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Nitrated fatty acids: from diet to disease

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Fatty acids not only provide caloric energy in our diets and building blocks of lipids but are also precursors of potent signaling molecules. Fatty acids can undergo enzymatic and non-enzymatic transformations to form autocrine and paracrine signaling molecules that regulate energy balance and metabolic homeostasis. A new class of lipid signaling mediators known as nitro-fatty acids (NO_2-FAs) have recently been identified. These NO_2-FAs are generated endogenously through non-enzymatic reactions of secondary products of nitrite and nitric oxide and are readily detected in human plasma and urine. NO_2-FAs are potent anti-inflammatory and antioxidant cell signaling mediators and exert protective effects in numerous pre-clinical animal models of disease including cardiovascular, pulmonary and renal fibrosis. Chronic unresolved inflammation is a common key feature underlying most fibrotic disorders. Two signaling pathways that converge on inflammation and oxidative stress are nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and nuclear factor kappa B (NF-κB). NO_2-FAs are pleiotropic signaling modulators that target both of these pathways providing a therapeutic strategy directed toward an integrated decrease in inflammation. This review summarizes the latest findings and understanding of the formation, signaling and anti-fibrotic effects of NO_2-FA.

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Introduction

The need for fatty acids goes far beyond the obligatory role of building blocks for fat and dietary sources for fuel in our bodies. The discovery of leukotrienes and prostaglandins, as enzymatic oxygenation products of unsaturated fatty acids, advanced the field demonstrating that fatty acid mediators are potent autocrine and paracrine signaling molecules. The technical advances provided by mass spectrometry-based approaches led to renewed interest and discoveries in this area of research. It has become clear that this large group of newly discovered bioactive molecules derived from polyunsaturated fatty acids executes physiological responses that are central to tissue homeostasis. Among them, NO_2-FAs are formed that participate in anti-inflammatory and tissue protective actions through activation of Nrf2-dependent-signaling and concomitant inhibition of NF-κB-derived inflammation. The latest advances are provided in areas of NO_2-FA formation and role in fibrotic disorders with a focus on unresolved inflammation, which appears to be a common feature of fibrosis across all tissues, and examining the signaling actions of NO_2-FAs as a novel therapeutic.

