

The specificity of endogenous fatty acid nitration: only conjugated substrates support the in vivo formation of nitro-fatty acids

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ABSTRACT

Through multiple pathways, nitrogen dioxide ($\bullet\text{NO}_2$) is the main species involved in endogenous nitration reactions. Early studies in the field primarily explored tyrosine nitration, a dominant reaction in the field. It was later shown that lipids are also nitration targets and generate an array of reaction products. Conjugated fatty acids are the preferential substrates of lipid nitration in vivo, generating electrophilic nitro-fatty acids (NO_2 -FAs), which serve as pleiotropic signaling modulators. In contrast, exposure of bisallylic fatty acids, including linoleic, linolenic and arachidonic acid, to $\bullet\text{NO}_2$ does not lead, under biological conditions, to the formation of nitrated species. This review focuses on the reaction mechanisms and products of lipid nitration and substrate specificity, focusing on the differential reactivity of conjugated dienes and bisallylic alkenes.

1. Introduction

Nitrogen dioxide ($\bullet\text{NO}_2$) is the radical responsible for most nitration reactions under pathophysiological conditions. Several endogenous redox mechanisms lead to the generation of $\bullet\text{NO}_2$, making it available to drive nitro- and oxidative modifications on biomolecules [1–3]. These reactions and the products are tightly regulated by the concentration of substrates and $\bullet\text{NO}_2$. The relevance of $\bullet\text{NO}_2$ as a nitrating free radical is exemplified by 3-nitrotyrosine, a well-accepted biomarker of oxidative stress. As a nitric oxide ($\bullet\text{NO}$) oxidation product, $\bullet\text{NO}_2$ plays an important role during inflammatory and oxidative processes through adaptive modifications, which can be either detrimental or have a protective role in cells. The seminal finding of the formation of peroxynitrite from the diffusion-limited reaction of superoxide radical and $\bullet\text{NO}$ led to the discovery of tyrosine nitration [1,4,5]. The field rapidly grew and gained relevance through observations of nitrotyrosine-regulated enzymatic function and affirmation of the in vivo presence of these modifications as a consequence of inflammatory reactions and dysregulated oxidative stress. The early development of specific antibodies allowed for the interrogation of nitrotyrosine formation in cells and tissues stressed by inflammatory conditions or a specific disease, which supported critical biological, biochemical, and chemical discoveries.

While most of the effort centered around tyrosine nitration, it became clear that $\bullet\text{NO}_2$ could participate in the nitration of other biomolecules, including GTP (giving rise to NO_2 -cGMP) and lipids [6].

The effect and products of lipid exposure to $\bullet\text{NO}_2$ were first explored in the early 1980s in environmental pollution settings [7]. Years later, with the advent of $\bullet\text{NO}$ and peroxynitrite, the evaluation of these reactions regained interest when seminal work showed that $\bullet\text{NO}$ could interrupt the propagation phase of lipid peroxidation and result in nitrated species [8]. These efforts ultimately led to the discovery of endogenous anti-inflammatory lipid mediators called nitro-fatty acids (NO_2 -FAs) [9]. Due to their electrophilic character, NO_2 -FAs reversibly alkylate nucleophilic amino acids, mainly cysteine and, to a lesser extent histidine, to regulate the function of transcription factors and redox-sensitive proteins [10–12]. This post-translational modification (defined as nitroalkylation) activates anti-inflammatory, anti-fibrotic, and cytoprotective signaling cascades both in vitro and in vivo [13–15]. Some of the major pathways targeted by NO_2 -FAs include NF- κB , Nrf2/Keap1, STING/cGAS, Calcineurin, STAT3, HSP, and PPAR γ , which are covered in another review of these series [16–21]. Since the initial discovery in 2003, endogenous fatty acid nitration and subsequent NO_2 -FA formation remains an ongoing area of investigation. Favorable preclinical outcomes have translated into the clinic through

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Phase I and II clinical trials to develop treatments for inflammatory and fibrotic diseases [22]. This review will discuss the chemical characteristics of fatty acid nitration, including the reactions that generate $\bullet\text{NO}_2$ and NO_2 -FA and the particularity of its substrates and products.

