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Nitric Oxide. 2018 September 01; 79: 38–44. doi:10.1016/j.niox.2018.07.003.**Nitro-fatty acid Formation and Metabolism****Gregory R. Buchan**^{1,*}, **Gustavo Bonacci**^{2,*}, **Marco Fazzari**^{1,3}, **Sonia Salvatore**¹, and **Stacy Gelhaus Wendell**^{1,4}¹Department of Pharmacology & Chemical Biology, University of Pittsburgh, Pittsburgh, PA 15261, USA²CIBICI – CONICET, Departamento de Bioquímica Clínica Facultad de Ciencias Químicas (U.N.C.) Haya de la Torre y Medina Allende Ciudad Universitaria, Córdoba C.P. N°: X5000HUA, República Argentina³Fondazione Ri.MED, Via Bandiera 11, 90133 Palermo, Italy⁴Clinical Translational Science Institute, University of Pittsburgh, Pittsburgh, PA 15261, USA**Abstract**

Nitro-fatty acids (NO₂-FA) are pleiotropic modulators of redox signaling pathways. Their effects on inflammatory signaling have been studied in great detail in cell, animal and clinical models primarily using exogenously administered nitro-oleic acid. While we know a great deal about their signaling, endogenous NO₂-FA formation and metabolism is relatively unexplored. This review will cover what is currently known regarding proposed mechanisms of formation, dietary modulation of endogenous NO₂-FA levels, and pathways of metabolism and detection of NO₂FA and corresponding metabolites.

Keywords

nitric oxide; nitrogen dioxide; nitration; nitro-fatty acid; metabolism; nitro-conjugated linoleic acid; diet

Nitro-Fatty Acid Formation and Metabolism

Metabolism of nitric oxide (*NO) and the production of nitrogen oxides under pathophysiological conditions generates nitrosating (R-NO) and nitrating (R-NO₂) species that react with proteins, unsaturated fatty acids and thiol-containing small molecules such as glutathione (GSH) (1–4). The nitration of unsaturated fatty acids by the radical nitrogen dioxide (*NO₂) generates electrophilic, bioactive lipids that form covalent Michael addition

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adducts with nucleophilic amino acids, predominantly cysteine, found in transcriptional regulatory proteins and enzymes involved in metabolism, cell signaling and redox homeostasis (5,6). Post translational modification (PTM) by NO₂-FA has been demonstrated to lead to profound functional changes (7,8). Because of this, there has been a growing interest over the last decade in the pleiotropic signaling of nitro-fatty acids (NO₂-FA) in numerous disease models that has led to substantial increases in our understanding of the reactive pathways that lead to their formation as well as the complexity of their adsorption, metabolism, and accurate analysis (5). This review will summarize what we know thus far regarding the *in vitro* and *in vivo* formation and metabolism of electrophilic NO₂-FA.

Nitration of fatty acids

Although much is known about the enzymatic and non-enzymatic oxidation of unsaturated fatty acids (9–13), fatty acid nitration is less understood. The structural characteristics of the unsaturated fatty acid define the reaction products and their formation is largely dependent on the differing chemical activities between bis-allylic and conjugated diene systems. Two different mechanisms have been proposed for the nitration of polyunsaturated bis-allylic fatty acids (14). The first mechanism is initiated by radical (*OH, *OOH, *NO₂) hydrogen abstraction from a bis-allylic carbon to yield an alkyl radical (Figure 1A-a). This is a common reaction in the formation of other non-enzymatic lipid oxidation products including isoprostanes and hydroperoxides (11). Hydrogen abstraction is followed by double bond rearrangement and the insertion of molecular oxygen (O₂) to yield a peroxy radical or insertion of *NO₂ to generate the non-electrophilic nitroalkane-alkene product (Figure 1A-b) (15). The second mechanism for bis-allylic polyunsaturated fatty acid nitration is the direct addition of *NO₂ to generate a carbon-centered radical (Figure 1A-c). This radical can be oxidized further with or without *NO₂ to form the electrophilic nitro-alkene (Figure 1A-e, d, f). Reacting with another molecule of *NO₂ can form an unstable nitro-nitrito or dinitro compound that will rapidly decompose (releasing HNO₂) to form an electrophilic nitro-alkene (Figure 1A-f) (16). However, biological or physiological conditions seem to be unfavorable for the *in vivo* generation of electrophilic products from the bis-allylic-containing fatty acids (i.e. linoleic and arachidonic) through the proposed mechanisms (17). While these NO₂-FA species have been observed under a few varying conditions, none have been as extensively characterized and observed as often as nitro-conjugated linoleic acid (NO₂-cLA). Studies consistently show nitration of cLA as the major NO₂-FA formed in murine studies and human clinical trials (17–19).

Endogenous nitro fatty acid formation in animal models

During the last decade, important advances in the knowledge of *in vitro* and *in vivo* lipid nitration have been achieved. In this regard, the first relevant report for the *in vivo* formation of NO₂-FA was in a murine model of focal cardiac ischemia-reperfusion (I/R) (20). In this model, the profile of NO₂-FA detected was attributed to nitration of oleic acid (NO₂-OA) and linoleic acid (NO₂-LA), predominantly the *trans*-NO₂-LA isoform. The formation of NO₂-FA was a consequence of the reoxygenation-induced tissue damage that involves hypoxia, acidification and the formation of reactive oxygen and nitrogen species; a process that was not observed when myocardial ischemia occurred without reperfusion. Similarly, Nadtochiy *et al.* demonstrated the presence of NO₂-FA in the experimental model of

