Nitro-fatty acid pharmacokinetics in the adipose tissue compartment

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Abstract  Electrophilic nitro-FAs (NO₂-FAs) promote adaptive and anti-inflammatory cell signaling responses as a result of an electrophilic character that supports posttranslational metabolic actions that could be expected for this chemically-reactive class of endogenous signaling mediators and synthetic drug candidates.—Fazzari, M., N. K. H. Khoo, S. R. Woodcock, D. J. Jorkasky, L. Li, F. J. Schopfer, and B. A. Freeman. Nitro-fatty acid pharmacokinetics in the adipose tissue compartment. J. Lipid Res. 2017. 58: 375–385.

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Unsaturated FAs of cellular membranes, lipoproteins, and dietary fats are susceptible to oxidation and nitration by reactive oxygen species and nitrogen oxides during inflammatory and metabolic stress (1–3). In particular, the reaction between unsaturated FAs and the nitric oxide and nitrite (NO⁻)–derived radical species nitrogen dioxide (NO₂) generates electrophilic FA nitro-alkene byproducts (NO₂-FAs) (4). These molecules are endogenously detected and have been observed to mediate salutary responses in models of inflammatory injury and oxidative stress. Current data supports that NO₂-FAs predominantly signal via posttranslational protein modifications, specifically of functionally significant Cys residues of nuclear transcription factor erythroid 2-related factor 2 (Nrf2), soluble epoxide hydrolase (sEH), p65 subunit of nuclear factor kappa B (NF-kB), transient receptor potential cation channel subfamily V member 1 (TRPV1), and heat shock factor-1 (HSF-1) (5–9).

NO₂-FAs have been detected in a variety of species, including mammals and plants, as both free acid and as complex lipid-esterified species (3, 10, 11). For example, NO₂-FAs are detected at micromolar concentrations in rodent cardiac mitochondria subjected to an episode of ischemia-reperfusion and at nanomolar concentrations in healthy human plasma and urine (12–14). Additionally, NO₂-FAs have been detected in Arabidopsis, fresh olives, and

Abbreviations:  BLQ, below the limit of accurate quantification; CE, cholesterol ester; CLA, conjugated linoleic acid [octadeca-(9Z,11E)-dioenoic acid]; HFD, high-fat diet; MAG+DAG, mono- and diacylglyceride; MRM, multiple reaction monitoring; NF-kB, nuclear factor kappa B; ·NO₂, nitro oxide; NO₂, nitrite; NO₃, nitrate; NO₂-CLA, nitro-conjugated linoleic acid; NO₂-LnA, nitro-linolenic acid; NO₂-FAs, electrophilic nitro-FA; 10-NO₂-OA, 10-nitro-oleic acid (10-nitro-octadec-9-enoic acid); 10-NO₂[¹³C]OA, 10-nitro-[¹³C]oleic acid radiolabeled at carbon 10; NO₂-OA, nitro-oleic acid; ·NO₂, nitro oxide; ·NO₂, nitro oxide (isomeric mixture of 10-nitro- and 9-nitro-octadec-9-enoic acid); NO₂-[¹³C]OA, nitro-[¹³C]octadec-9-enoic acid; NO₂-SA, nitro-stearic acid; NO₂-[¹⁵N/D₄]OA, [¹⁵N]-nitro-[D₄]octadecenoic acid; NO₂-[¹⁵N/D₄]OA, [¹⁵N]-nitro-[D₄]octadecenoic acid; Nrf2, nuclear transcription factor erythroid 2-related factor 2; OA, oleic acid; PC, phosphatidylcholine; QWBBA, quantitative whole-body autoradiography; sEH, soluble epoxide hydrolase; TAG, triacylglycerides. The designations “9-NO₂-” and “10-NO₂-” OA are used herein to describe the position of the nitro group in the fatty acid chain and do not refer to the free acid or esterified FAs. NO₂-FAs preferentially incorporated in monoaoyl- and diacylglycerides, while reduced metabolites were highly enriched in triacylglycerides. This differential distribution profile was confirmed in vivo in the adipose tissue of NO₂-OA-treated mice. This pattern of NO₂-FAs deposition lends new insight into the unique pharmacokinetics and pharmacologic actions that could be expected for this chemically-reactive class of endogenous signaling mediators and synthetic drug candidates—Fazzari, M., N. K. H. Khoo, S. R. Woodcock, D. J. Jorkasky, L. Li, F. J. Schopfer, and B. A. Freeman. Nitro-fatty acid pharmacokinetics in the adipose tissue compartment. J. Lipid Res. 2017. 58: 375–385.