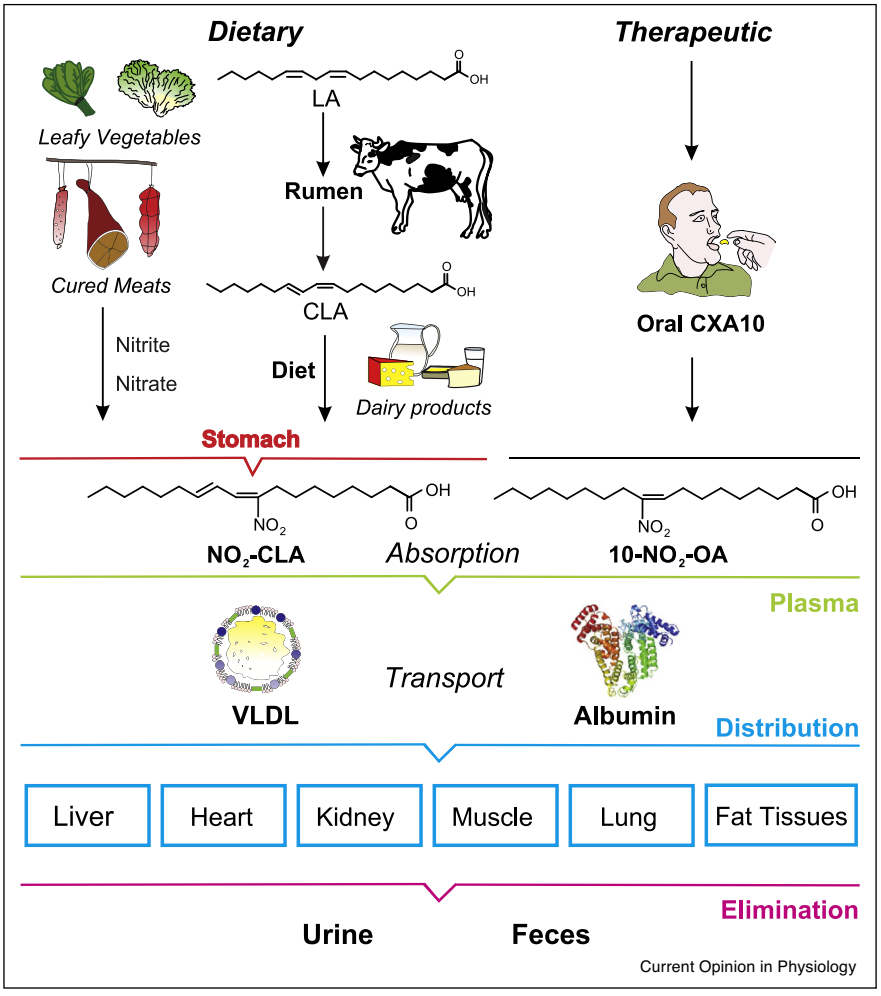
Formation of nitro-fatty acids

Dietary interventions have been shown to be effective in a broad range of pathologies including diabetes and metabolic syndrome [1]. In addition to providing the nutrients to support metabolism, certain dietary components modulate metabolic processes. Moreover, these can be further metabolized and processed by the microbiome, providing a new set of modified molecules with unique signaling properties that regulate metabolism. These microbial transformations are important [2,3•], the microbiome density and composition has also been implicated in the alteration of physiological responses, while a less known type of transformation and bio-activation of molecules occurs in the stomach [4,5]. The stomach is a bioreactor that combines highly acidic gastric fluid with digestive enzymes to facilitate the break down of large biomolecules. In addition to providing optimal conditions for digestion, the acidic pH also promotes additional chemical reactions, some of which involve conjugated fatty acids and nitrite [6]. Conjugated fatty acids are mostly found in dairy and meat products and originate in the rumen during bacterial biohydrogenation reactions of bis-allylic fatty acids [7]. The most abundant product is conjugated linoleic acid (CLA), for which beneficial and anti-obesogenic effects have been reported in rodents and to a lesser extent in humans [8]. Nitrite, in contrast, is acquired directly from leafy vegetables, processed food or by reduction of dietary nitrate in the oral cavity [9] (Figure 1).

Stomach as a bioreactor

The acidic conditions of the stomach during digestion result in its protonation and the secondary generation of nitric oxide (NO) through reductive reactions, the nitrosating species dinitrogen trioxide (N_2O_3) and nitrating species nitrogen dioxide ($^{\bullet}NO_2$) [9]. Initial studies evaluating the intake effects of high doses of nitrite

Figure 1



Therapeutic and dietary NO_2-FA. The main components for the gastric formation of NO_2-CLA come directly from the diet. Nitrite is contributed by leafy vegetables and cured meats while CLA is provided by dairy products. Therapeutic 10-NO_2-OA (CXA-10) is orally administered. Both follow absorption and packing into triglycerides as the main transport and distribution mechanism. Free acid NO_2-FA are also transported bound to albumin's hydrophobic pockets. Once distal target tissues are reached and the signaling is activated, NO_2-FA are deactivated and excreted mainly via urine with a yet uncharacterized participation of fecal excretion Nitrite (NO_2^-); Nitrate (NO_3^-).

demonstrated the formation of these reactive species in the stomach to be responsible for the formation of tumorigenic nitrosamines [10], prompting stringent standards for nitrite content in foods. Conversely, emerging data revealed that dietary levels of nitrite are beneficial and modulate physiological responses such as vasodilation and blood pressure while promoting oxygen delivery to tissues in the capillaries. Moreover, it is becoming clear that the initial observations of dietary nitrite-induced formation of nitrosamines and their participation in gastrointestinal tumors had been overestimated [11]. Nitrite has been shown to participate in the nitration of protein and peptides in the stomach (e.g. pepsin) with a role in the regulation of gastrointestinal function [5]. However, proteins are not the only target of nitrating species in the stomach, as conjugated fatty acids have been shown to be major substrates for nitration in the gastric milieu [6]. This reaction involves an initial addition of $^{\bullet}NO_2$ and resonance stabilization of the resulting nitrated fatty acid radical. This, in turn, is oxidized generating nitro-conjugated linoleic acid (NO_2-CLA) when the substrate CLA was used. Yet, the most abundant unsaturated fatty acids lack conjugated double bonds. Therefore, these abundant unsaturated fatty acids are very poor targets of nitration, as they are unable to stabilize further the initial radical intermediate reaction product formed upon $^{\bullet}NO_2$ addition [12].

