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Comparison of HNO reactivity with tryptophan and cysteine in small peptides

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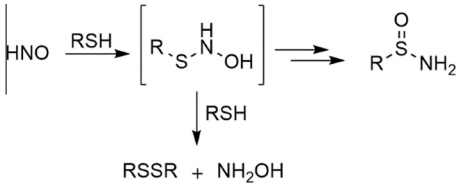
ABSTRACT

Recent discoveries of important pharmacological properties have drawn attention to the reactivity of HNO (azanone, nitroxyl) with biologically relevant substrates. Apart from its role in thiol oxidation, HNO has been reported to have nitrosative properties, for example, with tryptophan resulting in *N*-nitrosotryptophan formation. We have investigated the reactivity of HNO with tryptophan and small peptides containing either tryptophan or both a tryptophan and a cysteine residue. Our results point to the more reactive nature of cysteine towards HNO compared with tryptophan.

HNO (azanone, nitroxyl) is the protonated, one-electron reduced form of NO (nitric oxide), with its own important biological and pharmacological properties.[1–3] It has received significant attention due to its positive inotropic/lusitropic effects in normal and failing hearts, making it a potential heart failure therapeutic.[4,5] Moreover, we and others have shown that HNO targets several proteins involved in Ca^{2+} cycling.[6–9] Recent reviews also highlight the possible benefits of HNO in conditions such as vascular dysfunction, cancer, and alcoholism.[2,3] Thiols constitute one of the major biological targets of HNO.[3,10,11] Although HNO and NO are redox siblings, the thiol reactivity of HNO differs significantly from that of NO and normally results in the formation of a disulfide or a sulfinamide depending on the concentration of thiol (Scheme 1).[10,11] Additionally, recent reports point to the nitrosative role of HNO in generating *N*-nitrosoindole species (Scheme 2).[12,13] Although HNO-derived *N*-nitrosotryptophan (TrpNO) formation might be important in HNO pharmacology, information about this reactivity is scarce.

To gain more insight into HNO reactivity, we have investigated TrpNO formation in the presence of a nearby cysteine residue. For this purpose, we used the synthetic peptide, AGSCWA, which includes the active site sequence of the cysteine protease, papain, as well as several of its variants (CGSAWA, ACSAWA, AGCAWA, AGSAWC, GSAWCA, and AGSAWA). The small molecule *N*-acetyl-L-tryptophan was also employed for comparison.

As seen in Figure 1, treatment of the non-cysteine-containing peptide, AGSAWA, with different HNO-donors (Angeli's salt (AS) or *N*-hydroxy-2-(methylsulfonyl)benzenesulfonamide (2-MSPA)) at physiological pH and temperature results in the formation of the corresponding TrpNO-containing peptides (*m*/*z* 591) (Fig. 1a–c). Due to the reported instability of TrpNO species to mass spectrometry conditions,[14] electrospray ionization mass spectrometry (ESI-MS) data could be obtained only at relatively low temperatures (110 °C). Upon treatment of the sample with the HNO-donor byproducts (nitrite or 2-(methylsulfonyl)benzenesulfinic acid (2-MSSA)) no TrpNO species are detected. Also, the use of a ^{15}N-labeled HNO-donor, ^{15}N-2-MSPA, provides the corresponding Trp^{15}NO-containing peptide (*m*/*z* 592) (Fig. 1d). Similarly, the characteristic TrpNO peak was observed by UV–visible spectroscopy at 335 nm upon incubation of *N*-acetyl-L-tryptophan with the HNO



Scheme 1. Reaction of HNO with thiols.

Abbreviations: ACN, acetonitrile; AS, Angeli's salt; 2-BrPA, 2-bromo-*N*-hydroxybenzenesulfonamide; DMSO, dimethyl sulfoxide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTPA, diethylenetriamine pentaacetic acid; ESI-MS, electrospray ionization mass spectrometry; FT-NMR, Fourier-transform nuclear magnetic resonance; HPLC, high pressure liquid chromatography; 2-MSPA, *N*-hydroxy-2-(methylsulfonyl)benzenesulfonamide; 2-MSSA, 2-(methylsulfonyl)benzenesulfinic acid; TFA, trifluoroacetic acid; TrpNO, *N*-nitrosotryptophan.