2. Nitration in biology: from tyrosine to fatty acids

The profound role of $\bullet\text{NO}_2$ in redox biology can be appreciated through the formation of 3-nitrotyrosine. In the early 1990s, peroxynitrite (ONOO^-), a product of the diffusion-controlled reaction between $\bullet\text{NO}$ and superoxide radical ($\text{O}_2\bullet^-$) ($k = 1.9 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$), emerged as a potent oxidizing agent [23–25]. The efficiency of this reaction is supported by the paramagnetic nature of both radical species. $\bullet\text{NO}$ will readily diffuse in a biological medium until it encounters $\text{O}_2\bullet^-$ to form ONOO^- [26]. Upon ONOO^- protonation occurring at physiological pH values (pKa 6.8), the resulting peroxynitrous acid either directly reacts and oxidizes biomolecules (particularly cysteines and selenocysteines) or decomposes via homolytic scission into the oxidizing $\bullet\text{OH}$ and nitrating $\bullet\text{NO}_2$ radicals [27]. Although these species can recombine to form nitric acid, the reaction is kinetically unfavored, thereby facilitating the oxidation and nitration of biomolecules, primarily proteins and lipids [28].

Tyrosine nitration occurs via a two-step mechanism. The amino acid undergoes a one-electron oxidation through hydrogen atom abstraction from the hydroxyl group of the phenyl ring by cellular oxidants and peroxynitrite-derived radicals (e.g., carbonate, $\bullet\text{NO}_2$, and $\bullet\text{OH}$). This oxygen radical can be delocalized across the aromatic ring through resonance, resulting in a tyrosyl radical, which subsequently reacts with $\bullet\text{NO}_2$ to generate 3-nitrotyrosine. The biological consequences of this post-translational modification are still being studied, but it represents the first reported mechanism for biologically relevant biomolecule nitration. Similar to tyrosine nitration, fatty acid nitration also requires stabilization of the intermediate radical, which will be reviewed in detail. To put this reaction in context, from a chemical perspective, fatty acid nitration is the favored mechanism, even in the presence of high levels of tyrosine. In fact, the nitration of specific fatty acids proceeds over the formation of 3-nitrotyrosine even with a 400-fold excess of tyrosine [29]. While multiple factors may influence the extent of either reaction, this observation underscores both the prevalence and relevance of fatty acid nitration *in vivo*.

3. The endogenous formation of NO_2 -FAs requires conjugated dienes

In cells and tissues, fatty acids are mostly esterified to complex lipids, pointing to triglycerides and phospholipids as the major putative nitration targets [30,31]. Unsaturated fatty acids enriched within these lipids provide the largest pool of nitration substrates. Nonetheless, most studies characterizing lipid nitration evaluated the levels of non-esterified NO_2 -FAs, largely motivated by analytical challenges and complexity of the analysis of esterified nitrated products [32–34]. Despite the potential reaction between $\bullet\text{NO}_2$ and esterified fatty acids, under physiological conditions, this nitration reaction is highly selective towards conjugated fatty acids [29]. The term conjugated fatty acid broadly describes unsaturated fatty acids with at least one pair of conjugated double bonds. Like essential fatty acids (i.e., linoleic acid), these non-canonical substrates can only be obtained through dietary sources, mainly dairy products [35,36]. Conjugated (9Z,11E)-linoleic acid (CLA) is the most abundant and studied conjugated fatty acid in humans, followed by rumelenic acid (RLA, found in dairy products) and conjugated trienoic acids that include conjugated linolenic acid (CLnA) isomers exemplified by punicic and eleostearic acid [37]. The latter are found in pomegranate seeds (30%) and bitter melon (70%) [38,39]. Consumption of CLA and other conjugated species supports the distribution into different tissues where they serve as a nitration substrate upon nitro-oxidative stress. Unlike bisallylic targets, conjugated fatty acids do

not participate in typical peroxidation reactions and serve as a sink for $\bullet\text{NO}_2$ in cell membranes, preventing the propagation phase through the formation of nitroalkenes. In a seminal work, Rubbo et al. demonstrated the ability of $\bullet\text{NO}$ to blunt lipid peroxidation, ultimately leading to the discovery of nitrated species [8].

