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Conjugated Linoleic Acid Is a Preferential Substrate for Fatty Acid Nitration*[S]

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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 10^5 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 NO_2-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-(9*Z*,11*E*)-dienoic and octadeca-(10*E*,12*Z*)-dienoic acid, referred to herein as conjugated linoleic acid (CLA)[3] (6). Notably, CLA is distinct from octadeca-(9*Z*,12*Z*)-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

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[3] The abbreviations used are: CLA, octadeca-(9*Z*,11*E*)-dienoic acid; CID, collision-induced dissociation; β-ME, β-mercaptoethanol; DTPA, diethylenetriaminepentaacetic acid; •NO, nitric oxide; $^{\bullet}NO_2$, nitrogen dioxide; HNO_2, nitrous acid; OA, oleic acid; LA, linoleic acid; NO_2-FA, nitrated fatty acid; NO_2-OA, nitro-oleic acid; NO_2-LA, nitro-linoleic acid; NO_2-CLA (equimolar mixture of 9-NO_2-CLA [9-nitro-octadeca-9,11-dienoic acid] and 12-NO_2-CLA [12-nitro-octadeca-9,11-dienoic acid]); 10-NO_2-CLA [10-nitro-octadeca-10,12-dienoic acid]; 13-NO_2-CLA [13-nitro-octadeca-10,12-dienoic acid]; NO_2-oxo-OA, nitro-oxo-octadec-10-enoic acid; NO_2-OH-OA, nitro-hydroxyoctadec-10-enoic acid; PPAR, peroxisome proliferator-activated receptor; MPO, myeloperoxidase; MRM, multiple reaction monitoring. The designations "9-NO_2-" and "12-NO_2-" CLA are used herein to describe position of the nitro group in conjugated dienes and do not refer to IUPAC nomenclature.

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 H_2S (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 ($^{\bullet}NO_2$), nitrite (NO_2^-), and peroxynitrite ($ONOO^-$), all products of nitric oxide ($^{\bullet}NO$) oxidation or the dietary consumption and further reactions of NO_2^- and nitrate (NO_3^-) (16). Nitric oxide does not directly nitrate protein or lipids, rather its oxidation to the proximal nitrating species $^{\bullet}NO_2$ is essential. Multiple mechanisms can account for endogenous $^{\bullet}NO_2$ generation, including the following: (*a*) oxidation of $^{\bullet}NO$ by oxygen (O_2); (*b*) reaction of $^{\bullet}NO$ with superoxide ($O_2^{\bullet-}$) to yield $ONOO^-$; (*c*) the one-electron oxidation of NO_2^- by heme peroxidases (*i.e.* myeloperoxidase (MPO) and eosinophil peroxidase); and (*d*) the acid-catalyzed protonation of NO_2^- to nitrous acid (HNO_2). 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, NO_2^--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 $^{\bullet}NO$, salutary responses to NO_2^--derived oxides of nitrogen may also be transduced by the concomitant generation of electrophilic nitro-fatty acids (NO_2-FA).

Herein, we report the formation of previously undescribed endogenous NO_2-FA species, the nitro derivatives of CLA (9- and 12-nitro-octadeca-(9,11)-dienoic acid), termed NO_2-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 $^{\bullet}NO$ and NO_2^-. Moreover, rodents supplemented with CLA and the stable isotope $[^{15}N]O_2^-$ display elevated tissue, plasma, and urine content of $[^{15}N]O_2$-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 $^{\bullet}NO$, NO_2^-, and CLA (21–23).

EXPERIMENTAL PROCEDURES

Materials—All chemicals and fatty acids were purchased from Sigma and Nu-Chek Prep, Inc. (Elysian, MN), respectively. Internal standards ($[^{13}C_{18}]NO_2$-OA and $[^{13}C_{18}]NO_2$-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 C57Bl6 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/H_2O. 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 H_2O, 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 (Velos Orbitrap, ThermoScientific). NO_2-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).

NO_2-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 $NaNO_2$ or $Na[^{15}N]O_2$ (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 $ONOO^-$—(9*Z*,11*E*)-CLA, (10*E*,12*Z*)-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 H_2O_2 (100 μM), $NaNO_2$ (100 μM) for 0–2 h at 37 °C, followed by addition of internal standard ($[^{13}C_{18}]NO_2$-LA) prior to extraction. $ONOO^-$ (0–20 μM, $\epsilon_{302\,nm} = 1.67\ mM^{-1}, cm^{-1}$) 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

$ONOO^-$ 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 INFγ (200 units/ml) for 12 h; media were replaced with HBSS containing 10 μM 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 $^{\bullet}NO_2$ Gas—(9*Z*,11*E*)-CLA or (9*Z*,12*Z*)-LA (100 μM) in 50 mM sodium phosphate, pH 6.0, 100 μM DTPA was exposed to 5.6 ppm $^{\bullet}NO_2$ 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 $^{\bullet}NO_2$ radicals) in a two-step solvent extraction with hexane. Quantification was performed by LC-ESI-MS/MS using $[^{15}N]O_2$-CLA as an internal standard. Artifactual nitration during extraction procedures was controlled in parallel by adding a large excess (100 μM) of $Na[^{15}N]O_2$ and 1 mM 1,3-hexadiene to plasma and following the formation of $[^{15}N]O_2$-CLA in the presence of $[^{13}C_{18}]NO_2$-LA as an internal standard.

