Fatty Acid Transduction of Nitric Oxide Signaling

MULTIPLE NITRATED UNSATURATED FATTY ACID DERIVATIVES EXIST IN HUMAN BLOOD AND URINE AND SERVE AS ENDOGENOUS PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR LIGANDS**

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Mass spectrometric analysis of human plasma and urine revealed abundant nitrated derivatives of all principal unsaturated fatty acids. Nitrated palmitoleic, oleic, linoleic, linolenic, arachidonic and eicosapentaenoic acids were detected in concert with their nitrohydroxyl derivatives. Two nitoalkene derivatives of the most prevalent fatty acid, oleic acid, were synthesized (9- and 10-nitro-9-cis-octadecenoic acid; OA-NO2), structurally characterized and determined to be identical to OA-NO2 found in plasma, red cells, and urine of healthy humans. These regiosomers of OA-NO2 were quantified in clinical samples using 13C isotope dilution. Plasma free and esterified OA-NO2 concentrations were 619 ± 52 and 302 ± 36 nM, respectively, and packed red blood cell free and esterified OA-NO2 was 59 ± 11 and 155 ± 65 nM. The OA-NO2 concentration of blood is ~50% greater than that of nitrated linoleic acid, with the combined free and esterified blood levels of these two fatty acid derivatives exceeding 1 μM. OA-NO2 is a potent ligand for peroxisome proliferator activated receptors at physiological concentrations. CV-1 cells co-transfected with the luciferase gene under peroxisome proliferator-activated receptor (PPAR) response element regulation, in concert with PPARγ, PPARα, or PPARδ expression plasmids, showed dose-dependent activation of all PPARs by OA-NO2. PPARγ showed the greatest response, with significant activation at 100 nM, while PPARα and PPARδ were activated at ~300 nM OA-NO2. OA-NO2 also induced PPARγ-dependent adipogenesis and deoxyglucose uptake in 3T3-L1 preadipocytes at a potency exceeding nitrolinoleic acid and rivaling synthetic thiazolidinediones. These data reveal that nitrated fatty acids comprise a class of nitric oxide-derived, receptor-dependent, cell signaling mediators that act within physiological concentration ranges.

The oxidation of unsaturated fatty acids converts lipids, otherwise serving as cellular metabolic precursors and structural components, into potent signaling molecules including prostaglandins, leukotrienes, isoprostanes, and hydroxy- and hydroperoxyeicosatetraenoates. These enzymatic and auto-catalytic oxidation reactions yield products that orchestrate immune responses, neurotransmission, and the regulation of cell growth. For example, prostaglandins are cyclooxygenase-derived lipid mediators that induce receptor-dependent regulation of inflammatory responses, vascular function, initiation of parturition, cell survival, and angiogenesis (1). In contrast, the various isoprostane products of arachidonic acid auto-oxidation exert vasoconstrictive and pro-inflammatory signaling actions via receptor-dependent and -independent mechanisms (2). A common element of these diverse lipid signaling reactions is that nitric oxide (NO) and other oxides of nitrogen significantly impact lipid mediator formation and bioactivities.

The ability of NO and NO-derived species to oxidize, nitrosate, and nitrate biomolecules serves as the molecular basis for how NO influences the synthesis and reactions of bioactive lipids (3–5). Interactions between NO and lipid oxidation pathways are multifaceted and interdependent. For example, NO regulates both the catalytic activity and gene expression of prostaglandin H synthase (6). Conversely, leukotriene products of lipoxygenases induce nitric-oxide synthase-2 expression and increase inflammatory NO production (7). The free radical reactivity of NO lends an ability to inhibit the autocatalytic chain propagation reactions of lipid peroxyl radicals during membrane and lipoprotein oxidation (8). Of relevance, reactions between NO-derived species, unsaturated fatty acids, and lipid oxidation intermediates yield a spectrum of fatty acid oxidation and nitrination products (3). Recently, the nitroalkene derivative of linoleic acid (LNO2) was detected in human blood at concentrations sufficient to induce biological responses (~500 nM; Refs. 9–12). Compared with other NO-derived species such as nitrite (NO2−), nitrosothiols (RSNO), and heme-nitrosyl complexes, LNO2 alone represents the single most abundant pool of bioactive oxides of nitrogen in the healthy human vasculature (9, 13–16).

In vitro studies have shown that LNO2 mediates cGMP-dependent vascular relaxation, cGMP-independent inhibition of neutrophil degranulation and superoxide formation, and inhibition of platelet acti-

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6 The abbreviations used are: NO, nitric oxide; LNO2, nitrated linoleic acid; PPAR, peroxisome proliferator-activated receptor; OA-NO2, nitrated oleic acid; ONOO−, peroxynitrite; MPO, myeloperoxidase; HPLC ESI MS/MS, high performance liquid chromatography electrospray ionization triple quadrapole mass spectrometry; MRM, multiple reaction monitoring; CID, collision-induced dissociation; PPARE, PPAR response elements; DTPA, diethylenetriaminepentacacetate; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum.
The identification of the cell signaling actions of LNO₂, which include (a) robust endogenous PPARγ ligand activity that acts within physiological concentrations (17), (b) an ability to decay in aqueous conditions to release ‘NO (20), and (c) reactivity as an electrophile, motivated a search for other nitrated fatty acids that might serve related signaling actions. Herein, we report that nitoalkene derivatives of all principal unsaturated fatty acids are present in human blood and urine. Of the fatty acid content in red cells, linoleic acid and oleic acid comprise ~ 8% and ~ 18% of total, respectively (21). Due to its prevalence and structural simplicity, oleic acid was evaluated as a potential candidate for nitration. The synthesis, structural characterization, and cell signaling activities of 9- and 10-nitro-9-cis-octadecenoic acids are described (nitrated oleic acid, OA-NO₂; Fig. 1). OA-NO₂ regioisomers were measured in human blood and urine at levels exceeding those of LNO₂. Furthermore, OA-NO₂ activates PPARγ with a greater potency than LNO₂. These data reveal that nitrated unsaturated fatty acids represent a class of lipid-derived, receptor-dependent signaling mediators.

