

This is an Open Access document downloaded from ORCA, Cardiff University's institutional repository: <https://orca.cardiff.ac.uk/id/eprint/111610/>

This is the author's version of a work that was submitted to / accepted for publication.

Citation for final published version:

Freeman, Bruce A., O'Donnell, Valerie B. and Schopfer, Francisco J. 2018. The discovery of nitro-fatty acids as products of metabolic and inflammatory reactions and mediators of adaptive cell signaling. *Nitric Oxide: Biology and Chemistry* 77 , pp. 106-111. 10.1016/j.niox.2018.05.002

Publishers page: <http://dx.doi.org/10.1016/j.niox.2018.05.002>

Please note:

Changes made as a result of publishing processes such as copy-editing, formatting and page numbers may not be reflected in this version. For the definitive version of this publication, please refer to the published source. You are advised to consult the publisher's version if you wish to cite this paper.

This version is being made available in accordance with publisher policies. See <http://orca.cf.ac.uk/policies.html> for usage policies. Copyright and moral rights for publications made available in ORCA are retained by the copyright holders.



The Discovery of Nitro-Fatty Acids as Products of Metabolic and Inflammatory Reactions and Mediators of Adaptive Cell Signaling

Bruce A. Freeman^{1*}, Valerie B. O'Donnell² and Francisco J. Schopfer^{1*}

¹ Department of Pharmacology and Chemical Biology, University of Pittsburgh, Pittsburgh, PA, USA;

² Systems Immunity Research Institute and Division of Infection and Immunity, Cardiff University, Cardiff, UK.

Running title: *Nitro-fatty acid discovery and signaling actions*

* **Correspondence:** Bruce A. Freeman (Email: freerad@pitt.edu), Francisco J. Schopfer (Email: fjs2@pitt.edu)

Keywords: Nitroalkene, nitro-fatty acid, nitric oxide, nitrite, peroxynitrite, eicosanoid, prostaglandin, signaling, proliferation, fibrosis, inflammation, transcriptional regulation

ABSTRACT

Foundational advances in eicosanoid signaling, the free radical biology of oxygen and nitric oxide and mass spectrometry all converged to enable the discovery of nitrated unsaturated fatty acids. Due to the unique biochemical characteristics of fatty acid nitroalkenes, these species undergo rapid and reversible Michael addition of biological nucleophiles such as cysteine, leading to the post-translational modification of low molecular weight and protein thiols. This capability has led to the present understanding that nitro-fatty acid reaction with the alkylation-sensitive cysteine proteome leads to physiologically-beneficial alterations in transcriptional regulatory protein function, gene expression and in vivo rodent model responses to metabolic and inflammatory stress. These findings motivated the preclinical and clinical development of nitro-fatty acids as new drug candidates for treating acute and chronic metabolic and inflammatory disorders.

Highlights

- **The discovery of nitro-fatty acids (NO₂-FA) was motivated by new insight into the biochemistry of nitration reactions and the signaling actions of oxidized fatty acids.**
- **Four main areas of discovery supported the notion that NO₂-FA serve as mediators of physiological homeostasis and in pure form as pharmacologically-active agents:**
 - **The reversible reaction of electrophilic nitro-fatty acids with cysteine and the central role of this reaction in modulating key signaling and gene expression responses.**
 - **The identification of conjugated diene-containing fatty acids such as conjugated linoleic acid as main substrate for nitration.**
 - **The digestive and inflammatory formation of NO₂-FA in humans and rodents.**
 - **The protective anti-inflammatory and anti-fibrotic actions of NO₂-FA in a wide range of preclinical animal models of metabolic and inflammatory disease.**

Introduction

Three foundational discoveries helped direct us down the experimental pathway leading to the discovery of fatty acid nitroalkene derivatives (nitro-fatty acids, NO₂-FA). Prostaglandins and leukotrienes were identified as unsaturated fatty acid oxygenation products that mediate receptor-dependent regulation of inflammation, metabolism, and vascular function (1). Prior to this discovery, fatty acids and complex lipids were typically viewed as sources of metabolic energy and structural constituents of membranes, rather than as substrates for signaling mediator biosynthesis. At about the same time, seminal discoveries that led to the fields of free radical biology and redox signaling were being made. Specifically, the generation of superoxide (O₂^{•-}), hydrogen peroxide (H₂O₂) and other oxygen-derived species was identified in microbes, plants, fish and mammals, along with the existence of small molecule and enzymatic antioxidant networks that regulate cell levels of these oxidizing species (2). This led to the rapid acceptance of “reactive oxygen species” as mediators of xenobiotic toxicity and host defense and later, as cell signaling mediators. Finally, the free radical gas nitric oxide (•NO) was identified as a product of nitric oxide synthase-catalyzed arginine oxidative deamination and the roles of •NO as a mediator of endothelial-dependent vascular relaxation and neurotransmission were described (3). With this critical perspective in mind, we discovered that convergent reactions of unsaturated fatty acids and reactive species derived from oxygen, nitric oxide and nitrite (NO₂⁻) yield a family of chemically-reactive products that mediate pleiotropic metabolic and anti-inflammatory signaling actions. Moreover, synthetic homologs of NO₂-FA may have pharmacologic utility, as present data indicates oral bioavailability, good pharmacokinetics, signaling pathway engagement and a promising safety profile in model systems and humans. There were a number of critical steps that had to be taken and pitfalls to overcome in this process of discovery:

1. Overcoming an initial bias that •NO-derived reactive species primarily mediated pro-inflammatory and pathogenic oxidation and nitration reactions.

We had discovered that the toxicity of O₂^{•-} and •NO could be transduced by peroxynitrite (ONOO⁻), the product of their radical-radical reaction, and by the product of ONOO⁻ and carbon dioxide (CO₂) reaction, nitrosoperoxocarbonate (ONOOCO₂). These nitrogen oxides can react directly or rapidly undergo homolytic scission (as ONOOH) to yield nitrogen dioxide (•NO₂), hydroxyl radical (•OH) and, in the case of ONOOCO₂, carbonate radical (CO₃^{•-})(4-7). We also had discovered that the neutrophil-derived heme protein myeloperoxidase (MPO), upon degranulation and reaction with H₂O₂, catalytically consumes •NO and further oxidizes NO₂⁻ to the nitrating species nitrogen dioxide (•NO₂)(8-10). Because of the facile ability of MPO to generate •NO₂ during inflammatory responses and the unique ability of MPO to become anatomically “locked” in place by high affinity glycosaminoglycan binding, we also discovered a strong spatial co-distribution between MPO and nitrated biomolecules (11,12). More recently, we demonstrated that during •NO autooxidation, NO₂⁻ directly participates in fatty acid nitration reactions at neutral pH via the formation of symmetrical dinitrogen trioxide (ONONO)(13). These reactions, all operative in cell and murine models of inflammation and clinical observations, support the physiological relevance of these diverse mechanisms of nitro-oxidative stress (13,14).

