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Generation and esterification of electrophilic fatty acid nitroalkenes in triacylglycerides

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Abstract

Electrophilic fatty acid nitroalkenes (NO₂-FA) are products of nitric oxide and nitrite-mediated unsaturated fatty acid nitration. These electrophilic products induce pleiotropic signaling actions that modulate metabolic and inflammatory responses in cell and animal models. The metabolism of NO₂-FA includes reduction of the vinyl nitro moiety by prostaglandin reductase-1, mitochondrial β -oxidation and Michael addition with low molecular weight nucleophilic amino acids. Complex lipid reactions of fatty acid nitroalkenes are not well defined. Herein we report the detection and characterization of NO₂-FA-containing triacylglycerides (NO₂-FA-TAG) via mass spectrometry-based methods. In this regard, unsaturated fatty acids of dietary triacylglycerides are targets for nitration reactions during gastric acidification, where NO₂-FA-TAG can be detected in rat plasma after oral administration of nitro-oleic acid (NO₂-OA). Furthermore, the characterization and profiling of these species, including the generation of beta oxidation and dehydrogenation products, could be detected in NO₂-OA supplemented adipocytes. These data revealed that NO₂-FA-TAG, formed by either the direct nitration of esterified unsaturated fatty acids or the incorporation of nitrated free fatty acids into triacylglycerides, contribute to the systemic distribution of these reactive electrophilic mediators and may serve as a depot for subsequent mobilization by lipases to in turn impact adipocyte homeostasis and tissue signaling events.

Supplementary key words

triglycerides; nitro fatty acids; conjugated linoleic acid; nitrite; nitrate; mass spectrometry; anti-inflammatory; nitro-oleic acid; adipocytes; nitroalkane

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INTRODUCTION

Fatty acid nitroalkene derivatives (NO₂-FA) are formed by the addition reaction of nitrogen dioxide (*NO₂), a byproduct of reactions of nitric oxide (*NO) and nitrite (NO₂⁻)-derived species, with unsaturated fatty acids (1). The electron withdrawing nitro group renders the β alkenyl carbon electron deficient with a partial positive charge and promotes reversible Michael addition reactions of electrophilic NO₂-FA with cellular nucleophiles such as cysteine and histidine-containing peptides and proteins (2–4). These reactions can modulate the activity of functionally-significant proteins, especially during the conditions of metabolic stress and inflammation that promote fatty acid nitration. In particular, there is a significant impact on gene expression responses regulated by nuclear factor kappa B (NF-κB) and nuclear transcription factor erythroid 2-related factor 2/Kelch-like ECH-associated protein 1 (Nrf2/Keap1) and the heat shock response-regulating heat shock factor-1, as well as the activity of ion channels such as the transient receptor potential cation channel sub-family V member 1 (TRPV1) (5–8).

The generation of NO₂-FA is promoted by oxidative inflammatory responses and in the gastrointestinal tract, where acidic conditions promote the formation of nitrous acid (HNO₂) and downstream nitration of unsaturated fatty acids (9–11). Fatty acid nitration has been predominantly studied using free fatty acids such as oleic acid (OA), conjugated linoleic acid (CLA) and arachidonic acid (12) because of challenges in the direct mass spectrometric analysis of complex lipids and nitroalkene instability during de-esterification reactions. This is a limitation, as dietary and endogenous free fatty acid levels are proportionately very low (1–5 % of total) when compared to the levels of esterified fatty acids in tissue compartments and foods. In adipose tissues, where NO₂-FA exert critical signaling responses (13), and in foodstuffs the triacylglyceride (TAG) content is very high, suggesting that the unsaturated fatty acids in TAG could be both nitration targets and intermediates in nitroalkene-mediated signaling reactions *in vivo*. For example, exposure of the unsaturated fatty acid-rich TAG in extra virgin olive oil to nitrite and to the acidic conditions of digestion results in extensive fatty acid nitration (14). Gastric-induced fatty acid nitration mediates physiologically-relevant responses, as evidenced by the generation of NO₂-FA products that in turn inhibit soluble epoxide hydrolase activity and downstream suppression of angiotensin II-induced hypertension in mice fed with CLA and NO₂⁻, key precursors to NO₂-FA generation (15).

The adipose tissue and liver are involved in dietary lipid trafficking, homeostasis and storage. Dysregulation of lipid handling and oxidation are hallmarks of several pathological conditions including atherosclerosis, obesity, type 2 diabetes and metabolic syndrome (16–18). After a meal, TAG are hydrolyzed, then fatty acids are absorbed, re-esterified and packaged into chylomicrons that are processed by the liver and delivered as very low density lipoproteins (VLDL) to distal organs including white adipose tissue. Upon entry into adipocytes, free fatty acids derived from TAG participate in β-oxidation, reduction and elongation reactions and are re-esterified into TAG for storage. It is evident that >50% of this pool of esterified fatty acids is very active in TAG remodeling reactions on a daily basis (19, 20).

The detection and characterization of TAG typically involves separation by thin-layer chromatography (TLC) or liquid chromatography (LC), then hydrolysis and derivatization for analysis by gas chromatography/mass spectrometry (GC/MS) (21). Recently, high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) has become a powerful tool for characterizing TAG in biological samples, facilitating the analysis of the large number of structural and positional isomers displayed by TAG. This strategy also avoids GC-induced thermal decomposition of TAG (22). TAG are commonly analyzed in positive ion mode as ammonium $[M+NH_4]^+$ or alkali metal $[M+metal]^+$ adducts by electro spray ionization (ESI) or atmospheric pressure chemical ionization (APCI) methods (23, 24). In general, fragmentation of $[M+NH_4]^+$ yields three “diglyceride ions” $[M-R_1COOH-NH_3]^+$, $[M-R_2COOH-NH_3]^+$ and $[M-R_3COOH-NH_3]^+$, that correspond to neutral losses of fatty acyl substituents as free carboxylic acid and ammonia from the *sn*-1, *sn*-2 and *sn*-3 positions of the glycerol backbone, respectively. These diglyceride product ions generate the corresponding acylium $[R_nCO]^+$ and monoglyceride $[R_nC=O+74]^+$ ions after MS³ fragmentation (25, 26). Structural information at this level is of relevance, as the position of the esterified fatty acyl substituents affects both lipid absorption and metabolism, and can modulate atherogenic events (27, 28). In this regard, the detection and characterization of NO₂-FA-TAG, while not highly abundant, can mediate the regulation of adaptive cell signaling responses. Herein, we report the nitration of TAG under gastric conditions and the preferential incorporation of nitroalkanes over nitroalkenes into adipocyte and rat plasma TAG by directly measuring products both before and after hydrolysis using HPLC-mass spectrometry analysis.

