Effects of Uric Acid on Nitrosation of *N*-Acetylcysteine by Diethylamine NONOate and *N*-Acetyl-*N*-nitrosotryptophan

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Uric acid of human plasma concentration accelerated nitrosation of *N*-acetylcysteine by diethylamine NONOate at neutral pH, but diminished that of *N*-acetyltryptophan. Uric acid also accelerated nitrosation of *N*-acetylcysteine by *N*-acetyl-*N*-nitrosotryptophan, having a nitroso group on the nitrogen atom of the indole ring. *N*-Acetyl-*S*-nitrosocysteine was stable even in the presence of uric acid and *N*-acetyltryptophan at neutral pH, while decomposition of *N*-acetyl-*N*-nitrosotryptophan was accelerated by uric acid and *N*-acetylcysteine. The results indicate that uric acid receives a nitroso group from diethylamine NONOate or *N*-acetyl-*N*-nitrosotryptophan, and passes it to the thiol group of *N*-acetylcysteine resulting in *N*-acetyl-*S*-nitrosocysteine. This implies that uric acid may act as an effective transporter of nitric oxide to thiols resulting in accumulation of nitrosothiols in humans.

Key words uric acid; nitric oxide; nitrosation; *N*-acetylcysteine; *N*-acetyltryptophan

Nitric oxide (NO) is synthesized in various types of cells by the enzyme nitric oxide synthase and is involved in numerous biological functions, including vasodilation, neurotransmission, and inflammation.[3,4)] Although NO is a radical, the reactivity of NO *per se* is relatively low. However, in the presence of O_2, NO is converted to a reactive nitrosating reagent, dinitrogen trioxide (N_2O_3).[5)] N_2O_3 can react with thiols (RSHs) such as free cysteine and cysteine residues of peptides or proteins resulting in nitrosothiols (RSNOs).[6,7)] RSNOs are relatively stable and can release NO when they encounter transition metals or other reducing agents.[8—10)] Therefore, it can be seen that RSHs act as a NO buffering system, controlling the intracellular and extracellular activities of NO. N_2O_3 can also react with amino and imino groups in various biological molecules resulting in corresponding *N*-nitroso compounds.[11)] The imino group on the indole ring of free tryptophan and tryptophan residues of peptides or proteins readily reacts with N_2O_3 resulting in their *N*-nitroso derivatives.[12,13)] The reactivity of tryptophan residues of proteins is thought to be high enough to compete effectively with cysteine residues of protein for N_2O_3. The *N*-nitroso derivatives of free tryptophan and tryptophan residues are relatively unstable and can pass a nitroso group to other compounds. Thus, it is possible that these *N*-nitrosoindoles play some role in the NO buffering system. Uric acid (UA) is the end metabolite of purine nucleobases in humans since the enzyme uricase (also known as urate oxidase), that catalyzes the conversion of UA to a further oxidation product allantoin, was lost in evolution.[14—17)] UA has been proposed as an important antioxidant in humans since it reacts with various reactive oxygen species such as hydroxyl radical and peroxynitrite resulting in their safe elimination.[18—20)] However, it was thought that NO did not react with UA even in the presence of O_2.[21,22)] Thus, little attention was paid to the reaction of UA with NO. Recently, we have reported the identification and characterization of a reaction product of UA with NO.[23)] When UA was treated with NO gas in a neutral solution under aerobic conditions, UA was consumed, yielding an unknown product. The product was identified as a nitrosated UA (NO-UA) from mass spectrometric data, although the position of the nitroso group on the molecule was not determined. NO-UA decomposed to several compounds including UA with a half-life of 2.2 min at pH 7.4 and 37 °C. The incubation of NO-UA with glutathione resulted in the formation of *S*-nitrosoglutathione. NO-UA was also formed in the reaction with a NO donor, diethylamine NONOate (DEA-NO). NO-UA was detected in human serum and urine by *in vitro* treatment with DEA-NO. In the reaction of glutathione with DEA-NO, the addition of UA caused a 2-fold increase in the yield of *S*-nitrosoglutathione. These results indicate that UA can react readily with N_2O_3 generating its nitroso derivative, which can give a nitroso group to glutathione. However, there is no information about the effects of UA on the nitrosation reactions of amino and imino groups forming *N*-nitroso compounds. In the present study, we investigated the effects of UA on the reactions of *N*-acetyltryptophan (AcTrp) with DEA-NO generating *N*-acetyl-*N*-nitrosotryptophan (NO-AcTrp) and compared to those on the reactions of *N*-acetylcysteine (AcCys) with DEA-NO resulting in *N*-acetyl-*S*-nitrosocysteine (NO-AcCys). It has been reported that NO-AcTrp reacts with AcCys resulting in NO-AcCys *via* transnitrosation.[13)] However, there is no information about the effects of UA on the transnitrosation reactions. We also studied the effects of UA on the reactions of NO-AcTrp with AcCys generating NO-AcCys. These results may provide some information for roles of UA in NO dynamics in humans.