ischemic preconditioning (IPC) in isolated rat hearts; however, the nitration products differed from the nitrated products obtained in the I/R model. Thus, while a partially characterized *trans*-NO₂-LA was identified in cardiac tissue of the I/R model, the *cis*-NO₂-LA conformation was primarily measured in addition to NO₂-OA in the IPC model. Initially, isobaric *cis* or *trans* bis-allylic LA (18:2) were designated as the substrate for *in vivo* nitration reactions; however, using high resolution liquid chromatography mass spectrometry (LC-MS), it was established that nitration preferentially occurs on the isomer of LA characterized by conjugated double bonds (cLA) instead of the bis-allylic methylene group present in LA (17). Thus, initial identification of nitration products were incorrectly identified as *cis*- and *trans*-isomers of LA and the retrospective analysis of the original samples from Rudolph's animal model of myocardial focal I/R confirmed the presence of NO₂-cLA (17). The conjugated dienes (principally 9-*cis*, 11-*trans*-cLA and 10-*trans*, 12-*cis*-cLA isomers) are the main endogenous targets for *in vivo* nitration and display reactivity with [•]NO₂ ~5 orders of magnitude higher than the bis-allylic LA (9-*cis*, 12-*cis*-LA) (17). Both the bis-allylic and conjugated-diene configuration allows for direct addition of [•]NO₂; however, the conjugated system allows for the direct addition at the C-9 or C-12 positions and to a lesser extent at C-10 and C-11 positions, thus generating a delocalized allylic radical that is more stable than its bis-allylic counterpart (21). After [•]NO₂ addition (Figure 1B-a), this radical could follow several mechanisms of reaction; i) oxidation of the radical to reform the conjugated diene system with a vinyl NO₂ group (Figure 1B-b), ii) the addition of a second [•]NO₂ to the allyl radical intermediate, yielding an unstable dinitro or nitro-nitrito product. The nitrito would undergo rapid decomposition (releasing HNO₂) to form a vinyl nitro product (Figure 1B-c), and iii) oxidation of the NO₂-cLA radical intermediate to a nitro-peroxy followed by reduction of the peroxy to an alkoxy radical mediated by the oxidation of [•]NO to [•]NO₂. This intermediate undergoes another round of reduction to the final product, nitro-hydroxy-OA (NO₂-OH-OA) whereas an environment with higher oxygen tension would promote the formation of nitro-oxo-OA (NO₂-oxo-OA) (Figure 1B-d). These species expand the profile of fatty acids nitrated by [•]NO₂ and increases the diversity of molecules that can impact in the biological function of NO₂-FA (17). Oxygenated nitro fatty acids have not been well characterized in animal models or human trials and warrant further investigation.

The biological significance of the cLA findings were tested in a series of *in vitro* and *in vivo* experiments that involved the generation of the nitrating species [•]NO₂ through (a) reaction of [•]NO with superoxide (O₂^{•-}) to yield peroxynitrite (ONOO⁻) and its homolytic cleavage products ([•]NO₂ and [•]OH), (b) the one electron oxidation of nitrite (NO₂⁻) by heme peroxidases (i.e. myeloperoxidase), (c) direct nitration by [•]NO₂ gas, and (d) the acid-catalyzed protonation of NO₂⁻ to HNO₂ followed by its oxidation to [•]NO₂ (22). This complex set of reactions demonstrates a variety of different nitrating pathways, yet [•]NO₂ is the main intermediate in most of these reactions. Nitration of cLA was achieved in the cellular environment through inflammatory activated macrophages and also in the gastric lumen after oral administration of cLA and NO₂⁻ as was reflected by the increase of the endogenous levels in plasma and urine of treated mice (17,23). This mechanism may involve the protonation of NO₂⁻ to HNO₂ and yields the nitrating [•]NO₂ from dinitrogen trioxide (N₂O₃) and dinitrogen tetroxide (N₂O₄) homolysis, a process that is favored by the acidic

condition of gastric compartment leading to nitration of cLA in the gastrointestinal tract (5). These concepts are of significance to other cellular or tissue compartments with acidic pH that can support NO_2^- protonation (pKa 3.4) to HNO_2 such as lysosomes, ischemic or inflammatory tissue and mitochondria (17,24).

NO in nitration and nitrosation reaction.

In this complex scenario where $\text{NO}_2^-/\bullet\text{NO}_2$ participate extensively in the mechanisms of lipid nitration, it is important to note that $\bullet\text{NO}$ does not participate as a direct nitrating species, rather its autoxidation gives rise to the nitrating intermediate $\bullet\text{NO}_2$ (25). In this context, Vitturi and coworkers showed that $\bullet\text{NO}$ was essential for $\bullet\text{NO}_2$ -mediated cLA nitration induced by LPS and $\text{INF}\gamma$ macrophage activation. Incubation of activated macrophages with a selective inhibitor of iNOS (1400W) in the presence of NO_2^- abolished cLA nitration revealing that $\bullet\text{NO}$ is required for cellular NO_2^- -dependent nitration (23). Using an experimental strategy with stable isotopes of nitrogen (^{15}N and ^{14}N) the authors described incorporation of ^{15}N into both $^{15}\text{NO}_2$ -cLA (nitration) and GS^{15}NO (glutathione *S*-nitrosation) as a result of a potential mechanism involving the oxidation of $^{15}\text{NO}_2^-$ to $^{15}\bullet\text{NO}_2$ (Figure 2A, **reactions 1-4**), followed by the reaction of $^{15}\bullet\text{NO}_2$ with $^{14}\bullet\text{NO}$ to generate N_2O_3 containing a mix of ^{15}N and ^{14}N . These are the first findings to show that the formation of symmetrical N_2O_3 is possible. The *sym* N_2O_3 contains two equivalent nitroso groups connected to a central oxygen by identical N-O bonds that generate a random distribution of the ^{15}N or ^{14}N atom giving rise to $^{14}\bullet\text{NO}$ or $^{15}\bullet\text{NO}$, thus accounting for $^{15}\bullet\text{NO}_2^-$ -derived GS^{15}NO formation (Figure 2B). In the case of *asym* N_2O_3 the homolytic scission of the N-N bond would regenerate $^{14}\bullet\text{NO}$ and $^{15}\bullet\text{NO}_2$ that could not explain the formation GS^{15}NO from $^{15}\text{NO}_2^-$. Thus, two mechanisms can be predicted where NO_2^- can generate *sym* N_2O_3 ; a) via oxidation of NO_2^- to $\bullet\text{NO}_2$ followed by reaction with $\bullet\text{NO}$, or b) a direct nucleophilic substitution (Figure 2). Taken together, this report confirms previous findings and describes the concept that $\bullet\text{NO}$ oxidation plays an important role during *in vivo* nitration reactions and that nitration reactions are likely to occur anywhere *S*-nitrosation takes place (23).