The development of rigorous analytical methods afforded the detection of NO_2 -FAs in cellular systems, preclinical models, and humans [15,20,32,40–44]. Despite recent success with detection and characterization, early work in this field failed to support the endogenous generation of nitroalkenes. Seminal work by Pryor and Balazy reported that the reaction between unsaturated fatty acids and $\bullet\text{NO}_2$ promoted lipid oxidation and double bond isomerization, only leading to limited quantities of nitrogen-containing lipids at exceedingly high concentrations of $\bullet\text{NO}_2$ [45,46]. This failure to simulate nitration in synthetic conditions contrasted evidence showing the presence of NO_2 -FAs in human and rodent samples [14]. It took years to solve this apparent contradiction when, in 2012, Bonacci et al. described the high nitration yields obtained when using conjugated fatty acids as substrates. This formative work characterized the generation of NO_2 -CLA in synthetic systems and activated macrophages and confirmed its presence in cardiac mitochondria in mice and human urine [9,14,29]. Therefore, CLA was established as the primary endogenous nitration target. More recently, the substrates and products of fatty acid nitration in humans have expanded with reports showing alternative NO_2 -FAs in humans, including nitrated CLnA and RLA [32,43]. Reflecting on progress made in the last decade, we have a more comprehensive understanding of fatty acid nitration. However, the initial studies describing the interactions of biological alkenes and $\bullet\text{NO}_2$ provide critical mechanistic support for why only conjugated dienes are nitrated under biological conditions.

4. Only conjugated fatty acids stabilize nitrated intermediate radicals under biologically relevant conditions

Originally, *cis* or *trans* bisallylic 18:2 fatty acids were proposed to be the endogenous substrates for nitration reactions [14]. These assumptions were based on the endogenous abundance of non-conjugated fatty acids and the labile bisallylic C–H bonds. Bisallylic C–H bonds present in many non-conjugated fatty acids like linoleic acid (LA), linolenic acid (LnA), and arachidonic acid (AA), show a lower bond dissociation energy (BDE) of 76 kcal/mol compared to the allylic hydrogen of an isolated alkene (BDE \approx 88 kcal/mol) [47], facilitating hydrogen abstraction. Bonacci et al. proposed a modification of the dogma by reporting a preferential reactivity of $\bullet\text{NO}_2$ towards CLA to generate NO_2 -CLA. This report demonstrated that CLA nitration yields five orders of magnitude more nitrated products than oleic acid (OA) and LA [29]. This observation came two decades after pivotal experiments by Pryor, which focused on the mechanism of $\bullet\text{NO}_2$ driven alkene autooxidation.

Before elucidating the mechanism of oxidation, the *chemical* addition of $\bullet\text{NO}_2$ to alkenes was extensively studied, whereby the $\bullet\text{NO}_2$ radical adds across an olefin, resulting in a carbon radical subsequently quenched by either another molecule of $\bullet\text{NO}_2$ or $\bullet\text{NO}$ [48,49]. The reactions were carried out under higher concentrations of $\bullet\text{NO}_2$ (~ 0.5 mol/L) in hexanes. Even though, from a chemical standpoint, $\bullet\text{NO}_2$ is capable of adding across an isolated alkene (e.g., hexenes and cyclohexenes), the levels do not model those normally achieved in a biological environment. As Pryor reported, the addition of $\bullet\text{NO}_2$ to cyclohexene gave way to hydrogen abstraction under low to medium concentrations of $\bullet\text{NO}_2$ (< 1000 ppm) [46]. For reference, the levels of $\bullet\text{NO}_2$ in polluted air do not exceed 10 ppm, which aligns with reports of elevated levels of oxidized pulmonary lipids following $\bullet\text{NO}_2$ exposure [50,51]. The concentration threshold required for isolated alkene nitration will not be reached in physiology, even considering the lens effect of lipids and oxygen in $\bullet\text{NO}$ autooxidation [52]. In reality, the carbon radical formed from hydrogen abstraction is captured by molecular oxygen, generating a peroxy radical that supports propagation

reactions involving oxidation (Scheme 1). Ultimately, under physiological or pathological conditions, LA is oxidized by $\bullet\text{NO}_2$ without the appearance of nitrated products. $\bullet\text{NO}_2$ serves as the radical initiator for lipid peroxidation of bisallylic targets, whereas the NO_2 -CLA derived from conjugated fatty acids will switch the continuation of detrimental oxidation reactions off. Later demonstrated by Pryor and d'Ischia, only high $\bullet\text{NO}_2$ concentrations will lead to nitrated products for LA [53,54]. These products do not display the nitroalkene moiety and, thus, are not electrophilic and do not resemble endogenously found nitrated fatty acids.

