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ARTICLE *in* CHEMICAL RESEARCH IN TOXICOLOGY · FEBRUARY 1999

Impact Factor: 3.53 · DOI: 10.1021/tx980207u · Source: PubMed

Nitration of Unsaturated Fatty Acids by Nitric Oxide-Derived Reactive Nitrogen Species Peroxynitrite, Nitrous Acid, Nitrogen Dioxide, and Nitronium Ion

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Received August 31, 1998

Reactive nitrogen species derived from nitric oxide are potent oxidants formed during inflammation that can oxidize membrane and lipoprotein lipids in vivo. Herein, it is demonstrated that several of these species react with unsaturated fatty acid to yield nitrated oxidation products. Using HPLC coupled with both UV detection and electrospray ionization mass spectrometry, products of reaction of $ONOO^-$ with linoleic acid displayed mass/charge (*m*/*z*) characteristics of LNO_2 (at least three products at *m*/*z* 324, negative ion mode). Further analysis by MS/MS gave a major fragment at *m*/*z* 46. Addition of a NO_2 group was confirmed using [^{15}N]$ONOO^-$ which gave a product at *m*/*z* 325, fragmenting to form a daughter ion at *m*/*z* 47. Formation of nitrated lipids was inhibited by bicarbonate, superoxide dismutase (SOD), and Fe^{3+}−EDTA, while the yield of oxidation products was decreased by bicarbonate and SOD, but not by Fe^{3+}−EDTA. Reaction of linoleic acid with both nitrogen dioxide ($^{\bullet}NO_2$) or nitronium tetrafluoroborate (NO_2BF_4) also yielded nitrated lipid products (*m*/*z* 324), with HPLC retention times and MS/MS fragmentation patterns identical to the *m*/*z* 324 species formed by reaction of $ONOO^-$ with linoleic acid. Finally, reaction of HPODE, but not linoleate, with nitrous acid (HONO) or isobutyl nitrite (Bu^iONO) yielded a product at *m*/*z* 340, or 341 upon reacting with [^{15}N]HONO. MS/MS analysis gave an NO_2^- fragment, and ^{15}N NMR indicated that the product contained a nitro (RNO_2) functional group, suggesting that the product was nitroepoxylinoleic acid [$L(O)NO_2$]. This species could form via homolytic dissociation of LOONO to $LO^{\bullet}$ and $^{\bullet}NO_2$ and rearrangement of $LO^{\bullet}$ to an epoxyallylic radical $L(O)^{\bullet}$ followed by recombination of $L(O)^{\bullet}$ with $^{\bullet}NO_2$. Since unsaturated lipids of membranes and lipoproteins are critical targets of reactive oxygen and nitrogen species, these pathways lend insight into mechanisms for the formation of novel nitrogen-containing lipid products in vivo and provide synthetic strategies for further structural and functional studies.

Introduction

Nitric oxide ($^{\bullet}NO$)[1] is a free radical species that mediates a variety of biological actions, including blood pressure modulation, platelet activation, neurotransmission, and pathogen killing. The biological chemistry of $^{\bullet}NO$ is largely dominated by interaction with transition metals and radical−radical reactions, many of which can yield potent nitrating and nitrosating species. Under aerobic conditions, $^{\bullet}NO$ reacts rapidly with O_2 to form nitrogen dioxide ($\cdot NO_2$), a oxidizing and nitrating agent (eq 1, $k = 2 \times 10^6$ M^{-2} s^{-1}):

$$2^{\bullet}NO + O_2 \rightarrow 2^{\bullet}NO_2 \quad (1)$$

Reaction of $^{\bullet}NO_2$ with another $^{\bullet}NO$ produces dinitrogen trioxide (N_2O_3), a potent nitrosating agent. In the aqueous phase, N_2O_3 rapidly hydrolyzes to form two nitrite (NO_2^-) molecules (eq 2). Therefore, NO_2^- (discovered more than 100 years ago and recently described in ref *1*) is the major decomposition product of $^{\bullet}NO$ in aqueous buffers:

$$^{\bullet}NO_2 + {}^{\bullet}NO \rightarrow N_2O_3 + H_2O \rightarrow 2NO_2^- + 2H^+ \quad (2)$$

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[1] Abbreviations: $^{\bullet}NO$, nitric oxide; $^{\bullet}NO_2$, nitrogen dioxide; N_2O_3, dinitrogen trioxide; NO_2^-, nitrite; NO_3^-, nitrate; $O_2^{\bullet-}$, superoxide; $ONOO^-$, peroxynitrite; ONOOH, peroxynitrous acid; HONO, nitrous acid; $LOO^{\bullet}$, lipid peroxyl radical; $LO^{\bullet}$, lipid alkoxyl radical; LONO, nitritolinoleate; LNO_2, nitrolinoleate; $LONO_2$, linoleate nitrate; NO_2^+, nitronium ion; HODE, hydroxyoctadecadienoic acid; HPODE, hydroperoxyoctadecadienoic acid; NO_2BF_4, nitronium tetrafluoroborate; HPLC, high-pressure liquid chromatography; DTPA, diethylenetriaminepentaacetic acid; BHT, butylated hydroxytoluene; EDTA, ethylenediamenetetraacetic acid; SOD, superoxide dismutase; LC/MS, liquid chromatography/mass spectrometry; $L(O)^{\bullet}$, epoxyallylic radical.

10.1021/tx980207u CCC: $18.00
Published on Web 12/18/1998

In vivo, the predominant route for •NO removal from the vasculature is via reaction with oxyhemoglobin to form methemoglobin and nitrate (NO_3^-). In addition, recent evidence indicates that reaction between •NO and superoxide ($O_2^{\bullet-}$) also accounts for removal of a significant proportion of endogenous •NO (*2*, *3*) (eq 3). Reaction between •NO and $O_2^{\bullet-}$ to form peroxynitrite ($ONOO^-$) occurs at near diffusion-controlled rates and can outcompete superoxide dismutases for $O_2^{\bullet-}$ (*4*, *5*) (eq 3, $k = 6.7 \times 10^9$ M^{-1} s^{-1}; *4*):

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

Peroxynitrite anion ($ONOO^-$) and its conjugate acid, peroxynitrous acid (ONOOH, $pK_a = 6.8$ at 37 °C), are oxidizing species capable of reaction with a wide variety of biological targets, including lipids, thiols, amino acid residues, DNA bases, and low-molecular weight antioxidants (*6*−*10*). In addition, $ONOO^-$ can nitrate aromatic amino acids (e.g., tyrosine, tryptophan, and phenylalanine) in vitro and possibly in vivo (*11*−*13*). The half-life for $ONOO^-$ is pH-dependent, with the protonated form undergoing rapid decomposition to form NO_2^- and NO_3^- (*14*, *15*). Formation of a reactive intermediate with CO_2 (nitrosoperoxicarbonate anion, $ONOOCO_2^-$) leads to a redirection of $ONOO^-$ reactivity, thus enhancing nitration and limiting oxidation reactions (*16*−*20*). Reaction with CO_2 to form $ONOOCO_2^-$ lowers the half-life of $ONOO^-$ (*17*). Movement of $ONOO^-$ across intact erythrocyte membranes at pH 7.0 occurs by both passive diffusion of the protonated form, ONOOH, and transport of $ONOO^-$ through the anion channel (*21*, *22*), indicating that ONOOH can diffuse into hydrophobic lipid compartments.

