Nitro-fatty Acid Metabolome: Saturation, Desaturation, β-Oxidation, and Protein Adduction*

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Nitrated derivatives of fatty acids (NO2-FA) are pluripotent cell-signaling mediators that display anti-inflammatory properties. Current understanding of NO2-FA signal transduction lacks insight into how or if NO2-FA are modified or metabolized upon formation or administration in vivo. Here the disposition and metabolism of nitro-9-cis-octadecenoic (18:1-NO2) acid was investigated in plasma and liver after intravenous injection in mice. High performance liquid chromatography-tandem mass spectrometry analysis showed that no 18:1-NO2 or metabolites were detected under basal conditions, whereas administered 18:1-NO2 is rapidly adducted to plasma thiol-containing proteins and glutathione. NO2-FA are also metabolized via β-oxidation, with high performance liquid chromatography-tandem mass spectrometry analysis of liver lipid extracts of treated mice revealing nitro-7-cis-hexadecenoic acid, nitro-5-cis-tetradeconoic acid, and nitro-3-cis-docosenoic acid and corresponding coenzyme A derivatives of 18:1-NO2 as metabolites. Additionally, a significant proportion of 18:1-NO2 and its metabolites are converted to nitroalkane derivatives by saturation of the double bond, and to a lesser extent are desaturated to diene derivatives. There was no evidence of the formation of nitrohydroxy or conjugated ketone derivatives in organs of interest, metabolites expected upon 18:1-NO2 hydration or nitric oxide (NO) release. Plasma samples from treated mice had significant extents of protein-adducted 18:1-NO2 detected by exchange to added β-mercaptoethanol. This, coupled with the observation of 18:1-NO2 release from glutathione-18:1-NO2 adducts, supports that reversible and exchangeable NO2-FA-thiol adducts occur under biological conditions. After administration of [3H]18:1-NO2, 64% of net radiolabel was recovered 90 min later in plasma (0.2%), liver (18%), kidney (2%), adipose tissue (2%), muscle (31%), urine (6%), and other tissue compartments, and may include metabolites not yet identified. In aggregate, these findings show that electrophilic FA nitroalkene derivatives (a) acquire an extended half-life by undergoing reversible and exchangeable electrophilic reactions with nucleophilic targets and (b) are metabolized predominantly via saturation of the double bond and β-oxidation reactions that terminate at the site of acyl-chain nitration.

The reaction of unsaturated fatty acids with nitric oxide (NO)- and nitrite (NO2)-derived species, including nitrogen dioxide (NO2), peroxynitrite (ONOO−), and nitrous acid (HNO2), yields a complex array of oxidized and nitrated products (1–4). The mechanisms of biological fatty acid nitration, the structural isomer distribution of nitrated fatty acids (NO2-FA) and the signaling actions of specific NO2-FA regioisomers remain incompletely characterized. Current data reveal that, during fatty acid oxidation and nitration, vinyl nitro regioisomers represent a component of these products that display distinctive chemical reactivities and receptor-dependent signaling actions. Here, we investigate the metabolic fate of the nitroalkene derivative of oleic acid (1, 2).

Unsaturated fatty acid nitration was first described in model studies of air-pollutant-induced lipid oxidation where lipids were exposed to high concentrations of NO2 (5, 6). More recently nitrated unsaturated fatty acids have been reported as products of acidic reactions of NO2-, radical chain termination reactions induced by NO (7–10), and the oxidation of NO2 to NO2 by the leukocyte-derived enzyme myeloperoxidase (1). Various mechanisms can mediate the formation of nitroalkene derivatives of unsaturated fatty acids (11), including homolytic attack of NO2 (12), reaction of NO2 with a pre-existing fatty acid carbon-centered radical (2, 13), and the protonation of nitrite (NO2−) under acidic conditions (pH 5.5 and lower) to yield an array of HNO2-derived nitrating species (3, 14). The conditions promoting fatty acid nitration by NO and NO2 -derived species (low oxygen tension, radical formation, and low pH) are not expected to be broadly distributed systemically (e.g. in plasma or extracellular fluids). Rather, nitration reactions will preferably occur during inflammatory or metabolic stress in microenvironments such as the intermembrane space of mitochondria, the low pH environment of the digestive tract,

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‡ The abbreviations used are: NO2-FA, nitrated fatty acid; HPLC, high-performance liquid chromatography; ESI-MS, electrospray ionization-mass spectrometry; MS/MS, tandem MS; CID, collision-induced dissociation; MRM, multiple reaction monitoring; HBSS, Hanks’ balanced salt solution; BME, β-mercaptoethanol; EPI, enhanced product ion.

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and activated macrophage and neutrophil-rich compartments. Moreover, the acidic, NO$_2$-replete and low O$_2$ tension conditions that promote nitrination reactions are characteristic of inflammatory loci. Although multiple reactions leading to accelerated formation of nitrating species occur at specific anatomic sites, plasma levels of nitrated fatty acids are expected to be low due to events described herein.

Robust electrophilic reactivity and avid nuclear lipid receptor ligand activity have conferred to the class of fatty acid nitroalkene derivatives potent anti-inflammatory properties that occur predominantly via non-cGMP-dependent mechanisms. Nitro derivatives of oleic and linoleic acid inhibit leukocyte and platelet activation (15), vascular smooth muscle proliferation (16), lipopolysaccharide-stimulated macrophage cytokine secretion (17), activate peroxisome proliferator-activated receptor-γ (1, 18), and induce endothelial heme oxygenase 1 expression (19). NO$_2$-FA also potently modulate nuclear factor-erythroid 2-related factor 2/Kelch-like ECH-associating protein 1 (Nrf2/Keap1) (16, 17) and nuclear factor κB (NFκB)-regulated inflammatory signaling (17). Previous observations of the NO$^-$-mediated, cGMP-dependent vessel relaxation induced by NO$_2$-FA were made under serum- and lipid-free conditions. More recently, it has been appreciated that micellar and membrane stabilization of NO$_2$-FA prevents Nef-like aqueous decay reactions and consequent NO release, supporting that the predominant signaling actions mediated by NO$_2$-FA are NO and cGMP-independent (20, 21).

