Formation of *N*-Nitrosamines and *N*-Nitramines by the Reaction of Secondary Amines with Peroxynitrite and Other Reactive Nitrogen Species: Comparison with Nitrotyrosine Formation

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Reactive nitrogen species, including nitrogen oxides (N_2O_3 and N_2O_4), peroxynitrite ($ONOO^-$), and nitryl chloride (NO_2Cl), have been implicated as causes of inflammation and cancer. We studied reactions of secondary amines with peroxynitrite and found that both *N*-nitrosamines and *N*-nitramines were formed. Morpholine was more easily nitrosated by peroxynitrite at alkaline pH than at neutral pH, whereas its nitration by peroxynitrite was optimal at pH 8.5. The yield of nitrosomorpholine in this reaction was 3 times higher than that of nitromorpholine at alkaline pH, whereas 2 times more nitromorpholine than nitrosomorpholine was formed at pH <7.5. For the morpholine−peroxynitrite reaction, nitration was enhanced by low concentrations of bicarbonate, but was inhibited by excess bicarbonate. Nitrosation was inhibited by excess bicarbonate. On this basis, we propose a free radical mechanism, involving one-electron oxidation by peroxynitrite of secondary amines to form amino radicals ($R_2N^•$), which react with nitric oxide ($^•NO$) or nitrogen dioxide ($^•NO_2$) to yield nitroso and nitro secondary amines, respectively. Reaction of morpholine with $NO^•$ and superoxide anion ($O_2^{•-}$), which were concomitantly produced from spermine NONOate and by the xanthine oxidase systems, respectively, also yielded nitromorpholine, but its yield was <1% of that of nitrosomorpholine. $NO^•$ alone increased the extent of nitrosomorpholine formation in a dose-dependent manner, and concomitant production of $O_2^{•-}$ inhibited its formation. Reactions of morpholine with nitrite plus HOCl or nitrite plus H_2O_2, with or without addition of myeloperoxidase or horseradish peroxidase, also yielded nitration and nitrosation products, in yields that depended on the reactants. Tyrosine was nitrated easily by synthetic peroxynitrite, by $NaNO_2$ plus H_2O_2 with myeloperoxidase, and by $NaNO_2$ plus H_2O_2 under acidic conditions. Nitrated secondary amines, e.g., *N*-nitroproline, could be identified as specific markers for endogenous nitration mediated by reactive nitrogen species.

Introduction

Nitric oxide ($NO^•$), produced by nitric oxide synthase, is an important signal molecule that regulates various physiological functions in the cardiovascular, nervous, and immune systems (*1*, *2*). Excess production of $NO^•$, however, has been implicated as a cause of diverse pathophysiological conditions such as inflammation, neurodegenerative and cardiovasular diseases, and cancer. These detrimental effects of $NO^•$ have been attributed to reactive nitrogen species (RNS)[1] such as NOx and peroxynitrite ($ONOO^-$), which are formed by the reaction of $NO^•$ with oxygen and superoxide ($O_2^{•-}$), respectively. Recent studies have demonstrated that RNS are also formed by the reaction of nitrite and hypochlorous acid (HOCl), the latter being generated from hydrogen peroxide (H_2O_2) and chloride ion (Cl^-) by myeloperoxidase (MPO) (*3*). Furthermore, oxidases such as MPO and horseradish peroxidase (HPO) can form RNS in the presence of H_2O_2 and nitrite (*4*, *5*).

RNS can react with biomolecules such as proteins, DNA, and lipids through nitration and nitrosation reactions, thus altering their functions. The presence of 3-nitrotyrosine (NTYR) in human tissues and body fluids has been used as a marker of peroxynitrite formation in vivo, because peroxynitrite is a strong nitrating agent

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[1] Abbreviations: carboxy-PTIO, 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1*H*-imidazol-1-yloxy 3-oxide, potassium salt; DTPA, diethylenetriaminepentaacetic acid; HPO, horseradish peroxidase; MPO, myeloperoxidase; NO-MOR, *N*-nitrosomorpholine; NO_2-MOR, *N*-nitromorpholine; NTYR, 3-nitrotyrosine; RNS, reactive nitrogen species; TEA, thermal energy analyzer.

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that very easily nitrates some aromatic compounds, including tyrosine (*6*, *7*). However, other RNS, such as those formed from nitrous acid, $NaNO_2$ plus HOCl, and $NaNO_2$ plus H_2O_2 in the presence of MPO and HPO, can react with tyrosine to form NTYR (*3*−*5*, *8*, *9*). The relative importance of different RNS in NTYR formation in vivo has not been elucidated.

Carcinogenic nitrosamines have been reported to be formed by NO•, which is produced by inducible NO• synthase expressed in cultured cells, including macrophages, hepatocytes, and neutrophils, upon activation with lipopolysaccharide and γ-interferon (*10*−*15*). Increased endogenous nitrosation has been also demonstrated in humans with chronic infection and inflammatory conditions that have been associated with an increased risk for cancer at various sites (*16*). NO• and reactive oxygen species produced in infected and/or inflamed tissues could contribute to the process of carcinogenesis by several mechanisms, including the formation of carcinogenic nitrosamines from excess NO• (*16*−*18*). NO• is oxidized by oxygen to NO_2• which reacts further with NO• and NO_2• to form the nitrosating agents N_2O_3 and N_2O_4, respectively. However, it has not been determined whether peroxynitrite and other RNS react with amines to yield carcinogenic *N*-nitroso compounds.

In the study presented here, we found that both nitrosamines and nitramines are formed by the reaction of secondary amines with peroxynitrite and other RNS. On this basis, we propose that nitrated secondary amines, e.g., *N*-nitroproline, could be identified as a new and specific marker for endogenous nitration mediated by peroxynitrite and other RNS.

