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Nitro-fatty acids: novel anti-inflammatory lipid mediators

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Abstract

Nitro-fatty acids are formed and detected in human plasma, cell membranes, and tissue, modulating metabolic as well as inflammatory signaling pathways. Here we discuss the mechanisms of nitro-fatty acid formation as well as their key chemical and biochemical properties. The electrophilic properties of nitro-fatty acids to activate anti-inflammatory signaling pathways are discussed in detail. A critical issue is the influence of nitroarachidonic acid on prostaglandin endoperoxide H synthases, redirecting arachidonic acid metabolism and signaling. We also analyze *in vivo* data supporting nitro-fatty acids as promising pharmacological tools to prevent inflammatory diseases.

Key words: Nitro-fatty acids; Antioxidants; Nitric oxide; Inflammation

Introduction

Nitric oxide ($^{\bullet}NO$)-derived species (NOx) react with unsaturated fatty acids to yield a variety of oxidized and nitrated products (1). In particular, nitroalkenes (NO_2-FA) have been detected, identified, and quantified in plasma as well as in cell membranes and tissue (2,3). The mechanisms of fatty acid nitration *in vivo* remain unknown, with suggested pathways including reactions of unsaturated fatty acids with secondary products of $^{\bullet}NO$ oxidation such as nitrogen dioxide ($^{\bullet}NO_2$), nitrite (NO_2^-), and peroxynitrite ($ONOO^-$; Figure 1). Nitrogen dioxide can be formed from $^{\bullet}NO$ autoxidation (4). Alternatively, $^{\bullet}NO_2$ can be formed from NO_2^-, since NO_2^- is present in physiological fluids at high concentrations (5,6). Moreover, NO_2^- should be exposed to low pH in the gastric compartment as well as in phagocytic lysosomes to generate $^{\bullet}NO_2$; indeed, the human stomach is a source of $^{\bullet}NO$ and bioactive nitrogen oxides from precursors present in food and saliva (6). A critical step is the protonation of NO_2^- in the gastric lumen, thereby forming nitrous acid (HNO_2), which can also form $^{\bullet}NO$, $^{\bullet}NO_2$, and other nitrogen oxides (6). An additional lipid nitration mechanism involves peroxynitrite. Peroxynitrite anion ($ONOO^-$) and peroxynitrous acid (ONOOH) are potent one- and two-electron oxidants that can react with unsaturated fatty acids (4). Homolysis of ONOOH yields $^{\bullet}NO_2$ and hydroxyl radical ($^{\bullet}OH$). In fact, $ONOO^-$, ONOOH, and/or their derived radicals have been observed to readily diffuse through membranes to mediate fatty acid oxidation and nitration (1,7-10). Several reports support NO_2-FA formation *in vivo* (11-14). In fact, nitroalkenes are present endogenously as free, esterified, and nucleophilic-adducted species (12), and although reports about *in vivo* concentrations have changed from the micromolar (2) to the picomolar range (15) in the past few years, their presence has been shown to be greatly increased in inflammatory models (12,14,16). During macrophage activation by an inflammatory stimulus, one of the major esterified lipid components, cholesteryl linoleate (CL), becomes nitrated at the fatty acid moiety (12). The formation of cholesteryl nitrolinoleate (NO_2-CL) by activated macrophages is prevented by nitric oxide synthase (NOS) inhibitors, supporting the contribution of $^{\bullet}NO$-derived species toward CL nitration. More recently, it has been demonstrated that NO_2-FA is both present and formed in mitochondria from cardiac ischemia/reperfusion (13) or ischemic preconditioned (14) hearts.

Nitric oxide and arachidonic acid signaling are linked through nitro-fatty acids

It has been well established that arachidonic acid (AA) signaling cascades and $^{\bullet}NO$ pathways are intrinsically related (17). Nitration of AA yields a nitroalkene, nitroarachidonic acid (NO_2-AA). In activated macrophages,