2. How we overcame bias and preconceived notions.

In testing the concept that $\cdot\text{NO}$ exacerbates oxidative inflammatory responses in more biologically-relevant model systems, we evaluated biochemical, cellular and *in vivo* responses to the co-generation of $\text{O}_2^{\cdot-}$, H_2O_2 and $\cdot\text{NO}$. It was first observed that $\cdot\text{NO}$ more potently inhibited the oxidation of membranes and plasma lipoproteins than α -tocopherol (15,16). We then extended these studies to more biological model systems by showing that elevated rates of $\cdot\text{NO}$ biosynthesis or the introduction of $\cdot\text{NO}$ donors led to protection of pulmonary and vascular cells having elevated rates of $\text{O}_2^{\cdot-}$ and H_2O_2 generation. Similarly, rodents inhaling 95% oxygen (thus enhancing rates of pulmonary $\text{O}_2^{\cdot-}$ and H_2O_2 generation) were protected from pulmonary oxygen toxicity by the introduction of 8 ppm $\cdot\text{NO}$, a concentration of inhaled $\cdot\text{NO}$ that is within the range of that used clinically to treat pulmonary hypertension (17-19). In these studies, anti-inflammatory, antioxidant and tissue-protective responses prevailed that were contrary to dogma at the time regarding the biochemical effects of $\cdot\text{NO}$ during oxidative inflammatory reactions (20).

3. New perspective was gained regarding the tissue-protective and anti-inflammatory actions of $\cdot\text{NO}$ during oxidative-stress.

The antioxidant actions of $\cdot\text{NO}$ were first ascribed to its kinetically rapid reaction with lipid peroxy radicals, thus terminating autocatalytic free radical-mediated chain propagation reactions (15,16,21). It had become apparent that, *depending on concentration and the nature of the local free radical milieu, the reactions of $\cdot\text{NO}$ could promote both pro-inflammatory and anti-inflammatory responses.* This was exemplified in a biochemical reaction system where rates of $\cdot\text{NO}$ introduction and enzymatic $\text{O}_2^{\cdot-}$ and H_2O_2 generation were varied inversely (15). The continuous variation of the $\cdot\text{NO}/\text{O}_2^{\cdot-}$ ratios showed that when $\cdot\text{NO}$ concentrations exceeded those of $\text{O}_2^{\cdot-}$ and consequent $\text{ONOO}^-/\text{ONOOH}$ formation, lipid peroxidation was inhibited. The HPLC-MS/MS analysis of the different reaction conditions in this study also gave the first mass spectra showing the nitration of unsaturated fatty acids by oxidative inflammatory conditions. Further studies of linoleic acid reaction with ONOO^- , $\cdot\text{NO}_2$, NO_2^+ or $\text{NO}_2^-/\text{HONO}$ also revealed both linoleate oxidation and nitration products (22,23). Previously, photochemical air pollution-related studies of gaseous nitrogen dioxide ($\cdot\text{NO}_2$) reaction with fatty acids and phospholipids had also shown the formation of nitration products (24-26). Prior to appreciating that NO_2 -FA induce cell signaling responses via the PTM of nucleophilic protein targets, additional understanding of the chemical reactions that led to unsaturated fatty acid nitration was acquired (27-30). The fact that nitroalkene-containing hydrocarbons, released at high pressure by a termite soldier gland, act as a termite chemical warfare armament for establishing turf domain also suggested that fatty acid nitroalkenes might have some unique reactivities (31).

4. Nitric oxide and its secondary products were observed to regulate lipid signaling by modulating the enzymatically-catalyzed oxygenation of unsaturated fatty acids.

The small molecular radius, lipophilicity and free radical character of $\cdot\text{NO}$ all contribute to the broad range of actions that both $\cdot\text{NO}$ and its secondary nitrogen oxides will exert on the oxidative generation of bioactive unsaturated fatty acid products. These effects have been extensively reviewed and include the regulation of the gene expression and changes in the catalytic activities and oxygenated lipid product profiles of cyclooxygenase-1 and -2, multiple lipoxygenases, CYP450s and soluble epoxide hydrolase (32-34). When catalyzing fatty acid oxidation, cyclooxygenase-1 and -2 and lipoxygenases were observed to catalytically consume

\cdot NO and impair downstream cGMP-dependent signaling actions (10,35-37). Moreover, electrophilic NO₂-FA species inhibit cyclooxygenase and lipoxygenase catalysis and gene expression (38,39). These observations affirmed to us that there is a very strong and diverse array of biochemical linkages between lipid and \cdot NO signaling.

5. The organic synthesis of nitro-oleic, nitro-linoleic and nitro-arachidonic acid provided the key to unlocking the analytical, biochemical and pharmacological characteristics of nitro-fatty acids.

The characterization of nitration products of unsaturated fatty acids in model system reactions prioritized the first NO₂-FA to be synthesized. This was first accomplished by a selenium-catalyzed nitration reaction that gave mixed regioisomers of linoleic and oleic acid nitroalkenes (40-42). Later, the synthesis of specific nitro-oleic acid regioisomers by the Henry nitro-aldol reaction further facilitated the discovery of the pleiotropic signaling actions of NO₂-FA and the definition of structure-function relationships in the responses of signaling networks to different fatty acid nitroalkene derivatives (43-49). Moreover, these synthetic approaches allowed the synthesis of isotopically-labeled NO₂-FA (¹³C, ¹⁵N and ¹⁸O), permitting the development of HPLC-based isotopic dilution mass spectrometry methods. Overall, these capabilities and reagents were crucial for defining the endogenous generation, tissue levels, metabolism, and signaling actions of this new class of mediators (22,43,44,50-54). Nonetheless, some mistakes and incorrect assumptions were made in early studies of the structure and concentrations of NO₂-FA species in biological systems (15,50). These analytical challenges were similar to the those faced in other studies of electrophilic fatty acid derivatives, for example 15-deoxy-prostaglandin J₂ (15d-PGJ₂), 4-hydroxy-2-nonenal and α,β -unsaturated fatty acid ketone derivatives, when trying to establish their endogenous tissue and plasma levels, with net tissue concentrations of these species still remaining controversial. The later development of improved sample preparation, chromatographic separations, and more refined mass spectrometric analyses improved NO₂-FA structural and concentration determinations (51,55-63). Also, better understanding of the bond scission mechanisms operative in mass spectrometer-based fragmentation studies permitted the definition of NO₂-conjugated linoleic acid as the principal nitrated fatty acid regioisomer present in cellular models of inflammation, and endogenously in both animal models and humans (13,14,55-57,64). The availability of solid analytical approaches and critical reagents for studying lipid nitroalkene pharmacology were crucial for obtaining consistent results between investigators and labs focused on defining the mechanisms of NO₂-FA generation, metabolism and how these species impact cell and organ function. As discussed in the translational chapter of this series, these goals were accomplished in parallel with the acquisition of intellectual property protection, studies of NO₂-FA actions in preclinical models of inflammatory and metabolic diseases and the attainment of investment for supporting new drug development activities. Importantly, freely sharing reagents, standards, analytical expertise and ideas with colleagues was crucial for correcting mistakes, improving methods, replicating results and advancing understanding.

6. Critical issues and novel insights regarding the unique nature of NO₂-FA as endogenous mediators and new drug candidates.

