8-Nitro-2′-deoxyguanosine, a Specific Marker of Oxidation by Reactive Nitrogen Species, Is Generated by the Myeloperoxidase−Hydrogen Peroxide−Nitrite System of Activated Human Phagocytes†

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ABSTRACT: Reactive intermediates generated by phagocytes damage DNA and may contribute to the link between chronic inflammation and cancer. Myeloperoxidase, a heme protein secreted by activated phagocytes, is a potential catalyst for such reactions. Recent studies demonstrate that this enzyme uses hydrogen peroxide (H_2O_2) and nitrite (NO_2^-) to generate reactive nitrogen species which convert tyrosine to 3-nitrotyrosine. We now report that activated human neutrophils use myeloperoxidase, H_2O_2, and NO_2^- to nitrate 2′-deoxyguanosine, one of the nucleosides of DNA. Through HPLC, UV/vis spectroscopy, and mass spectrometry, the two major products of this reaction were identified as 8-nitroguanine and 8-nitro-2′-deoxyguanosine. Nitration required each component of the complete enzymatic system and was inhibited by catalase and heme poisons. However, it was independent of chloride ion and little affected by scavengers of hypochlorous acid, suggesting that the reactive agent is a nitrogen dioxide-like species that results from the one-electron oxidation of NO_2^- by myeloperoxidase. Alternatively, 2′-deoxyguanosine might be oxidized directly by the enzyme to yield a radical species which subsequently reacts with NO_2^- or $NO_2^{\bullet}$ to generate the observed products. Human neutrophils stimulated with phorbol ester also generated 8-nitroguanine and 8-nitro-2′-deoxyguanosine. The reaction required NO_2^- and was inhibited by catalase and heme poisons, implicating myeloperoxidase in the cell-mediated pathway. These results indicate that human neutrophils use the myeloperoxidase−H_2O_2−NO_2^- system to generate reactive species that can nitrate the C-8 position of 2′-deoxyguanosine. Our observations raise the possibility that reactive nitrogen species generated by myeloperoxidase and other peroxidases contribute to nucleobase oxidation and tissue injury at sites of inflammation.

Reactive oxidants generated by phagocytic white blood cells play critical roles in host defenses by destroying invading pathogens and malignant cells. A well-characterized source of such intermediates is myeloperoxidase, a secreted heme enzyme (*1*−*5*). Myeloperoxidase uses hydrogen peroxide (H_2O_2) generated by neutrophils and monocytes to produce potent cytotoxins. The major product at plasma concentrations of chloride ion is generally thought to be hypochlorous acid (HOCl) (*6*, *7*).

$$Cl^- + H_2O_2 + H^+ \rightarrow HOCl + H_2O \qquad (1)$$

This bactericidal oxidant bleaches aromatic compounds (*8*), inactivates sulfhydryl groups (*9*), chlorinates and oxygenates unsaturated lipids (*10*, *11*), and converts amino acids into chloramines that can decompose into reactive aldehydes (*12*−*14*). In a one-electron oxidation reaction that does not involve chloride, myeloperoxidase converts tyrosine to highly reactive tyrosyl radical (*15*, *16*). This species cross-links tyrosine residues in proteins and initiates lipid peroxidation by a pathway that is independent of free metal ions (*17*, *18*).

Nitric oxide ($^{\bullet}NO$) is another potential precursor of reactive intermediates (*19*, *20*). It reacts with oxygen to form N_2O_3, a nitrosating agent (*21*). It also reacts with superoxide to form peroxynitrite, a potent oxidant that hydroxylates and nitrates biological targets (*22*). Recent studies demonstrate, however, that myeloperoxidase also can generate reactive nitrogen species (*23*−*27*). This reaction requires nitrite (NO_2^-), a degradation product of NO, and is thought to involve two different mechanisms. The first is mediated by HOCl and yields nitryl chloride (NO_2Cl) (*25*−*27*):

$$HOCl + NO_2^- \rightarrow NO_2Cl + HO^- \qquad (2)$$

The second produces nitrogen dioxide radical ($NO_2^{\bullet}$) through direct one-electron oxidation of NO_2^- by compound I, a

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complex of myeloperoxidase and H_2O_2 (*25*−*27*):

$$NO_2^- + \text{compound I} + H^+ \rightarrow NO_2^{\bullet} + H_2O + \text{compound II} \quad (3)$$

These reactions may be physiologically relevant because human neutrophils use the myeloperoxidase−H_2O_2−NO_2^- system to chlorinate and nitrate free tyrosine and a fluorescent probe in vitro (*25*, *26*).

Reactive intermediates are potentially damaging to host proteins, lipids, and nucleic acids. Given that chronic inflammation is characterized by phagocytic infiltration and is strongly associated with an increased cancer risk (reviewed in refs *28*, *29*), it is reasonable to suspect that phagocytic products may be one important agent for the damage of nucleic acids. Moreover, phagocytic oxidants induce breaks in DNA strands (*30*, *31*), mutate bacterial DNA (*32*−*36*), cause cytogenetic changes in mammalian cells (*37*), and promote malignant transformation (*38*). One potential pathway involves reactive nitrogen species derived from NO, which is known to nitrosate, deaminate, and oxidatively damage DNA (*21*, *39*−*44*). This includes peroxynitrite, a mutagenic oxidant (*45*−*47*) which forms when •NO reacts with superoxide (*22*, *48*). Peroxynitrite converts guanine into 8-nitroguanine and oxidizes 2′-deoxyguanosine into 4,5-dihydro-5-hydroxy-4-(nitrosooxy)-2′-deoxyguanosine (*49*−*52*). Whether these pathways are physiologically relevant is not yet established.

In the current studies, we explored the ability of the myeloperoxidase−H_2O_2−NO_2^- system to modify nucleic acids, using the nucleoside 2′-deoxyguanosine as a model target. Through mass spectrometry, UV/vis spectroscopy, and chromatography, we then identified 8-nitroguanine and 8-nitro-2′-deoxyguanosine as the major products. Activated white blood cells also nitrated 2′-deoxyguanosine. The reaction required NO_2^- and was inhibited by catalase and heme poisons, implicating myeloperoxidase in the cellular pathway. These observations suggest that reactive nitrogen intermediates generated by myeloperoxidase might damage nucleic acids in vivo.

EXPERIMENTAL PROCEDURES

Materials

Fisher Chemical supplied sodium phosphate and H_2O_2 (30%; ACS grade). Sodium nitrite, ammonium formate, 2′-deoxyguanine, diethylenetriaminepentaacetic acid (DTPA[1]), superoxide dismutase, β-phorbol myristate acetate, and sodium azide were obtained from Sigma Chemical Co. (St. Louis, MO).

Methods

Isolation of Myeloperoxidase. Myeloperoxidase was extracted from HL60 cells with cetyltrimethylammonium bromide and subjected to lectin affinity chromatography and size-exclusion chromatography (*15*, *53*). Myeloperoxidase prepared by this method had an A_{430}/A_{280} ratio of 0.76. Enzyme concentration was determined spectrophotometrically ($\epsilon_{430} = 170\ mM^{-1}\ cm^{-1}$) (*54*).

Human Neutrophils. Neutrophils were prepared by density gradient centrifugation and suspended at 7×10^5 cells/mL in Hank's buffered salt solution (Ca^{2+}-, Mg^{2+}- and phenol red-free; GibcoBRL, Life Technology Inc., Grand Island, NY) supplemented with 2 mM 2′-deoxyguanosine (*17*). The cells (2 mL) were incubated at 37 °C for 60 min and maintained in suspension with intermittent inversion. The reaction was terminated by centrifugation of the cells at 400*g* for 10 min. The supernatant was concentrated to dryness under vacuum, solubilized in 0.3 mL of H_2O, centrifuged at 14000*g* for 10 min, and subjected to HPLC analysis.