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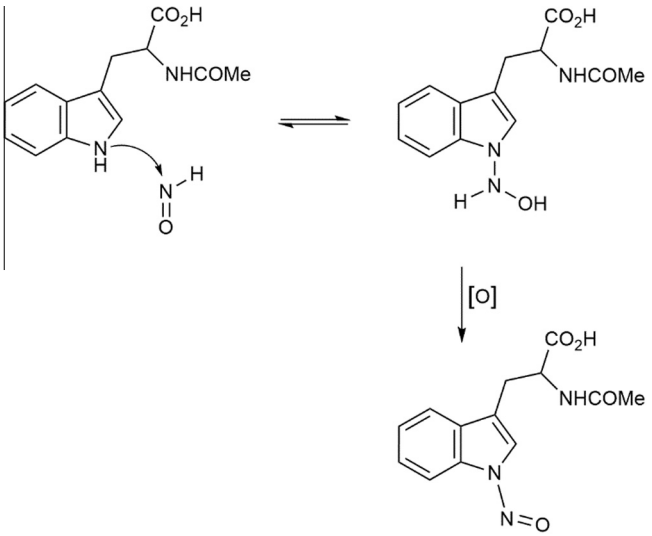

Scheme 2. Reaction of HNO with tryptophan.[13]

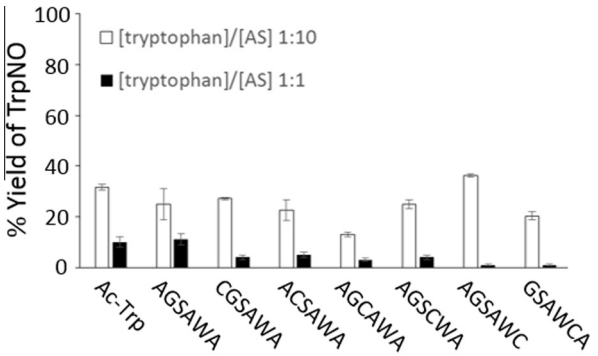

Figure 2. Percent yield of *N*-nitrosotryptophan (TrpNO) observed upon incubation of *N*-acetyl-L-tryptophan (Ac-Trp) or synthetic peptides with 10-fold excess ((□), [tryptophan] = 0.1 mM, [AS] = 1 mM) or equimolar ((■), [tryptophan] = 0.3 mM, [AS] = 0.3 mM) amounts of HNO-donor in 10 mM phosphate buffer with 50 μM DTPA (pH 7.4) at 37 °C for 30 min. The percent yield of TrpNO was determined by UV–visible spectroscopy with respect to the amount of tryptophan in the control samples (SEM ±6%, $n \geqslant 2$).

donor, 2-bromo-*N*-hydroxybenzenesulfonamide (2-BrPA)[15] under the same conditions (data not shown). These results are consistent with previous reports suggesting the formation of HNO-derived TrpNO,[12,13] and demonstrate that this modification is not specific to AS, but also takes place with other HNO-donors.

To assess the feasibility of an HNO-derived tryptophan modification in the vicinity of a free thiol, we treated the peptides containing both a tryptophan and a cysteine residue with different