EXPERIMENTAL PROCEDURES

Materials

Chemicals were of analytical grade and purchased from Sigma unless otherwise stated (St. Louis, MO). Artificial gastric fluid was from Fisher (Fisher Scientific International Inc., Hampton, NH). 1,3-dipalmitoylglycerol (1,3-DPG), oleic acid (OA) and conjugated-9,11-linoleic acid (CLA, cat. no. UC-60A) were from Nu-Check Prep, Inc. (Elysian, MN). Nitro-oleic acid (NO₂-OA) and NO₂-[¹³C₁₈]OA were synthesized as previously described (29). NO₂-OA concentration was measured spectrophotometrically in methanol before each experiment ($\epsilon_{257\text{ nm}} = 7000\text{ M}^{-1}\text{cm}^{-1}$, (29)). The synthesis of nitro-stearic acid (NO₂-18:0) was performed by selectively reducing the nitroalkene present in NO₂-OA to a nitroalkane with sodium borohydride. NO₂-[¹⁵N/D₄]18:0 was obtained by reducing NO₂-[¹⁵N/D₄]18:1 in the same manner. The full structural characterization and synthetic procedures for generating this labeled standard will be separately reported. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Hank's balanced salt solution (HBSS) and antibiotic- antimycotic solutions were from Corning Cellgro (Herndon, VA). Strata NH₂ solid phase extraction (SPE) columns (55 μm , 70A) were from Phenomenex (Torrance, CA). Solvents used for extractions and mass spectrometric analyses were from Burdick and Jackson (Muskegon, MI).

Synthesis of dipalmitoyl-conjugated-linoleoyl-glycerol (CLA-TAG) standards

Two mixed triacyl precursors were generated by reaction of 1,2-dipalmitoylglycerol (1,2-DPG) and 1,3-DPG with CLA in the presence of N,N'-dicyclohexylcarbodiimide (DCC) as coupling reagent. The 1,2-DPG and 1,3-DPG (50 mg) were dissolved in 10 ml dichloromethane, followed by 40 mg CLA. To the stirred flask, 34 mg DCC were added along with a few milligrams of 4-(dimethylamino)pyridine (DMAP) as catalyst. The flask was sealed under nitrogen and stirred at room temperature overnight. Then, the suspension was filtered to remove byproducts, the solids washed with dichloromethane and the solvents combined, evaporated and purified by column chromatography on silica gel (eluent: 0–5 % ethyl acetate/hexanes). The final yield (70 %) of 51 mg 1,2-dipalmitoyl-3-conjugated-linoleoyl-glycerol (3-CLA-TAG) and 41 mg (49 %) 1,3-dipalmitoyl-2-conjugated-linoleoyl-glycerol (2-CLA-TAG) were analyzed by ^1H nuclear magnetic resonance (NMR). 2-CLA-TAG and 3-CLA-TAG isomeric compositions were confirmed by MS/MS. Although 1,2-diacylglycerides are prone to rearrangement to 1,3-diacylglycerides (30), analysis of the product 3-CLA-TAG indicated that no acyl migration occurred (Supplementary Fig. 1).

In vitro nitration of 3-CLA-TAG and 2-CLA-TAG standards by gastric fluid

Synthetic nitrated triacylglyceride standards were generated by performing independent reactions of 100 μM 3-CLA-TAG or 2-CLA-TAG with 2 mM sodium nitrite (NaNO_2) in artificial gastric fluid for 1 h at 37 $^\circ\text{C}$, under continuous agitation. The lipid fraction was extracted by hexane, dried under a stream of nitrogen gas and dissolved in 0.2 ml ethyl acetate.

Cell culture

3T3-L1 fibroblasts were propagated and differentiated into adipocytes as previously (31). Fully differentiated adipocytes were then treated with 5 μM OA (n=3) or NO_2 -OA (n=5) in HBSS, to avoid Michael addition reaction with nucleophilic protein residues present in fetal bovine serum (FBS). Aliquots of media were obtained at 1, 2, 4, 8, and 24 h, spiked with NO_2 -[$^{13}\text{C}_{18}$]OA internal standard, extracted using the Bligh and Dyer method (32), dried under a stream of nitrogen and reconstituted in 0.2 ml methanol for HPLC-ESI-MS/MS analysis. At the end of the treatment, adipocytes were rinsed with cold phosphate buffered saline (PBS), scraped and lipids extracted.

Animal study

Male Sprague-Dawley rats (6-8 week old) were gavaged with NO_2 -OA (100 mg/kg) dissolved in sesame oil. Blood samples were obtained at different time points and plasma obtained by centrifugation. A sample volume of 200 μl of plasma was extracted. Plasma samples were provided by Complexa Inc. under a material transfer agreement #00000401.

Adipocyte and rat plasma lipid extraction

Adipocyte and rat plasma lipids were extracted according to Bligh and Dyer (32), dried under a stream of nitrogen and dissolved in 0.5 ml hexane/methyl tert-butyl ether/acetic acid (HBA, 100:3:0.3 v/v/v). Nitrated species were not detected in the OA-treated adipocytes (control) or untreated rat plasma. Adipocyte and rat plasma lipid classes were further

resolved using SPE Strata NH₂ columns 500 mg/6 ml and 100 mg/1 ml, respectively (33). Briefly, columns were washed twice with 2 ml acetone/water (7:1, v/v) and pre-conditioned twice with 2 ml hexane. The samples solubilized in HBA were loaded on the columns and cholesterol esters, TAG, mono-diglycerides and free fatty acids were sequentially eluted with 12 ml (for adipocyte lipids) or with 2 ml (for rat plasma lipids) of hexane, hexane/chloroform/ethyl acetate (100:5:5, v/v/v), chloroform/2-propanol (2:1, v/v) and diethyl ether/2 % acetic acid, respectively. The solvents were evaporated under a stream of nitrogen and TAG and free fatty acid fractions were dissolved in 0.2 ml ethyl acetate and methanol, respectively. Detection of NO₂-FA-TAG was performed by HPLC-HR-MS/MS and HPLC-APCI-MS/MS while NO₂-FA were analyzed by HPLC-ESI-MS/MS.

Enzymatic hydrolysis of triacylglycerides

Lipid extracts containing adipocyte and rat plasma TAG were spiked with NO₂-[¹³C₁₈]OA and NO₂-[¹⁵N/D₄]18:0 internal standards respectively, dried and incubated in presence of 1 ml porcine pancreatic lipase (0.4 mg protein/ml) in 50 mM phosphate buffer pH 7.4 and 10 µl sodium cholate (40 mg/ml), at 37 °C for 3 h under agitation. After hydrolysis, NO₂-FA were extracted using the Bligh and Dyer method (32), dried under a stream of nitrogen and reconstituted in 0.2 ml methanol for HPLC-MS/MS analysis.

Chromatography

Analysis of NO₂-FA was performed by HPLC-ESI-MS/MS using an analytical C18 Luna column (2 × 100 mm, 5 µm, Phenomenex) at a 0.6 ml/min flow rate, with a gradient solvent system consisting of water containing 0.1 % acetic acid (solvent A) and acetonitrile containing 0.1 % acetic acid (solvent B). Samples were 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.

Analysis of NO₂-FA-TAG was performed by HPLC-HR-MS/MS and HPLC-APCI-MS/MS using a C18 Luna column (2 x 150 mm, 3 µm, Phenomenex) at a flow rate of 0.45 ml/min. The gradient solvent system consisted of acetonitrile (solvent A) and ethyl acetate (solvent B), in presence of 66 µM ammonium acetate to generate [M+NH₄]⁺ species (34). NO₂-FA-TAG were eluted over 28 min using the following gradient: 0-30 % solvent B (0-3 min); 30-65 % solvent B (3-24 min); 65-90 % solvent B (24-26 min); 90 % solvent B (26-28 min) to then re-equilibrate to initial conditions for an additional 2 min.