Formation of reactive nitrogen species has been detected in cultured endothelial cells, human neutrophils, and murine macrophages (*23*−*25*). Evidence of their formation in vivo includes the detection of 3-nitrotyrosine in clinical problems such as atherosclerosis, sepsis, acute lung injury, and chronic organ rejection (*26*−*30*). Other •NO-dependent mechanisms can yield species capable of nitration and or nitrosation in vivo. For example, peroxidase-mediated oxidation of NO_2^- and reaction of NO_2^- with HOCl have been shown to result in tyrosine nitration via formation of $^{\bullet}NO_2$ and NO_2Cl, respectively (*31*, *32*). These reactions may be particularly important in inflammatory injury following phagocyte activation and secretion of myeloperoxidase. Also, acidification of NO_2^- forms nitrous acid (HONO) that in turn yields nitrosating species, a reaction possible in the low-pH environment of phagocytic lysosomes and the gastric compartment (*33*). Finally, nitrosation of several species with •NO, including tyrosyl, lipid peroxyl, and alkoxyl radicals, may occur (*34*, *35*).

Free and unesterified unsaturated fatty acids, such as arachidonate and linoleate, constitute major components of cell membranes, blood lipoproteins, and pulmonary surfactant. Oxidation of unsaturated lipids is a pivotal event in the pathogenesis of atherosclerosis; however, the mechanisms leading to lipid oxidation in vivo remain to be determined. Lipid oxidation also leads to disruption of membrane integrity and formation of both reactive intermediates and bioactive products, including peroxyl (LOO•), alkoxyl (LO•), and epoxyallylic radicals, lipid hydroperoxides, isoprostanes, and aldehydes. Reaction of •NO with peroxidizing lipid mixtures leads to termination of radical chain propagation reactions and formation of nitrogen-containing lipid species ($LONO/LNO_2$ and $LOONO/LONO_2$), the structural characteristics of which remain to be defined (*27*, *36*−*40*). In addition, reaction of linoleate and linolenate methyl esters with $^{\bullet}NO_2$ yields allylic nitro and nitrito derivatives (*41*, *42*). The nitration reactions of other biological •NO-derived products with unsaturated lipids are not well defined. Herein, we determine and compare nitration products formed upon reaction of $ONOO^-$, $^{\bullet}NO_2$, NO_2^+, or NO_2^-/HONO with linoleate, to understand mechanisms underlying their formation in vivo.

Experimental Procedures

Materials. All chemicals were from Sigma Chemical Co. (St. Louis, MO), unless otherwise stated. Linoleic acid was obtained from Nu-Chek Prep (Elysian, MN). 13-Hydroxy[*S*-(*E*,*Z*)]-9,11-octadecadienoic acid [13(*S*)HODE] and 13-hydroperoxy[*S*-(*E*,*Z*)]-9,11-octadecadienoic acid [13(*S*)HPODE] were from Cayman Chemical (Ann Arbor, MI). Nitronium tetrafluoroborate (NO_2BF_4) was from Aldrich Chemical Co. (Milwaukee, WI). HPLC grade diethyl ether was from Fluka Chemika (Buchs, Switzerland). HPLC grade acetonitrile, chloroform, and methanol were from Fisher Scientific (Pittsburgh, PA). Nitrogen dioxide (12 ppm in oxygen-free N_2) was from Scott Speciality Gases (Plumsteadville, PA). Sodium [^{15}N]nitrite was from Cambridge Isotope Laboratories (Andover, MA).

Peroxynitrite Treatment of Linoleic Acid. Peroxynitrite was synthesized from either sodium [^{14}N]- or [^{15}N]nitrite and acidified hydrogen peroxide using a quenched-flow reactor as previously described (*6*) and its concentration determined spectrophotometrically ($\epsilon_{302nm} = 1.67$ mM^{-1} cm^{-1}). Residual hydrogen peroxide was removed by treatment with granular manganese dioxide. Peroxynitrite in 100 μL of 0.1 M NaOH was infused at 100 μM/min into 2 mL of linoleic acid (3.8 mM) in 50 mM potassium phosphate (pH 7.4), containing sodium cholate (0.2%) and DTPA (100 μM), at 37 °C while the mixture was being stirred for 15 min using a Hamilton syringe and KD Scientific infusion pump (Fisher Scientific). To ensure that the pH was kept constant throughout the experiments, an equal volume of 0.1 M HCl was simultaneously infused using a parallel Hamilton syringe. Decomposed $ONOO^-$ was generated by neutralizing $ONOO^-$ with 1 M HCl at pH 7 and 20 °C for 10 min before infusion into linoleic acid mixtures. Since propagating radicals (e.g., LOO• and LO•) are likely to be present after $ONOO^-$ is gone, reactions were terminated by addition of 500 μM butylated hydroxytoluene (BHT), and samples were immediately cooled to 5 °C. Following acidification (pH 3), the internal standard (20 nmol of heptadecanoic acid) was added, and lipid products were immediately extracted with diethyl ether (2 × 2 volumes). Extracts were dried over anhydrous sodium sulfate (4 °C for 30 min), evaporated to dryness, and reconstituted in 200 μL of methanol for chromatographic analysis. For some samples, 5−20 mM bicarbonate, 0.5 mM Fe^{3+}−EDTA, or 10 μM superoxide dismutase (bovine, CuZn SOD, 4380 units/mg, OXIS International, Portland, OR) was added before $ONOO^-$ infusion. When Fe^{3+}−EDTA was added to samples, DTPA was omitted. When bicarbonate was added, dissolved CO_2 concentrations were determined using an Instrumentation Laboratory 1306 pH/blood gas analyzer (Lexington, MA) both before and after $ONOO^-$ infusion.

Nitration of Linoleic Acid with Nitronium Tetrafluoborate or Nitrogen Dioxide. Linoleic acid was oxidized and nitrated either by electrophilic substitution using nitronium tetrafluoroborate (NO_2BF_4) (*43*) or by free radical mechanisms using nitrogen dioxide ($^{\bullet}NO_2$) (*38*). Linoleic acid (100 mg, 0.36 mmol) was dissolved in chloroform (100 mL of $CHCl_3$) to a final concentration of 3.6 mM and placed in a round-bottom flask, and both the $CHCl_3$ and headspace were purged with anhydrous N_2 (99.999%) to remove oxygen. While the purging with N_2 was

being carried out, solid NO_2BF_4 (0.72 mmol) was added over the course of 15 min while the mixture was vigorously stirred. Then the headspace was purged with N_2 and the flask sealed with a glass stopper and stirred for 8 h at room temperature under N_2. Separately, linoleic acid (3.6 mM in $CHCl_3$) was deoxygenated with N_2 and bubbled with gaseous $^{\bullet}NO_2$ (12 ppm in oxygen-free N_2) for 24 h. In both cases, reactions were quenched with deionized H_2O (20 mL) to hydrolyze unreacted NO_2BF_4 and/or to separate contaminating anions (NO_2^-, NO_3^-, and BF_4^-) from the lipids. The biphasic solutions were transferred to separatory funnels, and 20 mL of saturated NaCl was added. Following acidification to pH 2−3 with 3 N HCl, the lipids were immediately extracted with $CHCl_3$ (3 × 50 mL). Pooled $CHCl_3$ extracts were then dried (24 h at 4 °C) over anhydrous sodium sulfate and evaporated to dryness in vacuo using a rotary evaporator. Following reconstitution into $CHCl_3$, samples were analyzed by reversed-phase HPLC and negative-ion electrospray ionization mass spectrometry.