Oxidation of 2′-Deoxyguanosine by Myeloperoxidase. Reactions were carried out for 30 min at 37 °C in 50 mM sodium phosphate buffer (pH 7.4) that was treated with Chelex resin to remove metal ions and supplemented with 100 μM DTPA.

Reduction of 2′-Deoxyguanosine Oxidation Products with Dithionite. HPLC fractions containing peaks 1 and 2 were collected, dried under vacuum, and resuspended in 50 mM ammonium acetate. A small amount of dithionite was added to each suspension until the yellow color of the solution disappeared. The reduced materials were then subjected to HPLC and collected for GC/MS analysis.

Reverse-Phase HPLC Analysis of 2′-Deoxyguanosine Oxidation Products. Products from the myeloperoxidase−H_2O_2−NO_2^- system were analyzed with a Beckman reverse-phase HPLC system equipped with a Ultrasphere ODS column (4.6 mm × 25 cm; 5 μm; Beckman Instruments, Berkeley, CA). Compounds were resolved by isocratic elution with 20 mM ammonium formate buffer at a flow rate of 1.0 mL/min (*49*). Nucleobases were detected by monitoring absorbance at 254 and 390 nm, corresponding to the absorbance maxima of 2′-deoxyguanosine and the nitrated product, respectively. Ultraviolet/visible spectra of eluting peaks were obtained by scanning from 200 to 500 nm on a Beckman photodiode array detector. HPLC fractions of interest were collected and concentrated under vacuum. For some experiments, collected products were again subjected to HPLC fractionation on the ODS reverse-phase column using the ammonium formate isocratic system.

Gas Chromatography/Mass Spectrometry (GC/MS). Trimethylsilyl (TMS) and *tert*-butyldimethylsilyl (BDMS) derivatives of nucleobases were produced with excess N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA; Regis Technologies, Morton Grove, IL) and *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (Regis Technologies, Morton Grove, IL) in acetonitrile (3:1; v:v) at 110 °C for 1 h. Products of derivatization reactions were concentrated to dryness under nitrogen gas and reconstituted in pyridine for analysis.

Reaction products were analyzed on a Varian Star 3400 CX gas chromatograph interfaced with an extended-mass range Finnigan MAT SSQ 7000. Gas chromatographic separations were carried out utilizing a 12 m DB-1 capillary column (J&W Scientific; 0.2 mm i.d., 0.33 μm film thickness) with helium as the carrier gas. Source temperature, filament current, and electron multiplier voltage were set at 150 °C, 700 μA, and 1200 V, respectively. The injector and

[1] Abbreviations: BDMS, *tert*-butyldimethylsilyl; BSTFA, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide; GC, gas chromatography; DTPA, diethylenetriaminepentaacetic acid; $M^{\bullet+}$, molecular ion; MS, mass spectrometry; *m*/*z*, mass-to-charge ratio; 8-nitro-dG, 8-nitro-2′-deoxyguanosine; 8-nitroG, 8-nitroguanine; TMS, trimethylsilyl; ESI, electrospray ionization.

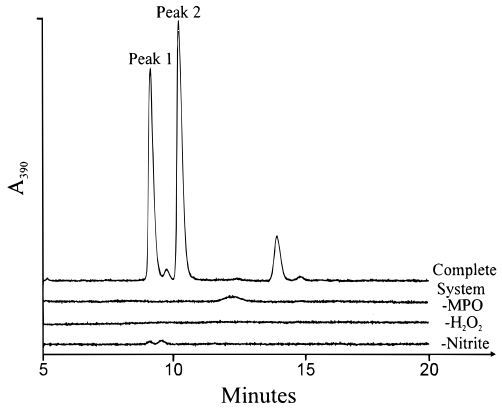


FIGURE 1: Reverse-phase HPLC analysis of 2′-deoxyguanosine exposed to the myeloperoxidase−H_2O_2−NO_2^- system. The complete system consisted of 2 mM 2′-deoxyguanosine, 20 nM myeloperoxidase, 100 μM H_2O_2, 30 μM NO_2^-, and 100 μM DTPA in 50 mM sodium phosphate buffer (pH 7.4). Where indicated, myeloperoxidase (MPO), H_2O_2, or NO_2^- (nitrite) was omitted from the complete system. After a 30 min incubation at 37 °C, the reaction mixture was subjected to HPLC as described under Methods. Products were detected by monitoring absorbance at 390 nm.

interface temperatures were 250 and 270 °C. The initial GC oven temperature of 120 °C was maintained for 1 min, followed by a 25 °C/min increasing ramp to 250 °C.

Electrospray Ionization MS. Full mass scanning, selected ion monitoring, zoom scanning, and low-energy collisionally activated dissociation were carried out on a Finnigan LCQ. A 5 μL portion of sample was injected at a flow rate of 3 μL/min. Samples were introduced with an electrospray source. The electrospray needle was held at 4500 V and the counter electrode was held at ground potential. Methanol/water/acetic acid (50:49:1; v/v/v) was used to dissolve samples and as a carrier solvent. The flow rate was typically 3 μL/min. Helium was used as a damping gas and collision activation partner. The flow of gas (1 mL/min) into the mass analyzer cavity was regulated by a pressure regulator and a capillary restrictor. Flow rates were matched to adjust the partial pressure of helium in the mass analyzer cavity to approximately 10^{-3} Torr. The temperature of heated capillary was 200 °C. The collision energy was varied by changing the resonance excitation RF voltage. In the full scan mode (m/z 100−400), 10 scans were signal averaged and the background from the same number of scans was subtracted from the full scan mass spectrum.

RESULTS

Myeloperoxidase Converts 2′-Deoxyguanosine into Oxidation Products by a Reaction Requiring H_2O_2 and NO_2^-. In preliminary studies, addition of purified myeloperoxidase to phosphate buffer (pH 7.4) containing H_2O_2, NO_2^-, and 2′-deoxyguanine immediately generated a visible yellow color. A similar reaction occurred when guanine was substituted for 2′-deoxyguanosine. This color change was not observed when adenine, thymine, uracil, cytosine, and their respective nucleosides were substituted for 2′-deoxyguanosine.

Reverse-phase HPLC analysis of 2′-deoxyguanosine exposed to the complete myeloperoxidase−H_2O_2−NO_2^- system in phosphate buffer revealed two major products (Figure 1). Their generation required H_2O_2, 2′-deoxyguanosine, NO_2^-, and active enzyme. The peaks of material eluting at 9 min

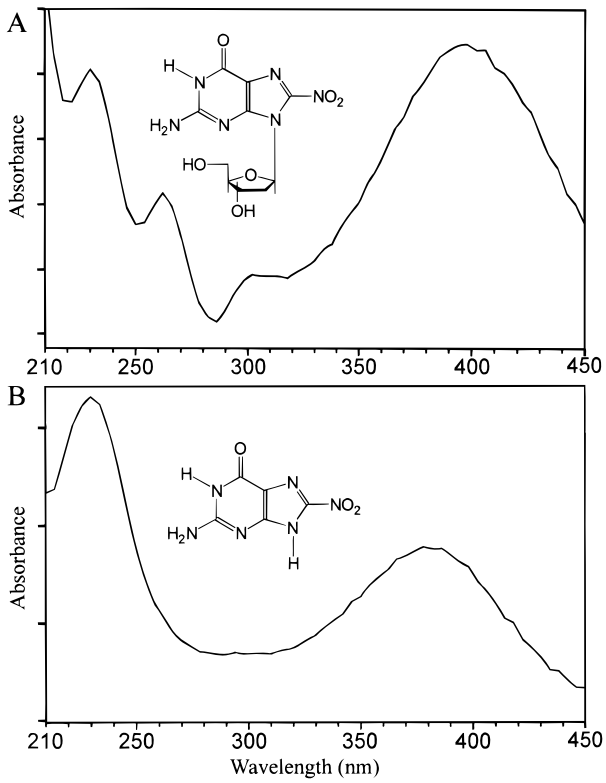


FIGURE 2: Absorption spectra of peak 1 (A) and peak 2 (B) produced by the myeloperoxidase−H_2O_2−NO_2^- system. Spectra were obtained with a diode array detector during HPLC analysis of 2′-deoxyguanosine exposed to the myeloperoxidase−H_2O_2−NO_2^- system as described in the legend to Figure 1.