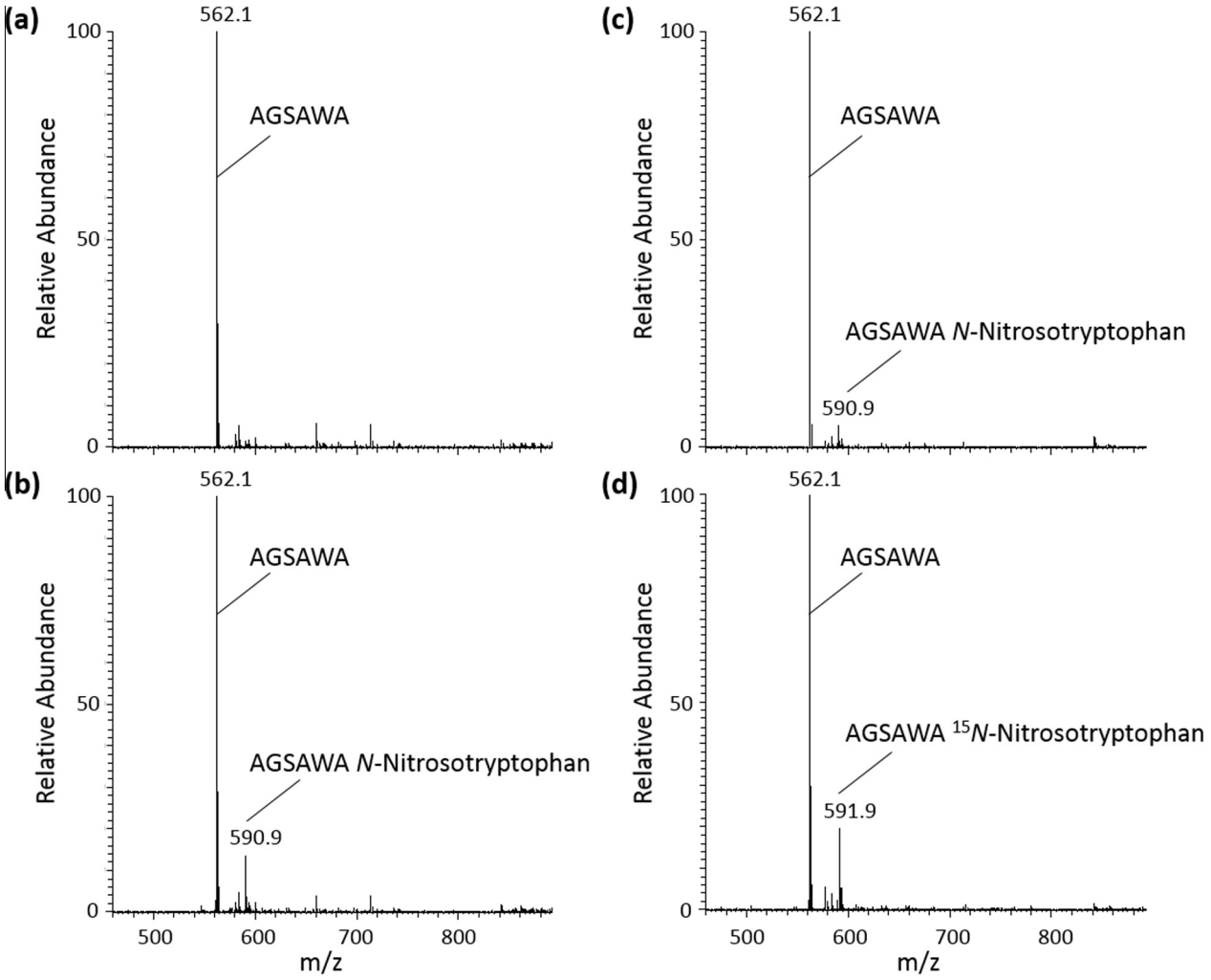

Figure 1. Selected region of ESI-MS spectra showing AGSAWA (0.3 mM) (a) untreated or treated with (b) 1 mM AS, (c) 1 mM 2-MSPA, and (d) 1 mM ^{15}N-2-MSPA in 10 mM phosphate buffer with 50 μM DTPA (pH 7.4) at 37 °C for 30 min.