Synthesis of 13(*S*)-Hydroperoxyoctadecadienoic acid [13(*S*)HPODE]. 13(*S*)-Hydroperoxyoctadecadienoic acid [13(*S*)-HPODE] was synthesized as previously described (*44*) with slight modification. Briefly, 100 mg of linoleate in 25 mL of 0.019 M NH_4OH was stirred for 30 min at 25 °C, following addition of 80 mL of soybean lipoxygenase 1 (1.6×10^6 units, Sigma) in 0.1 M borate buffer (pH 9.0). Then, the pH was adjusted to 3.0 and 20 g of NaCl added. Lipids were extracted twice into 150 mL of diethyl ether and dried using anhydrous $MgSO_4$, and solvent was removed in vacuo using rotary evaporation at 25 °C. The products were dissolved in diethyl ether and loaded onto a silicic acid column [4 g of equilibrated with diethyl ether/petroleum ether (1:9, v/v)]. Unreacted linoleate was eluted with 50 mL of diethyl ether/petroleum ether (1:9, v/v), and HPODE was eluted with diethyl ether/petroleum ether (1:4, v/v). Following removal of solvent using a stream of N_2, HPODE products were dissolved in 1 mL of methanol and stored at −80 °C under N_2. Product analysis using both normal-phase HPLC (Spherisorb S5W, Phase-Sep, 250 mm × 4.6 mm, 5 μm, with *n*-hexane/2-propanol/acetic acid, 100:2:0.1, v/v at 1 mL/min) and chiral-phase HPLC (Chiralcel OD, J. T. Baker, 250 mm × 4.6 mm, 5 μm, with *n*-hexane/2-propanol/acetic acid, 100:2:0.1, v/v at 1 mL/min) indicated the HPODE products were 97% 13(*S*)-HPODE and 3% 13(*R*)HPODE.

Reactions of Linoleic Acid or 13(*S*)HPODE with Nitrous Acid. Sodium nitrite (either [^{14}N] or [^{15}N]) was added to 2 mL of either 575 μM linoleate or 13(*S*)HPODE in 50 mM phosphate (pH 7.4), containing 0.2% sodium cholate. Following acidification to pH 3.0 using HCl and incubation at 25 °C for specified times, the internal standard (20 nmol of heptadecanoic acid) was added and lipids were extracted as described for $ONOO^-$-dependent reactions. Titrations were carried out by adjustment to the noted pH using HCl prior to NO_2^- addition. Following incubation, samples were extracted using diethyl ether without further acidification. The internal standard (20 nmol of heptadecanoic acid) was added before extraction to control for effects of pH variation on extraction yield. Samples were analyzed using both reversed-phase HPLC and LC/MS.

High-Pressure Liquid Chromatography of Linoleate Oxidation Products. For quantification of oxidized lipids, reaction products were separated by reversed-phase HPLC on a 150 mm × 4.6 mm i.d., 5 μm C_{18} column (Microsorb, Rainin, MA) using a gradient of 50 to 90% B over the course of 20 min (A is 75:25:0.1 v/v water/acetonitrile/acetic acid, B is 60:40:0.1 v/v methanol/acetonitrile/acetic acid) at a flow rate of 1 mL/min. Absorbance was monitored at 235 (conjugated dienes) and 205 nm (linoleic acid). Products were identified and quantified using 13(*S*)HPODE and 13(*S*)HODE standards from Cascade Biochemistry (Reading, England), with standard curves for both standards being linear over the concentration range examined, and between-day variation for 13(*S*)HPODE at 6%.

Liquid Chromatography/Mass Spectrometry. Mass spectroscopic analyses were performed on an API III triple-quadrupole mass spectrometer (PE-Sciex, Concord, ON) following reversed-phase HPLC on (i) a 100 mm × 2.1 mm i.d., Aquapore C_8 column (Perkin-Elmer Biosystems, Foster City, CA) with a linear 50 to 100% methanol gradient in 1% aqueous acetic acid at 0.2 mL/min. Under these conditions, nitration products with either *m*/*z* 324 or 340 eluted as a single peak, enabling integration and normalization with the internal standard or (ii) a 150 mm × 4.6 mm i.d., 5 μm C_{18} column (Microsorb) using a gradient of 50 to 90% B over the course of 20 min (A is 75:25:0.1 water/acetonitrile/acetic acid, B is 60:40:0.1 methanol/acetonitrile/acetic acid) at a flow rate of 1 mL/min. Using these conditions, groups of nitration products were separated (shown in Figure 1). The column eluent was split, with one-tenth going to the IonSpray interface. Negative ion mass spectra were recorded with an orifice potential of −60 V. Daughter ion mass spectra were obtained by selecting the parent molecular ion with the first quadrupole, colliding it with a mixture of 10% N_2-90% Ar in the second quadrupole and analyzing the fragment ions in the third quadrupole.

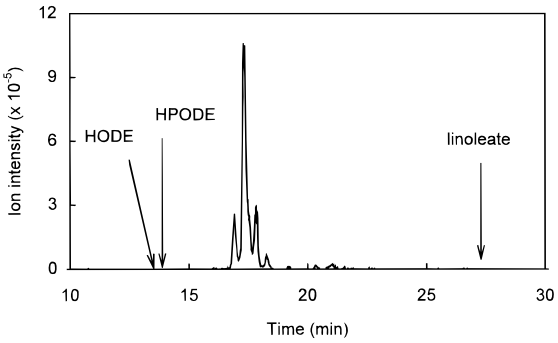


Figure 1. Elution profile of nitrated linoleate species by LC/MS. Following reaction of 3.8 mM linoleate with 100 μM/min $ONOO^-$ over the course of 15 min and solvent extraction, products were separated by reversed-phase HPLC using a C_{18} column and monitored using negative ion electrospray ionization mass spectrometry. Products with ions at *m*/*z* 324 are shown. The retention times of other major constituents of this sample are indicated by arrows: HPODE, hydroperoxyoctadecadienoic acid; HODE, hydroxyoctadecadienoic acid.