Endogenous NO_2-FA

While NO_2-CLA detection is well established and its levels are modulated by diet and inflammation, the remaining classes of NO_2-FAs still need to be explored

[13••,14]. Nitro-oleic acid (NO_2-OA), which has long been used as a surrogate to investigate the actions of endogenous NO_2-FA [15], has been reported in the subnanomolar level in plasma [16] but is usually below the limit of detection and/or quantification. In contrast to NO_2-CLA, NO_2-OA and its metabolites are normally not detected in human urine (unpublished observation). Similarly, nitro-arachidonic acid and its metabolites are absent from human plasma and urine (unpublished observation). Finally, while nitrated products of linolenic acid have been reported in plants [17], they have not yet been evaluated in humans.

Nitro-fatty acid reactivity

The interest in the physiology and pharmacology of NO_2-FA is fueled by the investigation of their anti-inflammatory and protective actions reported in several preclinical animal models of disease by NO_2-OA, which is used as a surrogate to interrogate the effects of endogenous NO_2-FA [15,18]. NO_2-FAs are electrophilic molecules that contain a nitroalkene group that reacts mainly with cysteines through Michael addition reactions [19]. While cysteine adducts are the most relevant to NO_2-FA signaling and regulation of enzymatic activity [20], HPLC-MSMS proteomic approaches also show the formation of histidine adducts upon protein tryptic digestion [21]. Interestingly, the formation of these adducts is not detected when these reactions are carried out in test tubes (unpublished data) suggesting that the microenvironments and surrounding amino acids in tertiary protein structures catalyze the formation of such adducts through stabilization of intermediate reaction products. Although it has not been directly measured, histidine adducts may display a lower k_{off} (slower elimination reaction) when compared to cysteine adducts, leading to more stable addition products and as a consequence their preferential detection by proteomic approaches. Under *in vivo* conditions, it is believed that cysteine adducts drive both the signaling and inactivation of NO_2-FA. The highly reversible adducts formed with cysteines may lead to a 'ping pong' type mechanism that is thought to sequentially hit regulatory cysteines in a variety of proteins, a cycle that ends with the formation of glutathione adducts resulting in cellular export through ATP binding cassette transporters and deactivation of the electrophilic signaling [22].

Nitro-fatty acid signaling

The reversibility of the NO_2-FA reaction is central to their pleiotropic signaling activity. While initially nuclear factor (erythroid-derived 2)-like 2 (Nrf2), heat shock response (HSR) activation and nuclear factor kappa B (NF-κB) inhibition were proposed as main drivers of their signaling mechanisms, emerging evidence reveals new pathways that are inhibited, specifically STING, epoxide hydrolase and angiotensin II receptor [23–25]. The current understanding of the signaling of NO_2-FA points toward cysteine modifications that subsequently impact signaling pathways, metabolic regulation, inflammatory and immune responses. Overall, these changes modulate global responses to injury, impact pathophysiological processes and regulate paracrine signaling.

Perhaps the most common outcome of chronic injury and tissue repair is fibrosis, a mechanism that involves cell differentiation and de-differentiation, inflammatory responses and tissue remodeling in an attempt to regain tissue structure, function and homeostasis. Multiple cell types and signaling pathways pose a therapeutic challenge and have precluded the development of efficacious treatments aimed at a single molecular target in this complex disease. In this context, the pleiotropic actions ascribed to NO_2-FA provide further support and rationale for their use and effectiveness in fibrotic diseases, as successfully demonstrated in kidney, cardiovascular and pulmonary preclinical models [26••,27]. Although Nrf2, HSR, and NF-κB are the commanding signaling pathways of NO_2-FA actions, their relative individual contribution to the different pathological conditions, organ and temporal responses are expected to be different. Thus, from a therapeutic perspective, a one-dose-fits-all-strategy is not expected to be effective. Therefore, doses to treat different pathological conditions will need to be established individually for different diseases. In addition, electrophiles characteristically display hormetic responses as previously demonstrated for dimethyl fumarate and Nrf2 activators further highlighting the need for tailored therapeutic approaches [28,29].

