating at 210 nm. The products were eluted using a gradient mobile phase: 92:8, phosphate buffer, 50 mM, pH 4:acetonitrile to 87:13 over 10 min to 40:60 over 15 min. Under these conditions, PECA eluted at 19.2 min and PEU eluted at 14.9 min. Quantitation was accomplished by comparison of the detector response with that of known standard solutions of PECA and PEU.

Analysis and quantitation of N_2O were accomplished by gas chromatographic analysis utilizing a Hewlett Packard 5710A gas chromatograph equipped with a thermoconductivity detector and a 2 m × 1.9 mm Porapak Q column operating at 60° and at a flow rate of approximately 30 mL/min. Under these conditions, N_2O has a retention time of 2.05 min. Confirmation of this peak as N_2O was accomplished by mass spectral analysis as previously described [11]. Thus, the reaction headspace was sampled utilizing a gas-tight syringe and injected directly into the GC. Quantitation was performed by direct comparison of the GC response to that obtained with standards.

Analysis of NO in reaction headspace was performed as previously described [11]. Briefly, sampling of the headspace with a gas-tight syringe followed by direct analysis of the gas using a chemiluminescence detector (Antek 720, Houston, TX) allowed for the detection of NO. Quantitation of NO in these samples was accomplished by direct comparison of the detector response with that of NO standards made up under conditions identical to the reactions.

Analysis for NO_2^- was performed on anaerobic reactions utilizing the method previously described by Green *et al.* [12]. Briefly, after the NO/*N*-hydroxyguanidine reaction was complete, N_2O analysis was performed as described above. The solution was then degassed on a vacuum line using a series of evacuation-N_2 purge cycles to remove any unreacted NO (since NO will react with O_2 to give, eventually, NO_2^-, degassing the solution is essential if you are measuring nitrogen oxides

Table 1. Generation of N_2O from the reaction of NO with *N*-hydroxyguanidine*

Reaction conditions	Time (hr)	Yield of N_2O† (%)
5 mM *N*-Hydroxyguanidine		
+2 equiv. NO gas	0	0
+2 equiv. NO gas	3	6
+2 equiv. NO gas	24	30
NO gas only	24	0
5 mM *N*-Hydroxyguanidine only	24	0

* All reactions were carried out under anaerobic conditions (see Materials and Methods).

† Yield of N_2O is based on *N*-hydroxyguanidine.

generated from the reaction). Then 0.2 mL of the reaction solution was added to a 2-mL cuvette along with 0.9 mL of a 0.1% naphthylethylenediamine · 2HCl solution in distilled water and 0.9 mL of a 1% sulfanilamide solution in 5% H_3PO_4. The assay mixture was allowed to stand at room temperature for 5 min. The absorbance at 546 nm was then determined using a visible spectrophotometer (Uvikon 810, San Diego, CA). Quantitation of NO_2^- levels in the reaction samples was determined by comparison to a standard curve generated from authentic $NaNO_2$ samples of known concentration. As controls, identical reactions that lacked either NO or *N*-hydroxyguanidine were also analyzed for NO_2^-. Also, to assure that *N*-hydroxyguanidine did not interfere with NO_2^- assay, a comparison of the absorbance at 546 nm for the standards in the absence and presence of added *N*-hydroxyguanidine was made. The addition of *N*-hydroxyguanidine made no significant difference in the absorbance.