These findings become relevant at neutral pH where NO_2^- cannot generate HNO_2 and the formation of nitrating and nitrosating species may be the consequence of metal catalysis. Nitration of cLA was described in a mouse model of acute peritonitis induced by intra-peritoneal injection of LPS and isotopically labeled $^{15}\text{N}^{18}\text{O}_2^-$. In this study NO_2 -cLA was formed by endogenously generated $^{14}\bullet\text{NO}_2$, but the administration of $^{15}\text{N}^{18}\text{O}_2^-$ revealed a dose dependent generation of $^{15}\text{N}^{18}\text{O}_2$ -cLA in addition to a scramble of isotopologues ($^{15}\text{N}^{18}\text{O}^{16}\text{O}$ -cLA, $^{14}\text{N}^{18}\text{O}^{16}\text{O}$ -cLA and $^{15}\text{N}^{16}\text{O}^{16}\text{O}$ -cLA) indicating that endogenously generated $\bullet\text{NO}$ reacts with $^{15}\text{N}^{18}\text{O}_2^-$ to form *sym* N_2O_3 *in vivo* (23). This result highlights a novel role for $\text{NO}_2^-/\bullet\text{NO}_2/\bullet\text{NO}$ system as a precursor of NO_2 -FA and nitrosating intermediates, independent of pH and the presence of metal centers, via the formation of *sym* N_2O_3 and its stochastic homolysis. A deeper understanding of nitroalkene formation in animal models will aid in the clinical development of NO_2 -FA, especially in the context of using dietary supplements of their precursors (i.e. cLA and $\text{NO}_2^-/\text{NO}_3^-$) to drive endogenous formation and evoke their signaling potential (3,5).

NO₂-FA formation in humans

The reactivity, metabolism and distribution of NO₂-FA make accurate quantification inherently challenging. Early reports of *in vivo* levels of NO₂-FA described the detection of a NO₂-FA profile that included nitrated species of palmitoleic (16:1), oleic (18:1), linoleic (18:2), linolenic (18:3), arachidonic (20:4) and eicosapentaenoic (20:5) acids. Also reported for the first time were quantitative values of free NO₂-OA upwards of 600 nM and esterified levels that approximated 300 nM in human plasma (26). Several years later these reports were refuted by Tsikas *et al.* who developed a stable isotope gas chromatography mass spectrometry method to accurately and precisely quantify the 9-NO₂- and 10-NO₂- regioisomers of NO₂-OA in plasma (27). The levels reported were three orders of magnitude lower than the original report by Baker *et al.* (27,28). Based on follow up studies and further investigation we now know these reports, which quantified sub-nanomolar levels of NO₂-OA are more accurate. Additionally, numerous studies investigating fatty acid nitration *in vitro* and in animal models have provided a solid foundation for understanding the nitration of cLA in humans. Under basal metabolic conditions, normal plasma NO₃⁻ levels range from 20-40 μM while NO₂⁻ levels are substantially lower (50-300 nM) (4). Conversion of NO₃⁻ to NO₂⁻ is an insufficient process in mammals as they lack the required nitrate reductases; however, commensal bacteria in the gastrointestinal tract and on body surfaces use NO₃⁻ as an alternative electron acceptor during anaerobic respiration to produce ATP (29). Dietary intake of leafy and root vegetables, and herbs account for 60-80% of total NO₃⁻ intake. Other sources include drinking water (15-20%) and other animal-based products (15-20%) in which NO₃⁻ and NO₂⁻ are used as preservatives (Figure 3) (4,30–33).

Diet is also the main source of cLA where it is found primarily in dairy products and meat and was originally recognized for its metabolic effects on obesity, body composition, insulin sensitivity, and its association with reduced risk of cardiovascular events (34). cLA is a product of microbiome-promoted isomerization of LA to cLA in humans and animals (35) and rumen also have bacteria with ⁹-desaturase activity capable of metabolizing vaccenic acid (*trans*-oleic acid) to cLA (36). The predominant dietary isomer is 9-*cis*, 11-*trans*-cLA whereas commercial preps made from sunflower oil contain a mixture of 9-*cis*, 11-*trans* and 10-*trans*, 12-*cis*-cLA (34). Confirmed now by several studies, cLA is the primary endogenous fatty acid to be nitrated at levels 5 orders of magnitude higher than LA to form NO₂-cLA (17). NO₂-cLA is formed under basal metabolic conditions in humans and rodents. Furthermore, dietary intake or supplement administration can increase NO₂-cLA levels most likely due to the mechanisms of nitration described above (18,37). Nitration of conjugated linolenic acid (cLna, punicic acid, 18:3) also occurs and is a primary fatty acid found in pomegranate (~72%); however, cLna is absorbed at much lower levels in the diet compared to cLA (38) (Figure 3).