Unsaturated FAs of cellular membranes, lipoproteins, and dietary fats are susceptible to oxidation and nitration by reactive oxygen species and nitrogen oxides during...
and extra virgin olive oil (15, 16). Notably, plasma and urinary NO₂-FA concentrations in humans are increased following oral supplementation with NO₂⁻, nitrate (NO₃⁻), and conjugated linoleic acid (CLA) (17, 18).

Recently, NO₂-FA-containing phospholipids have been identified in cardiac mitochondria isolated from an animal model of type 1 diabetes (10), and NO₂-FA-containing triacylglycerides (TAGs) have been detected in nitro-oleic acid (NO₂-OA)-supplemented adipocytes and plasma of rats after gavage with NO₂-OA (3). The analytical advances permitting the detection of FA nitro-alkene derivatives in complex lipids provide new opportunities for better understanding NO₂-FA pharmacokinetics, metabolism, and potential toxicology. To date, the identification and quantitation of NO₂-FAs in complex lipids has been limited by: 1) the instability of NO₂-FAs during enzymatic and basic hydrolysis; 2) the diversity of potential structures wherein NO₂-FAs can be incorporated (e.g., sterols, phospholipids, glycolipids, glyceralipids); 3) challenges of the analysis NO₂-FA-containing complex lipids and their relative low abundance in cells and tissues; and 4) the lack of synthetic standards (19, 20).

Herein, we show the preferential distribution of orally administered 10-nitro-[14C]oleic acid radiolabeled at carbon 10 (10-NO₂-[14C]OA) in adipose tissue of rats over a 2 week period via quantitative whole-body autoradiography (QWBA). Then, after lipid class fractionation, we report the quantitative analysis of the differential incorporation of NO₂-FA and metabolites into cultured adipocytes before and after acid hydrolysis, using HPLC-MS/MS. We observed the preferential incorporation of electrophilic versus nonelectrophilic NO₂-FAs in adipocyte mono- and diacylglycerides (MAGs/DAGs), a phenomenon confirmed in adipose tissue obtained from NO₂-OA-treated mice. These findings reveal tissue-specific pharmacokinetics and the preferential role of adipose tissue in distribution and metabolism of NO₂-FAs in vivo.