Mass spectrometry

NO₂-FA were analyzed by HPLC-MS/MS using an API4000 Q-trap triple quadrupole mass spectrometer (Applied Biosystems, San Jose, CA) equipped with an electrospray ionization source (ESI) in negative mode. The following parameters were used: declustering potential (DP) -75 V, collision energy (CE) -35 eV and a desolvation temperature of 650 °C. The following multiple reaction monitoring (MRM) transitions were used for the different NO₂-FA metabolites: nitro-stearic acid (NO₂-18:0, 328.3/46), nitro-oleic acid (NO₂-OA, NO₂-18:1, 326.3/46), nitro-hexadecanoic acid (NO₂-16:0, 300.3/46), nitro-hexadecenoic acid (NO₂-16:1, 298.3/46), nitro-tetradecanoic acid (NO₂-14:0, 272.3/46), nitro-tetradecenoic acid (NO₂-14:1, 270.3/46) and nitro-dodecanoic acid (NO₂-12:0, 244.3/46).

(35). Elongation products of NO₂-OA, β -oxidation products shorter than C₁₂ and nitro-dodecenoic acid (NO₂-12:1) were not detected. Relative concentrations of NO₂-FA in cell media over 24 h were reported as area ratio NO₂-FA/NO₂-[¹³C₁₈]OA and relative concentrations of NO₂-FA at 24 h in cell media and extracts (free fatty acid fraction and lipase hydrolyzed TAG fraction) were reported as an area ratio of NO₂-FA/NO₂-OA. Quantification of non-electrophilic NO₂-FA in rat plasma was performed by stable isotopic dilution analysis using NO₂-18:0 calibration curves in the presence of the NO₂-[¹⁵N/D₄]18:0 internal standard (MRM 333.3/47).

Structural and positional analysis of adipocyte NO₂-FA-TAG was performed by HPLC-HR-MS/MS using an LTQ Velos Orbitrap (Thermo Scientific) equipped with a HESI II electrospray source, in positive ion mode. The following parameters were used: source temperature 250 °C, capillary temperature 300 °C, sheath gas flow 9, auxiliary gas flow 5, sweep gas flow 2, source voltage 5 kV, S-lens RF level 70 %. Full mass scan experiments were carried out from 700 to 1000 *m/z* at a resolving power of 7500 full width at half maximum (FWHM). Ions with odd mass numbers, corresponding to potential NO₂-FA-TAG, were selected and subjected to data dependent MS² and MS³ fragmentations. The instrument FT-mode was calibrated using the manufacturer's recommended calibration solution.

Analysis of NO₂-FA-TAG in rat plasma was performed by HPLC-APCI-MS/MS using an API4000 Q-trap triple quadrupole mass spectrometer (Applied Biosystems, San Jose, CA) equipped with an atmospheric pressure chemical ionization source (APCI) in positive mode. The following parameters were used: declustering potential (DP) 85 V, collision energy (CE) 35 eV, desolvation temperature 300°C and a nebulized current of 5 μ A.

Statistical analysis

A Pearson's product correlation was used to determine an association between intra and extracellular content of NO₂-FA in adipocytes supplemented with NO₂-OA. Values are expressed as means $\pm$ standard deviation.

RESULTS

Mass spectrometry characterization of NO₂-CLA-TAG generated under gastric conditions

The acidic gastric compartment is a physiological catalyst for NO₂⁻ mediated nitration and nitrosation reactions subsequent to the protonation of NO₂⁻ to nitrous acid (HNO₂) (pKa 3.4) and the secondary formation of dinitrogen trioxide (N₂O₃) and its products (9). To test if CLA-containing-TAG is a substrate for acidic nitration, 3-CLA-TAG and 2-CLA-TAG standards were incubated in artificial gastric fluid in the presence of 2 mM NaNO₂. Several nitrated products were generated, including: NO₂-CLA-TAG (*m/z* 893.7), NO₂-oxo-OA-TAG (*m/z* 909.7), NO₂-OH-OA-TAG (*m/z* 911.7) and NO₂-OOH-OA-TAG (*m/z* 927.7) (Supplementary Fig. 2). The presence of oxygen during reactions suppresses the yield of NO₂-CLA and promotes the formation of oxygenated NO₂-CLA products (9). However, the gastric compartment, low in oxygen when compared to air-equilibrated experimental conditions, will favor the generation of products that are just nitrated rather than both

oxidized and nitrated species *in vitro*. Since NO₂-CLA is the principal nitrated free fatty acid derivative detected in human plasma and urine (36), focus in the present study was placed on further characterization of the ion m/z 893.7. This species displayed two peaks having HPLC retention times of 14.2 and 14.5 min (Fig. 1A) that were consistent with NO₂-CLA-TAG (confirmed at the 1 ppm level) (inset in Fig. 1A).

The NO₂-CLA-TAG species were further structurally characterized via MS² and MS³ fragmentation. MS² analysis generated three “diglyceride ions”: two characteristic even mass-containing ions m/z 620.48 (containing one nitrogen) and one odd mass-containing ion m/z 551.50 (containing no nitrogen atoms) which corresponded to the neutral losses of the palmitoyl- and nitro-conjugated-linoleoyl free fatty acids and ammonia, respectively (Fig. 1B). The relative intensity of the product enabled the determination of positional isomers, since the loss of fatty acyl substituents from *sn*-1/*sn*-3 positions is energetically favored over losses at the *sn*-2 position (24, 37–39). Based on this observation, the nitro-conjugated-linoleoyl- substituent was assumed to be in the *sn*-2 position of the glycerol backbone, as the odd ion m/z 551.5 [M-NO₂-CLA-NH₃]⁺ was about 4-times less abundant than the even ions m/z 620.4 [M-16:0-NH₃]⁺.

MS³ analysis on the diglyceride product ions provided further structural information. Collision induced dissociation (CID) fragmentation of m/z 620.4 gave two characteristic ions m/z 480.3 and m/z 493.3, generated by neutral losses of 140.2 amu and 127.2 amu respectively (Fig. 1C). These product ions correspond to the specific neutral losses characteristic of nitroalkene carbon chain fragmentation of 9-NO₂-CLA and 12-NO₂-CLA respectively (Fig. 1A) (9). Further fragmentation analysis of product ion m/z 620.4, showed formation of monoglyceride and acylium ions, such as [(NO₂-CLA)C=O+74]⁺ (m/z 382.25), [(NO₂-CLA)CO]⁺ (m/z 308.22), [(16:0)C=O+74]⁺ (m/z 313.27), and their corresponding dehydration products (m/z 602.4, m/z 364.2, m/z 290.2 and m/z 295.2) (Fig. 1C). The MS³ analysis of the fragment m/z 551.5 gave the expected product ion m/z 239.3 [(16:0)CO]⁺, confirming the presence of palmitoyl-substituents in *sn*-1 and *sn*-3 positions of the glycerol backbone (Fig. 1D).