To better understand how diet contributes to NO₂-cLA formation and signaling, a pilot study administering oral ¹⁵N-labeled Na¹⁵NO₃⁻ (1 g) and Na¹⁵NO₂⁻ (20 mg) without (Trial 1) and with cLA (3 g, Trial 2) was conducted in healthy volunteers (18,39). Volunteers were randomized to either NO₃⁻ or NO₂⁻ (Trial 1) and after a ~7 day washout period subjects received the opposite nitrogen oxide. In Trial 2 cLA supplementation was introduced concurrently with NO₃⁻ or NO₂⁻ supplementation. The endogenous ¹⁴NO₂-cLA and ¹⁵NO₂-

cLA derived from the dietary supplementation, along with corresponding metabolites, were measured in plasma and urine by LC-MS. In Trial 1, $^{15}\text{NO}_2\text{-cLA}$ was only detected in plasma at the 24 hr time point after $^{15}\text{NO}_3^-$ administration. These results indicated that cLA is rate limiting and its simultaneous presence in the gastric compartment along with NO_3^- or NO_2^- is required for the formation of $\text{NO}_2\text{-cLA}$. Hence, in Trial 2, simultaneous cLA supplementation led to the formation of $^{15}\text{NO}_2\text{-cLA}$ after both $^{15}\text{NO}_3^-$ and $^{15}\text{NO}_2^-$ administration at time points earlier than in Trial 1. $^{15}\text{NO}_2^- + \text{cLA}$ resulted in detectable, sustained levels of $^{15}\text{NO}_2\text{-cLA}$ from 1 to 6 hr after supplementation where as $^{15}\text{NO}_2\text{-cLA}$ was detected 2 hr after $^{15}\text{NO}_3^- + \text{cLA}$ supplementation and levels continued to increase over the 24 hr period. Formation of $^{15}\text{NO}_2\text{-cLA}$ after $^{15}\text{NO}_2^- + \text{cLA}$ supplementation tracked with peak levels of free cLA in plasma. Urinary cysteine adducts of $^{14}\text{NO}_2\text{-cLA}$, $^{15}\text{NO}_2\text{-cLA}$ and β -oxidation metabolites were also measured, thus confirming the electrophilic reactivity of the parent molecules and metabolites (18).

In this study the clinical responses were investigated to explore the impact of dietary lipid on nitrogen oxide metabolism and cardiovascular responses. Dietary supplementation of $^{15}\text{NO}_3^-$ and $^{15}\text{NO}_2^-$ support $^{15}\text{*NO}$ formation as measured by electroparamagnetic resonance spectroscopy detection of ^{15}NO -deoxyhemoglobin complexes. $^{15}\text{*NO}$ formation resulted in decreased systolic and mean arterial blood pressures and inhibited platelet function in Trial 1. Co-administration of cLA in Trial 2 reduced plasma NO_3^- and NO_2^- levels, decreased $^{15}\text{*NO}$ -deoxyhemoglobin formation, NO_2^- inhibition of platelet activation, and the vasodilatory properties of NO_2^- while enhancing the formation of $^{15}\text{NO}_2\text{-cLA}$. Thus the co-administration of the dietary supplement, cLA, significantly redirects cardiac responses attributed to $\text{NO}_2^-/\text{*NO}$ to alternate pathways (39).

The discovery of endogenous $\text{NO}_2\text{-cLA}$ formation in humans under basal conditions and its elevated formation and signaling during inflammation and metabolic stress expands the pharmacokinetics and scope of *NO signaling beyond guanylate cyclase-dependent cGMP production. Although mechanisms of $\text{NO}_2\text{-cLA}$ formation and signaling have been investigated *in vitro* and in animal models and to some extent now in humans, there is still much we do not know. Further studies are warranted to investigate how diet, precursor supplementation, and the microbiome influence $\text{NO}_2\text{-cLA}$ formation and signaling compared to the administration of the pre-formed $\text{NO}_2\text{-FA}$ as the chemistry and pathway targets of $\text{NO}_3^-/\text{NO}_2^- + \text{cLA}$ and $\text{NO}_2\text{-cLA}$ will greatly vary.

$\text{NO}_2\text{-FA}$ metabolism and distribution

The formation, metabolism, distribution and excretion of $\text{NO}_2\text{-FA}$ comprise a metabolic network capable of regulating steady state levels and signaling activity of these molecules under both basal and pathological conditions (Figure 4). The detection and measurement of $\text{NO}_2\text{-FA}$ and metabolites is inherently challenging because they form reversible covalent adducts with nucleophilic amino acids in proteins and cysteines in thiol containing molecules, such as GSH. Additionally as fatty acid species, they bind fatty acid binding pockets of carrier proteins like albumin (40) and participate in normal lipid metabolism and transport as they undergo saturation, β -oxidation, and are esterified into complex lipids (41–44). A complete knowledge of their metabolism will be required to fully understand their

role as novel therapeutics for a variety of inflammatory diseases and recent studies are beginning to unravel their pharmacokinetic profile.