Current data indicate that electrophilic adduction of biological targets primarily accounts for NO$_2$-FA signal transduction. The high electronegativity of NO$_2$ substituents, when bound to an alkanyl carbon of fatty acids, confers an electrophilic nature to the adjacent β-carbon and enables Michael addition reaction with nucleophiles such as protein His and Cys residues. This process, termed nitroalkylation (22), results in the clinically detectable and reversible adduction of the nucleophilic thiol of glutathione (GSH) and both cysteine and histidine residues of glyceraldehyde-3-phosphate dehydrogenase (23). Furthermore, inhibition of NFκB signaling occurs via nitroalkylation of p65 subunit thiols (17), and recent findings reveal that NO$_2$-FA activation of peroxisome proliferator-activated receptor-γ is uniquely induced by covalent nitroalkylation of the ligand binding domain Cys-285.

Multiple reports support the endogenous generation and presence of nitrated fatty acids (1, 24), first observed in bovine papillary muscles as a vicinal nitrohydroxyecosatetraenoic acid (25). Nitrolinoleate has been detected in human blood plasma and cholesteryl nitrolinoleate in human plasma and lipoproteins (4, 26), with hyperlipidemic and post-prandial conditions elevating plasma levels of NO$_2$-FA. Further support for the inflammatory generation of NO$_2$-FA comes from lipopolysaccharide and interferon-γ-activated murine J774.1 macrophages, where increased nitration of the acyl chain of cholesteryl linoleate was paralleled by increased macrophage expression and activity of nitric-oxide synthase 2 (27).

To date, insight into the mechanisms of nitroalkene signaling actions overshadows knowledge of the generation, trafficking, and metabolism of nitroalkenes in vivo. Appreciating that NO$_2$-FA derivatives are detectable clinically, and that their levels increase following NO-dependent oxidative reactions (4, 28), challenges still exist in their routine detection. Because the in vivo administration of NO$_2$-FA may exert anti-inflammatory benefit, the disposition and metabolite profiles of these species in vivo is of relevance. Here we report that only 2.4% of nitro-octadecenoic acid (18:1-NO$_2$) is immediately detectable in the vascular compartment as native 18:1-NO$_2$ upon intravenous injection in mice, with the remaining pool of 18:1-NO$_2$ (a) reversibly bound to plasma and tissue thiols via Michael addition; (b) metabolized to nitro-octadecanoic acid (18:0-NO$_2$) and nitro-octadecadienoic acid (18:2-NO$_2$); and (c) catabolized by hepatic β-oxidation following thioester formation with coenzyme A.

EXPERIMENTAL PROCEDURES

Materials—A synthesis producing equal yields of 9- and 10-nitro-9-cis-octadecenoic acid regioisomers (collectively termed 18:1-NO$_2$) and [13C]18:1-NO$_2$ was conducted as previously shown (1, 29). In some experiments, [3H]18:1-NO$_2$ was utilized, prepared by a similar synthetic and purification strategy using 9,10-[3H]-cis-octadecenoic acid as the starting material. The 9- and 10-nitro regioisomers of octadecenoic acid were not differentiated for the present study and for shorter acyl chain length β-oxidation products, the NO$_2$ position was assumed to remain on these carbons (e.g. becoming 3-nitro-3-cis-dodecenoic acid and 4-nitro-3-cis-dodecenoic acid). CoA- heptadecenoic acid (17:0-CoA) was from Sigma. Solvents used for extractions and mass spectrometric (MS) analysis were from Burdick and Jackson (Muskegon, MI). C57/Bl6 mice were from Jackson Laboratory (Bar Harbor, ME). Insulin syringes for tail vein injections were from BD Biosciences.

Experimental Preparations—All animal studies were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (Approval 0605735-A3). Male C57BL/6 mice, 8–10 weeks of age (Jackson Laboratories, Bar Harbor, ME), were used for all described procedures. 18:1-NO$_2$ or [13C]18:1-NO$_2$ were solvated in 30 μl of 20% ethanol to obtain a final concentration of 10 μM for measurements involving free and plasma constituent-adducted 18:1-NO$_2$ and to a final concentration of 60 μM for measurement of hepatic NO$_2$-FA CoA derivatives. Because of limited amounts of [13C]18:1-NO$_2$, this molecule was not utilized for hepatic metabolite studies. Injection solutions were prepared freshly for every animal and administered immediately via the tail vein. Injection of 30 μl of vehicle was administered to control mice. Blood samples were collected from the saphenous vein prior to 18:1-NO$_2$ injection and then at 5, 15, 30, and 60 min post injection. Mice were anesthetized using intraperitoneal injection of Nembutal® sodium solution (65 mg/kg, Ovation Pharmaceuticals, Deerfield, IL) after 90 min to obtain liver specimens and final blood samples by right ventricular cardiac puncture. Blood samples were transferred to heparinized tubes and stored on ice for further processing. Samples were then stored at −80 °C.

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until further analysis. Liver specimens were frozen in liquid nitrogen and stored at −80 °C for further analysis.