Do NO₂-FA generate \cdot NO? We initially viewed that NO₂-FA might serve as an endogenous reserve of \cdot NO that would be formed by metabolic and inflammatory reactions, and that after decay or metabolism would subsequently mediate cGMP-dependent vascular relaxation. To

this extent, we and others have documented very low stoichiometric levels of $\cdot\text{NO}$ release by $\text{NO}_2\text{-FA}$ under aqueous conditions (40,44,65-70). While the mechanism of $\cdot\text{NO}$ release is still a matter of debate (via a modified Nef reaction or a rearrangement to a nitrite ester and N-O bond homolysis), these potential reactions are inhibited in membranes and in the presence of plasma constituents such as protein and lipoproteins (44,65,70). Further lines of evidence coming from more biologically relevant systems such as rodent model and clinical studies also do not support the occurrence of $\cdot\text{NO}$ -mediated, cGMP-dependent signaling actions of $\text{NO}_2\text{-FA}$. For example, acute intravenous infusion of low to high concentrations of $\text{NO}_2\text{-FA}$ does not affect blood pressure or heart rate in rodents, dogs and humans (71). Still, there are other biochemical and cellular studies that still suggest that fatty acid nitroalkenes yield $\cdot\text{NO}$. We view that additional experimental evidence is needed, that must be supported by the reactions of ^{15}N -labeled $\text{NO}_2\text{-FA}$ and the subsequent detection of $^{15}\text{NO}_2^-$ and/or ^{15}NO . These approaches are important for eliminating the confounding effects of adventitious NO_2^- , contamination and the concomitant modulation of cellular or *in vivo* sources of $\cdot\text{NO}$ generation. In the latter regard, $\text{NO}_2\text{-FA}$ a) induce endothelial nitric oxide synthase gene expression and catalytic activity and b) promote the upregulation of multiple antioxidant mechanisms that will “preserve” $\cdot\text{NO}$ by limiting its oxidative inactivation and the subsequent generation of secondary nitrogen oxides.(72)

$\text{NO}_2\text{-FA}$ displays a unique pharmacology that is dissimilar from $\cdot\text{NO}$. In the early 2000s, two cell biological studies documented non-cGMP-dependent inhibition of platelet and neutrophil function by nitro-linoleic acid (73,74). This work significantly transformed our understanding of the mechanisms accounting for the potential signaling actions $\text{NO}_2\text{-FA}$ signaling. Both of these studies revealed that, in the absence of cytotoxicity, $\text{NO}_2\text{-FA}$ induced novel anti-inflammatory signaling actions at very low concentrations. This data affirmed that there were effects of $\text{NO}_2\text{-FA}$ on calcium homeostasis and cAMP/adenyl cyclase signaling that occurred in the absence of transcriptional responses and reactions that could be attributable to $\cdot\text{NO}$ /cGMP.

Michael acceptor properties – a crucial mechanism of action underlying $\text{NO}_2\text{-FA}$ signaling. The cGMP-independent signaling actions of $\text{NO}_2\text{-FA}$ pointed to non-canonical signaling actions being defined by the nitroalkene group of $\text{NO}_2\text{-FA}$. This functionality confers unique electrophilic character to the β -carbon of nitro-activated alkenes. *The $-\text{NO}_2$ substituent is one of the most electron-withdrawing moieties in chemistry, thus when bonded to an alkene it promotes a kinetically rapid and reversible $\text{NO}_2\text{-FA}$ Michael addition with cysteine (Cys), a reaction that induces the post-translational modification (PTM) of proteins and potentially alters target protein structure and function (59,75,76).* These biochemical characteristics (*kinetically rapid and reversible*) differentiate $\text{NO}_2\text{-FA}$ from most other endogenous signaling electrophiles such as cyclopentanone prostaglandins and aldehydic lipid oxidation products (e.g., 15d-PGJ₂ and 4-hydroxy-2-nonenal). These non-nitrated lipid electrophiles, while potentially abundant, react slowly with nucleophiles, do not readily β -eliminate and can form irreversible Schiff's base products. Unlike fatty acid cyclopentenone and lipid aldehyde derivatives, $\text{NO}_2\text{-FA}$ will not accumulate as thiol addition products or promote toxicity at low concentrations. The irreversible reaction of many Michael adducts under biological conditions can be a main cause of cell toxicity (77). Current model systems, preclinical pharmacokinetics and toxicology studies and Phase 1 safety evaluation in healthy and obese individuals continue to support that $\text{NO}_2\text{-FA}$ such as 10-nitro-oleic acid is safe in humans at pharmacologically-active doses. A single exception to this generalization comes from the topical application of $\text{NO}_2\text{-FA}$ in a model of

allergic contact dermatitis. While oral and subcutaneous NO₂-FA administration inhibits dermal responses to hapten-induced inflammation, the topical administration of solvated NO₂-FA results in a sustained neutrophil-dependent inflammation (78,79). Even though the inflammatory milieu of psoriasis in humans actually increases dermal production and levels of NO₂-FA, it appears that the high local epithelial concentrations that would result from dermal administration of solvated NO₂-FA induces pro-inflammatory responses.

NO₂-FA detection approaches evolved in response to new understanding of NO₂-FA electrophilic character and metabolism. HPLC-MS/MS was crucial for resolving and detecting fatty acids having NO₂ substituents. Early bioanalysis of NO₂-FA was occasionally complicated by acid-catalyzed nitration occurring by the formation of nitrous acid from the protonation of NO₂⁻ to nitrous acid (HNO₂) during lipid extractions and de-esterification reactions, leading to further generation of NO₂-FA. We learned that one could control for this artifact by including ¹⁵NO₂⁻ during tissue handling and extractions, with the formation of ¹⁵NO₂-FA indicating unwanted processing-induced nitration. Upon realizing that these species were electrophilic, new strategies were developed for “fishing out” protein-adducted NO₂-FA for MS-based structural and quantitative analysis. Three techniques have been particularly revealing. The first involves addition of a high concentration of β-mercaptoethanol to cell preparations and tissue homogenates to force the β-elimination of adducted NO₂-FA and reaction with excess β-mercaptoethanol, yielding higher molecular weight adducts that would yield a fragment ion of m/z 78. A second approach is the addition of HgCl₂ to biological fluid or tissue samples. Since NO₂-FA form reversible adducts with thiols, after an “off reaction” (β-elimination of NO₂-FA from a thiol) Hg will react with the free thiol formed by NO₂-FA dissociation, resulting in the inhibition of subsequent thiol reactions. Overall, this results in a net increase in detectable “free” NO₂-FA and still-electrophilic NO₂-FA metabolites. The chemistry of this reaction has not been studied in depth and direct interactions of Hg with the NO₂-FA adduct should not be excluded. Applying this approach to healthy human urine, the Hg-induced NO₂-FA displacement strategy increases detectable “free” NO₂-FA levels by 10-20 fold (57). More recently, the clinical administration of ¹⁵N-labeled nitrite and nitrate has convincingly shown that fatty acid nitration occurs in humans during digestion (**Fig. 1**) (80,81). This observation in turn motivated the proposal that the cardiovascular benefits of a Mediterranean-like diet, rich in both vegetable-derived NO₂⁻ and NO₃⁻ and marine or olive oil-derived unsaturated fatty acids, could be (in part) ascribed to increased NO₂-FA generation and the downstream elevation of vasoactive and anti-inflammatory lipid mediators (82).

In intracellular environments, where the concentration of low molecular weight and protein thiols can exceed 15 mM, the concentration of free NO₂-FA is expected to be below 1% of total available NO₂-FA, given the calculated equilibrium constants (83). This underscores the need to identify the specific protein targets of these bioactive electrophilic lipids. While biotin derivatives of the carboxylate of NO₂-FA were initially used, these suffer from increased bulkiness, the intrinsic problem of esterase cleavage and disruption of potential CoA ester formation, potentially impairing detection and altering intracellular distribution and pharmacokinetics. To address this, ω-terminal azido derivatives of NO₂-FA were synthesized for click chemistry-based definition of protein targets. Finally, NO₂-FA alkylation increases HPLC retention times of modified peptides, allowing for more facile target identification and providing an alternative mass spectrometry-based approach for target protein determinations (84).

