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Inflammatory signaling and metabolic regulation by nitro-fatty acids

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Abstract

The addition of nitrogen dioxide (•NO_2) to the double bond of unsaturated fatty acids yields an array of electrophilic nitro-fatty acids (NO_2-FA) with unique biochemical and signaling properties. During the last decade, NO_2-FA have been shown to exert a protective role in various inflammatory and metabolic disorders. NO_2-FA exert their biological effects primarily by regulating two central physiological adaptive responses: the canonical inflammatory signaling and metabolic pathways. In this mini-review, we summarize current knowledge on the regulatory role of NO_2-FA in the inflammatory and metabolic response *via* regulation of nuclear factor kappa B (NF-κB) and peroxisome proliferator-activated receptor γ (PPARγ), master regulators of inflammation and metabolism. Moreover, the engagement of novel signaling and metabolic pathways influenced by NO_2-FA, beyond NF-κB and PPAR signaling, is discussed herein.

Graphical Abstract

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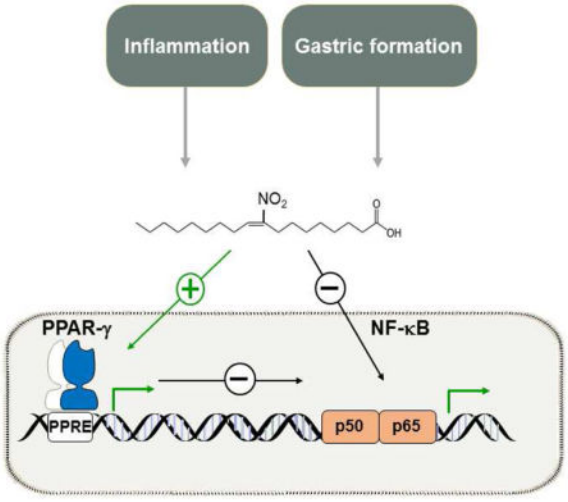

Keywords

Nitro-fatty acids; Inflammation; Metabolism; NF-κB; PPARγ

1. Introduction

Endogenous nitro-fatty acids (NO_2-FA) are generated during inflammation and digestion through non-enzymatic reactions of unsaturated fatty acids with nitrogen dioxide ($^{\bullet}NO_2$) [1]. Conjugated linoleic acid (CLA) is the preferred fatty acid target of nitration reactions due to the highly reactive external flanking carbons in the conjugated diene, yielding electrophilic nitro-conjugated linoleic acid (NO_2-CLA), the predominant endogenous isomer detected *in vivo* [2].

A large body of evidence has accumulated during the last decade supporting a protective role for NO_2-FA in numerous experimental settings [3]. These include endotoxin-induced vascular inflammation, endotoxemia and multi-organ injury [4; 5], inflammatory bowel disease (IBD) [6], allergic airway disease [7], renal ischaemia and reperfusion (I/R) injury and diabetic kidney disease [8; 9], pulmonary arterial hypertension (PAH) [10; 11], myocardial I/R injury [12], hypertension [13; 14], and atherosclerosis [15]. Beyond the above experimental models, the safety and pharmacokinetics of NO_2-FA have been clinically examined in four successfully completed phase I trials (NCT02127190, NCT02248051, NCT02460146, NCT02313064) [16; 17; 18; 19]. Currently, NO_2-FA administration is entering phase II clinical trials for the treatment of focal segmental glomerulosclerosis (FSGS), PAH and obese asthmatics.

The above protective effects on NO_2-FA are meditated by their pluripotent cell signaling capabilities, affecting various intracellular pathways. First, NO_2-FA modulate nitric oxide (•NO) signaling by yielding low concentrations of •NO (*via* Nef reaction) that mediate cGMP-dependent cell signaling activities and *via* a non cGMP-dependent manner in which NO_2-FA regulate endothelial and inducible nitric oxide synthase (eNOS and iNOS)-mediated •NO generation and reactions [20; 21; 22]. Second, NO_2-FA can potently regulate