**Analysis of 18:1-NO2 Metabolites**—For lipid extraction, 40 µl of cold (−20 °C) acetonitrile were added to 10 µl of whole blood. Samples were mixed well and centrifuged at 2500 rpm for 15 min at 4 °C, and the supernatant was collected. For quantification purposes [13C]18:1-NO2 and [13C]nitro-9-cis-12-cis-octadecadienoic acid ([13C]linoleic acid) were added as internal standards to samples obtained from animals treated with saline and [13C]18:1-NO2 prior to extraction with acetonitrile. Qualitative and quantitative lipid analyses were conducted by using high-performance liquid chromatography-electrospray ionization mass spectrometry (HPLC-ESI MS/MS) using either a hybrid triple quadrupole mass spectrometer (API 4000) or a triple quadrupole mass spectrometer (API 5000, Applied Biosystems/MS Scieix, Framingham, MA). NO2-FA molecular species were resolved by integrated reversed-phase HPLC (Shimadzu CBM20A, Japan) employing a 150-µm ˟ 2-mm C18 Luna column (particle size, 3 µm, Phenomenex, Belmont, CA) at a flow rate of 0.25 ml/min using a gradient elution with 0.1% acetic acid as solvent A and 0.1% acetic acid in 100% acetonitrile as solvent B. Elution was carried out with the following gradient profile: 0–3 min 3% of B, 3–6 min of 3–50% B, 6–45 min 50–99% of B, 45–53 min 99% of B, and 53.1–65 min 3% of B. Electrospray voltage was −4.5 kV, and the source temperature was set at 550 °C. Mass spectrometric detection of NO2-FA was first performed using the precursor ion scan mode set to detect molecules that, upon collision-induced dissociation (CID), generate a fragment corresponding to NO2 (m/z 46). The precursor masses of molecules containing a nitro functional group were identified, and multiple reaction monitoring (MRM) transitions were used to detect and quantify NO2-FA molecular species using a collision energy of −32.0 eV. The mass transition of m/z 326/46 was used to detect 18:1-NO2, with the appearance of 46 atomic mass units being consistent with the formation of NO2. Mass transitions for β-oxidation metabolites of 18:1-NO2 were calculated according to expected differences in mass, i.e. to account for each loss of an ethyl moiety (−CH2-CH₂-) as to be expected in the course of β-oxidation a mass of 28 was subtracted for Q1 (e.g. 326–28 = 298 for nitro-7-cis-hexadecenoic acid), whereas Q3 remained unaltered (Table 1). Similarly, monitoring for 18:0-NO2 and 18:2-NO2 was performed allowing for the respective changes in masses (Table 1). Additionally, expected MRM transitions of nitrohydroxyl and conjugated ketone derivatives were employed. Structural confirmation of observed compounds was carried out by MS/MS analysis using the same HPLC settings described earlier. After confirmation of structure, quantification of biological samples was performed using a 20-˟ 2-mm reversed-phase column (Mercury MS Gemini 3µ C18, 110 Å, Phenomenex, Torrance, CA) with a flow rate of 0.75 ml/min and a linear gradient of solvent B (11–99% in 3.5 min). For quantification of 18:1-NO2 and 18:2-NO2 peak areas were assessed using Analyst 1.4.2 quantification software (Applied Biosystems/MS Scieix, Thornhill, Ontario, Canada), and ratios of analytes to internal standard were calculated for determination of concentration. Peak areas for 18:0-NO2 were determined as for 18:1-NO2. An external standard curve of nitro-octadecanoic acid was used to determine concentration. The same approaches for quantification were used to approximate concentrations of the metabolites of 18:1-NO2 and 18:0-NO2. Because no standards were available for these metabolites standards for 18:1-NO2 and 18:0-NO2, respectively, were used to correct for any losses and values reported as area ratio.

**Metabolism of 18:1-NO2 to 18:0-NO2 Acid in Vitro**—Peripheral human blood was collected by venipuncture into heparinized tubes with Institutional Review Board approval (number 0606145). Blood was centrifuged (2500 rpm, 4 °C, 15 min) to obtain plasma. NO2-OA was added to a final concentration of 200 nM and incubated for 15 min at 37 °C. Controls were performed using oleic acid (final concentration, 200 nM) or an equal volume of saline to separate plasma samples and incubated for 15 min at 37 °C. Sample processing for HPLC-ESI MS/MS analysis was then performed as for whole blood. Conversion of 18:1-NO2 to 18:0-NO2 was assessed by scanning for the corresponding transitions using HPLC-ESI MS/MS in the MRM scanning mode. Separately, bovine aortic endothelial cells (passages 7–9) were grown to confluence on 6-well plates and incubated at 37 °C for 90 min with 0.15 M NaCl, oleic acid (5 µM, as control), 18:1-NO2 (5 µM), or [13C]18:1-NO2 (5 µM) in 3 ml of Hanks’ buffered salt solution (HBSS). Cell medium (200 µl) was collected at baseline and after 5, 15, 30, and 90 min. After 90 min, cells were washed twice with HBSS and collected by scraping in 200 µl of HBSS. Media samples, cells, and a parallel HBSS solution, which was also incubated with lipids at 37 °C for 90 min, were all treated with acetonitrile as above to deproteinize and extract lipids. Further analysis was performed by HPLC-ESI MS/MS.

**Analysis of NO2-FA Adduction**—Serum samples obtained 90 min after injection of 18:1-NO2 were used to investigate nitroalkylation of plasma components. Free 18:1-NO2 was measured as above using HPLC-ESI MS/MS after lipid extraction with acetonitrile. For evaluation of the presence of glutathione (GSH)-adducted 18:1-NO2 (GS-18:1-NO2) the same HPLC-ESI MS/MS approach was employed. The positive mass transition of m/z 633.3/306.3 was used in the MRM scan mode, where 306.3 is the mass of glutathione and 633.3 is the mass of the adduct of 18:1-NO2 to glutathione. To assess the total amount of 18:1-NO2 (free and adducted to any plasma components) [13C]18:1-NO2 was added to serum samples as internal standard, and samples were treated with 500 µM β-mercaptoethanol (BME) in phosphate-buffered saline for 1 h at 37 °C. Under these conditions, nitroalkylated adducts underwent an exchange reaction where the nitroalkylated moiety transnitroalkylates with BME to form BME adducts (BME-18:1-NO2), and the original protein amino acid moiety is restored to its reduced form. Samples were then analyzed by HPLC-ESI MS/MS using the same chromatographic gradient as for quantification of free NO2-FA. Detection of BME-adducted NO2-FA was performed in MRM scan mode using mass transitions of m/z x + 78 to m/z x (where x = the mass of the nascent NO2-FA and 78 is the atomic mass units of a neutral loss of BME).