(termed peak 1, Figure 2A) and at 11 min (termed peak 2, Figure 2B) were yellow and had absorbance maxima at 390 and 380 nm, respectively. The minor peak that eluted at 14 min exhibited an absorbance maximum at 385 nm; this material proved difficult to characterize and was not further investigated. These results suggested that reactive nitrogen intermediates generated by myeloperoxidase had reacted with the aromatic ring of guanine to yield a nitroso (−NO), nitro (−NO_2), nitrosooxy (−ONO), or nitrate (−NO_3) function.

Nucleosides are hydrolyzed to nucleobases and ribose under acidic conditions. When we isolated peak 1 by HPLC and exposed it to formic acid, we obtained a nearly quantitative yield of peak 2. 8-Nitroguanine in DNA undergoes spontaneous depurination (*49*), and we found that the amount of peak 2 relative to peak 1 increased when the reaction mixture was incubated for prolonged periods of time. These results suggest that the myeloperoxidase−H_2O_2−NO_2^- system initially converts 2′-deoxyguanosine into a modified nucleoside (peak 1), which then hydrolyses spontaneously or during exposure to acidic conditions to yield the respective modified nucleobase (peak 2).

The Myeloperoxidase−H_2O_2−NO_2^- System Generates 8-Nitroguanine. Reactive nitrogen species might generate multiple guanine isomeric products. We therefore investigated the nature and regiospecificity of the guanine adduct(s) generated by myeloperoxidase by subjecting the TMS derivative of peak 1 to GC/MS analysis. The total ion chromatogram of this material exhibited a single major peak, suggesting that a single major isomer was present. As expected for nitroguanine or nitrosooxyguanine (the compound released from nitrated or nitrosooxygenated 2′-

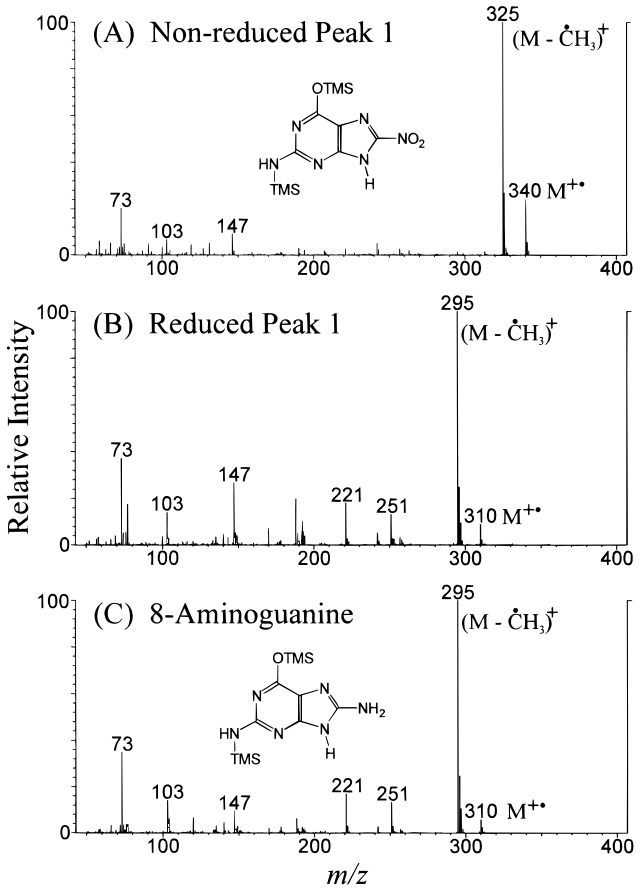


FIGURE 3: Mass spectra of the TMS derivatives of peak 1 (A), reduced peak 1 (B), and 8-aminoguanine (C). 2′-Deoxyguanosine was oxidized by the myeloperoxidase−H_2O_2−NO_2^- system. Peak 1 was isolated by reverse-phase HPLC, derivatized with BSTFA, or reduced with dithionite and then derivatized with BSTFA, and analyzed by positive ion electron−ionization GC/MS. The derivatization procedure removes the sugar moiety of the nucleoside. (A) Ions in the mass spectrum consistent with a bis-TMS derivative of nitroguanine were observed at *m*/*z* 340 ($M^{+\bullet}$) and *m*/*z* 325 [M − $^{\bullet}CH_3]^+$ (B,C). The mass spectra of the TMS derivatives of reduced peak 1 and authentic 8-aminoguanine are identical, suggesting that the attachment site of the nitro group in peak 1 is the C-8 position in the purine ring.

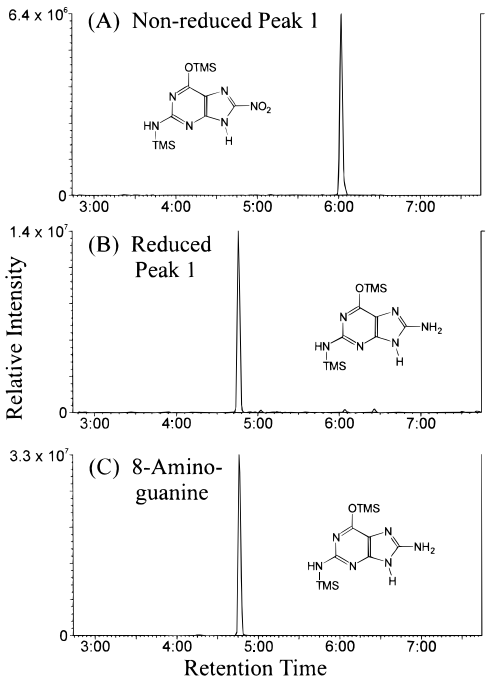


FIGURE 4: Positive ion chromatogram of the TMS derivative of peak 1 (A), reduced peak 1 (B), and 8-aminoguanine (C). 2′-Deoxyguanosine was oxidized by the myeloperoxidase−H_2O_2−NO_2^- system. Peak 1 was isolated by reverse-phase HPLC, derivatized with BSTFA, or reduced with dithionite and then derivatized with BSTFA, and analyzed by electron ionization GC/MS with selected ion monitoring of ions ([M − $^{\bullet}CH_3]^+$) of *m*/*z* 325 (A) or *m*/*z* 295 (B,C).