Results

To reveal the effect of UA on the nitrosation reaction of AcCys and AcTrp by a NO donor, AcCys or AcTrp (300 μM each) was treated with DEA-NO (300 μM) in 100 mM potassium phosphate buffer (pH 7.4, 37 °C) in the absence or presence of UA (300 μM) and the concentrations were monitored by reversed phase (RP)-HPLC. First, to know the time course of the concentration changes of NO-UA, UA alone is incubated with DEA-NO (Fig. 1A). The NO-UA concentration increased up to 2.5 min and then decreased. Figure 1B shows the time course of the concentration changes of NO-AcCys when AcCys is incubated with DEA-NO. The concentration of NO-AcCys increased up to 10 min and then reached a

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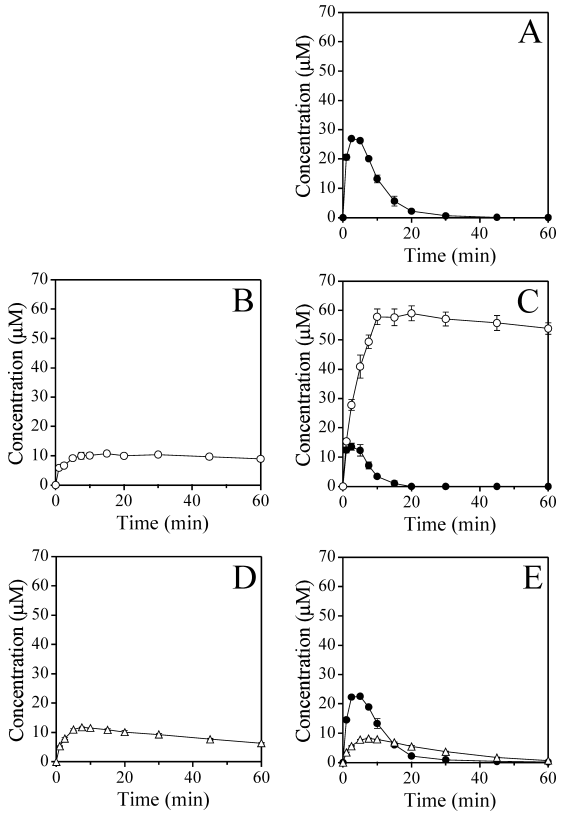

Fig. 1. Effects of UA on the Nitrosation Reactions of AcCys and AcTrp by DEA-NO

Time course of the concentrations of formed NO-UA (closed circle), NO-AcCys (open circle), and NO-AcTrp (open triangle) is shown. (A) UA (300 μM) is incubated with DEA-NO (300 μM). (B) AcCys (300 μM) is incubated with DEA-NO (300 μM). (C) UA (300 μM) and AcCys (300 μM) are incubated with DEA-NO (300 μM). (D) AcTrp (300 μM) is incubated with DEA-NO (300 μM). (E) UA (300 μM) and AcTrp (300 μM) are incubated with DEA-NO (300 μM). The samples were incubated in 100 mM potassium phosphate buffer (pH 7.4) at 37 °C. The concentrations were determined by RP-HPLC analysis. Means±S.D. (n=3) are presented.