RESULTS

The possible reaction between NO and *N*-hydroxyguanidine was investigated under anaerobic conditions. It was found that a degassed 5 mM solution of *N*-hydroxyguanidine in pH 7.4 buffer reacted with a 2-fold excess of NO, added to the reaction headspace to give, as the only detectable gaseous product, N_2O. The yield of N_2O was determined to be approximately 6%, based on *N*-hydroxyguanidine, after 3 hr and 30% after 24 hr (Table 1). Control experiments lacking *N*-hydroxyguanidine or NO gave no detectable N_2O. Thus, *N*-hydroxyguanidine is capable of reducing NO to N_2O, although the reaction appears to be fairly slow under anaerobic conditions. Also, analysis of the anaerobic reaction mixtures for NO_2^- indicated that no significant NO_2^- was formed even though significant levels of N_2O were reached. That is, less than 0.01 molar equivalents of NO_2^- were generated per mole of N_2O formed. Preliminary experiments performed under aerobic conditions (data not shown) indicated that the generation of N_2O was markedly faster in the presence of oxygen. To evaluate the ability of O_2 to accelerate the reduction of NO by *N*-hydroxyguanidine, the generation of N_2O was monitored under both aerobic and anaerobic conditions in otherwise identical systems. As expected, the reduction of NO to N_2O by *N*-hydroxyguanidine was found to be markedly faster in the presence of O_2 (Fig. 2).

To determine whether NO reduction was specific for *N*-hydroxyguanidine or a general reaction of the *N*-hydroxyguanidine function, a phenethyl-substituted deriv-

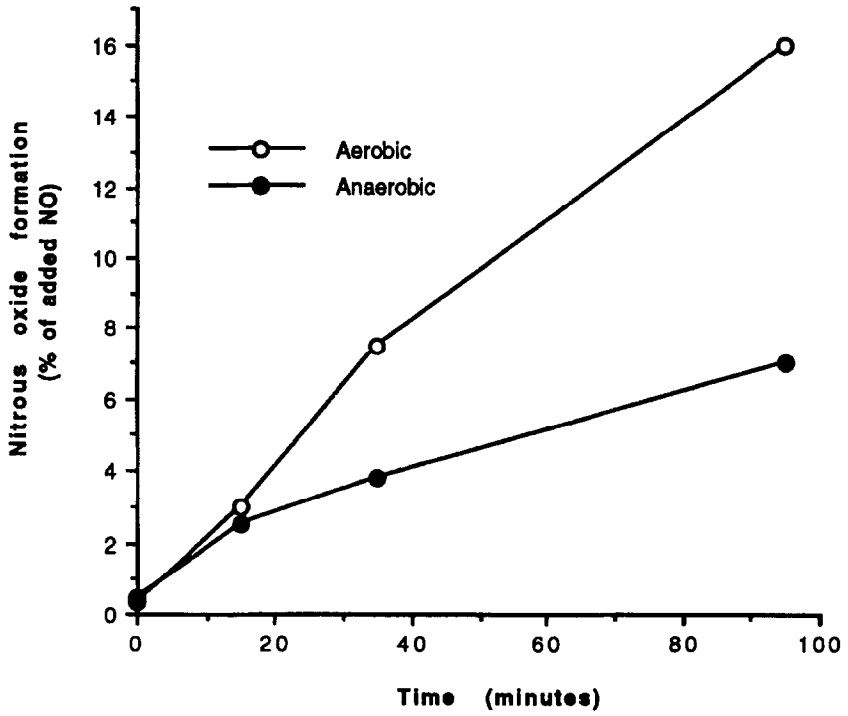

Fig. 2. Effect of oxygen on the generation of N_2O from the reaction of NO (0.11 mmol) with *N*-hydroxyguanidine (0.1 mmol).

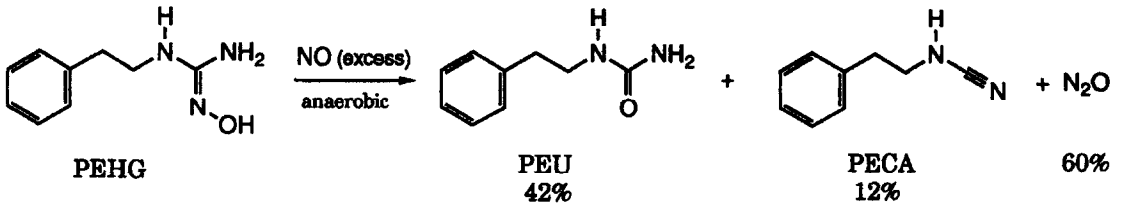

Fig. 3. Formation of PECA, PEU and N_2O from the reaction of NO with PEHG. Reported yields are the averages of three separate experiments and are based on PEHG.