**MATERIALS AND METHODS**

**Materials**

Oleic acid (OA), CLA (catalog number UC-60A), and α-linolenic acid were from Nu-Check Prep, Inc. (Elysian, MN). The [15N]labeled OA was obtained from Spectra Stable Isotopes (Columbia, MD). The [15N]labeled sodium nitrate was purchased from Cambridge Isotope Laboratories (Tewksbury, MA). Deuteron-labeled 1-bromononone-6,6,7,7-d₄ was obtained from CDN Isotopes (Quebec, Canada). The 10-NO₂-[14C]OA (labeled at carbon 10) was synthesized by ABC Laboratories, Inc. (Columbia, MO). Nitro-linolenic acid (NO₂-LnA), nonspecific 9- and 10-nitrooctadec-9-enoic acid (NO₂-OA), and isotopically labeled analog nitro-[15N₁₃C₉]octadec-9-enoic acid (NO₂-[15N]₁₃C₉OA) were synthesized by direct alkene nitroselenation of the corresponding native FAs, as previously described (21). Nitro-CLA (NO₂-CLA) was synthesized according to a previously described method for conjugated diene nitration (11). The [15N]nitro-[13C₁₀]octadecenoic acid (NO₂-[15N]₁₃C₁₀OA) was synthesized from the corresponding isotopically labeled precursor, 1-[^15N]nitrononone-6,6,7,7-d₄ (obtained by nucleophilic substitution of [¹⁵N]labeled sodium nitrate and 1-bromononene-6,6,7,7-d₄) and a nine-carbon aldehyde-ester via a previously described nitroaldol procedure (21, 22). Nitro-stearic acid (NO₂-SA) and its isotopically labeled analog, [¹⁵N]nitro-[D₄]octadecanoic acid (NO₂-[¹⁵N/D₄]SA), were synthesized by selective reduction with sodium borohydride of the nitro-alkene double bond in NO₂-OA and NO₂-[¹⁵N/D₄]OA, respectively. Nitro-alkene standards of various lengths (C₁₀, C₁₁, C₁₂, and C₁₃) were synthesized by nitroselenation (21) of the corresponding native FAs and used as calibrators to normalize for MS responses. Before each experiment, NO₂-OA, NO₂-CLA, and NO₂-LnA concentrations were measured spectrophotometrically in methanol using the following extinction coefficients: ε₁₈₀ = 11,200 M⁻¹ cm⁻¹ for NO₂-OA, ε₉₅₇ = 7.000 M⁻¹ cm⁻¹ for NO₂-OA, and ε₉₅₀ = 7.000 M⁻¹ cm⁻¹ for NO₂-Ln (21). DMEM, FBS, HBSS, and antibiotic-antimycotic solutions were from Corning Cellgro (Herndon, VA). Strata NH₂ solid phase extraction columns (55 μm, 70 A) were from Phenomenex (Torrance, CA). Solvents were LC-MS grade from Burdick and Jackson (Muskegon, MI). Chemicals were of analytical grade and purchased from Sigma (St. Louis, MO) unless otherwise stated.

**Cell culture**

The 3T3-L1 preadipocytes were maintained and differentiated into adipocytes as previously (23). Fully differentiated adipocytes were then treated with 100 μM CLA or 5 μM OA, NO₂-SA, 10-nitro-oleic acid (10-NO₂-OA), NO₂-CLA, NO₂-LnA in HBSS. Aliquots of cellular media were obtained at 1, 2, 4, 8, and 24 h, spiked with 20 pmol NO₂-[15N]₁₃C₁₀OA and NO₂-[¹⁵N/D₄]SA as internal standards, extracted using the Bligh and Dyer method (24), dried under a stream of nitrogen and reconstituted in 0.2 ml methanol for HPLC-MS/MS analysis. At the end of the treatment, adipocytes were rinsed with cold PBS, scraped, and lipids extracted.

**Quantitative whole-body autoradiography analysis**

The 10-NO₂-[14C]OA (labeled at carbon 10) was administered by a single oral gavage as a solution in sesame oil to male Sprague-Dawley rats (8–10 weeks old, n = 8) at a dose level of 30 mg/4 MBLq/2 ml sesame oil per kilogram body weight. Rats were euthanized at 1, 6, 24, 48, 72, 120, 168, and 236 h after dose administration and QWBA was then carried out on the carcass of n = 1 animal for each time point. A frozen carcass was set in a block of 2% (w/v) aqueous carboxymethyl cellulose at −80°C. Samples of whole blood reference standards containing six different concentrations of radioactive activity were placed into holes drilled into the block to facilitate signal calibration. The block was mounted onto the stage of a microtome in a cryostat maintained at −20°C. Sagittal sections (~90 μm) were obtained at six different levels through the carcass of each animal: 1) kidney; 2) intraorbital lacrimal gland; 3) hardier gland; 4) adrenal gland; 5) half brain and thyroid; and 6) brain and spinal cord. The sections, mounted on sectioning tape, were freeze-dried using a Lysolab B freeze-drier. One section from each level was exposed to imaging plates and an adjacent freeze-dried section at each level was mounted and used for reference purposes when evaluating the images. After exposure in a refrigerated lead-lined exposure box for 3 days, imaging plates were scanned using a FLA5000 radioluminography system. The electronic images were analyzed using an image analysis package (Sescan Densitometry software, version 2.0). The limits of quantification for the procedure corresponded to the lowest and highest calibration standards (0.12 to 528 μg equivalents of 10-NO₂-OA per gram). Wherever possible, the maximum area for each tissue within a single autoradiogram was defined for measurement. These radiolabeling experiments were conducted at Huntingdon Life Sciences (Cambridgeshire,
Liquid scintillation analysis