Materials and Methods

Caution: *Many nitrosamines and nitramines are carcinogenic in experimental animals and should be handled with caution.*

All reactions were carried out at 37 °C and at least in duplicate.

Chemicals. We purchased spermine NONOate and 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1*H*-imidazol-1-yloxy 3-oxide, potassium salt (carboxy-PTIO), from Cayman Chemical Co. (Ann Arbor, MI). MPO (human leukocytes) were from Alexis Co. (San Diego, CA), and xanthine oxidase was from Boehringer Mannheim (Mannheim, Germany); desferrioxamine was from Ciba-Geigy Laboratoire (Rueil-Malmaison, France), and morpholine, aminopyrine, dimethylamine, diethylamine, methylbenzylamine, proline, *N*-methylurea, and other chemicals were from Sigma (St. Louis, MO), Aldrich (Milwaukee, WI), or Merck (Darmstadt, Germany). Nitrosamines were purchased from Sigma or synthesized in our laboratory by the reaction of secondary amines with sodium nitrite ($NaNO_2$) under acidic conditions. Nitramines were synthesized from corresponding nitrosamines using 50% H_2O_2 (a gift from Elf Atochem, Pierre-Bénite, France) and trifluoroacetic acid (*19*). The structures and purity of the synthesized nitrosamines and nitramines were verified by gas chromatography/mass spectrometry (GC/MS) (see below). Peroxynitrite was synthesized in a quenched-flow reactor, and excess H_2O_2 was destroyed by granular manganese dioxide (*20*).

Reaction of Secondary Amines with Peroxynitrite. Peroxynitrite solution was prepared in cold 0.5 N NaOH. The effect of pH was studied by incubating 1 mL of 50 mM sodium phosphate buffer (pH 3−12) containing 0.05 mM diethylenetriaminepentaacetic acid (DTPA, a metal chelator), 1 mM morpholine, and 1 mM peroxynitrite for 15 min. We examined the effect of incubation time on nitrosation and nitration of morpholine by peroxynitrite. At both pH 7.4 and 9.5, the reactions were complete within 2 min after addition of peroxynitrite and no further increase or decrease in yields of the products was observed. On this basis, the 15 min incubation time was used for all reactions with peroxynitrite. The pH values were adjusted with HCl or NaOH. The reaction was initiated by adding peroxynitrite to the mixture, and the final pH was read using an aliquot of the mixture. The effect of morpholine and peroxynitrite concentrations was studied similarly by reacting 1 mL of 50 mM sodium phosphate buffer (pH 7.5 or 9.5) containing 0.05 mM DTPA, morpholine (0−10 mM), and peroxynitrite (0−10 mM). The effect of CO_2 was studied using 1 mM morpholine, 1 mM peroxynitrite, and 0−30 mM sodium bicarbonate in 50 mM sodium phosphate buffer containing 0.05 mM DTPA (pH 7.5 or 9.5). Similarly, •HO scavengers, antioxidants, an NO• scavenging compound (carboxy-PTIO), and methylurea (final concentrations of 1 and 10 mM) were examined for their effects on nitrosation and nitration of morpholine (1 mM) by peroxynitrite (1 mM) at pH 7.5. Various amines and tyrosine (1 mM) were also reacted with 1 mM peroxynitrite in 50 mM sodium phosphate buffer and 0.05 mM DTPA (pH 7.5 or 9.5). Reverse order-of-addition control experiments were performed with peroxynitrite added to buffer first, followed 5 min later by amines.

Reaction of Morpholine with NO• and O_2•$^-$. Reactions were carried out at 37 °C for 3 h in 50 mM sodium phosphate buffer, containing 0.05 mM DTPA, 1 mM morpholine, spermine NONOate (prepared in 0.01 N NaOH), an O_2•$^-$ generation system (xanthine oxidase with either 200 μM acetaldehyde or 200 μM hypoxanthine as a substrate), and an appropriate amount of HCl to neutralize the NaOH in the spermine NONOate solution (final volume of 1 mL and final pH of 7.5) (*21*−*24*). The final concentrations of spermine NONOate and xanthine oxidase were 0−800 μM and 0−800 milliunits/mL, respectively. Under these conditions, NO• and O_2•$^-$ were generated at rates of 0−16 nmol/min, which were measured spectrophotometrically as described previously (*24*, *25*).

Reaction of Morpholine with $NaNO_2$ plus HOCl or $NaNO_2$ plus H_2O_2. Reactions were carried out at 37 °C for 3 h by incubating 1 mM morpholine in 50 mM sodium phosphate buffer (final pH of 7.5) containing 0.05 mM DTPA, $NaNO_2$ (0−5 mM), and HOCl (0−5 mM). Other reactions were carried out at 37 °C for 3 h by incubating 1 mM morpholine in sodium phosphate buffer (pH 3.5 or 4.5) containing 0.05 mM DTPA, 1 mM $NaNO_2$, and 1 mM H_2O_2. In some experiments, HPO (1 μM) or MPO (80 nM) was incubated in the reaction mixture (pH 7.5) containing 1 mM morpholine, 1 mM $NaNO_2$, and 1 mM HOCl or H_2O_2.

Analysis of Nitrosamines and Nitramines by Gas Chromatography Coupled with a Thermal Energy Analyzer (GC-TEA). Reactions were terminated by adding $^1/_{10}$ volume of 20% ammonium sulfamate in 3.6 N H_2SO_4. Internal standard (500 ng of *N*-nitrosodibutylamine in ethanol) was added to the reaction mixture, which was then extracted three times with an equal volume of CH_2Cl_2. The combined CH_2Cl_2 extracts were dried over anhydrous Na_2SO_4. In the case of reactions with proline, 500 ng of *N*-nitrosopipecolic acid in ethyl acetate was added as an internal standard to the reaction mixture, which was then extracted with ethyl acetate. Nitroso- and nitroproline were converted to their methyl derivatives with diazomethane before GC-TEA analysis (*26*).