Gastric Generation of NO_2-CLA—Mice fasted overnight were intraperitoneally injected with pentagastrin (5 mg/kg) 60 min prior to oral gavage with 100 nmol each of CLA and $Na[^{15}N]O_2$ (0.23 mg/kg) in 100 μl 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 $Na[^{15}N]O_2$ doses (100 μmol and 10 μmol, respectively, in 100 μl 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 μmol each of CLA and $Na[^{15}N]O_2$ in 100 μl of PEG 400. Control rats included gavage with vehicle, 100 μmol of CLA, or 100 μmol of NO_2^-. During the final 24 h of treatment, before euthanasia, animals were housed in metabolic cages, fasted overnight, and intraperitoneally injected with pentagastrin (5 mg/kg) 60 min prior to a final oral gavage with 100 μmol each of CLA and $Na[^{15}N]O_2$ (28). At various times, tissues and plasma were harvested and lipids extracted as described previously using $[^{13}C_{18}]NO_2$-LA as an internal standard and analyzed by HPLC-MS/MS. NO_2-CLA levels (^{14}N and ^{15}N) 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 μm) 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 ×20 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 μM $[^{15}N]O_2$-CLA, the specific isomer 9-NO_2-CLA, NO_2-LA, and 5 μM 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. 1*a*) (17). Importantly, these peaks displayed longer retention times when resolved on a C18 reversed phase column than the $[^{13}C_{18}]NO_2$-LA used as an internal standard. This standard consisted of the 9-,10-,12-, and 13-NO_2 positional isomers of $[^{13}C_{18}]NO_2$-LA (Fig. 1*a*). 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 -$[^{15}N]O_2$ groups, respectively), displayed the common losses of H_2O and the distinctive anionic and neutral losses of NO_2^- and HNO_2, both indicative of fatty acid nitroalkene derivatives (Fig. 1*b*) (30). The mitochondrial generation of these species was dependent on both pH and NO_2^- concentrations, with a 10-fold increase in fatty acid nitration at pH 6.0 (1.1 pg of NO_2-FA/mg of mitochondrial protein), compared with pH 7.0 (0.1 pg of NO_2-FA/mg of mitochondrial protein). This is consistent with the reactions of secondary oxides of nitrogen formed upon NO_2^- protonation (Fig. 1*c*). This supports that metabolic and inflammatory conditions having increased NO_2^- levels and decreased pH will catalyze mitochondrial lipid nitration.

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

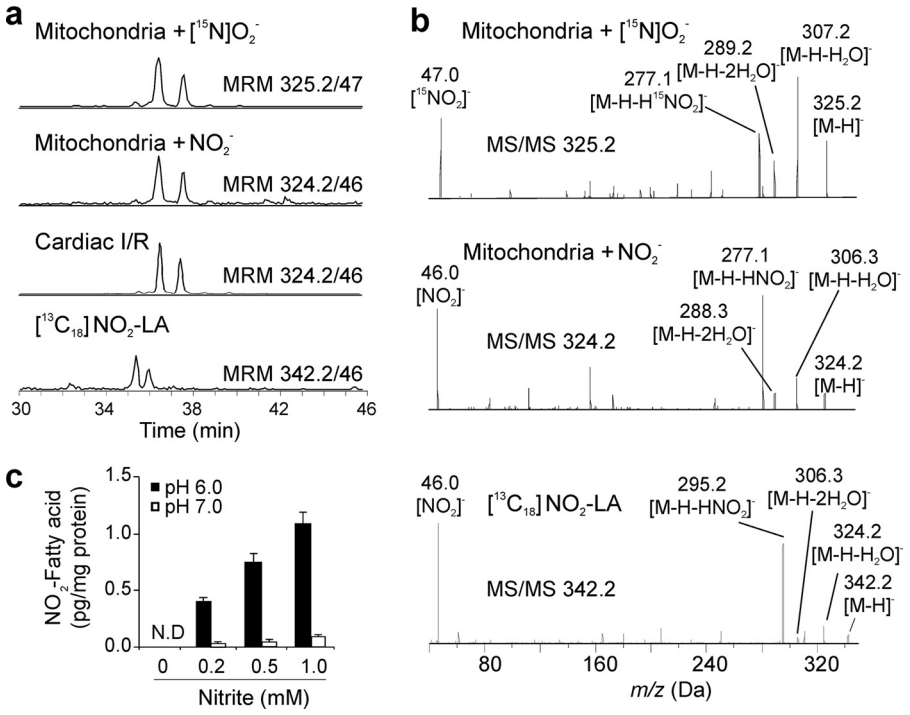


FIGURE 1. **Nitrite induces mitochondrial formation of NO_2-FA.** Mitochondria were incubated for 2 h with NO_2^- (0.2–1.0 mM) in phosphate buffer (50 mM, pH 6). *a*, chromatogram showing nitration of 18:2 fatty acids in mitochondria by $[^{15}N]O_2^-$ or NO_2^-, cardiac tissue from mice subjected to focal myocardial I/R and $[^{13}C_{18}]NO_2$-LA internal standard, respectively. Product ions of *m/z* 46 (NO_2^-) and *m/z* 47 ($[^{15}N]O_2^-$) were followed upon fragmentation of ions *m/z* 324.2 and 325.2, respectively. *b*, MS-MS spectra of mitochondrial NO_2-FA showing characteristic losses of H_2O and NO_2^- derived from organic nitro groups. *c*, NO_2^- and pH dependence of mitochondrial fatty acid formation (followed as 324.2/46 MRM transition). *ND*, not detected. Results represent the mean of three independent experiments ± S.D.