^{15}N Nuclear Magnetic Resonance Spectroscopy. $^{15}NO_2^-$ (2 mM) was added to 75 mL of 575 μM 13(*S*)HPODE in 50 mM phosphate (pH 3) containing 0.2% sodium cholate. Following incubation at 25 °C for 15 min, lipids were extracted using diethyl ether and dried in vacuo. The sample was dissolved in 500 μL of methanol-d_4 with 4 mM $GdCl_3$ added to decrease the nitrogen relaxation time. The spectrum was collected on a Bruker AM600 spectrometer with the following acquisition parameters: 131 072 scans, 1 s recycle time, composite-pulse decoupling during the acquisition time (0.33 s), 30° pulse width, 6097 Hz spectral width, and 278 K; results are displayed as the chemical shift, and not chemical shielding, which has the opposite sign (*45*, *46*). Chemical shifts were referenced to 10% nitromethane in methanol. The ^{15}N sensitivity of the AM-600 NMR system was confirmed using a solution of nitromethane in methanol.

Results

Peroxynitrite Reaction with Linoleic Acid. Infusion of [^{14}N]$ONOO^-$ into linoleate emulsified in cholate/DTPA-containing phosphate buffer led to formation of both oxidized and nitrated products. Significant quantities of hydroperoxyoctadecadienoic (HPODE, *m*/*z* 311) and hydroxyoctadecadienoic (HODE, *m*/*z* 295) acids were identified by LC/MS and quantified using standards on reversed-phase HPLC with UV detection at 234 nm (Table 1). Negative-ion electrospray ionization MS revealed multiple products characteristic of nitritolinoleate (LONO) or nitrolinoleate (LNO_2), e.g., with *m*/*z* 324, eluting just after HPODE, at 16.5−18.5 min (Figures 1

Table 1. Influence of Fe^{3+}−EDTA or CuZn SOD on the Yield of Peroxynitrite-Induced Linoleate Nitration and Oxidation Products[a]

	HODE (nmol)	HPODE (nmol)	LNO_2 (*m/z* 324)
control	6.44	184	0.78
Fe^{3+}−EDTA (0.5 mM)	16.8	256	0.07
SOD (10 μM)	1.17	71.9	0

[a] Peroxynitrite was infused (100 μM/min over the course of 15 min) into 2 mL of 3.8 mM linoleate, and products were separated using a C_{18} column (oxidation products, detected using absorbance at 235 nm) or a C_8 column (nitration products, detected using negative ion electrospray mass spectrometry). Oxidized products (HODE and HPODE) were quantified by integration of peak areas and comparison with standards. Standard curves for both were linear over the concentration range examined, with between-day variation for 13(*S*)HPODE at 6%. Since no standards are available for nitrated lipids, comparisons between samples were made by normalizing total integrated peak area for the *m/z* species of interest with that of an internal standard (heptadecanoic acid, *m/z* 269). When Fe^{3+}−EDTA was added, DTPA was omitted. Results of a typical experiment which was replicated at least three times are shown.

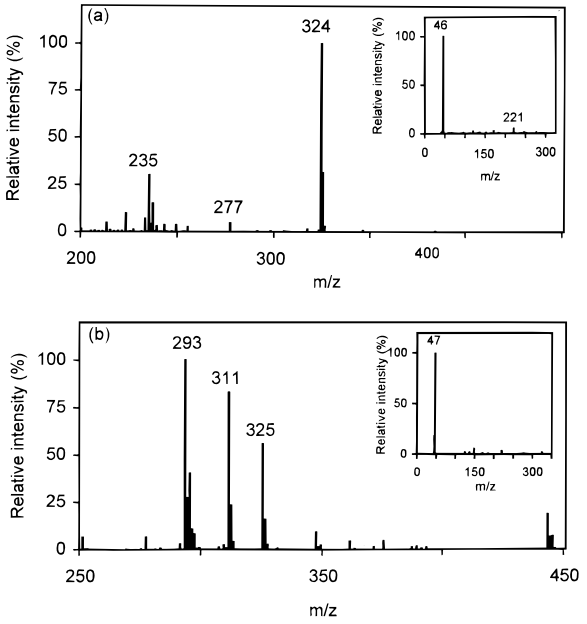


Figure 2. Mass spectra and MS/MS fragmentation patterns of LNO_2 derived from reaction of $ONOO^-$ with linoleate. Samples prepared as described in the legend of Figure 1 using either $ONOO^-$ (a) or [^{15}N]$ONOO^-$ (b) were chromatographed using reversed-phase HPLC, and elution was monitored using negative ion electrospray ionization mass spectrometry. (a) Representative mass spectrum during elution of a species with an ion at *m/z* 324. (Inset) MS/MS fragmentation pattern of the species with an ion at *m/z* 324 shown in panel a. (b) Representative mass spectrum during elution of a species with an ion at *m/z* 325. (Inset) MS/MS fragmentation pattern of the species with an ion at *m/z* 325 shown in panel b.

and 2a). MS/MS fragmentation yielded a major peak at *m/z* 46, suggestive of NO_2^- (Figure 2 inset). To confirm that these species contain an NO_2^- group, [^{15}N]$ONOO^-$ was synthesized and reacted with linoleate. Here, the products had an ion at *m/z* 325 and fragmented by MS/MS to give a daughter ion at *m/z* 47 (Figure 2b). These data indicate that products at *m/z* 324 are nitrated lipids, containing the N atom of $ONOO^-$. When [^{14}N]$ONOO^-$ was decomposed by neutralizing to pH 7 before infusion and subsequent lipid extraction under acidic conditions, no oxidation or nitration products were observed (Figure 3). A second nitration product (*m/z* 340) was also observed

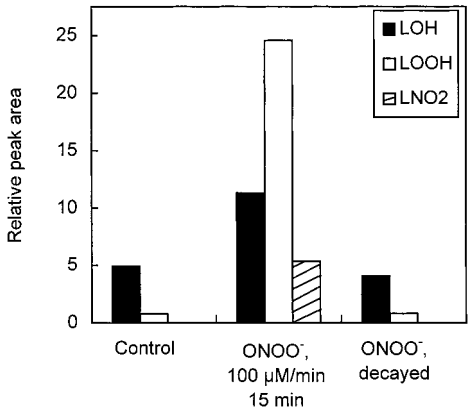


Figure 3. Comparison of total ion intensity of the major $ONOO^-$-dependent oxidation and nitration products of linoleate. Following reaction of $ONOO^-$ with linoleate and organic extraction (as described in the legend of Figure 1), samples were separated by reversed-phase HPLC and monitored using negative ion electrospray ionization mass spectroscopy. Normalized area refers to total integrated ion intensity eluting from the column for each species with the noted *m/z*, relative to the internal standard (heptadecanoic acid). Decayed $ONOO^-$ was prepared by decreasing the pH to 7, for 10 min.

in these reaction mixtures (not shown). Control experiments demonstrated that this species was not a direct product of reaction of $ONOO^-$ with linoleate, rather most likely originated from reaction of NO_2^-, a product of both $ONOO^-$ synthesis and decomposition, with the LOOH formed by $ONOO^-$ oxidation of linoleate during acidic extraction (see below for characterization of this reaction). Control experiments determined that omission of BHT during extractions gave greater quantities of oxidation products with no effect on nitration product yields.