plateau. When AcCys was incubated with DEA-NO in the presence of UA, the concentration of NO-AcCys was increased (Fig. 1C). At the reaction time of 60 min, the concentration of NO-AcCys was 6-fold greater than in the reaction without UA (Fig. 1B). The maximum concentration of NO-UA was 2-fold smaller than in the reaction of UA alone (Fig. 1A). Figure 1D shows the time course of the concentration changes of NO-AcTrp when AcTrp is incubated with DEA-NO. The NO-AcTrp concentration increased up to 7.5 min and then decreased slowly. The concentration of NO-AcTrp was comparable to that of NO-AcCys. When AcTrp was incubated with DEA-NO in the presence of UA, the concentrations of NO-AcTrp decreased slightly at the initial stage up to 10 min, compared to the reaction of AcTrp alone (Fig. 1E). After that, NO-AcTrp decreased greatly. At 60 min incubation, the concentration of NO-AcTrp was 9-fold smaller than that in the reaction of AcTrp alone (Fig. 1D). The concentrations of NO-UA also decreased slightly at the initial stage compared to the reaction of UA alone (Fig. 1A). To study the effect of UA on the nitrosation reaction from NO-AcTrp to AcCys, the synthesized NO-AcTrp (300 μM) was incubated with AcCys (300 μM each) in the absence or presence of UA (300 μM). First, to clarify whether UA can react with NO-AcTrp, UA is incubated with NO-AcTrp. Figure 2A shows the time course of the concentration changes of NO-

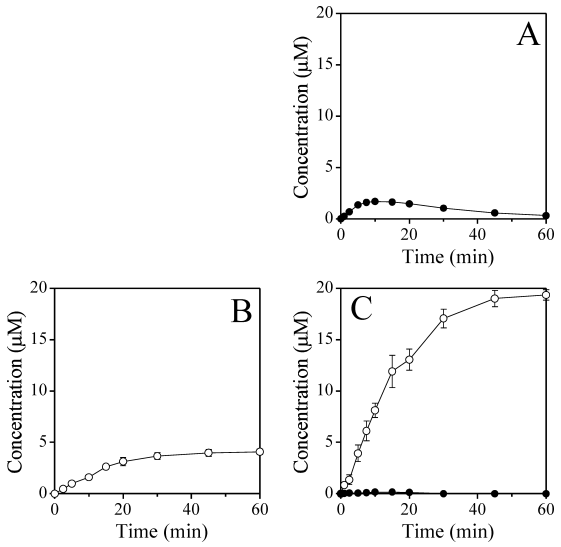

Fig. 2. Effects of UA on the Nitrosation Reaction of AcCys by NO-AcTrp

Time course of the concentrations of formed NO-UA (closed circle) and NO-AcCys (open circle) is shown. (A) UA (300 μM) is incubated with NO-AcTrp (300 μM). (B) AcCys (300 μM) is incubated with NO-AcTrp (300 μM). (C) UA (300 μM) and AcCys (300 μM) are incubated with NO-AcTrp (300 μM). The samples were incubated in 100 mM potassium phosphate buffer (pH 7.4) at 37 °C. The concentrations were determined by RP-HPLC analysis. Means±S.D. (n=3) are presented.

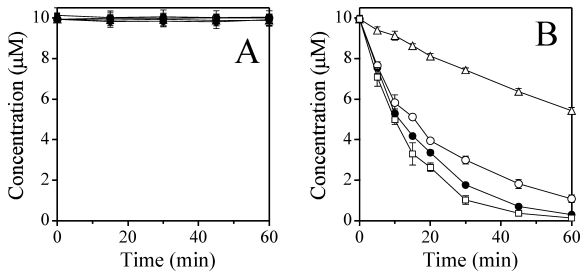

Fig. 3. Stabilities of NO-AcCys and NO-AcTrp

(A) Time course of the concentration of NO-AcCys. NO-AcCys (10 μM) was incubated with none (open circle), 300 μM AcTrp (open triangle), 300 μM UA (closed circle), and both 300 μM AcTrp and 300 μM UA (closed square). (B) Time course of the concentration of NO-AcTrp. NO-AcTrp (10 μM) was incubated with none (open triangle), 300 μM AcCys (open circle), 300 μM UA (closed circle), and both 300 μM AcCys and 300 μM UA (open square). The samples were incubated in 100 mM potassium phosphate buffer (pH 7.4) at 37 °C. The concentrations were determined by RP-HPLC analysis. Means±S.D. (n=3) are presented.