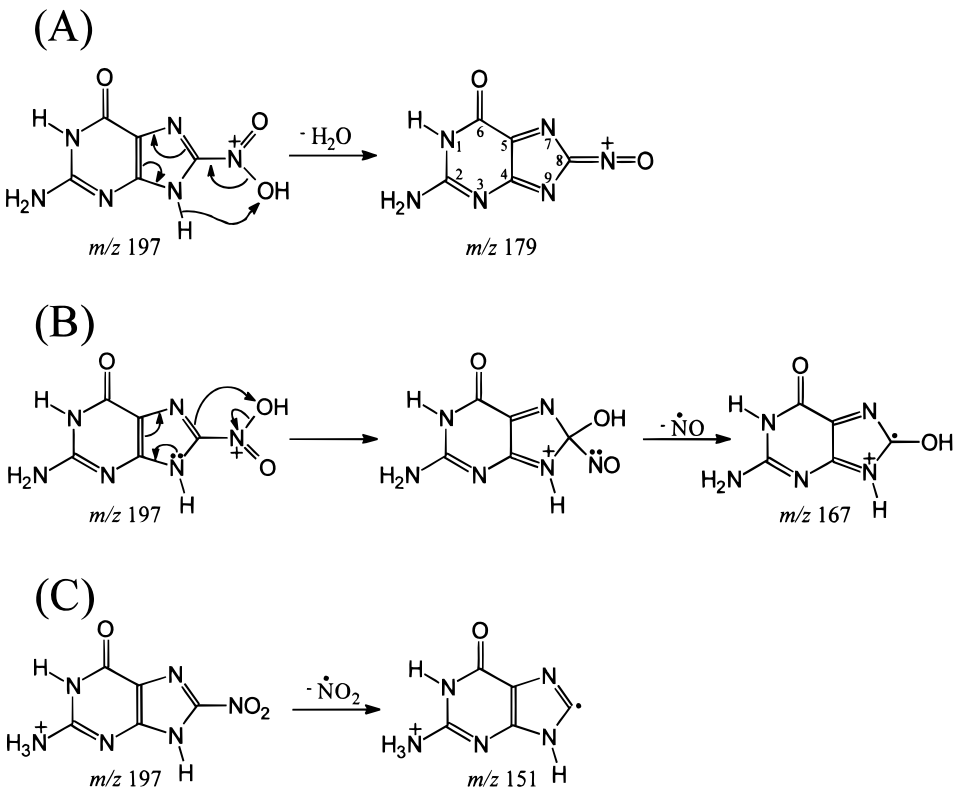
deoxyguanine by derivatization, respectively), the positive ion electron ionization mass spectrum (Figure 3A) demonstrated major ions at *m*/*z* 340 ($M^{+\bullet}$) and *m*/*z* 325 ([M − $^{\bullet}CH_3]^+$). In contrast, ions consistent with modification by nitroso, nitrate, or nitrosooxy plus hydroxyl groups were not observed in the mass spectra of peak 1 (Figure 3A) and peak 2 (data not shown). These results suggest that nitration or nitrosooxygenation was the major reaction catalyzed by myeloperoxidase under our experimental conditions. The GC retention time and mass spectrum of derivatized peak 2 was indistinguishable from that of peak 1. Because derivatization of nucleosides with TMS hydrolyzes the N-glycosyl bond, this again suggests that the materials in peak 1 and peak 2 contain the same modified nucleobase, and that one of the peaks is the nucleobase and the other is the nucleoside.

To determine whether myeloperoxidase nitrates or nitrosooxygenates 2′-deoxyguanosine, we isolated peak 1 by reverse-phase HPLC and exposed it to dithionite (*55*), which reduces aromatic nitro groups to primary amino groups and is anticipated to reduce nitrosooxy groups to an alcohol. The TMS derivative of the reduced material was analyzed by electron ionization GC/MS. The GC retention time (Figure 4B) and positive ion mass spectrum for TMS-derivatized and reduced peak 1 (Figure 3B) were essentially identical to those of TMS-derivatized authentic 8-aminoguanine (Figures 4C and 3C). These results suggest that peak 1 contained guanine with a single nitro substituent at the C-8 position.

To confirm that myeloperoxidase nitrates 2′-deoxyguanosine in the C-8 position, we hydrolyzed both reduced peak 1 material and authentic 8-aminoguanosine to the corresponding nucleobases with formic acid. Then we determined the mass spectra and GC retention times of the DMTBS derivatives of the free bases. The full scan mass spectrum of authentic 8-aminoguanine was consistent with a *bis*-DMTBS derivative, demonstrating a base peak at *m*/*z* 337 [M − $C_4H_9]^+$ and a small peak at *m*/*z* 379 [M − $CH_3]^+$. The molecular ion of the bis-DMTBS derivative of 8-aminoguanine was undetectable in both the full scanning and selected ion monitoring modes. The GC retention times and mass spectra of the DMTBS derivatives of hydrolyzed 8-aminoguanosine and reduced, hydrolyzed peak 1 material were identical (data not shown). These results provide further evidence that peak 1 contained guanine with a single nitro substituent located at the C-8 position of the purine ring.

The Myeloperoxidase-H_2O_2−NO_2^- System Does Not Generate Nitroso or Nitrosooxy Adducts of Guanine. GC/MS analysis might fail to detect nonvolatile or poorly ionized derivatives of nitrosated products of guanine and 2′-deoxyguanosine. To avoid this potential oversight, we subjected the materials in peaks 1 and 2 to electrospray ionization mass spectrometric analysis. This method of ionization is ap-

Scheme 1: Proposed Mechanism for the Generation of Fragment Ions in Low Energy Collisionally Activated Dissociation of the Protonated Molecular Ion of 8-Nitroguanine[a]



[a] The *m*/*z* 197 ion (protonated peak 2) is decomposed to generate the product ion at *m*/*z* 179 by hydride transfer from N-9 to the protonated oxygen of the nitro group together with a rearrangement that eliminates neutral water (A). A two-step fragmentation of the *m*/*z* 197 ion also produces an ion at *m*/*z* 167 (B). In the first step, the double bond in the ring migrates to the 5−7 position, the unpaired electrons on N^9 move to the C-4 position, and the hydroxyl group of the nitro group migrates to C-8. In the second, a homolytic cleavage between C-8 and the NO group removes •NO and generates a distonic radical species. The product ion at *m*/*z* 151 (C) is generated from a molecular ion with a protonated exocyclic amine group by the elimination of nitrogen dioxide.

plicable to a broad array of biological molecules and introduces compounds directly into the gas phase without the requirement for derivatization.

We isolated peaks 1 and 2 generated by the myeloperoxidase−H_2O_2−NO_2^- system using reverse-phase HPLC. Positive ion electrospray ionization mass spectrometry of peak 2 revealed a major ion at *m*/*z* 197, consistent with the protonated molecular ion of 8-nitroguanine (Figure 5A). Control experiments performed with the carrier solvent demonstrated that the other major peaks seen in Figure 5A were derived from impurities that coeluted with peak 2. The electrospray ionization mass spectrum of peak 1 demonstrated a major ion at *m*/*z* 313 that was consistent with a protonated molecular ion $[M + H]^+$ of 8-nitro-2′-deoxyguanosine (data not shown). A less abundant ion at *m*/*z* 335 was consistent with a sodiated molecular ion $[M + Na]^+$ of 8-nitro-2′-deoxyguanosine. In contrast, major ions consistent with guanine adducts of nitroso, nitrate, hydroxyl, or nitrosooxy plus hydroxyl groups were not observed in the electrospray ionization mass spectra of either peak 1 or peak 2.