For assessment of 18:1-NO2 adducted to albumin, serum proteins were separated by gel electrophoresis (Criterion XT Precast Gel, Bio-Rad, Hercules, CA). After separation, bands of albumin were detected by Coomassie staining, excised, and cut
in 1-mm³ cubes in 400 μl of phosphate buffer (50 mM, pH 7.4) containing [13C]18:1-NO₂ as an internal standard. Subsequently, BME was added to a final concentration of 500 mM and samples were incubated for 2 h to transnitroalkylate 18:1-NO₂ from albumin nucleophiles to BME. Finally, BME-adducted and free, non-GSH-adducted 18:1-NO₂ was assessed after extraction with acetonitrile (see “Experimental Procedures” for details). Scan shows precursor ions of 46 atomic mass units corresponding to the mass of NO₂⁻ and identified peaks.

FIGURE 1. Representative example of precursor ion scan of 46 atomic mass units of whole blood samples. Fatty acids were extracted from whole blood samples of mice treated with vehicle (A) or 30 μl of 10 mM 18:1-NO₂ (B) using acetonitrile (see “Experimental Procedures” for details). Scan shows precursor ions of 46 atomic mass units corresponding to the mass of NO₂⁻ and identified peaks.

in 1-mm³ cubes in 400 μl of phosphate buffer (50 mM, pH 7.4) containing [13C]18:1-NO₂ as an internal standard. Subsequently, BME was added to a final concentration of 500 mM, and samples were incubated for 2 h to transnitroalkylate 18:1-NO₂ from albumin nucleophiles to BME. Finally, BME-adducted 18:1-NO₂ was quantified after extraction with acetonitrile by HPLC-ESI MS/MS as above. To estimate the concentration of 18:1-NO₂-adducted to albumin a plasma albumin concentration of 30 mg/ml was assumed.

To evaluate the reversibility of 18:1-NO₂-nucleophile alkylation reactions further, glutathione-adducted 18:1-NO₂ (GS-18:1-NO₂) was synthesized and purified. Glutathione (GSH) (300 mM) was solvated in 500 mM potassium phosphate buffer (final pH 7.4) and treated with 1.5 mM of 18:1-NO₂ at 37 °C for 30 min. The reaction was stopped by acidification with formic acid at a final pH of 2.0. GS-18:1-NO₂ was purified from residual GSH by reversed-phase chromatography. Samples were loaded onto PrepSep™ C18 columns (Fisher Scientific, Pittsburgh, PA) and equilibrated with 0.1% formic acid. After washing, adducts were eluted with 0.1% formic acid in methanol and fractions concentrated in vacuo.

Synthetic GS-18:1-NO₂ was added to 2 ml of phosphate buffer (50 mM, pH 7.4) and incubated for 6 h at 37 °C. Release of free, non-GSH-adducted 18:1-NO₂ was assessed after 0 min, 30 min, 1 h, 3 h, and 6 h. For this, 100 μl were collected from the phosphate buffer solution, acidified to pH 4 using 10% formic acid and diluted with acetonitrile. GSH-adducted and free 18:1-NO₂ were measured with HPLC-ESI MS/MS as indicated above. To test for the characteristic electrophilic activity of reversibly released 18:1-NO₂ samples were incubated with 500 mM BME for 30 min at 37 °C. Subsequently, BME-adducted 18:1-NO₂ was assessed by HPLC-ESI MS/MS.

Analysis of CoA Derivatives of Nitro-fatty Acids—For measurement of NO₂-FA metabolites, liver specimens dissected from anesthetized mice 90 min after injection of lipid derivatives were frozen with liquid nitrogen and stored at −80°C until further analysis. For lipid extraction, specimens were homogenized (sample weight between 620 and 710 mg), 1 ml of water containing 5 mM 17:0-CoA as an internal standard was added. Thereafter NO₂-FA derivatives were extracted using 4 ml of cold acetonitrile, centrifuged at 2500 rpm for 15 min at 4 °C, and supernatants were collected. HPLC-ESI MS analysis was performed as described previously for fatty acyl-CoA derivatives (30). Briefly, NO₂-FA-CoA derivatives were resolved by HPLC (CBM20A, Shimadzu, Japan) with a 150-×- 2-mm C18 Luna column (particle size, 3 μm; Phenomenex) at a flow rate of 0.25 ml/min. A linear gradient elution was carried out using 0.1% NH₄OH (solvent A) and 0.1% NH₄OH in acetonitrile (solvent B, 0–48% of B) over 45 min. Mass spectrometric analysis was conducted in the positive ion mode using the MRM scan mode (30). Mass transitions for NO₂-FA-CoA derivatives were calculated according to the expected masses for the different species and the theoretical fragments corresponding to the difference from the mass of 17:0-CoA, which was determined as m/z 1020.3/513.3 (Table 1). The description of CoA derivatives was qualitative, because no internal standards were used.

Assessment of Tissue Distribution Using [3H]18:1-NO₂—30-μl aliquots of 10 mM 18:1-NO₂ containing ~0.4 μCi of [3H]18:1-NO₂ (18:1 in 20% ethanol) were injected intravenously into the tail vein of C57BL/6 mice. After 90 min mice were anesthetized, and blood was taken by cardiac puncture to obtain serum. Specimens of liver, kidney, fat, muscle, spleen, and feces were weighed and homogenized in phosphate buffer (50 mM, pH 7.4). The tissue solubilizer Soluene® 350 (PerkinElmer Life Sciences) was added to each homogenate, and the mixture was incubated 6 h at 50 °C. After 6 h samples were cooled to room temperature, 200 μl of 30% hydrogen peroxide was added in four aliquots, and the mixture was incubated for 30 min at 50 °C. Then, 5 ml of scintillation fluid (Hionic-Fluor, PerkinElmer Life Sciences) was added to each vial. Samples were measured after 1 h of dark adaptation. This procedure was repeated three times for each tissue. For calculation of the percentage of recovered specific activity per organ, values were either normalized to the net weight of the organ or, in the case of fat and muscle total weight, were estimated according to expected normal values (1.25 g for fat, 10 g for muscle).