NO₂-OA metabolism, distribution and incorporation into TAG in adipocytes

The differentiation of 3T3-L1 cells to mature adipocytes provides a platform to model primary adipocyte lipid metabolism and the distribution and incorporation of NO₂-OA into TAG (40). The analysis of NO₂-OA added to the culture medium of adipocytes over 24 h showed that cellular NO₂-18:1 was rapidly metabolized within the first two hours by β-oxidation reactions, yielding NO₂-16:1 and NO₂-14:1 (Fig. 2A–C). A second phase of metabolism was evident from 8 to 24 h, where the reduction of the nitroalkene to non-electrophilic nitroalkanes NO₂-18:0, NO₂-16:0, NO₂-14:0 and NO₂-12:0, with NO₂-18:0 being the most abundant as evidenced by its relative area ratio (Fig. 2A–D). Of note, a mass balance study of metabolites was not performed, limiting the analysis of the data shown in Fig. 2 to comparisons of the relative levels obtained for each species at the different time points.

To evaluate the cell distribution of NO₂-FA metabolites, their incorporation into TAG, the intracellular (cell lysate), extracellular (cell media) and TAG esterified fractions (lipase-

hydrolyzed TAG species) were analyzed. Nitroalkane metabolites were preferentially esterified as opposed to nitroalkene species, as evidenced by the higher area ratios of the various NO₂-FA ions to NO₂-OA (Fig. 3A–C). Nonetheless, NO₂-OA was also esterified in the TAG fraction of adipocytes (Fig. 3A). Intracellular nitroalkanes had a higher area ratio than nitroalkanes detected extracellularly (Fig. 3B,C). This is most likely due to the Michael addition of electrophilic nitroalkenes with intracellular nucleophiles (*e.g.*, glutathione, protein cysteine- and histidine-residues) and the relative concentrations of these species in these two compartments (35, 41). Overall, the relationship between intracellular and extracellular NO₂-FA area ratio was strongly correlated ($r^2=0.98$, $P<0.001$, Fig. 3D) with a preferential intracellular distribution of short and saturated chain length nitroalkane metabolites. In contrast, TAG-esterified species and intracellular NO₂-FA area ratios were not strongly correlated, rather there was a significant esterification of longer chain nitroalkanes (*e.g.*, NO₂-16:0 and NO₂-18:0) into TAG (Fig. 3E).

Mass spectrometry analysis NO₂-FA-TAG in adipocytes

The detection of NO₂-FA after hydrolysis of adipocyte TAG motivated the direct detection and characterization of NO₂-FA-TAG. Purified TAG fractions from cultured adipocytes were subjected to HPLC-HR-MS analysis in positive mode and the most intense odd mass ions were selected for MS² fragmentation to identify NO₂-FA-TAG and discriminate structural and positional isomers. Candidate ions of TAG containing a nitro group were selected based on confirmation of atomic composition using high resolution mass spectrometry at the 2 ppm level. Fragmentation of these ions generated even and odd “diglyceride ions” that allowed the identification of the fatty acyl chains present in the NO₂-FA-TAG. Furthermore, the presence of an odd mass diacyl product ion, generated by the neutral loss of the nitro-fatty acyl substituent as free carboxylic acid and ammonia, allowed the identification of even mass fragments and confirmation of NO₂-FA-TAG fatty acyl substituents and structure. This was further validated using a synthetic NO₂-CLA-TAG standard. As an example, an ion with an m/z 893.7 and molecular composition in agreement with a NO₂-FA-TAG (confirmed at the 1 ppm level, inset in Fig. 4A), presented two chromatographic peaks eluting at 12.5 and 13.2 min (Fig. 4A). MS² analysis of the first and second peak unequivocally confirmed the presence of non-electrophilic NO₂-FA-TAG isomers 16:1/NO₂-18:0/16:1-TAG and NO₂-16:0/16:1/18:1-TAG, respectively (Fig. 4A–C). The fragmentation of the first peak generated two abundant even mass products ions with m/z 622.5 [M-16:1-NH₃]⁺ and one odd fragment with m/z 547.4 [M-NO₂-18:0-NH₃]⁺. These correspond to the neutral losses of palmitoleoyl- and nitro-stearoyl-substituents as free carboxylic acid and ammonia in *sn*-1/*sn*-3 and *sn*-2 positions of the glycerol backbone, respectively (Fig. 4B). The MS² analysis of the second peak generated two even product ions m/z 622.5 [M-16:1-NH₃]⁺, m/z 594.4 [M-18:1-NH₃]⁺, and one odd ion at m/z 575.5 [M-NO₂-16:0-NH₃]⁺, stemming from the neutral loss of palmitoleoyl-, oleoyl- and nitro-palmitoyl- substituents as free carboxylic acid and ammonia from the glycerol backbone, respectively (Fig. 4C). In this case, the presence of a chromatographically co-eluting NO₂-FA-TAG isomer (NO₂-16:0/17:1/17:1-TAG), sharing the isobaric product ion m/z 575.5, did not allow a specific positional isomers discrimination (42).

To gain structural insight into non-electrophilic (nitroalkane) NO₂-FA-TAG species, MS³ analysis of product ions was performed (Fig. 4D). Fragmentation of the even mass “diglyceride ions” with m/z 622.5 confirmed loss of water, generating the ion m/z 604.5, and monoglyceride and acylium ions [(NO₂-18:0)C=O+74]⁺, [(NO₂-18:0)CO]⁺ and [(16:1)C=O+74]⁺. In addition, the nitro-containing species [(NO₂-18:0)C=O+74]⁺ and [(NO₂-18:0)CO]⁺ also displayed loss of water, generating the ions m/z 368.2 and m/z 294.2, respectively. Interestingly, three new fragments m/z 591.4, m/z 573.4 and m/z 339.2 were identified. The first and the second ions were generated from a 31 amu mass loss (HNO) of the product ion m/z 622.5 and m/z 604.4 respectively, while m/z 339.2 originated from a 47 amu mass loss (HNO₂) of the parent fragment ion [(NO₂-18:0)C=O+74]⁺. MS³ analysis of the odd “diglyceride ion” m/z 547.4 showed the expected formation of [(16:1)C=O+74]⁺ and [(16:1)CO]⁺, confirming the presence of palmitoleyl-substituents in *sn*-1 and *sn*-3 positions of the glycerol backbone (Fig. 4E). This workflow was applied to characterize the predominant nitrated TAG detectable in adipocytes (Table 1). The analysis of positional isomers, on the basis of the relative intensities of the “diglyceride ions”, showed that nitroalkanes were preferentially incorporated into the *sn*-2 position of TAG. Accurate mass determination at the 2 ppm level for MS and MS² analysis confirmed the molecular and fatty acid composition of these species, respectively. Overall, non-electrophilic medium to long chain NO₂-FA metabolites (nitroalkane species) were TAG-esterified, nitroalkene-containing-TAG were not detectable and there was an absence of nitro-fatty acyl chains longer than C₁₈ and shorter than C₁₂ (Supplementary Table 1). NO₂-FA incorporated into TAG were mostly saturated, in conjunction with saturated and monounsaturated C₁₅-C₁₇ (Supplementary Table 1).