Similar to other fatty acids, saturation and β -oxidation products as well as NO₂-FA-Coenzyme A conjugates (NO₂-FA-CoA) have been detected and well characterized by LC-MS/MS (44). Many PK studies have utilized NO₂-OA as a model NO₂-FA since it has one double bond, thus making metabolism easier to characterize; although, increasing evidence suggests NO₂-cLA metabolism follows a very similar pattern. The predominant free fatty acid species resulting from NO₂-OA metabolism is nitro-stearic acid (NO₂-SA) (44). NO₂-SA is a non-electrophilic nitroalkane formed from the saturation of the electrophilic double bond of NO₂-OA by prostaglandin reductase 1 (PTGR1) (45). NO₂-OA and NO₂-SA can undergo several rounds of β -oxidation to form dinor (C16), tetranor (C14) and hexanor (C12) species. Similar metabolites have been described for NO₂-cLA and these species have been detected in animal models and human plasma and urine under basal conditions and after supplementation (37). Other oxidized and nitrated products (*e.g.* nitro-nitrate, hydroxyl-NO₂-FA) have been observed in various studies, but have not been explored in detail (46–48) (Figure 4).

A recent study gave an even more detailed report of NO₂-FA metabolism and discovered taurine and sulfo- adducts along with ω -oxidation products as evidenced by dicarboxylic metabolites of reduced and electrophilic NO₂-FA (49). Many of these products are found in both rodents and humans. NO₂-FA readily form adducts with GSH and these adducts are exported from cells by multi-drug resistance transporters (50,51) and metabolites of GSH adduction are detected in urine as NO₂-FA adducts of N-acetylcysteine and cysteine (37,49). These products are in equilibrium with free NO₂-FA and corresponding metabolites in urine and can be readily displaced by thiol reaction with mercury chloride, increasing the free NO₂-FA pool by an order of magnitude (37) (Figure 4).

The intricacies of NO₂-FA metabolism and distribution are slowly being unraveled, as the incorporation of NO₂-FA into complex lipids in cells and *in vivo* have been reported (Figure 4). The formation of NO₂-FA-containing triglycerides (TG) have been analyzed during *in vitro* acidic gastric digestion of TG and in adipocytes and rat plasma after NO₂-OA supplementation (41) whereas endogenously formed NO₂-FA and nitro-oxidized-containing phospholipids were detected in isolated cardiac mitochondria and cardiomyoblasts in a type 1 diabetes mellitus animal model (48). These data demonstrate that NO₂-FA-containing complex lipids can be generated after gastric digestion and inflammatory conditions.

An exhaustive quantitative body distribution analysis of radiolabeled [¹⁴C]-10-NO₂-OA has been reported after oral administration in rats. Upon absorption, radioactivity was mainly distributed in kidney, liver, lungs, and heart, while long lasting accumulation was detected in brown and white adipose tissue over two weeks (42). However, the radioactivity measured in tissues by autoradiography could result from the [¹⁴C]-10-NO₂-OA or its ¹⁴C-containing metabolites. To better understand NO₂-OA metabolism and incorporation profiling into complex lipids, fractionated lipid classes of adipocytes and murine adipose tissue supplemented with NO₂-OA were hydrolyzed and analyzed by HPLC-MS/MS. Interestingly, it was demonstrated that non-electrophilic NO₂-OA metabolites, such as NO₂-SA, were

preferentially esterified into TGs, while NO₂-OA was incorporated into monoglycerides (MG) and diglycerides (DG) to a larger extent. This trend was further confirmed by supplementing adipocytes with NO₂-cLA, NO₂-LnA and NO₂-SA. In this regard, the differential incorporation into MG+DG and TG of NO₂-FA may be a consequence of their cellular availability, trafficking, and metabolism. In fact, the free intracellular pool of unsaturated NO₂-FA is influenced by the electrophilic reactivity toward GSH and the formation of NO₂-FA-GSH adducts, which are exported extracellularly by the multi-drug resistance proteins (50,52). In addition, intracellular NO₂-FA are metabolized by tissue-specific PTGR1 and long-chain acyl-CoA synthetase (ACSL) isoforms, generating saturated NO₂-FA and NO₂-FA-CoA species, respectively. Of note, ACSL isoforms have shown FA preferences (53) and could differentially esterify NO₂-FA to form the CoA derivatives. Furthermore, the resultant NO₂-FA-CoA may be preferentially used by different enzymes to generate phospholipids and glycerolipids. Then, the esterification of unsaturated NO₂-FA into MG+DG fractions may generate a depot of potentially bioactive lipids, which could store and release electrophilic signaling mediators following an inflammatory response. Instead, the incorporation of saturated NO₂-FA into TG fractions confirms their role as depot of inactive non-electrophilic metabolites. Esterified NO₂-OA was ~18× more abundant in MG + DG vs. TG. The profile for NO₂-cLA was similar to NO₂-OA; however, overall esterification was 10-20% lower. Overall there were lower levels of incorporation into phospholipids compared to glycerolipids (41). These data demonstrate that NO₂-FA and metabolites are distributed and incorporated into complex lipids, which can release electrophilic species after lipase hydrolysis and be delivered to remote tissue as lipoproteins, thus modulating cell homeostasis and tissue signaling.

Conclusions and Future Studies

The mechanisms of fatty acid nitration *in vivo* remain unclear; however, during recent years important advances have been made to elucidate the molecular intermediaries involved in nitration, its molecular targets and the resulting reaction products. Confirmation of cLA as the primary endogenous NO₂-FA, correct measurement of free endogenous NO₂-OA as well as new analyses of NO₂-FA incorporation into complex lipids have helped shape the overall picture of NO₂-FA formation and have provided better insight into the pharmacokinetics and pharmacodynamics of these pleiotropic anti-inflammatory therapies. Analysis of excreted radioactivity and metabolites in urine showed that 10-NO₂-OA is extensively absorbed and rapidly metabolized within 24 hr (49). Future studies focused on NO₂-FA intestinal absorption and the evaluation of hepatic (first-pass) metabolism is required as they need to solve the discrepancy between initial reports of NO₂-OA bioavailability (~6%) (unpublished data) and the 35% urinary recovery reported in the [¹⁴C]-NO₂-OA rat study (49). It may be that a significant portion of NO₂-FA is stored in complex lipids and distributed throughout various tissues. These studies along with further characterization of novel NO₂-FA metabolites and the investigation of next generation NO₂-FA derivatives (54) as therapeutics will continue to expand our knowledge of the formation and metabolism of this pleiotropic class of therapeutic signaling molecules.