RESULTS

Detection and Identification of Free 18:1-NO₂ and Its Metabolites—A complete, representative chromatogram showing the precursor ions of NO₂⁻ (46 atomic mass units) from a vehicle-treated and 18:1-NO₂-treated animal is shown in Fig. 1, A and B. Assessment of blood samples in the MRM
scan mode allowed identification of 18:1-NO$_2$ as well as all predicted metabolites of β-oxidation, i.e. nitro-7-cis-hexadecenoic acid (16:1-NO$_2$), nitro-5-cis-tetradecenoic acid (14:1-NO$_2$), and nitro-3-cis-dodecenoic acid (12:1-NO$_2$) when monitoring for the calculated mass transitions shown in Table 1. Products of β-oxidation with shorter fatty acid chain length were not...
observed. The identity of these metabolites was confirmed by the analysis of $^{13}$C$\cdot$18:1-NO$_2$ and by MS/MS analysis. Treatment of mice with $^{13}$C$\cdot$18:1-NO$_2$ allowed identification of $^{13}$C-labeled metabolites with elution profiles identical to the metabolites of $^{12}$C$\cdot$18:1-NO$_2$ (Fig. 2A). Concurrent MRM scanning also revealed metabolites exhibiting a mass that was 2 atomic mass units greater than for each of the observed nitroalkenes, reflecting the reduction of the double bond. These metabolites were confirmed by MS/MS analysis as the nitroalkanes 18:0-NO$_2$, nitro-hexadecanoic acid (16:0-NO$_2$), nitro-tetradecanoic acid (14:0-NO$_2$), and nitro-dodecanoic acid (12:0-NO$_2$, Figs. 2B and 3). 18:0-NO$_2$ was further confirmed by comparison with the synthetic derivative. Additional metabolites were detected that displayed a mass 2 atomic mass units less than observed for the nitroalkene derivative, reflecting an additional monounsaturation step, and was confirmed as the corresponding nitroalkadienes by MS/MS analysis and comparison with the synthetic derivative (Figs. 2B and 3). Because desaturation of fatty acids in mammals occurs between the existing double bond and the C terminus, typically at positions 6 and 7 (31–34), these metabolites were assumed to be nitro-6-cis-9-cis-octadecadienoic acid (18:2-NO$_2$) and the corresponding β-oxidation metabolites nitro-4-cis-7-cis-hexadecadienoic acid (16:2-NO$_2$) and nitro-2-cis-5-cis-tetradecadienoic acid.
Identification and structural characterization of 18:1-NO₂ and its metabolites by MS/MS analysis. MS/MS analysis was performed for all observed metabolites. Representative examples of each species are shown. The left column displays HPLC elution profiles acquired by MRM monitoring of transitions shown in Table 1. In the right column identifying MS/MS fragmentation patterns, which were used for characterization of the different metabolites, are illustrated. The top panel displays the HPLC elution profile and MS/MS spectrum of the 18:1-NO₂-injection solution. Relative intensities are displayed, which do not allow for quantity relative to the other profiles.

Concentrations of 18:1-NO₂ and 18:0-NO₂ in whole blood over the time course of 90 min after intravenous injection of 18:1-NO₂. Venous blood of treated mice was extracted and prepared for mass spectrometric analysis as described under "Experimental Procedures." Concentrations of 18:1-NO₂ were calculated using [¹³C]18:1-NO₂ as internal standard, which was added during sample preparation to correct for any losses. 18:0-NO₂ was quantified using an external standard curve of nitro-octadecanoic acid, which was linear over four orders of magnitude (0.08–80.00 nM). Top left panel, a two-phase decline of the 18:1-NO₂ concentration with the first phase (5–15 min) predominantly reflecting distribution of the compound into extraplasmatic compartments and the second phase (15–90 min) predominantly reflecting elimination. 18:0-NO₂ concentration already after 5 min was detectable first after 15 min. Peak areas for later time points showed a continuous increase (B). An isotopic peak of 18:1-NO₂ was observed in the mass transition m/z 328/46, which co-eluted exactly with 18:1-NO₂ and showed a decrease over the time course of 90 min.

Retention times for nitroalkanes were slightly increased compared with 18:1-NO₂. As expected, because of the increased degree of unsaturation, nitroalkadienes eluted earlier than corresponding nitroalkenes.

Quantification of free 18:1-NO₂ and Its Metabolites—Concentrations of whole blood 18:1-NO₂ at different times after administration and its metabolite 18:0-NO₂ are shown in Fig. 4. The peak concentration of 18:1-NO₂ was 212 ± 25 nM, occurring 5 min after injection. The peak concentration of 18:0-NO₂ was also observed 5 min after injection of 18:1-NO₂ (85 ± 39 nM) attaining ~40% of 18:1-NO₂ concentration at this time point. Convergence of the concentration of both species was observed within the remaining 90 min. β-Oxidation metabolites of nitroalkanes displayed higher area ratios than nitroalkene metabolites, however these differences were not statistically significant. In contrast to 18:1-NO₂ and 18:0-NO₂, which already peaked after 5 min, peak concentrations of β-oxidation metabolites occurred 60 min after injection. 18:2-NO₂ and its β-oxidation metabolites yielded considerably lower concentrations compared with 18:0 and 18:1 metabolites (peak concentration of 1.8 ± 0.9 nM 5 min after injection, not shown).

Saturation of 18:1-NO₂ to 18:0-NO₂—Saturation of 18:1-NO₂ to 18:0-NO₂ was induced by human plasma ex vivo (not shown). Incubation of bovine aortic endothelial cell with 18:1-NO₂, however, yielded increasing levels of 18:0-NO₂ over 90 min, with the metabolite 18:0-NO₂ also accumulating in media within 15 min (Fig. 5, A and B). Lipid extracts of cells treated with 18:1-NO₂ revealed an isotope peak of 18:1-NO₂ in the MS
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**FIGURE 6.** Identification of NO₂-FA species without electrophilic reactivity. The top panel shows the HPLC elution profile for the mass transition m/z 326/46 of 18:1-NO₂. Peak 2 represents the characteristic peak of 18:1-NO₂. To test for electrophilic reactivity the extracted blood sample, which gave the HPLC elution profile illustrated in panel A, was treated with BME as demonstrated in B. As expected, the characteristic peak of 18:1-NO₂ disappeared in the mass transition m/z 326/46 (arrow) and a new peak eluted shortly before (*) whereas peaks 1 and 3 remain unaltered suggesting a lack of electrophilic reactivity. As demonstrated in C this new peak co-eluted with the BME-adducted 18:1-NO₂ (*), which can be explained by partial in-source fragmentation of the BME-adducts resulting in the release of the free fatty acid ion, which then is detectable in its actual mass transition m/z 326/46. D and E illustrate HPLC elution profiles of free and BME-adducted [¹³C]18:1-NO₂. Although peak 1 is most likely explained by an undefined noncovalent adduct of 12:0-NO₂, we propose the presence of a “nitroalkane-alkene” as a result of saturation of the 9-cis-double bond with concomitant desaturation of the bond between carbons 6 and 7 (see Scheme 1) as explanation for peak 3.