Nitrosamines and nitramines were identified by GC-TEA (model 546, Thermoelectron) (*15*). A glass column (3 mm in diameter × 2 m in length), packed with 5% FFAP on chromosorb W-HP (80−100 mesh), was used with a carrier gas, argon, at a flow rate of 30 mL/min. The oven temperature was 120−190 °C, depending on the nitrosamine that was analyzed, and injection port temperature was set 50 °C higher than that of the oven temperature. The TEA pyrolyzer temperature was 700 °C. Under these conditions, not only nitrosamines but also nitramines could be analyzed by the TEA (*8*).

GC/MS Analysis. Nitrosated and nitrated products were analyzed on an HP-1 column (cross-linked methyl siloxane, 25 m in length × 0.18 mm in diameter, 0.18 μm film thickness)

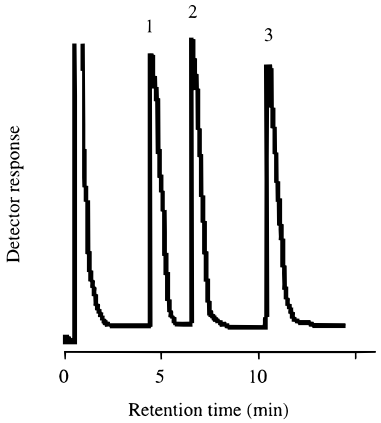

Figure 1. Typical GC-TEA chromatogram from the analysis of reaction products of morpholine with peroxynitrite. Peak 1 is *N*-nitrosodibutylamine (added as an internal standard), and peaks 2 and 3 are NO-MOR and NO_2-MOR, respectively, which are formed by the reaction of morpholine with peroxynitrite.

using a Hewlett-Packard 5890 gas chromatograph with a mass-selective detector (model 5971A). The GC conditions were as follows: injection port temperature, 250 °C; detector temperature, 250 °C; interface temperature, 280 °C; GC oven temperature, 50 °C for 1 min, up to 230 °C at a rate of 20 °C/min; and splitless injection.

Analysis of NTYR by HPLC. Reactions were carried out using L-tyrosine and various RNS. NTYR was analyzed by a Spectraphysics HPLC system (model SP 8810) equipped with a reverse-phase column (4.6 mm × 250 mm Ultrasphere ODS column, 5 μm, Beckman) under isocratic conditions with a mobile phase of 15% methanol in 20 mM ammonium formate buffer (pH 6.0) at a flow rate of 1 mL/min. NTYR was detected by a Waters LC spectrophotometer (model 481) at 390 nm.

Results

Formation of Nitrosamines and Nitramines by the Reaction of Secondary Amines with Peroxynitrite. Figure 1 shows a typical GC-TEA chromatogram obtained from the analysis of reaction products of morpholine with peroxynitrite. In addition to an internal standard peak (*N*-nitrosodibutylamine, peak 1), two peaks (peaks 2 and 3) were observed. Peak 2 coeluted with authentic *N*-nitrosomorpholine (NO-MOR). Increase in the pyrolyzer temperature of the TEA from 500 to 700 °C increased the response for peak 3, suggesting that it was an *N*- or *C*-nitro compound (*8*). The products were analyzed by GC/MS. Peak 2 exhibited ions at *m*/*z* 116 (relative intensity, 12%), 86 (14%), and 56 (54%), whereas peak 3 exhibited ions at *m*/*z* 132 (3%), 86 (16%), and 56 (74%). The mass spectra of peaks 2 and 3 were in good agreement with those of authentic NO-MOR and *N*-nitromorpholine (NO_2-MOR), respectively. As peroxynitrite is a strong oxidant, it is possible that nitrosamines were oxidized to nitramines by peroxynitrite. We incubated NO-MOR with peroxynitrite, but no NO_2-MOR was detected. On this basis, we concluded that NO_2-MOR was formed by the reaction of morpholine with peroxynitrite.

Conditions for the reaction between secondary amines and peroxynitrite were varied. Figure 2A shows the effect of pH on this reaction. The NO-MOR yield was 3 times higher than that of NO_2-MOR under alkaline conditions (pH 8.5−10.5), whereas below pH 7.5, twice as much NO_2-MOR as NO-MOR was formed (Figure 2A). Aminopyrine reacted with peroxynitrite rapidly to form nitrosodimethylamine but not nitrodimethylamine (Figure 2B). Between pH 5 and 9.5, peroxynitrite reacted with aminopyrine to form nitrosodimethylamine, whereas decomposed peroxynitrite did not yield this compound. Below pH 4.5, peroxynitrite and decomposed peroxynitrite yielded similar amounts of nitrosodimethylamine, possibly due to acid-catalyzed nitrosation of aminopyrine by nitrite present in peroxynitrite solutions.

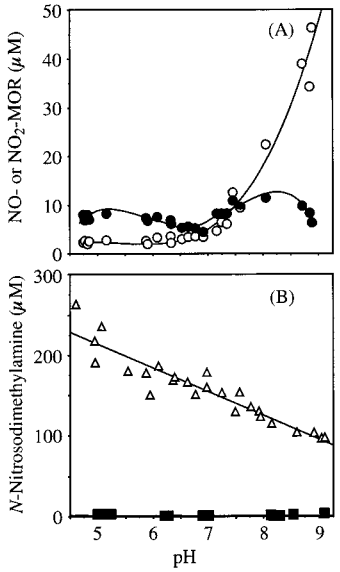

Figure 2. (A) Effect of pH on the yields of NO-MOR (○) and NO_2-MOR (●) in the peroxynitrite−morpholine reaction. (B) Yield of *N*-nitrosodimethylamine for the reaction of aminopyrine with peroxynitrite (△) and decomposed peroxynitrite (■).