Complexities of fibrosis

Fibrosis is a complex and vital program to repair injured tissue. It occurs after repetitive insults to the epithelium and is defined by the accumulation of extracellular matrix (ECM) molecules such as collagen and fibronectin [30]. Under normal conditions that lead to wound healing following an injury, the fibrotic ECM is degraded, the epithelium is repaired and fibrosis is resolved. In a fibrotic state, however, the normal repair and resolution mechanisms are dysfunctional leading to scarring and eventually impaired organ function [31]. Excessive tissue scarring is a highly relevant unmet clinical need as fibrosis contributes to an estimated ~45% of deaths in the developed world [30]. Fibrosis affects vital organs including lung, liver, kidney, heart, eye, and skin among others. A core feature across most tissue fibrotic disorders is that it is triggered by inflammation and oxidative stress. This promotes myofibroblast activation and secretion of ECM proteins, which in turn drive altered cytokine overproduction [31]. The initial stimulus that provokes the inflammatory response may be tissue-specific and be largely resolved by the time chronic inflammation is established. However, the mechanistic definition of common fibrotic pathways may provide the therapeutic clues

that lead treatments that prevent and/or reverse existing fibrotic lesions in all tissues.

The need for novel therapeutics: NO_2-FA

Electrophilic drugs inhibit pro-inflammatory signaling mediators and have been shown to provide a valuable approach in several pre-clinical fibrosis models. NO_2-OA and other electrophiles, such as dimethyl fumarate and 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO), protect against kidney and pulmonary animal models of fibrosis [32–35]. In cardiovascular disease, NO_2-OA reverses hypoxia-induced right ventricular (RV) pressure and fibrotic RV remodeling in a pulmonary arterial hypertension model [27]. Additionally, NO_2-OA inhibits angiotensin II-mediated atrial fibrosis and fibrillation [36] and myocardial fibrosis [26••]. A majority of the effects mediated by these electrophiles have centered on the anti-inflammatory and antioxidant protective actions although the exact mechanism(s) of action is largely unknown. NO_2-FAs potently antagonize NF-κB and activate Nrf2 signaling.

NF-κB inhibition

Under basal conditions, NF-κB is inactive as it is complexed with inhibitory κB (IκB)-α subunit, which contains a strong nuclear export signal, in the cytoplasm. In the canonical pathway, most stress signals activate NF-κB by controlling the redox-sensitive serine-specific IkB kinase (IKK). The activation of the IKK complex facilitates IκB-α phosphorylation (Ser-32 and Ser-36) by IκB kinase β (IKK-β) leading to IκB-α ubiquitination and proteasomal degradation resulting in NF-κB transactivation [37•]. Typically, NF-κB is a rapid transient signal, as soon as *de novo* IκB-α is synthesized, it retrieves nuclear NF-κB and turns off the signal once shuttled to the cytoplasm [38].

Proteolytic degradation of NF-κB halts the signaling initiated by this ubiquitously expressed transcription factor that is central to the regulation of inflammation, proliferation and cell survival pathways [38]. NO_2-FAs control the redox-sensitive transcription factor NF-κB activity on multiple levels. NF-κB activity is directly inhibited by nitroalkylating (adducting) p65's Cys38 resulting in proteasomal degradation [39]. Moreover, NO_2-FAs covalently modify IKK-β, blocking IKK-β phosphorylation and preventing IκB-α phosphorylation and its subsequent degradation [22]. Lastly, the examination of upstream NF-κB signaling cascade events revealed that NO_2-FAs decrease the expression of Toll-like receptor 4 (TLR4) and recruitment of TLR4 and TNF receptor-associated factor 6 (TRAF6) to the lipid rafts compartments. This subsequently suppressed IKK-β phosphorylation and the phosphorylation and ubiquitination of IκB-α [40]. Combined, all of these actions inhibit downstream NF-κB signaling and activity as NO_2-FAs suppress lipopolysaccharide (LPS)-induced secretion of interleukin 6 (IL-6), tumor necrosis factor α (TNF-α) and monocyte chemoattractant protein 1 (MCP-1) in macrophages [13••,39]. NO_2-FAs downregulate pro-inflammatory signaling molecules expression and/or activity induced by various stimuli including IL-6, LPS, transforming growth factor β, and TNF-α [13••,22,26••,39–41].