**SCHEME 1.** Proposed mechanism for the generation of a “nitroalkane-alkene” from 18:1-NO₂. The nitroalkane-alkene can be formed either via the oxidation of 18:1-NO₂ to 18:2-NO₂ and the subsequent desaturation of the 9,10-double bond or via reduction of the 9,10-double bond of 18:1-NO₂ and subsequent oxidation of 18:0-NO₂ in the 6,7-position.

The observation that 18:1-NO₂ is either reduced to 18:0-NO₂ or further desaturated to 18:2-NO₂ motivated the experiment illustrated in Fig. 6, which was performed to characterize the peaks typically eluting before and after 18:1-NO₂ when monitoring for the mass transition m/z 326/46. Incubation of a blood sample from an 18:1-NO₂-treated animal with BME after lipid extraction revealed a lack of electrophilic reactivity of the compounds eluting before and after 18:1-NO₂. Although the earlier eluting peak (peak 1 in Scheme 1) is most likely an undefined non-covalent adduct of 12:0-NO₂, a possible explanation for the peak eluting later could be the presence of a nitroalkane configuration of an 18-carbon alkenyl derivative, which would result from desaturation and subsequent saturation of 18:1-NO₂ (peak 3 in Scheme 1).

**FIGURE 7.** Concentrations of free 18:1-NO₂ and adduction to plasma components. A, serum obtained 90 min after injection was used to assess adduction of nitro-9-cis-octadecenoic acid to plasma samples. Samples were either treated directly with BME to acquire total 18:1-NO₂ or only albumin was incubated with BME after protein separation by gel electrophoresis to obtain albumin-adducted 18:1-NO₂.Both samples were analyzed without BME treatment to assess free 18:1-NO₂. B, the bar graph demonstrates that only 5.7% of 18:1-NO₂ is present in its free form. C, the left-hand panel shows the elution profile of BME-18:1-NO₂ as assessed in MRM scan mode. The product ion scan of this moiety is displayed with the major fragments representing the parent ion (404.1), 18:1-NO₂ (326.1), 18:1-NO₂-NO₂ (279.3), and NO₂ (45.8). C, chromatogram assessed using MRM scan mode showing the elution profile for GSH-adducted 18:1-NO₂ (2.71 min). On the right-hand side the product ion scan of GSH-18:1-NO₂ is displayed. Fragments represent the parent ion (633.3), GSH-18:1-NO₂-glutamic acid (504.3), GSH (305.9), GSH-H₂O (287.9), GSH-[H₂O + NH₃] (253.9).

**FIGURE 8.** Reversible reaction of 18:1-NO₂ with thiols. A, release of 18:1-NO₂ from synthesized GSH-18:1-NO₂ in phosphate buffer. The decrease of GSH-18:1-NO₂ and the concomitant increase of free 18:1-NO₂ over time is shown. No detectable concentrations of BME-adducted 18:1-NO₂ could be obtained. B, after treatment of samples with BME, GSH-18:1-NO₂ and free 18:1-NO₂ were no longer detectable. Equal levels of BME-adducted 18:1-NO₂ for all time points suggest complete transfer of 18:1-NO₂ to BME. C, synthesized GSH-18:1-NO₂ spontaneously decomposes to GSH and 18:1-NO₂ demonstrating the reversibility of the electrophilic adduction of 18:1-NO₂. In the presence of BME, free and GSH-adducted 18:1-NO₂ were added to this stronger nucleophile.
Determination of Electrophilic NO$_2$-FA Adduction—The total concentration of 18:1-NO$_2$ in serum 90 min after injection as assessed with BME pretreatment was 541.0 nM, whereas free 18:1-NO$_2$ had a concentration of 30.9 nM, which was consistent with the concentration of free 18:1-NO$_2$ measured in whole blood (Fig. 7, A and B). β-Oxidation metabolites of 18:1-NO$_2$ and 18:2-NO$_2$ were also found to be adducted to BME (data not shown). After separation of plasma proteins by gel electrophoresis, it was possible to quantify adduction of 18:1-NO$_2$ to albumin. The concentration of 18:1-NO$_2$ adducted to albumin was estimated to be 287.5 nM (Fig. 7A). Furthermore HPLC-ESI-MS/MS allowed qualitative assessment of GSH-adducted 18:1-NO$_2$ (Fig. 7C). Incubation of previously synthesized GS-18:1-NO$_2$ in phosphate buffer revealed the reversibility of the covalent adduction of 18:1-NO$_2$ to GSH (Fig. 8, A and B).

Detection and Identification of NO$_2$-FA-CoA Derivatives—HPLC-ESI-MS using MRM scan mode analysis revealed the presence of the CoA derivative of nitro-9-cis-octadecenoic acid (18:1-NO$_2$-CoA). Furthermore, the CoA derivatives of the β-oxidation metabolites nitro-9-cis-hexadecenoic acid (16:1-NO$_2$-CoA), nitro-9-cis-tetradecenoic acid (14:1-NO$_2$-CoA), nitro-9-cis-dodecenoic acid (12:1-NO$_2$-CoA) were observed (Fig. 9) using mass transitions shown in Table 1. EPI scan mode was used for structural confirmation of all observed peaks, assuming a similar fragmentation pattern for NO$_2$-FA than for synthetic 17:0-CoA. All fragmentation patterns were manually confirmed (Fig. 10, A and B). Similarly, the presence of CoA derivatives for all observed nitroalkane- and nitroalkadiene-β-oxidation metabolites (Fig. 9) could be demonstrated and confirmed by EPI (examples shown in Fig. 10B).