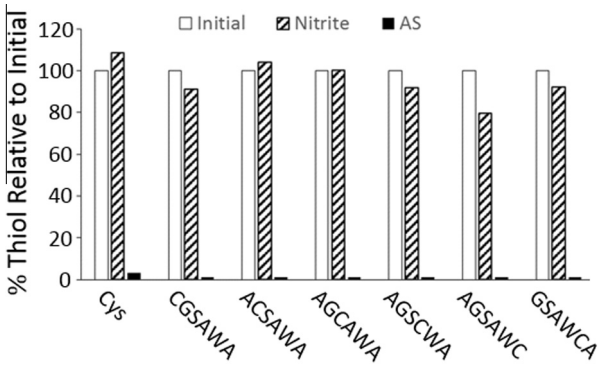

Figure 3. Standard DTNB analyses of L-cysteine (Cys) or synthetic peptides (0.3 mM) initially (□), or following treatment with nitrite ((▨), 0.3 mM) and AS ((■), 0.3 mM) in 10 mM phosphate buffer with 50 μM DTPA (pH 7.4) at 37 °C for 30 min. The amounts of free thiol were normalized with respect to that detected in the initial samples.

ratios of HNO-donor in 10 mM phosphate buffer with 50 μM metal chelator at pH 7.4 (Fig. 2). The amount of thiol in the unmodified peptide samples was found to be similar based on a standard 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) assay (data not shown).[16] Following a 30 min-incubation with AS at physiological pH and temperature, the presence of TrpNO was analyzed by UV–visible spectroscopy (ε_{335} = 6100 $M^{-1}cm^{-1}$).[17]

TrpNO is observed in all peptides upon treatment of 10-fold excess or equimolar amount of HNO-donor. As expected, the percent yield of TrpNO is significantly higher in the presence of excess HNO-donor (ca. 24% vs ca. 3%) (Fig. 2 and Supplementary data). Side-by-side control experiments conducted with the AS byproduct, nitrite, did not produce any TrpNO peaks in either case (data not shown). Comparisons with *N*-acetyl-L-tryptophan and AGSAWA indicate that although similar TrpNO yields are obtained for the samples in the presence of excess AS, the TrpNO yields are significantly lower in cysteine-containing peptides upon treatment with an equimolar amount of AS (Fig. 2). These results suggest that the reaction of HNO with tryptophan is approximately 1–2 orders of magnitude slower than the corresponding reaction with cysteine. It should also be noted that no significant TrpNO modification was observed under anaerobic conditions, consistent with previous reports (data not shown).[13] Although the formation of TrpNO requires both HNO and oxygen, the mechanism for this reaction is not well understood, and ongoing efforts are focused on addressing this issue.

Since HNO is known to be very thiophilic (Scheme 1), the extent of HNO-derived cysteine modifications in the above samples was determined by a DTNB assay. As seen in Figure 3, treatment of the cysteine and tryptophan-containing peptides with equimolar amounts of AS results in the complete depletion of thiols, indicating that all cysteine residues are modified to the corresponding sulfinamide or disulfide species. Consistent with these results, no free thiol is detected following incubation of the peptides with excess HNO-donor (data not shown). Importantly, comparable quantities of free thiol are observed in the untreated and nitrite-treated samples, suggesting that the depletion in thiol is due to HNO treatment (Fig. 3). Overall, these results reveal that cysteine is more reactive towards HNO than tryptophan.

Since cysteines were found to be completely modified upon HNO treatment, all the TrpNO-containing peptides are also expected to carry an HNO-derived cysteine modification. To gain more information about the products formed, we first analyzed the peptide samples with our ^{15}N-edited nuclear magnetic resonance (NMR) method for sulfinamide detection.[18] In this method, application of an isotope filter for ^{15}N allows the selective

