Conjugated Linoleic Acid Is a Preferential Substrate for Fatty Acid Nitration

Background: Nitroalkene fatty acids are electrophilic cell metabolites that mediate anti-inflammatory signaling actions.

Results: Conjugated linoleic acid is the preferential unsaturated fatty acid substrate for nitration reactions during oxidative inflammatory conditions and digestion.

Conclusion: Nitro-fatty acid formation in vivo occurs during metabolic and inflammatory reactions and modulates cell signaling.

Significance: Nitro-conjugated linoleic acid transduces signaling actions of nitric oxide, nitrite, and conjugated linoleic acid.

The oxidation and nitration of unsaturated fatty acids by oxides of nitrogen yield electrophilic derivatives that can modulate protein function via post-translational protein modifications. The biological mechanisms accounting for fatty acid nitration and the specific structural characteristics of products remain to be defined. Herein, conjugated linoleic acid (CLA) is identified as the primary endogenous substrate for fatty acid nitration in vitro and in vivo, yielding up to 105 greater extent of nitration products as compared with bis-allylic linoleic acid. Multiple enzymatic and cellular mechanisms account for CLA nitration, including reactions catalyzed by mitochondria, activated macrophages, and gastric acidification. Nitroalkene derivatives of CLA and their metabolites are detected in the plasma of healthy humans and are increased in tissues undergoing episodes of ischemia reperfusion. Dietary CLA and nitrite supplementation in rodents elevates NO2-CLA levels in plasma, urine, and tissues, which in turn induces heme oxygenase-1 (HO-1) expression in the colonic epithelium. These results affirm that metabolic and inflammatory reactions yield electrophilic products that can modulate adaptive cell signaling mechanisms.

Products of oxidative inflammatory reactions mediate both the progression and resolution of inflammation (1). Notably, unsaturated fatty acids can induce adaptive cell signaling responses and modulate the resolution of inflammation by the redox-dependent formation and secondary reactions of noneicosanoid oxo and nitroalkene derivatives (2). These signaling actions are transduced in vitro and in vivo via the post-translational modification of functionally significant nucleophilic amino acids of susceptible transcription factors and enzymes (3, 4).

Multiple clinical trials support that the increased dietary intake of ω-3 unsaturated fatty acids promotes a broad range of physiological benefits (5). The beneficial actions of conjugated diene-containing fatty acids in both animal models and clinical studies have also been reported. The predominant conjugated diene species found clinically are octadeca-(9Z,11E)-dienoic and octadeca-(10E,12Z)-dienoic acid, referred to herein as conjugated linoleic acid (CLA) (6). Notably, CLA is distinct from octadeca-(9Z,12Z)-dienoic acid (linoleic acid, LA) by having a conjugated rather than methylene-interrupted diene. Oxidative inflammatory reactions also give rise to elevated levels of conjugated diene-containing lipids that are generated from bis-allylic dienes following hydrogen abstraction (7). The conjugation of double bonds promotes radical addition reactions to both CLA and other conjugated diene-containing species (8).

Both ω-3 fatty acids and CLA have been proposed to exert anti-inflammatory actions by serving as endogenous peroxisome proliferator-activated receptor (PPAR) ligands and inhibitors of nuclear factor-κB (NF-κB)-dependent cytokine expression (9, 10). Notably, electrophilic derivatives of these unsaturated fatty acids (i.e. nitroalkene and α,β-unsaturated nitroalkene fatty acids) are preferential substrates for fatty acid nitration reactions during oxidative inflammatory conditions and digestion.
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carbonyl species) are critical proximal mediators of these signaling actions, because electrophilic fatty acids are orders of magnitude more potent than native unsaturated fatty acids in modulating these and other key tissue-protective and adaptive signaling mechanisms (2, 3, 11). Electrophilic lipids mediate signaling responses via Michael addition, inducing post-translational protein modifications (2). These often reversible reactions can be modulated by relative concentrations of competing tissue nucleophiles such as GSH and H2S (12). In human coronary artery endothelium for example, fatty acid nitroalkenes significantly influence the expression of ~400 metabolic and anti-inflammatory-related genes (13). Specific cellular nitroalkylation targets include functionally significant thiol residues in the transcriptional regulatory proteins PPARγ (14), Keap1/Nrf2 (Kelch-like ECH-associated protein 1 (Keap1)/regulator of nuclear factor (erythroid-derived-2)-like 2 (Nrf2)) (15), heat shock factor-1 (HSF-1), and NF-κB (4).

In model systems, unsaturated fatty acid nitration is induced by oxides of nitrogen (NOx) such as nitrogen dioxide (NO2), nitrite (NO2−), and peroxynitrite (ONOO−), all products of nitric oxide (NO) oxidation or the dietary consumption and further reactions of NO2 and nitrate (NO3−) (16). Nitric oxide does not directly nitrate protein or lipids, rather its oxidation to the proximal nitrating species NO2 is essential. Multiple mechanisms can account for endogenous NO2 generation, including the following: (a) oxidation of NO by oxygen (O2); (b) reaction of NO with superoxide (O2−) to yield ONOO−; (c) the one-electron oxidation of NO2 by heme peroxidases (i.e. myeloperoxidase (MPO) and eosinophil peroxidase); and (d) the acid-catalyzed protonation of NO2− to nitrous acid (HNO2). These oxidizing and nitrating species are produced both basally and at increased rates during inflammation and metabolic stress.