MATERIALS AND METHODS

Materials—9-Octadecenoic acid (oleic acid) was purchased from Nu-Check Prep (Elysian, MN). [13C₁₈]Oleic acid (> 98% isotopic purity) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). OA-NO₂ and [13C₁₈]OA-NO₂ were synthesized as described below. Phenylselenium bromide, HgCl₂, NaNO₂, anhydrous tetrahydrofuran (THF), CH₃CN, CDCl₃, insulin, dexamethasone, and 3-isobutyl-1-methylxanthine were obtained from Sigma. Peroxynitrite (ONOO⁻) was prepared as described (22). Silica gel H and HF thin layer chromatography plates (250 and 2000 μm) were from Analtech (Newark, DE). Methanolic BF₃, horseradish peroxidase-linked goat anti-rabbit IgG, and Coomasie Blue were from Pierce. Myeloperoxidase (MPO) derived from human polymorphonuclear leukocytes was obtained from Calbiochem. Synthetic solvents were of HPLC grade or better from Fisher Scientific. Solvents used for extractions and mass spectrometric analyses were from Burdick and Jackson (Muskegon, MI). Anti-PPARγ and anti-β-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-ap2 antibody was from Chemicon International Inc. (Temecula, CA).

Synthesis of OA-NO₂—Oleic acid and [13C₁₈]oleic acid were nitrated as described (9, 12), with modifications. Oleic acid, HgCl₂, phenylselenium bromide, and NaNO₂ (1:1.3:1.1, mol/mol) were combined in THF/acetonitrile (1:1, v/v) with a final concentration of 0.15 M oleic acid. The reaction mixture was stirred (4 h, 25 °C), followed by centrifugation to sediment the precipitate. The supernatant was recovered, the solvent evaporated in vacuo, the product mixture redissolved in THF (original volume), and the temperature reduced to 0 °C. A 10-fold molar excess of H₂O₂ was slowly added with stirring to the mixture, which was allowed to react in an ice bath for 20 min followed by a gradual warming to room temp (45 min). The product mixture was extracted with hexane, the organic phase collected, the solvent removed in vacuo, and lipid products solvated in CH₃OH. OA-NO₂ was isolated by preparative TLC using silica gel HF plates developed twice in a solvent system consisting of hexane/ether/acetic acid (70:30:1, v/v). The region of silica containing OA-NO₂ was scraped and extracted (23). Based on this synthetic rationale, two regioisomers are generated: 9- and 10-nitro-9-cis-octadecenoic acids (generically termed OA-NO₂). Preparative TLC does not adequately resolve the two isomers. [13C₁₈]OA-NO₂ was synthesized using [13C₁₈]oleic acid as a reagent. All nitrated fatty acid stock solutions were diluted in MeOH, aliquoted, and stored under argon gas at ~ 80 °C. Under these conditions, OA-NO₂ isomers remain stable for > 3 months.

The nitralkene positional isomers are described as cis throughout this article based on the configuration of the carbon skeleton, which correlates the cis alkene stereochemistry in the nitralkenes with the corresponding cis alkene stereochemistry in naturally occurring oleic acid. The IUPAC nomenclature of the nitralkenes has the opposite stereochemical terminology, because it focuses on the relationship of the higher priority nitro group to the carbon substituents on the alkene. For example, the 9-nitro isomer has the carbon chains cis to each other on the nitralkene, but the official IUPAC nomenclature designates this compound as E (or trans) because the nitro group on C-9 and the carbon chain on C-10 have the E (entgegen) or trans relationship to each other.

Quantitation of Synthetic OA-NO₂—The concentrations of synthetic OA-NO₂ stock solutions were determined using chemiluminescent nitrogen analysis (Antek Instruments, Houston, TX), a quantitative measure of nitrogen content in synthetic and biological samples (24, 25). Briefly, purified synthetic nitralkene preparations were subjected to complete pyrolysis (> 1000 °C). The nitrogen-containing OA-NO₂ reacts with O₂ to ultimately yield ‘NO at a ratio of one mole ‘NO for every mole of nitrogen present in OA-NO₂. The generated ‘NO reacts with O₂ to yield nitrogen dioxide (“NO₂, O₂, and h_, the latter of which is sensitively detected with a photomultiplier). Concentrations were calculated using caffeine as the standard.

Stability of OA-NO₂ and LNO₂—The relative stabilities of OA-NO₂ and LNO₂ in MeOH and phosphate buffer (100 mM, KPO₄ containing 100 μM DTPA, pH 7.4) were determined by electrospray ionization triple quadrupole mass spectrometry (ESI MS/MS) using the quantitative methodology detailed below. OA-NO₂ and LNO₂ (3 μM each) were incubated at 37 °C in either MeOH or phosphate buffer, and aliquots were taken over time. The aliquots were extracted as described (23), with 1 μM [13C₁₈]LNO₂ added during the monophase stage of the extraction procedure as an internal standard, and analyzed for non-degraded OA-NO₂ and LNO₂. In aqueous buffer, nitrated lipids degrade more rapidly than in organic solvents (20); thus, their stability in phosphate buffer was measured over 2 h. The stability of nitrated fatty acids solvated in MeOH at 37 °C was measured over the course of 1 month.