NO₂-FA reaction with the cysteine proteome regulates protein expression by the electrophile-responsive genome. Affinity labeling and mass spectrometry studies have identified susceptible NO₂-FA protein targets and now focus has been placed on understanding the biochemical and cellular consequences of NO₂-FA-protein reactions. Current perspective holds that *transient post-translational modification (PTM) of hyperreactive protein thiols by NO₂-FA modulates signaling pathways involved in cell proliferation and inflammatory responses*(85). This occurs as a result of the alkylation of functionally-significant Cys residues in transcriptional regulatory proteins, including the Kelch-like ECH-associated protein-1 (Keap1) regulator of nuclear factor (erythroid-derived-2)-like 2 (Nrf2) signaling, the nuclear lipid receptor peroxisome proliferator-activated receptor γ (PPAR γ), heat shock factor-1 (HSF-1) and NF- κ B (48,86,87). Of relevance to the anti-inflammatory actions of NO₂-FA is the potent and multifaceted inhibition of NF- κ B-mediated signaling, as demonstrated in diverse cell and murine models of cancer, metabolic syndrome, cardiopulmonary disease and both acute and chronic renal disease (87-90). Unbiased gene expression response studies in human vascular endothelial and smooth muscle cells affirm the broad and pleiotropic modulation of adaptive cell responses by NO₂-FA that would be anticipated from the PTM of key transcriptional regulatory proteins (91,92). Functional enrichment analysis of differentially expressed genes reveals multiple cellular processes will be affected, including cell proliferation, lipid metabolism, antioxidant and inflammatory-related gene expression responses.

NO₂-FA esterification in complex lipids. Since the initial demonstration of increased cholesterol nitrooleate levels in post-prandial human plasma, further advances related to the characterization and quantitation of esterified NO₂-FA have been scarce (53,61). These analyses are complicated by the instability of NO₂-FA in most hydrolysis conditions, ion suppression by native complex lipids during direct mass spectrometric analysis and the complexity of the different molecular species expected from various esters of NO₂-FA in phospholipids, glycerides and sterols. Fortunately, recent advances in the detection and characterization of esterified NO₂-FA have been made. While phospholipid analysis has not yet been translated into *in vivo* measurements, the presence of NO₂-FA containing triglycerides has been reported in animal models supplemented with NO₂-FA (93,94). Of importance, triglycerides are an important storage depot and distribution mechanism for remote organ deposition of NO₂-FA. In this regard, the hydrophobic environment of membranes, lipid droplets, and lipoproteins abrogates any nitroalkene reactivity with thiols, even those present in small molecules such as β -mercaptoethanol, cysteine and glutathione. In addition to these accessibility limitations for Michael addition by esterified NO₂-FA, current data indicates that NO₂-FA also do not add to thiolates in organic solvents, supporting that acid-base chemistry for NO₂-FA nucleophile reactivity.

7. Conclusions

The study of interactions between [•]NO-derived species and lipid peroxidation intermediates motivated the discovery of a functionally unique class of endogenous mediators (lipid nitroalkenes). The unique biochemical qualities of the electrophilic nitroalkene substituent prompted the evaluation of downstream signaling responses, the acquisition of better understanding of the pharmacokinetics and potential toxicity of orally and intravenously-administered NO₂-FA and the *in vivo* testing of NO₂-FA pharmacology in preclinical models of metabolic and inflammatory disease. Moreover, the detection of fatty acid nitroalkenes in plants

and their linkage with plant stress responses expands the scope of actions of this class of mediators. These data, reinforced by the observation that NO₂-FA are endogenously-generated during digestion, inflammatory responses and metabolic stress, has motivated their evaluation as therapeutic agents for treating pathogenic cell proliferation, metabolic and inflammatory-related diseases in humans.

Declaration of interest

BAF, VOD and FJS acknowledge financial interest in Complexa Inc.

Acknowledgments

This article was written when we were supported by the following agencies, NIH R37HL058115, P01-HL103455, R01-HL132550 (BAF), Wellcome Trust (094143/Z/10/Z), British Heart Foundation (RG/12/11/29815), European Research Council (VBOD), and R01-GM125944, American Heart Association 17GRN33660955 (FJS). We also gratefully acknowledge the contributions of the many innovative and dedicated colleagues to this area of discovery and development, many of whom are cited below in this abbreviated reference list.

References

1. Hamberg, M., and Samuelsson, B. (1967) On the mechanism of the biosynthesis of prostaglandins E-1 and F-1-alpha. *The Journal of biological chemistry* **242**, 5336-5343
2. McCord, J. M., and Fridovich, I. (1969) Superoxide dismutase. An enzymic function for erythrocyte protein (hemocuprein). *J. Biol. Chem.* **244**, 6049-6055
3. Ignarro, L. J., Buga, G. M., Wood, K. S., Byrns, R. E., and Chaudhuri, G. (1987) Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. U.S.A* **84**, 9265-9269
4. Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., and Freeman, B. A. (1990) Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. U.S.A* **87**, 1620-1624
5. Radi, R., Beckman, J. S., Bush, K. M., and Freeman, B. A. (1991) Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. *J. Biol. Chem.* **266**, 4244-4250
6. Radi, R., Beckman, J. S., Bush, K. M., and Freeman, B. A. (1991) Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. *Arch. Biochem. Biophys.* **288**, 481-487
7. Denicola, A., Freeman, B. A., Trujillo, M., and Radi, R. (1996) Peroxynitrite reaction with carbon dioxide/bicarbonate: kinetics and influence on peroxynitrite-mediated oxidations. *Arch. Biochem. Biophys.* **333**, 49-58
8. Eiserich, J. P., Hristova, M., Cross, C. E., Jones, A. D., Freeman, B. A., Halliwell, B., and Van der Vliet, A. (1998) Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature* **391**, 393-397
9. Eiserich, J. P., Baldus, S., Brennan, M. L., Ma, W., Zhang, C., Tousson, A., Castro, L., Luscis, A. J., Nauseef, W. M., White, C. R., and Freeman, B. A. (2002) Myeloperoxidase, a leukocyte-derived vascular NO oxidase. *Science* **296**, 2391-2394
10. Clark, S. R., Coffey, M. J., Maclean, R. M., Collins, P. W., Lewis, M. J., Cross, A. R., and O'Donnell, V. B. (2002) Characterization of nitric oxide consumption pathways by normal, chronic granulomatous disease and myeloperoxidase-deficient human neutrophils. *J Immunol* **169**, 5889-5896
11. Baldus, S., Eiserich, J. P., Mani, A., Castro, L., Figueroa, M., Chumley, P., Ma, W., Tousson, A., White, C. R., Bullard, D. C., Brennan, M. L., Luscis, A. J., Moore, K. P., and Freeman, B. A. (2001) Endothelial transcytosis of myeloperoxidase confers specificity to vascular ECM proteins as targets of tyrosine nitration. *J Clin Invest* **108**, 1759-1770
12. Baldus, S., Eiserich, J. P., Brennan, M. L., Jackson, R. M., Alexander, C. B., and Freeman, B. A. (2002) Spatial mapping of pulmonary and vascular nitrotyrosine reveals the pivotal