The mechanisms underlying biological fatty acid nitration and the structural characteristics of products require additional characterization (17–19). Importantly, NO2−-supplemented diets are associated with a variety of beneficial anti-inflammatory and metabolic actions, including the regulation of mitochondrial function, adipogenesis, oxygen delivery to tissues, and blood pressure (20). Although these events can in part be attributed to the generation of NO, salutary responses to NO2−-derived oxides of nitrogen may also be transduced by the concomitant generation of electrophilic nitro-fatty acids (NO2-FA).

Herein, we report the formation of previously undescribed endogenous NO2-FA species, the nitro derivatives of CLA (9- and 12-nitro-octadeca-(9,11)-dienoic acid), termed NO2-CLA, and we show their presence in the plasma of healthy individuals. CLA is the preferential endogenous substrate for the fatty acid nitration reactions promoted by NO and NO2. Moreover, rodents supplemented with CLA and the stable isotope [15N]O2 display elevated tissue, plasma, and urine content of [15N]O2-CLA. These electrophilic nitroalkene derivatives, formed endogenously during inflammatory conditions and gastric acidification, mediate transcriptional regulatory responses that can account for a component of the tissue-protective and anti-inflammatory actions attributed to NO, NO2, and CLA (21–23).

EXPERIMENTAL PROCEDURES

Materials—All chemicals and fatty acids were purchased from Sigma and Nu-Chek Prep, Inc. (Elyssian, MN), respectively. Internal standards ([13C18]NO2-OA and [13C18]NO3-LA) were synthesized as described previously (18, 25). For animal studies, male Sprague-Dawley rats (Harlan Lab, Indianapolis, IN), 200–250 g body mass, and C57B16 mice were housed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (publication number 85-23, revised 1996), and all procedures were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh (protocol numbers 0911852, 0901770B-4, and 0905750B-7).

Lipid Extraction from Urine—In rodent studies, urine was collected over a 24-h period by capture in a metabolic cage. Lipids were extracted using C-18 solid phase extraction (SPE) columns conditioned with 100% MeOH, followed by equilibration with 5% MeOH/H2O. Urine containing 5% MeOH and 3.0 ng/ml internal standard (equilibrated at 4 °C for 30 min prior to extraction) was loaded and sequentially washed with 2 column volumes each of H2O, 5% MeOH, and 50% MeOH. Lipids were eluted with 3 ml of MeOH, dried, and dissolved in MeOH for quantitative HPLC-MS/MS analysis.

Mass Spectrometry—Quantification and structural analysis of nitrated species were conducted by high performance liquid chromatography-electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) using a triple quadrupole mass spectrometer in positive and negative ion mode (API 5000 and API QTrap 4000, Applied Biosystems, Framingham, MA) and a high resolution hybrid mass spectrometer (Velas Orbitrap, ThermoScientific). NO2-FA were separated with a C18 reversed phase column (2 × 150 mm, 3 μm, Phenomenex, Torrance, CA) using a water/acetonitrile solvent system containing 0.1% acetic acid. Reactivity toward β-ME and the Dess Martin reaction were used to characterize electrophilicity and the presence of hydroxy groups, respectively (26).

NO2-FA Formation by Mitochondria and Cardiac Tissue—Rat liver mitochondria from male Sprague-Dawley rats were isolated as described previously (27). For all reactions, 2 mg of liver mitochondrial protein was incubated in the presence or absence of NaNO2 or Na[15N]O2 (0–1000 μM) for 0–4 h at 37 °C with or without fatty acid supplementation. Similar results were obtained for heart mitochondria. The ischemia/reperfusion data were obtained upon MS analysis of archived murine cardiac tissue that was subjected to 30 min of ligation of the coronary artery and 30 min of reperfusion as described previously (17).

CLA Nitration by MPO and ONOO−(9Z,11E)-CLA, (10E,12Z)-CLA, and LA (1 μM) were subjected to nitration by MPO (50 ng) in 50 mm sodium phosphate, pH 7.2, 100 μM DTPA, in the presence of H2O2 (100 μM), NaN3O2 (100 μM) for 0–2 h at 37 °C, followed by addition of internal standard ([13C18]NO2-LA) prior to extraction. ONOO− (0–20 μM, ε302 nm = 1.67 mm−1 cm−1) was added to CLA or LA (10 μM) in 50 mm potassium phosphate, pH 7.2, containing 100 μM DTPA. Reverse order additional studies were carried out by adding...
ONOO\textsuperscript{−} to buffers for 30 s to promote decomposition prior to CLA or LA addition.