the expression of key inflammatory, cell proliferation and differentiation-related genes [4; 23; 24; 25; 26; 27]. Third, NO_2-FA are endogenous ligands for peroxisome proliferator-activated receptors (PPARs), mainly PPARγ, centrally involved in lipid and glucose homeostasis as well as inflammation [28; 29; 30]. Finally, NO_2-FA facilitate reversible adduction by nucleophilic targets (e.g. Cys and His protein residues), leading to the post-translational modifications (PTM) of proteins [31; 32]. Particularly, the electrophilic nature of NO_2-FA results in nitroalkylation of nuclear factor kappa B (NF-κB), the master regulator of the immune and inflammatory response, inhibiting its DNA binding activity and repressing inflammatory gene expression, underlying a key role for NO_2-FA as endogenous anti-inflammatory signaling mediators [24]. This mini-review focuses on NO_2-FA regulation of NF-κB and PPARγ, key regulators of inflammation and metabolism, and the implication for inflammatory and metabolic disorders.

2. NO_2-FA promote cellular anti-inflammatory responses *via* NF-κB inhibition

NO_2-FA exerts potent anti-inflammatory actions, primarily by antagonizing the activities of NF-κB, and signal transducers and activators of transcription (STATs) [25; 33], while activating PPARγ and the anti-inflammatory nuclear factor E2-related factor 2 (Nrf2) [23; 28; 34]. Yet, a key mechanism behind the role of NO_2-FA is their ability to inhibit NF-κB activity by preventing the phosphorylation of inhibitor of kappa B (IκB) and its subsequent degradation as well as by activating PPARγ (Fig. 1). Studies using affinity labeling and mass spectrometry strategies reveal that NO_2-FA derivatives inhibit NF-κB *via* nitroalkylation of Cys residues in the NF-kB p65 subunit *in vitro* and a similar reaction with p65 in macrophages treated with NO_2-FA [24]. Both nitro-oleic acid (NO_2-OA) and nitro-linoleic acid (NO_2-LA) or novel NO_2-FA derivatives inhibit lipopolysaccharide (LPS)-induced macrophage secretion of pro-inflammatory cytokines including interleukin 6 (IL-6), tumor necrosis factor α (TNF-α) and monocyte chemoattractant protein 1 (MCP-1) as well as iNOS expression [24; 35; 36]. NO_2-FA down-regulate expression or activity of pro-inflammatory signaling molecules induced by various stimuli (e.g., TNF-α, LPS, IL6, TGF-β) [24; 26].

Further experimental evidence reveals that NO_2-FA regulate the NF-κB signaling pathway at multiple levels (Fig 1). These include reduced membrane expression of Toll-like receptor 4 (TLR4) and impaired recruitment of TLR4 and TNF receptor associated factor 6 (TRAF6) to the lipid rafts compartment with subsequent inhibition of of IκB kinase β (IKKβ) phosphorylation as well as phosphorylation and ubiquitination of IκB-α, [4]. In addition, alkylation the NF-κB p65/RelA by NO_2-FA, targeting RelA for proteasomal degradation has been demonstrated in triple negative breast cancer (TNBC) cells [37].

The anti-inflammatory properties of NO_2-FA *via* NF-κB suppression resemble that of known NF-κB inhibitors. For instance, TNBC cells treated with NO_2-OA or the IKKβ inhibitor 3-[(4-methylphenyl)sulfonyl]-(2E)-propenenitrile (Bay 11-7082) show a similar inhibitory effect on TNFα-induced IKKβ phosphorylation and IκB degradation [37]. Also, the oleanane triterpenoid 2-cyano-3,12-dioxooleana-1,9,-dien-28-oic acid methyl ester

(CDDO-Me, Bardoxolone methyl) contains α,β-unsaturated carbonyl in the A-ring that forms reversible adducts with thiol nucleophiles. CDDO-Me inhibits the NF-κB pathway by interacting with Cys-179 in the IKKβ activation loop [38]. Although CDDO-Me showed beneficial effects of improving renal function in patients with chronic kidney disease and type 2 diabetes [39], a phase III trial of CDDO-Me was terminated early due to safety concerns associated with increased rate of cardiovascular events in these patients [40]. While both NO_2-FA and CDDO-Me are equally effective in the activation of Nrf2-dependent signaling, NO_2-FA more efficiently inhibits NF-κB than CDDO-Me [36], which may serve as an advantage over bardoxolone. NO_2-FA are endogenously-generated NF-κB inhibitors suggesting improved safety as shown in past and ongoing phase II trials [16; 17; 18; 19].

