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Convergence of Nitric Oxide and Lipid Signaling: Anti-Inflammatory Nitro-Fatty Acids

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Abstract

The signaling mediators nitric oxide (NO) and oxidized lipids, once viewed to transduce metabolic and inflammatory information via discrete and independent pathways, are now appreciated as interdependent regulators of immune response and metabolic homeostasis. The interactions between these two classes of mediators result in reciprocal control of mediator synthesis that is strongly influenced by the local chemical environment. The relationship between the two pathways extends beyond co-regulation of NO and eicosanoid formation to converge *via* the nitration of unsaturated fatty acids to yield nitro derivatives (NO₂-FA). These pluripotent signaling molecules are generated *in vivo* as an adaptive response to oxidative inflammatory conditions and manifest predominantly anti-inflammatory signaling reactions. These actions of NO₂-FA are diverse, with these species serving as a potential chemical reserve of NO, reacting with cellular nucleophiles to post-translationally modify protein structure, function and localization. In this regard these species act as potent endogenous ligands for peroxisome proliferator activated receptor γ . Functional consequences of these signaling mechanisms have been shown in multiple model systems, including the inhibition of platelet and neutrophil functions, induction of heme oxygenase-1, inhibition of LPS-induced cytokine release in monocytes, increased insulin sensitivity and glucose uptake in adipocytes and relaxation of pre-constricted rat aortic segments. These observations have propelled further *in vitro* and *in vivo* studies of mechanisms of NO₂-FA signaling and metabolism, highlighting the therapeutic potential of this class of molecules as anti-inflammatory drug candidates.

Keywords

Nitrated fatty acids; Nitric oxide; Oxidized lipids; Signal transduction; Inflammation

Introduction

Two signaling pathways, both mediated by labile species, are central modulators of inflammatory responses and metabolic homeostasis: one regulated by nitric oxide (NO) and the other via oxidized lipids (e.g., eicosanoids). These two pathways biochemically converge at multiple levels, resulting in alterations of steady state concentrations of mediators, extents

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of gene expression of biosynthetic enzymes and the formation of bioactive secondary reaction products, all combining to modulate metabolic and inflammatory responses. Nitric oxide is a soluble mediator of vasodilation, inflammatory cell function and neurotransmission that acts via stimulation of soluble guanylate cyclase (sGC). Nitric oxide and its metabolites also induce cyclic guanosine monophosphate (cGMP)-independent actions in host defense mechanisms and cell signaling. Reactions of $\cdot\text{NO}$ -derived species with eicosanoids and the impact of $\cdot\text{NO}$ on expression and activity of eicosanoid biosynthetic enzymes (i.e., cyclooxygenase (COX) and lipoxygenase (LOX)) are significant elements in the modulation of diverse inflammatory responses. Furthermore, reactions between $\cdot\text{NO}$ -derived species and lipid oxidation products yield nitrated (NO_2 -containing) lipid species that confer novel reactivities to fatty acids and thereby potentially modulate cell function and gene expression. Thus, a new view of $\cdot\text{NO}$ and eicosanoid signaling is emerging wherein a broader and more dynamic reactivity of $\cdot\text{NO}$ is manifested. Depending on the local oxidation-reduction environment, pH and $\cdot\text{NO}$ concentration, conditions exist *in vivo* that both modulate eicosanoid signaling and enable the formation of nitrated lipids—found as either modified fatty acids, eicosanoids or esterified components of complex lipids (e.g., glycerophospholipids, di- and tri-glycerides and cholesterol esters).

Herein the interactions of $\cdot\text{NO}$ and eicosanoid signaling are addressed. A convergence of these signaling pathways in the formation of nitrated fatty acids ($\text{NO}_2\text{-FA}$), which are detected *in vivo* and display distinct anti-inflammatory signaling properties is presented. Current understanding reveals that lipid nitration provides a means by which the pro-inflammatory and potentially cytotoxic aspects of reactive oxygen and nitrogen species and eicosanoids are down-regulated by a product of fatty acid reaction with oxides of nitrogen.

Generation of $\cdot\text{NO}$ and reactions with oxygen metabolites

Nitric oxide is synthesized from the guanidino nitrogen of L-arginine via three different isoforms of NOS (NOS1, NOS2 and NOS3) (1). Its biological effects are diverse and result from both direct interactions with target molecules (e.g., guanylate cyclase) and from the generation and distinct bioactivities of secondary oxides of nitrogen such as peroxynitrite (ONOO^-) and nitrosothiols (RSNO) (2). This discussion focuses on the formation and pro-oxidative reactivity of $\cdot\text{NO}$ metabolites and the pro- and anti-oxidant activity of $\cdot\text{NO}$ itself. The specific species formed are dictated by the local oxidative inflammatory environment. Depending on the reduction/oxidation (redox) state, the chemical equilibrium can shift such that proteins and polyunsaturated fatty acids (PUFAs) are oxidized, nitrosated and/or nitrated.

Nitric oxide-derived species

Many of the non-cGMP-dependent signaling actions of $\cdot\text{NO}$ are founded in its conversion to secondary reactive species. The reactivity and effects of $\cdot\text{NO}$ depend on the balance between different biological factors, including the presence of other free radical species, metal centers, the local redox environment, concentration(s) and ambient pH (3). The biological half-life of $\cdot\text{NO}$ is several seconds (4), which is long enough for the molecule to diffuse to the hydrophobic milieu of the cell membrane (5). Its small molecular radius and lipophilic nature allows $\cdot\text{NO}$ to diffuse into bilayers or lipoproteins ($k_d = 2 \times 10^{-5} \text{ cm}^2\text{s}^{-1}$ (6)), where it concentrates up to 20-fold (7,8). Molecular oxygen also concentrates in hydrophobic regions, resulting in a ~30-fold accelerated reaction rate (9) to form secondary $\cdot\text{NO}$ -derived species capable of oxidation, nitrosation and nitration reactions (5). Both in or near these hydrophobic tissue compartments, $\cdot\text{NO}$ and $\cdot\text{NO}$ -derived products undergo a rich spectrum of reactions with molecular oxygen, superoxide ($\text{O}_2^{\cdot-}$), peroxidases, transition metals, thiols, lipids and a variety of organic radicals. These multifaceted reactions yield reactive species that transduce $\cdot\text{NO}$ signaling and modulate tissue inflammatory responses.

Nitric oxide falls roughly in the middle of the series of redox-related nitrogen oxides and can be oxidized to nitrite (NO_2^-) and nitrate (NO_3^-) or reduced to nitroxyl (HNO) or hydroxylamine (H_2NOH). It readily reacts with other free radical species to form secondary oxides of nitrogen (Fig. 1). A reaction of particular biological relevance is the reaction of $\cdot\text{NO}$ with $\text{O}_2^{\cdot-}$, a molecule whose concentration increases up to $0.1\ \mu\text{M}$ during host inflammatory responses (10), to form OONO^- (11). At neutral pH, OONO^- becomes protonated to form ONOOH and undergoes homolytic scission to yield $\cdot\text{NO}_2$, which in turn can further react with $\cdot\text{NO}$ to form N_2O_3 and N_2O_4 , species that can oxidize, nitrate or nitrosate target molecules. In the presence of CO_2 , OONO^- reacts to form a transient nitrosoperoxocarbonate intermediate (ONOOCO_2^-), which further amplifies OONO^- -mediated oxidation and nitration reactions (12). Furthermore, OONO^- can react directly with thiols and metal centers—reaction with the latter can lead to the formation of oxo-metal complexes that may participate in hydrogen abstraction reactions in PUFAs (for review, see (13)). The formation of OONO^- thus results in diverse biological consequences, including oxidation of thiols and lipids (14–16) and nitrosation and/or nitration of both lipids and proteins (17,18). The spectrum of products stemming from OONO^- -mediated reactions is dependent on the balance of $\cdot\text{NO}$ and $\text{O}_2^{\cdot-}$ within the local chemical environment (19–21).

