Comparison of the chemical reactivity of synthetic peroxynitrite with that of the autoxidation products of nitroxyl or its anion

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ABSTRACT

Donors of nitroxyl (HNO) exhibit pharmacological properties that are potentially favorable for treatment of a variety of diseases. To fully evaluate the pharmacological utility of HNO, it is therefore important to understand its chemistry, particularly involvement in deleterious biological reactions. Of particular note is the cytotoxic species formed from HNO autoxidation that is capable of inducing double strand DNA breaks. The identity of this species remains elusive, but a conceivable product is peroxynitrous acid. However, chemical comparison studies have demonstrated that HNO autoxidation leads to a unique reactive nitrogen oxide species to that of synthetic peroxynitrite. Here, we extend the analysis to include a new preparation of peroxynitrite formed via autoxidation of nitroxyl anion (NO^-). Both peroxynitrite preparations exhibited similar chemical profiles, although autoxidation of NO^- provided a more reliable sample of peroxynitrite. Furthermore, the observed dissimilarities to the HNO donor Angeli's salt substantiate that HNO autoxidation produces a unique intermediate from peroxynitrite.

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1. Introduction

Donors of nitroxyl (HNO) have been shown to induce pharmacological responses, including enhancement of myocardial contractility [1], preconditioning against ischemia/reperfusion injury [2], induction of apoptosis and suppression of tumor angiogenesis [3], inhibition of alcohol metabolism [4–6] and analgesia [7]. The clinical efficacy of HNO donors has been demonstrated with cyanamide (NH_2CN) in the treatment of alcohol abuse [4,6]. A more recent discovery that HNO donors may also be beneficial in the treatment of heart failure [8–10] has stimulated the development of a variety of structurally diverse HNO donors and related prodrugs [11,12].

As with all pharmacological agents, the therapeutic usefulness of HNO donors depends on the ability to elicit beneficial responses without inducing harmful side effects. Cyanamide, which

upon metabolic activation is converted to HNO and CN^-, illustrates the potential complications of using donors of HNO. Nevertheless, such species are necessary due to the self consumption of HNO through a dimerization pathway [13] (Eq. 1; 8×10^6 M^{-1} s^{-1} [14]).

$$2HNO \rightarrow [HONNOH] \rightarrow N_2O + H_2O \tag{1}$$
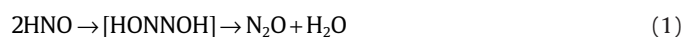
Given the structural diversity of HNO donors, the potential side effects of the byproducts of HNO donor decomposition are likely to be highly variable and thus will require tailored analysis. The production of HNO itself may also induce diverse responses, depending on a variety of factors including production rates and levels as well as environment. Analysis may be simplified by the relation of HNO to the well-studied signaling molecule nitric oxide (NO).

While endogenous formation of NO regulates physiological processes in the cardiovascular, nervous and immune systems [15–17], pathophysiological effects are also associated with NO production [18]. This dichotomy is often explained by considering the chemical modifications that occur through direct reaction of NO with a biological target or following oxidation of NO [19]. The deleterious effects of NO are generally associated with the nitrogen oxides that form upon reaction with molecular oxygen or superoxide (O_2^-). These products of NO oxidation are known to induce oxidative or nitrosative stress responses that NO itself does not elicit.

Similarly, the pharmacological effects of HNO donors are associated with direct reactions of HNO, particularly with heme proteins and thiols [20–22]. Although, HNO does not react with O_2^-, it is
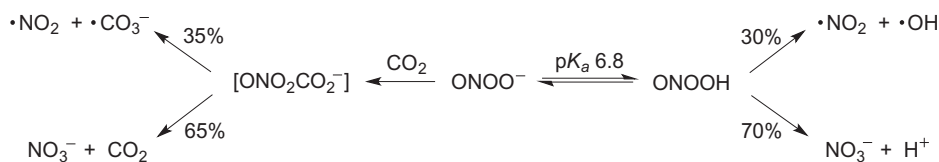
Scheme 1. Decomposition pathways for ONOO⁻ (adapted from reference 36).

autoxidized to a cytotoxic species [23]. Of particular note is the capability of this autoxidation product to cleave double strand DNA [23–26]. The clinical use of cyanamide as well as numerous pharmacological studies and the slow kinetics of the reaction of HNO and O_2 (3×10^3 M^{-1} s^{-1} [21]) indicate that HNO autoxidation is likely to be limited relative to other reactions. In addition, HNO autoxidation may only be relevant in environments with relatively high O_2 concentrations such as membranes. Nonetheless, full elucidation of HNO autoxidation is critical to the continued development of the pharmacology of HNO donors.

Despite extensive analysis, the identity of the HNO autoxidation product remains elusive. Peroxynitrous acid (ONOOH) has been considered as an obvious candidate [26–28]. This species is also relevant to the chemical biology of NO, since reaction of NO and O_2^- produces peroxynitrite (ONOO⁻) (Eq. 2) [29,30] at a nearly diffusion controlled rate ($4–7 \times 10^9$ M^{-1} s^{-1} [31,32]).

$$NO + O_2^- \rightarrow ONOO^- \qquad (2)$$

Although Eq. 2 is often considered to be an antioxidant pathway due to consumption of O_2^-, it can nonetheless initiate deleterious reactions, including one- and two-electron oxidation, hydroxylation and nitration, causing damage to cellular components [33].