To firmly establish the attachment site of the nitro group, we subjected the ion observed at *m*/*z* 197 in the electrospray mass spectrum to low energy collisionally activated tandem mass spectrometric analysis (Figure 5B). The $[M + H]^+$ ion of the nitroguanine adduct (*m*/*z* 197) decomposed into major ions at *m*/*z* 179 $(M + H - H_2O)^+$, *m*/*z* 167 $(M + H - {}^{\bullet}NO)^{+\bullet}$, and *m*/*z* 151 $(M + H - {}^{\bullet}NO_2)^{+\bullet}$. The failure to detect

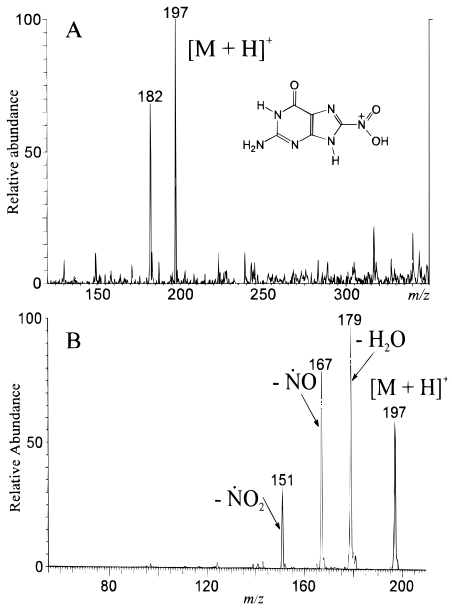


FIGURE 5: Full scan positive ion electrospray ionization mass spectra of 8-nitroguanosine (A) and low energy collisionally activated tandem mass spectrum of the $[M + H]^+$ ion of 8-nitroguanine (B). Peak 2 was isolated by HPLC and subjected to electrospray ionization mass spectrometry as described under Methods.

a major ion at *m*/*z* 180, representing loss of neutral NH_3, suggests that there is little protonation of the exocyclic amine

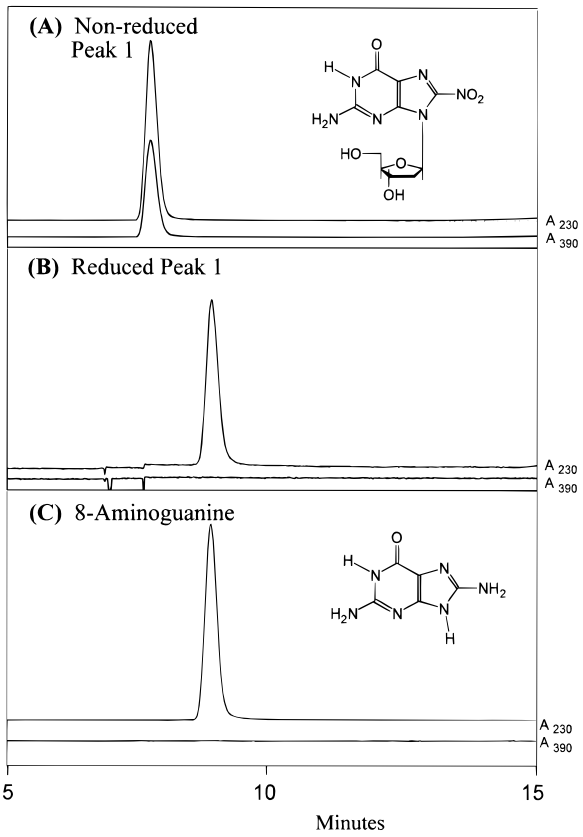


FIGURE 6: Reverse-phase HPLC analysis of peak 1 (A), reduced peak 1 (B), and hydrolyzed 8-aminoguanosine (C). 2-Deoxyguanosine was oxidized with the complete myeloperoxidase−H_2O_2−NO_2^- system as described in the legend to Figure 1. Peak 1 isolated from the reaction mixture by HPLC, peak 1 isolated from the reaction mixture by HPLC and reduced with dithionite, and 8-aminoguanosine subjected to formic acid treatment were then subjected to reverse-phase HPLC analysis with monitoring of absorbance at 230 and 390 nm as described under Methods.

of the nitrated guanosine molecular ion. Instead, the most likely location for the charge is an oxygen atom in the nitro group or carbonyl group of guanine. Scheme 1 rationalizes the ion chemistry of peak 2 observed during electrospray ionization tandem mass spectrometry. We were unable to rationalize the ion chemistry of peak 2 for other isomers of nitroguanine or for nitrosooxyguanine, suggesting that the C-8 position is the major target for nitration by the myeloperoxidase system.

8-Nitro-2′-deoxyguanosine Accounts Quantitatively for the Formation of Peak 1 by the Myeloperoxidase−H_2O_2−NO_2^- System. Chemical reduction of peak 1 with sodium dithionite (*55*) eliminated absorbance of the material at 390 nm and changed the HPLC retention time from 6.8 to 8.9 min (Figure 6A,B). Consistent with hydrolysis and reduction of a nitro group to an amino group, the reduced material comigrated with authentic 8-aminoguanosine (Figure 6C). Moreover, peak 1 was quantitatively converted into a compound that comigrated with 8-aminoguanine. These results confirm the mass spectrometric analyses, which suggested that peak 1 contained 8-nitro-2′-deoxyguanosine. Moreover, they indicate that 8-nitro-2′-deoxyguanosine is likely to account for most of the material in peak 1. Because peak 1 was quantitatively converted into peak 2 by formic acid, it is likely that most of the material in peak 2 is 8-nitroguanine (Scheme 2; next page). Therefore, the C-8 position of guanine appears to be the major target for nitration by the myeloperoxidase-H_2O_2−NO_2^- system.

Nitration of 2′-Deoxyguanosine by Myeloperoxidase Is Optimal at Neutral pH and Proceeds at Physiological Levels of NO_2^-. We used HPLC to investigate the optimal conditions for nitration of 2′-deoxyguanosine by myeloperoxidase, assessing the reaction as the sum of 8-nitroguanine (peak 2) and 8-nitro-2′-deoxyguanosine (peak 1) in the mixture. Addition of 20 nM myeloperoxidase to phosphate buffer (pH 7.4) containing 2′-deoxyguanosine, DTPA, H_2O_2, and NO_2^- led to nitration of 2′-deoxyguanosine. Production of 8-nitroguanine and 8-nitro-2′-deoxyguanosine increased rapidly over the first 10 min and gradually over the next 100 min (Figure 7A). Nitration of 2′-deoxyguanosine required each component of the complete myeloperoxidase−H_2O_2−NO_2^- system and was inhibited by heme poisons (3-aminotriazole and azide) and by catalase, a scavenger of H_2O_2 (Table 1). Nitration of 2′-deoxyguanosine was optimal at neutral pH (Figure 7D) and at physiologically plausible concentrations of H_2O_2 (100 μM), with a decrease in yield at higher oxidant concentrations (Figure 7B).

Plasma contains 1−50 μM NO_2^- under various inflammatory conditions (*56*−*59*). Nitration of 2′-deoxyguanosine by myeloperoxidase was directly proportional to the concentration of NO_2^- up to 100 μM. Higher concentrations resulted in less efficient nitration (Figure 7C).