Reactions of $\cdot\text{NO}$ with eicosanoid enzymes and effects on eicosanoid synthesis

The synthesis and actions of eicosanoids are essential steps in immune responses. These potent mediators regulate a myriad of inflammatory responses and vascular cell functions and, consequently, their synthesis is regulated by multiple components of the inflammatory milieu. The first step of eicosanoid synthesis is the hydrolysis of arachidonate from intact phospholipids by a phospholipase A_2 (PLA_2). Once released, arachidonic acid (eicosatetraenoic acid; AA) is oxygenated at various positions by LOX, prostaglandin endoperoxide H synthases 1 and 2 (PGHS1/2) or cytochrome P450 to form bioactive lipid species (Fig. 2). The synthesis of eicosanoids typically involves the formation of enzyme-bound lipid radical intermediates, including alkyl and peroxy radicals. It is now appreciated that $\cdot\text{NO}$ and its derivatives influence eicosanoid activity and product distribution via multiple mechanisms. For example, alkyl and peroxy radical intermediates of eicosanoid synthesis react with $\cdot\text{NO}$ at diffusion-limited rates (22,23). Also, $\cdot\text{NO}$ readily reacts with critical tyrosyl radical catalytic intermediates and reduced iron centers within the synthetic enzymes thereby modulating eicosanoid formation. The latter metal center interactions, however, only occur in the presence of supra-physiological concentrations of $\cdot\text{NO}$ and are thus not viewed as biologically relevant (22,23).

Prostaglandin endoperoxide H synthase 1 and 2

The committed step of prostaglandin synthesis is catalyzed by PGHS-1 and -2 [also termed cyclooxygenase-1 and -2 (COX-1 and -2)] (24). Both isozymes have two catalytic activities: a COX activity that incorporates O_2 into PUFAs and a peroxidase activity. These occur at distinct sites within the enzyme and are functionally and structurally interconnected (25). Prostaglandin production is controlled by the availability of free AA, which in turn is regulated by PLA_2 . Once AA is hydrolyzed and available for reaction, both PGHS-1 and -2 are subject to multiple levels of NO-dependent regulation (Fig. 3). First, $\cdot\text{NO}$ is catalytically consumed by PGHS-1 via serving as a reducing peroxidase substrate. This occurs with both purified PGHS-1 and also during AA, thrombin, or A23187 activation of platelets, thus representing a pro-aggregatory function for PGHS-1 and a mechanism for the regulated removal of vascular $\cdot\text{NO}$ (22). *In vivo* studies of NOS2-competent and deficient rodents expand this concept, by revealing that PGE_2 formation by macrophages from NOS2-deficient animals is decreased compared with cells from control animals. Also, platelet thromboxane B_2 production upon

aggregation increases in NOS2 deficient mice, supporting that $\cdot\text{NO}$ and its metabolites modulate PGHS activity and eicosanoid production (26). Secondary $\cdot\text{NO}$ -derived species also modulate PGHS activity, with outcome influenced by the local chemical environment, which in turn dictates the spectrum of reactive species being formed (26–35). Specifically, $\cdot\text{NO}$ can indirectly activate PGHS activity upon the co-generation of $\text{O}_2^{\cdot-}$ and $\cdot\text{NO}$ and the downstream formation of ONOO^- (36,37). Peroxynitrite then directly activates PGHS by serving as a peroxidase substrate thus inducing PGHS “priming” via heme oxidation, which promotes subsequent fatty acid oxygenation reactions (36). In the absence of AA, or under conditions of high ONOO^- concentration (i.e., likely non-physiological), ONOO^- can also nitrate and inhibit PGHS. Recent studies have also shown that the heme moiety of PGHS may also catalyze the ONOO^- -dependent nitration of the catalytic tyrosine-285 of PGHS-1 (38). In summary, during host inflammatory responses where the respiratory burst produces $\text{O}_2^{\cdot-}$ and NOS2 generates $\cdot\text{NO}$, conditions exist that favor the formation of ONOO^- , thus triggering the activation of prostanoid formation; however at higher concentrations, ONOO^- can inhibit PGHS activity.

Lipoxygenases

Lipoxygenases catalyze the specific dioxygenation of PUFAs to yield reactive lipid peroxides such as leukotrienes and HETEs (39). These oxidized species are generally pro-inflammatory, acting as chemotactic and chemokinetic agents. Recent studies reveal a more complex structure/function relationship between LOX products and signaling events. Lipoxins, resolvins and hepoxins—all LOX products—are lipid mediators that appear to mediate receptor-dependent and -independent anti-inflammatory actions (40). Lipoxygenases, like PGHS, are also subject to $\cdot\text{NO}$ regulation (23,41). Nitric oxide reversibly inhibits LOX, concomitantly being catalytically consumed via reaction with an enzyme-bound lipid peroxy radical intermediate. LOX catalytic turnover thus inhibits $\cdot\text{NO}$ -dependent activation of sGC and acts as catalytic sink of $\cdot\text{NO}$ in the vasculature (42).

Under conditions in which $\cdot\text{NO}$ is converted to the more powerful oxidant ONOO^- , LOX isoforms are inhibited by tyrosine nitration at concentrations as low as 1 μM (43,44), although it remains to be determined whether such ONOO^- concentrations occur physiologically. Lipoxygenase exposure to ONOO^- induces a dose-dependent tyrosine nitration and S-nitrosylation of recombinant 5-LOX. Studies on the effects of ONOO^- on prostacyclin synthase yielded similar results (45). Interestingly, prostacyclin (PGI_2) is a vasodilator; thus, the reaction of $\cdot\text{NO}$ with $\text{O}_2^{\cdot-}$ not only depletes $\cdot\text{NO}$ levels, which results in decreased vasodilation, but also generates ONOO^- , which causes a decrease in synthesis of PGI_2 . Together, these data illustrate the delicate balance of vasoactive and inflammatory signaling by reactive species. Subtle changes in the redox environment, such as fluctuations in the balance of $\cdot\text{NO}/\text{O}_2^{\cdot-}$ levels within the vasculature can induce dramatic changes in the production of lipid mediators.

4. Reciprocal regulation of enzyme expression by $\cdot\text{NO}$ and eicosanoids

The reactions of $\cdot\text{NO}$ and $\cdot\text{NO}$ metabolites not only influence catalytic reactions in eicosanoid synthesis, they also modulate the gene expression of related enzymes. Conversely, eicosanoids contribute to the regulation of $\cdot\text{NO}$ biosynthetic enzyme expression. This coordinated regulation of gene expression enables both synergistic activation and feedback inhibition of eicosanoid biosynthesis and thus establishes a mechanism allowing reciprocal control between $\cdot\text{NO}$ and eicosanoid signaling mechanisms.

Inducible-nitric oxide synthase

The transcription factor NF- κB mediates inducible NOS (NOS2) expression in LPS-activated macrophages (46,47). Eicosanoids, also produced at elevated rates following LPS exposure,

independently induce NOS2 expression. For example, leukotrienes induce NOS2 expression in PMN (specifically LTB₄, LTC₄ and LTD₄) (48), murine macrophages (LTB₄) (49) and RAW 267.4 cells (LTB₄ and LTC₄) (50). The induction of NOS2 expression by leukotrienes occurs at nM concentrations and is inhibited by leukotriene receptor antagonists (48–50). Leukotriene receptor antagonists display the same effect as NOS inhibitors by leading to dose-dependent inhibition of cellular NO and nitrite formation (50) and suggest that NOS2 gene expression occurs upon activation of leukotriene receptor(s). As for LPS-induced NOS2 expression, NF-κB up-regulates leukotriene biosynthetic enzyme gene transcription. Inhibitors that specifically block NF-κB binding to DNA, also block NOS2 expression (50). Peroxynitrite also induces NOS2 expression (51), a reaction blocked by pyrroline dithiocarbamate, an NF-κB inhibitor. The induction of NOS2 by ONOO[−], leukotrienes or both, indicates that during inflammatory responses, feed-forward mechanisms exist that potentiate both NO and ONOO[−] formation.