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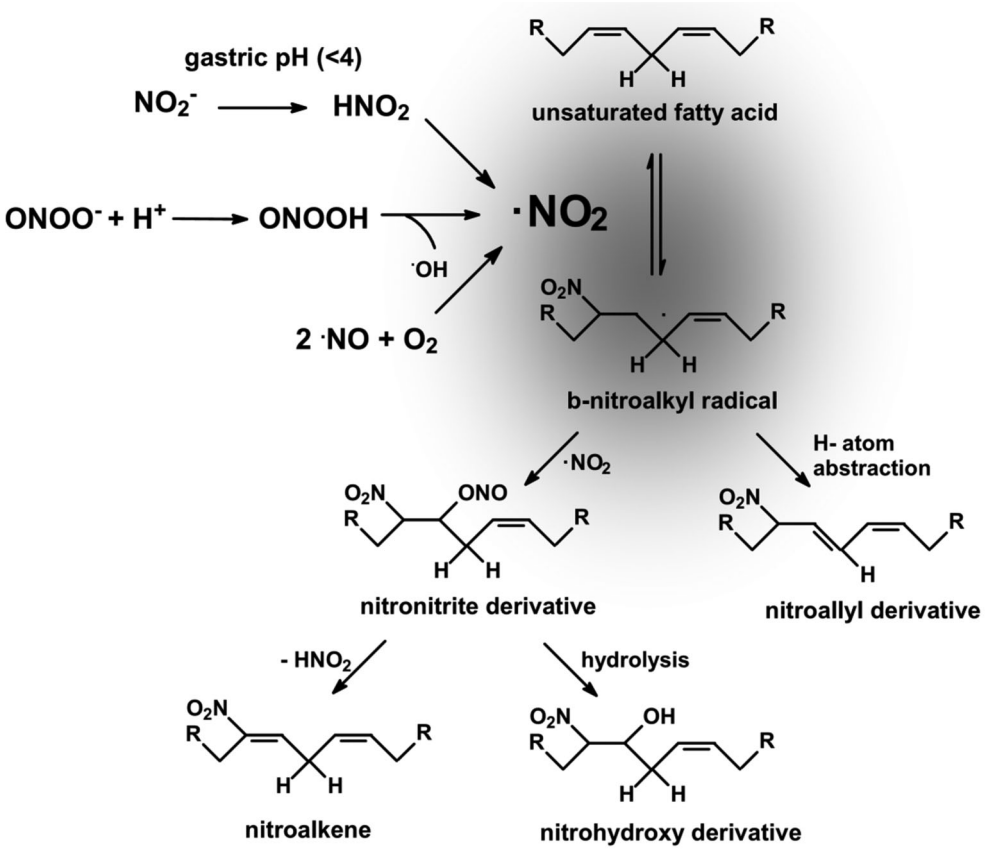

Figure 1. Mechanisms of unsaturated fatty acid nitration. Nitrogen dioxide can be formed by at least three major biologically relevant mechanisms (see text) and react with unsaturated fatty acids to preferentially form (at low oxygen tensions) nitro-alkenes (nitro group bonded at the double bond) and nitro-allyl derivatives (nitro group bonded at a single bond). NO_2-FA alkylates susceptible thiols of multiple transcriptional regulatory proteins, affecting downstream gene expression and the metabolic and inflammatory responses under their regulation.

NO_2-AA exerts a protective anti-inflammatory action, diminishing NOS-2 expression and secretion of proinflammatory cytokines (18). Downregulation of NOS-2 by nitroalkenes should contribute to the physiological shutdown of inflammatory responses in macrophages. Both NO_2-AA and its methylated form (Met6-NO_2-AA) increased cGMP levels in treated endothelial cells, suggesting that guanylate cyclase was activated directly or via *NO/*NO-like species (19). Nitroalkenes react with nucleophilic residues in proteins (16); they are potent electrophiles, and the addition of a nitro group (-NO_2) to a double bond at the carbon chain of the unsaturated fatty acids leads to an alkenyl nitroconfiguration with electrophilic reactivity of the β-carbon adjacent to the nitro-bonded carbon. Through Michael addition reactions, nitroalkenes can react with nucleophiles (i.e., Cys or His residues), yielding a new carbon-carbon or carbon-heteroatom bond framework (20-22). Biochemical studies reveal that NO_2-FA rapidly and reversibly undergoes Michael addition with thiols and, to a lesser extent, primary and secondary amines (20-22). In contrast to other lipid-derived electrophiles, nitroalkylation of Cys and His is reversible (16,20,22). Through this mechanism, NO_2-FA alkylate susceptible thiols of multiple transcriptional regulatory proteins affect downstream gene expression and the metabolic and inflammatory responses under their regulation.