adducts ($[M + Li]^+$) of mitochondrially induced fatty acid nitration 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. 1*a*). In contrast to the ion fragmentation of mitochondrial fatty acid nitration products, synthetic bis-allylic NO_2-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 mitochondrially derived fatty acid nitration products, along with the appearance of the 14 atomic mass units shift in product ion fragments upon CID (*m/z* 219.1 *versus* 205.1), indicated that the NO_2 group of mitochondrial nitroalkenes is placed at positions C-9 or C-12, with unsaturations at C-9 and C-11, respectively (supplemental Fig. 1*b*). To confirm this proposed isomeric structure, negative ion ESI-MS/MS product ions were evaluated using high resolution mass spectrometry (30). The atomic composition of the parent ions formed by mitochondria were $C_{18}H_{30}NO_4^-$ (theoretical *m/z* 324.2180, measured *m/z* 324.2182 (0.52 ppm)). CID induced gas-phase fragmentation of mitochondrially nitrated fatty acids, confirmed by high accuracy mass determinations at the 2 ppm level, revealed four characteristic product ions (*m/z* 157.1 (*a*), 171.1 (*b*), 195.1 (*d*), and 213.1 (*c*)) for the peak at RT for 36.5 min and three product ions (*m/z* 168.1 (*e*), 210.2 (*f*), and 224.2 (*g*)) for the peak at RT for 37.5 min (Fig. 2, *a* and *b*). These distinctive product ions were indicative of fragments derived from parent ions containing a nitro group at position C-12 or C-9 (Fig. 2*a* and supplemental Table 2), confirming the structural determinations from Li^+ adduct analysis. Further structural confirmation of mitochondrial NO_2-FA was obtained by comparing HPLC elution and MS/MS fragmentation characteristics of products generated by acidic nitration of pure (9,11)- or (10,12)-CLA and synthetic NO_2-LA. These results confirmed that the double bonds were present on carbons C-9 and 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-NO_2-CLA and 10-NO_2-CLA, minor nitration products of the less abundant mitochondrial pool of (10*E*,12*Z*)-CLA was also detected by CID analysis in positive (supplemental Fig. 1, *c* and *d*) and negative mode (supplemental Fig. 2, *a* 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, 209, and 227 (13-NO_2-CLA) and 182, 224, and 238 (10-NO_2-CLA), respectively, consistent with their predicted fragmentation (supplemental Fig. 2, *a* 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-$[^{15}N]O_2$-CLA isomer mixture, with molecular structures confirmed by MS and ^1H NMR analysis (Fig. 5*a* and supplemental Fig. 3). Finally, the *de novo* synthesis of the specific isomer 9-NO_2-CLA (to be reported separately) provided a standard for confirming the identification of the nitrated fatty acids detected in mitochondria (supplemental Fig. 2*c*).

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-

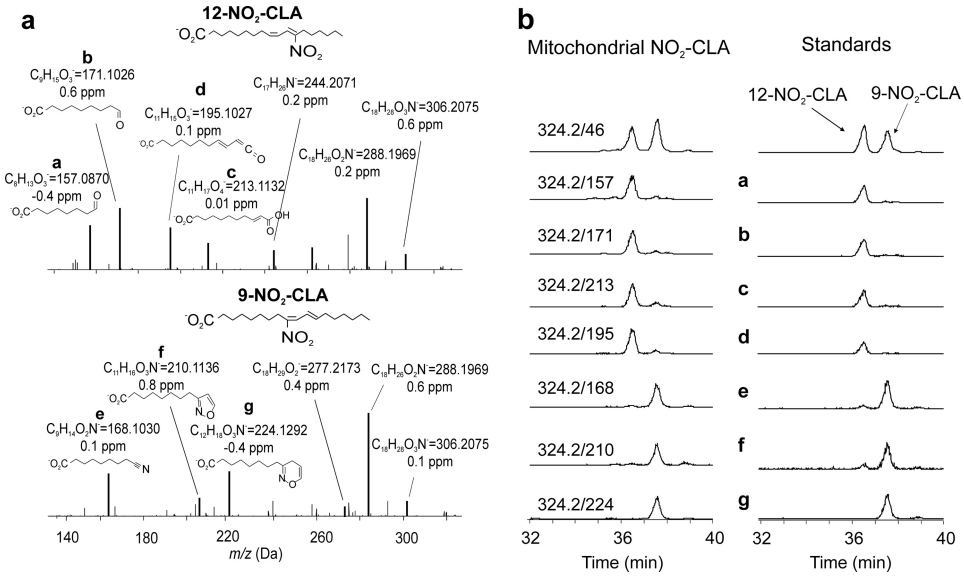


FIGURE 2. **Structural analysis of NO_2-FA by collision-induced dissociation in the negative ion mode.** *a*, MS/MS spectra of ions with *m/z* 324.2 (RT for 36.3 and 37.4 min) obtained from mitochondria exposed to NO_2^-. Specific product ions were determined at a 1 ppm level for 12-NO_2-CLA (*m/z* 157.08 (*a*), 171.10 (*b*), 195.10 (*d*), and 213.11 (*c*)) and for 9-NO_2-CLA (*m/z* 168.10 (*e*), 210.11 (*f*), and 224.11 (*g*)). *b*, elution profile of NO_2-CLA. MRM chromatograms of mitochondrial lipid extracts and CLA acidic nitration (*a*–*g*), analyzed by LC-ESI-MS/MS in the negative ion mode using specific transitions for NO_2-CLA isomers, were determined in *a*.

ferent free fatty acids and NO_2^-. The addition of both oleic acid (OA) and LA (1 μM each) did not impact the extent of fatty acid nitration and did not increase yields of nitrated products. Supplementation with CLA (1 μM) induced ~26-fold increase in NO_2-CLA generation (Fig. 3*a*). NO_2-CLA formation was time-, pH-, and [NO_2^-]-dependent and correlates with decreased mitochondrial levels of free CLA (Fig. 3*b*). 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 $^{\bullet}NO$ and NO_2^- (32); thus, macrophage-induced CLA nitration was evaluated in this context. Activation of RAW 264.7 mouse macrophages with LPS/INFγ in the presence of (9*Z*,11*E*)-CLA yielded both 9-NO_2-CLA and 12-NO_2-CLA. These products co-eluted with 9- and 12-[^{15}N]O_2-CLA internal standards and yielded characteristic product ions (Fig. 3*c*). No additional nitration products were detected upon LA supplementation, further affirming the susceptibility of CLA to nitration. Addition of the NOS inhibitor L-NAME decreased macrophage-dependent CLA nitration by 30%, in concert with a 50% reduction in media NO_2^- levels, revealing a contribution of $^{\bullet}NO$-derived species to CLA nitration (Fig. 3*d*).

Nitration of CLA by Myeloperoxidase and Peroxynitrite—During inflammation, neutrophil MPO catalyzes biomolecule nitration (33). The reaction of LA with MPO, NO_2^-, and H_2O_2 yielded oxidation products, with nitrated products undetectable under these conditions. Similar treatment of (9*Z*,11*E*)-CLA yielded 9- and 12-NO_2-CLA and the further oxidized nitration products NO_2-OH-OA (MRM 342.3/46) and NO_2-oxo-OA (MRM 340.3/46) (Fig. 3*e*). MPO/H_2O_2/NO_2^--catalyzed nitration of (9*Z*,11*E*)-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 M excess of tyrosine over CLA increased CLA nitration, most likely a consequence of reactions between tyrosyl and nitroalkenyl radical intermediates (Fig. 3*f*). This indicates that although LA is a very poor substrate, CLA is a preferential target for nitration by $^{\bullet}NO_2$, even in the presence of other nitration-susceptible species.