Platelet-activating factor

In many cell types, arachidonate is predominantly incorporated in ether-linked phospholipids. The PLA₂-mediated hydrolysis of AA from these phospholipid pools not only enables eicosanoid synthesis but also directly, or indirectly via CoA-independent transacylase activity, contributes to the formation of lyso-PAF, the immediate precursor to platelet activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PAF) (52). Nitric oxide antagonizes PAF synthesis in multiple model systems (53–55). In mesangial cells, inhibition of NO synthesis increases PAF synthesis; and reciprocally, NO generation inhibits PAF synthesis (56). This complementarity suggests a balance between the signaling actions of PAF and NO, with PAF promoting and NO inhibiting leukocyte adhesion. Other studies indicate cooperative actions between PAF and NO (55,57), although the mechanisms of interaction between NO and PAF are unclear. *In vivo* rodent studies indicate that PAF contributes to LPS-mediated induction and activation of NF-κB (58), suggesting that PAF also contributes to NOS2 induction. Collectively, these findings indicate that in NO-replete inflammatory conditions, where leukotriene and PAF derived species are elevated, NO can serve to down-regulate initial lipid-mediated signaling events.

Prostaglandin endoperoxide H synthase-1 and -2

The high-output NO production of NOS2 can either increase or inhibit PGHS-1/2 activity, depending on concomitant rates of production of partially reduced oxygen species. In addition, NO also influences prostaglandin synthesis by altering PGHS-2 expression. The majority of reports, particularly the more recent, indicate that NO increases PGHS-2 mRNA and protein levels in a variety of cell types (59–64). However, a global view for NO regulation of PGHS-2 expression remains unclear as to whether and to what extent NO increases or decreases PGHS-2 mRNA and/or protein levels. Current discrepancies may be explained by species and cell type differences, differences in cell culture techniques, the relative degree of stimulation and differences in experimental conditions (e.g., serum concentration, substrate availability) that could affect how NO and its reactive metabolites influence PGHS-2 expression. The redox environment also likely contributes to disparate results thus far described (65). Individual studies do not allow the generation of a set of rules by which the effects of NO on PGHS-2 can be predicted. Collectively, however, they indicate that the effects of NO and other nitrogen oxides must be evaluated in the context of the system. With this in mind, it appears that experimental conditions that favor NO as the predominant nitrogen oxide species promotes induction of PGHS-2 expression in inflammatory-activated non-phagocytic cells.

Lipid Nitration

The interdependency of $\cdot\text{NO}$, its redox-derived metabolites and lipid signaling pathways is now appreciated to extend well-beyond regulating the eicosanoid gene expression and biosynthetic enzyme activity. Free radicals generated during inflammatory processes can react with unsaturated fatty acids to generate a myriad of oxidized bioactive lipid species. Isoprostanes, for example, are a product of arachidonic acid auto-oxidation that exert vaso-constrictive and pro-inflammatory signaling actions via receptor-dependent and -independent mechanisms (66). Indeed, the majority of oxidized lipid species—enzymatically-derived or otherwise—are generally pro-inflammatory. Most recently, there is growing evidence that oxidized fatty acids also display anti-inflammatory signaling actions. For example, free-radical-oxidized docosahexaenoic and arachidonic acid derivatives can also play a role in the resolution of inflammation (67). The concept that lipid mediators participate in both the onset and resolution of inflammatory responses is an emerging paradigm that highlights the complex structure/function relationship that exists between diverse oxidized lipid molecular species and their signaling actions.

A class of lipid mediators has recently been discovered that differs significantly from previously characterized oxidized fatty acids in that one or more carbons of the lipid backbone are covalently attached to a nitro (NO_2) functional group (Table 1). The concept of fatty acid nitration via NO -dependent free radical reactions stems from the seminal observation that protein tyrosine and tryptophan residues are nitrated by ONOO^- and nitrite/heme peroxidase-mediated reactions. Since their detection and characterization *in vitro* and *in vivo*, nitrated lipids have emerged as pluripotent signaling mediators whose functional outcomes are strongly anti-inflammatory. In the following sections, mechanisms of lipid nitration and the anti-inflammatory properties of these species are presented (Fig. 4).

Nitration Chemistry

Many of the actions of $\cdot\text{NO}$ and secondary RNS and ROS are rooted in their capacity to covalently modify biological macromolecules and alter their structure and function. Protein tyrosine nitration is now recognized to be a mediator of pathological conditions (e.g., atherosclerosis, sepsis, acute lung injury and chronic organ rejection (68–70)) and possibly oxidative signaling reactions (71,72). The extensive evidence that tyrosine residues are nitrated *in vivo* during inflammatory processes support that other biomolecules can be nitrated as well, including lipids, other amino acids such as tryptophan, DNA bases and low molecular weight antioxidants (13,73–75). Current understanding of the mechanisms of biomolecule nitration primarily stems from studies on tyrosine nitration *in vitro* and *in vivo* and support that fatty acids are endogenously generated by similar chemical reactions.

Lipid reactions with $\cdot\text{NO}$ and other oxides of nitrogen

The reaction of nitrogen dioxide gas ($\cdot\text{NO}_2$) with unsaturated fatty acids of phosphatidylcholine was the first lipid nitration event to be investigated (76,77). The effects of this photochemical air pollutant were assessed in a model system designed to evaluate reactions between $\cdot\text{NO}_2$ and unsaturated fatty acids in pulmonary surfactant. Two mechanisms of nitration were identified: addition reactions and $\cdot\text{NO}_2$ -mediated hydrogen abstraction. The competitive equilibrium between these two outcomes is dependent on the concentration of $\cdot\text{NO}_2$. At relatively high $\cdot\text{NO}_2$ concentrations, addition reactions generate nitro- or dinitro-alkanes; however, as $\cdot\text{NO}_2$ levels decrease to the low ppm range (and relative O_2 levels increase), hydrogen abstraction reactions are favored, resulting in the formation of allylic hydroperoxides, allylic nitrohydroperoxides and nitrate esters (76–82). These data are complemented with studies investigating nitration of ethyl linoleate by $\cdot\text{NO}$ (83), wherein O_2 is required for lipid nitrite

and nitrates to be formed. In the presence of O_2 , $\cdot NO$ auto-oxidizes to form $\cdot NO_2$, which nitrates via the above described mechanisms.

Protonation of NO_2^- under acidic conditions produces nitrous acid (HNO_2 , $pK_a \sim 3$), a reaction possible in the gastric compartment (pH 1.0–5.0), phagolysosomes (pH 4.0–6.0), airways of asthmatics and possibly ischemic (i.e., acidotic) microenvironments. Decomposition of HNO_2 in aqueous media proceeds via a series of reactions to ultimately yield $\cdot NO_2$, N_2O_3 and N_2O_4 , all viable candidates for mediating nitration reactions. Although acidic nitration is not kinetically rapid, the residence time of lipids, proteins and NO_2^- in acidic tissue compartments suggests that this may be significant pathway for nitrating reactions. Since diet (e.g., cured meats) and inflammatory states are a considerable source of NO_2^- , formation of NO_2Tyr or nitration of lipids could readily occur via this pathway. Biomolecule nitration *via* nitrous acid is also likely to be enhanced by H_2O_2 , via the reaction of acidified NO_2^- with H_2O_2 to yield $ONOO^-$. This reaction may be relevant in phagolysosomes where increased H_2O_2 production from the respiratory burst oxidase also occurs.