OA-NO₂ Spectrophotometric Characterization—OA-NO₂ stock solution concentrations derived from chemiluminescent nitrogen analysis were utilized to determine dilution concentrations for subsequent spectral analysis. An absorbance spectrum of OA-NO₂ from 200–450 nm was generated using 23 μM OA-NO₂ in phosphate buffer (100 mM, pH 7.4) containing 100 μM DTPA. The extinction coefficients (ε) for OA-NO₂ and the isotopic derivative [13C₁₈]OA-NO₂ were measured (λ₂₇₀) using a UV-visible spectrophotometer (Shimadzu, Japan).

FIGURE 1. Nitrated oleic acid (OA-NO₂). Two regioisomers of OA-NO₂ were synthesized by nitrosenylation of oleic acid yielding 9- and 10-nitro-9-cis-octadecenoic acids.
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Absorbance values for increasing concentrations of OA-NO2 and [13C18]OA-NO2 were plotted against concentration to calculate e.

NMR Spectrometric Analysis of OA-NO2—1H and 13C NMR spectra were acquired using a Varian INOVA 300 and a 500 MHz NMR and recorded in CDCl3. Chemical shifts are in 6 units (ppm) and referenced to residual proton (7.26 ppm) or carbon (77.28 ppm) signals in deuterated chloroform. Coupling constants (J) are reported in Hertz (Hz).

Structural Characterization of OA-NO2 by ESI MS/MS—Qualitative analysis of OA-NO2 by ESI MS/MS was performed using a hybrid triple quadrupole-linear ion trap mass spectrometer (4000 Q trap, Applied Biosystems/MD Sciei). To characterize synthetic and endogenous OA-NO2, a reverse-phase HPLC methodology was developed using a 150 × 2 mm C18 Phenomenex Luna column (3 μm particle size). Lipids were separated and eluted from the column using a gradient solvent system consisting of CH3CN:H2O:NH4OH (85:15:0.1, pH 7.2) in the presence of 2% sodium cholate (26). The pH was adjusted to 7.2 prior to red cell and plasma lipid purification and analysis, which permitted the MS detection of possible 13C-labeled OA-NO2 formation. Also, 200 μM NO2− was included in initial lipid extractions to determine whether separations or analysis-induced nitration reactions might be supported by physiological NO2− levels that can exceed 200 nM (14, 15).

In no case did we detect artifactual synthesis of oleic acid due to sample processing and analysis.

Qualitative Analysis of Nitro and Nitrohydroxy Adducts of Fatty Acids—Using HPLC ESI MS/MS in the presence of the negative ion mode, blood and urine samples were evaluated for the presence of nitroalkene derivatives other than LNO2. HPLC separations using the qualitative gradient elution methodology were performed similarly to those used to characterize OA-NO2, with some modifications. Alternative MRM transitions were used to detect other potential nitroalkene derivatives. Theoretical MRM transitions were determined for the CID-induced loss of the nitro group from nitrated palmitoleic (16:1-NO2), linolenic (18:3-NO2), arachidonic (20:4-NO2), and eicosapentaenoic (20:5-NO2) acids. MRM transitions for nitrohydroxy adducts were also monitored: 16:1(OH)-NO2, 18:1(OH)-NO2, 18:2(OH)-NO2, 18:3(OH)-NO2, 20:4(OH)-NO2, and 20:5(OH)-NO2.

In Vitro Formation of OA-NO2—Three different conditions were examined for an ability to induce nitration of oleic acid: acidic nitration, treatment with peroxynitrite, and treatment with MPO in the presence of H2O2 and nitrite. Briefly, for acidic nitration, oleic acid (1 mM) and sodium nitrite (100 μM) were prepared in phosphate buffer (50 mM, pH 7.2) in the presence of 2% sodium cholate (26). The pH was adjusted to 7.2, and the reaction mixture was incubated with stirring (40 min; 25 °C). The reaction was stopped by solvent extraction, and OA-NO2 levels were measured by HPLC ESI MS/MS. For peroxynitrite-induced nitration, oleic acid (1 mM) was suspended in phosphate buffer (100 mM, pH 7.2), and ONOO− was infused via syringe pump into a stirred chamber (100 μM/min; 15 min) (26). Decayed ONOO− (pH 7.4, 10 min) was added as a control. Products were extracted and analyzed for OA-NO2.

For MPO-induced nitration, oleic acid (1 mM) was incubated in phosphate buffer (100 mM; pH 7.2) in the presence of MPO (50 nM), sodium nitrite (100 μM), and hydrogen peroxide (100 μM) as described (27). The reaction proceeded for 90 min with additional aliquots of hydrogen peroxide added at 30-min intervals. The reaction was stopped by lipid extraction, and OA-NO2 was measured by HPLC ESI MS/MS. Significance of difference between treated and control groups was determined using a one-tailed, paired Student’s t test.