Plasma and blood cell radioactivity was measured by liquid scintillation analysis using Wallac 1409 automatic liquid scintillation counters. Radioactivity in amounts less than twice that of the background concentration in the samples was considered to be below the limit of accurate quantification (BLQ).

Administration of NO2-OA in high-fat diet mice

All murine studies were conducted with the approval of the University of Pittsburgh Institutional Animal Care and Use Committee (25). In brief, 6–8 week old male C57Bl/6j mice were subjected to high-fat diet (HFD) purchased from Research Diets Inc. (D12492; New Brunswick, NJ) for 20 weeks. Age-matched controls were maintained on a standard rodent Chow diet (Pro Lab RHM 3000 rodent diet; PMI Feeds, Inc., St. Louis, MO). Mice were fed ad libitum for 20 weeks and given free access to water. At week 13.5 of the HFD study, mice were anesthetized with isoflurane before Alzet osmotic pumps (Cupertino, CA) containing vehicle (polyethylene glycol/ethanol, 92:8) or 9- and 10-NO2-OA were implanted subcutaneously in the back region. The osmotic mini pump was set to deliver 8 mg NO2-OA / kg/day. At the end of the 20th week, mice were euthanized and epididymal fat pads were quickly removed (n = 9 per treatment group), snap-frozen, and stored at −80°C. Sections of adipose tissues (~100 mg) were homogenized in a bullet blender for 5 min in 0.8 ml phosphate buffer 50 mM pH 7.4, and lipids were extracted.

Adipocyte lipid analysis

Adipocyte and adipose tissue lipids were extracted according to Bligh and Dyer (24), dried under a stream of nitrogen, and dissolved in 0.5 ml hexane/methyl tert-butyl ether/acetic acid (100:39.3 v/v/v). Lipid classes were further resolved chromatographically using solid phase extraction Strata NH2 columns (100:3:0.3 v/v/v). Lipid classes were further resolved chromatographically resolved using the following gradient program: 45–100% solvent B (0–8 min); 100% solvent B (8–10 min) followed by 2 min re-equilibration to initial conditions. NO2-FAs were detected using an API4000 Q-trap triple quadrupole mass spectrometer (AB Sciex, San Jose, CA) equipped with an ESI source in negative mode. The following parameters were used: declustering potential, −75 V; collision energy, −35 eV; and a desolvation temperature of 650°C. NO2-FAs and their corresponding metabolites were detected using the multiple reaction monitoring (MRM) transitions shown in supplemental Table S1. Quantification of NO2-FAs in cell media over 24 h in adipocytes and adipose tissue was performed by stable isotopic dilution analysis using NO2-OA and NO2-SA calibration curves in the presence of NO2-[13C18]OA (MRM 344.3/46) and NO2-[15N/D2]SA (MRM 333.3/47) internal standards. Nitro-FAs of various lengths (C16, C15, C14, C13, and C12) followed as MRM transitions (supplemental Table S1). Tissue distribution of radiolabeled NO2-OA