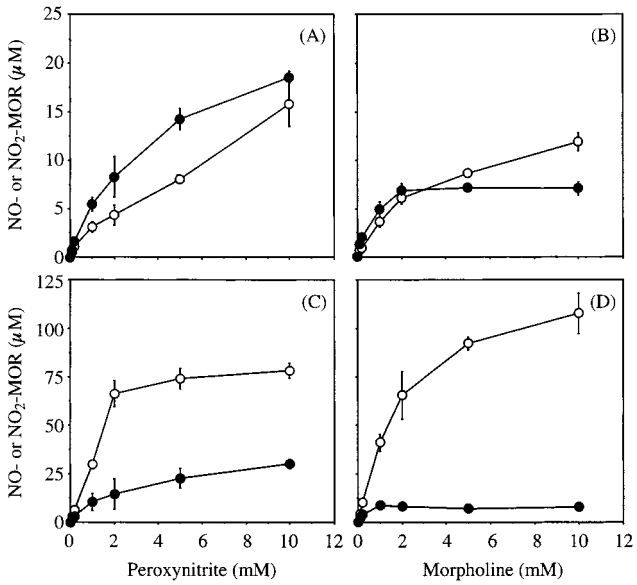

Figure 3. Effect of varying concentrations of (A) peroxynitrite and (B) morpholine at pH 7.3 and (C) peroxynitrite and (D) morpholine at pH 9.2 on the yields of NO-MOR (○) and NO_2-MOR (●).

Increased concentrations of peroxynitrite dose-dependently increased the yields of both NO-MOR and NO_2-MOR at neutral pH (Figure 3A); their yields reached a plateau at 2 mM peroxynitrite, and only slight increases in NO-MOR and NO_2-MOR yields were observed with >2 mM peroxynitrite at alkaline pH (Figure 3C). When increased concentrations of morpholine were reacted with 0.5 mM peroxynitrite, the NO-MOR yield increased dose-dependently at both neutral and alkaline pH; however, the yield of NO_2-MOR reached a maximum with 2 mM

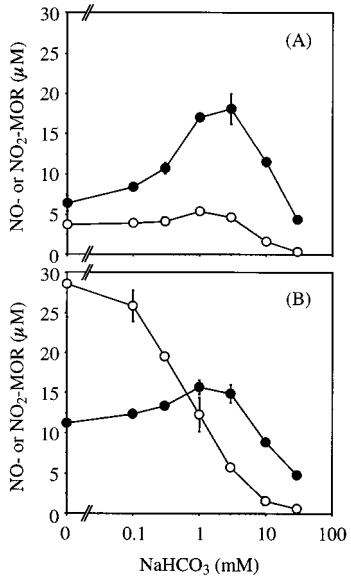

Figure 4. Effect of bicarbonate on the yield of NO-MOR (○) and NO_2-MOR (●) at (A) pH 7.3 and (B) pH 9.5.

morpholine, and no further increase was observed at either neutral or alkaline pH (panels B and D of Figure 3). Under neutral conditions (pH 7.3), the presence of 0.1−3 mM $NaHCO_3$ dose-dependently enhanced nitration of morpholine up to 3-fold, whereas concentrations of $NaHCO_3$ above 10 mM inhibited this reaction (Figure 4A). The extent of NO-MOR formation was slightly increased in the presence of low concentrations of $NaHCO_3$ (0.1−3 mM), but was inhibited by concentrations above 10 mM. Under alkaline conditions (pH 9.5), NO-MOR formation was inhibited dose-dependently by $NaHCO_3$, whereas NO_2-MOR formation was enhanced 1.5-fold with 1−3 mM $NaHCO_3$ (Figure 4B).

Eight secondary and tertiary amines with different pK_a values were reacted with peroxynitrite under neutral and alkaline conditions (Table 1). At pH 9.5, less basic amines (morpholine and aminopyrine), whose pK_a values are lower than 9.5, were more easily nitrosated than they were nitrated, whereas more basic amines (pyrrolidine, diethylamine, and dimethylamine), whose pK_a values are higher than 9.5, were more easily nitrated than they were nitrosated. Methylbenzylamine (pK_a = 9.54) was nitrosated and nitrated to equal extents. At pH 7.4, the yields of nitramines were about twice as high as those of nitrosamines. In general, the sum of the amounts of nitroso and nitro derivatives formed from the reactions of secondary amines and peroxynitrite was inversely related to the basicity of the amines that were tested, although the yields were not directly proportional to pK_a values. Exceptions were proline, triethylamine, and aminopyrine. Proline was less easily nitrosated and nitrated by peroxynitrite than were other secondary amines, possibly because it posseses a carboxyl group that affected the reactions. Triethylamine yielded more nitrodiethylamine than nitrosodiethylamine under both neutral and alkaline conditions. Aminopyrine yielded nitrosodimethylamine but not nitrodimethylamine.

Effects of Scavengers, Antioxidants, Carboxy-PTIO, and *N*-Methylurea on Formation of NO- and NO_2-MOR by Peroxynitrite. We examined effects of various hydroxyl radical (•HO) scavengers, antioxidants, and an NO• trapping agent on the reaction of morpholine (1 mM) with peroxynitrite (1 mM) under neutral conditions (pH 7.5). Uric acid [an antioxidant and free radical scavenger (*27*)] and glutathione strongly inhibited the formation of NO- and NO_2-MOR, the 50% inhibitory concentrations (IC_{50}) being about 0.05 and 0.08 mM, respectively. As shown in Figure 5, *N*-acetylcysteine, ascorbic acid, selenomethionine, and desferrioxamine at concentrations of 1 mM strongly inhibited nitration of morpholine, but inhibited its nitrosation by only 60−80%. In contrast, •HO scavengers such as D-mannitol, dimethyl sulfoxide, and ethanol inhibited nitration weakly and did not inhibit nitrosation. Interestingly, the NO• trapping agent carboxy-PTIO (1 or 10 mM), which oxidizes NO• to NO_2• (*28*), enhanced nitrosation 8-fold, but completely inhibited nitration. Similar stimulation of nitrosation in the presence of carboxy-PTIO has been reported for the *S*-nitrosation of glutathione by peroxynitrite (*29*). *N*-Methylurea (10 mM), which may also be *N*-nitrosated or *N*-nitrated by peroxynitrite, did not affect nitrosation, but slightly (20−30%) inhibited nitration of 1 mM morpholine by 1 mM peroxynitrite.