The product distribution of fatty acids nitrated by acidified NO_2^- has been structurally characterized (84–86); with the $\cdot NO_2$ generated by HNO_2 decomposition nitrating PUFAs via homolytic addition to the methylene-interrupted double bond(s) of the carbon chain, yielding β -nitroalkyl radicals. Free radical recombination with another molecule of $\cdot NO_2$ generates 1,2-nitronitrite intermediates that undergo HNO_2 elimination and/or hydrolysis to generate nitroalkenes and/or 1,2-nitrohydroxy derivatives. Unlike the reactions between $\cdot NO_2$ gas and PUFAs, there does not appear to be an oxygen-dependence on the reaction equilibrium with acidic nitration, with a similar product distribution generated in the presence or absence of O_2 (84–86). Hydrogen abstraction reactions by $\cdot NO_2$ also occur, generating 5-nitro-1,3-diene products (84). Depending on the concentration of $\cdot NO_2$, these species further react with $\cdot NO_2$ to form dinitro adducts. Nitrosation/nitration of lipid hydroperoxides (LOOH) by HNO_2 forms alkyl nitrates ($LONO_2$) (87).

Synthesis of nitroalkenes

Considering the diversity and potent inflammatory signaling capacities of many oxidized lipids, nitrated lipids were hypothesized to manifest unique reactivities and signal transduction reactions. In order to assess the cellular effects of these novel species and to obtain standards for the identification of biologically-occurring nitrated lipids, synthetic regioisomers of nitrated free fatty acids and complex lipids have been prepared via multiple methods (84–86). Initial efforts yielded multiple nitrated species requiring extensive purification, while a more directed synthetic approach such as nitrosenylation generates greater quantities of more defined nitrated products (88). For example, nitrosenylation of linoleic acid generates a mixed isomer preparation of four positional isomers of nitrated linoleic acid (LNO_2) (75,89,90). The synthetic isomers are unified by several structural features including: a) they maintain the same methylene-interrupted, *cis*-double bond arrangement as endogenous fatty acids, b) the nitro group is located at a site of unsaturation (hence, the classification of this type of nitrated fatty acid as a 'nitroalkene') and c) the fatty acids are not esterified. Using this technique, the stable isotopes $[^{13}C_{18}]LNO_2$ and $[^{15}N]LNO_2$ are readily synthesized in gram quantities and can be used as standards for *in vivo* quantitation (91,92). Nitrated arachidonic acid ($AA-NO_2$) and cholesteryl linoleate regio-isomers have been synthesized via preparative-scale acidic nitration followed by TLC purification; however, due to the complex array of isomers formed under acidic nitration conditions, structural characterization has been more limited (92). Acidic nitration has also been used to nitrate cholesteryl linoleate to form multiple species of nitro-cholesterol linoleate ($CLNO_2$) (93). Recent reports also detail the more directed synthesis of specific regio-isomers of nitrated oleic acid ((*E*)- and (*Z*)-, 9- and 10-nitro-octadeca-9-enoic acids) (94,95).

In vivo detection and characterization of NO₂-FA

Nitrated lipids were first reported in mammalian tissue with the characterization of nitrohydroxyeicosatrienoic acid [AA(OH)NO₂] in hydrolyzed extracts of bovine cardiac tissue (96). A standard was made, structurally characterized by product ion analysis (MS/MS) and used as a diagnostic tool to identify similar species *in vivo*. A species that co-eluted with a deuterated internal standard by GC/MS that had the correct mass-to-charge ratio (*m/z*) was detected; however, there was no MS/MS structural confirmation performed on the molecule. An ancillary complication may be that lipid extractions were performed under acidic conditions (pH 3.5 to 4) (97), which can nitrate lipids in the presence of adventitious nitrite (87).

A subsequent study identified stable, linoleic acid-derived nitrated products in human plasma, with levels increasing in hyperlipidemic subjects (98). Also identified was the nitrohydroxy-derivative of linoleic acid (L(OH)NO₂). The cholesteryl ester derivative cholesteryl linoleate (CLNO₂) has been identified in human plasma and lipoproteins of normolipidemic subjects (99). The concentrations of OA-NO₂ and LNO₂ whole blood as free and esterified species were initially reported to be ~500 nM (75,100). These previous estimates of free and esterified *cis*-nitroalkene derivatives are now viewed to be overestimated in concentration due to electrophilic adduction of biological nucleophiles (101) and the co-elution of an isobaric molecule that is not covalently nitrated. Recent analyses of rodent mitochondria and healthy human specimens reveal a much more complex array of NO₂-FA molecular species are present *in vivo*, including trans isomers, shorter chain nitroalkenes (putative β -oxidation products of 18 carbon and longer precursors), nitroalkenes that have been further oxidized and nitroalkanes presumably derived from nitroalkene precursors (unpublished).

Because multiple endogenous NO₂-FA molecular species have been identified, their formation appears to be mediated via random free radical mechanisms rather than by enzyme-catalyzed processes that would yield a more limited array of regioisomers. This hypothesis is strengthened by the formation of LNO₂ and other nitrated and/or oxidized fatty acid species in mitochondria isolated from rat cardiac tissue exposed to short, repeated cycles of ischemia/reperfusion, a phenomenon known as ischemic preconditioning (IPC) (102). This event is cardioprotective against future ischemic episodes (103). During IPC, reactive oxygen and NO-derived species are generated to an increased extent, inducing NOS-dependent fatty acid nitration in mitochondria. As a consequence of IPC, LNO₂ concentrations rise to ~800 nM (102), a concentration shown to protect mitochondrial function in *in vitro* models of ischemia. Similarly, LPS and cytokine-activated macrophages induce the NOS-dependent acyl chain nitration of the complex lipid cholesteryl linoleate (93). It is expected that, due to the random free radical nature of lipid nitration, the further oxidation and catabolism of nitrated lipids and the multitude of different lipid species present in tissues (up to 180,000 per cell), that the array of regioisomers found biologically will be abundant and diverse.

Cell signaling properties of nitrated lipids

The detection of NO₂-FA species *in vivo* introduces a novel, NO-derived class of cell signaling molecules. Understanding the role these molecules play in the orchestrated and yet stochastic events occurring during inflammation and metabolic regulation requires characterization of the physical and chemical nature of fatty acids modified by the presence of a nitro group. Although the physiological role of this class of molecules is still relatively unclear, evidence collected thus far indicate that these species display anti-inflammatory signaling actions. The chemical properties of biomimetic synthetic nitroalkene derivatives and how these reactivities translate to signaling actions are presented below and are mediated by multiple mechanisms, including a) NO₂-FA decay reactions, b) electrophilic reactions and c) receptor-dependent interactions. These mechanisms appear to be interdependent, resulting in a complex array of signaling actions.

Nitric oxide release by NO₂-FA

The generation of 'NO or 'NO-like byproducts by organic oxides of nitrogen (e.g., organic nitrates and nitrites) is long-established; however, the mechanisms involved remain controversial. The nitrate ester nitroglycerin has been used as a vasodilator since the mid-nineteenth century for the treatment of angina pectoris, but its therapeutic mechanism of action was only realized more than a century later with the determination that one of its metabolites is 'NO [For review, see (104)]. Nitric oxide activates sGC, an enzyme that converts guanosine triphosphate (GTP) to cGMP, which induces vessel relaxation via protein kinase-mediated signaling pathways (105). Other biological and synthetic molecules that release 'NO include: protein heme-nitrosyl, NONOates, organic nitrites (RONO; e.g., isopentyl nitrite), nitroprusside, nitrosothiols (RSNO), *N*-nitrosamine derivatives (RNNO) and most recently, NO₂-FA (Fig. 5). As with nitroglycerin metabolism, the mechanisms of 'NO release by these molecules are not fully understood; however, it is hypothesized that protein metal centers or thiols may play a role. The release of 'NO from these molecules has been shown to occur spontaneously, and it is viewed that they play a role as physiological 'NO reserves.