The reaction of LA (1 μM) with $ONOO^-$ (0–20 μM) also gave no detectable nitrated species, whereas $ONOO^-$ induced a dose-dependent nitration of (9*Z*,11*E*)-CLA (Fig. 3*g*). The addition of $ONOO^-$ to reaction systems prior to lipid target, to allow for its decay to NO_3^-, did not support CLA nitration (Fig. 3*g*). Notably, all biochemical conditions tested (mitochondria, plasma, cardiac tissue after focal I/R, activated RAW cells, acidic NO_2^-, MPO/H_2O_2/NO_2^-, $ONOO^-$, and pure $^{\bullet}NO_2$ gas) yielded the same NO_2-CLA regioisomers (supplemental Fig. 5). In addition to NO_2-CLA, NO_2-oxo-OA and NO_2-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 $^{\bullet}NO_2$ Gas and the Electrophilic Reactivity of NO_2-CLA—The electrophilic nitroalkene of NO_2-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 NO_2-CLA electrophilicity, reactivity toward β-ME was evaluated (26). NO_2-CLA reacted rapidly, yielding products detected by the neutral loss of β-ME (78 atomic mass units) upon CID fragmentation (reverse Michael addition) to give NO_2-CLA (MRM 402.4/324.2), NO_2-oxo-OA (MRM 418.4/340.3), and [$^{13}C_{18}$]NO_2-LA (MRM 420.4/342.2, Fig. 4*a*). Further structural characterization of the ion *m/z* 340.3 revealed a nitroalkane with an α,β-unsaturated carbonyl that also conferred electrophilic character. No β-ME

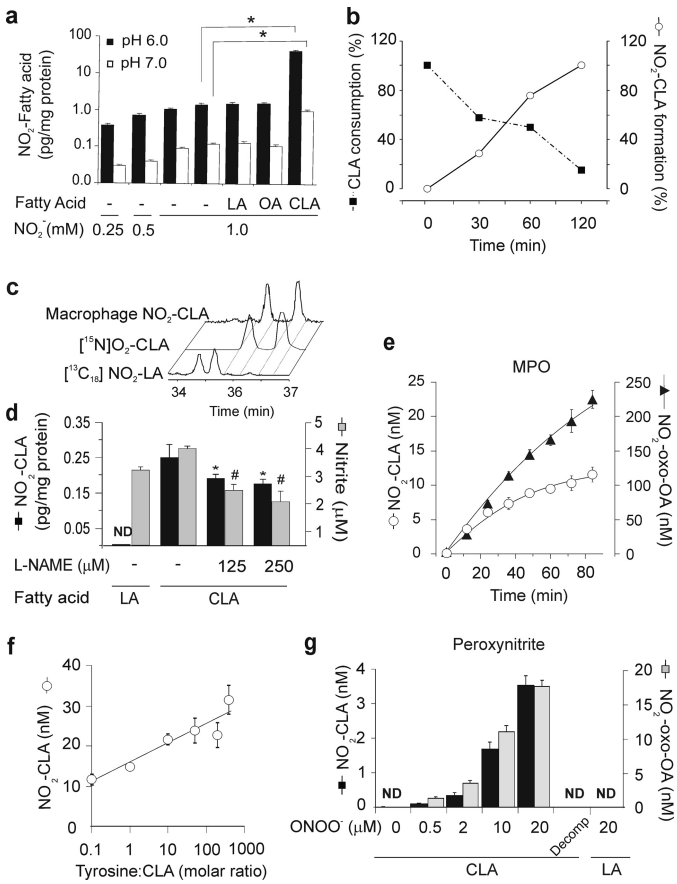
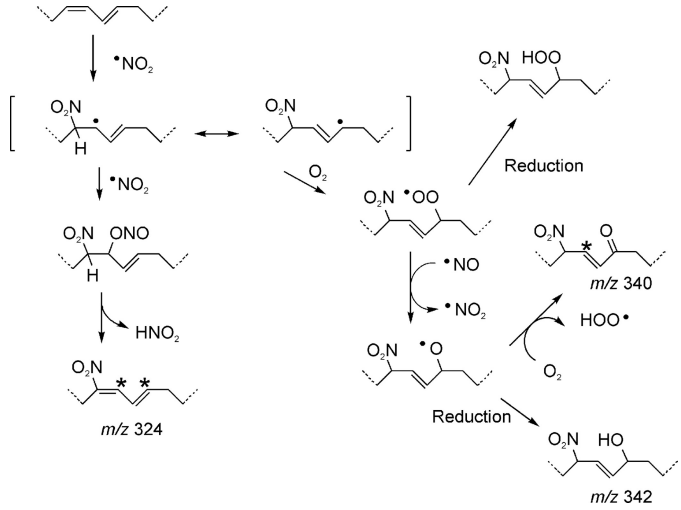


FIGURE 3. **CLA nitration by mitochondria, activated RAW 264.7 macrophages, MPO, and $ONOO^-$.** *a,* mitochondria (2 mg) were incubated in the presence of NO_2^- (0.25–1.0 mM) and supplemented with various fatty acids (1 μM). Lipids were extracted, and NO_2-FA was quantified by HPLC-MS/MS using [$^{13}C_{18}$]NO_2-LA as internal standard. Results represent the mean ± S.D. ($n = 3$), * indicates significantly different ($p < 0.05$) from control without fatty acid addition. *b,* NO_2-CLA formation from endogenous CLA and the concomitant CLA consumption was quantified by HPLC-MS/MS in mitochondria (2 mg) incubated with NO_2^- (1 mM) at pH 6 ($n = 3$). *c,* chromatogram showing formation of NO_2-CLA by activated macrophages in the presence of CLA and internal standards ([^{15}N]O_2-CLA and [$^{13}C_{18}$]NO_2-LA). *d,* incubation with the NOS inhibitor L-NAME decreased NO_2^- (*gray bars*) and NO_2-CLA (*black bars*) levels in media. *e,* CLA (10 μM) nitration induced by MPO/H_2O_2/NO_2^- (30 min). Nitration (NO_2-CLA, *m/z* 324.2) and oxidation (NO_2-oxo-OA, *m/z* 340.3) products were quantified using [$^{13}C_{18}$]NO_2-LA as internal standard. *f,* CLA (10 μM) nitration reactions by MPO were competed using increasing molar ratios of tyrosine (1–400 mol eq, Tyr/CLA). *g,* $ONOO^-$ induced formation of NO_2-CLA (*m/z* 324.2) and NO_2-oxo-OA (*m/z* 340.3). Results represent the mean of three independent experiments ± S.D. *ND,* not detected. *d,* * and # are significantly different from CLA control ($p < 0.05$) in the absence of L-NAME (analysis of variance post hoc Tukey's test).