Nitration of 2′-Deoxyguanosine by Myeloperoxidase Is Independent of HOCl. The in vitro studies outlined above were performed in phosphate buffer that lacked chloride ion. Previous studies have suggested that the reaction of HOCl with NO_2^- generates NO_2Cl, a nitrating and chlorinating intermediate (*24*, *27*). In addition, both chloride and NO_2^- bind to myeloperoxidase, and it is likely that their binding to the active site is competitive (*26*). We therefore investigated whether chloride ion affected the nitration of 2′-deoxyguanosine by the myeloperoxidase−H_2O_2−NO_2^- system. In contrast to the ion's inhibitory effect on tyrosine oxidation (*15*), chloride concentrations ranging from 30 μM to 300 mM had little effect on the myeloperoxidase-catalyzed nitration of 2′-deoxyguanosine (Figure 8A). These results suggest that chloride ion neither stimulates nor inhibits nitration by myeloperoxidase under our experimental conditions.

Two potent scavengers of HOCl, taurine and methionine (*9*), also failed to inhibit nitration of 2′-deoxyguanosine by the myeloperoxidase−H_2O_2−NO_2^- system supplemented with 100 mM chloride ion (as the sodium salt) (Figure 8B,C), even though their concentrations ranged from 10 μM to 1 mM, three times the concentration of H_2O_2 in the reaction mixture. Collectively, these results suggest that HOCl-dependent reactions are not major contributors to the NO_2^--dependent nitration of 2′-deoxyguanosine by myeloperoxidase.

Activated Human Neutrophils Employ the Myeloperoxidase−H_2O_2−NO_2^- System to Nitrate 2′-Deoxyguanosine. Reverse-phase HPLC analysis revealed that human neutrophils activated with β-phorbol myristate acetate in a physiological salt solution generated significant levels of 8-nitroguanine and 8-nitro-2′-deoxyguanosine (Table 2). Nitration of 2′-deoxyguanosine required the presence of cells, NO_2^-, and an activating stimulus. Addition of superoxide dismutase, which speeds the conversion of superoxide to

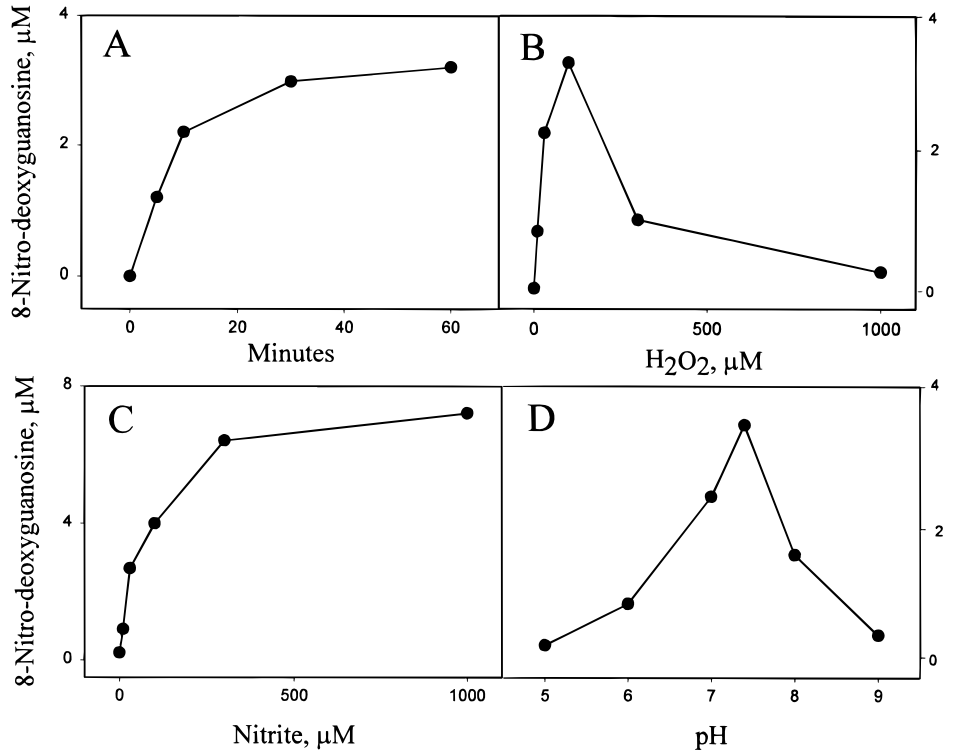
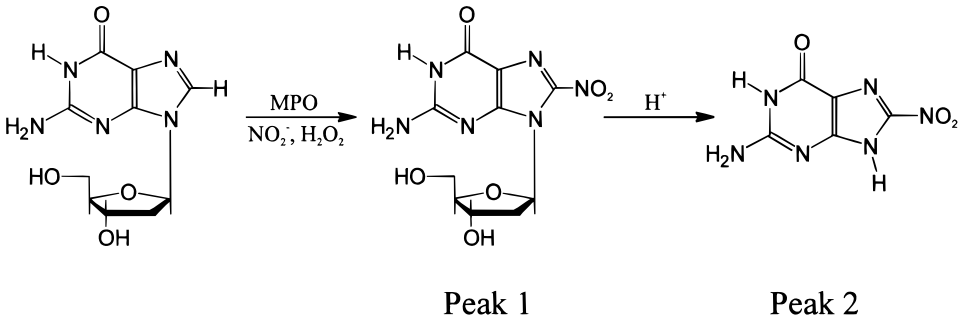


FIGURE 7: Oxidation of 2′-deoxyguanosine by the myeloperoxidase−H_2O_2−NO_2^- system. The reaction was initiated by the addition of myeloperoxidase (20 nmol) to 125 μL of buffer (pH 7.4) containing 2 mM 2′-deoxyguanosine, 100 μM DTPA, 100 μM H_2O_2, and 30 μM NO_2^-. At the indicated times (A) or after a 30 min incubation (B−D) at 37 °C, the reaction products were analyzed by reverse-phase HPLC. Conditions were varied by assaying the reaction mixture with the indicated final concentrations of H_2O_2 (B), NO_2^- ions (C), and hydrogen ions (D). 2′-Deoxyguanosine oxidation was terminated in the time course experiment by adding 200 nM catalase to the reaction mixture.

Scheme 2



H_2O_2, increased the yields of 8-nitroguanine and 8-nitro-2′-deoxyguanosine, perhaps by increasing the supply of peroxide or by protecting myeloperoxidase from inactivation (*5*). Nitration of 2′-deoxyguanosine by human neutrophils was inhibited by heme poisons (azide and cyanide) and by catalase. These results implicate the myeloperoxidase−H_2O_2−NO_2^- system in the cellular nitration of 2′-deoxyguanosine.

Reverse-phase HPLC was used to investigate product yield of 8-nitroguanine and 8-nitro-2′-deoxyguanosine in 2′-deoxyguanosine oxidized by activated human neutrophils. Neutrophils (7×10^5/mL) were incubated at 37 °C in medium supplemented with 2 mM 2′-deoxyguanosine and the indicated final concentration of NO_2^-. Following a 60 min incubation, the reaction was terminated by pelleting the cells by centrifugation, and the total product yield of 8-nitroguanine and 8-nitro-2′-deoxyguanosine in the supernatant was determined by reverse-phase HPLC. Nitration of 2′-deoxyguanosine by human neutrophils was directly proportional to the concentration of NO_2^- up to 100 μM. As with myeloperoxidase, higher concentrations resulted in less efficient nitration (Figure 9).

To confirm the identity of the products generated by activated neutrophils, we isolated the peak 1 and 2 materials from the reaction mixture by reverse-phase HPLC. After converting these products to their TMS derivatives, they were subjected to electron ionization GC/MS analysis. The full scan mass spectra were virtually identical to those of the compounds generated by the myeloperoxidase−H_2O_2−NO_2^- system. Moreover, the GC retention times and relative ion intensities of the major ions observed in peak 1 (Figure 10; *m*/*z* 325 ($[M - {}^{\bullet}CH_3]^+$) and *m*/*z* 340 ($M^{+\bullet}$) ions; compare with Figure 3A) and peak 2 (data not shown) were indistinguishable from those of peak 1 and peak 2 generated by the complete myeloperoxidase system. These results provide strong evidence that activated human neutrophils employ the myeloperoxidase−H_2O_2−NO_2^- system to generate 8-nitro-2′-deoxyguanosine.