QWBA revealed the tissue distribution of NO2-OA over time. After oral administration of a single dose of 10-NO2-[13C]OA (30 mg/kg) to rats, radioactivity was readily absorbed from the gastrointestinal tract and widely distributed throughout the animal body. The vast majority of tissues reached maximum radiolabel distribution by 6 h after dosing (Fig. 1, supplemental Table S2), with radioactivity concentrations declining in most tissues by 24 h. Notably, brown and abdominal white adipose tissue displayed the highest levels of radioactivity 72 h postdosing in comparison with other organs, affirming that NO2-FAs and potential metabolites preferentially accumulate in adipose tissue (Fig. 1). NO2-FA distribution and metabolism in cultured adipocytes

To better characterize the distribution of NO2-FAs and metabolites in cellular lipid fractions, cultured 3T3-L1 adipocytes were supplemented with the biologically relevant mono- and poly-unsaturated nitro-alkanes, 10-NO2-OA, NO2-CLA, and NO2-LnA, and the saturated nitro-alkane, NO2-SA. The quantitation of NO2-FAs before and after acid hydrolysis of adipocyte lipids revealed specific patterns of incorporation, intracellular distribution, and metabolism in each lipid fraction. As expected, free acid nitrated species

RESULTS

Tissue distribution of radiolabeled NO2-OA

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were only detectable in the FFA fraction before hydrolysis. Neither elongation products nor metabolites shorter than C₁₂ were observed for all NO₂-FA treatments.

NO₂-OA was principally reduced to NO₂-SA and metabolized to its corresponding dinor, tetranor and hexanor β-oxidation products (3, 29). Esterified NO₂-OA was ~18 times more abundant in MAG+DAG than in TAG (Fig. 2A), while the nonelectrophilic NO₂-SA and its β-oxidation metabolites showed the opposite distribution. The NO₂-SA metabolite and its dinor, tetranor, and hexanor β-oxidation

![Fig. 1. Distribution of radioactivity in rats after a single oral administration of radiolabeled 10-NO₂-OA. Whole-body autoradiograms of rats euthanized at 1 h (A), 6 h (B), 24 h (C), 48 h (D), 72 h (E), 120 h (F), 168 h (G), and 336 h (H) after an oral administration of 30 mg/kg 10-NO₂-[¹⁴C]OA (labeled at carbon 10). Radioactivity appears in black (n = 1 for each time point).]
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products were more abundant in TAG than in MAG+DAG fractions (Fig. 4B–D). Consistent with this trend, adipocytes supplemented with the trienoic nitro-alkene, NO₂-LnA, exhibited even lower levels of esterification compared with mono- and di-unsaturated nitro-alkenes. The NO₂-LnA levels in TAG and MAG+DAG fractions were similar (Fig. 5A).

NO₂-dihydro-LnA, the nonelectrophilic reduced metabolite of NO₂-LnA, and its β-oxidation product dinor, NO₂-di-hydro-LnA, had levels and lipid distributions that were similar to those of NO₂-SA and reduced NO₂-CLA metabolites (Fig. 5B, C). Notably, lower extents of NO₂-FA incorporation occurred in glycerophospholipid fractions as opposed to glycerolipid fractions (Figs. 2–5), with PC showing the highest levels among phospholipids likely due to its greater abundance (30, 31).

Fig. 2. Metabolism and distribution of NO₂-OA in lipid fractions of NO₂-OA-supplemented adipocytes. Fractionated lipid classes from adipocytes were analyzed by HPLC-MS/MS in negative ion mode after acid hydrolysis to determine the cellular content (picomoles per milligram protein) of NO₂-OA (A), NO₂-SA (B), dinor NO₂-SA (C), tetranor NO₂-SA (D), and hexanor NO₂-SA (E). FFA fraction was not hydrolyzed. Two independent experiments were performed. Range (minimum-maximum) and mean of one representative experiment (n = 4) are shown.