Analysis of NO₂-FA-TAG in rat plasma

The detection of NO₂-FA-TAG in adipocytes motivated the *in vivo* detection and characterization of TAG-adducted species in rat plasma after a single oral dose of the nitroalkene NO₂-OA. Lipase hydrolysis of TAG-containing fractions from rat plasma, obtained at different time points after administration, showed the presence of non-electrophilic nitroalkanes NO₂-18:0, NO₂-16:0 and NO₂-14:0 confirming the preferential and rapid incorporation of these reduced species in TAG (Fig. 5A–C). NO₂-18:0 was the most abundant nitroalkane and displayed a concentration in the range of 16–113 nM (Fig. 5A). This result encouraged the detection of individual NO₂-18:0-TAG species in rat plasma. As an initial approach, MRM screening for transitions for potential NO₂-18:0-TAG was performed following the neutral loss of 346 amu (NO₂-18:0+NH₃), as NO₂-18:0 was the most prominent non-electrophilic NO₂-FA detected in plasma after lipase reaction.

Two candidate ions indicative of NO₂-18:0-containing TAG were observed by evaluating the co-eluting MRM transitions (MRM 921.8/648.5, 921.8/624.5, 921.8/575.5 and 945.8/648.5, 945.8/599.5). These two species, with peaks at 12.6 min (peak 1 in Fig. 5D lower panel) and 10.7 min (peak 2 in Fig. 5F lower panel) were only present in NO₂-OA gavaged plasma samples and were not detected in untreated samples (Fig. 5D,F upper panels). Structural confirmation of NO₂-18:0/16:0/18:2-TAG and NO₂-18:0/18:2/18:2-TAG was obtained by full scan MS/MS analysis of ions displaying m/z 921.8 and m/z 945.8 respectively (Fig. 5E,G). The fragmentation of m/z 921.8 generated two even mass products

ions with m/z 648.5 $[M-16:0-NH_3]^+$, m/z 624.5 $[M-18:2-NH_3]^+$ and one odd fragment with m/z 575.5 $[M-NO_2-18:0-NH_3]^+$. These correspond to the neutral losses of ammonia and acyl chains (palmitoyl-, linoleoyl- and nitro-stearoyl- respectively) from the glycerol backbone (Fig. 5E). Fragmentation of m/z 945.8 generated two even mass products ions with m/z 648.5 $[M-18:2-NH_3]^+$ and one odd fragment with m/z 599.5 $[M-NO_2-18:0-NH_3]^+$ corresponding to the neutral losses of linoleoyl- and nitro-stearoyl- substituents as free carboxylic acid and ammonia from the glycerol backbone, respectively (Fig. 5G).

DISCUSSION

Enzymatic and non-enzymatic fatty acid oxidation reactions generate a wide array of products, some of which can act as signaling mediators rather than as pathogenic byproducts of inflammation. In addition to the oxidation of the free fatty acids, many oxidized fatty acid species are also found esterified in polar and neutral complex lipids. This includes esterified isoprostanes, hydroxyl fatty acid derivatives and lipid oxidation chain scission products such as 4-hydroxy-2-nonenal (4-HNE) (43–45). In many instances, it is not well defined if oxidative fatty acid modifications occurred directly with esterified species or if a modified free fatty acid is subsequently esterified into a complex lipid following metabolic or inflammatory reactions.

NO_2 -FA, formed by oxidative inflammatory reactions and in the acidic gastric compartment (9), are pleiotropic signaling mediators that induce reversible post-translational protein modifications via reversible Michael addition reactions (2). Recent data also support that NO_2 -FA can be formed during digestion. For example, the exposure of extra virgin olive oil to NO_2^- under acidic conditions, followed by pancreatic lipase hydrolysis, resulted in significant extents of NO_2 -FA generation (14). This also suggested that unsaturated fatty acid-containing-TAG could be substrates for nitration. Dietary TAG, a key source of fatty acids, can undergo multiple cycles of hydrolysis and re-esterification. Lipases hydrolyze TAG in the lumen of the duodenum to permit free fatty acids to be absorbed, re-esterified in the liver and delivered to remote tissues by lipoproteins. Tissue uptake by adipocytes involves hydrolysis, internalization, and further esterification into TAG. Re-esterification requires the formation of coenzyme A (CoA) derivatives, central for directing mitochondrial fatty acid transport and supporting β -oxidation and acylation reactions. These events motivated the synthesis and characterization of a NO_2 -CLA-TAG and the present study of the uptake and metabolism of NO_2 -FA, as free and as complex lipid esterified- NO_2 -FA, in adipocytes and rat plasma. This is of relevance, since the formation of NO_2 -FA by gastric nitration of dietary lipids could represent a key source of NO_2 -FA-TAG that in turn could represent a pool of metabolic and inflammatory signaling mediators.

Vegetable oils and dairy products are rich in unsaturated fatty acid-containing TAG containing both essential and non-essential fatty acids (46, 47). Moreover, leafy vegetables, fruits and cured meat are excellent sources of NO_2^- and nitrate (NO_3^-) (48), which can be reduced to NO_2^- via entero-salivary anaerobic bacteria (49, 50). In the gastric compartment, the low pH induces protonation of NO_2^- , the generation of nitrogen dioxide (*NO_2) (11, 51, 52) and the subsequent nitration of unsaturated fatty acids (10). This is confirmed by the nitration of both synthetic 3-CLA-TAG and 2-CLA-TAG standards exposed to *in vitro*

gastric conditions in presence of NO_2^- (Fig. 1). The resultant NO_2 -CLA-TAG products share the same general MS^2 fragmentation patterns as TAG, displaying the characteristic neutral losses of the fatty acyl chains and ammonia from *sn*-1, *sn*-2 and *sn*-3 positions. The application of the nitrogen rule allows for rapid screening of NO_2 -containing-products, as their ammonium adducts display odd mass values compared to even m/z values for $[\text{TAG} + \text{NH}_4]^+$. In addition, $[\text{TAG} + \text{NH}_4]^+$ fragmentation generates three odd “diglyceride ions” as previously reported (25), while NO_2 -CLA-TAG results in two even and one odd “diglyceride ion” products. The odd diacyl product ion, after CID fragmentation, reflects the neutral loss of a NO_2 -containing-fatty acyl chain.

The differentiation and characterization of the alternative positional isomers of NO_2 -CLA-TAG (acyl chain and nitro group positions) is of biochemical and biological relevance, as this will reflect fatty acyl substrates, shed light on the reaction mechanisms and can impact downstream cell signaling reactions. Of note, ^1H NMR analysis of 3-CLA-TAG and 2-CLA-TAG resulted in identical spectra and did not allow for positional isomers evaluation (Supplemental Fig. 1A,C). In contrast, MS^2 fragmentation of 3-CLA-TAG and 2-CLA-TAG standards permitted the determination of positional isomers by the relative abundance of diacyl product ions (24, 38), as neutral losses of *sn*-1 and *sn*-3 acyl chains are energetically favored over *sn*-2 fatty acyl chain (37, 39). Herein, the synthesis of 3-CLA-TAG and 2-CLA-TAG gave products with CLA esterified to the *sn*-1/3 positions (Supplementary Fig. 1B) and *sn*-2 position (Supplementary Fig. 1D), respectively. Nonetheless, MS^2 analysis of NO_2 -CLA-TAG products displayed the same spectra suggesting acyl migration of NO_2 -CLA substituents to the *sn*-2 position during acidic nitration reactions (Fig. 1B and Supplementary Fig. 1E). It is also possible that the presence of the nitro group induced changes in fragmentation efficiencies, disfavoring neutral loss of NO_2 -CLA+ NH_3 .