Nitrated fatty acid [i.e., AA(OH)NO₂] stimulated cGMP-dependent vasorelaxation in pre-contracted bovine coronary aortic rings via the generation of 'NO (96). The nitroalkene LNO₂ has also been confirmed to be an 'NO donor in multiple studies (89,106,107). LNO₂ induced significant relaxation in endothelium-intact and endothelium-denuded precontracted rat thoracic aortic rings (89). Relaxation was inhibited by ODQ as well as oxyhemoglobin (oxyHb), a 'NO scavenger; furthermore, cGMP levels increased in both cultured rat aortic smooth muscle cells and aortic segments, confirming sGC activation.

Direct evidence that 'NO is released by nitroalkenes in aqueous milieu was obtained by electron paramagnetic resonance (EPR) using 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), a selective spin trap for 'NO (106,107). The apparent rate constant for 'NO release from LNO₂ was calculated to be $k = 9.67 \times 10^{-6} \text{ s}^{-1} \text{ M}^{-1}$ in aqueous buffer (pH 7.4). Importantly, 'NO release is abrogated in hydrophobic environments such as lipid micelles and bilayers indicating a requirement for water (107). Multiple nitrohydroxy adducts and a series of isomers with an *m/z* consistent with the loss of HNO from LNO₂ are detected. From this, 'NO is postulated to be released from LNO₂ via a modified Nef reaction mechanism wherein deprotonation of the carbon gamma to the nitro group leads to the formation of an unstable nitroso intermediate that rapidly degrades to release either HNO, the predicted Nef reaction product, or 'NO, a product considered likely due to the weak C—N bond present on the hydroxyl-nitroso intermediate (107,108). Another aspect of this mechanism considers the strong electrophilic nature of the carbon adjacent to the nitroalkene moiety. Due to the acidity of its bound hydrogen, the nitroalkene can interconvert to a vicinal nitrohydroxy fatty acid under aqueous conditions; this equilibrium reaction is affected by pH, whereby basic conditions favor nitrohydroxy adduct formation. This mechanism can also account for the release of 'NO by AA(OH)NO₂ (96), where the nitrohydroxy adduct of AA interconverts to the nitroalkene AA-NO₂, which then releases 'NO by the above mechanism.

An alternative mechanism to the modified Nef reaction has also been hypothesized, which predicts that the nitroalkene rearranges to a nitrite ester, followed by N—C bond homolysis to form 'NO and a lipid radical (106). A recent study using multiple, model synthetic compounds compared the two postulated pathways and called into question whether either mechanism is viable *in vivo*, especially in light of the very low yields of 'NO formation measured by chemiluminescence methods, potentially indicative of artifactual 'NO formation (109). However, vessel relaxation studies clearly indicate the generation of 'NO; the authors suggest that the functional and structural similarity of nitroalkenes to nitroglycerin may suggest similar reductive or hydrolytic metabolic pathways of 'NO formation *in vivo*. Parenthetically, aqueous decomposition studies using OA-NO₂ do not support significant 'NO release from

monounsaturated nitroalkenes (100,110). Under aqueous conditions, these molecules are significantly more stable than polyunsaturated nitroalkenes, due to the absence of acidic hydrogens that are present on the methylene carbon located between bis-allylic double bonds. While it is viewed that $\cdot\text{NO}$ generation by nitroalkenes will only occur in stringent aqueous conditions not found biologically due to micellar and membrane stabilization, further studies are warranted to determine the precise mechanism of $\cdot\text{NO}$ release *in vitro* and *in vivo*. Furthermore, considering the β -carbon adjacent to the nitroalkene is strongly electrophilic and reacts covalently with protein thiols (73,90,101), the release of $\cdot\text{NO}$ is likely a very minor component, if at all, of the diverse biological actions of nitroalkenes.

Anti-inflammatory activity of $\text{NO}_2\text{-FA}$

The motivation to detect, identify and quantify $\text{NO}_2\text{-FA}$ *in vivo* was spurred by reports demonstrating the ability of synthetic LNO_2 to inhibit neutrophil (polymorphonuclear leukocytes; PMN) and platelet function (111,112) (Table 2). Neutrophil function *in vivo* is regulated in part via $\cdot\text{NO}$ - and eicosanoid-dependent mechanisms (113,114), thus it was hypothesized that these pathways may converge via the generation of nitrated unsaturated lipids that would uniquely influence PMN activity (111). Current data indicate that $\cdot\text{NO}$ does not transduce the signaling actions of $\text{NO}_2\text{-FA}$ in PMN, rather it is attributed to an increase in levels of cAMP that inhibit fMLP-stimulated $\text{O}_2^{\cdot-}$ production (114).

Nitro-linoleic acid also inhibits thrombin-induced human platelet aggregation. Platelet cAMP levels increase upon LNO_2 treatment, and inhibitors of adenylate cyclase restore thrombin-induced aggregation, indicating a role for cAMP (112). The inhibition of thrombin-induced platelet activation by LNO_2 is mediated by cAMP-dependent phosphorylation of Ser-159 in vasodilator-stimulated phosphoprotein (VASP) and attenuation of calcium mobilization. It is not yet clear how LNO_2 increases cAMP levels, but evidence indicates that it induces adenylate cyclase activity and causes a decrease in cAMP hydrolysis. A recent study reveals that AA- NO_2 inhibits LPS- and $\text{IFN}\gamma$ -stimulated $\cdot\text{NO}$ formation (92), with decreased $\cdot\text{NO}$ production due to inhibition of NOS2 expression rather than a result of decreased enzyme activity. The observation that $\text{NO}_2\text{-FA}$ abrogate activation of multiple inflammatory cell-types has encouraged further studies aimed at how $\text{NO}_2\text{-FA}$ affect specific inflammatory signaling events, including the modulation of enzymatic activities, gene expression and defining $\text{NO}_2\text{-FA}$ concentrations necessary required to elicit biological activity.

During inflammation, adaptive and protective responses are elicited by vascular and other tissues to protect the host from its own mechanisms directed at destroying invading pathogens. Recent studies have shown that HO-1 plays a central role in vascular inflammatory signaling and mediates a protective response to inflammatory stresses such as atherosclerosis, acute renal failure, vascular restenosis, transplant rejection and sepsis [for review, see (115)]. Heme oxygenase 1 catalyzes the degradation of heme to biliverdin, iron and CO, the last of which has been shown to display diverse, adaptive biological properties, including anti-inflammatory, anti-apoptotic and vasodilatory actions (116,117). During inflammation, HO-1 gene expression is up-regulated, with induction typically occurring transcriptionally. Nitrated linoleic acid potently induces HO-1 expression by a $\cdot\text{NO}$ - and $\text{PPAR}\gamma$ -independent mechanism in human aortic endothelial cells (118) (Fig. 6). Similarly, CLNO_2 suppresses inflammatory responses in LPS-stimulated macrophages (91). Though less potent than LNO_2 , CLNO_2 inhibits NOS2 expression and $\text{Nf-}\kappa\text{B}$ signaling, and induces HO-1 expression. Activated macrophages form CLNO_2 , which may thus represent an adaptive response to inflammation, leading to eventual resolution.