**CLA Nitration by Activated Macrophages**—RAW 264.7 macrophages were activated with LPS (100 ng/ml) and INF\(\gamma\) (200 units/ml) for 12 h; media were replaced with HBSS containing 10 \(\mu M\) CLA or LA for 6 h in the presence or absence of L-NAMe. Media NO\(_2\) and NO\(_2\)-FA determinations were performed using a Sievers nitric oxide analyzer 280i (General Electric, Boulder, CO) and mass spectrometry, respectively.

**Fatty Acid Nitration by \(\cdot\)NO\(_2\) Gas**—(9Z,11E)-CLA or (9Z,12Z)-LA (100 \(\mu M\)) in 50 mM sodium phosphate, pH 6.0, 100 \(\mu M\) DTPA was exposed to 5.6 ppm NO\(_2\) gas flowing over the headspace at 690 ml/min for 60 min in the dark using \(N_2\) (~10 mm Hg residual O\(_2\) was measured using a blood gas analyzer) or air (20.9% oxygen) as the carrier gas.

**Detection of NO\(_2\)-CLA in Healthy Human Plasma**—Lipids from human plasma (1.5 ml) (University of Pittsburgh IRB approval PRO07110032) were extracted using 3.5 ml of hexane, propan-2-ol, 1 M formic acid (30:20:2, v/v/v) in the presence of 1 mM 1,3-hexadiene (added to quench possible NO\(_2\) radicals) in a two-step solvent extraction with hexane. Quantification was performed by LC-ESI-MS/MS using [15N]O2-CLA as an internal standard. Artifactual nitration during extraction procedures was controlled in parallel by adding a large excess (100 \(\mu M\)) of Na\(^{15N}\)O\(_2\) and 1 mM 1,3-hexadiene to plasma and following the formation of [15N]O2-CLA in the presence of \(^{13C}_{18}\)NO\(_2\)-LA as an internal standard.

**Gastric Generation of NO\(_2\)-CLA**—Mice fasted overnight were intra-peritoneally injected with pentagastrin (5 mg/kg) 60 min prior to oral gavage with 100 nmol each of CLA and Na\(^{15N}\)O\(_2\) (0.23 mg/kg) in 100 \(\mu l\) of PEG 400. Typical dietary consumption of NO\(_2\) by humans (based on an average weight of 60 kg) is 0.33 mg/kg. Other groups of mice were given higher CLA and Na\(^{15N}\)O\(_2\) doses (100 \(\mu M\) and 10 \(\mu M\), respectively, in 100 \(\mu l\) of PEG 400), which equates to 23 mg of NO\(_2\)/kg. Animals were euthanized at various times, and stomach, small intestine, and colon were harvested and frozen in liquid nitrogen, pulverized, and lipids extracted for MS analysis.

Similar experiments were performed with rats treated via oral gavage every 24 h for 4 days with 100 \(\mu M\) of CLA and Na\(^{15N}\)O\(_2\) in 100 \(\mu l\) of PEG 400. Control rats included gavage with vehicle, 100 \(\mu M\) of CLA, or 100 \(\mu M\) of NO\(_2\). During the final 24 h of treatment, before euthanasia, animals were housed in metabolic cages, fasted overnight, and intra-peritoneally injected with pentagastrin (5 mg/kg) 60 min prior to a final oral gavage with 100 \(\mu M\) of each of CLA and Na\(^{15N}\)O\(_2\) (28). At various times, tissues and plasma were harvested and lipids extracted as described previously using \(^{13C}_{18}\)NO\(_2\)-LA as an internal standard and analyzed by HPLC-MS/MS. NO\(_2\)-CLA levels (\(^{14N}\) and \(^{15N}\)) in urine obtained from the cages were collected and quantified.

**HO-1 Immunocytochemistry**—The colon from vehicle, CLA, NO\(_2\), and CLA + NO\(_2\)-treated rats was divided to provide tissue for both lipid and immunocytochemical analyses. In addition, a group of rats received NO\(_2\)-CLA by gavage (770 nmol) 8 h prior to colon dissection. The proximal colon was cut into 2–3-cm segments and fixed. Sections (5 \(\mu M\)) were mounted on slides and processed for immunocytochemical analyses. HO-1 was detected with a specific antibody (rabbit, Abcam) and visualized with anti-rabbit IgG labeled with Cy3 (Jackson ImmunoResearch). Actin distribution was detected with Alexa488-labeled phalloidin (Invitrogen) and the nuclei with Hoechst dye (Invitrogen). Images were collected with an Olympus FV1000 confocal microscope using a \(\times20\) oil immersion objective. All settings (laser power, photomultiplier tube voltage, and offset) were kept constant for the collection of all images.

**HO-1 Expression in RAW 264.7 Macrophages**—RAW 264.7 cells were grown to 90% confluence on 6-well plates and incubated with 1–5 \(\mu M\) [15N]O2-CLA, the specific isomer 9-NO\(_2\)-CLA, NO\(_2\)-LA, and 5 \(\mu M\) CLA and LA as fatty acid controls for 12 h. Protein samples were harvested, homogenized in lysis buffer, resolved by SDS-PAGE, and transferred to PVDF membranes (Bio-Rad).