Assessment of Tissue Distribution of [³H]18:1-NO$_2$—Ninety minutes after intravenous injection of [³H]-labeled 18:1-NO$_2$, the greatest proportion of specific activity was recovered in muscle (30.6%) and liver (17.9%). In contrast, all other organs contained <5% of net administered [³H]-label. Plasma accounted for 0.5% of administered [³H]-labeled 18:1-NO$_2$. Around 9% of specific activity was excreted within 90 min (5.6% in urine, 3.5% in feces, 0.9% in bile) (Fig. 11). Because only liver and plasma were investigated in detail, we do not exclude the potential formation of alternative metabolites in other tissue compartments. The use of [³H]18:1-NO$_2$ to assess extents of protein adduction was complicated by protein aggregation and quenching by reagents upon liquid scintillation counting.

**DISCUSSION**

Nitro-9-cis-octadecenoic acid undergoes multiple metabolic modifications and biochemical reactions after intravenous injection: (i) A significant amount of 18:1-NO$_2$ is saturated to...
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18:0-NO₂ 5 min after injection. Modest extents of desaturation to 18:2-NO₂ were also observed, with plasma levels only 1% of 18:1-NO₂. (ii) β-Oxidation metabolites of 18:1-NO₂, 18:0-NO₂, and 18:2-NO₂ along with respective CoA derivatives are formed, with metabolite ion intensities 80- to 130-fold lower than their respective 18-carbon parent molecule for the different time points. (iii) Over 90% of nitro-9-cis-octadecenoic acid in the circulation is not present in the free form, but rather is rapidly adducted to plasma macromolecules via Michael addition. This reaction is reversible, indicating these adducts serve as a reservoir of NO₂-FA.

Blood levels of "free" 18:1-NO₂ decrease after intravenous injection via biphasic kinetics. The first phase, between 5 and 15 min, reflects the rapid distribution into extravascular compartments that is typical of lipophilic compounds. The second phase involves elimination of these compartments after saturation of extravascular compartment levels. A peak concentration of 18:1-NO₂, 212 nM, was measured 5 min after injection and displayed a half-life of ~8 min.

Capture of plasma 18:1-NO₂ with BME permitted differentiation of free and adducted species, and revealed that only 6% of 18:1-NO₂ was in free form; with the majority adducted to plasma components. Accordingly, 18:1-NO₂ was adducted to albumin at an estimated concentration of 287.5 nM, corresponding to 53% of total 18:1-NO₂. Of note, the electrophilic adduction of 18:1-NO₂ to protein thiols is reversible. In support of this, spontaneous release of free 18:1-NO₂ from previously synthesized GSH-adducted 18:1-NO₂ was observed, affirming the reversibility of nitroalkylation reactions. The transnitroalkylation of 18:1-NO₂ from albumin to BME also supports the reversibility of 18:1-NO₂ adduction to plasma proteins. Plasma protein-adducted 18:1-NO₂ thus represents a reservoir of NO₂-FA that temporarily restrains electrophilic reactivity that can subsequently release NO₂-FA when equilibria are shifted. More generally, because reversibility of signaling reactions is a prerequisite for signal transduction, this finding further corroborates the evolving role of electrophilic NO₂-FA as signaling mediators and affirms previous reports regarding electrophilic adduction as a signaling mechanism (22, 35, 36).

Robust evidence supports protein conjugation of NO₂-FA in the cell, an event in part regulated by multidrug resistance protein-1-mediated efflux of penultimate GSH-NO₂-FA adducts (23, 37).

Conversion of 18:1-NO₂ to 18:0-NO₂ was detectable 5 min after intravenous injection of 18:1-NO₂, reaching 40% of the concentration of the injected 18:1-NO₂ at this time. The extent of 18:1-NO₂ saturation in vivo and the observation that this reaction is mediated by bovine aortic endothelial cells in vitro,
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but not human plasma, indicates an enzymatically catalyzed rather than spontaneous reaction. Because saturation of 18:1-NO₂ to 18:0-NO₂ leads to loss of electrophilic reactivity, this represents a mechanism for cellular inactivation of reactive electrophiles. Although enzymes competent to catalyze the saturation of nitroalkenes have been reported, including the flavin mononucleotide-containing NADPH oxido-reductase “old yellow enzyme” (38), these enzymes are only reported for yeast, plants, and bacteria (39). The identity of enzymes responsible for nitroalkene reduction in mammalian cells remains to be defined.

In comparison to nitroalkane formation, nitroalkadienes were generated to much lower extents after intravenous injection of 18:1-NO₂. Because mammals typically desaturate fatty acids between the carboxyl group and an already existing olefin the additional desaturation of 18:1-NO₂ is most likely inserted between carbons 6 and 7 (31–34). Conversion of nitroalkenes to nitroalkadienes suggests the inclusion of nitroalkenes into synthetic pathways for polyunsaturated fatty acids. The presence of nitrated linolenic, arachidonic, and eicosapentaenoic acids, all of which are kinetically more likely to become nitrated than oleic acid, has been reported in vivo (1). The observation that 18:1-NO₂ acid undergoes saturation and desaturation also provides a possible explanation for the origin of the non-electrophilic isobaric species that elutes after the 18:1-NO₂ peak when monitoring for the characteristic mass transition m/z 326/46 both in Fig. 6 and biological samples (not shown). This peak is commonly observed when treating rodents and cells with 18:1-NO₂. Saturation of the double bond between carbons 9 and 10, along with desaturation at another location, e.g. between carbons 6 and 7, could result in a nitroalkene-alkane, (e.g. 9-nitro-6-cis-octadecenoic acid), that exhibits the same mass transition as 9-nitro-9-cis-octadecenoic acid but displays a different HPLC retention time and no electrophilic reactivity (Scheme 1).