Nitro-fatty acid derivatives also potently inhibit stimulus-induced cytokine release from a variety of cells, highlighting the diversity of mechanisms by which nitroalkenes mediate signal transduction. Using the macrophage cell line THP-1, LNO_2 and OA- NO_2 inhibited LPS-

induced cytokine release, with significant reductions observed in interleukin-6 (IL-6), tumor necrosis factor alpha (TNF α), and monocyte chemoattractant protein-1 (MCP-1) levels versus cells treated with stimulus alone (119). The inhibition of cytokine release was shown to be NO-, PPAR γ - and HO-1 independent. Nitroalkenes also inhibit LPS-induced phosphorylation of the pro-inflammatory signal transducer and activator of transcription (STAT), thereby suppressing expression of its target genes including NOS2 and MCP-1. This inhibition appears to be mediated in part by the induction and activation of mitogen activated protein kinase phosphatase 1 (120).

Nitroalkene signaling is in part due to electrophilic reactions

Upon peroxidation, PUFAs are converted from hydroperoxides into electrophilic products via enzymatic and non-enzymatic pathways (121). These products include 15d-PGJ₂, 4-hydroxy-2-nonenal (4-HNE) and a variety of isoprostane derivatives (122,123). Electrophilic lipids react with cellular nucleophiles such as cysteine, histidine and lysine by Michael addition (121). For example, 4-HNE reacts with sulfhydryl groups (124), the imidazole moiety of histidine (125) and the ϵ -amino group of lysine residues (125). Covalent, post-translational modifications by electrophilic fatty acid derivatives alter the structure, trafficking and catalytic activity of proteins such as cathepsin B (126), Keap1 (127), insulin (128) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (129,130). For example, a key tissue defense against xenobiotics and oxidants is the regulated expression of phase II proteins (enzymes of glutathione (GSH) synthesis and transfer, quinone reductase, epoxide hydrolase, thioredoxin, transferrin, catalase, superoxide dismutase and HO-1) (131). This widespread mechanism, conserved in both plants and animals, protects against pathogens and metabolic or inflammatory stress. Phase II protein expression is regulated by the antioxidant response element (ARE), a *cis*-acting DNA regulatory element also referred to as the electrophile response element (EpRE). An elegant thiol oxidation-mediated transcription factor mechanism controls ARE-dependent electrophile responses, consisting of the pivotal Nrf2 (nuclear factor erythroid 2-related factor 2, a member of the basic-leucine zipper NF-E2 family of transcription factors) and b) the electrophile-reactive, cysteine-rich cytoplasmic suppressor protein Keap1 (Kelch-like ECH-associating protein).

The location of an electron-withdrawing nitro functional group on a carbon-carbon double bond makes the carbon beta to the nitro group electron poor, enabling electrophilicity and nucleophilic reactivity. Oxidized species such as lipid aldehyde derivatives can also react with nucleophilic amino acid residues to form Schiff's base products (132). Alternatively, protein adducts can be formed by a Michael addition reaction with protein thiolate anions reacting with the β -carbon centers of unsaturated carbonyls. The post-translational modification of proteins by oxidized electrophilic lipids has been reported in several instances, wherein electrophilic lipids covalently modify target proteins to induce altered protein structure/function (127, 133–136). Because the nitro group is one of the strongest electron-withdrawing functional groups known, the conjugation of the nitro group with an alkene confers highly electrophilic properties to the nitroalkene β -carbon, enabling robust Michael addition reactions with nucleophiles. In light of their bioactivity and presence *in vivo*, it is hypothesized that many if not most of the signaling actions of nitroalkenes are mediated by forming covalent adducts with nucleophilic sites on proteins (e.g., Cys residues), a reaction termed nitroalkylation (101).

Nitroalkylation is a biologically-relevant reaction between nitroalkenes and thiols (90,101). For example, LNO₂ reacts with GSH *in vitro* via Michael addition to form a covalent adduct (GS-LNO₂) in a time-dependent manner (90). The concerted actions of GSH and the multi-drug resistance protein-1 (MRP-1) have been shown to play an important role in regulating cellular nitroalkene levels. Confirmation that GSH reacts with LNO₂ and OA-NO₂ to form

covalent adducts comes from the identification of endogenous GS-OA-NO₂ and GS-LNO₂ in healthy human red blood cells (101). Comparison of the reaction rate constants between nitroalkenes, 15d-PGJ₂ and other biological electrophiles with GSH and cysteine revealed LNO₂ and OA-NO₂ to be significantly more electrophilic than 15d-PGJ₂, 4-HNE and the isoprostane 8-*iso* prostaglandin A₂ (73).

The avid reactivity of nitroalkenes with GSH prompted studies to determine whether proteins would also react with nitroalkenes to form adducts. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is useful as a model protein to study protein-thiol nitroalkylation for multiple reasons: (a) there is a catalytic thiol that is essential for activity (137), (b) GAPDH is inhibited by other electrophilic lipids (129,130) and (c) post-translational modification of GAPDH affects its sub-cellular localization and activity (138). Treatment of purified GAPDH with OA-NO₂ inhibits enzymatic activity (101) in a manner reversible by addition of GSH, resulting in the formation of GS-OA-NO₂ and non-adducted GAPDH (a process termed trans-nitroalkylation). Structural analysis revealed multiple covalent adducts, with five identified His and Cys residues modified by the nitroalkene. Of note, a peptide containing the catalytic Cys residue (Cys149) was identified as nitroalkylated, thus providing a mechanism for GAPDH inhibition by OA-NO₂. Incubating GAPDH in the presence or absence of OA-NO₂ in prepared liposomes demonstrated translocation of soluble GAPDH to membrane fractions upon treatment with OA-NO₂, suggesting post-translational modification by nitroalkenes *in vivo* may modulate protein sub-cellular localization. Studies have confirmed that nitroalkenes post-translationally modify proteins *in vivo* (101). It has been proposed that free nitroalkenes, derived from either lipid bilayer partitioning or from PLA₂-mediated lipolysis, react with proteins to modulate their activity and/or mediate membrane or lipoprotein translocation.

The discrete signaling actions of nitroalkylation are exemplified by the inhibition of LPS-stimulated cytokine release by nitroalkenes (119). The transcription factor NF-κB plays a pivotal role in orchestrating inflammatory responses via regulation of genes that encode pro-inflammatory cytokines, including IL-6, TNFα, MCP-1. In unstimulated cells, NF-κB is associated with an inhibitory protein (IκB); upon stimulation, IκB is degraded and NF-κB migrates to the nucleus to mediate gene expression (139). NF-κB is comprised of two subunits, p50 and p65; both contain a Cys residue in the DNA binding pocket that can be alkylated by electrophiles, including 15d-PGJ₂, sesquiterpene lactone, ethyl pyruvate and *N*-ethyl maleimide, resulting in the inhibition of NF-κB-mediated gene expression (140–143). Both OA-NO₂ and LNO₂ nitroalkylate p65 and inhibit DNA binding (119). Mitochondria isolated from IPC-treated rat hearts and LNO₂-treated cardiomyocytes also reveal protein nitroalkylation (102). In this instance, LNO₂ stimulated cardiac mitochondrial H⁺ leak, a process shown to be cardioprotective, via post-translational modification of the adenine nucleotide transporter and mitochondrial uncoupling protein-2 (144–146).

In summary, the post-translational modification of proteins by Michael addition reactions of nitroalkene derivatives appears to be a primary mechanism by which NO₂-FA exert their potent bioactivity. It is not clear yet whether nitroalkylation accounts for LNO₂- and OA-NO₂-mediated inhibition of PMN and platelet activation, HO-1 induction and smooth muscle cell proliferation. However, considering the strong electrophilic nature of the nitroalkene moiety, the abundance of critical nucleophiles as catalytic residues in both protein kinases and phosphatases of signaling pathways regulating the expression and activity of these processes, this mechanism must be considered when evaluating these aspects of NO₂-FA bioactivity. Indeed, nitroalkylation is also emerging as an important element in the strong protein/ligand interaction that is appreciated to occur between nitroalkenes and peroxisome proliferator-activated receptors (PPARs).