Electrophilic prostaglandin metabolites such as the cyclopentenone 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (15d-PGJ_2) is another class of lipid mediators that inhibit NF-κB with comparable actions to NO_2-FA [41]. In spite of the similar reactivity (e.g. Michael addition) [42], differences in the electrophilic potential or accessibility to nucleophilic thiols likely account for the divergent biological outcomes. 15d-PGJ_2 inhibits NF-κB transcriptional activity *via* covalent modification of Cys 62 in p50 [43]. 15d-PGJ_2 do not readily dissociate from the sites of adduction, accumulate and promote toxicity at low concentrations [44], while NO_2-FA allows rapid Cys adduct formation as well as dissociation, possibly *via* transalkylation of different thiol sites [45]. For a more comprehensive discussion, see [41].

In addition to inhibiting NF-κB *via* direct PTM of the p65 subunit or upstream phosphorylation of IKK or IκB, NO_2-FA may inhibit NF-κB signaling through the activation of PPAR-mediated transrepression of inflammatory responses, term coined to describe the ability of nuclear receptors to antagonize the expression of pro-inflammatory transcription factors, such as NF-kB [46]. As for PPARγ, this occurs *via* ligand-dependent and -independent transrepression mechanisms. For example, PPARγ can directly interact with NF-κB subunits p50 and p65 to inhibit pro-inflammatory signaling events [47]. Additionally, the sumoylation of specific lysine residues in the PPARγ ligand binding domain (LBD) does not allow clearance of corepressors which results in protein complexes bound to promoters in the actively repressed state [48]. Specifically, the sumoylation of PPARγ results in the corepressor NCoR complex bound to iNOS promoter preventing transcriptional activation in response to LPS [49]. Transrepression signaling by NO_2-FA has been experimentally observed under inflammatory stimuli. Co-immunoprecipitation analysis of PPARγ and NF-κB has indeed been described in endothelial cells exposed to inflammatory insult and treatment with NO_2-OA further promotes PPARγ/NF-κB interaction [7].

3. NO_2-FA regulate metabolism and inflammation as PPARγ ligands

NO_2-FA are potent endogenous ligands for PPARs, in particular PPARγ [28; 29; 30]. A comparative analysis of equimolar concentrations of several proposed endogenous PPARγ agonists (15d-PGJ_2, linoleic acid, conjugated linoleic acid, 16:0 lysophosphatidic acid, 18:1 lysophosphatidic acid, 1-*O*-hexadecyl-2-azelaoyl-sn-glycero-3-phosphocholine and 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine) or exogenous PPARγ agonists thiazolidinediones (TZDs, rosiglitazone) revealed a similar activation by NO_2-LA compared

to rosiglitazone and exceeded all of the endogenous agonists [28]. A common structural feature of PPARγ binding domain is the relatively large binding pocket resulting in a rather promiscuous affinity for lipids [50]. Yet, structure-function relationships prioritized electrophilic lipids as preferential PPARγ endogenous ligands, including unsaturated ketones and most selectively NO_2-FA derivatives [51]. Cys 285 at the PPARγ LBD is critical for covalent binding of NO_2-FA to PPARγ *via* Michael addition, while docking of rosiglitazone does not require reaction with Cys 285. This critical structural difference in the mode of interactions of endogenous NO_2-FA and rosiglitazone is reflected in the differential recruitment of coactivators. Both rosiglitazone and NO_2-FA share a similar displacement of the NCoR ID2 corepressor from PPARγ. However, unlike rosiglitazone, NO_2-FA does not induce coactivator Trap220/Drip2 recruitment upon PPARγ binding and activation [52] (Fig. 2). Further studies applying mutation analysis of key PPARγ LBD residues demonstrated that NO_2-LA interaction with PPARγ and its activation by NO_2-LA differ from rosiglitazone [29].

The discovery of unique PPARγ agonism by NO_2-FA, suggests that NO_2-FA can induce different physiologic responses than TZDs, giving hope that NO_2-FA can be used as a therapeutic to improve insulin sensitivity without causing undesirable TZDs-mediated side-effects such as weight gain, edema and increased adverse cardiovascular events [53; 54; 55]. Unlike rosiglitazone which improves glucose homeostasis, while promoting weight gain, administration of NO_2-OA increases insulin sensitivity without undesirable weight gain in leptin deficient mice [52]. Similarly, administration of NO_2-OA to mice with high-fat diet (HFD)-induced adiposity, hyperglycemia and PAH improves glucose tolerance without altering body weight [11].