Reaction of Morpholine with NO• and O_2•⁻. As peroxynitrite is formed from NO• and O_2•⁻, we examined whether concurrent production of these radicals has an effect similar to that of peroxynitrite itself. We carried out reactions of morpholine with NO• and O_2•⁻ which were generated concomitantly from spermine NONOate and xanthine oxidase with acetaldehyde or hypoxanthine as the substrate (*21*−*24*). When the rate of O_2•⁻ generation was kept constant at 4.4 nmol/min, the extent of nitrosation of morpholine increased with increasing amounts of spermine NONOate (Figure 6A). Under these conditions, the extent of nitration was highest with a rate of NO• release of 1.5−2 nmol/min, whereas increasing the NO• flux above 4 nmol/min resulted in a decreased level of nitration (Figure 6B). In contrast, when the NO• flux was constant at 4.4 nmol/min, increasing the O_2•⁻ flux inhibited the nitrosation of morpholine (Figure 6C). The extent of nitration of morpholine was highest for an O_2•⁻ release rate of 4 nmol/min, whereas increasing the O_2•⁻ flux above 8 nmol/min resulted in a decreased nitration level (Figure 6D). In general, yields of NO_2-MOR were 100 times lower than those of NO-MOR. The yield of NO_2-MOR was also lower by 50−90% with hypoxanthine as the substrate for xanthine oxidase than with acetaldehyde as the substrate, suggesting that the reaction was inhibited by hypoxanthine and/or uric acid. Because $NaHCO_3$ enhanced nitration of morpholine by peroxynitrite, we examined the effects of $NaHCO_3$ on the reaction of morpholine with NO• and O_2•⁻ (each 4.4 nmol/min generated from spermine NONOate and xanthine oxidase/acetaldehyde). The presence of up to 30 mM $NaHCO_3$ had no effect on nitrosation. However, $NaHCO_3$ inhibited nitration dose-dependently (about 10 and 50% inhibition by 0.1 and 0.3 mM $NaHCO_3$, respectively, with complete inhibition at >1 mM) (data not shown).

Effects of MPO and HPO on the Reaction of Morpholine with $NaNO_2$ plus HOCl or $NaNO_2$ plus H_2O_2. Because it has been reported that nitrating agents such as nitrogen dioxide (•NO_2), peroxynitrite, and nitryl chloride (NO_2Cl) are formed by reactions of $NaNO_2$ with H_2O_2 or HOCl in the presence or absence of oxidases such as MPO and HPO (*4*, *5*), we examined the reaction of morpholine with these reagents. As summarized in Table 2, small amounts of NO-MOR were formed by the reaction of morpholine with $NaNO_2$ and H_2O_2 under neutral conditions. The yield increased 3- or 4-fold in the

Table 1. Relationship between the Basicity of Amines (pK_a Values) and Formation of Their *N*-Nitrosated and *N*-Nitrated Products by Peroxynitrite[a]

		yield of nitrosated and nitrated products (μM, mean ± SD)			
		pH 7.4		pH 9.5	
amino compound	pK_a[b]	nitrosamine	nitramine or NTYR	nitrosamine	nitramine or NTYR
pyrrolidine	11.27	0.63 ± 0.10	1.48 ± 0.22	1.81 ± 0.10	5.45 ± 1.77
diethylamine	10.93	0.69 ± 0.06	2.35 ± 0.27	1.06 ± 0.17	7.75 ± 0.57
triethylamine	10.75	+[c]	0.08 ± 0.02	0.08 ± 0.05	0.28 ± 0.01
dimethylamine	10.72	1.62 ± 0.22	3.03 ± 0.27	4.03 ± 2.20	8.48 ± 0.52
proline	10.64 (NH)	0.61 ± 0.24	0.10 ± 0.03	1.86 ± 1.64	0.31 ± 0.18
methylbenzylamine	9.54	1.55 ± 0.23	2.78 ± 0.32	10.9 ± 4.6	11.9 ± 1.4
morpholine	8.7	2.49 ± 0.03	5.03 ± 0.31	64.8 ± 6.1	8.03 ± 1.75
aminopyrine	5.04	137 ± 12	−[d]	117 ± 5	−[d]
tyrosine	9.11 (OH) 10.13 (NH_2)		66.8 ± 0.5[e]		46.5 ± 2.62[e]

[a] Reactions were carried out at 37 °C for 15 min by incubating 1 mL of 50 mM sodium phosphate buffer (pH 7.4 or 9.5) containing 0.05 mM DTPA, 1 mM amine, and 1 mM peroxynitrite. Values are the averages ± SD of triplicate analyses. [b] pK_a values were taken from refs *46* and *47*. [c] +, trace amounts (just above the detection limit). [d] −, not detected. [e] 3-Nitrotyrosine.