SCHEME 1. **Reaction mechanism of nitrogen dioxide-induced CLA nitration.** This pathway also depicts the formation of the hydroxy and keto containing NO_2-FA derivatives that can be generated in the presence of oxygen. * denotes electrophilic carbons.

adducts were formed by NO_2-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 NO_2-oxo-OA (*m/z* 340.2), now yielding a β-ME-reactive product (MRM 418.4/340.3) (Fig. 4*b*). Thus, although nitration of CLA generates electrophilic nitroalkenes and α-β-unsaturated ketone, NO_2-OH-OA requires further oxidation to gain electrophilic reactivity.

CLA nitration occurs via $^{\bullet}NO_2$ addition (Scheme 1 and Figs. 1–3), a precept more directly tested by treating CLA and LA in solution with pure $^{\bullet}NO_2$ using air or N_2 as the carrier gas. HPLC-ESI-MS/MS quantification revealed that CLA yielded ~10^5 greater nitration products than LA (1.5 μM NO_2-CLA *versus* 20 pM NO_2-LA formed, see Fig. 4, *c* and *d*). Reactions performed in aerobic conditions produced further oxidized nitration products, including NO_2-oxo-OA (*m/z* 340.3) and NO_2-OH-OA (*m/z* 342.2) in addition to NO_2-CLA (*m/z* 324.2). When reactions were conducted in N_2-saturated conditions, the formation of NO_2-CLA predominated (*m/z* 324, Fig. 4*c*). These data support the formation of a resonance-stabilized radical, upon addition of $^{\bullet}NO_2$ to the flanking carbons of the conjugated diene, that under aerobic conditions can further react with oxygen (Fig. 4*c* and Scheme 1).

Detection of NO_2-CLA in Healthy Human Plasma—The nitration of CLA *in vitro* by diverse inflammatory-related mechanisms motivated clinical investigation of endogenous NO_2-CLA. Analysis by HPLC-MS/MS revealed NO_2-CLA in healthy human plasma, with identification confirmed by comparison with the synthetic [^{15}N]O_2-CLA internal standard (Fig. 5*a*). Endogenous levels of NO_2-CLA in plasma ranged from 0.3 to 1.3 nM (average = 0.72 nM, median = 0.62 nM, $n = 7$, using [^{15}N]O_2-CLA as internal standard) (supplemental Fig. 6). Plasma NO_2-CLA was reactive with β-ME. Upon β-ME addition, the NO_2-CLA MRM elution profile (324.2/46) was lost (Fig. 5*a*, *lower panel*). Accurate mass determination, at resolution of 40,000 (<2 ppm), also confirmed the presence of NO_2-CLA in plasma (Fig. 5*b*, *upper panel*). The nitrated CLA species (9-NO_2-CLA and 12-NO_2-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 NO_2-CLA from plasma CLA was tested by adding 0.5 mM Na[^{15}N]O_2 to plasma under acidic conditions for 30 min (Fig. 5*b*, *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[^{15}N]O_2 (well above physiological level) plus 1 mM hexadiene in plasma showed no artifactual CLA nitration occurred during extraction procedures (supplemental Fig. 7*c*).

Gastric Nitration of CLA—The *ex vivo* acid-catalyzed nitration of CLA suggested that this reaction might occur *in vivo* in