Effect of Fe^{3+}−EDTA, SOD, and Bicarbonate on $ONOO^-$-Mediated Nitration of Linoleate. In the presence of Fe^{3+}−EDTA, increases in both HPODE and HODE yields were observed; however, the degree of formation of nitrated lipid species was significantly decreased (Table 1). Inclusion of 10 μM SOD led to decreased HODE and HPODE yields, while nitrated products were undetectable (Table 1). Addition of 5−20 mM bicarbonate to lipid reaction systems before $ONOO^-$ infusion also led to significant decreases in the yields of oxidation products and the extent of inhibition of nitrated product formation (Table 2).

Nitration of Linoleate by Nitronium Tetrafluoroborate or Nitrogen Dioxide. To gain insight into mechanisms of $ONOO^-$-mediated fatty acid nitration, products of reaction of linoleate with NO_2BF_4 and $^{\bullet}NO_2$ were examined. Following reaction of linoleate with NO_2BF_4, a group of $LONO/LNO_2$ products with ions at *m/z* 324 (three or four peaks) were eluted by HPLC with retention times (15−18 min) similar to those of $ONOO^-$-derived *m/z* 324 products. These species also yielded an ion at *m/z* 46 by MS/MS analysis (Figure 4a). For comparison, reaction of linoleate with $^{\bullet}NO_2$ also resulted in formation of $LONO/LNO_2$ (*m/z* 324) products that eluted with retention times similar to those of both $ONOO^-$ and NO_2BF_4-derived products. MS/MS fragmentation also gave a major *m/z* 46 peak (Figure 4b). No oxidation products were formed under these experimental conditions, since all reactions were conducted anaerobically (not shown).

pH-Dependent Reaction of NO_2^-/HONO with 13(*S*)-HPODE. While HONO or NO_2^- did not directly nitrate

Table 2. Influence of Bicarbonate on the Yield of Peroxynitrite-Induced Linoleate Nitration and Oxidation Products[a]

sample	CO_2 (mM) at start	CO_2 (mM) at end	oxidized product (nmol/sample) HODE	oxidized product (nmol/sample) HPODE	nitrated product (normalized to standard) LNO_2 (*m/z* 324)
$ONOO^-$	0.16	0.16	2.78	146	0.78
$ONOO^-$ and 5 mM bicarbonate	0.50	0.43	2.85	117	0
$ONOO^-$ and 10 mM bicarbonate	0.62	0.50	2.26	80	0
$ONOO^-$ and 20 mM bicarbonate	0.95	0.60	1.66	50	0

[a] Peroxynitrite was infused (100 μM/min over the course of 15 min) into 2 mL of 3.8 mM linoleate, and products were separated by HPLC using a C_{18} column (oxidation products) or a C_8 column (nitration products). The carbon dioxide (CO_2) concentration was determined using a blood gas analyzer in samples both before and after $ONOO^-$ addition. Oxidized products (HODE and HPODE) were quantified by integration of peak areas and comparison with standards. Since no standards are available for nitrated lipids, comparisons between samples were made by normalizing total integrated peak areas for the *m/z* 324 species with that of an internal standard (heptadecanoic acid, *m/z* 269). Results of a typical experiment which was replicated at least three times are shown.

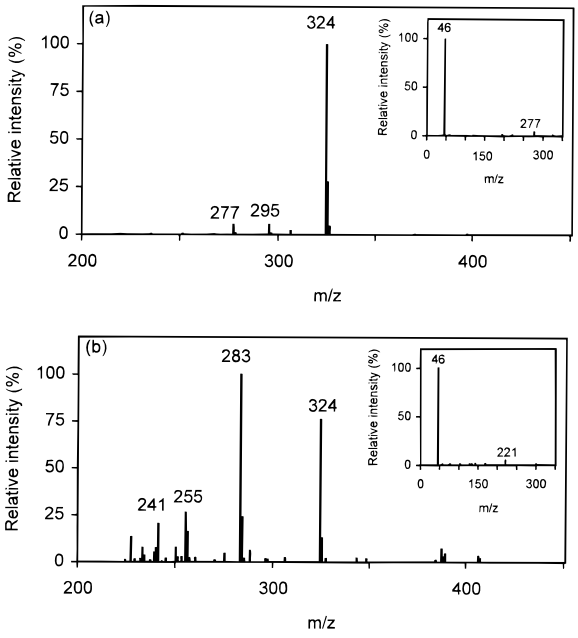


Figure 4. Mass spectra and MS/MS fragmentation patterns of lipid products derived from reaction of NO_2BF_4 or $^{\bullet}NO_2$ with linoleate. Samples (prepared as described in Experimental Procedures) were separated by reversed-phase HPLC, and elution was monitored using negative ion electrospray ionization mass spectrometry. (a) Mass spectrum during elution of a species with an ion at *m/z* 324 formed from reaction of NO_2BF_4 with linoleate. (Inset) Representative MS/MS fragmentation pattern of the species with anion at *m/z* 324 shown in panel a. (b) Mass spectrum during elution of a species with an ion at *m/z* 324 formed from reaction of $^{\bullet}NO_2$ with linoleate. (Inset) Representative MS/MS fragmentation pattern of the species with an ion at *m/z* 324 shown in panel b.

or oxidize linoleate (data not shown), reaction with HPODE yielded a series of products having ions at *m/z* 340 [e.g., LOONO, $LONO_2$, or $L(O)NO_2$] that eluted at 10−12 min and fragmented on MS/MS to give a major peak at *m/z* 46 (Figure 5a). Experiments in which $^{15}NO_2^-$ was used yielded products with ions at *m/z* 341

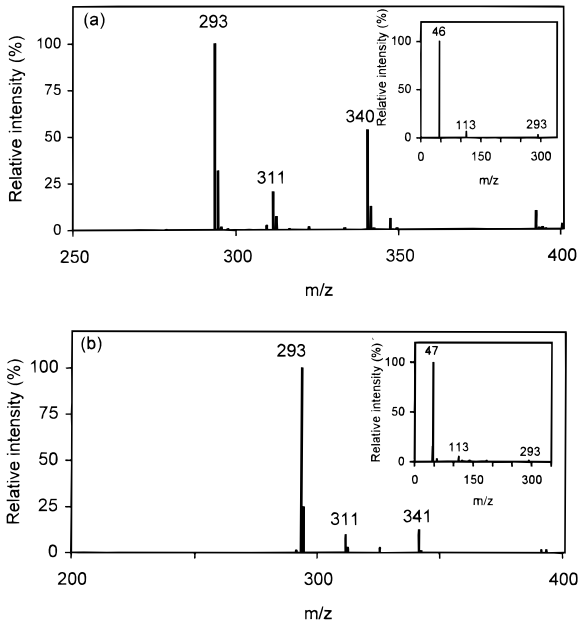


Figure 5. Mass spectra and MS/MS fragmentation patterns of lipid products derived from reaction of HONO with linoleate. Samples were prepared as described in Experimental Procedures utilizing a 10 min incubation at pH 3 with NO_2^- (a) or $^{15}NO_2^-$ (b). Products were separated by reversed-phase HPLC and monitored using negative ion electrospray ionization mass spectrometry. (a) Mass spectrum during elution of a species with an ion at *m/z* 340. (Inset) MS/MS fragmentation pattern of the species with an ion at *m/z* 340 shown in panel a. (b) Mass spectrum during elution of a species with an ion at *m/z* 341. (Inset) MS/MS fragmentation pattern of the species with an ion at *m/z* 341 shown in panel b.

which fragmented on MS/MS to give a daughter ion at *m/z* 47 (Figure 5b). These data confirm that these products are nitrated lipids, fragmenting on MS/MS to give a NO_2^- daughter ion. The degree of formation of these species was concentration-dependent for NO_2^- up to at least 500 μM (Figure 6a) and pH-dependent, requiring a pH of ≤4 (Figure 6b).