In alkaline solution, ONOO⁻ is relatively stable, but once protonated (pK_a of 6.8 [34]) rapid, spontaneous decomposition ($t_{1/2}$ 1.9 s at pH 7.4, 37 °C [35]) occurs via competing pathways. Homolytic cleavage of the O—O bond of ONOOH produces nitrogen dioxide (NO₂) and the hydroxyl radical (·OH). This pathway accounts for ~30% of decomposition products while the major product is the isomer nitrate (NO₃⁻; ~70%) (Scheme 1) [37–39]. In the presence of CO₂, decomposition of ONOO⁻ yields NO₂ and the carbonate radical (·CO₃⁻, ~35% [39]) through the initial formation of the nitrosoperoxycarbonate adduct (ONO₂CO₂⁻) [40].

Autoxidation of the nitroxyl anion (NO⁻) also produces ONOO⁻ (Eq. 3) at a nearly diffusion controlled rate (3×10^9 M^{-1} s^{-1} [14,41]).

$$NO^- + O_2 \rightarrow ONOO^- \qquad (3)$$

This reaction has historical importance in the study of HNO chemistry, as it provides a spectroscopic marker at high pH where ONOO⁻ is stable to decomposition. In fact degradation of HNO donors in aerobic, alkaline solution visibly produces the yellow product ONOO⁻ [14,28,41,42].

The acid–base relationship of HNO and NO⁻ is atypical because HNO exists in a singlet ground state while NO⁻ is a triplet. The necessity of a spin change significantly impacts the rate of proton transfer ($k_f = 5 \times 10^4$ M^{-1} s^{-1} and $k_r = 1 \times 10^2$ s^{-1} at pH 7 and 22 °C [14]). Near neutral pH, these slow rates coupled with self-consumption pathways (Eq. 1) impede interconversion, such that HNO does not significantly deprotonate. The rate of deprotonation becomes progressively more efficient with increased alkalinity, as OH⁻ rather than water serves as the base (see reference 43 for a detailed discussion).

In aerobic, highly alkaline solution, the relative rates suggest that deprotonation of HNO occurs prior to autoxidation. In a prior analysis of the common HNO donor Angeli's salt (Na₂N₂O₃), we demonstrated that under these conditions, ONOO⁻ is produced by competitive pathways in which O₂ reacts with not only NO⁻ but also

directly with Angeli's dianion, $N_2O_3^{2-}$ [28]. We also verified experimentally and by quantum mechanical analysis that at physiological pH, O₂ reacts with HNO only after the spontaneous decomposition of Angeli's salt (Eq. 4) (half-life of 2.5 min at pH 7.4 and 37 °C [44]). However, we were unable to identify the subsequent product.

$$N_2O_3^{2-} + H^+ \rightarrow HNO + NO_2^- \qquad (4)$$
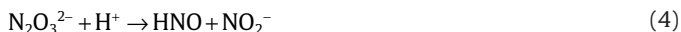
Formation of ONOOH is difficult to assess directly due to short lifetime and low absorbance intensity. To determine indirectly if autoxidation of HNO produces ONOOH, we also compared the chemical profiles of Angeli's salt and synthetic ONOO⁻ toward various trapping agents [26,27]. The observed dissimilarity, particularly in one-electron oxidation and hydroxylation, led to the conclusion that HNO autoxidation produces a unique reactive nitrogen oxide species from that formed by protonation of ONOO⁻.

Although the synthetic ONOO⁻ preparation is convenient, it involves neither NO nor NO⁻ as reactants (Eqs. 2 and 3) (see reference 45 for mechanistic details). We therefore wondered if the chemical profile of ONOO⁻ produced by autoxidation of NO⁻ (Eq. 3) would be more similar to that observed previously for Angeli's salt or the synthetic ONOO⁻ preparation. Angeli's salt proved to be unsuitable to answer this question due to the slow rate of decomposition at high pH [46], the lack of significant accumulation of ONOO⁻ [28], and the inherent release of nitrite (NO₂⁻; Eq. 4), which is bioactive [47].

We recently determined that unlike Angeli's salt, which has a degradation pathway that is increasingly inhibited at higher pH [46], above pH 8, the HNO donor IPA/NO (Na[(CH₃)₂CHNH(N(O)NO], sodium 1-(N-isopropylamino)diazen-1-ium-1,2-diolate) decomposes through a pH-independent, internal tautomerization (Scheme 2; 6×10^{-4} s^{-1} at pH 13) [42]. The resulting nitrosamine is rapidly converted to the related alcohol following loss of N₂ [48].

Preparation of ONOO⁻ by degradation of IPA/NO in aerobic, alkaline solution is attractive since it spontaneously decomposes to produce HNO in a pH-independent manner above pH 8 without direct production of nitrite [42]. Furthermore, that both IPA/NO and Angeli's salt are from the same class of HNO donors (diazeniumdiolates) facilitates comparison of the chemical profiles of the reaction of HNO or NO⁻ with O₂.

2. Materials and methods

2.1. Chemicals

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich and were used without further purification. Unless stated, stock solutions were prepared daily in Barnstead Nanopure Diamond filtered water. The assay buffer was calcium and
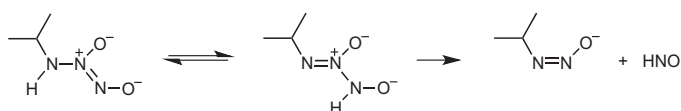
Scheme 2. Decomposition of IPA/NO.