UA when UA (300 μM) was incubated with NO-AcTrp. The NO-UA concentration increased up to 10 min and then decreased. Figure 2B shows the time course of the concentration changes of NO-AcCys when AcCys was incubated with NO-AcTrp. The concentration of NO-AcCys increased gradually up to 60 min. When AcCys was incubated with NO-AcTrp in the presence of UA, the concentration of NO-AcCys was increased (Fig. 2C). At the reaction time of 60 min, the concentration of NO-AcCys was 5-fold greater than in the reaction without UA (Fig. 2B). The concentration of NO-UA was much smaller than in the reaction of UA alone. To evaluate the stability of NO-AcCys and NO-AcTrp, each (10 μM) was incubated in potassium phosphate buffer (pH 7.4) at 37 °C and the concentrations were monitored by RP-HPLC. Figure 3A shows time course of the concentration change of NO-AcCys. NO-AcCys was stable and no concentration change was observed even with 300 μM UA and/or 300 μM AcTrp up to the incubation time of 60 min. Figure 3B

shows the time course of the concentration change of NO-AcTrp. When NO-AcTrp alone was incubated, the concentration of NO-AcTrp decreased with the first-order rate constant (k) $1.35\times10^{-4}\,s^{-1}$ ($t_{1/2}$=85.7 min). In the presence of 300 μM AcCys, the decomposition was accelerated with $k=8.79\times10^{-4}\,s^{-1}$ ($t_{1/2}$=13.1 min). In the presence of 300 μM UA, NO-AcTrp disappeared with $k=9.63\times10^{-4}\,s^{-1}$ ($t_{1/2}$=12.0 min), which is faster than in the presence of AcCys. When NO-AcTrp was incubated both with AcCys and UA, the decomposition was further accelerated with $k=1.16\times10^{-3}\,s^{-1}$ ($t_{1/2}$=10.0 min).

Discussion

For the reaction of DEA-NO with UA (Fig. 1A), AcCys (Fig. 1B), or AcTrp (Fig. 1D), the maximum concentration of NO-UA was the highest, and those of NO-AcCys and NO-AcTrp were comparable. At pH 7.4 and 37 °C, the half-life of NO-UA has been reported to be 2.2 min.[21] NO-AcCys was stable and showed no decrease for 1 h (Fig. 3A). The half-life of NO-AcTrp was 85.7 min (Fig. 3B). That is, unstable NO-UA showed the highest maximum yield, and relatively stable NO-Cys and NO-Trp showed comparative maximum yields. This suggests that the reactivity of UA to N_2O_3 is the highest among them and those of AcCys and AcTrp were comparable. Addition of the physiological relevant dose of UA (300 μM) in the AcCys/DEA-NO system increased the concentration of NO-AcCys, while the concentration of NO-UA was smaller than in the reaction of UA alone (Fig. 1C). This implies that UA receives NO from DEA-NO and passes it to AcCys effectively. At pH 7.4 and 37 °C, the half-life of NO-UA (2.2 min) is comparable to that of DEA-NO (2 min).[24] If NO released from NO-UA reacts with AcCys resulting in NO-AcCys, the concentration of NO-AcCys should be similar to that without UA. This suggests that NO is passed from NO-UA to AcCys *via* transnitrosation. In contrast, when AcTrp was incubated with DEA-NO in the presence of UA, the concentration of NO-AcTrp decreased, and long incubation caused a large decrease of NO-AcTrp concentration (Fig. 1E). Decomposition of NO-AcTrp was accelerated by addition of UA (Fig. 3B). The results indicate that UA slightly inhibits the nitrosation of AcTrp and greatly accelerates the decomposition of NO-AcTrp. For the reaction of NO-AcTrp with UA (Fig. 2A) or AcCys (Fig. 2B), considerable amounts of NO-UA or NO-AcCys were formed, respectively. At the initial stage of the reactions (up to 5 min), the concentration of NO-UA was greater than that of NO-AcCys, meaning that the reactivity of UA to NO-AcTrp is greater than that of AcCys. When NO-AcTrp was incubated both with UA and AcCys, the formation of NO-AcCys greatly enhanced but the concentration of NO-UA was low (Fig. 2C). This implies that UA receives NO from NO-AcTrp and passes it to AcCys effectively.