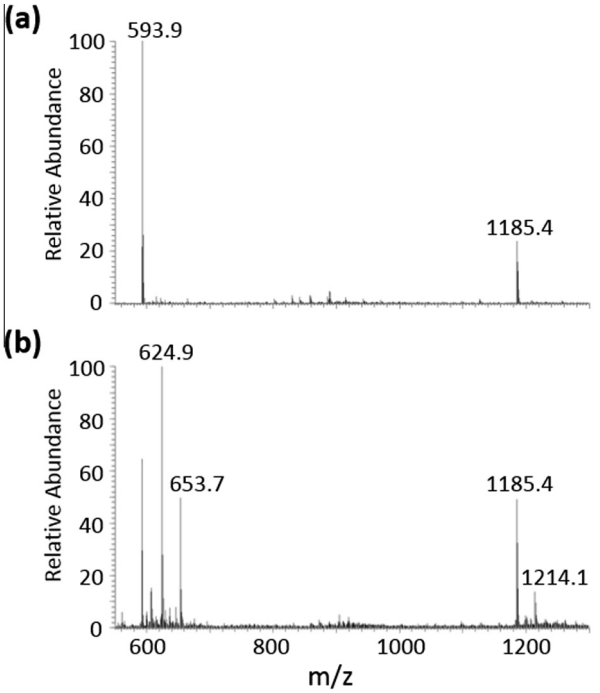

Figure 4. Selected region of ESI-MS spectra showing CGSAWA (0.1 mM) treated with (a) nitrite (1 mM) and (b) AS (1 mM) in 10 mM phosphate buffer with 50 μM DTPA (pH 7.4) at 37 °C for 30 min. The peaks were assigned to the unmodified peptide (*m/z* 593.9 ± 0.1), peptide sulfinamide (*m/z* 624.9 ± 0.1), peptide sulfinamide carrying a TrpNO modification (*m/z* 653.7 ± 0.1), peptide disulfide (*m/z* 1185.4 ± 0.1), and peptide disulfide carrying a TrpNO modification (*m/z* 1214.1 ± 0.1).

detection of protons attached to the ^{15}N nuclei and provides simplified NMR spectra.[18,19] Consistent with the expected reactivity of HNO, the corresponding sulfinamides are observed for all cysteine-containing peptides upon treatment with 10-fold excess H^{15}NO-donor (Supplementary data),[10,11,20] except for AGSAWC, which has a C-terminal cysteine and possesses an atypical reactivity, which we have recently examined in detail.[21] With the use of an internal standard, ^{15}N-labeled benzamide, the approximate relative yields of sulfinamides were estimated; they follow the order AGSCWA ≈ GSAWCA > ACSAWA > AGCAWA > CGSAWA.

To investigate the presence of doubly modified-peptides, we examined HNO-treated AGSCWA and CGSAWA by ESI-MS. These results indicate that the HNO-induced TrpNO modification is observed on both sulfinamide and disulfide-containing peptides (Fig. 4 and Supplementary data). Further studies are required to determine if TrpNO formation has a preference for either one of these thiol modifications. Treatment with nitrite did not produce any modifications under these conditions.

Since TrpNO is known to undergo denitrosation upon incubation with thiols,[22–24] we have also examined the aerobic denitrosation of TrpNO-containing peptides in the presence of excess L-cysteine (5 mM). The observed rates for TrpNO disappearance under pseudo-first-order conditions were determined by UV–visible spectroscopy. Similar results (k_{obs} ≈8 × 10^{-4} s^{-1}) were obtained for all TrpNO-containing peptides, indicating the presence of a nearby HNO-derived cysteine modification does not have a significant effect on denitrosation under these conditions (Table 1 and Supplementary data). Comparison with k_{obs} for *N*-acetyl-*N*-nitroso-tryptophan (ca. 1.2 × 10^{-3} s^{-1}), which is in agreement with the reported values for glutathione and other nucleophiles,[23,25] demonstrates that the observed rates of denitrosation are slightly slower for peptide samples examined (Table 1), potentially due to steric hindrance.[23] Under aerobic conditions, the kinetic behavior

Table 1
Observed rates for denitrosation of HNO-derived *N*-nitrosotryptophan species in the presence of 5 mM L-cysteine[a]