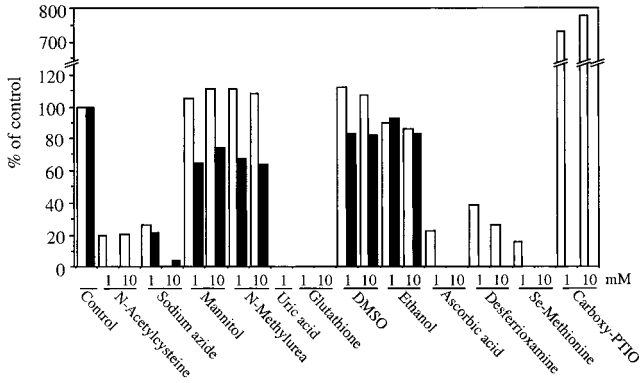

Figure 5. Effect of various scavengers and antioxidants on the formation of NO-MOR (white bars) and NO_2-MOR (black bars) mediated by peroxynitrite. Hydroxyl radical scavengers, antioxidants, an NO• scavenger (carboxy-PTIO), and other compounds (final concentrations of 1 and 10 mM) were examined for their effects on nitrosation and nitration of morpholine (1 mM) by peroxynitrite (1 mM) at pH 7.5. The yields (mean ± SD) of NO-MOR and NO_2-MOR in the absence of scavengers (controls) were 3.56 ± 0.52 and 9.63 ± 1.07 μM, respectively. Experiments were carried out in triplicate. A lack of bars indicates that no nitrosation or nitration products were detected.

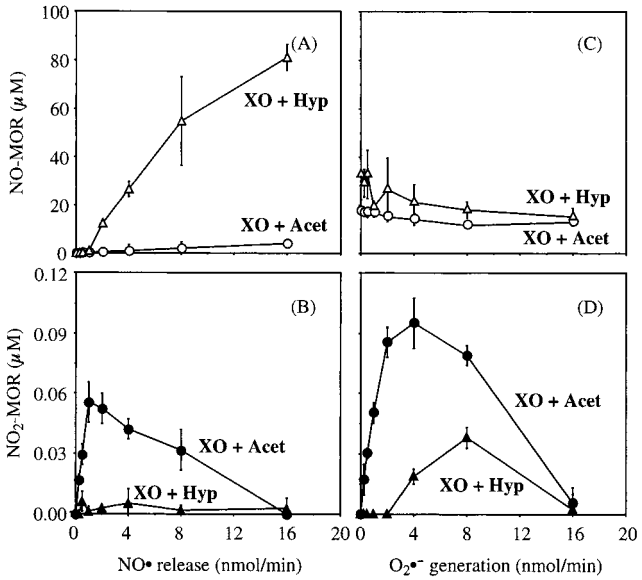

Figure 6. Formation of NO-MOR and NO_2-MOR by NO• and superoxide generated simultaneously at varying rates. NO-MOR [○ and △, formed by NO• plus superoxide generated with xanthine oxidase (XO) and hypoxanthine (Hyp) or acetaldehyde (Acet) as the substrate, respectively] or NO_2-MOR (● and ▲, formed by NO• plus superoxide generated with XO and Hyp or Acet as the substrate, respectively).

presence of HPO or MPO, respectively. NTYR formation in the presence of $NaNO_2$ and H_2O_2 was enhanced 90-fold by MPO, whereas it was enhanced only 3-fold by HPO. NO- and NO_2-MOR were also detected after the reaction of morpholine with $NaNO_2$ and HOCl. The presence of MPO or HPO decreased the extent of NO-MOR formation by $NaNO_2$ and HOCl (Table 2). As peroxynitrite may be formed from $NaNO_2$ and H_2O_2 under acidic conditions (*20*), we examined NO- and NO_2-MOR formation under acidic conditions (Table 3). Large amounts of NO-MOR were formed with $NaNO_2$ at pH 3.5, whereas the presence of H_2O_2 inhibited nitrosation. In contrast, NTYR formation was enhanced 2- and 7-fold at pH 3.5 and 4.5, respectively, in the presence of H_2O_2 and $NaNO_2$, relative to that in the presence of $NaNO_2$ alone.

Discussion

Peroxynitrite-Mediated Nitrosation and Nitration of Secondary Amines. We have shown that peroxynitrite reacts with secondary amines to form both nitrosated and nitrated products (Table 1). In general, yields of both products were higher at alkaline pH than at neutral and acidic pH, except in the case of aminopyrine. This compound reacted with peroxynitrite to form more *N*-nitrosodimethylamine at neutral pH than at alkaline pH. The optimal pH for morpholine nitration by peroxynitrite was 8.5, whereas nitrosation occurred more rapidly at alkaline pH than at neutral or acidic pH (Figure 2A). A similar pH effect on the nitration and nitrosation of phenol by peroxynitrite has been reported (*30*). The yields of nitrosation and nitration products formed by peroxynitrite were related to amine basicity. More nitramines than nitrosamines were formed at neutral pH (Table 1). Similarly at pH 9.5, more nitramines than nitrosamines were formed in reactions of peroxynitrite with secondary amines (pyrrolidine, diethylamine, and dimethylamine), whose pK_a values are higher than 9.5. Methylbenzylamine (pK_a = 9.5) yielded equal amounts of nitramine and nitrosamine, whereas less basic amines (morpholine and aminopyrine), whose pK_a values are lower than 9.5, were more easily nitrosated than nitrated. These results suggest that unprotonated amines undergo nitrosation more easily than nitration. However, under both alkaline and neutral conditions, yields of nitrosamines and nitramines were