The dienoic nitro-alkene, NO₂-CLA, showed a profile of complex lipid partitioning that was similar to NO₂-OA, with the proviso that net extents of neutral lipid esterification were 10- to 20-fold lower (Fig. 4A). As for the distribution of NO₂-SA metabolites, the reduced metabolite of NO₂-CLA (NO₂-dihydro-CLA) and its dinor and tetranor β-oxidation products were more abundant in TAG than in MAG+DAG fractions (Fig. 4B–D). Consistent with this trend, adipocytes supplemented with the trienoic nitro-alkene, NO₂-LnA, exhibited even lower levels of esterification compared with mono- and di-unsaturated nitro-alkenes. The NO₂-LnA levels in TAG and MAG+DAG fractions were similar (Fig. 5A). NO₂-dihydro-LnA, the nonelectrophilic reduced metabolite of NO₂-LnA, and its β-oxidation product dinor, NO₂-dihydro-LnA, had levels and lipid distributions that were similar to those of NO₂-SA and reduced NO₂-CLA metabolites (Fig. 5B, C). Notably, lower extents of NO₂-FA incorporation occurred in glycerophospholipid fractions as opposed to glycerolipid fractions (Figs. 2–5), with PC showing the highest levels among phospholipids likely due to its greater abundance (30, 31).
mainly the reduced metabolite, NO$_2$-dihydro-LnA (Fig. 5D), and dinor and tetranor $\beta$-oxidation products (Fig. 5E, F).

**NO$_2$-OA esterification and metabolism in adipose tissue in vivo**

The QWBA study coupled with the distribution and metabolism of nitro-alkenes and nitro-alkanes in cultured adipocytes encouraged testing whether NO$_2$-FAs could be detected in adipose tissue in vivo in a clinically-relevant model system. A HFD-induced murine model of obesity demonstrates inflammatory responses akin to humans, in part characterized by the increased generation of reactive nitric oxide and oxygen-derived reactive species, which could potentially give rise to esterified NO$_2$-FAs (32–34). However, analysis of lipid fractions from adipose tissue of HFD-control mice did not show the presence...
Nitro-fatty acid pharmacokinetics specifically the incorporation of NO\textsubscript{2}-OA and its metabolites into adipose tissue complex lipids after NO\textsubscript{2}-OA supplementation. Notably, as for in vitro study observations, NO\textsubscript{2}-OA was esterified to neutral glycerolipids and more abundant in the MAG+DAG than in the TAG fraction (Fig. 6A), while its nitro-alkane metabolites, such as NO\textsubscript{2}-SA and dinor, tetranor, and hexanor NO\textsubscript{2}-SA, were preferentially distributed in TAG fractions (Fig. 6B–E). No NO\textsubscript{2}-FAs were detected in the phospholipid fraction, either before or after hydrolysis. Very low levels of NO\textsubscript{2}-OA were present in the FFA fraction (Fig. 6A), along with reduced and β-oxidation products. Similarly, low amounts of NO\textsubscript{2}-OA were detected in CE fractions.

Fig. 4. Distribution and metabolic profile of NO\textsubscript{2}-CLA in NO\textsubscript{2}-CLA-supplemented adipocyte lipids and media. Cellular content of NO\textsubscript{2}-CLA (A), NO\textsubscript{2}-dihydro-CLA (B), dinor NO\textsubscript{2}-dihydro-CLA (C), and tetranor NO\textsubscript{2}-dihydro-CLA (D) at 24 h was established on fractionated lipid classes after acid hydrolysis. FFA fraction was not subjected to acid hydrolysis. Concentration of nitro-FA species was determined in media for the following species: NO\textsubscript{2}-CLA (filled square) and NO\textsubscript{2}-dihydro-CLA (open square) (E) and dinor NO\textsubscript{2}-CLA (filled square) and dinor NO\textsubscript{2}-dihydro-CLA (open square) (F) over a period of 24 h. Range (minimum-maximum) and mean (A–D) and mean ± SD (E, F) are shown. Two independent experiments were performed (n = 4).