ative of *N*-hydroxyguanidine, PEHG, was synthesized, and its reaction with NO examined. As with the *N*-hydroxyguanidine, this alkyl-substituted analog was also capable of reducing NO to N_2O. Thus, it appears that NO can be reduced by *N*-hydroxyguanidines, in general, to give N_2O. In this reaction, NO appears to be acting as an oxidizing agent, and therefore products of *N*-hydroxyguanidine oxidation should be generated. To fully characterize this reaction, the organic products of the reaction of NO with PEHG were characterized, and the stoichiometry of the reaction was determined after 24 hr. It was found that the only detectable reaction products were the corresponding cyanamide, PECA, the corresponding urea, PEU, and N_2O (Fig. 3). In a typical 24-hr reaction under anaerobic conditions, the yields of PECA, PEU and N_2O were determined to be 12, 42 and 60%, respectively, based on PEHG. In control reactions lacking either NO or PEHG, no N_2O or other products were detected. That is, PEHG was stable under the reaction conditions in the absence of NO. Also, it was determined that PECA was not the precursor to PEU since PECA was stable with respect to water addition under the conditions of the reaction. Interestingly, under anaerobic conditions, the amount of N_2O generated was always greater than the amount of the combined organic products, PECA plus PEU. Stoichiometric determinations were not performed under aerobic conditions due to the potential confounding reactions of O_2 with potential radical reaction intermediates and the spontaneous reaction of O_2 with NO.

DISCUSSION

There appears to be little doubt that NO is capable of oxidizing *N*-hydroxyguanidines resulting in, eventually, the reduced nitrogen oxide, N_2O. Since N_2O can be formed from the dimerization-dehydration of HNO [13], reaction 1 below, N_2O detection is often used as an indication of nitroxyl (HNO) generation.

$$2HNO \Rightarrow HON{=}NOH \Rightarrow N_2O + H_2O \qquad (1)$$

However, mechanisms can be envisoned for this reaction which can account for at least some of the N_2O production without the intermediacy of HNO (discussed below). The other products of this reaction include both the corresponding cyanamide and urea (Fig. 3). Since the formal oxidation state of carbon in these compounds is the same as that of *N*-hydroxyguanidine starting material, oxidation must have occurred at the hydroxy-substituted nitrogen. Possible mechanisms for the formation of N_2O from the reaction of NO with *N*-hydroxyguanidines are shown in Fig. 4. The mechanisms presented herein propose that NO is initially reduced by the *N*-hydroxyguanidine to give HNO, which can dimerize and be responsible for at least some of the observed N_2O. Although NO is typically not considered to be a potent H-atom abstractor, it has been demonstrated previously that it can abstract a hydrogen atom from hydroxylamine [14] and alkyl hydroxylamines [15] especially under basic conditions. Mechanism **A-B-C-D** and **A-B-C-E** (Fig. 4) would require that NO act as a radical trap for the nitroxide to form a dinitroso-intermediate and that all the observed N_2O comes from the dimerization-dehydration of HNO. Mechanisms **F-G-H** and **F-I-J** (Fig. 4) are similar to a previously proposed mechanism for NO reduction by hydroxylamine in that the H-atom abstraction from a nitrogen center by NO is the first step [14]. Subsequent trapping of NO by the nitrogen centered radical results in N-nitrosated species that can decompose to give N_2O directly. As indicated in Fig. 4, hydrogen abstraction from either oxygen or nitrogen can result in formation of the observed cyanamide and urea products.

Another possible mechanism by which N_2O can be formed as a result of initial HNO generation is via the process shown below [13].*

$$^{-}NO + 2NO \Rightarrow N_3O_3^{-} \Rightarrow N_2O + NO_2^{-} \qquad (2)$$

However, analysis of the anaerobic NO/*N*-hydroxyguanidine reaction solutions indicated that no significant NO_2^{-} was formed. Thus, under the conditions of our experiments, this reaction is not likely to be responsible for N_2O generation.