Nitroalkene activation of anti-inflammatory signaling pathways

Under physiological conditions, intracellular levels of glutathione can detoxify NOx, favoring NO_2-FA formation to activate anti-inflammatory signaling pathways (Figure 2). When high production rates of NOx such as peroxynitrite occur related to inflammation, antioxidant mechanisms are compromised; then, protein tyrosine nitration (and oxidation) increase and participate in events such as mitochondrial cytochrome *c* release and apoptosis (Figure 2). Even under this unfavorable biochemical scenario, NO_2-FA may serve as a cytoprotective agent,

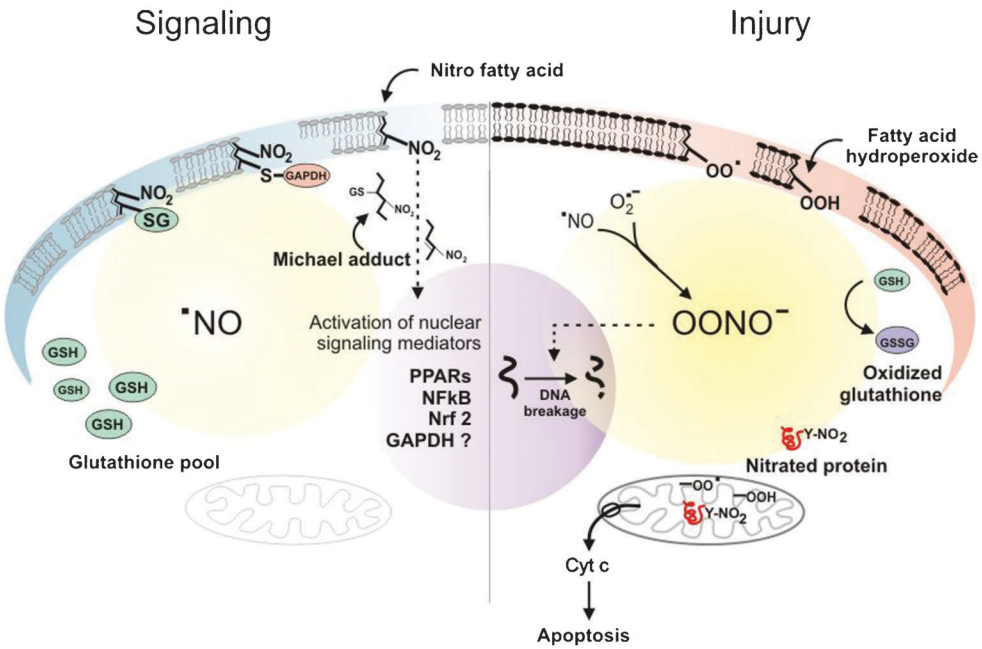

Figure 2. Nitroalkene-activation of anti-inflammatory signaling pathways. On the left side of the scheme, the signaling role of nitroalkenes and nitroalkene thiol adducts on transcription factors is indicated in a cell with normal levels of GSH. On the right side, cytotoxic events predominate as a result of an increase in NOx, leading to pro-oxidant pathways that include GSH depletion, protein tyrosine nitration and lipid oxidation, triggering apoptotic cascades. Adapted from Ref. 47, with permission.

partially counteracting the proinflammatory effects of oxidant exposure, thus inhibiting the propagation of lipid oxidation and protein nitration, in part by attenuating the oxidant-dependent inflammatory response. Key anti-inflammatory mechanisms include the following.

Peroxisome proliferator-activated receptor (PPAR) activation

The transcriptional factor PPAR has been found to serve as a nuclear receptor capable of selectively binding NO_2-FA. Nitroalkenes are able to potently regulate the expression of multiple PPAR target genes (23-25). The effects of NO_2-FA on full-length PPAR were then tested in transfection assays using a PPARγ response reporter, where nitroalkenes potently activated all PPAR subtypes, having a stronger activity on PPARγ than PPARα and PPARβ/δ (11). This is of significance since PPARγ has been associated with anti-inflammatory actions such as modulation of expression of several proinflammatory cytokines and chemokines in activated macrophages.