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Highlights

- Nitro-Fatty Acid (NO₂-FA) formation is highly dependent on environmental factors including pH, oxygen tension and the presence of reactive species.
- NO₂-cLA is the most abundant, endogenous NO₂-FA.
- NO₂-cLA is generated during metabolism and under conditions of inflammatory stress.
- Absolute measurement of NO₂-FA is complicated by protein adduction, metabolism (reduction, β-oxidation, conjugation), and incorporation into complex lipids.

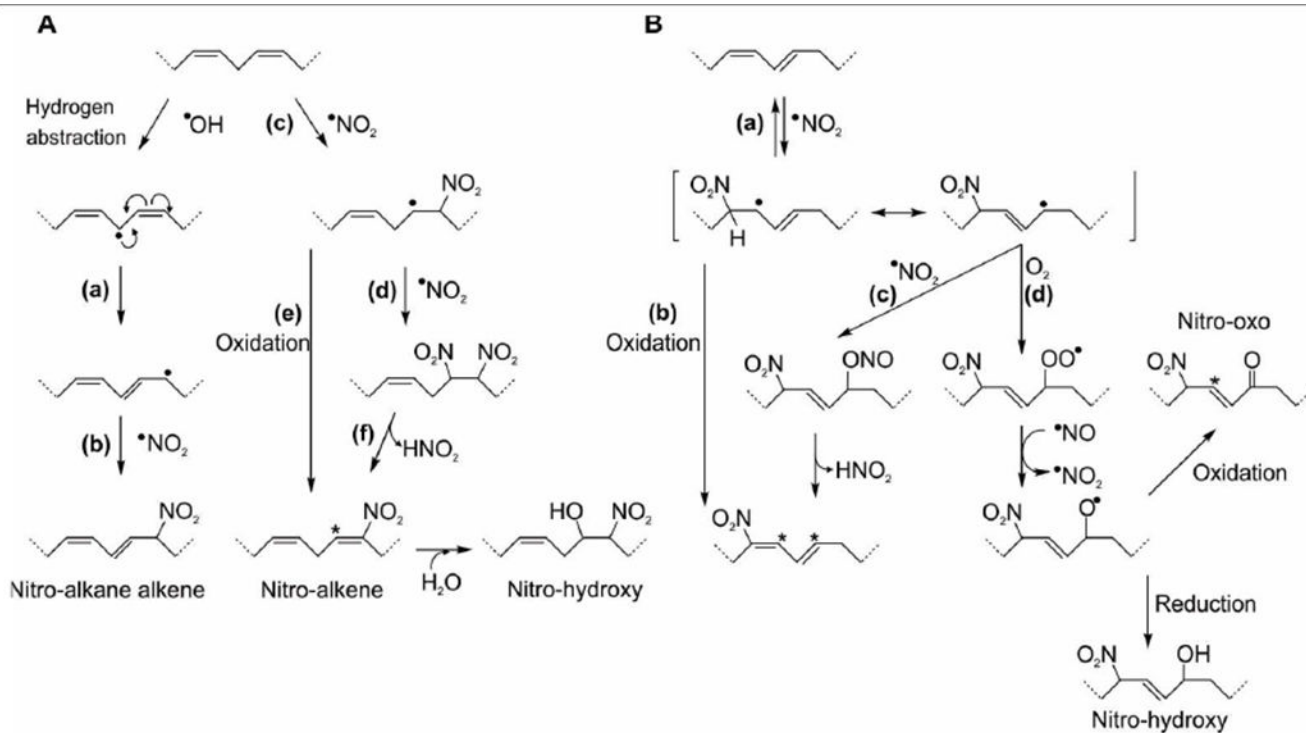


Figure 1: Proposed mechanisms of NO_2 -FA formation.

(A) NO_2 -FA formation from bis-allylic unsaturated fatty acids. (B) Nitration of conjugated linoleic acid. Asterisk indicate reactive electrophilic carbons.