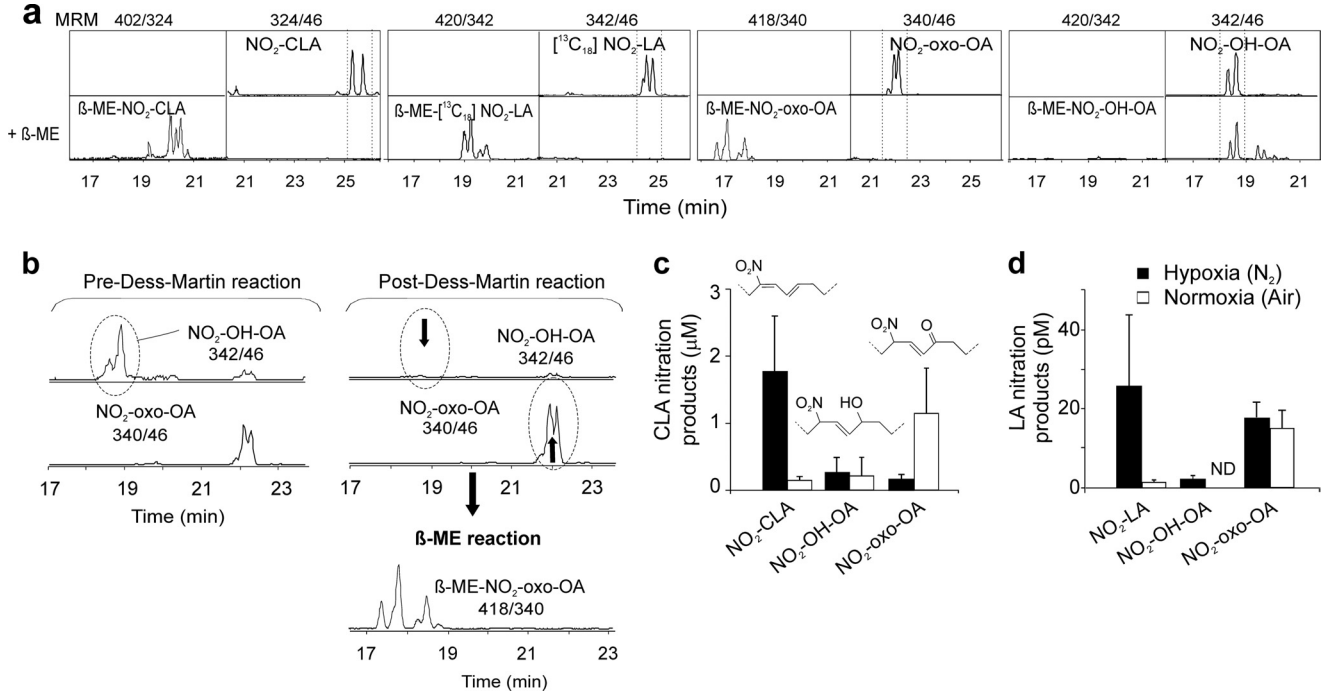


FIGURE 4. **Nitrogen dioxide-mediated nitration of CLA and electrophilic reactivity of NO_2-CLA.** *a,* MS/MS chromatograms of NO_2-CLA, NO_2-OH-OA, and NO_2-oxo-OA pre- and post-β-ME (1 mM) reaction. Chromatograms of β-ME adducted NO_2-FA followed the neutral loss of 78 atomic mass units (β-ME) (β-ME-NO_2-CLA, 402.3/324.2; β-ME-NO_2-oxo-OA, 418.3/340.3; β-ME-[$^{13}C_{18}$]NO_2-LA, 420.3/342.2) and show the characteristic shift in the elution time when compared with pre-β-ME reaction NO_2-FA. NO_2-OH-OA does not display electrophilic reactivity. *b,* Dess Martin reaction (to oxidize hydroxy groups to ketones) of CLA nitration products shows specific oxidation only for NO_2-OH-OA to NO_2-oxo-OA and its subsequent gaining of β-ME reactivity. Product displays the same chromatographic profile as NO_2-oxo-OA shown in *a*. Oxygen tension modulates FA nitration product distribution during reaction with $^{\bullet}NO_2$. CLA (100 μM) (*c*) or LA (100 μM) (*d*) nitration was induced by pure $^{\bullet}NO_2$ gas (5.6 ppm) during 60 min under hypoxia (*black bars*) and normoxia (*white bars*). Products were quantified by HPLC-MS/MS in the presence of [$^{13}C_{18}$]NO_2-LA. *ND*, not detected. Results represent the mean of three independent experiments ± S.D.

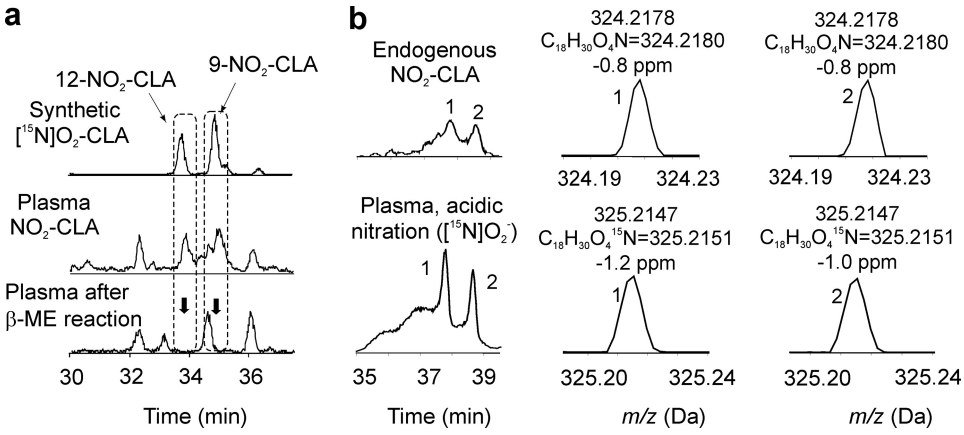
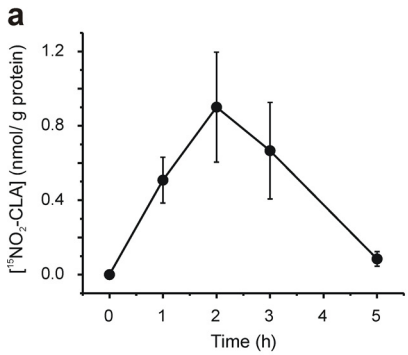


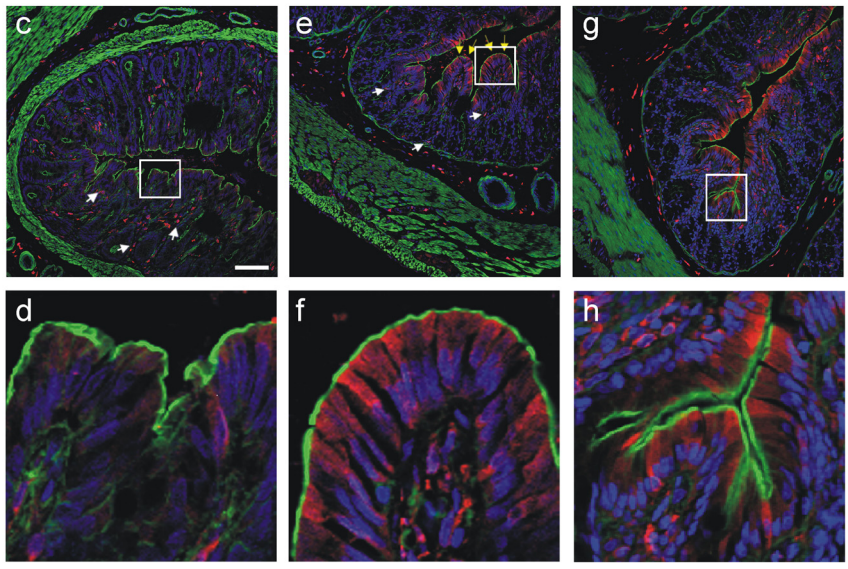
FIGURE 5. **Detection of NO_2-CLA in human plasma.** The presence of NO_2-CLA was identified and quantified using 9-[^{15}N]O_2-CLA and 12-[^{15}N]O_2-CLA as internal standards in plasma from seven healthy subjects. *a,* chromatograms show endogenous NO_2-CLA (324/46, *middle panel*) and internal standard [^{15}N]O_2-CLA (325.3/47, *upper panel*). Confirmation of electrophilic reactivity of the endogenous NO_2-CLA was followed upon β-ME reaction (*lower panel*). *b,* accurate mass determinations (1 ppm level) of endogenous NO_2-CLA and plasma subjected to acidic nitration using Na[^{15}N]O_2 (0.5 mM) were determined for both 9- and 12-NO_2-CLA.