Nrf2 activation

NO_2-FA derivatives activate Nrf2-dependent gene transcription by alkylating two functionally significant cysteine residues (Cys-273 and Cys-288) of Kelch-like ECH-associated protein (Keap)-1 in the cytoplasm. This inhibits Keap1-dependent Nrf2 degradation and results in nuclear translocation of *de novo* synthesized Nrf2 protein, binding to antioxidant response element and transactivation of gene signaling [41,42]. Nrf2-regulated genes are critical in protecting against oxidative stress, by expressing genes that limit inflammation- and reactive oxygen species -induced cell injury. Nrf2 activation protects against fibrosis in many organs including kidney, liver and lung [43–45]. Targeting Nrf2 can be a novel therapy to limit inflammation and oxidative/nitrosative stress in fibrosis.

Crosstalk between Nrf2 and NF-κB pathways

Not only does Nrf2 activate antioxidant gene expression but Nrf2 directly limits inflammation by binding to promoter regions of pro-inflammatory cytokines [46]. Additionally, Nrf2 inhibits RNA polymerase II recruitment to the transcription start site of IL-6 and IL-1β genes without altering the required recruitment of NF-κB [46]. In this case, Nrf2 and NF-κB signaling appear to be acting individually. However, there is considerable crosstalk between these two redox-regulated transcription factors NF-κB and Nrf2 pathways in response to stress [47•]; and this coordinated regulation helps maintain cellular and redox homeostasis. The first insight into the interplay between these pathways was observed in global Nrf2-knockout mice exhibiting increased pro-inflammatory cytokine expression [48]. Cell culture studies using Nrf2−/− mouse embryonic fibroblasts (MEFs) discovered that NF-κB activity was increased due to enhanced IKK-β activity resulting in IκB-α phosphorylation and its subsequent degradation [49]. Additionally, oxidative/nitrosative stress stimulates IκB-α phosphorylation resulting in NF-κB transactivation. Yet, Nrf2 is an adaptive mediator that limits oxidative/nitrosative stress by increasing the expression of genes that display antioxidant and protective roles. Nrf2 drives synthesis of glutathione and glutathione-dependent enzymes to promote conditions that control and reduce the damage exerted by oxidative stress. Therefore, the pharmacologic or genetic activation of Nrf2 will downregulate NF-κB activity indirectly as a consequence of reducing oxidative stress and decreased IκB-α phosphorylation [50,51]. Furthermore, direct inhibition of NF-κB pro-inflammatory

activity is observed when Keap1, an E3 ubiquitin ligase, directly binds to IKKβ, which causes IKKβ degradation through ubiquitination [52].

Summary and perspective

Unresolved inflammation is the backbone of many diseases, especially during the progression to fibrosis. Electrophilic NO_2-FAs have been proven safe in Phase I clinical trials and provide new dietary and pharmacological approaches to target inflammatory and fibrotic disorders by reversibly regulating signaling pathways. The decrease of inflammation and oxidative stress through Nrf2 and NF-κB pathways provides the conditions to return to homeostasis. The use of electrophiles is not free of challenges, as they display hormetic responses and in most cases, need a tailored dosing regimen for acute and chronic conditions. Nevertheless, the possibility of targeting several pathways involved in the pathophysiology of disease at once opens challenging but exciting opportunities to combat these diseases in the near future.

Conflict of interest statement

F.J.S. has financial interest in Complexa Inc. N.K.H.K. has no conflicts of interest to declare.

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