- role of myeloperoxidase as a catalyst for tyrosine nitration in inflammatory diseases. *Free radical biology & medicine* **33**, 1010
13. Vitturi, D. A., Minarrieta, L., Salvatore, S. R., Postlethwait, E. M., Fazzari, M., Ferrer-Sueta, G., Lancaster, J. R., Jr., Freeman, B. A., and Schopfer, F. J. (2015) Convergence of biological nitration and nitrosation via symmetrical nitrous anhydride. *Nature chemical biology* **11**, 504-510
 14. Villacorta, L., Minarrieta, L., Salvatore, S. R., Khoo, N. K., Rom, O., Gao, Z., Berman, R. C., Jobbagy, S., Li, L., Woodcock, S. R., Chen, Y. E., Freeman, B. A., Ferreira, A. M., Schopfer, F. J., and Vitturi, D. A. (2018) In situ generation, metabolism and immunomodulatory signaling actions of nitro-conjugated linoleic acid in a murine model of inflammation. *Redox biology* **15**, 522-531
 15. Rubbo, H., Radi, R., Trujillo, M., Telleri, R., Kalyanaraman, B., Barnes, S., Kirk, M., and Freeman, B. A. (1994) Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation. Formation of novel nitrogen-containing oxidized lipid derivatives. *J. Biol. Chem.* **269**, 26066-26075
 16. O'Donnell, V. B., Chumley, P. H., Hogg, N., Bloodsworth, A., Darley-Usmar, V. M., and Freeman, B. A. (1997) Nitric oxide inhibition of lipid peroxidation: kinetics of reaction with lipid peroxy radicals and comparison with alpha-tocopherol. *Biochemistry* **36**, 15216-15223
 17. Freeman, B. A., and Crapo, J. D. (1981) Hyperoxia increases oxygen radical production in rat lungs and lung mitochondria. *J. Biol. Chem.* **256**, 10986-10992
 18. Gutierrez, H. H., Nieves, B., Chumley, P., Rivera, A., and Freeman, B. A. (1996) Nitric oxide regulation of superoxide-dependent lung injury: oxidant-protective actions of endogenously produced and exogenously administered nitric oxide. *Free Radic Biol Med* **21**, 43-52
 19. Hill, N. S., Preston, I. R., and Roberts, K. E. (2015) Inhaled Therapies for Pulmonary Hypertension. *Respir Care* **60**, 794-802; discussion 802-795
 20. Darley-Usmar, V. M., Patel, R. P., O'Donnell, V. B., Freeman, B. A., and Ignarro, L. J. (2000) Antioxidant actions of nitric oxide. in *Nitric oxide : biology and chemistry*, Academic Press. pp 265-276
 21. Rubbo, H., Parthasarathy, S., Barnes, S., Kirk, M., Kalyanaraman, B., and Freeman, B. A. (1995) Nitric oxide inhibition of lipoxygenase-dependent liposome and low-density lipoprotein oxidation: termination of radical chain propagation reactions and formation of nitrogen-containing oxidized lipid derivatives. *Arch. Biochem. Biophys.* **324**, 15-25
 22. O'Donnell, V. B., Eiserich, J. P., Chumley, P. H., Jablonsky, M. J., Krishna, N. R., Kirk, M., Barnes, S., Darley-Usmar, V. M., and Freeman, B. A. (1999) Nitration of unsaturated fatty acids by nitric oxide-derived reactive nitrogen species peroxynitrite, nitrous acid, nitrogen dioxide, and nitronium ion. *Chemical research in toxicology* **12**, 83-92

23. O'Donnell, V. B., Eiserich, J. P., Bloodsworth, A., Chumley, P. H., Kirk, M., Barnes, S., Darley-USmar, V. M., and Freeman, B. A. (1999) Nitration of unsaturated fatty acids by nitric oxide-derived reactive species. *Methods in enzymology* **301**, 454-470
24. Kobayashi, T. (1983) The reaction of nitrogen dioxide with lung surface components: The reaction with cis-9-octadecenoic acid. *Chemosphere* **12**, 1317-1325
25. Pryor, W. A., and Lightsey, J. W. (1981) Mechanisms of nitrogen dioxide reactions: initiation of lipid peroxidation and the production of nitrous Acid. *Science* **214**, 435-437
26. Finlayson-Pitts, B. J., Sweetman, L. L., and Weissbart, B. (1987) A Fourier transform infrared spectrometry study of the reactions of phosphatidylcholines with gaseous N₂O₅ and NO₂. *Toxicol Appl Pharmacol* **89**, 438-448
27. d'Ischia, M., Rega, N., and Barone, V. (1999) Medium-dependent competitive pathways in the reactions of polyunsaturated fatty acids with nitric oxide in the presence of oxygen. Structural characterisation of nitration products and a theoretical insight. *Tetrahedron* **55**, 9297-9308
28. d'Ischia, M. (1996) Oxygen-dependent nitration of ethyl linoleate with nitric oxide. *Tetrahedron Lett* **37**, 5773-5774
29. Napolitano, A., Camera, E., Picardo, M., and d'Ischia, M. (2000) Acid-promoted reactions of ethyl linoleate with nitrite ions: Formation and structural characterization of isomeric nitroalkene, nitrohydroxy, and novel 3-nitro-1,5-hexadiene and 1,5-dinitro-1,3-pentadiene products. *J. Org. Chem.* **65**, 4853-4860
30. Napolitano, A., Crescenzi, O., Camera, E., Giudicianni, I., Picardo, M., and d'Ischia, M. (2002) The acid-promoted reaction of ethyl linoleate with nitrite. New insights from N-15-labelling and peculiar reactivity of a model skipped diene. *Tetrahedron* **58**, 5061-5067
31. Spanton, S. G., and Prestwich, G. D. (1981) Chemical self-defense by termite workers: prevention of autotoxication in two rhinotermitids. *Science* **214**, 1363-1365
32. Bloodsworth, A., O'Donnell, V. B., and Freeman, B. A. (2000) Nitric oxide regulation of free radical- and enzyme-mediated lipid and lipoprotein oxidation. *Arterioscler Thromb Vasc Biol* **20**, 1707-1715
33. O'Donnell, V. B., and Freeman, B. A. (2001) Interactions between nitric oxide and lipid oxidation pathways: implications for vascular disease. *Circulation research* **88**, 12-21
34. Baker, P. R., Schopfer, F. J., O'Donnell, V. B., and Freeman, B. A. (2009) Convergence of nitric oxide and lipid signaling: anti-inflammatory nitro-fatty acids. *Free radical biology & medicine* **46**, 989-1003
35. Anning, P. B., Coles, B., Morton, J., Wang, H., Uddin, J., Morrow, J. D., Dey, S. K., Marnett, L. J., and O'Donnell, V. B. (2006) Nitric oxide deficiency promotes vascular side effects of cyclooxygenase inhibitors. *Blood* **108**, 4059-4062