A key mechanism for the metabolism of NO_2 -FA is reduction of the vinyl nitro substituent by prostaglandin reductase 1, yielding nitroalkane species lacking electrophilic reactivity and the capability to post-translationally modify the activity of critical transcriptional regulatory protein and enzyme targets (53, 54). Mass spectrometric characterization via MS^3 analysis of NO_2 -CLA-TAG identified the nitro-conjugated linoleoyl- substituent. In addition to the commonly-observed acylium $[\text{RnCO}]^+$ and $[\text{RnC=O}+74]^+$ ions (26), MS^3 analysis of the even diacyl product ions of NO_2 -CLA-TAG generated two characteristic nitrosamine and aldehyde ions that confirmed the presence of two positional isomers, the 9- NO_2 -CLA-TAG and 12- NO_2 -CLA-TAG respectively. This data is in agreement with fragmentation pathways previously reported for $[9\text{-NO}_2\text{-CLA}+\text{Li}]^+$ and $[12\text{-NO}_2\text{-CLA}+\text{Li}]^+$ (9). While NO_2 -CLA-TAG did not generate product ions stemming from HNO_2 neutral losses typical for nitro-containing fatty acids (Fig. 1C) (9, 55), the fragmentation of the non-electrophilic NO_2 -18:0-TAG displayed this typical neutral loss (Fig. 4D). Moreover, MS^3 analysis of NO_2 -18:0-TAG revealed a previously unrecognized neutral loss of HNO associated with a dehydration reaction that leads to products with m of 31, 18 and 49 amu (Fig. 4D) that were not observed in free fatty acid fragmentation analysis (55). Notably, HNO loss was specific for nitroalkanes and not for nitroalkenes, a result confirmed by comparison with synthetic nitro-stearic and nitro-oleic standards (Supplementary Fig. 3).

The cellular distribution of NO₂-FA and their metabolites will impact physiological responses. Also, fatty acid CoA thioesterification will significantly modulate pathways of intracellular processing (56, 57). The analysis of free NO₂-FA and TAG-esterified NO₂-FA levels in adipocyte studies showed a preferential extracellular partitioning of β-oxidized nitroalkene metabolites (NO₂-16:1 and NO₂-14:1) (inset, Fig. 3D). Michael addition reactions will be favored in the thiol-rich intracellular compartment, predominantly mediating the formation of glutathione (GSH) adducts that are exported via the multidrug resistance protein-1 (35, 41, 58). In this regard, the extracellular milieu is low in reduced thiols compared with intracellular GSH (10–40 μM vs 8 mM GSH respectively) and protein thiols (59). As GSH-NO₂-FA adducts are reversible (2), a new equilibrium will be established in the extracellular compartment that will promote β-elimination release of free NO₂-FA in extracellular milieu. The short-chain and more hydrophilic β-oxidized and reduced nitroalkane metabolites (NO₂-12:0 and NO₂-14:0) were enriched in the intracellular compartment (inset in Fig. 3D) as opposed to the longer chain and more hydrophobic nitro-containing species (NO₂-16:0 and NO₂-18:0). This partitioning based on fatty acyl chain length may be a consequence of differential trans-membrane transport, thioesterase activities that modulate pools of CoA or substrate preferences of diglyceride acyltransferases (DGATs), all of which could impact relative metabolite levels. The C₁₆ fatty acids are the major components of TAG and preferential substrates for TAG synthesis in adipocytes, which is confirmed by the significant incorporation of NO₂-16:0 following the addition of NO₂-OA (Fig. 3E) (60–62). Interestingly, while NO₂-OA was detected as free NO₂-FA after lipase hydrolysis of adipocyte TAG, there was no evidence of electrophilic NO₂-FA-TAG. Several factors may impair the detection of these species via the direct analysis of NO₂-FA-TAG. The chromatographic resolution of free fatty acids is greater than for TAG. This is of relevance, since the combination of a high NO₂-FA/NO₂-OA relative ratio, up to 50 for C₁₆-C₁₈ chain length nitroalkanes (Fig. 3A), and the lower chromatographic resolution could mask the detection of co-eluting low abundance species. In addition, the analysis of NO₂-FA-TAG was performed using a high resolution MS to obtain structural data, while the analysis of NO₂-FA used a triple quadrupole MS, further enhancing an ability to detect less abundant species with high sensitivity.

In adipocytes, non-electrophilic NO₂-FA-TAG mainly consisted of saturated, monounsaturated C₁₄-C₁₆-C₁₈ followed by C₁₅-C₁₇ chain length fatty acids (Supplementary Table 1), in agreement with reports that 3T3-L1 adipocytes contain more than 20% of odd chain fatty acids (60). The positional analysis of the most abundant non-electrophilic NO₂-FA-TAG suggested preferential incorporation of nitroalkane substituents in *sn*-2 position (Table 1). However, the co-elution of structural isomers, containing common diacyl product ions, did not allow any further positional analysis (Supplementary Fig. 4A) (42). In some cases, the minor contribution of the co-eluting NO₂-FA-TAG to the common MS² fragment allowed the precise identification of positional isomers (Supplementary Fig. 4B) (24, 42).

Oral administration of labeled or modified fatty acids has been widely used to assess the formation of TAG *in vivo* (63, 64). The analysis of plasma NO₂-FA-TAG after oral gavage resulted in a spectrum of similar TAG species as when triacylglycerides were obtained from NO₂-OA supplemented adipocytes, with non-electrophilic NO₂-FA being the predominant

species detected in both cases. Even though NO₂-18:0-containing- plasma TAG were confirmed in these experiments, the detection of these species remains challenging as overall they represent a small percentage of the total TAG in plasma. Moreover, chromatographic separations result in a high number of species having different possible acyl chain and isomeric compositions.