of NO\textsubscript{2}-FAs (data not shown). Recent reports indicate that NO\textsubscript{2}-OA promotes beneficial metabolic and anti-inflammatory responses by modulating Nrf2-dependent antioxidant gene expression, sEH activity, and TLR4/NF-kB signaling (6–8). The administration of pure synthetic NO\textsubscript{2}-OA induces beneficial signaling actions and physiological responses in animal models of metabolic, vascular, renal, and pulmonary disease (12). The safety of NO\textsubscript{2}-OA use as a drug candidate in humans has been tested by multiple phase I studies. With Food and Drug Administration approval, NO\textsubscript{2}-OA is now undergoing phase II clinical trials. For these reasons, we considered it important to better understand FA nitro-alkene pharmacokinetics, specifically the incorporation of NO\textsubscript{2}-OA and its metabolites into adipose tissue complex lipids after NO\textsubscript{2}-OA supplementation. Notably, as for in vitro study observations, NO\textsubscript{2}-OA was esterified to neutral glycerolipids and more abundant in the MAG+DAG than in the TAG fraction (Fig. 6A), while its nitro-alkane metabolites, such as NO\textsubscript{2}-SA and dinor, tetranor, and hexanor NO\textsubscript{2}-SA, were preferentially distributed in TAG fractions (Fig. 6B–E). No NO\textsubscript{2}-FAs were detected in the phospholipid fraction, either before or after hydrolysis. Very low levels of NO\textsubscript{2}-OA were present in the FFA fraction (Fig. 6A), along with reduced and β-oxidation products. Similarly, low amounts of NO\textsubscript{2}-OA were detected in CE fractions.
NO$_2$-FAs are metabolized by various reactions, such as mitochondrial $\beta$-oxidation, Michael addition, enzyme-catalyzed reduction, and esterification into complex lipids (3, 14, 45, 46). Both nitro-alkenes and nitro-alkanes are metabolized by mitochondrial $\beta$-oxidation generating (C$_2$H$_4$)$_n$-shorter metabolites (14, 29, 47). In humans and rodents, metabolites as short as C$_8$ have been observed in urine (48). However, no nitro-C$_{12}$-alkenes and nitro-C$_{10}$-alkanes were detected in adipocytes, indicating that adipocytes lack the necessary mitochondrial or peroxisomal enzymatic machinery to further process nitro-alkenes through $\beta$-oxidation cycles when the nitro group is proximal to the carboxyl moiety (3, 14, 29). The electrophilic nature of nitro-alkenes undergoes:

**DISCUSSION**

Nitration of unsaturated FAs and the corresponding generation of electrophilic NO$_2$-FA occur during acidic conditions of digestion and oxidative inflammatory conditions (3, 4, 17, 18, 35). The electrophilic properties of NO$_2$-FA induce anti-inflammatory and cytoprotective actions via reversible posttranslational modification of transcriptional regulatory proteins, such as NF-kB, Keap1/Nrf2, and PPAR-$\gamma$, and enzymes such as xanthine oxidoreductase and sEH (6–8, 36–38). Beneficial metabolic and anti-inflammatory effects of NO$_2$-FAs have been shown in animal models of fibrosis, atherosclerosis, renal and cardiac ischemia reperfusion, restenosis, and diabetes (12, 39–44).