Table 2. Effect of Myeloperoxidase (MPO) and Horseradish Peroxidase (HPO) on Nitrosation and Nitration of Morpholine and Tyrosine by $NaNO_2$ plus H_2O_2 or $NaNO_2$ plus HOCl[a]

		yield of nitrosated and nitrated products (μM, mean ± SD)		
		NO-MOR	NO_2-MOR	NTYR
with MPO (80 nM)	$NaNO_2 + H_2O_2$	0.08 ± 0.01	0.002 ± 0.001	60.0 ± 1.00
	$NaNO_2$ + HOCl	0.07 ± 0.04	0	0.55 ± 0.01
	$NaNO_2$ alone	0.02 ± 0.01	0	0
with HPO (1 μM)	$NaNO_2 + H_2O_2$	0.06 ± 0.02	0	2.46 ± 0.13
	$NaNO_2$ + HOCl	0.09 ± 0.02	0.001 ± 0.002	1.24 ± 0.43
	$NaNO_2$ alone	0.02 ± 0.01	0	0
no enzyme	$NaNO_2 + H_2O_2$	0.02 ± 0.01	0	0.69 ± 0.10
	$NaNO_2$ + HOCl	0.16 ± 0.04	0.004 ± 0.004	0.75 ± 0.26
	$NaNO_2$ alone	0.02 ± 0.01	0	0

[a] Reactions were carried out at 37 °C for 3 h, by incubating 50 mM phosphate buffer (pH 7.45) containing 0.05 mM DTPA, 1 mM morpholine or tyrosine, and 500 μM $NaNO_2$ with or without 500 μM H_2O_2 or HOCl. All experiments were carried out in triplicate.

Table 3. Formation of NO-MOR, NO_2-MOR, and NTYR by Reaction of $NaNO_2$ with Morpholine or Tyrosine with or without H_2O_2 under Acidic Conditions[a]

		yield of nitrosated and nitrated products (μM, mean ± SD)		
reaction	pH	NO-MOR	NO_2-MOR	NTYR
$NaNO_2 + H_2O_2$	3.50	2.26 ± 0.42	0	19.3 ± 0.18
$NaNO_2 + H_2O_2$	4.50	0.87 ± 0.06	0	6.95 ± 0.79
$NaNO_2$ alone	3.50	9.98 ± 0.27	0	9.33 ± 0.41
$NaNO_2$ alone	4.50	1.23 ± 0.11	0	0.98 ± 0.03

[a] Reactions were carried out at 37 °C for 3 h, by incubating 50 mM phosphate buffer (pH 3.5 or 4.5) containing 0.05 mM DTPA, 1 mM morpholine or tyrosine, and 1 mM $NaNO_2$ with or without 1 mM H_2O_2. All experiments were carried out in triplicate.

not directly proportional to the pK_a values of the secondary amines (Table 1). Low concentrations of $NaHCO_3$ (0.1−3 mM) enhanced peroxynitrite-mediated nitration of secondary amines at neutral pH (Figure 4), as reported for nitration of tyrosine and guanine (*31*, *32*). In contrast, high concentrations of $NaHCO_3$ (>10 mM) inhibited the nitration of secondary amines at neutral pH and their nitrosation at neutral and alkaline pH. High concentrations of $NaHCO_3$ have been reported to inhibit the nitrosation of phenol by peroxynitrite (*33*) as well as the nitrosation of morpholine by NO• (*34*).

On the basis of these observations, as well as those reported in the literature on mechanisms for the nitrosation of phenol by peroxynitrite (*30*, *33*), we propose here a free radical mechanism for nitrosation and nitration of secondary amines by peroxynitrite. At neutral and acidic pH, $^{\bullet}NO_2$ and $^{\bullet}OH$ can be generated by homolytic decomposition of peroxynitrous acid (*35*). These radicals are capable of one-electron oxidation of secondary amines to form amino radicals ($R_2N^{\bullet}$), which can then react with $^{\bullet}NO$ or $^{\bullet}NO_2$ to yield nitroso and nitro secondary amines, respectively. Amino radicals could also be formed by the reaction of amines with $CO_3^{\bullet-}$ which is generated from the peroxynitrite−CO_2 adduct ($ONOOCO_2^-$) (*31*) as well as with $^{\bullet}NO_2$ generated from N_2O_3 and N_2O_4 (*36*). N_2O_3 and N_2O_4 could be formed from peroxynitrite by the reactions of $^{\bullet}NO_2$ with either $^{\bullet}NO$ or $^{\bullet}NO_2$ (*35*, *37*).

In addition to this free radical mechanism, nitrosamines might be formed by direct nucleophilic nitrosation of amines by peroxynitrite anion (eq 1) in a manner similar to that for the formation of *S*-nitrosoglutathione by peroxynitrite (*38*), though direct oxidation of secondary amino groups may be more difficult than that of thiol groups.

$$R_2NH + O{=}N{-}O{-}O^- \rightarrow R_2NNO + HOO^- \quad (1)$$

Furthermore, at alkaline pH, N_2O_3 can be formed from peroxynitrite anion (*35*, *37*) and can react with unprotonated amines to yield nitrosamines (*36*). On the other hand, at pH <7, peroxynitrous acid may decompose homolytically to generate $^{\bullet}NO_2$ and $^{\bullet}OH$ (*35*). Two molecules of $^{\bullet}NO_2$ react to form dinitrogen tetroxide (N_2O_4), which is a strong nitrosating agent. N_2O_4, generated by radiolysis of aqueous nitrate ($NaNO_3$), has been reported to react with secondary amines to produce both nitrosamines and nitramines (*39*). Nucleophiles, such as amines, react with the symmetrical and asymmetrical tautomers of N_2O_4 ($O_2N{-}NO_2$ and $ON{-}NO_3$, respectively) to form both nitrosated and nitrated products (*36*, *39*−*42*). Furthermore, peroxynitrous acid may decompose heterolytically into HO^- and NO_2^+ (*43*), and the latter species may react with amines to form nitramines (eqs 2 and 3).

$$ONOOH \rightarrow HO^- + NO_2^+ \quad (2)$$

$$R_2NH + NO_2^+ \rightarrow R_2NNO_2 + H^+ \quad (3)$$

The peroxynitrite−CO_2 adduct ($ONOOCO_2^-$) may also form a nitrating agent ($O_2N{-}OCO_2^-$), which reacts with amines to form nitramines (eqs 4−6) (*31*, *44*).