PPAR Transient Transfection Assay—CV-1 cells from the ATCC (Manassas, VA) were grown to ~85% confluence in DMEM/F-12 supplemented with 10% FBS and 1% penicillin-streptomycin. Twelve hours before transfection, the medium was removed and replaced with antibiotic-free medium. Cells were transiently co-transfected with a plasmid containing the luciferase gene under the control of three tandem PPAR response elements (PPRE) (PPRE × 3 TK-luciferase and PPARγ, PPARα, or PPARβ expression plasmids, respectively (provided by Ron Evans, Salk Institute). In all cases, a green fluorescence protein (GFP) expression plasmid was co-transfected as the control for transfection efficiency. Twenty-four hours after transfection, cells were returned to Opti-MEM (Invitrogen) for 24 h and then treated as indicated for another 24 h. Reporter luciferase assay kits from Promega (Madison, WI) were used to measure the luciferase activity according to the manufacturer’s instructions with a luminometer (Victor II, PerkinElmer Life Sciences). Luciferase activity was normalized by GFP units. Each condition was performed in triplicate for each experiment (n ≥ 3).

3T3-L1 Differentiation and Oil Red O Staining—3T3-L1 preadipocytes were propagated and maintained in DMEM containing 10% FBS. To induce differentiation, 2-day post-confluent preadipocytes (designated day 0) were cultured in DMEM containing 10% FBS plus 1 and 3 μM OA-NO2 for 14 days. The medium was changed every 2 days. Rosiglitazone (3 μM) and oleic acid (3 μM) were used as positive and nega-
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TABLE ONE

| Fatty acid      | Carbons-double bonds | Nitro adduct (-NO₂) | Nitrohydroxy adduct (L(OH)-NO₂) |
|-----------------|----------------------|---------------------|---------------------------------|
| Palmitoleic     | 16:1                 | 298/251             | 316/269                         |
| Oleic           | 18:1                 | 326/279             | 344/297                         |
| Linoleic        | 18:2                 | 324/277             | 342/295                         |
| Linolenic       | 18:3                 | 322/275             | 340/293                         |
| Arachidonic     | 20:4                 | 348/301             | 366/319                         |
| Eicosapentaenoic| 20:5                 | 346/299             | 364/317                         |

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Spectral Characterization of Synthetic OA-NO₂—The absorbance spectrum of OA-NO₂ was acquired in phosphate buffer in the presence of the iron chelator DTPA (Fig. 4A). The maximum at 270 nm was ascribed to photon absorption by π electrons in the nitro functional group. Extinction coefficients for OA-NO₂ and [13C₁₈]OA-NO₂ were determined by plotting absorbance (A_m) versus concentration, giving m = A_U cm⁻¹·m⁻³ and a calculated e = 8.22 and 8.23 cm⁻¹·m⁻³, for OA-NO₂ and [13C₁₈]OA-NO₂, respectively (Fig. 4B).

Stability of OA-NO₂—OA-NO₂ was found to be fully stable for >3 months when stored at ~80 °C in MeOH (data not shown). However, some decay was observed in MeOH at 37 °C, showing ~10% decay after 1 month (Fig. 5). In phosphate buffer, OA-NO₂ decayed much faster, with ~40% loss after 2 h. In both solvent environments, LNO₂ was much less stable than OA-NO₂, with this attributed to the greater reactivity of the bisalicylic bond arrangement in LNO₂.

Characterization and Quantitation of Endogenous OA-NO₂ by ESI MS/MS—Using the gradient HPLC elution protocol described under "Materials and Methods," synthetic OA-NO₂ regioisomers eluted from the reverse-phase column as two partially overlapping peaks (Fig. 6). The HPLC elution profiles for synthetic OA-NO₂ and [13C₁₈]OA-NO₂ were identical (Fig. 6A, left panels). Concurrent product ion analysis of the overlapping peaks showed spectra consistent with OA-NO₂-derived species (Fig. 6A, right panels), with major fragments identified in 18:2, and 18:3; however, for 16:1, 20:4, and 20:5, the injection peak was much less stable than OA-NO₂, with this attributed to the greater reactivity of the bisalicylic bond arrangement in LNO₂.

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TABLE TWO. Using these same parameters and machine settings, lipid extracts of packed red cells and plasma were analyzed (Fig. 6B). The product ion spectra for the OA-NO2 present in red cells and plasma were identical to those obtained from synthetic OA-NO2, revealing that OA-NO2 is endogenously present in healthy human blood. Interestingly, the HPLC elution profiles for plasma- and blood-derived OA-NO2 acquired during qualitative analysis show single peaks rather than overlapping species as seen for the synthetic standard, suggesting the possibility that only one regioisomer is present in vivo. The peaks in the elution profiles for both urine and plasma have the same retention times as the second peak of the synthetic standard.

To quantitate OA-NO2 content in red cells and plasma, lipid extracts were separated using an isocratic HPLC elution protocol. Analytes co-eluted and MRM transitions for OA-NO2 and [13C18]OA-NO2 were monitored (data not shown). The concentration of OA-NO2 in biological samples was determined from the ratio of analyte to internal standard peak areas using an internal standard curve that is linear over 4 orders of magnitude. The limit of quantitation (LOQ determined as ten times the standard deviation of the noise) was calculated to be 1.2 fmol/µl packed cells. Blood samples obtained from 10 healthy human volunteers (5 female, 5 male, ages ranging from 24 to 53) revealed free OA-NO2 in red cells (i.e. OA-NO2 not esterified to glycerophospholipids or neutral lipids) to be 59 ± 11 pmol/ml packed cells (TABLE THREE). Total free and esterified OA-NO2, the amount present in saponified samples, was 214 ± 76 pmol/ml packed cells. Thus, ~75% of OA-NO2 in red cells is esterified to complex lipids (9). In plasma, the free and esterified OA-NO2 concentrations were 619 ± 52 and 302 ± 369 nM, respectively, and thus are more abundant than linoleic acid nitration products (9). Control studies revealed that the extraction and analysis conditions do not induce OA-NO2 formation. Present data also show that saponification reactions induce loss of fatty acid nitro derivatives (data not shown), suggesting that current quantitative results may be an underestimation of actual pool sizes of esterified fatty acid nitroalkene adducts.