N-Nitrosotryptophan species	$k_{obs} \times 10^4$,[b] (s^{-1})	$k_{obs} \times 10^4$,[c] (s^{-1})
Ac-Trp	12 ± 0.1	5.3
AGSAWA	7.9 ± 0.1	4.8
CGSAWA	9.2 ± 0.1	6.7
ACSAWA	9.3 ± 0.2	N/A
AGCAWA	8.0 ± 0.4	N/A
AGSCWA	8.1 ± 0.5	5.0
AGSAWC	8.9 ± 0.6	4.8
GSAWCA	7.7 ± 0.3	4.5

[a] The HNO-derived *N*-nitrosotryptophan samples were treated with excess L-cysteine (5 mM) at physiological pH and temperature. The observed rates are reported as mean ± SEM ($n = 2$).
[b] Data were collected under aerobic conditions.
[c] Data were collected under argon-saturated conditions. The observed rates are reported as single values.

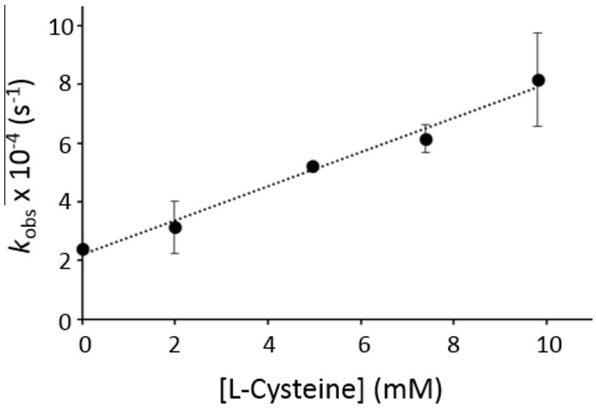

Figure 5. Observed rates of denitrosation of HNO-derived *N*-acetyl-*N*-nitrosotryptophan as a function of L-cysteine concentration in 10 mM phosphate buffer with 50 μM DTPA (pH 7.4) at 37 °C. The values represent the mean of two independent experiments.

of HNO-derived *N*-acetyl-*N*-nitrosotryptophan is the same as the synthetic standard (data not shown), with the observed rates reaching a limiting value in the presence of ca. 1 mM L-cysteine, consistent with the literature.[22,23,25,26]

Consistent with the suggested role of oxygen,[23] the observed rates for denitrosation of all TrpNO-containing peptides were slower (ca. 5×10^{-4} s^{-1}) upon treatment with 5 mM cysteine under anaerobic conditions (Table 1). By monitoring the observed rates of denitrosation of HNO-derived *N*-acetyl-*N*-nitrosotryptophan as a function of L-cysteine concentration (0–10 mM), the second-order rate constant for the anaerobic reaction of *N*-acetyl-*N*-nitrosotryptophan with L-cysteine (k_{cys}) can be estimated from the pseudo-first-order equation $k_{obs} = k_0 + k_{cys}$[cysteine], where k_0 is the rate of *N*-acetyl-*N*-nitrosotryptophan decay in the absence of L-cysteine. This analysis (Fig. 5) gives $k_{cys} = 5.8 \times 10^{-2}$ M^{-1} s^{-1} under argon-saturated conditions.

In summary, the observation of TrpNO formation with different HNO-donors confirms that this modification of tryptophan is due to HNO reactivity. We have examined HNO reactivity with tryptophan in peptides in the presence or absence of a nearby cysteine residue. Our results confirm the highly reactive nature of cysteine towards HNO compared with that of tryptophan. Consequently, in the presence of cysteine, excess HNO is required for significant amounts of TrpNO formation.

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Supplementary data

Supplementary data (the experimental methods for the preparation of synthetic peptides, incubation of tryptophan-containing species with HNO-donors, and NMR, ESI-MS and UV–visible spectroscopy analyses as well as UV–visible and NMR spectra of HNO-treated peptides) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.07.014.

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