acidic physiological environments such as the gastric compartment. Also, $^{\bullet}NO_2$ generation from NO_2^- 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}N]O_2 (100 nmol of each), resulting in an increase in NO_2-CLA content in stomach tissue that reached a maximum concentration 2 h after gavage (0.9 ± 0.3 nmol of NO_2-CLA/g of tissue) (Fig. 6*a*). When higher doses of CLA and Na[^{15}N]O_2 were given to nonfasting mice (100 and 10 μmol, respectively), 33 ± 22 nmol of NO_2-CLA/g of tissue was detected in the stomach. Increases in NO_2-CLA were also detected in the small intestine and colon at 1.5 ± 1.1 and 1.2 ± 1.7 nmol/g tissue, respectively. All treated animals showed detectable NO_2-CLA levels throughout the gastrointestinal tract, which was absent in control animals and animals gavaged with CLA alone. Upon administration of NO_2^- alone, NO_2-CLA (0.4 ± 0.2 nmol/g stomach) was detected, supporting the presence of significant native CLA and indicating that ingested NO_2^- levels are a factor in the digestive formation of NO_2-CLA.



b

Tissue	[NO_2-CLA] (nmol/mg tissue) Control	[$^{15}NO_2$-CLA] (nmol/mg tissue) Control	[$^{15}NO_2$-CLA] (nmol/mg tissue) CLA + $^{15}NO_2^-$
Stomach	0.065 ± 0.055	ND	27.4 ± 8.5
Intestine	ND	ND	0.63 ± 0.28
Colon	ND	ND	1.65 ± 0.64
Kidney	ND	ND	ND
Liver	ND	ND	0.013 ± 0.004
	[NO_2-CLA] (nM)		[$^{15}NO_2$-CLA] (nM)
Plasma	ND	ND	23.01 ± 11.21
Urine	1.19 ± 0.83	ND	7.32 ± 1.30



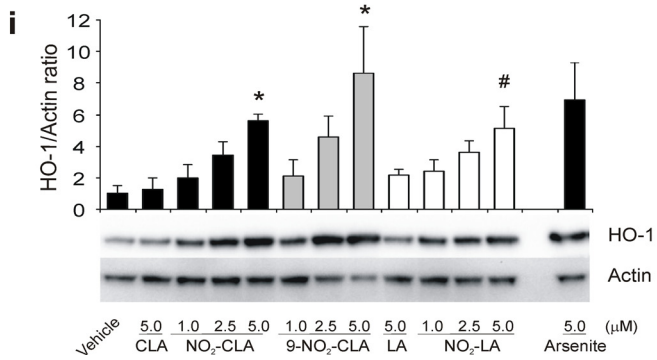


FIGURE 6. **Gastric formation of [^{15}N]O_2-CLA modulates HO-1 expression in gut epithelium and levels in plasma, tissue, and urine.** *a,* fasted mice were injected with pentagastrin (5 mg/kg) 1 h prior to gavage with CLA (100 nmol) and Na[^{15}N]O_2 (100 nmol). Lipids were extracted from stomach tissue and analyzed by HPLC-MS/MS. *b,* rats were fed CLA and Na[^{15}N]O_2 via oral gavage once per day for 4 days (100 μmol each), and urine was collected over the final 24 h of treatment. Extracted lipids from urine and different tissues were analyzed by HPLC-MS/MS, revealing endogenous formation of labeled [^{15}N]O_2-CLA (*m/z* 325/47). *ND*, not detected. *c–h*, gastric generation of NO_2-CLA stimulates expression of HO-1 in rat colon epithelium. Representative images of colon tissue from vehicle controls (*c* and *d*), CLA + NO_2^- (*e* and *f*), and synthetic NO_2-CLA treatment (*g* and *h*) are shown. The actin labeling (*green*) delineates the adventitia and the apical surface of epithelium. The nuclei are shown in *blue* (DAPI staining). *e* and *g* and *f* and *h* (high power magnification), there is intense HO-1 immunoreactivity (*red*) in the subapical cytoplasm of the epithelial cells (*yellow arrowheads*). This labeling is absent in the vehicle-treated control tissue (*c* and *d*). There is some discrete labeling within the lamina propria in both treated and control animals (*white arrows*), which corresponds to labeling that typically occurs in the macrophages and occasional dendritic cells found in this locale. *Bar,* 100 μm. *i,* NO_2-CLA induces HO-1 expression in the mouse macrophage RAW 264.7 cells. Cells were incubated with [^{15}N]O_2-CLA (9- and 12-NO_2-CLA isomer mixture), 9-NO_2-CLA (specific isomer), bis-allylic NO_2-LA, and control native fatty acids (CLA and LA) for 12 h. Results represent the mean of three independent experiments ± S.E.; * and # are significantly different from CLA control ($p < 0.05$) and LA control ($p < 0.05$) (analysis of variance post hoc Tukey's test).

Electrophilic NO_2-oxo-CLA was also detected throughout the gastrointestinal tract of CLA and NO_2^--gavaged mice.