Table 1: Requirements of the Myeloperoxidase−H_2O_2−NO_2^- System for Conversion of 2′-Deoxyguanosine into 8-Nitro-2′-deoxyguanosine[a]

condition	8-nitroguanine + 8-nitro-2′-deoxyguanosine (μM)
complete system	1.30
complete system minus	
myeloperoxidase	0.00
H_2O_2	0.00
NO_2^-	0.00
complete system plus	
NaCl (100 mM)	1.07
aminotriazole (20 mM)	0.00
azide (10 mM)	0.00
catalase (200 nM)	0.02
heat-inactivated catalase (200 nM)	1.00

[a] The complete system consisted of 20 nM myeloperoxidase, 2 mM 2′-deoxyguanosine, 30 μM NO_2^-, 100 μM DTPA, and 100 μM H_2O_2 in 50 mM sodium phosphate buffer (pH 7.4). The reaction was initiated by the addition of H_2O_2. After a 30 min incubation at 37 °C, the reaction was terminated with catalase, and the concentrations of 8-nitroguanine and 8-nitro-2′-deoxyguanosine were determined by HPLC analysis. Catalase was heat-inactivated by boiling for 10 min. Values are means of duplicate determinations and are representative of the results found in three independent experiments.

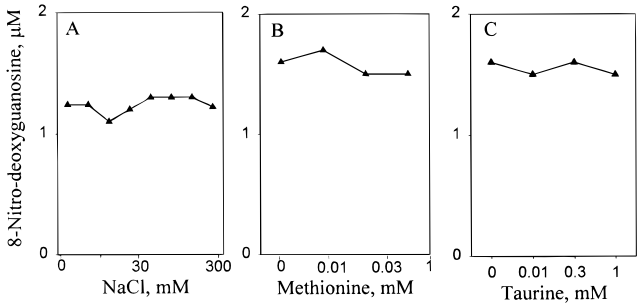


FIGURE 8: Effects of chloride (A), methionine (B), and taurine (C) on nitration of 2′-deoxyguanosine by the myeloperoxidase−H_2O_2−NO_2^- system. Reactions and HPLC were carried out as described in the legend to Figure 7. For reactions involving methionine or taurine, the reaction mixture was supplemented with 100 mM NaCl.

DISCUSSION

In this study, we investigated the ability of the myeloperoxidase−H_2O_2−NO_2^- system to generate reactive nitrogen intermediates that nitrate nucleic acids. Multiple lines of evidence implicate myeloperoxidase and NO_2^- in the production of 8-nitroguanine by activated phagocytes. First, when myeloperoxidase, H_2O_2, and NO_2^- oxidized 2′-deoxyguanosine, two major peaks seen during HPLC analysis exhibited absorption spectra characteristic of nitrated aromatic compounds. Electron ionization GC/MS analysis and electrospray ionization MS analysis of these peaks were consistent with the presence of nitroguanine adducts. Second, reduction of peaks 1 and 2 with dithionite yielded amino compounds as assessed by GC/MS analysis, strongly supporting the notion that the initial adduct was a nitro group. Third, HPLC analysis revealed that acid hydrolysis quantitatively converted peak 1 into peak 2, which in turn was quantitatively converted into 8-aminoguanine by dithionite. Electrospray ionization MS analysis suggested that peaks 1 and 2 represented 8-nitro-2′-deoxyguanosine and 8-nitroguanine, respectively, consistent with depurination of the nitrated nucleoside. Tandem mass spectrometric analysis of peak 2 strongly supported the structural assignment of the compound as 8-nitroguanine. These results indicate that the nitro group was located on C-8 of guanine and strongly suggest that 8-nitroguanine accounted for the majority of the modified base in peaks 1 and 2. Finally, activated neutrophils nitrated 2′-deoxyguanosine as assessed by HPLC and GC/MS analysis. The reaction required H_2O_2 and NO_2^- and was inhibited by heme poisons, implicating myeloperoxidase in the cellular pathway. Collectively, these results demonstrate that 8-nitroguanine and 8-nitro-2′-deoxyguanosine are products of 2′-deoxyguanosine oxidation by reactive nitrogen intermediates generated by myeloperoxidase.

Previous studies have suggested that myeloperoxidase generates reactive nitrogen intermediates from NO_2^- by two

Table 2: Requirements for the Conversion of 2′-Deoxyguanosine into 8-Nitro-2′-deoxyguanosine by Human Neutrophils[a]

condition	8-nitroguanine + 8-nitro-2′-deoxyguanosine (nM)
complete system	48
complete system minus	
phorbol myristate acetate	0
cells	0
NO_2^-	0
complete system plus	
azide (10 mM)	0
cyanide (10 mM)	0
superoxide dismutase (100 nM)	98
catalase (200 nM)	0

[a] The complete system consisted of 2 mM 2′-deoxyguanosine, 30 μM NO_2^-, 200 nM phorbol myristate acetate, and 7×10^5 cells/mL in Hank's buffered salt solution. Cells were incubated at 37 °C for 60 min and maintained in suspension by intermittent inversion. The reaction was terminated by pelleting the cells by centrifugation, and the concentrations of 8-nitroguanine and 8-nitro-2′-deoxyguanosine in the supernatant were determined by HPLC analysis. Values are means of duplicate determinations and are representative of the results found in three independent experiments.

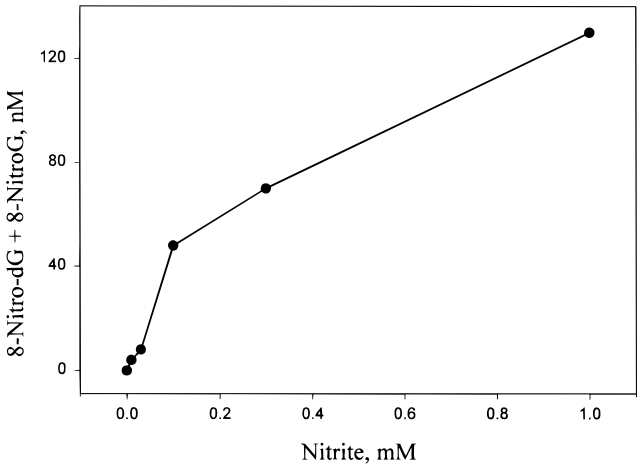


FIGURE 9: Product yield of 8-nitroguanine and 8-nitro-2′-deoxyguanosine production by activated human neutrophils. Neutrophils (7×10^5/mL) were incubated at 37 °C in Hank's buffered saline solution supplemented with 2 mM 2-deoxyguanosine and the indicated final concentration of NO_2^-. Following a 60 min incubation, the reaction was terminated by the addition of 200 nM catalase, the cells were pelleted by centrifugation, and the total product yields of 8-nitroguanine and 8-nitro-2′-deoxyguanosine in the supernatant were determined by reverse-phase HPLC as described under Methods: 8-nitro-2′-deoxyguanosine, 8-nitro-dG; 8-nitroguanine, 8-nitroG.