Nitroalkenes, as well as nitroalkanes and nitroalkadienes, undergo β-oxidation. Thus, β-oxidation metabolites for all three species were detectable that displayed expected mass transitions and the decreasing retention time characteristic of smaller molecules. In the tissue compartments of interest, concentrations for these metabolites were 80- to 130-fold less than concentrations of the parent NO₂-FA, based on the assumption that fragmentation efficiencies between metabolites are comparable. Collision-induced product fragmentation via MS/MS confirmed these metabolites. The detection and characterization of CoA derivatives of metabolites detected in liver samples of treated mice further support these findings. No metabolites with chain lengths shorter than 12 carbons were detected for free NO₂-FA and their CoA derivatives. The metabolite of the two unsaturated species at this stage would be nitro-3-cis-dodecenoic acid. For this acid to be further oxidized by β-oxidation, a Δ⁵-3-cis-Δ⁴-trans-enoxy-CoA isomerase must convert the 3-cis-double bond to a 2-trans-double bond. The presence of the nitro group that is located either on carbon 3 or 4, depending on whether it is a metabolite of the 9- or 10-NO₂ regiosomer of 18:1-NO₂, prevents this enzymatic step and therefore any further β-oxidation. In the case of nitroalkanes, further oxidation of 12:0-NO₂ to nitrodecanoic acid was expected. As previously noted, nitroselenation-catalyzed synthesis of 18:1-NO₂ yields two regiosomers, 9-nitro-9-cis-octadecenoic acid and 10-nitro-9-cis-octadecenoic acid, which at this stage of metabolism would result in either 3- or 4-nitro-dodecanoic acid. The former metabolite is unlikely to be further β-oxidized, because the nitro-bonded carbon would be destined as the carboxylate carbon of the product. Because no nitrated fatty acid metabolite of chain length <12 carbons can be formed by β-oxidation, relatively greater levels of 12:1-NO₂ and 12:0-NO₂ are expected and were observed (Fig. 4).

The finding that NO₂-FA undergo β-oxidation has multiple implications. First, as a consequence of shorter chain length, β-oxidation metabolites will be less hydrophobic. This will not only influence partitioning between hydrophobic and hydrophilic compartments and consequent anatomic distribution, but can also affect chemical reactivity and pharmacological profiles by altering accessibility to reaction targets. This concept is reminiscent of the differential regulation of myocyte and pancreatic β-cell ATP-sensitive K⁺-channels by acyl-CoA esters depending on respective chain length (40–42). Second,
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modified fatty acids undergo β-oxidation with kinetics that differ from the parent native fatty acid. For example, 5-hydroxy-decanoyl-CoA exhibits a 5-fold lower $V_{\text{max}}$ at the penultimate step of β-oxidation compared with the corresponding non-hydroxylated fatty acid, eventually resulting in inhibition of the β-oxidation of decanoyl-CoA (43). Whether NO$_2$-FA acts in a similar fashion is of relevance to the response of tissues to ischemic insult and warrants further investigation. Finally, the fact that NO$_2$-FA undergo β-oxidation upon formation of CoA thioester derivatives affirms that these species gains intramitochondrial access. Studies of HEK-293 cell mitochondrial fractions reveal that mitochondria contain a myriad of protein targets that selectively interact with thiol-reactive electrophiles (44). Esterification of 18:1-NO$_2$ to membrane and lipoprotein phospholipids is also a candidate metabolic disposition of 18:1-NO$_2$ in vivo, but was not addressed in herein.

The tissue distribution of specific activity after injection of $[^{3}H]$18:1-NO$_2$ showed liver having the greatest organ specific radioactivity, and the greatest percentage of administered radioactivity per whole organ was in muscle and liver. This indicates that 18:1-NO$_2$ traffics much like a native fatty acid in vivo. The observation of only 0.5% of administered radioactivity in the plasma compartment 90 min after administration agrees with independent mass spectrometry-based quantitation. Thus, intravenous administration of 300 nmol of $[^{3}H]$18:1-NO$_2$ gave a net concentration of 18:1-NO$_2$ per BME “capture” of adducted species, of 541 nM 18:1-NO$_2$ in blood. Assuming a blood volume of 4 ml in mice, this is ~2.2 nmol or 0.7% of the administered amount. Quantitative limitations apply to the interpretation of these data, because the measured radioactivity reflects not only 18:1-NO$_2$ but also its metabolites.

No basal 18:1-NO$_2$ was detected in plasma and liver of the C57BL/6 cohort of mice used for the present metabolism study. We and others have detected nitro-oleate in rodents and humans in other instances, as well as metabolites reported herein. There are a number of mitigating factors in the detection and levels of fatty acid nitration products. For example, gastric acidification results in nitrination of dietary fatty acids present in rodent chow, an event subject to dietary NO$_2$ and unsaturated fatty acid levels. Also, plasma and organ levels of oleate and linoleate nitration products are affected by underlying inflammatory conditions (e.g. lipopolysaccharide treatment (27) and ischemic preconditioning). Finally, the present study reveals that Michael addition reactions and metabolism (saturation, desaturation, and β-oxidation) affect detectable levels of “free” fatty acid nitration products. The initial report of ~500 nM nitro-oleate in human plasma (1) is now viewed to be higher than current measurements, with the original value complicated by non-covalent complexes of nitrite and oleate.

The metabolism of exogenously administered 18:1-NO$_2$ was evaluated to reveal the spectrum of reactions that endogenously produced nitro-fatty acid derivatives can undergo. Due to these rapid and diverse reactions, it is expected that specific organs, cells, and subcellular compartments responsible for fatty acid nitrination will display levels higher than those detected in plasma. Thus, the reactions described herein are reflective of the trafficking and metabolic events expected for endogenous fatty acid nitration products. In summary, 18:1-NO$_2$ undergoes a rapid and substantial modification that affects subsequent chemical reactivity and signaling actions. Specifically, the reversible addition of 18:1-NO$_2$ to biological nucleophiles and conversion to 18:0-NO$_2$ induces a rapid and transient neutralization of electrophilic reactivity. This addition of nitroalkenes to nucleophilic targets thus both modifies the timing and sites of NO$_2$-FA signaling and accounts for many of the anti-inflammatory and adaptive signaling actions of these species.

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