**Statistics**—A one-way analysis of variance test with post hoc Tukey test was used to analyze data.

**RESULTS**

**Mitochondrial Fatty Acid Nitration**—Isolated rat liver mitochondria were incubated with NO\(_2\) at pH ranges reflective of tissue ischemia (29). HPLC-MS/MS precursor ion scanning of lipid extracts revealed the loss of an organic nitro group as NO\(_2\) (m/z 46). This sentinel fragment ion for nitrated species was predominantly from parent ions with m/z 324.2. Two peaks (36.3 and 37.4 min) were detected by following the multiple reaction monitoring (MRM) transition 324.2/46 and displayed the same retention time as nitrated fatty acids generated in cardiac tissue homogenates after rodent hearts were subjected to focal myocardial ischemia-reperfusion (I/R) (Fig. 1a) (17). Importantly, these peaks displayed longer retention times when resolved on a C18 reversed phase column than the \(^{13C}_{18}\)NO\(_2\)-LA used as an internal standard. This standard consisted of the 9-,10-,12-, and 13-NO\(_2\) positional isomers of \(^{13C}_{18}\)NO\(_2\)-LA (Fig. 1a). Product ions obtained upon MS/MS fragmentation of mitochondrially generated NO\(_2\)-FA (m/z 324.2 and 325.2, for species having -NO\(_2\) and -[15N]O2 groups, respectively), displayed the common losses of H\(_2\)O and the distinctive fragmentation of mitochondrially generated NO\(_2\)-FA (Fig. 1a) (23). Product ions formed using a Sievers nitric oxide analyzer 280i (General Electric, Boulder, CO) and mass spectrometry, respectively. Mitochondria were incubated with NO\(_2\)-FA (100 \(\mu M\)), which equates to 23 mg of NO\(_2\)/kg. Animals were euthanized at various times, and stomach, small intestine, and colon were harvested and frozen in liquid nitrogen, pulverized, and lipids extracted for MS analysis.

Similar experiments were performed with rats treated via oral gavage every 24 h for 4 days with 100 \(\mu M\) of each of CLA and Na\(^{15N}\)O\(_2\) in 100 \(\mu l\) of PEG 400. Control rats included gavage with vehicle, 100 \(\mu M\) of CLA, or 100 \(\mu M\) of NO\(_2\). During the final 24 h of treatment, before euthanasia, animals were housed in metabolic cages, fasted overnight, and intra-peritoneally injected with pentagastrin (5 mg/kg) 60 min prior to a final oral gavage with 100 \(\mu M\) of each of CLA and Na\(^{15N}\)O\(_2\) (28). At various times, tissues and plasma were harvested and lipids extracted as described previously using \(^{13C}_{18}\)NO\(_2\)-LA as an internal standard and analyzed by HPLC-MS/MS. NO\(_2\)-CLA levels (\(^{14N}\) and \(^{15N}\)) in urine obtained from the cages were collected and quantified.

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**Mass Spectrometric Characterization of Mitochondrial NO\(_2\)-FA**—To gain further insight into the structural characteristics of the nitrated fatty acid species having longer HPLC elution times than the synthetic standard, MS\(^2\) analysis in the positive ion mode was performed. Upon collision induced dissociation (CID), lithium adducts of fatty acid nitroalkenes generate specific product ions predictive of the overall nitroalkene position in the parent ion (31). The MS\(^2\) spectrum of lithium nitroalkene NO\(_2\) radicals was performed by LC-ESI-MS/MS using [15N]O2-CLA as an internal standard. Artifactual nitration during extraction procedures was controlled in parallel by adding a large excess (100 \(\mu M\)) of Na\(^{15N}\)O\(_2\) and 1 mM 1,3-hexadiene to plasma and following the formation of [15N]O2-CLA in the presence of \(^{13C}_{18}\)NO\(_2\)-LA as an internal standard.
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FIGURE 1. Nitrite induces mitochondrial formation of NO2-FA. Mitochondria were incubated for 2 h with NO2- (0.2–1.0 mM) in phosphate buffer (50 mM, pH 6). a, chromatogram showing nitration of 18:2 fatty acids in mitochondria by [15NO2] or NO2-, cardiac tissue from mice subjected to focal myocardial I/R and [15NO2]-LA internal standard, respectively. Product ions of m/z 46 (NO2-) and m/z 47 ([15NO2]-LA) were followed upon fragmentation of ions m/z 324.2 and 322.2, respectively. b, MS-MS spectra of mitochondrial NO2-FA showing characteristic losses of H2O and NO2 derived from organic nitro groups. c, NO2- and pH dependence of mitochondrial fatty acid formation (followed as 324.2/46 MRM transition). NO2- not detected. Results represent the mean of three independent experiments ± S.D.