To further examine the structure of nitrogen-containing oxidized lipid products, ^{15}N NMR was performed on samples prepared using [^{15}N]HONO. Since NMR is typically 1000−10000 times less sensitive than mass spectrometry, reaction volumes were scaled up to obtain sufficient material for analysis. Since a mixture of lipid products was present, it was not possible to utilize more sensitive ^1H NMR structural determination. Analysis of products of reaction of [^{15}N]HONO with 13(*S*)HPODE in methanol-d_4 by ^{15}N NMR showed one major species with a chemical shift of 16.0 ppm relative to nitromethane, suggesting the presence of a NO_2 group directly bonded to a carbon (e.g., $C-NO_2$, Figure 7). Minor components at 13−14 ppm are also visible. If samples were analyzed in $CdCl_3$, the chemical shift was observed at 14.0 ppm (not shown). No signals were found elsewhere in the spectrum, particularly around −40 to −50 ppm, or 180−210 ppm, where alkyl nitrates ($RONO_2$) and alkyl nitrites (RONO), respectively, are typically detected. The sensitivity of the ^{15}N NMR spectra is not what is expected for typical labeled nitro compounds, indicating a low final concentration of the ^{15}N-containing reaction product.

Discussion

Reactions of linoleate with species that form in biological systems following secondary reactions of $^{\bullet}NO$ yielded

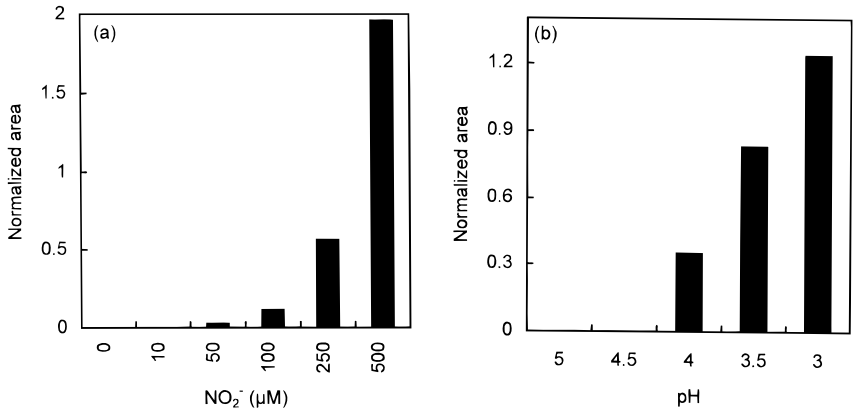


Figure 6. Concentration and pH dependence of HONO nitration of LOOH. Samples (prepared as described in Experimental Procedures) were chromatographed using a C_{18} reversed-phase column, and the elution of products was monitored using negative ion electrospray ionization mass spectrometry. Normalized area refers to total integrated ion intensity eluting from the column for an ion at *m*/*z* 340, relative to the internal standard (heptadecanoic acid). (a) The concentration of NO_2^- was varied from 0 to 500 μM, and samples were incubated for 10 min at pH 3 before extraction. (b) The pH was varied from 3 to 5, and samples were incubated for 2 h with 500 μM NO_2^- before extraction.

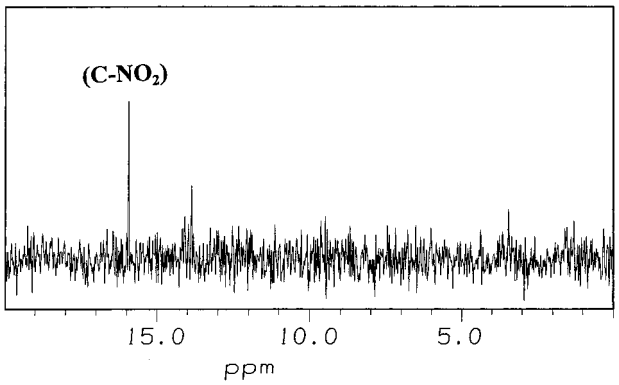


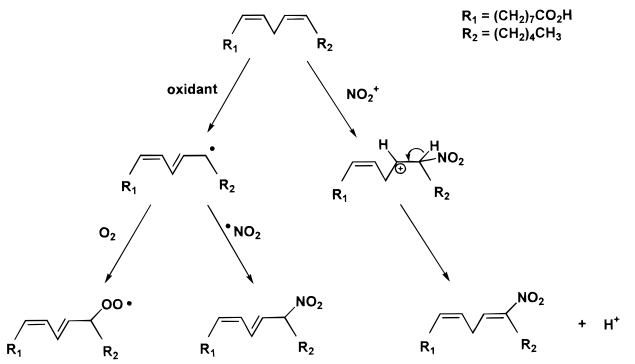
Figure 7. ^{15}N NMR spectrum of products of reaction of [^{15}N]-HONO with 13(*S*)HPODE. Sample preparation and spectral acquisition parameters were as described in Experimental Procedures.

two main nitration product groups, LNO_2 (formed by reaction of $ONOO^-$, $^\bullet NO_2$, or NO_2^+ with linoleate) and $L(O)NO_2$ [formed by reaction of HONO with 13(*S*)-HPODE].

Reaction of peroxynitrite with unsaturated lipids yielded at least three nitrated products, all with ions at *m*/*z* 324, designated LONO/LNO_2. These likely represent a mixture of stereo- and positional isomers (i.e., at C_9 or C_{13}, and/or cis−trans isomers) or different functional group orientations (e.g., ONO or NO_2). It was not possible to determine whether products were allylic nitrite or nitro compounds (e.g., LONO or LNO_2, respectively) from the mass spectroscopic data. Since it is known that allylic nitrites hydrolyze to alcohols at low pH, such as that used for the extraction and chromatography conditions herein (*42*), it is likely that *m*/*z* 324 products from $ONOO^-$ attack on linoleate observed in our experiments are nitro derivatives, LNO_2. Further structural characterization will resolve these issues.