Although this reaction can occur anaerobically, the presence of O_2 greatly accelerates the formation of N_2O (Fig. 2). The observed acceleration of NO reduction by *N*-hydroxyguanidine in the presence of O_2 is apt to be significantly greater than indicated since NO is unstable with respect to O_2, and therefore the aerobic reaction undoubtedly contained considerably less NO compared with the anaerobic reaction. A possible explanation for the observed rate enhancement by O_2 is that the first step in the reaction, H-atom abstraction by NO, is slow (either steps **A** or **F** in Fig. 4), and oxidation products from the reaction of NO with O_2, such as ONOO· or NO_2 (reactions 3 and 4, below), are more efficient at abstracting the hydrogen atom from either oxygen or nitrogen. Alternatively, the addition of O_2 may lead to the generation of N_2O_3 (reactions 3–5), which, in turn, may act as a nitrosating agent (reaction 6) [16].

$$NO + O_2 \Leftrightarrow ONOO\cdot \qquad (3)$$

* We are indebted to a reviewer for mentioning this mechanistic possibility for N_2O generation from initial formation of HNO.

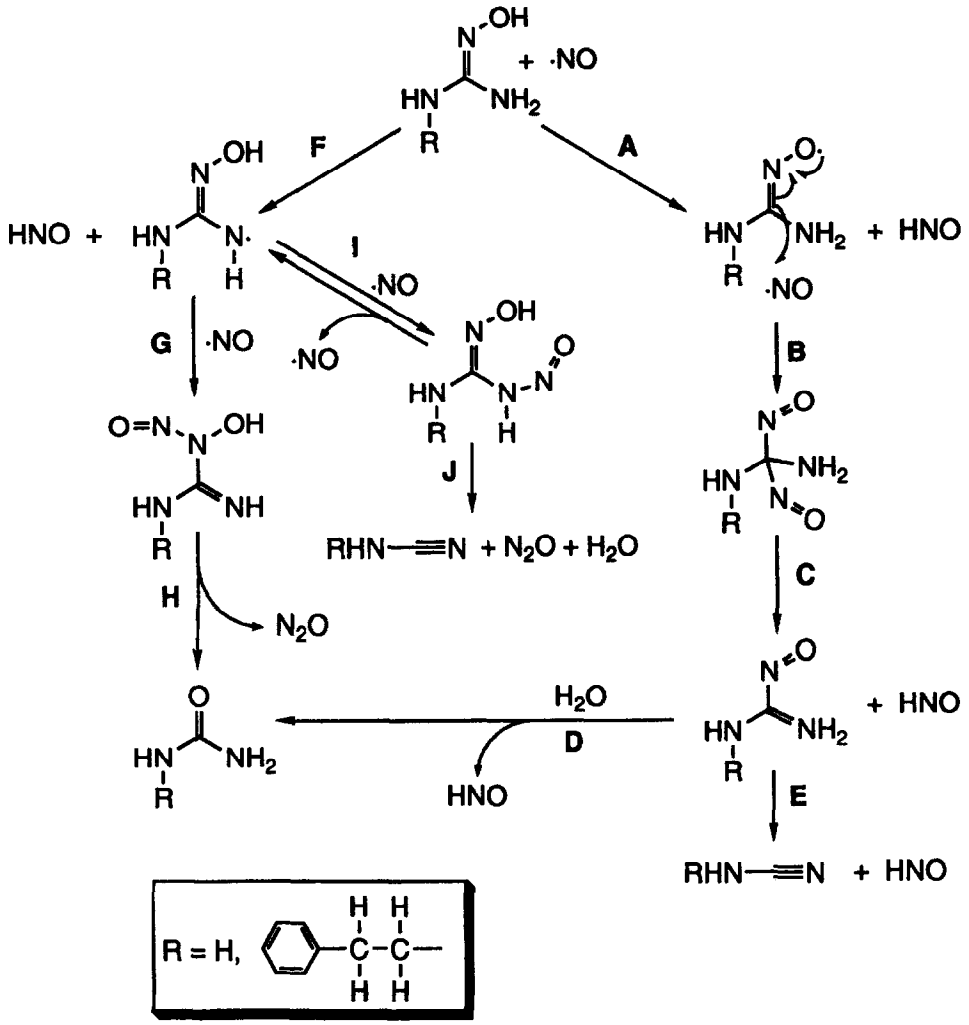

Fig. 4. Possible mechanisms for the oxidation of *N*-hydroxyguanidine by NO.