adducts ([M + Li]+) of mitochondrial adducts ([M + Li]+) of mitochondrial fatty acid induction products (m/z 332.2, chromatographic peaks at 36.3 and 37.4 min) showed unique product ions with m/z 205.2 (RT 36.3 min) and m/z 192.2 (RT 37.4 min) (supplemental Fig. 1a). In contrast to the ion fragmentation of mitochondrial fatty acid nitration products, synthetic bis-allylic NO2-LA presented product ions with m/z 219.1 (34.6 min) and m/z 192.2 (35.8 min) (supplemental Table 1). The differences in the elution time between synthetic standard and mitochondrial derived fatty acid nitration products, along with the appearance of the 14 carbon mass units shift in product ion fragments upon CID (m/z 219.1 versus 205.1), indicated that the NO2 group of mitochondrial nitroalkenes is placed at positions C-9 or C-11, consistent with the nitration of conjugated linoleic acid rather than bis-allylic LA, which contains methylene-interrupted double bonds (supplemental Tables 1 and 2). The formation of 13-NO2-CLA and 10-NO2-CLA, minor nitration products of the less abundant mitochondrial pool of (10E,12Z)-CLA was also detected by CID analysis in positive (supplemental Fig. 1c and d) and negative mode (supplemental Fig. 2a and b). Production analysis of these isobaric species eluting at RT of 36.25 and 36.59 min gave specific transitions at m/z 171, 185, and 209, and 227 (13-NO2-CLA) and 182, 224, and 238 (10-NO2-CLA), respectively, consistent with their predicted fragmentation (supplemental Fig. 2a and b). These results affirm that nitration of (9,11)-CLA in mitochondria prevails over the less abundant (10,12)-CLA. Final structural confirmation came from the synthesis and purification of the 9- and 12-[15NO2]-CLA isomer mixture, with molecular structures confirmed by MS and 1H NMR analysis (Fig. 5a and supplemental Fig. 3). Finally, the de novo synthesis of the specific isomer 9-NO2-CLA (to be reported separately) provided a standard for confirming the identification of the nitrated fatty acids detected in mitochondria (supplemental Fig. 2c).

CLA Is the Substrate for Mitochondrially Induced Fatty Acid Nitration—To further define fatty acid substrate selectivity for nitration, isolated mitochondria were supplemented with dif-
different free fatty acids and NO2-\textsuperscript{-}. The addition of both oleic acid (OA) and LA (1 \textmu M each) did not impact the extent of fatty acid nitration and did not increase yields of nitrated products. Supplementation with CLA (1 \textmu M) induced ~26-fold increase in NO2-CLA generation (Fig. 3a). NO2-CLA formation was time-, pH-, and [NO2-] dependent and correlates with decreased mitochondrial levels of free CLA (Fig. 3b). CLA was quantified by derivatization with 4-phenyl-1,2,4 triazoline-3,5-dione and HPLC-MS/MS analysis against standards (supplemental Fig. 4). This analysis revealed that CLA is present in mitochondrial membranes and is a preferential substrate for nitration reactions.

**Nitration of CLA by Macrophages**—Nitration of free and protein-associated tyrosine is a hallmark of oxidative inflammatory reactions stemming from ‘NO and NO2- (32); thus, macrophage-induced CLA nitration was evaluated in this context. Activation of RAW 264.7 mouse macrophages with LPS/INF\textgamma did not impact the extent of fatty acid nitration and did not increase yields of nitrated products. Supplementation with CLA (1 \textmu M) induced ~26-fold increase in NO2-CLA generation (Fig. 3a). NO2-CLA formation was time-, pH-, and [NO2-] dependent and correlates with decreased mitochondrial levels of free CLA (Fig. 3b). CLA was quantified by derivatization with 4-phenyl-1,2,4 triazoline-3,5-dione and HPLC-MS/MS analysis against standards (supplemental Fig. 4). This analysis revealed that CLA is present in mitochondrial membranes and is a preferential substrate for nitration reactions.

**Nitration of CLA by Myeloperoxidase and Peroxynitrite**—During inflammation, neutrophil MPO catalyzes biomolecule nitration (33). The reaction of LA with MPO, NO2-, and H2O2 yielded oxidation products, with nitrated products undetectable under these conditions. Similar treatment of (9Z,11E)-CLA yielded 9- and 12-NO2-CLA and the further oxidized nitration products NO2-OH-OA (MRM 342.3/46) and NO2-oxo-OA (MRM 340.3/46) (Fig. 3e). MPO/H2O2/NO2- catalyzed nitration of (9Z,11E)-CLA, even in the presence of high tyrosine concentrations, revealed that tyrosine does not compete with CLA for nitration. Moreover, the addition of a 50–400 \textmu M excess of tyrosine over CLA increased CLA nitration, most likely a consequence of reactions between tyrosyl and nitroalkenyl radical intermediates (Fig. 3f). This indicates that although LA is a very poor substrate, CLA is a preferential target for nitration by NO2-, even in the presence of other nitration-susceptible species.