Characterization of Nitrohydroxy Allylic Derivatives—Nitrohydroxy allylic derivatives of fatty acids are also present in plasma and urine (Fig. 2). This was confirmed by product ion analysis run concomitantly with MRM detection (Fig. 7, A–C). Structures of nitrohydroxy adducts are presented with diagnostic fragments and product ion spectra for 18:1(OH)-NO2, 18:2(OH)-NO2, and 18:3(OH)-NO2. Both the 9- and 10-nitro regioisomers of 18:1(OH)-NO2 are present in urine (Fig. 7A) and plasma (data not shown), as evidenced by the intense peak corresponding to m/z 171 and to a lesser extent m/z 202 (Fig. 7A). Also present are major fragments consistent with the loss of a NO2 group and H2O (m/z 297 and 326, respectively). The product ion spectrum obtained from 18:2(OH)-NO2 shows a predominant fragment (m/z 171), consistent with a hydration product of LNO2 nitrated at the 10-carbon (Fig. 7B). Diagnostic fragments for the three other potential regioisomers were not apparent. Finally, multiple regioisomers of 18:3(OH)-NO2 are present, again with an apparent preferential nitrination at C-10 (Fig. 7C).

In Vitro Nitration of Oleic Acid to OA-NO2—The in vivo detection of nitrated mono-unsaturated fatty acids raised question as to how these derivatives may be formed in vivo. To gain insight into potential mechanisms of formation of OA-NO2, in vitro reactions were performed to determine whether free radical or alternative mechanisms can generate this nitrated fatty acid species (Fig. 8). Treatment of oleic acid with MPO, H2O2 and NO2 yielded OA-NO2 with an HPLC elution profile identical to synthetic OA-NO2. Additionally, nitrohydroxy adducts were observed. Oleic acid treated under mild acidic conditions (pH 4.0) in the presence of NO2 also generated OA-NO2 with the same physical characteristics as OA-NO2 prepared by nitrosylation. Relatively less nitrohydroxy adducts were generated as compared with the amount generated by MPO. Finally, treatment of oleic acid with ONOO− resulted in significant formation of OA-NO2 and nitrohydroxy adducts.

Activation of PPARs by OA-NO2—Recently, LNO2 was identified as an endogenous PPAR ligand (17). Considering the even greater levels of OA-NO2 detected in vivo, OA-NO2 was compared with LNO2 as a PPARα, PPARγ, and PPARδ ligand. CV-1 cells were transiently cotransfected with a plasmid containing the luciferase gene under regulation by three PPREs in concert with PPARγ, PPARα, or PPARδ expres-
Dose-dependent activation by OA-NO₂ was observed for all PPARs (Fig. 9A), with PPARγ showing the greatest response (significant activation at 100 nM). PPARα and PPARδ showed significant activation at ~300 nM OA-NO₂. Nitrated oleic acid was consistently more potent than LNO₂ in the activation of PPARγ. A concentration of 1 μM OA-NO₂ typically induced the same degree of reporter gene expression as 3 μM LNO₂ and 1 μM rosiglitazone, with these activities partially inhibited by the PPARγ antagonist GW9662 (Fig. 9B). Native fatty acids did not activate PPARs at these concentrations (data not shown). The greater potency of OA-NO₂ as a PPARγ agonist, compared with LNO₂, motivated evaluation of the relative stability of these molecules. Current data indicate that LNO₂ decays in aqueous milieu to generate products.
that do not activate PPARs (17, 20). Compared with LNO₂, OA-NO₂ is relatively stable in aqueous conditions with only minimal decay occurring after 2 h (Fig. 5).

The signaling actions of OA-NO₂ as a PPAR/H₉₂₅ ligand were further assessed by evaluating its impact on adipocyte differentiation, as PPAR/H₉₂₅-dependent gene expression plays an essential role in the development of adipose tissue (28, 33). 3T3-L1 preadipocytes were treated with OA-NO₂ (3 μM), LNO₂ (3 μM), and negative controls for 2 weeks (Fig. 10). Adipocyte differentiation was assessed both morphologically and via oil red O staining, which indicated the accumulation of intracellular lipids. Vehicle, oleic acid and linoleic acid did not induce adipogenesis. In contrast, OA-NO₂ (3 μM) and LNO₂ (3 μM) induced 60% and 30% of 3T3-L1 preadipocyte differentiation, respectively. Rosiglitazone, a synthetic PPAR/H₉₂₅ ligand, also induced PPAR/H₉₂₅-dependent preadipocyte differentiation (Ref. 17 and data not shown). OA-NO₂ and rosiglitazone-induced pre-adipocyte differentiation resulted in expression of specific adipocyte markers (PPAR/H₂ and aP2); oleic acid had no effect on these gene products (Fig. 10B). PPAR/H₉₂₅ ligands also play a central role in glucose uptake and metabolism, with agonists widely used as insulin-sensitizing drugs. Consistent with its potent PPAR/H₉₂₅ ligand activity, OA-NO₂ induced an increase in the deoxyglucose uptake for the differentiated adipocytes (Fig. 11A). This effect of OA-NO₂ (1 μM) was almost paralleled by higher concentrations of LNO₂ (3 μM). The increased adipocyte glucose uptake, induced by nitrated fatty acids and the positive control rosiglitazone, was partially inhibited by GW9662 (Fig. 11B). In aggregate, these observations reveal that OA-NO₂ manifests well characterized PPAR/H₉₂₅-dependent signaling actions.