Inhibition of nuclear factor-kappa B (NF-κB)

Various mechanisms have been proposed to explain the protective actions exerted by nitroalkenes. One of them involves the inhibition of NF-κB translocation to the nucleus (11,13). In fact, NF-κB plays an important role during inflammatory responses, regulating genes that encode proinflammatory cytokines. Nitroalkenes can inhibit lipopolysaccharide-induced secretion of proinflammatory cytokines in macrophages [e.g., interleukin-6, tumor necrosis factor-α, and monocyte chemoattractant protein 1 (MCP-1)]. These observed effects resulted from the covalent alkylation of recombinant NF-κB p65 protein *in vitro* and from a similar reaction with the p65 subunit in macrophages. Inhibition of NF-κB migration to the nucleus inhibited DNA binding activity and repressed NF-κB-dependent target gene expression (11).

Induction of hemoxygenase-1 (HO-1)

HO-1 catalyzes the oxidative degradation of heme to biliverdin, exerting anti-oxidant and anti-inflammatory actions. Induction of HO-1 is an endogenous cytoprotective pathway triggered by a variety of stress-related signals and electrophilic species. Nitroalkenes induce HO-1 expression in endothelial cells (26), RAW264.7 (11), and J774.1 macrophages (12) by a PPARγ-independent mechanism as well as both $^{\bullet}NO$-dependent and $^{\bullet}NO$-independent mechanisms (26). Considering the vascular protective effects of HO-1 expression, induction of HO-1 represents a key novel cell-signaling action of nitroalkenes.

Activation of nuclear factor E2-related factor 2 (Nrf2)

Nrf2 is a mediator of antioxidant and phase II detoxifying enzyme expression (27-29). Nrf2 is a transcription factor that is in an inactive form in the cytosol due to the activity of Kelch-like ECH-associated protein 1

(Keap1). When activated Nrf2 migrates to the nucleus, it binds as a heterodimer to the antioxidant response element (ARE) in DNA, activating the expression of phase 2 enzymes (29). Potential activators for Nrf2 include lipid electrophiles that react with reactive Keap1 thiols, dissociating Nrf2 from ubiquitin E3 ligase complex and facilitating nuclear accumulation and downstream effects on gene transcription (29). Keap1 is highly reactive to nitroalkylation because it constitutes a cysteine-rich protein (27-29). Diverse functional studies using Keap1 mutants showed that Cys^{151} is an electrophile sensor residue whose adduction causes the dissociation of Keap1 from Cul3, preventing Nrf2 proteosomal degradation and allowing the activation of its target genes via binding to AREs (30-32). Nitro-fatty acids are Cys^{151}-independent Nrf2 activators, as Keap1 Cys^{151} mutants remain unaffected by nitroalkenes, enhancing, instead of diminishing, the binding of Keap1 with Cul3 (33). Like HO-1, Nrf2 activation protects various cell types against oxidative stress. In this way, vascular smooth muscle cell (VSMC) proliferation is inhibited by physiological levels of nitroalkenes (34). The signaling pathway that participates in this action involves the Nrf2/ARE system. During VSMC inhibition of proliferation, Nrf2 nuclear translocation is enhanced by NO_2-FA, suggesting that this signaling cascade is also involved in the observed anti-inflammatory actions of nitroalkenes (34). The role of NO_2-FA on the Nrf2 pathway has also been explored in human aortic endothelial cells (35). The expression of Nrf2-dependent genes, including HO-1 and glutamate cysteine ligase modifier subunit, was significantly stimulated by NO_2-FA; however, array analyses showed that the majority of NO_2-FA-regulated genes were regulated by Nrf2-independent pathways. More in depth studies demonstrated that the heat shock response is the major pathway activated by NO_2-FA (35). Regulation of the heat shock response is a novel anti-inflammatory and cytoprotective action of NO_2-FA in addition to the other protective cell signaling functions reported for nitroalkenes.