The reaction of LA (1 \textmu M) with ONOO\textsuperscript{-} (0–20 \textmu M) also gave no detectable nitrated species, whereas ONOO\textsuperscript{-} induced a dose-dependent nitration of (9Z,11E)-CLA (Fig. 3g). The addition of ONOO\textsuperscript{-} to reaction systems prior to lipid target, to allow for its decay to NO2-, did not support CLA nitration (Fig. 3g). Notably, all biochemical conditions tested (mitochondria, plasma, cardiac tissue after focal I/R, activated RAW cells, acidic NO2-, MPO/H2O2/NO2-, ONOO\textsuperscript{-}, and pure NO2-gas) yielded the same NO2-CLA regioisomers (supplemental Fig. 5). In addition to NO2-CLA, NO2-oxo-OA and NO2-OH-OA were also generated during CLA nitration reactions (Fig. 3 and Scheme 1), although no nitrated products were observed using its positional isomer LA as a substrate.

**Nitration of CLA by NO2 Gas and the Electrophilic Reactivity of NO2-CLA**—The electrophilic nitroalkene of NO2-FA mediates the signaling actions of these species through the post-translational protein modifications of cysteine and to a lesser extent histidine (15, 34). To probe NO2-CLA electrophilicity, reactivity toward \textbeta-ME was evaluated (26). NO2-CLA reacted rapidly, yielding products detected by the neutral loss of \textbeta-ME (78 atomic mass units) upon CID fragmentation (reverse Michael addition) to give NO2-CLA (MRM 402.4/324.2), NO2-oxo-OA (MRM 418.4/340.3), and \textsuperscript{13C15}NO2-LA (MRM 420.4/342.2, Fig. 4a). Further structural characterization of the ion m/z 340.3 revealed a nitroalkane with an \alpha,\beta-unsaturated carbonyl that also conferred electrophilic character. No \textbeta-ME application
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adducts were formed by NO₂-OH-OA, as predicted from a structure that infers lack of electrophilicity (Scheme 1). The presence of an OH group at the ion m/z 342.2 was confirmed by Dess Martin-mediated oxidation to NO₂-oxo-OA (m/z 340.2), now yielding a β-ME-reactive product (MRM 418.4/340.3) (Fig. 4b). Thus, although nitration of CLA generates electrophilic nitroalkenes and α-β-unsaturated ketone, NO₂-OH-OA requires further oxidation to gain electrophilic reactivity.

CLA nitration occurs via ‘NO₂ addition (Scheme 1 and Figs. 1–3), a precept more directly tested by treating CLA and LA in solution with pure ‘NO₂ using air or N₂ as the carrier gas. HPLC-ESI-MS/MS quantification revealed that CLA yielded ~10³ greater nitration products than LA (1.5 μM NO₂-CLA versus 20 pm NO₂-LA formed, see Fig. 4, c and d). Reactions performed in aerobic conditions produced further oxidized nitration products, including NO₂-oxo-OA (m/z 340.3) and NO₂-OO-OA (m/z 342.2) in addition to NO₂-CLA (m/z 324.2). When reactions were conducted in N₂-saturated conditions, the formation of NO₂-CLA predominated (m/z 324, Fig. 4c). These data support the formation of a resonance-stabilized radical, upon addition of ‘NO₂ to the flanking carbons of the conjugated diene, that under aerobic conditions can further react with oxygen (Fig. 4c and Scheme 1).

**Detection of NO₂-CLA in Healthy Human Plasma**—The nitration of CLA in vivo by diverse inflammatory-related mechanisms motivated clinical investigation of endogenous NO₂-CLA. Analysis by HPLC-MS/MS revealed NO₂-CLA in healthy human plasma, with identification confirmed by comparison with the synthetic [¹⁵N]O₂-CLA internal standard (Fig. 5a). Endogenous levels of NO₂-CLA in plasma ranged from 0.3 to 1.3 nm (average = 0.72 nm; median = 0.62 nm, n = 7, using [¹⁵N]O₂-CLA as internal standard) (supplemental Fig. 6). Plasma NO₂-CLA was reactive with β-ME. Upon β-ME addition, the NO₂-CLA MRM elution profile (324.2/46) was lost (Fig. 5a, lower panel). Accurate mass determination, at resolution of 40,000 (<2 ppm), also confirmed the presence of NO₂-CLA in plasma (Fig. 5b, upper panel). The nitrated CLA species (9-NO₂-CLA and 12-NO₂-CLA) were further confirmed by co-elution with synthetic standards, CID product ion fragmentation, and accurate mass determinations (supplemental Fig. 7, a and b). The de novo generation of NO₂-CLA from plasma CLA was tested by adding 0.5 mm Na[¹⁴N]O₂ to plasma under acidic conditions for 30 min (Fig. 5b, lower panel). As a control for artifactual nitration during hexane extraction and post-extraction acidic conditions, hexadiene was added to compete for nitration. These controls and the inclusion of 10 μM Na[¹⁴N]O₂ (well above physiological level) plus 1 mm hexadiene in plasma showed no artifactual CLA nitration occurred during extraction procedures (supplemental Fig. 7c).