**DISCUSSION**

The nitration of hydrocarbons has long been recognized (34). Following the more recent discovery of cell signaling actions of oxides of nitro-
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Nitroalkene derivatives of fatty acids were analyzed by electrospray-ionization tandem mass spectrometry. Product ion spectra from synthetic standards were obtained in the negative ion mode as described under “Materials and Methods.” Major fragments generated for each standard are listed below.

| Mass/charge (m/z) | OA-NO₂ | [¹³C₁₅]OA-NO₂ | LNO₂ |
|------------------|--------|--------------|------|
| 344              | [M - H]| [M - H]      | [M - H]|
| 326              | [M - (H + H₂O)] | [M - (H + H₂O)] | [M - (H + H₂O)]|
| 324              | [M - (H + H₂O)] | [M - (H + H₂O)] | [M - (H + H₂O)]|
| 306              | [M - (H + H₂O)] | [M - (H + H₂O)] | [M - (H + H₂O)]|
| 295              | [M - (H + HNO₂)] | [M - (H + HNO₂)] | [M - (H + HNO₂)]|
| 279              | [M - (H + HNO₂)] | [M - (H + HNO₂)] | [M - (H + HNO₂)]|
| 277              | [M - (H + HNO₂)] | [M - (H + HNO₂)] | [M - (H + HNO₂)]|
| 263              | [M - (H + H₂O + CO₂)] | [M - (H + H₂O + CO₂)] | [M - (H + H₂O + CO₂)]|
| 246              | [M - (H + H₂O + CO₂)] | [M - (H + H₂O + CO₂)] | [M - (H + H₂O + CO₂)]|

Nitroalkene derivatives of fatty acids were analyzed by electrospray-ionization tandem mass spectrometry. Product ion spectra from synthetic standards were obtained in the negative ion mode as described under “Materials and Methods.” Major fragments generated for each standard are listed below.

| Compartment | Fraction | OA-NO₂ | LNO₂ |
|-------------|----------|--------|------|
| Plasma (nmol) | Free | 619 ± 52 | 79 ± 35 |
|             | Esterified | 302 ± 369 | 550 ± 275 |
|             | Total | 921 ± 421 | 630 ± 240 |
| Packed red cells (nmol) | Free | 59 ± 11 | 50 ± 17 |
|             | Esterified | 155 ± 65 | 199 ± 121 |
|             | Total | 214 ± 76 | 249 ± 104 |
| Whole Blood (nmol) | Total | 639 ± 366 | 477 ± 128 |
| Urine (fmol/mg creatinine) | 5.40 ± 52 | 2.28 ± 0.84 |

Assuming a 40% hematocrit.

Nitrogen (1, 35), it was also appreciated that “NO-derived species can mediate the oxidation, nitrosation, nitrosylation, and nitration reactions of protein, DNA, and unsaturated fatty acids (36). These reactions frequently yield stable products that induce functional and structural modifications to target molecules that can (a) translate the signaling actions of ‘NO or (b) mediate pathogenic responses when occurring in ‘excess.’

The reactions of ‘NO and its redox-derived products with lipids are multifaceted. Model studies of photosynthetic air pollutant-induced lipid oxidation reveal that exceedingly high concentrations of nitrogen dioxide (‘NO₂) induce both oxidation and nitration of fatty acids in phosphatidylcholine liposomes and fatty acid methyl ester preparations (37–39). Subsequently, reaction systems were designed to explore the interactions of endogenous ‘NO and ‘NO-derived species with fatty acids, including the superoxide reaction product ONOO⁻ and the nitrite acidification product nitrous acid (HNO₂). These model studies of the inflammatory reactions of ‘NO with fatty acids supported that (a) ‘NO mediates potent inhibition of autokatalytic radical chain propagation reactions of lipid peroxidation (40, 41) and (b) ‘NO-derived species produce both nitrated and oxidized derivatives of unsaturated fatty acids (3, 42). One product of these reaction pathways, LNO₂, is present at ~500 nM concentration in healthy human red cells and plasma and serves as a ligand for the PPAR nuclear lipid receptor family (9, 17). This insight, coupled with the fact that oleic acid is the most abundant unsaturated fatty acid in living organisms, motivated the present search for other potential endogenous nitrated fatty acid derivatives that might translate tissue redox signaling reactions.

The structure of OA-NO₂ (Fig. 1) was defined on the basis of the synthetic rationale and NMR analysis (Fig. 3). Proton and ¹³C NMR spectra indicate that synthetic OA-NO₂ is comprised of two regioisomers, 9- and 10-nitro-9-cis-octadecenoic acids, with no trans-isomers apparent. Peaks characteristic of the nitroalkene and olefinic carbons in the ¹³C spectrum appear as doublets that are equal in intensity, indicating an equivalent distribution between regioisomers. HPLC ESI MS/MS further characterized synthetic OA-NO₂. The combined fragmentation pattern of OA-NO₂ regioisomers was obtained by CID, which provided a “molecular fingerprint” used to identify OA-NO₂ in biological samples (Fig. 6). ESI MS/MS analysis of lipid extracts derived from plasma and red cells yielded spectra with identical HPLC retention times and major product ions, confirming that OA-NO₂ exists endogenously. It is not possible from MS analysis, however, to determine the cis/trans conformation of OA-NO₂ regioisomers. Quantitative analysis of plasma and red cells showed that OA-NO₂ is present in the vasculature at net concentrations ~50% greater than LNO₂ (TABLE THREE). The combined concentrations of free and esterified OA-NO₂ and LNO₂ are well above 1 μM. Multiple in vitro studies support that this is a concentration range capable of eliciting robust cell signaling responses.