$$ONOO\cdot + NO \Rightarrow 2NO_2 \quad (4)$$

$$NO_2 + NO \Rightarrow N_2O_3 \quad (5)$$

$$N_2O_3 + RXH \Rightarrow RX\text{-}NO + NO_2^- + H^+ \ (X=N, C \text{ or } O) \quad (6)$$

Decomposition of such nitrosated species via pathways **C, H** or **J** could result in the observed cyanamide and urea products. Of special note, an accelerating effect of O_2 has also been observed in the reduction of NO by hydroxylamine, and this effect has been attributed to nitrosation of hydroxylamine by N_2O_3 [14]. To be sure, the mechanisms proposed herein are somewhat speculative and certainly other mechanisms can be envisioned. However, they do serve as a basis for explaining the generation of the observed products and may offer a reasonable explanation of the previously reported generation of other vasoactive species from the interaction of NO with *N*-hydroxyguanidines (discussed below).

These results indicate that *N*-hydroxyguanidines are capable of reducing NO with HNO and/or an NO-adduct being possible products. The observed pharmacological activity of the reaction product(s) may be due to either HNO directly or the release of NO from a reaction intermediate. The amount of HNO generated in our system would depend on the mechanism involved (see Fig. 4). Under anaerobic conditions, it is likely that at least 1 HNO is formed in an initial H-atom abstraction reaction. Possible NO adducts resulting from the trapping of NO by one-electron oxidized radical intermediates may also lead to the further generation of HNO (e.g. steps **B-C-E,** Fig. 4). Since it has been demonstrated previously that HNO is a potent vasorelaxant [17–19], the proposed chemistry may explain the generation of other vasoactive species from the reaction of NO with *N*-hydroxyguanidines as has been reported previously [5, 7, 8]. Also, the possible N-nitrosated species resulting from the trapping of NO by a nitrogen centered radical (step **G** or **I,** Fig. 4) may lead to the re-release of NO since compounds of analogous structure, *N*-nitrosoureas, have been shown to release NO by thermal homolytic cleavage of the N–N bond (reaction 7) [20].

$$ONNH - C(O) - NH_2 \Rightarrow ON\cdot + \cdot NH - C(O) - NH_2 \quad (7)$$

Therefore, the apparent lifetime of NO may be effectively prolonged by temporary "sequestration" by the

oxidized *N*-hydroxyguanidine intermediate. It should be mentioned that the previously observed biological activity associated with NO/*N*-hydroxyguanidine reaction products cannot be due to N_2O since it has been shown to lack vasorelaxing properties [17].

The potential physiological relevance of the chemistry proposed herein remains to be determined. This study is meant only to describe possible chemical interactions between NO and *N*-hydroxyguanidines since previous pharmacological studies have indicated that such a process occurs *in vitro*. Due to the possible pharmacological relevance of a chemical interaction between NO and *N*-hydroxyguanidine, the above mechanistic discussion has concentrated on the possible generation of species that may account for the previously observed biological activity. To be sure, the chemical mechanism(s) that would account for the observed reaction products as well as the biological activity is far from being understood. Certainly, the reactions described herein will occur only if reasonable concentrations of NO and *N*-hydroxy-L-arginine are attained. Significantly, it has been reported recently that levels of up to 37 μM *N*-hydroxy-L-arginine can be achieved in an *in vitro* system [21]. Also, physiological concentrations of NO in the micromolar range have been reported in rat brain [22] and could be significantly higher under pathophysiological conditions. Regardless, the results presented here indicate that NO is capable of acting as an oxidizing agent for easily oxidized substrates that may result in the generation of other biologically active species with varied pharmacokinetic properties.

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