Beyond the controversial aspects on PPARγ dependent and independent outcomes by TZDs and aligned side-effects [56], PPARγ has multiple functions in the immune system and is implicated in major inflammatory diseases [57; 58]. Recent reports demonstrate an essential role of PPAR in the gut responses to bacterial infections by generating endogenous ligands thus improving systemic energy metabolism [59]. Also, PPARγ controls cell development of type-2 immunity (including T lymphocytes and dendritic cells) [60]. Although some reports have demonstrated that the anti-inflammatory properties of NO_2-FA are independent of PPARγ activation [13; 24], other studies have shown that PPARγ regulation by NO_2-FA exert an anti-inflammatory response [6; 7; 61].

4. Regulation of lipid metabolism by NO_2-FA

Regulation of PPARγ activity by NO_2-FA has been proven to promote an anti-inflammatory response and to improve glucose homeostasis without inducing obesity [6; 7; 52], yet a putative role of NO_2-FA in lipid metabolism is intriguing. Administration of NO_2-OA to apolipoprotein E-deficient ($apoE^{-/-}$) mice reduces atherosclerosis and macrophage foam-cell formation without altering the lipoprotein profile [15]. Still, administration of NO_2-FA in pre-clinical models of metabolic disorders suggests an active role in lipid metabolism, with observed improved lipid profiles and liver steatosis, with decreased plasma triglycerides [62; 63] and reduced expression of lipoprotein-associated phospholipase A2 (Lp-PLA2), a biomarker of cardiovascular risk [64]. In macrophages, NO_2-FA prevents

triglyceride accumulation *via* down-regulation of diacylglycerol acyltransferase 1 (DGAT1), and induced expression of triglyceride hydrolysis enzymes, hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) [65]. Novel evidence demonstrates an active incorporation of free NO_2-FA into monoacyl-, diacyl- and triglycerides [66], suggesting that the aforementioned lipases might be putative targets of NO_2-FA nitroalkylation. Electrophilic NO_2-FA are preferentially incorporated into monoacyl- and diacylglycerides, while saturated and β-oxidized metabolites are more often incorporated into triglycerides [67]. Thus, NO_2-FA may influence fluxes of fatty acid interconversion between di-and triglycerides and subsequent metabolism by yet unidentified mechanisms [67]. These examples certainly pave the way for a deeper appreciation of the putative regulatory role of NO_2-FA on fatty acid distribution and gain further molecular insight on the regulation of key enzymatic activities involved in lipid metabolism [68; 69].

Given the intimate link between inflammation and metabolic derangements [70], understanding the mechanisms by which NO_2-FA regulates signaling pathways centrally involved in immune regulation and energy metabolism is highly provocative [71]. Whereas a role for NO_2-FA in innate immune responses has been well-established in models of acute inflammatory responses and sterile inflammation [4; 35; 72], a role of NO_2-FA in metabolic adaptation of immune cells rather than the classical inflammatory responses [73], is only starting to be explored.

5. Conclusions and perspectives

The pharmacokinetics and pharmacologic actions of NO_2-FA continue to actively be pursued. Biodistribution of nitrated fatty acid species indicates an active signaling role of NO_2-FA in adipose tissue, liver, kidney, lung, the immune and cardiovascular systems [66; 74]. To what extent NO_2-FA regulation of NF-κB and PPARγ signaling discussed herein contributes to the beneficial outcomes under disease conditions is largely unknown. While the role of NO_2-FA on inflammatory processes continues to be elucidated, with established protective role in chronic and acute inflammatory responses, their role in adaptive immunity remains to be explored with molecular mechanisms that expand beyond PPAR and NF-κB signaling [73]. Given that PTM of key signaling mediators by NO_2-FA continues to be uncovered [75; 76], it will be possible to assess these functions and mechanisms in further molecular detail *in vivo*. These remaining questions are relevant to establish the therapeutic efficacy of nitrated fatty acids and further elucidate their role on the mutual regulation of energy metabolism and immune function.