The NO₂ functional groups of OA-NO₂ and LNO₂ are located on olefinic carbons. This configuration imparts a unique chemical reactivity that enables the release of NO during aqueous decay of nitroalkenes via a modified Nef reaction (20). Furthermore, the β-carbon proximal to the alkenyl NO₂ group is strongly electrophilic and reacts with H₂O via a Michael addition-like mechanism to generate nitrohydroxy adducts (Figs. 2 and 7). Nitrohydroxyarachidonic acid species have been detected in bovine cardiac muscle (43), and nitrohydroxylinoleic acid has been identified in lipid extracts obtained from hypercholesterolemic and post-prandial human plasma, suggesting that this is a ubiquitous derivative (44). The present identification of a wide spectrum of nitrated fatty acids and corresponding nitrohydroxy fatty acid derivatives in human plasma and urine reveals that nitration reactions occur with all unsaturated fatty acids (Figs. 2 and 7). The hydroxyl moiety of nitrohydroxy fatty acid derivatives destabilizes the adjacent carbon-carbon bond, facilitating heterolytic scission reactions that generate predictable fragments during CID (Fig. 7). Present data indicate that nitrohydroxy...
adducts of LNO$_2$ and OA-NO$_2$ are not ligands for PPAR$\gamma$ (Ref. 17 and data not shown).

Multiple mechanisms can support the basal and inflammatory nitration of fatty acids by 'NO-derived species, including 'NO$_2$-initiated auto-oxidation of polyunsaturated fatty acids via hydrogen abstraction from the bis-allylic carbon (26, 38, 45-48). Of relevance to cell signaling, 'NO$_2$ is derived from multiple reactions. These include the homolytic scission of both peroxynitrous acid (ONOOH) and the reaction product of ONOO$^-$ with CO$_2$, nitrosoperoxocarbonate (ONOOCO$_2$). The oxidation of NO$_3^-$ by heme peroxidases, such as myeloperoxidase, is also a significant source of inflammatory 'NO$_2$ production (49, 50). These alkene nitration mechanisms yield nitrated fatty acids that are structurally similar or identical to the OA-NO$_2$ and nitrohydroxy adducts detected clinically (Fig. 8). Nitration by a free radical mechanism might suggest that all olefinic carbons within a fatty acid would be susceptible nitration targets, with the additional likelihood of double bond rearrangement and conjugation. The discovery of OA-NO$_2$ lends critical perspective to this issue, because monounsaturated fatty acids are less susceptible but still capable of oxidation reactions (51). In view of the present structural data regarding nitroalkene positional isomer distribution, alternative fatty acid nitration mechanisms may also occur. For example, nitration by an ionic addition reaction (e.g. nitronium ion, NO$_2^+$) can generate singly nitrated fatty acids with no double bond-rearrangement (26). Since NO$_2^+$ readily reacts with H$_2$O, this species may require localized catalysis (e.g. reaction of ONOO$^-$ with transition metals) to serve as a biologically relevant nitrating species. Finally, data in Fig. 8 indicate that acidic nitration reactions occur with both mono- and polyunsaturated fatty acids to yield non-conjugated nitroalkene derivatives of polyunsaturated fatty acids. This precept is also supported by acidified NO$_2^-$ and 'NO$_2$-mediated fatty acid methyl ester oxidation and nitration profiles (39, 48, 52).

Of relevance to mechanisms underlying fatty acid nitration in vivo, the nitrohydroxy adducts of 18 unsaturated fatty acids examined in the present study (18:1, 18:2, and 18:3) all yield a predominant CID fragment of m/z 171 (Fig. 7). This mass is consistent with 9-oxo-nonanoic acid, a CID fragment generated with standards when the NO$_2$ group is located at the 10-carbon and the hydroxyl moiety at the 9-carbon. There are several interpretations of these data. First, the differences in relative intensities of the CID products may be due to differential fragmentation efficiencies. Indeed, the m/z 171 product generated from the C-10

**FIGURE 7. Structural analysis of nitrohydroxy fatty acid adducts in urine.** The presence of nitrohydroxy fatty acids in urine was confirmed using HPLC ESI MS/MS in the negative ion mode by performing product ion analyses concurrent to MRM detection. Structures of possible adducts are presented along with their diagnostic fragments and product ion spectra for 18:1(OH)-NO$_2$ (A), 18:2(OH)-NO$_2$ (B), and 18:3(OH)-NO$_2$ (C). Some regions of the MS/MS fragmentation patterns are amplified, as indicated, to better convey structural information. The 10-nitro regioisomer of 18:1(OH)-NO$_2$ is present in urine, as evidenced by the intense peak corresponding to m/z 171; also present are fragments consistent with the 9-nitro regioisomer (m/z 202), loss of a nitro group (m/z 297), and water (m/z 326). 18:2(OH)-NO$_2$ also shows a predominant m/z 171 fragment, again consistent with an oxidation product of LNO$_2$ nitrated at the 10-carbon (B). Diagnostic fragments for the three other potential regioisomers were not apparent. Finally, multiple regioisomers of 18:3(OH)-NO$_2$ are present (C).
adduct is a 9-oxo-nonanoic anion, whereas the C-9 product (m/z 202) is 9-nitro-nonanoic anion. An alternative interpretation is that the C-10 nitrohydroxy adduct is more predominant, suggesting that either steric control or enzymatic mechanisms regulate the stereospecificity of biological fatty acid nitration. The nitration of ∆9 unsaturated fatty acids to C-10 nitroalkene derivatives, with retention of double bond arrangement, supports that stereospecific enzymatic reactions may mediate fatty acid nitration. It is also possible that nitrated fatty acids are made bioavailable from dietary sources consisting of stereospecific fatty acid nitroalkene derivatives. Further studies are currently under way to address this issue.