Thiols (RSHs) are thought to be important vehicles of NO *in vivo*. Common RSHs in blood are free cysteine (6 μM in plasma),[25] glutathione (GSH) (3 μM in plasma and 1.2 mM in whole blood),[26] and albumin (550 μM in plasma),[27] which contains a single free cysteine residue.[28] Nitrosothiols (RSNOs) are formed readily from RSHs by the reaction with NO and NO donors at pH 7.4 under aerobic conditions.[6,7] On the other hand, it is reported that AcTrp and a peptide containing a Trp also readily react with NO and NO donors under aerobic condition, resulting in *N*-nitrosation of the Trp residues.[12,29] The concentration of free Trp is 54 μM in plasma,[30] and albumin contains a single Trp residue.[28] However, it is difficult to detect nitrosated tryptophan residues in proteins formed *in vivo* by mass spectrometry. Recently, it has been reported that NO-AcTrp readily reacted with AcCys resulting in NO-AcCys *via* transnitrosation.[13] This transnitrosation might decrease the concentration of the nitrosated tryptophan residues. In addition, the present study showed that UA accelerates decomposition of NO-AcTrp and nitrosation of AcCys by NO-AcTrp. Thus, UA may further decrease the concentration of the nitrosated tryptophan residues in proteins and increase RSNOs in humans. Humans have a relatively higher concentration of UA in biological fluids and cells than other mammals. The human UA concentration ranges widely by individual. Although common human plasma concentrations of UA are 120—450 μM,[18,31] it varies from several dozen μM to over 1 mM.[32] Since the fate of NO formed in humans might be affected by UA, we should take into account UA concentrations for human studies of NO. For animals, it varies largely by species. The plasma UA concentrations are reported to be 113 μM for orangutans, 54 μM for mice (serum), 29 μM for cats, 11 μM for dogs, 10 μM for rabbits, 9 μM for horses, and 7 μM for pigs.[15,33] To get useful information for humans from data of animal studies of NO, we should take into account these differences of UA concentrations between humans and animals.

The present study showed that UA accelerated nitrosation of an RSH not only by a NO donor but also a nitrosoindole under aerobic conditions. It suggests that UA can collect NO from NO donors and nitrosoindoles, and pass it to RSHs efficiently, resulting in accumulation of RSNOs. UA may play a role as a NO collection vehicle in the NO buffering system including RSNOs in humans. We should pay attention to the concentrations of UA when we study NO behaviors in living bodies.

Experimental

Materials *N*-Acetylcysteine (AcCys), *N*-acetyltryptophan (AcTrp), and uric acid (UA) were obtained from Sigma (MO, U.S.A.). NO (99.7%) was purchased from Sumitomo Seika (Tokyo). Diethylamine NONOate (DEA-NO) was purchased from Cayman Chemical (MI, U.S.A.). All other chemicals of reagent grade were purchased from Sigma, Aldrich (WI, U.S.A.), Cica (Tokyo), and Nacalai Tesque (Osaka, Japan), and used without further purification. Water was purified with a Millipore Milli-Q deionizer.

HPLC and MS Conditions The high performance liquid chromatography (HPLC) system consisted of Shimadzu LC-10ADvp pumps and an SCL-10Avp system controller. On-line UV spectra were obtained with a Shimadzu SPD-M10Avp UV–vis photodiode-array detector. Absorbance spectra were collected from 200 to 500 nm. For the reversed phase HPLC (RP-HPLC), an Inertsil ODS-3 octadecylsilane column of 4.6×100 mm and particle size 5 μm (GL Science, Tokyo) was used. The eluent was 20 mM triethylamine acetate buffer (pH 7.0) with 10% methanol for detection of NO-UA and NO-AcCys or with 50% methanol for NO-AcTrp. The column temperature was 20 °C and the flow rate was 1.0 ml/min. The electrospray ionization time of flight mass spectrometry (ESI-TOF/MS) measurements were performed on a MicroTOF spectrometer (Bruker, Bremen, Germany) in the negative and positive mode. The sample isolated by RP-HPLC was directly infused into the MS system by a syringe pump without a column at a flow rate of 5 μl/min.

Synthesis of NO-UA, NO-AcCys, and NO-AcTrp NO-UA (nitrosated uric acid) was obtained from UA by bubbling NO gas through UA solution using the reported procedure,[23] slightly modified by us. A stirring UA solution (300 μM, 7 ml) of 100 mM potassium phosphate buffer (pH 7.4) was bubbled by the NO gas (6.5 ml/min) for 1 min at ambient temperature. The gas flow was controlled by a Model 3660 mass flow controller (KOFLOC,