Fig. 5. Distribution and metabolic profile of NO$_2$LnA in NO$_2$LnA-supplemented adipocyte lipids and media. Adipocyte cellular content of NO$_2$LnA (A), NO$_2$-dihydro-LnA (B), and dinor NO$_2$-dihydro-LnA (C) at 24 h, after acid hydrolysis of the different fractionated lipid classes. FFA fraction was analyzed without hydrolysis. Time-dependent concentration changes of NO$_2$LnA (filled square) and its metabolites NO$_2$-dihydro-LnA (open square) (D), dinor NO$_2$LnA (E), and tetranor NO$_2$LnA (F). Range (minimum-maximum) and mean (A–C) and mean ± SD (D–F) are shown. Two independent experiments were performed (n ≥ 3).
addition with GSH and cysteine-containing proteins (4, 49, 50); and 2) rapid metabolism by prostaglandin reductase-1 and resultant generation of inactive nitro-alkanes (47). These two metabolic reactions have a significant impact on the extra- and intra-cellular distribution of NO₂-FAs and downstream pharmacological effects. The time-dependent enrichment of nitro-alkene metabolites in the extracellular compartment could be a consequence of NO₂-FA-GSH adduct export via multidrug resistance protein-1. This, in the presence of low extracellular GSH concentrations, can more readily dissociate to regenerate free nitro-alkenes or be passively transported across the cellular membrane, a pathway also shared by nitro-alkane metabolites (49–51). Furthermore, the extracellular time-dependent decrease in nitro-alkane metabolites could reflect their cellular reuptake and esterification into complex lipids. In this regard, the incorporation of NO₂-FAs into CoA, phospholipid, and TAG has been reported (3, 10), but little is known about the amount and the differential esterification of NO₂-FA and metabolites into complex cellular lipids.

Notably, 10-NO₂-OA is now entering Food and Drug Administration-approved phase II clinical trials, motivating an even better understanding of pathways that might impact NO₂-FA pharmacokinetics. Herein, the QWBA analysis of 10-NO₂-[¹⁴C]OA (labeled at carbon 10) in rats revealed absorption into the systemic circulation and distribution throughout all tissue compartments, reaching the highest concentrations within 6 h after oral dosing and then
declining over the course of 2 weeks. Radiolabel distribution was prominent in brown and abdominal adipose tissue, in part due to the lipophilic nature of 10-NO2-OA. One limitation of the QWBA study is that the radioactivity measured in tissues by autoradiography could be either native 10-NO2-OA or metabolites that retain carbon 10. In order to better define the pharmacology of the parent molecule, its still-electrophilic metabolites, quantitative analysis of de-esterified NO2-FAs in adipocytes and adipose tissue by HPLC-MS/MS.

Pharmacokinetics studies of electrophilic FAs have principally focused on the FFA species. Recently, qualitative studies of the esterification of FA electrophiles into phospholipids and TAGs have been reported (3, 10, 14, 29, 47). The quantitative analysis of NO2-FA containing phospholipids and TAGs have been reported (3, 10, 14, 29, 47). The quantitative analysis of NO2-FA-containing phospholipids and TAGs have been reported (3, 10, 14, 29, 47). The quantitative analysis of NO2-FA-containing phospholipids and TAGs have been reported (3, 10, 14, 29, 47). The quantitative analysis of NO2-FA-containing phospholipids and TAGs have been reported (3, 10, 14, 29, 47). The quantitative analysis of NO2-FA-containing phospholipids and TAGs have been reported (3, 10, 14, 29, 47).

In the intracellular compartment of adipocytes, both nitro-alkenes and nitro-alkanes showed preferential incorporation into MAG+DAG and TAG lipids, respectively, which could be a result of differential NO2-FA metabolism, distribution, and incorporation into cellular lipids. In the intracellular compartment of adipocytes, both nitro-alkenes and nitro-alkanes showed preferential incorporation into MAG+DAG and TAG lipids, respectively, which could be a result of differential NO2-FA metabolism, distribution, and incorporation into cellular lipids.

In summary, the esterification of fatty acyl nitro-alkene derivatives into MAG+DAG and PG reveals a unique pharmacokinetic character of NO2-FAs, wherein adipose tissue MAG+DAG and to a lesser extent TAG represent an intermediate reservoir of still-electrophilic nitro-alkene species that can impact the signaling actions of this class of mediators.

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