To better evaluate the formation of NO_2-CLA, rats were also treated with CLA and Na[^{15}N]O_2, allowing for greater fluid volumes and tissue mass for the analysis of [^{15}N]O_2-CLA. The gastric formation, absorption, and distribution of [^{15}N]O_2-CLA after gavage with CLA and Na[^{15}N]O_2 (100 μmol each) was detected in the stomach, intestine, colon, plasma, liver, and

urine (Fig. 6*b*). Structural characterization of $[^{15}N]O_2$-CLA in the tissues and urine collected from treated rats showed that these species displayed *m/z* and HPLC retention times identical to synthetic $[^{15}N]O_2$-CLA. Accurate mass determination (*m/z* 325.2150 for $[^{15}N]O_2$-CLA (theoretical 325.2151) and 324.2181 for NO_2-CLA (theoretical 324.2180)) confirmed the atomic composition of these species at the 1 ppm level. The electrophilicity of these products was confirmed by β-ME reactivity (data not shown). These results support that dietary CLA and NO_2^- content can modulate levels of NO_2-CLA in tissues, plasma, and urine.

Colonic Mucosal HO-1 Expression Is Induced by CLA and Nitrite Treatment of Rats—The gastric formation of electrophilic NO_2-CLA motivated the evaluation of whether endogenously generated electrophilic fatty acids can regulate gene expression in tissues. The expression of HO-1, a target gene for NO_2-FA (15, 36), was analyzed by immunofluorescence microscopy. There was a significant increase in HO-1 expression in the cytosol of colonic epithelial cells of rats gavaged with CLA and NO_2^- (Fig. 6, *e* and *f*) as well as synthetic NO_2-CLA (Fig. 6, *g* and *h*), as compared with control rats fed vehicle (Fig. 6, *c* and *d*). Control studies showed that when no primary antibody was used, no staining or autofluorescence of tissue was detectable. No changes in basal HO-1 expression levels were observed in colonic epithelial cells of control rats that were gavaged with CLA or NO_2^- individually and not as a binary mixture. Moreover, the expression of HO-1 was not up-regulated in the lamina propria immune cells (macrophages and dendritic cells) of the different treatment groups. To further confirm that NO_2-CLA mediates signaling actions analogous to those reported for NO_2-LA and NO_2-OA (2, 15), HO-1 expression in RAW 264.7 macrophages was evaluated. Notably, Fig. 3, *c* and *d*, shows that RAW 264.7 macrophages mediate the nitration of CLA. In addition, $[^{15}N]O_2$-CLA (mixed 9- and 12-NO_2-CLA regioisomers) induced a dose- dependent up-regulation of HO-1 expression comparable with that induced by NO_2-LA and 9-NO_2-CLA (Fig. 6*i*).

DISCUSSION

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 $^{\bullet}NO_2$ 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 hydroxyl group, followed by radical rearrangement to a tyrosyl radical intermediate (Tyr$^{\bullet}$) that reacts with $^{\bullet}NO_2$ 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 $MPO/H_2O_2/NO_2^-$, 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$^{\bullet}$ formation *in vivo* and (*b*) differences in the chemical reactivity and compartmentalization of tyrosine and conjugated diene-containing fatty acids.

Nitrated 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 $^{\bullet}NO_2$-induced nitration several orders of magnitude greater than bis-allylic LA (Figs. 3 and 4, *c* and *d*). This preferential reactivity of $^{\bullet}NO_2$ toward CLA originates from the conjugated diene moiety, which in the gas phase is more reactive than simple monoalkenes by a factor of 10^3–10^4 (39). In carbon tetrachloride, the conjugated diene 2,5-dimethyl-2,4-hexadiene is 10^4–10^6 more reactive than monoalkenes (40), reinforcing that there will be a preferential reaction of $^{\bullet}NO_2$ 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 $^{\bullet}NO_2$ 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 $^{\bullet}NO_2$ radical addition, the fate of the acyl chain radical formed upon $^{\bullet}NO_2$ addition is unclear. Possible reactions that could result in carbonyl radical reduction include a radical reaction between a second $^{\bullet}NO_2$ and the allyl radical intermediate through the nitrogen or oxygen atoms of $^{\bullet}NO_2$, 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 NO_2-CLA radical intermediate by $^{\bullet}NO_2$ can generate HNO_2 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 NO_2-OH and NO_2-oxo products. The detection of NO_2-OH-OA (*m/z* 342.2) and NO_2-oxo-OA (*m/z* 340.3) further expands the potential product profiles of fatty acids nitrated by $^{\bullet}NO_2$. In aggregate, these results indicate that conditions where the extent of $^{\bullet}NO$, NO_2^-, and $^{\bullet}NO_2$ 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 $^{\bullet}NO_2$ (44). These include exposure to $^{\bullet}NO_2$ gas, the $O_2^{\bullet-}$-derived inflammatory by-products ONOOH and nitrosoperoxocarbonate ($ONOOCO_2$) (45), and heme protein-catalyzed oxidation of NO_2^- to $^{\bullet}NO_2$ (46). In activated macrophages and rodent hearts subjected to ischemia-reoxygenation, inhibition of nitric-oxide synthase (NOS) activity suppresses fatty acid nitration by ~50% (Fig. 3*d*) (19, 47). Because NO_2^- is a product of $^{\bullet}NO$ autoxidation, the inhibition of fatty acid nitration by NOS inhibitors could be a consequence of the following: (*a*) suppressing the formation of NO_2^- or (*b*) limiting the reaction of $O_2^{\bullet-}$ with $^{\bullet}NO$ and subsequent $ONOO^-$-dependent $^{\bullet}NO_2$ generation.

The protonation of NO_2^- (pK_a 3.4) to nitrous acid (HNO_2) also yields $^{\bullet}NO_2$ from N_2O_3 and N_2O_4 homolysis (48). The acidic conditions of digestion induced the NO_2^--dependent nitration of CLA, leading to increased levels of NO_2-CLA in gastrointestinal tract tissue. The administration of $[^{15}N]O_2^-$ by gavage permitted differentiation from endogenous NO_2-CLA species. After gastric CLA nitration and absorption from the gut, $[^{15}N]O_2$-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 NO_2^- protonation (pK_a 3.4) to HNO_2. 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 NO_2-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 (NO_2^- 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 (NO_3^-) reduction by commensal bacterial nitrate reductases in saliva and the gut (53) and is a product of $^{\bullet}NO$ oxidation. Nitrite serves as an *in vivo* reservoir for $^{\bullet}NO$ 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 NO_2-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 NO_2^- 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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