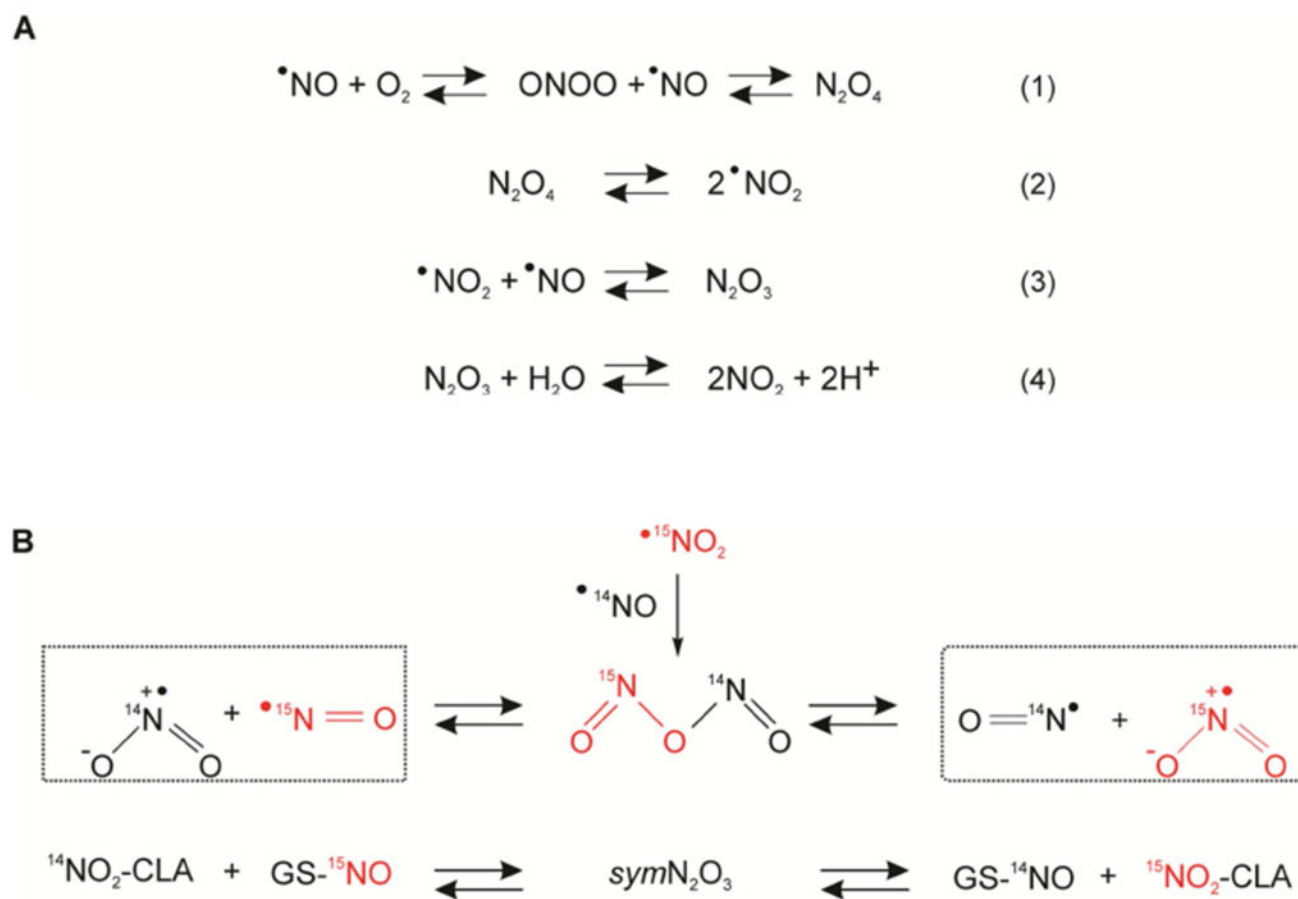


Figure 2: Generation of symN₂O₃.

(A) From NO₂⁻ oxidation to form [•]NO₂ followed by reaction with [•]NO (B) or direct nucleophilic substitution.

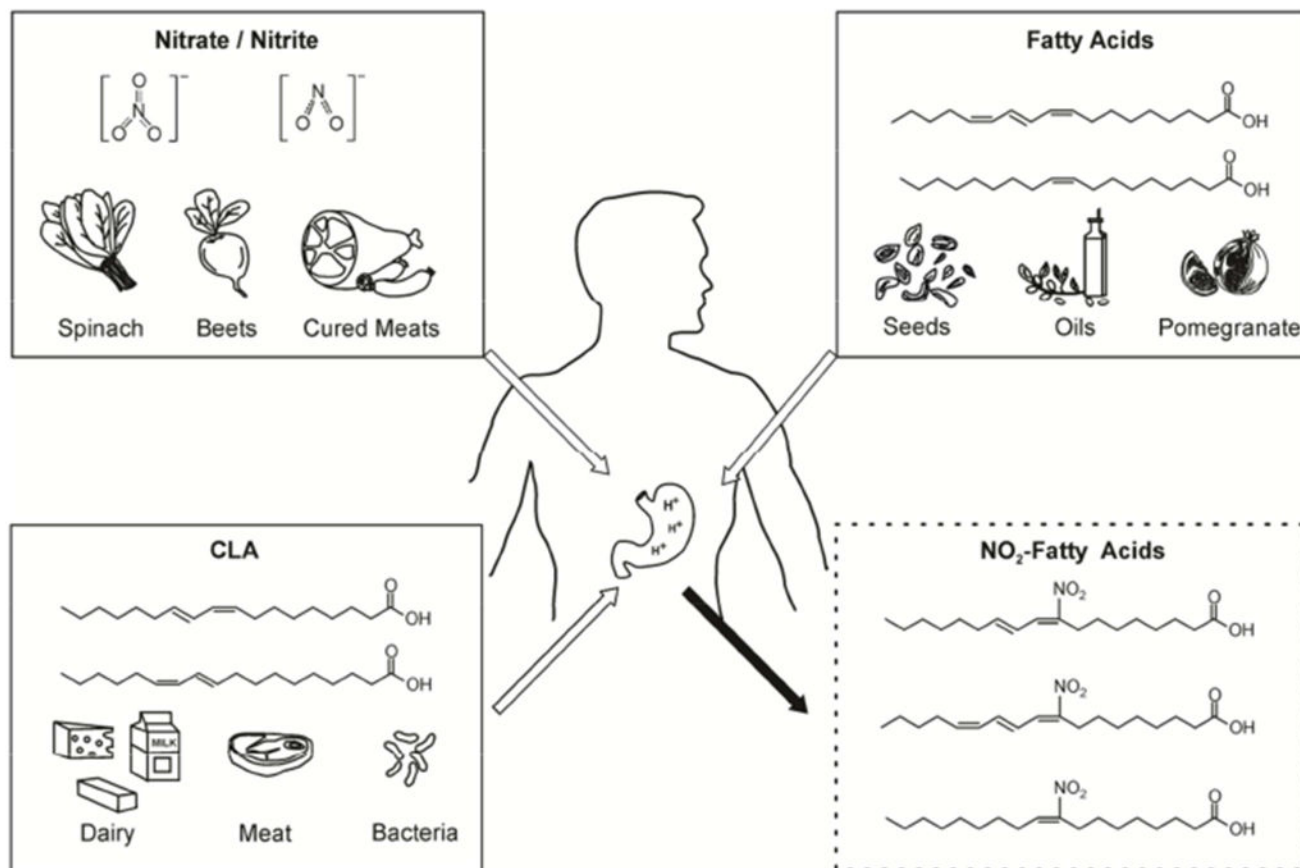


Figure 3: Dietary intake alters endogenous NO₂-FA formation.