**Gastric Nitration of CLA**—The ex vivo acid-catalyzed nitration of CLA suggested that this reaction might occur in vivo in
acidic physiological environments such as the gastric compartment. Also, NO2 generation from NO2 occurs in the stomach (35). Mice, which have higher gastric pH values than humans, were fasted overnight, and pentagastrin was administered to mimic feeding-induced gastric acid secretion. Mice were then gavaged with CLA and Na\(^{15}\text{N}\)O2 (100 nmol of each), resulting in an increase in NO2-CLA content in stomach tissue that reached a maximum concentration 2 h after gavage (0.9 ± 0.3 nmol of NO2-CLA/g of tissue) (Fig. 6a). When higher doses of CLA and Na\(^{15}\text{N}\)O2 were given to nonfasting mice (100 and 10 \(\mu\)mol, respectively), 33 ± 22 nmol of NO2-CLA/g of tissue was detected, supporting the presence of significant native CLA and indicating that ingested NO2 levels are a factor in the digestive formation of NO2-CLA.

![FIGURE 4. Nitrogen dioxide-mediated nitration of CLA and electrophilic reactivity of NO2-CLA.](image)

![FIGURE 5. Detection of NO2-CLA in human plasma.](image)
Electrophilic NO₂-oxo-CLA was also detected throughout the gastrointestinal tract of CLA and NO₂/H₂O₂-gavaged mice.

To better evaluate the formation of NO₂-CLA, rats were also treated with CLA and Na[¹⁵N]O₂, allowing for greater fluid volumes and tissue mass for the analysis of [¹⁵N]O₂-CLA. The gastric formation, absorption, and distribution of [¹⁵N]O₂-CLA after gavage with CLA and Na[¹⁵N]O₂ (100 μmol each) was detected in the stomach, intestine, colon, plasma, liver, and
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The conjugated diene-containing fatty acid CLA is a preferential substrate for nitration reactions mediated by mitochondria, digestion, and macrophage activation and following metabolic stress such as focal cardiac I/R (supplemental Fig. 5) (17). Biochemical reaction systems reported herein reinforce that the increased rates of generation of reactive inflammatory mediators (partially reduced oxygen species and various oxides of nitrogen), metabolic acidosis, and low oxygen tensions all contribute to unsaturated lipid nitration.

The propensity of reactions yielding 'NO₂ to induce the nitration of fatty acids is underscored by the lack of competition of tyrosine with CLA for nitration. The nitration of tyrosine occurs via a two-step mechanism, consisting of an initial hydrogen abstraction of the phenolic hydroxy group, followed by radical rearrangement to a tyrosyl radical intermediate (Tyr•) that reacts with 'NO₂ via radical-radical termination reaction (37). Herein, up to a 400-fold m excess of tyrosine over CLA did not compete for the nitration of CLA by MPO/H₂O₂/NO₂⁻, and actually promoted CLA nitration. Other biological factors that would influence relative extents of Tyr and CLA nitration under oxidative inflammatory conditions include the following: (a) the multiple independent and overlapping mechanisms leading to both lipid and Tyr• formation in vivo and (b) differences in the chemical reactivity and compartmentalization of tyrosine and conjugated diene-containing fatty acids.

Nitrosated fatty acids have been detected both in animal tissues subjected to inflammatory conditions and endogenously in healthy human plasma and urine (25, 38). Initially, isobaric cis or trans bis-allylic 18:2 fatty acids were proposed to be the substrate for nitration reactions, based on the abundance and the acidity of the bis-allylic hydrogen. Present data now reveal that CLA regioisomers are the principal targets of fatty acid nitration in vivo and in vitro. CLA is unique in that it displays a reactivity toward 'NO₂-induced nitration several orders of magnitude greater than bis-allylic LA (Figs. 3 and 4, c and d). This preferential reactivity of 'NO₂ toward CLA originates from the conjugated diene moiety, which in the gas phase is more reactive than simple monoalkenes by a factor of 10³−10⁴ (39). In carbon tetrachloride, the conjugated diene 2,5-dimethyl-2,4-hexadiene is 10⁴−10⁶ more reactive than monoalkenes (40), reinforcing that there will be a preferential reaction of 'NO₂ with CLA rather than bis-allylic dienes, even when conjugated dienes are present in ~100-fold lower concentrations than other bis-allylic unsaturated fatty acids in plasma (41).

CLA nitration proceeds through the free radical addition of 'NO₂ to one of the flanking alkenyl carbons, resulting in a radical product stabilized by electron resonance (Scheme 1). Thus, addition at the C-9 and C-12 positions prevails over additions at C-10 and C-11 and gives a delocalized allylic radical (42). Although consensus exists for the mechanism involved in the 'NO₂ radical addition, the fate of the acyl chain radical formed upon 'NO₂ addition is unclear. Possible reactions that could result in carbonyl radical reduction include a radical reaction between a second 'NO₂ and the allyl radical intermediate through the nitrogen or oxygen atoms of 'NO₂, thereby yielding unstable dinitro or nitro-nitrito products, respectively. Nitrito functional groups would undergo rapid hydrolysis to yield the corresponding alcohol, with further elimination of water, resulting in a vinyl nitro product (43). Alternatively, hydrogen abstraction of the NO₂-CLA radical intermediate by 'NO₂ can generate HNO₂ and reform the corresponding vinyl nitro product. Dinitro and nitro-nitrito products have been reported as reaction products during the nitration of LA at pH <3 (43), but no evidence for the formation of stable dinitro or nitro-nitrito CLA intermediates was apparent under the mild acidic conditions used herein (pH 5–7).