In summary, unsaturated fatty acid-containing TAG are targets for nitration reactions during digestive or inflammatory conditions and can become a substrate for subsequent mobilization of the free fatty acid upon lipase hydrolysis. The pool of NO₂-FA metabolites can also be re-esterified in adipocytes and, during enterohepatic transit, yield various NO₂-FA-TAG species preferentially consisting of nitroalkanes rather than nitroalkenes. New insight into the metabolism, distribution and incorporation of NO₂-OA showed that: 1) NO₂-OA is reduced by nitroalkene reductases and undergoes mitochondrial β -oxidation; 2) the electrophilicity and lipophilicity of nitroalkenes and nitroalkanes affect intra- and extracellular distribution; 3) NO₂-OA is esterified into TAG with the potential generation of electrophilic NO₂-FA-TAG; and 4) nitroalkanes were preferentially esterified into TAG as opposed to nitroalkenes. On this basis, unsaturated fatty acid nitration in the gastric compartment can contribute to the formation of esterified NO₂-FA *in vitro* and *in vivo* and participate in metabolic and anti-inflammatory signaling responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

8:0	octanoic acid
12:1	dodecenoic acid
14:0	tetradecanoic acid
14:1	tetradecenoic acid
15:0	pentadecanoic acid
15:1	pentadecenoic acid
16:0	hexadecanoic acid
16:1	hexadecenoic acid
17:0	heptadecanoic acid
17:1	heptadecenoic acid
18:0	stearic acid (octadecanoic acid)

18:1	oleic acid (octadec-9-enoic acid)
1,2-DPG	1,2-dipalmitoylglycerol
1,3-DPG	1,3-dipalmitoylglycerol
CID	collision induced dissociation
CLA	conjugated linoleic acid [octadeca-(9 <i>Z</i> ,11 <i>E</i>)-dienoic acid]
CLA-TAG	dipalmitoyl-conjugated-linoleoylglycerol
CoA	coenzyme A
DGAT	diglyceride acyltransferase
DMEM	Dulbecco's modified Eagle's medium
ESI	electro spray ionization
EPI	enhanced product ion scan
FBS	fetal bovine serum
FWHM	full width at half maximum
GC	gas chromatography
GSH	glutathione
HBA	hexane/methyl tert-butyl ether/acetic acid solution
HBSS	Hank's balanced salt solution
HNO₂	nitrous acid
HPLC-MS/MS	high performance liquid chromatography tandem mass spectrometry
HPLC-APCI-MS/MS	high performance liquid chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry
HPLC-ESI-MS/MS	high performance liquid chromatography-electrospray ionization-tandem mass spectrometry
HPLC-HR-MS/MS	high performance liquid chromatography-coupled high resolution-tandem mass spectrometry analysis
Keap1	Kelch-like ECH-associated protein 1
MRM	multiple reaction monitoring scan mode
MS	mass spectrometer
<i>m/z</i>	mass-to-charge ratio
NH₃	ammonia
N₂O₃	dinitrogen trioxide
NMR	nuclear magnetic resonance

NaNO₂	sodium nitrite
NF-κB	nuclear factor kappa B
•NO	nitric oxide
•NO₂	nitrogen dioxide
NO₂⁻	nitrite
NO₃⁻	nitrate
NO₂-oxo-OA-TAG	dipalmitoyl-nitro-oxo-octadec-10-enoylglycerol
NO₂-OH-OA-TAG	dipalmitoyl-nitrohydroxyoctadec-10-enoylglycerol
NO₂-OOH-OA-TAG	dipalmitoyl-nitrohydroperoxyoctadec-10-enoylglycerol
NO₂-CLA-TAG	1,3-dipalmitoyl-2-nitro-conjugated-linoleoylglycerol
NO₂-CLA	nitro-conjugated linoleic acid
NO₂-FA	electrophilic nitro-fatty acids
NO₂-FA-TAG	nitro-fatty acid-containing-triglycerides
NO₂-OA, NO₂-18:1	nitro-oleic acid (10-nitro-octadec-9-enoic acid)
NO₂-[¹³C₁₈]OA	nitro-[¹³ C ₁₈]octadec-9-enoic acid
NO₂-18:0	nitro-stearic acid
NO₂-[¹⁵N/D₄]18:0	[¹⁵ N]nitro-[D ₄]octadecanoic acid
NO₂-[¹⁵N/D₄]18:1	[¹⁵ N]nitro-[D ₄]octadecenoic acid
NO₂-16:0	nitro-hexadecanoic acid
NO₂-16:1	nitro-hexadecenoic acid
NO₂-14:0	nitro-tetradecanoic acid
NO₂-14:1	nitro-tetradecenoic acid
NO₂-12:0	nitro-dodecanoic acid
NO₂-12:1	nitro-dodecenoic acid
Nrf2	nuclear transcription factor erythroid 2-related factor 2
OA	oleic acid
SPE	solid phase extraction
TAG	triacylglycerides
TRPV1	transient receptor potential cation channel subfamily V member 1
9-NO₂-CLA	9-nitro-octadeca-(9,11)-dienoic acid
12-NO₂-CLA	12-nitro-octadeca-(9,11)-dienoic acid. The designations “9-NO ₂ -” and “12-NO ₂ -”CLA are used herein to describe the

position of the nitro group in conjugated dienes and do not refer to IUPAC nomenclature.

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- Triacylglycerides are nitrated under *in vitro* gastric conditions.
- Nitro-triacylglycerides characterization by HPLC-MS/MS.
- Nitro fatty acids are esterified into adipocyte and plasma triacylglycerides.
- Esterification of nitro-fatty acids favors nitroalkanes over nitroalkenes.

Fig. 1. Detection and characterization of NO₂-CLA-TAG generation by *in vitro* gastric conditions

(A) Mass chromatogram of NO₂-CLA-TAG as ammonium adduct (m/z 893.7) [M+NH₄]⁺ and inset with its atomic composition and isotopic abundance. (B) MS² spectral data of NO₂-CLA-TAG. (C) MS³ spectral data of the MS² even fragments m/z 620.4 [M-16:0-NH₃]⁺. (D) MS³ spectral data of the MS² odd fragment m/z 551.5 [M-NO₂-CLA-NH₃]⁺. Representative chemical structures are only shown for the 9-NO₂-CLA-TAG isomer and corresponding product ions.

Fig. 2. Time-dependent changes in extracellular NO₂-FA after supplementation of NO₂-OA in adipocytes

Values of electrophilic nitroalkenes (filled square) and non-electrophilic nitroalkanes (open square) are given as area ratio of (A) nitro- C_{18} -, (B) C_{16} -, (C) C_{14} - and (D) C_{12} - fatty acid/NO₂- $[^{13}C_{18}]OA$ over a period of 24 h. Data shown are the means $\pm$ SD of 3 independent experiments with 5 and 3 replicates each for NO₂-OA and OA treatment, respectively.

Fig. 3. Metabolism, distribution and esterification of NO₂-FA into TAG in adipocytes supplemented with NO₂-OA

Lipase-hydrolyzed TAG, cell lysate and cell media were analyzed by HPLC-ESI-MS/MS in negative mode to determine (A) esterified into TAG, (B) intracellular and (C) extracellular content of NO₂-FA at 24 h, respectively. A strong correlation ($r^2 = 0.98$, $P < 0.001$) was observed between (D) intracellular and extracellular but not between (E) esterified into TAG and intracellular content of NO₂-FA. Values are given as area ratio of NO₂-FA/NO₂-OA. Data shown are the means $\pm$ SD of 3 independent experiments with 5 and 3 replicates each for NO₂-OA and OA treatment, respectively.