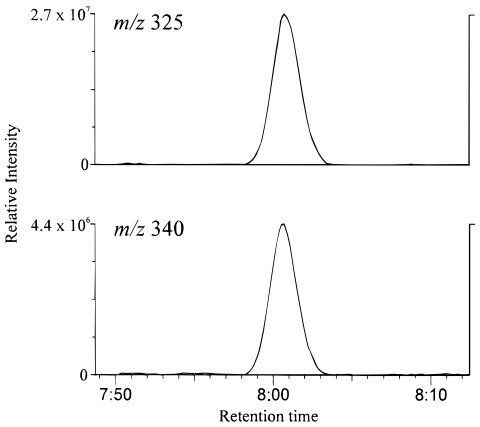


FIGURE 10: Positive ion chromatogram of the TMS derivative of 8-nitroguanine generated by activated human neutrophils. Peak 1 was isolated by reverse-phase HPLC from the medium of neutrophils incubated with phorbol myristate and NO_2^- as described in the legend to Table 2. The TMS derivative of peak 1 was then analyzed by electron ionization GC/MS with selected ion monitoring detection of ions at *m*/*z* 325 and 340.

distinct pathways (*24*−*27*). One involves the direct, one-electron oxidation of NO_2^- to $NO_2^\bullet$ by compound I, a complex of myeloperoxidase and H_2O_2. This pathway should not require chloride. The other involves the initial generation of HOCl by myeloperoxidase and the reaction of HOCl with NO_2^- to yield NO_2Cl , a nitrating and chlorinating intermediate. This pathway should be inhibited by sulfur- or amino-containing compounds that react with HOCl. We found that nitration of 2′-deoxyguanosine by myeloperoxidase was not dependent upon chloride ion and also was insensitive to scavengers of HOCl. Moreover, the oxidation potentials of NO_2^- and chloride ion are −0.99 and −1.36 V (*60*, *61*), respectively, suggesting that NO_2^- will be oxidized by myeloperoxidase. These observations indicate that a $NO_2^\bullet$-like species is likely to mediate the formation of 8-nitro-2′-deoxyguanosine by myeloperoxidase. Alternatively, 2′-deoxyguanosine might be oxidized directly by compound I to yield a radical species which subsequently reacts with NO_2^- or $NO_2^\bullet$ to generate the observed products. Indeed, certain oxidation reactions of myeloperoxidase take place at or near the sheltered active site (*1*−*5*). This might also represent a microenvironment inaccessible to low molecular weight thiols.

A key question is whether these reactive nitrogen products of myeloperoxidase damage biological targets in vivo. In healthy humans, the level of NO_2^- in plasma is 0.5−4 μM (*56*, *57*). Much higher levels are present in saliva, respiratory fluid, and gastric juice as well as in plasma during inflammation (*58*, *59*). Nitration of 2′-deoxyguanosine by myeloperoxidase was appreciable at plasma levels of NO_2^- and increased rapidly at higher concentrations of NO_2^-. These observations suggest that reactive nitrogen species generated by myeloperoxidase may execute oxidative reactions in vivo. One strategy for investigating this issue would be to analyze normal and inflamed tissue for stable end products of this pathway. The mass spectrometric methods we have developed for detecting 8-nitroguanine and 8-nitro-2′-deoxyguanosine should provide powerful tools for addressing this issue. We intend to adopt this approach to determine whether activated phagocytes can use the myeloperoxidase−H_2O_2−NO_2^- system to nitrate nucleic acids in cultured cells and animal tissues.

Another important issue is whether reactive nitrogen species generated by myeloperoxidase damage cellular DNA. Superoxide and $^\bullet NO$, the precursors of peroxynitrite, may be produced both intracellularly and extracellularly (*22*, *29*, *61*). In contrast, myeloperoxidase is a secreted heme protein that generates oxidants in the extracellular milieu (*1*, *2*, *5*). The reactive nitrogen species generated by the myeloperoxidase−H_2O_2−NO_2^- system may also be less effective than peroxynitrite at nitrating guanine. For the myeloperoxidase system, the yield of 8-nitro-2′-deoxyguanosine from 2′-deoxyguanosine was ∼0.01 mol/mol of H_2O_2. For a bolus addition of reagent peroxynitrite, the yield of 8-nitroguanine from guanine and peroxynitrite was 6-fold higher (∼0.06 mol/mol of peroxynitrite) (*48*). These studies may overestimate the yield of 8-nitroguanine from peroxynitrite; tyrosine is apparently nitrated much more effectively by peroxynitrite added as a bolus than by peroxynitrite generated continuously (*62*). Because DNA resides in the nucleus where it is surrounded by proteins and cellular antioxidants, these observations suggest that myeloperoxidase is unlikely to nitrate DNA directly. However, it is important to note that oxidized nucleotides are mutagenic in bacteria (*63*). Moreover, halogenated nucleobases, nucleosides, and nucleotides are incorporated into the DNA of cultured cells and tissues where they are mutagenic and clastogenic and induce sister-chromatid exchanges (*64*). It is therefore possible that nitration or chlorination of extracellular or cytosolic nucleic acids by myeloperoxidase-generated reactive intermediates may generate products that exert genotoxic or cytotoxic effects. It is also possible that peroxidases and heme proteins located close to the nucleus generate reactive nitrogen species that damage DNA directly.

Reactive phagocytic products may injure normal tissue under pathological conditions, and these species have been implicated in carcinogenesis, aging, and atherosclerosis (*28*, *29*, *65*−*67*). Moreover, myeloperoxidase is a component of human atherosclerotic tissue, where it co-localizes in part with tissue macrophages (*68*). The HOCl and tyrosyl radical it generates may be relevant to oxidative damage in the artery wall because elevated levels of 3-chlorotyrosine and dityrosine have been detected in this tissue (*69*, *70*). We also have shown that 3-nitrotyrosine levels are elevated in human atherosclerotic tissue (*71*). This raises the possibility that myeloperoxidase both nitrates and chlorinates host tissues in vivo.

Immunohistochemical detection of 3-nitrotyrosine in pathological disorders ranging from inflammatory bowel disease to rheumatoid arthritis to acute lung injury has been interpreted as evidence that the pathway for oxidative damage involves peroxynitrite, a product of the interaction of superoxide and NO. It is noteworthy that neutrophils, monocytes, and macrophages, all potential sources of myeloperoxidase, are present at high levels in these and other inflammatory conditions (*1*−*5*, *65*−*67*). In vitro studies suggest that myeloperoxidase will generate reactive nitrogen species such as $NO_2^\bullet$ and NO_2Cl, and demonstrate that activated human neutrophils nitrate tyrosine by a NO_2^--dependent pathway (*24*−*27*). Moreover, NO_2^- enhances bacterial killing by myeloperoxidase (*23*), and ingested bacteria undergo protein nitration in the phagolysosome (*72*), where high concentrations of H_2O_2 and myeloperoxidase are secreted. Our observations suggest that damage of nucleic

acids by myeloperoxidase may play a role in host defenses against invading pathogens. Collectively, these results provide strong evidence that multiple different pathways can potentially contribute to the generation of reactive nitrogen species and the nitration of biological targets in vivo.

In conclusion, we have demonstrated that myeloperoxidase can use NO_2^- and H_2O_2 to nitrate 2′-deoxyguanosine. The reaction appears to involve an $NO_2^{\bullet}$-like intermediate, and it proceeds at plasma concentrations of NO_2^- and chloride ion, suggesting potential physiological relevance. These observations, together with previous studies, raise the possibility that reactive nitrogen intermediates generated by myeloperoxidase and other peroxidases damage nucleic acids as well as proteins at sites of inflammation.

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