Nitroalkenes are potent, endogenous PPAR γ ligands

Peroxisome proliferator-activated receptor gamma (PPAR γ) belongs to the nuclear receptor superfamily that forms heterodimers with retinoid X receptors (RXR), which bind to DNA PPAR responsive elements (PPRE) and regulate target gene transcription in response to small lipophilic ligands. PPAR γ is expressed throughout the vasculature, including monocytes/macrophages, endothelial cells and vascular smooth muscle cells (147). Nuclear receptor/cofactor interactions play an important role in the pathology of disease, with PPAR γ established as a master regulator of metabolism, inflammation, adipogenesis and insulin sensitization (148). Moreover, these inherent functions of PPAR γ are driven by different subsets of target genes. PPAR γ ligands exert their effects by mediating conformational changes in the receptor. Most of the co-activators share a conserved LXXLL motif that mediates the interaction of the co-activator with the LBD (149,150). Co-repressors are complexed to the receptor during the non-ligand bound state or in the presence of antagonists. Upon activation, helix 12 (AF-2 located on the C-terminal of the ligand binding domain) of PPAR γ adopts an active conformation that promotes co-activator recruitment and co-repressor release (151,152). Two naturally occurring mutations in human PPAR γ have been proposed to destabilize the AF-2 helix and impair co-repressor recruitment, which results in severe insulin resistance, type II diabetes and early-onset hypertension (151,153). Moreover, a common polymorphism in PPAR γ (Pro12Ala) is associated with a decreased incidence in type II diabetes (154), and a mutation that prevents receptor phosphorylation (S112A) increases insulin sensitivity without the side effect of increased body weight (155). It has thus been proposed that ligands that can mimic the conformational change induced by phosphorylation of PPAR γ may avoid the documented deleterious effects of TZDs (155).

High, often non-physiological, concentrations (>50 μ M) of fatty acids, prostaglandin metabolites and oxidized fatty acid derivatives activate PPAR γ , α , and δ (156–158). Fatty acids containing an α - β unsaturated ketone as a core structural element, such as 15d-PGJ₂, also activate PPAR γ (159). Docking of 15d-PGJ₂ to the ligand binding domain (LBD) is not sufficient to activate the receptor; rather, a covalent Michael addition reaction (locking reaction) is required for activation. The PPAR γ receptor contains a critical thiol (Cys285) in the LBD, with covalent modification of this highly-conserved Cys285 (analogous to Cys313 in the PPAR γ 2 isoform) by thiol-reactive compounds sufficient to induce partial receptor activation (160,161). It is problematic that currently-proposed endogenous PPAR ligand candidates require concentrations 2–3 orders of magnitude greater than their physiological concentration ranges before even partial activation occurs. For example, 15d-PGJ₂ is present in plasma at less than a 5 nM concentration, is not a strong electrophile and has been discounted as an endogenous PPAR γ ligand (162).

The discovery of LNO₂ and OA-NO₂, and the appreciation that these molecules are electrophilic, motivated studies to determine whether they activate PPARs (100,163). Luciferase reporter transactivation assays demonstrated predominantly PPAR γ , but also PPAR α and δ activation by nitroalkenes. Significant activation of PPAR γ occurs within physiological concentrations. These studies were confirmed using OA-NO₂ as a PPAR γ ligand (100). Experiments designed to report down-stream effects of PPAR γ activation revealed both LNO₂ and OA-NO₂ induced PPAR γ 1 and 2, aP2 and CD36 expression in 3T3-L1 pre-adipocytes. Furthermore, both compounds increase glucose uptake and induce 3T3-L1 differentiation to adipocytes—factors that are specifically under the control of PPAR γ . In a study designed to compare the potency between Rosiglitazone and LNO₂ as PPAR γ activators, it was determined that at equal concentrations both drugs similarly and significantly increased glucose uptake; however, LNO₂ induced significantly lower adipogenesis than Rosiglitazone. One of the adverse side-effects of TZD treatment is weight gain; thus a therapeutic treatment

for type II diabetes that ameliorates glucose homeostasis without causing weight gain would be a significant advance in the treatment of this disease.

The mechanism by which LNO₂ activates PPAR γ has been determined with recent solution of the crystal structure of PPAR γ having LNO₂ occupancy in the LBD (164) (Fig. 8). The data show that the nitroalkene moiety is proximal to Cys285 and is anchored to the binding domain via hydrogen bonding between residues Arg288 and Glu343 and the nitro group in LNO₂. Mutations in Glu343 greatly reduced LNO₂ activation of PPAR γ in cell-based assays, further confirming the hydrogen bonds formed between LNO₂ and amino acids in the LBD are essential to stabilize the ligand/receptor complex and activate PPAR γ . Thus, LNO₂ and OA-NO₂ are endogenous molecules that have the ability to activate PPARs, with PPAR γ activation being the most prominent. Nitroalkenes and Rosiglitazone are equipotent in activating PPAR γ but appear to stabilize in the LBD by different mechanisms. Differential conformational changes to PPAR γ resulting from this unique endogenous ligand have the capacity to impart unique specificity to the downstream signaling events resulting from PPAR γ activation.

Conclusions

Signal transduction reactions, rather than occurring via discrete, linear mechanisms, are increasingly appreciated as interdependent, cascading events where 'cross talk' is rule rather than exception. The web of reactions that occur with one of the most structurally simple of signaling mediators, NO, paradoxically highlights this concept. Either directly, via activation of sGC, or indirectly via the redox-dependent formation of secondary oxides of nitrogen, NO broadly and profoundly impacts inflammation and metabolic homeostasis (Fig. 7). On one level, reactions between NO, eicosanoids and enzymes of eicosanoid synthesis provide reciprocal control of their respective pathways. On another level, however, the convergent reactions between these signaling pathways lead to the formation of a distinct class of signaling molecules, nitro-fatty acids, which are imbued with strong anti-inflammatory and metabolic signaling actions. Observations to date support that NO₂-FA contribute to the resolution of inflammation by mediating adaptive signaling reactions such as inhibition of p65 DNA binding, activation of Nrf2-dependent gene expression and stimulation of expression of PPAR γ -regulated genes.

A working model is emerging wherein during inflammation, NO₂-FA are either formed and released from a pool of complex lipid precursors by PLA₂, or NO₂-FA are generated directly from free radical-mediated oxidative and nitrative reactions. NO₂-FA then transduce signaling reactions by covalently modifying nucleophilic protein targets to alter structure/function of proteins such as enzymes, lipid receptors and transcription factors. The implications from the cell-based studies suggest this class of lipid signaling mediators could be used as a therapeutic strategy to treat metabolic diseases such as type II diabetes mellitus or ameliorate inflammatory conditions such as ischemia-reperfusion injury or arthritis. Biochemical foundations have thus established NO₂-FA as a distinct class of signaling mediators, now it remains important to translate this fundamental knowledge to the pharmacologic investigation of clinical problems.