Kyoto, Japan). A small aliquot of the reaction solution was analyzed by RP-HPLC. On the RP-HPLC chromatogram, a peak showing a UV spectrum with λ_{max}=294 and 378 nm at a retention time of 2.7 min was eluted. Negative-ion ESI-TOF/MS of the peak showed signals including *m/z* 196 and 166, which are attributable to a molecular ion $[M-H]^-$ and a denitrosated ion $[M-NO-H]^-$. On the basis of these data according with the reported values,[23] the product was identified as NO-UA. The concentration of NO-UA was determined assuming that NO-UA was converted into UA exclusively when NO-UA was incubated with 10 mM GSH in 100 mM potassium phosphate buffer at pH 7.4 and 37 °C for 2 h. NO-AcCys was synthesized from AcCys by bubbling a mixture of NO/O_2 through AcCys solutions using the reported procedure,[34] modified by us. A stirring AcCys solution (1 mM, 7 ml) was bubbled by the gas mixture ($NO:O_2$=6.5 : 6.5 ml/min) at ambient temperature. Small aliquots of the reaction solution were analyzed by RP-HPLC. On the RP-HPLC chromatogram a peak showing a UV spectrum with λ_{max}=335 nm at a retention time of 5.8 min was eluted. Positive-ion ESI-TOF/MS of the peak showed signals at *m/z* 193 and 163, which are attributable to a molecular ion $[M+H]^+$ and a denitrosated ion $[M-NO+H]^+$. On the basis of these data, the product was identified as *S*-nitrosated AcCys (NO-AcCys). The peak area of NO-AcCys in RP-HPLC chromatogram increased with increasing bubbling time up to 40 s and then reached a plateau. After 60 s bubbling of the NO/O_2 gas mixture, the solution was bubbled by N_2 gas for 10 min at room temperature to purge dissolved gases. Immediately after that, the NO-AcCys solution was used. The concentration of NO-AcCys solution obtained was considered to be equal to the starting concentration of AcCys (1 mM). NO-AcTrp was prepared from AcTrp with nitrous acid as previously reported.[13] Briefly, AcTrp (53 mg) and sodium nitrite (16 mg) were stirred in water (2 ml) for 2 h in the dark at room temperature. The mixture was cooled to 0 °C, and cold HCl (1 ml, 1 M, 0 °C) was added. A yellow precipitate was immediately extracted with ethyl acetate (6 ml, 0 °C). The cold organic layer was separated and dried by N_2 gas at room temperature. On the RP-HPLC chromatogram a peak showing a UV spectrum with λ_{max}=260 and 350 nm at a retention time of 5.0 min was eluted. Positive-ion ESI-TOF/MS of the peak showed signals at *m/z* 276 and 246, which are attributable to a molecular ion $[M+H]^+$ and a denitrosated ion $[M-NO+H]^+$. The product was identified as *N*-nitrosated AcTrp (NO-AcTrp). The concentration of NO-AcTrp was determined spectrophotometrically with a Multi-Spec-1500 UV–vis photodiode-array spectrometer (Shimadzu) using ε_{335} (ethanol)=6100 $M^{-1}\,cm^{-1}$.[13]

Reaction Conditions For time course experiments of formation of NO-UA (Fig. 1A), 300 μM UA was incubated with DEA-NO in 100 mM potassium phosphate buffer (1 ml, pH 7.4) at 37 °C in a capped microtube. The final pH was 7.5. For time course experiments of formation of NO-UA, NO-AcCys, NO-AcTrp (Figs. 1B—E), 300 μM AcCys or AcTrp was incubated with DEA-NO in the absence or presence of 300 μM UA in 100 mM potassium phosphate buffer (1 ml, pH 7.4) at 37 °C in a capped microtube. The final pH was 7.5. For experiments of nitrosation by NO-AcTrp (Figs. 2A—C), 300 μM UA or AcCys was incubated with 300 μM NO-AcTrp in 100 mM potassium phosphate buffer (1 ml, pH 7.4) at 37 °C in a capped microtube. The final pH was 7.3. For stability experiments of NO-AcCys and NO-AcTrp (Figs. 3A, B), 10 μM NO-AcCys or NO-AcTrp was incubated with or without 300 μM UA, AcCys, or AcTrp in 100 mM potassium phosphate buffer (1 ml, pH 7.4) at 37 °C in a capped microtube. The final pH was 7.3—7.4.

Quantitative Procedures The concentrations of all the compounds in the reaction mixture were evaluated from integrated peak areas on HPLC chromatograms compared with those of authentic standard solutions. The detection wavelengths were 300 nm for UA, 330 nm for NO-AcCys and NO-AcTrp, and 380 nm for NO-UA. All the reaction mixtures were analyzed by PR-HPLC immediately after the reactions. The first-order rate constants were calculated by using the nonlinear least-squares fitting algorithm in Igor Pro (Wavemetrics). All the experiments reported here were carried out independently three times. The results are expressed as means±S.D.

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