Fig. 4. Mass spectrometry analysis of adipocyte NO₂-FA-TAG

Purified TAG fractions from adipocytes treated with 5 μ M NO₂-OA for 24 h were subjected to HPLC-HR-MS analysis in positive mode. (A) Mass chromatogram of NO₂-FA-TAG as ammonium adducts (m/z 893.7) [M+NH₄]⁺, inset with atomic composition and isotopic abundance. (B) MS² spectral data of peak 1 in Fig. 4A. (C) MS² spectral data of peak 2 in Fig. 4A. (D) MS³ spectral data of the MS² even fragments m/z 622.5 [M-16:1-NH₃]⁺ in Fig. 4B. (E) MS³ spectral data of the MS² odd fragment m/z 547.4 [M-NO₂-18:0-NH₃]⁺ in Fig. 4B. ^a Order of notation does not connote regiochemistry. * Presence of a shared MS² fragment from a co-eluting NO₂-FA-TAG isomer (NO₂-16:0/17:1/17:1-TAG).

Fig. 5. Characterization of rat plasma NO_2 -FA-TAG

Kinetic analysis of (A) nitro- C_{18} -, (B) C_{16} -, (C) C_{14} - fatty acid concentration (nM) in lipase hydrolyzed rat plasma triacylglycerides after supplementation of NO_2 -OA. (D, F) MRM chromatograms for NO_2 -18:0/16:0/18:2-TAG and NO_2 -18:0/18:2/18:2-TAG, as ammonium adducts in positive mode, in (upper panel) untreated and (lower panel) treated rat plasma samples. (E) EPI analysis of peak 1 for m/z 921.8. (G) EPI analysis of peak 2 for m/z 945.8. Data shown are the means $\pm$ SD of a pharmacokinetic study performed on two treated and one control animal. ^a Order of notation does not connote regiochemistry.

Table 1
Positional analysis of the most abundant non-electrophilic NO₂-FA-TAG in adipocytes supplemented with NO₂-OA

Positional fatty acid distribution, specific product ion, accurate mass determination and retention time of non-electrophilic NO₂-FA-TAG in positive ion mode.

$\frac{[\text{NO}_2\text{-FA-TAG} + \text{NH}_4]^+}{sn-1/3}$		Diacylglyceride product ion [M-R _n COOH-NH ₃] ⁺		Chemical formula	Experimental mass	Theoretical mass	Retention time (min)
NO ₂ -18:0 ^a	16:1	16:1		C ₅₃ H ₁₀₃ N ₂ O ₈ ⁺	895.771	895.7709	
			NO ₂ -18:0/16:0	C ₃₇ H ₇₀ NO ₆ ⁺	624.5191	624.5198	14
			NO ₂ -18:0/16:1	C ₃₇ H ₆₈ NO ₆ ⁺	622.5041	622.5041	
			16:0/16:1	C ₃₅ H ₆₅ O ₄ ⁺	549.4872	549.4877	
NO ₂ -18:0 ^a	16:1	15:0		C ₅₂ H ₁₀₁ N ₂ O ₈ ⁺	881.7538	881.7552	
			NO ₂ -18:0/16:1	C ₃₇ H ₆₈ NO ₆ ⁺	622.5037	622.5041	13.1
			NO ₂ -18:0/15:0	C ₃₆ H ₆₈ NO ₆ ⁺	610.5045	610.5041	
			16:1/15:0	C ₃₄ H ₆₃ O ₄ ⁺	535.472	535.4721	
NO ₂ -18:0	16:1	16:1		C ₅₃ H ₁₀₁ N ₂ O ₈ ⁺	893.7561	893.7552	
			NO ₂ -18:0/16:1	C ₃₇ H ₆₈ NO ₆ ⁺	622.5036	622.5041	12.5
			16:1/16:1	C ₃₅ H ₆₃ O ₄ ⁺	547.4714	547.4721	
NO ₂ -16:0	16:1	16:1		C ₅₁ H ₉₇ N ₂ O ₈ ⁺	865.7244	865.7239	
			NO ₂ -16:0/16:1	C ₃₅ H ₆₄ NO ₆ ⁺	594.472	594.4728	11.5
			16:1/16:1	C ₃₃ H ₆₃ O ₄ ⁺	547.4711	547.4721	
NO ₂ -14:0	16:1	16:1		C ₄₉ H ₉₃ N ₂ O ₈ ⁺	837.6939	837.6926	
			NO ₂ -14:0/16:1	C ₃₃ H ₆₀ NO ₆ ⁺	566.4405	566.4415	10.9
			16:1/16:1	C ₃₅ H ₆₃ O ₄ ⁺	547.4715	547.4721	
NO ₂ -14:0	18:1	16:1		C ₅₁ H ₉₇ N ₂ O ₈ ⁺	865.7254	865.7239	
			NO ₂ -14:0/18:1	C ₃₅ H ₆₄ NO ₆ ⁺	594.4722	594.4728	12.3
			NO ₂ -14:0/16:1	C ₃₃ H ₆₀ NO ₆ ⁺	566.4406	566.4415	

$\frac{[\text{NO}_2\text{-FA-TAG} + \text{NH}_4]^+}{sn-1/3}$		Diacylglyceride product ion [M-R ⁿ COOH-NH ₃] ⁺		Chemical formula	Experimental mass	Theoretical mass	Retention time (min)
NO ₂ -12:0	18:1	16:1	16:1/18:1	C ₃₇ H ₆₇ O ₄ ⁺	575.5033	575.5034	11.7
				C ₄₉ H ₉₃ N ₂ O ₈ ⁺	837.6944	837.6926	
			NO ₂ -12:0/18:1	C ₃₃ H ₆₀ NO ₆ ⁺	566.4405	566.4415	
			NO ₂ -12:0/16:1	C ₃₁ H ₅₆ NO ₆ ⁺	538.4093	538.4102	
NO ₂ -12:0 ^a	16:0	16:1	16:1/18:1	C ₃₇ H ₆₇ O ₄ ⁺	575.5031	575.5034	11.8
				C ₄₇ H ₈₉ N ₂ O ₈ ⁺	811.6784	811.6777	
			NO ₂ -12:0/16:0	C ₃₁ H ₅₈ NO ₆ ⁺	540.4253	540.4259	
			NO ₂ -12:0/16:1	C ₃₁ H ₅₆ NO ₆ ⁺	538.4092	538.4102	
NO ₂ -12:0	16:1	16:1	16:0/16:1	C ₃₅ H ₆₅ O ₄ ⁺	549.4871	549.4877	10.3
				C ₄₇ H ₈₉ N ₂ O ₈ ⁺	809.6611	809.6613	
			NO ₂ -12:0/16:1	C ₃₁ H ₅₆ NO ₆ ⁺	538.4094	538.4102	
			16:1/16:1	C ₃₅ H ₆₃ O ₄ ⁺	547.4712	547.4721	
NO ₂ -12:0	16:1	14:1	NO ₂ -12:0/16:1	C ₄₅ H ₈₅ N ₂ O ₈ ⁺	781.6311	781.63	9
				C ₃₁ H ₅₆ NO ₆ ⁺	538.4095	538.4102	
			NO ₂ -12:0/14:1	C ₂₉ H ₅₂ NO ₆ ⁺	510.3786	510.3789	
			14:1/16:1	C ₃₃ H ₅₉ O ₄ ⁺	519.4402	519.4408	

^a Order of notation does not connote regiochemistry.