Modulation of prostaglandin endoperoxide H synthase (PGHS)

PGHS is a key enzyme of AA metabolism catalyzing the formation of prostaglandin H_2 (36-38). Two isoforms of PGHS (PGHS-1 and PGHS-2) are found in mammalian tissues. PGHS-1 is constitutively expressed, whereas PGHS-2 is an inducible enzyme. Both isoforms are of pharmacological importance because they are targets for nonsteroidal anti-inflammatory drugs (39). Prostaglandin H_2 formation by PGHS catalysis involves two separate reactions at different active sites (36-38). The first is oxidation of AA by the cyclooxygenase reaction to yield prostaglandin G_2, where two molecules of oxygen are added to AA, and the second is reduction of the hydroperoxyl group at C15 by the peroxidase reaction, yielding prostaglandin H_2 (37,38,40). We have recently evaluated the interaction of the nitrated derivative of AA with PGHS (41). Kinetic analysis showed that the inhibition of peroxidase activity exerted by NO_2-AA was time- and concentration-dependent in both PGHS-1 and PGHS-2, suggesting a two-step mechanism of inactivation: an initial reversible binding followed by a practically irreversible event leading to enzyme inactivation. Inactivation was associated with an irreversible disruption of heme binding to the protein. The observed effects for NO_2-AA were selective, since other nitroalkenes tested were unable to inhibit enzyme activity. In activated human platelets, NO_2-AA significantly decreased PGHS-1-dependent thromboxane B_2 formation in parallel with a decrease in platelet aggregation, thus confirming the biological relevance of this novel inhibitory pathway (41). These anti-platelet effects were cGMP-independent and did not involve Ca^{2+}-store-dependent mobilization, providing a possible novel mechanism for platelet regulation *in vivo*. Signaling downstream of protein kinase C (PKC), such as α-granule secretion and extracellular signal-regulated kinase 2 activation, was strongly inhibited by NO_2-AA (42). Inhibition of PKCα translocation to the plasma membrane represents a potential mechanism for platelet regulation *in vivo*.

Modulation of NADPH oxidase (NOX)

A novel additional mechanism by which NO_2-AA may have anti-inflammatory actions is the regulation of superoxide radical ($O_2^{\cdot-}$) production via NOX isoforms. In fact, nitroalkenes may alter the formation of $O_2^{\cdot-}$ during macrophage activation by modulating phagocytic NOX-2. Recent data show that NO_2-AA inhibits NOX-2-mediated $O_2^{\cdot-}$ production in activated macrophages (43). The mechanism involves prevention of migration of cytosolic subunits to the membrane, thus inhibiting correct assembly of the active enzyme. This inhibitory role of nitroalkenes observed during macrophage activation could facilitate the resolution of inflammation.

Therapeutic potential

While the biochemical mechanisms leading to lipid nitration are under active investigation, there is unambiguous evidence of their formation *in vivo* as well as their increase during inflammatory conditions. Thus, it is possible that, at the levels expected to be found *in vivo* during chronic inflammatory conditions, nitrated lipids may serve as anti-oxidant and anti-inflammatory agents, partially counteracting the proinflammatory effects of oxidant exposure. There are several reports using NO_2-FA as pharmacological modulators of inflammation-related diseases in animal models (13,14,44). Nitro-fatty acid subcutaneous administration to angiotensin II-treated mice significantly lowered the increase in blood pressure as well as the contractile responses through NO_2-FA binding to the AT1R, modulating intracellular signaling

cascades (inositol-1,4,5-trisphosphate and calcium mobilization) (44). These results show that NO_2-FA diminishes the pressor response to angiotensin II, inhibiting AT1R-dependent vasoconstriction and suggesting that NO_2-FA can be a pharmacologically relevant modulator of hypertension (44). NO_2-FA were also tested in C57BL/6 mice subjected to coronary artery ligation followed by 30-min reperfusion (I/R). When administered exogenously during an ischemic episode, NO_2-FA exerted protection against I/R injury, reducing the infarct size as well as preserving the left ventricular function (13). In an animal model of atherosclerosis, subcutaneous administration of NO_2-FA potently reduced atherosclerotic lesion formation (45). More recently, it has been demonstrated that acute administration of NO_2-FA is effective to reduce vascular inflammation *in vivo* (46). The mechanism involves a direct role for NO_2-FA in the disruption of the Toll-like receptor 4 signaling complex in lipid rafts, leading to resolution of proinflammatory activation of NF-κB in the vasculature.

Although beneficial effects of NO_2-FA have been clearly demonstrated in different *in vivo* models, there are still no reports evaluating the potential toxicity that this compound could exert when administered for longer periods of time. Further study is necessary to determine whether NO_2-FA supplementation would exert novel anti-inflammatory and tissue protective actions in human disease.

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