36. Coffey, M. J., Natarajan, R., Chumley, P. H., Coles, B., Thimmalapura, P. R., Nowell, M., Kuhn, H., Lewis, M. J., Freeman, B. A., and O'Donnell, V. B. (2001) Catalytic consumption of nitric oxide by 12/15- lipoxygenase: inhibition of monocyte soluble guanylate cyclase activation. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 8006-8011
37. O'Donnell, V. B., Taylor, K. B., Parthasarathy, S., Kuhn, H., Koesling, D., Friebe, A., Bloodsworth, A., Darley-Usmar, V. M., and Freeman, B. A. (1999) 15-Lipoxygenase catalytically consumes nitric oxide and impairs activation of guanylate cyclase. *Journal of Biological Chemistry* **274**, 20083-20091
38. Trostchansky, A., Bonilla, L., Thomas, C. P., O'Donnell, V. B., Marnett, L. J., Radi, R., and Rubbo, H. (2011) Nitroarachidonic acid, a novel peroxidase inhibitor of prostaglandin endoperoxide H synthases 1 and 2. *The Journal of biological chemistry* **286**, 12891-12900
39. Awwad, K., Steinbrink, S. D., Fromel, T., Lill, N., Isaak, J., Hafner, A. K., Roos, J., Hofmann, B., Heide, H., Geisslinger, G., Steinhilber, D., Freeman, B. A., Maier, T. J., and Fleming, I. (2014) Electrophilic fatty acid species inhibit 5-lipoxygenase and attenuate sepsis-induced pulmonary inflammation. *Antioxidants & redox signaling* **20**, 2667-2680
40. Lim, D. G., Sweeney, S., Bloodsworth, A., White, C. R., Chumley, P. H., Krishna, N. R., Schopfer, F., O'Donnell, V. B., Eiserich, J. P., and Freeman, B. A. (2002) Nitrolinoleate, a nitric oxide-derived mediator of cell function: synthesis, characterization, and vasomotor activity. *Proc. Natl. Acad. Sci. U.S.A* **99**, 15941-15946
41. Baker, P. R., Schopfer, F. J., Sweeney, S., and Freeman, B. A. (2004) Red cell membrane and plasma linoleic acid nitration products: synthesis, clinical identification, and quantitation. *Proc. Natl. Acad. Sci. U.S.A* **101**, 11577-11582
42. Hayama, T., Tomoda, S., Takeuchi, Y., and Nomura, Y. (1982) Synthesis of Conjugated Nitroalkenes Via Nitroselenenylation of Alkenes. *Tetrahedron Lett* **23**, 4733-4734
43. Woodcock, S. R., Marwitz, A. J., Bruno, P., and Branchaud, B. P. (2006) Synthesis of nitrolipids. All four possible diastereomers of nitrooleic acids: (E)- and (Z)-, 9- and 10-nitro-octadec-9-enoic acids. *Organic letters* **8**, 3931-3934
44. Gorczynski, M. J., Huang, J., and King, S. B. (2006) Regio- and Stereospecific Syntheses and Nitric Oxide Donor Properties of (E)-9- and (E)-10-Nitrooctadec-9-enoic Acids. *Organic letters* **8**, 2305-2308
45. Khoo, N. K. H., Li, L., Salvatore, S. R., Schopfer, F. J., and Freeman, B. A. (2018) Electrophilic fatty acid nitroalkenes regulate Nrf2 and NF-kappaB signaling: A medicinal chemistry investigation of structure-function relationships. *Scientific reports* **8**, 2295
46. Alexander, R. L., Wright, M. W., Gorczynski, M. J., Smitherman, P. K., Akiyama, T. E., Wood, H. B., Berger, J. P., King, S. B., and Morrow, C. S. (2009) Differential potencies of naturally occurring regioisomers of nitrolinoleic acid in PPARgamma activation. *Biochemistry* **48**, 492-498

47. Gorczynski, M. J., Smitherman, P. K., Akiyama, T. E., Wood, H. B., Berger, J. P., King, S. B., and Morrow, C. S. (2009) Activation of peroxisome proliferator-activated receptor gamma (PPARgamma) by nitroalkene fatty acids: importance of nitration position and degree of unsaturation. *J Med Chem* **52**, 4631-4639
48. Schopfer, F. J., Cole, M. P., Groeger, A. L., Chen, C. S., Khoo, N. K., Woodcock, S. R., Golin-Bisello, F., Motanya, U. N., Li, Y., Zhang, J., Garcia-Barrio, M. T., Rudolph, T. K., Rudolph, V., Bonacci, G., Baker, P. R., Xu, H. E., Batthyany, C. I., Chen, Y. E., Hallis, T. M., and Freeman, B. A. (2010) Covalent peroxisome proliferator-activated receptor gamma adduction by nitro-fatty acids: selective ligand activity and anti-diabetic signaling actions. *The Journal of biological chemistry* **285**, 12321-12333
49. Kansanen, E., Bonacci, G., Schopfer, F. J., Kuosmanen, S. M., Tong, K. I., Leinonen, H., Woodcock, S. R., Yamamoto, M., Carlberg, C., Yla-Herttuala, S., Freeman, B. A., and Levonen, A. L. (2012) Electrophilic Nitro-fatty Acids Activate NRF2 by a KEAP1 Cysteine 151-independent Mechanism. *J. Biol. Chem.* **286**, 14019-14027
50. Baker, P. R., Lin, Y., Schopfer, F. J., Woodcock, S. R., Groeger, A. L., Batthyany, C., Sweeney, S., Long, M. H., Iles, K. E., Baker, L. M., Branchaud, B. P., Chen, Y. E., and Freeman, B. A. (2005) Fatty acid transduction of nitric oxide signaling: multiple nitrated unsaturated fatty acid derivatives exist in human blood and urine and serve as endogenous peroxisome proliferator-activated receptor ligands. *J. Biol. Chem.* **280**, 42464-42475
51. Woodcock, S. R., Bonacci, G., Gelhaus, S. L., and Schopfer, F. J. (2013) Nitrated fatty acids: synthesis and measurement. *Free radical biology & medicine* **59**, 14-26
52. Trostchansky, A., Souza, J. M., Ferreira, A., Ferrari, M., Blanco, F., Trujillo, M., Castro, D., Cerecetto, H., Baker, P. R., O'Donnell V. B., and Rubbo, H. (2007) Synthesis, Isomer Characterization, and Anti-Inflammatory Properties of Nitroarachidonate. *Biochemistry*
53. Lima, E. S., Di Mascio, P., Rubbo, H., and Abdalla, D. S. (2002) Characterization of linoleic acid nitration in human blood plasma by mass spectrometry. *Biochemistry* **41**, 10717-10722
54. Ferreira, A. M., Ferrari, M. I., Trostchansky, A., Batthyany, C., Souza, J. M., Alvarez, M. N., Lopez, G. V., Baker, P. R., Schopfer, F. J., O'Donnell, V., Freeman, B. A., and Rubbo, H. (2009) Macrophage activation induces formation of the anti-inflammatory lipid cholesteryl-nitrolinoleate. *The Biochemical journal* **417**, 223-234
55. Bonacci, G., Ascutto, E. K., Woodcock, S. R., Salvatore, S. R., Freeman, B. A., and Schopfer, F. J. (2011) Gas-phase fragmentation analysis of nitro-fatty acids. *Journal of the American Society for Mass Spectrometry* **22**, 1534-1551
56. Bonacci, G., Baker, P. R., Salvatore, S. R., Shores, D., Khoo, N. K., Koenitzer, J. R., Vitturi, D. A., Woodcock, S. R., Golin-Bisello, F., Cole, M. P., Watkins, S., St Croix, C., Batthyany, C. I., Freeman, B. A., and Schopfer, F. J. (2012) Conjugated linoleic acid is a preferential substrate for fatty acid nitration. *The Journal of biological chemistry* **287**, 44071-44082