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Abbreviations

NO	nitric oxide
sGC	soluble guanylate cyclase
cGMP	cyclic guanosine monophosphate
COX	cyclooxygenase
LOX	lipoxygenase
NO₂-FA	nitrated fatty acids
NOS	nitric oxide synthase
ONOO⁻	peroxynitrite
RSNO	nitrosothiols
PUFAs	polyunsaturated fatty acids
O₂^{•-}	superoxide
NO₂⁻	nitrite
NO₃⁻	nitrate
HNO	nitrosyl
H₂NOH	hydroxylamine
ONOOCO₂⁻	nitrosoperoxocarbonate
PLA₂	phospholipase A ₂
AA	arachidonic acid
PGHS1/2	prostaglandin endoperoxide H synthase 1 and 2

PGI₂	prostacyclin
PAF	platelet-activating factor
NO₂	nitrogen dioxide
HNO₂	nitrous acid
LOOH	lipid hydroperoxides
LONO₂	alkyl nitrates
LNO₂	nitrated linoleic acid
AA-NO₂	nitrated arachidonic acid
CLNO₂	nitro-cholesterol linoleate
AA(OH)NO₂	nitrohydroxyeicosatrienoic acid
OA-NO₂	nitrated oleic acid
IPC	ischemic preconditioning
GTP	guanosine triphosphate
RNNO	nitrosamine
oxyHB	oxyhemeoglobin
cPTIO	2-(4-carboxyphenol)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
PMN	polymorphonuclear leukocytes
VASP	vasodilator-stimulated phosphoprotein
HO-1	hemeoxygenase-1
TNFα	tumor necrosis factor alpha

MCP-1	monocytes chemoattractant protein-1
STAT	signal transducer and activator of transcription
4-HNE	4-hydroxy-2-nonenal
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GSH	glutathione
ARE	antioxidant response element
EpRE	electrophilic response element
Nrf2	nuclear factor erythroid 2-related factor 2
Keap-1	Kelch-like ECH-associating protein
MRP-1	multi-drug resistant protein-1
PPAR	peroxisome proliferator-activated receptor

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Fig. 1. Sources of $\bullet NO_2$

During host inflammatory responses, the activities of NADPH oxidase and nitric oxide synthase-2 are increased elevating steady state levels of superoxide ($O_2^{\bullet -}$) and ($\bullet NO$). Reaction between these two free radicals generates peroxynitrite ($ONOO^-$), which when protonated at neutral pH, homolyzes to form the hydroxyl radical ($\bullet OH$) and nitrogen dioxide ($\bullet NO_2$). Also, reaction of $ONOO^-$ with CO_2 yields nitrosoperoxocarbonate ($ONOOCO_2^-$) that undergoes homolytic scission to carbonate radical ($HCO_3^{\bullet}$) and $\bullet NO_2$. Dietary-derived nitrite and nitrates can be converted to $\bullet NO_2$ in the presence of bacterial flora and acidic conditions found in the gastric compartment.

Fig. 2. Fatty acid oxidation pathways

The PLA₂-mediated arachidonic acid release from phospholipids provides the precursor to a diverse array of oxidized lipids including the prostaglandins, leukotrienes and isoprostanes. The eicosanoids and pathways illustrated herein are not comprehensive but are most directly relevant to this review. Virtually any fatty acid containing a 1,4-pentadienyl bond configuration (e.g., linoleic, linolenic, eicosapentaenoic and docosahexaenoic acids) can be oxidized via these oxidation pathways to generate a vast spectrum of lipid oxidation products. *The ribbon structure of PGHS is courtesy of Dr. R.M. Garavito, Michigan State University.*

Fig. 3. The effect of $\cdot\text{NO}$ on PGHS1/2 activity

A) Oxidation of arachidonic acid occurs at two separate but interdependent regions of PGHS that catalyze cyclooxygenation to PGG_2 and peroxidation to PGH_2 . B) The activity of PGHS1/2 is modulated by $\cdot\text{NO}$ and secondary oxides of nitrogen (i.e., ONOO^-) by acting as a substrate to the peroxidase activity, which 'primes' the cyclooxygenase activity of the enzyme. The level of ONOO^- depends on the balance of $\text{O}_2^{\cdot-}$ and $\cdot\text{NO}$ concentrations. At high and possibly non-biological concentrations of ONOO^- , however, PGHS becomes nitrated, resulting in inhibition of the enzyme.

Fig. 4. Fatty acid nitration

Unsaturated fatty acids can be nitrated by a variety of free radical-mediated mechanisms, the majority of which involve $\cdot\text{NO}_2$. The diversity of molecular species generated is driven by the complex redox environment that lipids can be exposed to, double bond rearrangement, secondary metabolism (i.e., β -oxidation, saturation, desaturation) and reaction with nucleophiles.

Fig. 5. Nitric oxide release by nitroalkenes

Two mechanisms have been proposed to explain the release of $\cdot NO$ from nitroalkenes in an aqueous environment. In mechanism I, a nitroso intermediate can be formed during aqueous decay. The C—N bond of the nitroso compound is expected to be very weak and can homolyze to form $\cdot NO$ and a carbon radical stabilized by the conjugated double bond and the hydroxyl group (107). Mechanism II proposes a nitroalkene rearrangement to form a nitrite ester. This species can also homolyze to form $\cdot NO$ and a stabilized radical (96).

Fig. 6. Induction of HO-1 in rat aortic endothelial and smooth muscle cells

Nitrated linoleic acid induces expression of HO-1, which catalyzes the breakdown of heme to CO, a molecule that also mediates anti-inflammatory signaling actions (118). Aortic segments were treated with vehicle or LNO₂ for 16 h, and prepared for imaging. Immunofluorescence detection of HO-1 protein (green) and DAPI nuclear staining was performed on sections with nonimmune rabbit IgG as a negative control (*Center*). (*Upper Panels*) A higher magnification of the endothelial layer is shown.

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Fig. 8. LNO₂ bound the ligand binding domain of PPAR γ

A) The ribbon structure of the ligand binding domain of PPAR γ is shown with bound LNO₂. The nitro-fatty acid ligand is stabilized by different amino acids than the synthetic ligand Rosiglitazone, which may explain the differential signaling actions and potencies of the two compounds. B) A closer view of bound LNO₂, which is presented in green.

Table 1

Nitroalkene Chemical Structures

Name	Formula	Structure
Nitrated Oleic Acid 9- and 10-nitro- <i>cis</i> -octadecenolic acids	OA-NO ₂	
Nitrated Linoleic Acid 9-, 10-, 12- and 13-nitro- <i>cis</i> -octadecadienoic acids	LNO ₂	
Nitrated Arachidonic Acid 5-, 6-, 8-, 9-, 11-, 12-, 14-, and 15-nitro- <i>cis</i> -eicosatetraenoic acids	AA-NO ₂	
Nitrated Cholesteryl Linoleate Cholesteryl-9-, 10-, 12- and 13-nitro- <i>cis</i> -octadecadienecates	CLNO ₂	

Table 2

Biological Activity of Nitroalkenes

Chemical Name	Biological Action/Activity	Reference
LNO ₂	γ Relaxes precontracted rat aortic rings	(88,104)
	γ Inhibits O ₂ ^{•−} release in PMN, platelet aggregation and macrophage degranulation	(110,111)
	γ Upregulates HO-1 expression	(117)
	γ Inhibits LPS-induced cytokine release	(119)
	γ Inhibit NF-κB mediated gene expression	(118)
	γ Binds and activates PPARγ	(162,163)
	γ Cardioprotective against ischemia-reperfusion injury	(100)
OA-NO ₂	γ Directs protein/membrane association	(109)
	γ Inhibits stimulus induced cytokine release	(119)
	γ Inhibits NF-κB mediated gene expression	(118)
	γ Activates PPARγ	(99)
	γ Increase glucose uptake and insulin sensitivity	(99)
AA-NO ₂	γ Decreases NOS2 expression	(91)
	γ Relaxes precontracted rat aortic vessels	(91)
CLNO ₂	γ Decreases NOS2 expression	(90)
	γ Upregulates HO-1 expression	(90)
	γ Inhibits stimulus induced cytokine release	(90)
	γ Inhibits NF-κB mediated gene expression	(90)