Designation of nitroalkene derivatives as a class of signaling molecules is contingent upon ascribing specific bioactivities to multiple members within the class at clinically relevant concentrations. Nitroilnoleate inhibits neutrophil and platelet function via cGMP-independent, cAMP-mediated mechanisms (10–12). Also, aqueous decay of LNO2 yields NO, a reaction facilitated by translocation of LNO2 from a hydrophobic to hydrophilic microenvironment, which in turn induces cGMP-dependent vessel relaxation (12, 20). LNO2 also serves as a robust ligand for PPARγ (17), a nuclear hormone receptor that binds lipophilic ligands and induces DNA binding of the transcription factor complex at DRI-type motifs in the promoter sites of target genes. Downstream effects of PPARγ activation include modulation of metabolic and cellular differentiation genes, regulation of inflammatory responses, adipogenesis, and glucose homeostasis (18, 19). In the vasculature, PPARγ is expressed in monocytes, macrophages, smooth muscle cells, and endothelium (53) and plays a central role in regulating the expression of genes related to lipid trafficking, cell proliferation, and inflammatory signaling. Herein we show that OA-NO2 also serves as a PPARγ, -α, and -δ ligand that exceeds the potency of LNO2 and rivals the potency of synthetic PPAR ligands such as fibrates and thiazolidinediones (Figs. 9–11). The greater potency of OA-NO2 as a PPAR ligand relative to LNO2, increased receptor affinity, or both.

The combined blood concentrations of OA-NO2 and LNO2 in healthy humans exceeds 1 μM (TABLE THREE); thus, they are present at concentrations capable of modulating inflammatory cell function and activation of PPAR receptors. Endogenous blood concentrations of nitroalkenes also far exceed those of previously proposed endogenous

**FIGURE 8. Nitration of oleic acid by inflammatory oxidants.** The potential nitration of the monounsaturated oleic acid by oxidants generated in an inflammatory milieu was explored by reaction with MPO, H2O2, and NO;2, peroxynitrite (ONO0)2. Each candidate nitrating condition included a negative control, as indicated. After reactions, lipids were extracted and analyzed for oleic acid nitration. Top panel, nitration reactions using MPO, acidic nitration, and ONOO− all resulted in significant extents of oleic acid nitration as compared with matched controls. Significance of difference between treated and control groups was determined using a one-tailed, paired Students t test, with p < 0.05 and indicated by *. Middle panel, by monitoring the MRM transition m/z 344/202, the generation of nitrohydroxy C-9 OA-NO2 was measured. Due to the lack of corresponding 13C internal standards, quantitative determinations were precluded, thus data were expressed as the peak ion intensity of C-9 OA(OH)-NO2 generation as a proportion of added [13C18]OA-NO2. All three reaction conditions generated nitrohydroxy C-9 OA-NO2. Greater peak intensities for each reaction condition suggest that the C-10 nitrated oleic acid is the predominant nitroalkene product of these reactions.

**FIGURE 9. OA-NO2 is a PPARγ agonist.** A, CV-1 cells transiently co-transfected with a plasmid containing the luciferase gene under the control of three tandem PPRE (PPRE × 3 TK-luciferase) and hPPARγ, hPPARδ, or hPPARα expression plasmids showed all three PPARs were activated by OA-NO2, with the relative activation of PPARγ > PPARδ > PPARα. All values are expressed as mean ± S.D. (n = 3). PPARγ activation was significantly different from vehicle at 100 nM OA-NO2, whereas PPARα and PPARδ activation were significantly different from vehicle at 300 nM and 1 μM OA-NO2, respectively (*, p ≤ 0.05; Student’s t test). B, OA-NO2 inducing a degree of PPARγ activation that was similar to that induced by 3 μM LNO2 versus control (*, p ≤ 0.05; Student’s t test). Nitroalkene activation of PPARα was partially blocked by the PPARγ antagonist GW9662 (#, p ≤ 0.05; Student’s t test).
PPARγ ligands (17). These data thus have broad implications for the NO and redox signaling reactions that play a crucial role in dysregulated cell growth and differentiation, metabolic syndrome, atherosclerosis, diabetes, and a variety of inflammatory conditions, all clinical pathologies that include a significant contribution from PPAR-regulated cell signaling mechanisms (54).

The regulation of inflammation by inhibiting eicosanoid synthesis is a well established and prevalent target of anti-inflammatory drug strategies. Much less well understood are the concerted cell signaling mechanisms by which inflammation is favorably resolved in vivo. While the composite in vivo tissue signaling activities of nitrated fatty acids remain to be defined, studies to date indicate that these pluriportant signaling mediators generally manifest salutary metabolic and anti-inflammatory actions (10–12, 17). The capability of redox-derived lipid signaling molecules to mediate the resolution of inflammation is a relatively new concept, with lipoxins and resolvins also representing new classes of lipid mediators that act in this manner (55, 56). Of note, endogenous concentrations of OA-NO2 and LNO2 are abundant and are increased by oxidative inflammatory reactions. Thus, nitrated fatty acids will exert both receptor-dependent (via PPAR ligand activity) and cyclic nucleotide-mediated roles in transducing the redox signaling actions of oxygen and NO, thereby regulating organ function, cell differentiation, cell metabolism, and systemic inflammatory responses.

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