57. Salvatore, S. R., Vitturi, D. A., Baker, P. R., Bonacci, G., Koenitzer, J. R., Woodcock, S. R., Freeman, B. A., and Schopfer, F. J. (2013) Characterization and quantification of endogenous fatty acid nitroalkene metabolites in human urine. *Journal of lipid research* **54**, 1998-2009
58. Fazzari, M., Khoo, N., Woodcock, S. R., Li, L., Freeman, B. A., and Schopfer, F. J. (2015) Generation and esterification of electrophilic fatty acid nitroalkenes in triacylglycerides. *Free radical biology & medicine*
59. Turell, L., Vitturi, D. A., Coitino, E. L., Lebrato, L., Moller, M. N., Sagasti, C., Salvatore, S. R., Woodcock, S. R., Alvarez, B., and Schopfer, F. J. (2017) The Chemical Basis of Thiol Addition to Nitro-conjugated Linoleic Acid, a Protective Cell-signaling Lipid. *J. Biol. Chem.* **292**, 1145-+
60. Lima, E. S., Di Mascio, P., and Abdalla, D. S. (2003) Cholesteryl nitrolinoleate, a nitrated lipid present in human blood plasma and lipoproteins. *Journal of lipid research* **44**, 1660-1666
61. Lima, E. S., Di, M. P., and Abdalla, D. S. (2003) Cholesteryl nitrolinoleate, a nitrated lipid present in human blood plasma and lipoproteins. *J.Lipid Res.* **44**, 1660-1666
62. Tsikas, D., Zoerner, A. A., Mitschke, A., and Gutzki, F. M. (2009) Nitro-fatty acids occur in human plasma in the picomolar range: a targeted nitro-lipidomics GC-MS/MS study. *Lipids* **44**, 855-865
63. Tsikas, D., Zoerner, A. A., and Jordan, J. (2011) Oxidized and nitrated oleic acid in biological systems: Analysis by GC-MS/MS and LC-MS/MS, and biological significance. *Biochim. Biophys. Acta* **1811**, 694-705
64. Rudolph, V., Rudolph, T. K., Schopfer, F. J., Bonacci, G., Woodcock, S. R., Cole, M. P., Baker, P. R., Ramani, R., and Freeman, B. A. (2010) Endogenous generation and protective effects of nitro-fatty acids in a murine model of focal cardiac ischaemia and reperfusion. *Cardiovascular research* **85**, 155-166
65. Lima, E. S., Bonini, M. G., Augusto, O., Barbeiro, H. V., Souza, H. P., and Abdalla, D. S. (2005) Nitrated lipids decompose to nitric oxide and lipid radicals and cause vasorelaxation. *Free radical biology & medicine* **39**, 532-539
66. Rudnicki, M., Faine, L. A., Dehne, N., Namgaladze, D., Ferderbar, S., Weinlich, R., Amarante-Mendes, G. P., Yan, C. Y., Krieger, J. E., Brune, B., and Abdalla, D. S. (2011) Hypoxia inducible factor-dependent regulation of angiogenesis by nitro-fatty acids. *Arterioscler Thromb Vasc Biol* **31**, 1360-1367
67. Mata-Perez, C., Sanchez-Calvo, B., Begara-Morales, J. C., Carreras, A., Padilla, M. N., Melguizo, M., Valderrama, R., Corpas, F. J., and Barroso, J. B. (2016) Nitro-linolenic acid is a nitric oxide donor. *Nitric oxide : biology and chemistry* **57**, 57-63
68. Mata-Perez, C., Sanchez-Calvo, B., Begara-Morales, J. C., Padilla, M. N., Valderrama, R., Corpas, F. J., and Barroso, J. B. (2016) Nitric oxide release from nitro-fatty acids in Arabidopsis roots. *Plant signaling & behavior* **11**, e1154255

69. Gorczynski, M. J., Huang, J., Lee, H., and King, S. B. (2007) Evaluation of nitroalkenes as nitric oxide donors. *Bioorganic & medicinal chemistry letters* **17**, 2013-2017
70. Schopfer, F. J., Baker, P. R., Giles, G., Chumley, P., Batthyany, C., Crawford, J., Patel, R. P., Hogg, N., Branchaud, B. P., Lancaster, J. R., Jr., and Freeman, B. A. (2005) Fatty acid transduction of nitric oxide signaling. Nitrolinoleic acid is a hydrophobically stabilized nitric oxide donor. *The Journal of biological chemistry* **280**, 19289-19297
71. Zhang, J., Villacorta, L., Chang, L., Fan, Z., Hamblin, M., Zhu, T., Chen, C. S., Cole, M. P., Schopfer, F. J., Deng, C. X., Garcia-Barrio, M. T., Feng, Y. H., Freeman, B. A., and Chen, Y. E. (2010) Nitro-oleic acid inhibits angiotensin II-induced hypertension. *Circulation research* **107**, 540-548
72. Khoo, N. K., Rudolph, V., Cole, M. P., Golin-Bisello, F., Schopfer, F. J., Woodcock, S. R., Batthyany, C., and Freeman, B. A. (2010) Activation of vascular endothelial nitric oxide synthase and heme oxygenase-1 expression by electrophilic nitro-fatty acids. *Free radical biology & medicine* **48**, 230-239
73. Coles, B., Bloodsworth, A., Clark, S. R., Lewis, M. J., Cross, A. R., Freeman, B. A., and O'Donnell, V. B. (2002) Nitrolinoleate inhibits superoxide generation, degranulation, and integrin expression by human neutrophils: novel antiinflammatory properties of nitric oxide-derived reactive species in vascular cells. *Circulation research* **91**, 375-381
74. Coles, B., Bloodsworth, A., Eiserich, J. P., Coffey, M. J., McLoughlin, R. M., Giddings, J. C., Lewis, M. J., Haslam, R. J., Freeman, B. A., and O'Donnell, V. B. (2002) Nitrolinoleate inhibits platelet activation by attenuating calcium mobilization and inducing phosphorylation of vasodilator-stimulated phosphoprotein through elevation of cAMP. *J. Biol. Chem.* **277**, 5832-5840
75. Baker, L. M., Baker, P. R., Golin-Bisello, F., Schopfer, F. J., Fink, M., Woodcock, S. R., Branchaud, B. P., Radi, R., and Freeman, B. A. (2007) Nitro-fatty acid reaction with glutathione and cysteine. Kinetic analysis of thiol alkylation by a Michael addition reaction. *J. Biol. Chem.* **282**, 31085-31093
76. Batthyany, C., Schopfer, F. J., Baker, P. R., Duran, R., Baker, L. M., Huang, Y., Cervenansky, C., Branchaud, B. P., and Freeman, B. A. (2006) Reversible post-translational modification of proteins by nitrated fatty acids in vivo. *The Journal of biological chemistry* **281**, 20450-20463
77. Lin, D., Saleh, S., and Liebler, D. C. (2008) Reversibility of covalent electrophile-protein adducts and chemical toxicity. *Chemical research in toxicology* **21**, 2361-2369
78. Mathers, A. R., Carey, C. D., Killeen, M. E., Salvatore, S. R., Ferris, L. K., Freeman, B. A., Schopfer, F. J., and Faló, L. D., Jr. (2018) Topical electrophilic nitro-fatty acids potentiate cutaneous inflammation. *Free radical biology & medicine* **115**, 31-42
79. Mathers, A. R., Carey, C. D., Killeen, M. E., Diaz-Perez, J. A., Salvatore, S. R., Schopfer, F. J., Freeman, B. A., and Faló, L. D., Jr. (2017) Electrophilic nitro-fatty acids suppress allergic contact dermatitis in mice. *Allergy* **72**, 656-664