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Abbreviations

CLA	conjugated linoleic acid
eNOS	endothelial nitric oxide synthase

IBD	inflammatory bowel disease
iNOS	inducible nitric oxide synthase
NCoR	Nuclear receptor co-repressor
NF-κB	nuclear factor kappa B
•NO	nitric oxide
NO_2^-	nitrite
$•NO_2$	nitrogen dioxide
NO_2-FA	nitro-fatty acids
NO_2-OA	nitro-oleic acid
NO_2-LA	nitro-linoleic acid
NO_2-CLA	nitro-conjugated linoleic acid
PAH	pulmonary arterial hypertension
PPARγ	peroxisome proliferator activating receptorγ
PTM	posttranslational modification
TZDs	thiazolidinediones

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Highlights

- NO_2-FA modulate key signaling pathways *via* posttranslational modifications.
- NO_2-FA inhibit NF-κB *via* direct p65 nitroalkylation and regulate Toll-like receptor signaling.
- NO_2-FA are PPARγ ligands that differ from thiazolidinediones in receptor affinity and extent of activation.
- This mini-review conveys the protective role of NO_2-FA in inflammatory responses and regulation of metabolic pathways.

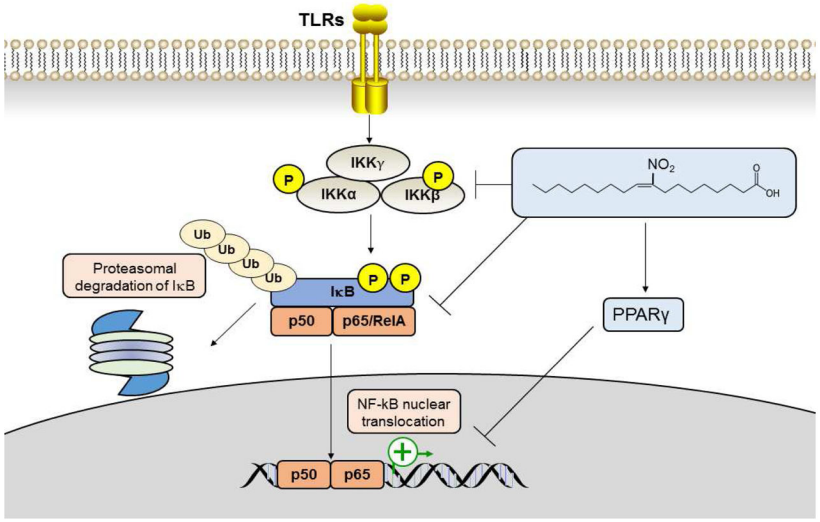

Fig. 1.

NO_2-FA promote cellular anti-inflammatory responses through NF-κB suppression *via* inhibition of IκB phosphorylation and its subsequent degradation, PTM (e.g. p65 nitroalkylation) or PPARγtransrepression signaling. NO_2-FA-induced NF-κB inhibition contributes to prevention of vascular inflammation and endotoxemia, allergic airway disease, myocardial I/R injury and myocardial fibrosis [3].

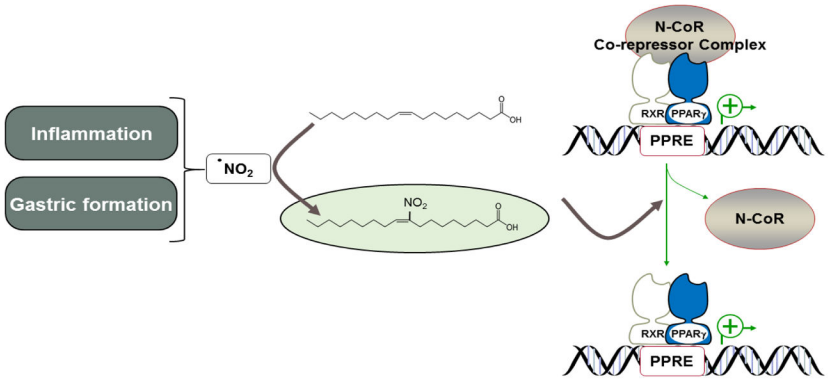

Fig. 2.

Endogenous generation of NO_2-FA and PPARγ transcriptional activation. Fatty acid nitration occurs primarily during inflammation and gastric formation *via* non-enzymatic reaction of $^{\bullet}NO_2$ with unsaturated fatty acids. NO_2-FA covalently bind to Cys 285 in the LBD of PPARγ *via* Michael addition, resulting in displacement of N-CoR corepressor complex yet with unique PPARγ agonism that induces different physiologic responses than known PPARγ agonists TZDs.