$$ONOO^- + CO_2 \rightarrow ONOOCO_2^- \quad (4)$$

$$ONOOCO_2^- \rightleftarrows O_2N{-}OCO_2^- \quad (5)$$

$$R_2NH + O_2N{-}OCO_2^- \rightarrow R_2NNO_2 + HCO_3^- \quad (6)$$

Reaction with Concomitant Generation of NO• and $O_2^{\bullet-}$. Pfeiffer and Mayer (*24*) recently studied nitration of free tyrosine by NO• and $O_2^{\bullet-}$ generated from spermine NONOate and from xanthine oxidase with hypoxanthine as a substrate. They found that nitration was most efficient with the NO• donor alone, and that concomitant generation of $O_2^{\bullet-}$ inhibited the nitration of tyrosine. These authors concluded that the species formed from NO• and $O_2^{\bullet-}$ at physiological pH differs from synthetic peroxynitrite (*24*). However, because tyrosine can be nitrated by both nitrosating and nitrating agents (*8*, *9*), it is impossible to distinguish nitrosation and nitration reactions using NTYR formation as a marker. For these reasons, we have studied nitration and nitrosation of morpholine using experimental protocols similar to those described by Pfeiffer and Mayer (*24*). We observed nitration of morpholine when the fluxes of NO• and $O_2^{\bullet-}$ were similar at 4 nmol/min (Figure 6). Increasing the relative flux of either NO• or $O_2^{\bullet-}$ resulted in a

decrease in the extent of nitration. Similar inhibitory effects of excess $NO^{\bullet}$ and $O_2^{\bullet-}$ have been reported for the oxidation of dihydrorhodamine (*22*). However, the yield of NO_2-MOR was >100 times lower than that of NO-MOR. These results indicate that $NO^{\bullet}$ and $O_2^{\bullet-}$ reacted to form peroxynitrite, but the yield of peroxynitrite or its reactivity with amines was low under our experimental conditions. In contrast, with a fixed flux of $O_2^{\bullet-}$, increasing the flux of $NO^{\bullet}$ resulted in higher yields of NO-MOR. When the flux of $NO^{\bullet}$ was fixed, increasing the flux of $O_2^{\bullet-}$ inhibited NO-MOR formation. These results suggest that nitrosation is mediated by nitrosating agents (probably N_2O_3) formed by the reaction of $NO^{\bullet}$ with $^{\bullet}NO_2$, the latter being formed via autoxidation of $NO^{\bullet}$. The presence of $O_2^{\bullet-}$ inhibits the formation of nitrosating agents due to a rapid reaction between $NO^{\bullet}$ and $O_2^{\bullet-}$ to form peroxynitrite. In general, our results (Figure 6) are analogous to those observed for the nitration of tyrosine by Pfeiffer and Mayer (*24*) and for the nitrosation of 2,3-diaminonaphthalene by Wink et al. (*23*).

Nitrosation and Nitration of Morpholine by Various RNS. We studied nitration and nitrosation of morpholine by various RNS and compared them with tyrosine nitration (Table 2). Under physiological conditions, $NaNO_2$ alone did not nitrosate nor nitrate morpholine well, but the addition of HOCl increased the levels of both nitrosation and nitration (Table 2). The presence of MPO and HPO increased the level of formation of nitrosamine mediated by $NaNO_2$ plus H_2O_2. These results indicate that carcinogenic nitrosamines could be formed in inflamed tissues, where increased levels of nitrite, OCl^-, and H_2O_2 could be present and MPO is secreted by activated neutrophils.

Comparison of Nitrosation and Nitration of Secondary Amines with Nitration of Tyrosine. Several possible mechanisms for NTYR formation have been reported, including (i) reaction of the tyrosyl radical with $NO^{\bullet}$, leading to formation of 3-nitroso- or *O*-nitrosotyrosine, followed by their two-electron oxidation and rearrangement to NTYR (*45*) and (ii) hydrogen abstraction from the aromatic ring by oxidant(s) to yield the tyrosyl radical, followed by addition of $^{\bullet}NO_2$ to form NTYR. In the case of the peroxynitrite-mediated reaction, tyrosine is more effectively nitrated at physiological pH than at alkaline pH, suggesting that the mechanism involving tyrosyl radical formation and nitration by peroxynitrous acid may be involved. In contrast, the reactions of secondary amines with peroxynitrite at alkaline pH could be mediated by the nucleophilic reaction between unprotonated amines and nitrosating agents, e.g., N_2O_3. Under physiological conditions, free radical mechanisms (amino radical fomation followed by addition of $NO^{\bullet}$ or $^{\bullet}NO_2$) may also be responsible for nitrosation and nitration of amines. Among the different RNS that were tested, $NaNO_2$ plus H_2O_2 in the presence of MPO was strikingly effective in the nitration of tyrosine, compared to the nitrosation and nitration of amines. We think this occurred because aromatic compounds such as tyrosine undergo one-electron oxidation easily to form aromatic radicals, which then react rapidly with RNS (NO_2Cl, $^{\bullet}NO_2$, and peroxynitrite) produced by MPO in the presence of $NaNO_2$, Cl^-, and H_2O_2 (*4*, *5*).

Conclusion

We have shown that peroxynitrite and other RNS react with secondary amines to form nitrated and nitrosated amines. Although NTYR has been identified as a marker of peroxynitrite formation in vivo, a variety of RNS, including nitrosating and nitrating agents, can nitrate tyrosine to form NTYR. In contrast, nitrated secondary amines (e.g., *N*-nitroproline) are formed more specifically by nitrating agents. Therefore, *N*-nitroproline could be identified in human urine as a specific marker for endogenous nitration, analogous to *N*-nitrosoproline in human urine, which has been analyzed as a marker for endogenous nitrosation (*26*).

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