Nitrate (NO₃⁻), nitrite (NO₂⁻) and fatty acids such as oleic (OA, 18:1), conjugated linoleic (cLA, 18:2) and linolenic (cLnA, 18:3) acid are found in dietary sources including vegetables, seeds and oils, meat and dairy. Further metabolism of NO₃⁻ and NO₂⁻ results in multiple nitrogen oxides including nitrogen dioxide (*NO₂). Fatty acids most susceptible to nitration by *NO₂ are those with a conjugated diene structure. Due to its abundance in the diet, cLA is the predominant endogenous fatty acid that is nitrated to form NO₂-cLA.

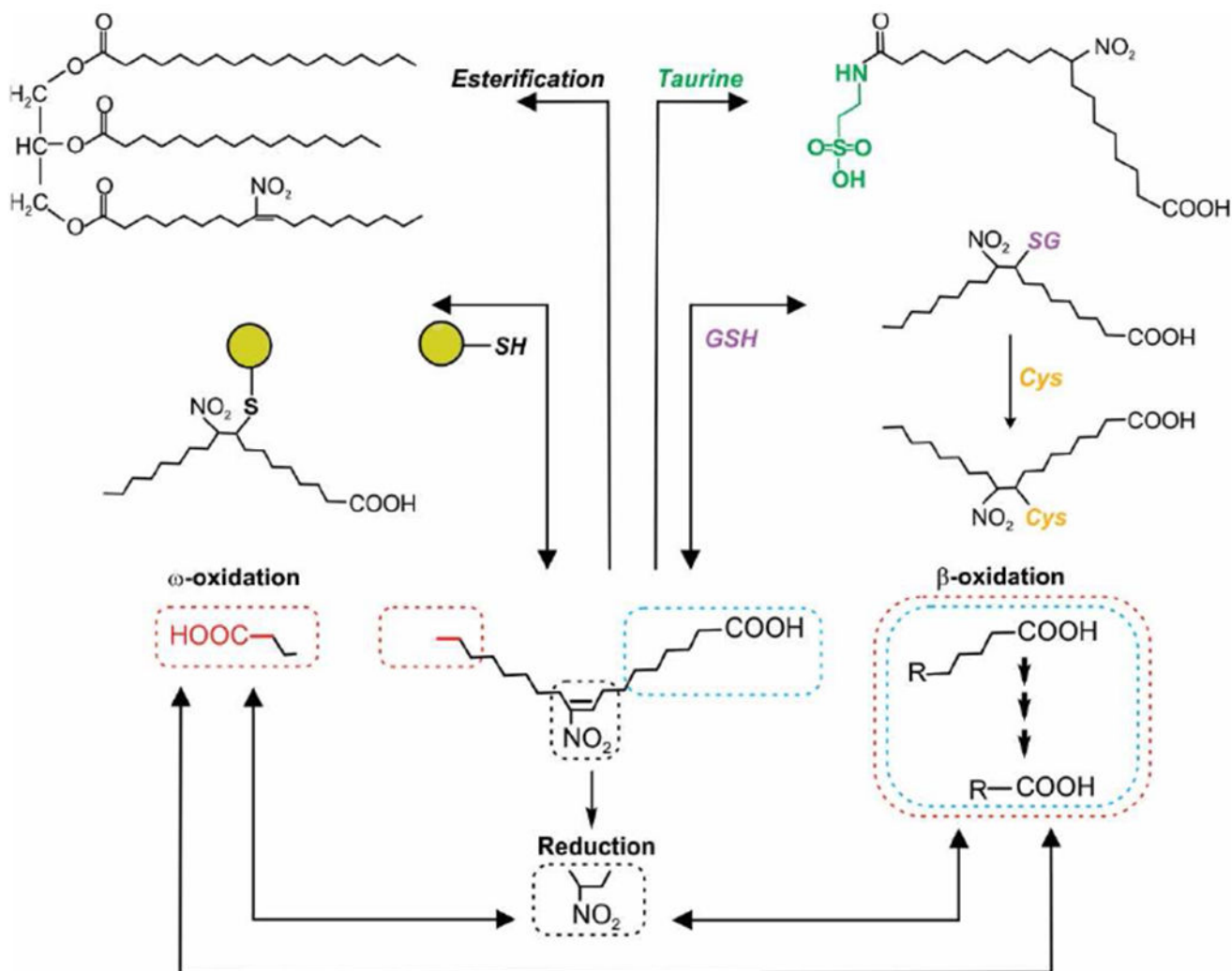


Figure 4: Routes of NO₂-FA metabolism.

NO₂-FA form reversible covalent adducts with reactive thiols and can also be incorporated into complex lipids. Free species of NO₂-FA and metabolites are detected in plasma, tissue and urine. NO₂-FA are reduced to non-electrophilic nitroalkanes by prostaglandin reductase and both nitroalkanes and alkenes undergo β-oxidation. In urine dicarboxylic acids resulting from ω oxidation were found as well as taurine and GSH derived conjugates.