Since $ONOO^-$ nitration reactions may result from either $^\bullet NO_2$- or NO_2^+-dependent mechanisms (*24*), products from both of these nitration strategies were compared with product profiles from reaction of $ONOO^-$ with linoleate. Nitronium (NO_2^+) salts have been extensively used for nitration of olefinic compounds (*43*). These reactions proceed under anhydrous conditions by electrophilic substitution to give the corresponding nitro and

Scheme 1. Mechanisms of Linoleate Nitration for Forming LNO_2[a]



[a] Nitration of linoleate to form LNO_2 (*m*/*z* 324) may occur by either $^\bullet NO_2$ (oxidation of LH followed by $^\bullet NO_2$ addition) or NO_2^+ (direct addition of NO_2^+ followed by deprotonation)-dependent mechanisms.

nitrito derivatives. Nitration of methyl linoleate and linolenate by $^\bullet NO_2$ proceeds via initial hydrogen abstraction to form a carbon-centered alkyl radical. At low oxygen tensions, this combines with $^\bullet NO_2$, after rearrangement to a conjugated diene, to form allylic nitro (RNO_2) products (Scheme 1; *41*, *42*). Using negative ion electrospray ionization mass spectrometry, a series of products with ions at *m*/*z* 324 that behaved in a manner identical to that of $ONOO^-$-derived products (*m*/*z* 324), with respect to LC retention time and MS/MS fragmentation, were observed following reaction of either $^\bullet NO_2$ or NO_2BF_4 with linoleic acid. Since products from either nitration strategy were analogous with respect to HPLC retention time and MS/MS fragmentation, it was concluded that either NO_2^+ or $^\bullet NO_2$-dependent mechanisms could be responsible for $ONOO^-$-mediated fatty acid nitration. One additional mechanism that could occur during $ONOO^-$ reaction is addition across a double bond to form a hydroxy nitrite ester, as observed for 2′-deoxyguanosine (*47*). Interestingly, extremely low yields of a product with an ion at *m*/*z* 342, consistent with this mechanism, were observed in these experiments (not shown). This suggested that addition reactions of $ONOO^-$ were also occurring.

As an alternative strategy for differentiating between either $^\bullet NO_2$- or NO_2^+-dependent mechanisms of $ONOO^-$

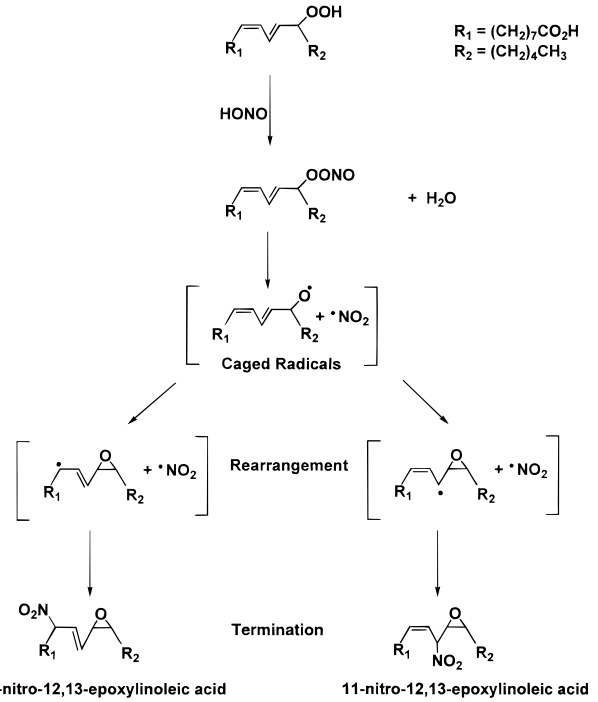
nitration, experiments were conducted in the presence of either Fe^{3+}−EDTA or CuZn SOD. Peroxynitrite-mediated nitration of phenolic compounds such as tyrosine and (4-hydroxyphenyl)acetic acid in aqueous media is enhanced by either Fe^{3+}−EDTA or CuZn SOD (*11*). These metal complexes enhance phenolic nitration by mediating electron withdrawal from the nitrogen of $ONOO^-$ and favoring heterolytic cleavage to a nitronium (NO_2^+)-like species that then nitrates phenols. During this process, nitration of tyrosine residues of SOD can result (*48*). In the presence of 0.5 mM Fe^{3+}−EDTA, the total yield of $ONOO^-$-dependent oxidation products (HPODE and HODE) was increased. This is likely due to metal-catalyzed decomposition of HPODE, which leads to formation of secondary products, that include HODE and HPODE. In contrast, Fe^{3+}−EDTA inhibited nitration of linoleate by $ONOO^-$. Addition of 10 μM CuZn SOD to reaction mixtures potently inhibited both oxidation and nitration of linoleic acid. Our observation of a decreased level of linoleate nitration in the presence of either Fe^{3+}−EDTA or CuZn SOD may suggest that this nitration reaction is mediated by $^{\bullet}NO_2$, rather than by NO_2^+. Alternatively, it is possible that complex formation between $ONOO^-$ and either Fe^{3+}−EDTA or CuZn SOD decreases the accessibility of $ONOO^-$ for hydrophobic linoleate oxidation sites buried within micellar structures.

Bicarbonate (5−20 mM, which gave a CO_2 concentration of 0.4−0.9 mM) inhibited oxidation and nitration of linoleate by $ONOO^-$. Formation of a CO_2−$ONOO^-$ adduct ($ONOOCO_2^-$) leads to a redirection of $ONOO^-$ reactivity, favoring nitration of phenolics and partially inhibiting oxidation reactions (*17*, *18*). Since $ONOOCO_2^-$ is a charged anion (in contrast to $^{\bullet}NO_2$, the most likely nitrating species in these experiments) and decomposes considerably faster than $ONOO^-$ (*17*), diffusion into lipid compartments may be restricted.

Reaction of $^{\bullet}NO_2$ with linoleate to form LNO_2 occurs following hydrogen abstraction by $^{\bullet}NO_2$ to form L$^{\bullet}$. Addition of a second $^{\bullet}NO_2$ gives the product LNO_2 (Scheme 1; *41*, *42*). Biologically, $^{\bullet}NO_2$ formation from $^{\bullet}NO$ will occur where O_2 concentrations are highest, for example, in the lung epithelial lining fluid. Alternatively, generation of $^{\bullet}NO_2$ from oxidation of NO_2^- by peroxidases, such as myeloperoxidase, also mediates nitration reactions (*23*). However, since $^{\bullet}NO_2$ can terminate oxygen-centered radicals [for example, it will react with ROO$^{\bullet}$ or RO$^{\bullet}$ to form $ROONO_2$ or $RONO_2$, respectively (*49*)], lipid propagation reactions will be inhibited, resulting in an overall low yield of oxidation products.