80. Hughan, K. S., Wendell, S. G., Delmastro-Greenwood, M., Helbling, N., Corey, C., Bellavia, L., Potti, G., Grimes, G., Goodpaster, B., Kim-Shapiro, D. B., Shiva, S., Freeman, B. A., and Gladwin, M. T. (2017) Conjugated linoleic acid modulates clinical responses to oral nitrite and nitrate. *Hypertension* **70**, 634-644
81. Delmastro-Greenwood, M., Hughan, K. S., Vitturi, D. A., Salvatore, S. R., Grimes, G., Potti, G., Shiva, S., Schopfer, F. J., Gladwin, M. T., Freeman, B. A., and Gelhaus Wendell, S. (2015) Nitrite and nitrate-dependent generation of anti-inflammatory fatty acid nitroalkenes. *Free radical biology & medicine* **89**, 333-341
82. Charles, R. L., Rudyk, O., Prysyzhna, O., Kamynina, A., Yang, J., Morisseau, C., Hammock, B. D., Freeman, B. A., and Eaton, P. (2014) Protection from hypertension in mice by the Mediterranean diet is mediated by nitro fatty acid inhibition of soluble epoxide hydrolase. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 8167-8172
83. Turell, L., Vitturi, D. A., Coitino, E. L., Lebrato, L., Moller, M. N., Sagasti, C., Salvatore, S. R., Woodcock, S. R., Alvarez, B., and Schopfer, F. J. (2017) The Chemical Basis of Thiol Addition to Nitro-conjugated Linoleic Acid, a Protective Cell-signaling Lipid. *The Journal of biological chemistry* **292**, 1145-1159
84. Batthyany, C., Schopfer, F. J., Baker, P. R., Duran, R., Baker, L. M., Huang, Y., Cervenansky, C., Branchaud, B. P., and Freeman, B. A. (2006) Reversible post-translational modification of proteins by nitrated fatty acids in vivo. *J. Biol. Chem.* **281**, 20450-20463
85. Freeman, B. A., Pekarova, M., Rubbo, H., and Trostchansky, A. (2017) *Electrophilic Nitro-Fatty Acids: Nitric Oxide and Nitrite-Derived Metabolic and Inflammatory Signaling Mediators*,
86. Kansanen, E., Bonacci, G., Schopfer, F. J., Kuosmanen, S. M., Tong, K. I., Leinonen, H., Woodcock, S. R., Yamamoto, M., Carlberg, C., Ylä-Herttuala, S., Freeman, B. A., and Levonen, A.-L. (2011) Electrophilic nitro-fatty acids activate NRF2 by a KEAP1 Cysteine 151-independent mechanism. *J. Biol. Chem.* **286**, 14019-14027
87. Cui, T., Schopfer, F. J., Zhang, J., Chen, K., Ichikawa, T., Baker, P. R., Batthyany, C., Chacko, B. K., Feng, X., Patel, R. P., Agarwal, A., Freeman, B. A., and Chen, Y. E. (2006) Nitrated fatty acids: Endogenous anti-inflammatory signaling mediators. *The Journal of biological chemistry* **281**, 35686-35698
88. Villacorta, L., Chang, L., Salvatore, S. R., Ichikawa, T., Zhang, J., Petrovic-Djergovic, D., Jia, L., Carlsen, H., Schopfer, F. J., Freeman, B. A., and Chen, Y. E. (2013) Electrophilic nitro-fatty acids inhibit vascular inflammation by disrupting LPS-dependent TLR4 signalling in lipid rafts. *Cardiovascular research* **98**, 116-124
89. Snyder, N. W., Golin-Bisello, F., Gao, Y., Blair, I. A., Freeman, B. A., and Wendell, S. G. (2015) 15-Oxoeicosatetraenoic acid is a 15-hydroxyprostaglandin dehydrogenase-derived electrophilic mediator of inflammatory signaling pathways. *Chemico-Biological Interactions* **234**, 144-153

90. Woodcock, C. C., Huang, Y., Woodcock, S. R., Salvatore, S. R., Singh, B., Golin-Bisello, F., Davidson, N. E., Neumann, C., Freeman, B. A., and Wendell, S. G. (2017) Nitro-fatty acid inhibition of triple negative breast cancer cell viability, migration, invasion and tumor growth. *The Journal of biological chemistry*
91. Li, S., Chang, Z., Zhu, T., Li, Y., Freeman, B. A., Chen, Y. E., and Zhang, J. (2018) Transcriptomic sequencing reveals diverse adaptive gene expression responses of human vascular smooth muscle cells to nitro-conjugated linoleic acid. *Physiological genomics*
92. Kansanen, E., Jyrkkanen, H. K., Volger, O. L., Leinonen, H., Kivela, A. M., Hakkinen, S. K., Woodcock, S. R., Schopfer, F. J., Horrevoets, A. J., Yla-Herttuala, S., Freeman, B. A., and Levonen, A. L. (2009) Nrf2-dependent and -independent responses to nitro-fatty acids in human endothelial cells: identification of heat shock response as the major pathway activated by nitro-oleic acid. *The Journal of biological chemistry* **284**, 33233-33241
93. Fazzari, M., Khoo, N. K., Woodcock, S. R., Jorkasky, D. K., Li, L., Schopfer, F. J., and Freeman, B. A. (2017) Nitro-fatty acid pharmacokinetics in the adipose tissue compartment. *Journal of lipid research* **58**, 375-385
94. Fazzari, M., Khoo, N., Woodcock, S. R., Li, L., Freeman, B. A., and Schopfer, F. J. (2015) Generation and esterification of electrophilic fatty acid nitroalkenes in triacylglycerides. *Free Radic Biol Med* **87**, 113-124

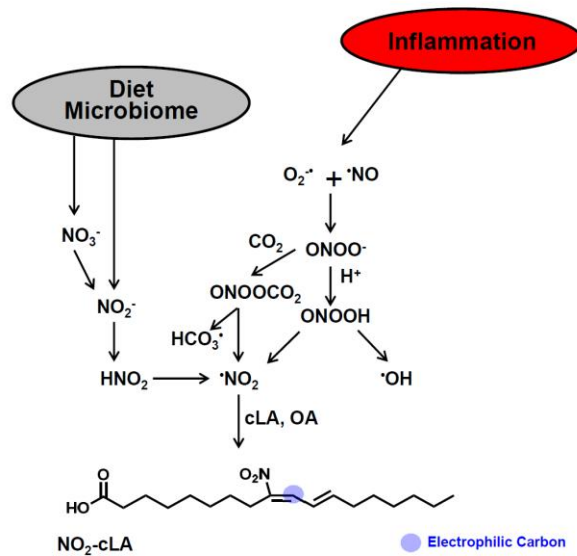


Figure 1. Fatty acid nitration is induced by nitrite and nitric oxide dependent mechanisms. The concentrations of nitrogen oxide precursors for nitrogen dioxide generation and levels of readily-nitrated unsaturated fatty acids such as conjugated linoleic acid will impact rates of formation and tissue concentrations of fatty acid nitroalkene derivatives.