The presence of oxygen during fatty acid nitration promotes higher yields of NO₂-OH and NO₂-oxo products. The detection of NO₂-OH-OA (m/z 342.2) and NO₂-oxo-OA (m/z 340.3) further expands the potential product profiles of fatty acids nitrated by 'NO₂. In aggregate, these results indicate that conditions where the extent of 'NO₂, NO₂⁻, and 'NO₂ generation change along with different degrees of tissue acidosis and oxygenation, the yields of specific nitrated CLA products will be impacted. This in turn will influence downstream signaling actions due to the different electrophilic reactivities displayed by these compounds.
The biochemical mechanisms by which fatty acid nitration occurs are those that give rise to NO2 (44). These include exposure to NO2 gas, the O2- derived inflammatory by-products ONOO and nitrosoperoxocarbonate (ONOOO2) (45), and heme protein-catalyzed oxidation of NO2 - to NO2 (46). In activated macrophages and rodent hearts subjected to ischemic-reoxygenation, inhibition of nitric-oxide synthase (NOS) activity suppresses fatty acid nitration by ~50% (Fig. 3d) (19, 47).

Because NO2 is a product of NO autooxidation, the inhibition of fatty acid nitration by NOS inhibitors could be a consequence of the following: (a) suppressing the formation of NO2 or (b) limiting the reaction of O2 with NO and subsequent ONOO- dependent NO2 generation.

The protonation of NO2 (pKa 3.4) to nitrous acid (HNO2) also yields NO2 from NO2O and NO2O homolysis (48). The acidic conditions of digestion induced the NO2-dependent nitration of CLA, leading to increased levels of NO2-CLA in gastrointestinal tract tissue. The administration of [15N]NO2 by gavage permitted differentiation from endogenous NO2-CLA species. After gastric CLA nitration and absorption from the gut, [15N]NO2-CLA was detected in plasma, liver, and urine. These observations are of relevance to other physiological compartments where pH levels are or can become sufficiently acidic to support NO2 protonation (pKa 3.4) to HNO2. This includes phagolysosomes, actively respiring mitochondria, and tissues subjected to ischemic episodes or inflammation.

Electrophilic fatty acid derivatives react with nucleophilic amino acids of proteins via Michael addition, thereby altering protein structure and function (2). These modifications have typically been viewed as toxic, but recent data affirm that very low concentrations or rates of generation of reversibly reactive electrophiles mediate transient and functionally significant reactions with susceptible protein targets, eliciting a broad range of responses. The consideration of the endogenous generation, metabolism, and reactions of electrophilic NO2-FA is of relevance because these species potently limit inflammation via multiple mechanisms, including PPARγ activation, the inhibition of expression of pro-inflammatory NF-κB-regulated genes, and up-regulation of HO-1 and other phase 2 genes regulated by Keap1/Nrf2. Thus, in murine models of metabolic and inflammatory injury, fatty acid nitroalkene administration at nanomolar concentrations prevents restenosis after vessel injury (49), limits weight gain and loss of insulin sensitivity in murine models of metabolic syndrome (14), protects against ischemia-reperfusion injury (17, 47, 50), reduces plaque formation in a rodent model of atherosclerosis (51), and inhibits the onset of chemically induced inflammatory bowel disease (52).

There is a provocative convergence of the actions attributed to both the precursors (NO2 and CLA) and the products (fatty acid nitroalkenes), as these species induce positive metabolic responses and anti-inflammatory actions. Nitrite is an inorganic anion that is enriched in vegetables and both fresh and cured meats. It is also the product of nitrate (NO3) reduction by commensal bacterial nitrate reductases in saliva and the gut (53) and is a product of NO oxidation. Nitrite serves as an in vivo reservoir for NO generation, alters mitochondrial function (54), and manifests a broad array of anti-inflammatory actions in model systems (22). Similarly, the putative PPARγ/α ligand activity and NF-κB inhibitory properties of CLA are proposed as the primary mechanisms of action accounting for its anti-cancer, anti-atherogenic, and immune regulatory effects (55).

Considering that nitroalkene fatty acid derivatives are readily generated from CLA and that NO2-FA are orders of magnitude more potent than native fatty acids in modulating similar transcriptional regulatory mechanisms of anti-inflammatory signaling (2, 24), Fig. 6 reveals that nitroalkenes can mediate the transduction of many of the salutary signaling actions noted for both NO2 and CLA.

In summary, conjugated dienes are a preferential target of nitration reactions during oxidative inflammatory conditions and digestion, leading to the formation of electrophilic products that act as potent transcriptional regulatory mediators. These fatty acid derivatives and their metabolites are detected in the plasma of healthy humans and are generated during digestion, metabolic stress, and inflammation.

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