The major product of $^{\bullet}NO$ formed by nitric oxide synthase (NOS) enzymes in vivo is nitrite (NO_2^-). While this species is stable at physiological pH, acidification yields nitrous acid (HONO), that in turn generates a complex mixture of oxidizing and nitrating/nitrosating intermediates (e.g., N_2O_3, $^{\bullet}NO$, $^{\bullet}NO_2$, and N_2O_4). Reaction of linoleate hydroperoxide (LOOH), but not linoleate, with HONO led to formation of a species with an ion at *m*/*z* 340, suggesting several possible structures, including LOONO, $LONO_2$, or L(O)NO_2. Initial nitrosation of LOOH during HONO decomposition likely yields LOONO, as noted for nitrosation of ROOH (*tert*-butyl or cumene hydroperoxide) by $^{\bullet}NO_2/N_2O_4$ (*50*). However, organic peroxynitrites are generally unstable with first-order rate constants for decomposition to caged radicals, LO$^{\bullet}$ and $^{\bullet}NO_2$, of 0.1−0.3 s^{-1}, which can recombine to form $LONO_2$

Scheme 2. Mechanism of L(O)NO_2 Formation from Reaction of HONO with LOOH



($k = 1.68 \times 10^9$ M^{-1} s^{-1}; *51*, *52*). Unsaturated lipid alkoxyl radicals (LO$^{\bullet}$) rearrange rapidly to yield carbon-centered epoxyallylic radicals [L(O)$^{\bullet}$, Scheme 2; *53*−*55*]. It is therefore possible that the *m*/*z* 340 species forms through termination of the epoxyallylic radical by $^{\bullet}NO_2$ (formed via either HONO or LOONO decomposition) to yield R(O)NO_2. Two lines of evidence support this concept.

The major daugher ion on MS/MS analysis is at *m*/*z* 46, characteristic of NO_2^-. This ion also forms on MS/MS analysis of nitrolinoleate (LNO_2) formed from reaction of either $ONOO^-$, $^{\bullet}NO_2$, or NO_2^+ with linoleate. In contrast, MS/MS fragmentation of nitrates such as glyceryl mononitrate ($RONO_2$) yields a daughter ion at *m*/*z* 62, characteristic of a NO_3^- leaving group, with no daughter ion visible at *m*/*z* 46 (*56*).

^{15}N NMR analysis of [^{15}N]HONO-treated HPODE shows a species with a chemical shift at 16.0 ppm in methanol or 14.0 ppm in chloroform, referenced to nitromethane. These are similar to those of other nitro compounds, for example, ethyl-NO_2 (4.1−11 ppm), *n*-propyl-NO_2 (3.8−10.1 ppm), isopropyl-NO_2 (14.7−19.5 ppm), and *tert*-butyl-NO_2 (21.8−28.2 ppm) in various solvents (*46*). In contrast, nitrates such as $MeONO_2$ (in methanol) or β-cellobiose octanitrate (in acetone) show a chemical shift at −35 to −50 ppm and nitrites such as methyl-ONO or *tert*-butyl-ONO (in methanol, DMSO, or chloroform) show a chemical shift at 180−205 ppm. Finally, HONO and $NaNO_2$ show chemical shifts of 180.5 and 227.6 ppm, respectively (*46*).

Interestingly, we have also seen this product (identical retention time, *m*/*z* and MS/MS daughter ion at *m*/*z* 46, not 62) form following reaction of *iso*-butyl-ONO with LOOH (data not shown), another reaction which is expected to proceed via initial generation of LOONO. This reinforces the concept that LOONO formed in unsaturated hydrocarbons rearranges to nitro species. Nitrosation of LOOH by HONO-derived RNS appeared to require a pH of ≤4.0. However, since these samples could not be

acidified before organic solvent extraction, a reduced degree of partitioning of products into the organic phase is likely. Thus, it remains unclear if the absence of detection of HPODE nitration above pH 4.0 is due to the lack of formation of nitrogen-containing products or a reduction in extraction efficiency. At least two biological compartments will experience pH conditions low enough to support HONO formation from NO_2^- in vivo. In the gastric compartment, pH varies from 2.5 to 4.5 during different stages of digestion (*33*) and the pH of neutrophil phagocytic vesicles has been reported to be as low as pH 3.0 (*57*). Nitrite concentrations in vivo range from 0.5 to 3.6 μM in plasma (*58*), 15 μM in respiratory tract lining fluid (*59*), and 30−210 μM in saliva (*60*), making HONO formation likely in many tissue compartments, especially during periods of excessive NO_2^- production (e.g., sepsis or inflammation).

Nitration of unsaturated lipid to form LOONO/L(O)-NO_2 or LONO/LNO_2 during $^{\bullet}$NO inhibition of peroxidation has been shown (*37*, *61*). Comparison of HPLC retention times and *m*/*z* values for products identified in these studies with those demonstrated herein indicates that these are likely to be the same groups of compounds. This is not unexpected, since the proposed mechanisms of nitrated product formation involving either $^{\bullet}$NO termination reactions or $ONOO^-$ nitration can converge at the following points.

(i) The initial product of HONO attack on LOOH will be LOONO (Figure 6), which is identical to the initial termination product of reaction of $LOO^{\bullet}$ with $^{\bullet}$NO.

(ii) Dissociation of LOONO to give $LO^{\bullet}$ and $^{\bullet}NO_2$ caged radicals, which recombine to give L(O)NO_2 (*m*/*z* 340) and further rearrangement products, is expected to occur regardless of how LOONO is formed (*35*, *50*).

(iii) In some experiments, the reaction of $O_2^{\bullet-}$ (where oxidation is intitiated by xanthine oxidase-derived $O_2^{\bullet-}$ production and metal-dependent decomposition of H_2O_2) with $^{\bullet}$NO will result in the generation of $ONOO^-$. Nitration of unsaturated lipid by this species will yield a nitrated lipid (*37*), demonstrated herein to be LNO_2.

In summary, several biologically relevant RNS have been shown to nitrate unsaturated lipids. Evidence implicating the formation of RNS in human diseases is illustrated by the detection of nitrotyrosine in numerous pathologies, including acute lung injury, atherosclerosis, and chronic organ rejection (*26*−*30*). Detection of nitrated lipids in vivo as well would implicate RNS as mediators of lipid oxidation during tissue injury and inflammation. Oxidation of linoleate by $ONOO^-$ also led to formation of both HPODE and HODE, species that display potent bioactivity at nanomolar concentrations. For example, 13(*S*)HPODE induces apoptosis in vitro and regulates prostaglandin synthesis in rabbit kidney medulla (*62*, *63*), while 13(*S*)HODE mediates airway hyperresponsiveness to histamine in guinea pigs in vivo (*64*). In vivo, nitrated lipid species may also possess unique signal transduction reactivities by (a) specific isomers acting as agonists or antagonists of eicosanoid signaling by interacting with specific receptors or (b) mediation of signal transduction via direct $^{\bullet}$NO donation or transnitrosation reactions. In support of this concept, analogous bioactive products are formed following reaction of $ONOO^-$ with glucose, glycerol, and other biomolecules (*56*, *65*).

Acknowledgment. This work was supported by NIH Grants P60 HL58418-1, PO1 HL40456, and RO1 HL51245 (B.A.F. and V.M.D.-U.), The Parker B. Francis Foundation (V.B.O.), and The American Lung Association (J.P.E.). The PE Sciex API 3 mass spectrometer was purchased with funds from a NIH Shared Instrumentation Grant (SIORR06487). Operation of the mass spectrometry and NMR shared facilities is supported in part by a Core Research Support Grant to the UAB Comprehensive Cancer Center (P30 CA13148-26).

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TX980207U