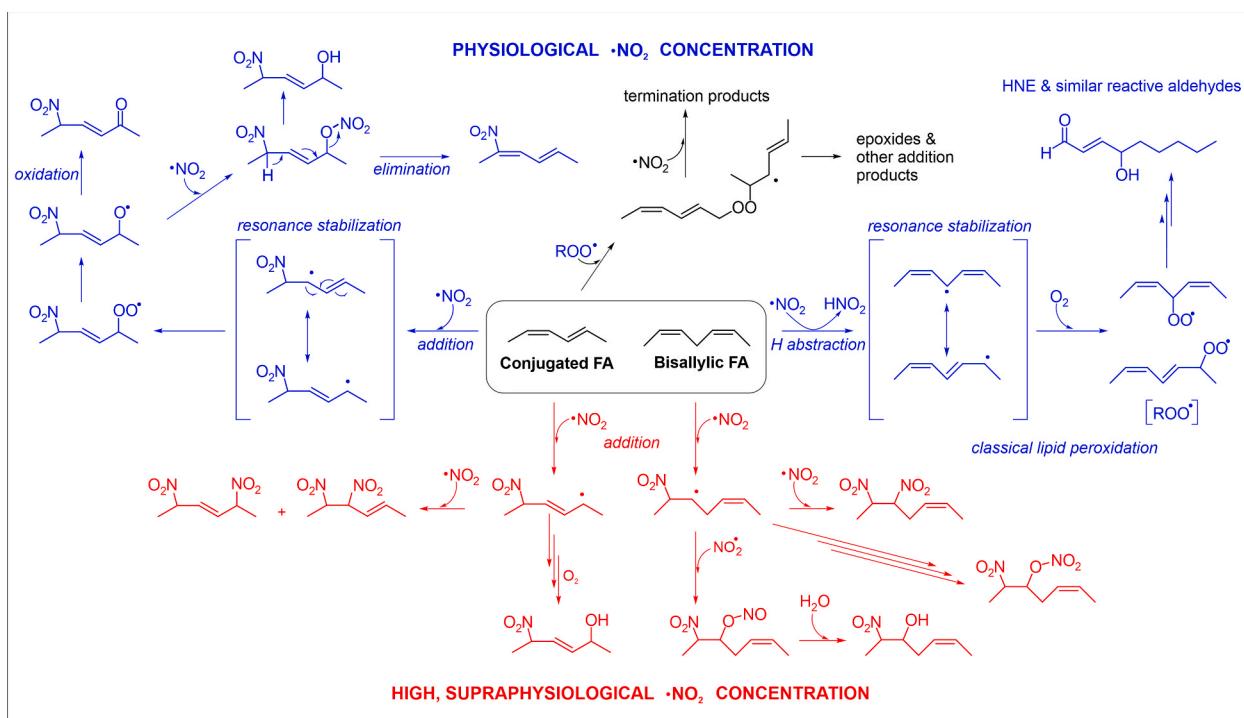
As shown in Scheme 1, the distinctive reactivity between conjugated and bisallylic fatty acids becomes evident through the initial step, highlighted by the blue pathways, where both substrates are exposed to the radical $\bullet\text{NO}_2$. Conjugated fatty acids facilitate $\bullet\text{NO}_2$ addition due to radical stabilization achieved across the conjugation system. In contrast, $\bullet\text{NO}_2$ prefers bisallylic C-H abstraction due to its reduced BDE [47]. Consequently, conjugated fatty acids incorporate $\bullet\text{NO}_2$ into derivatives such as nitro-keto, nitro-hydroxy, and notably nitroalkenes [29]. Conversely, upon hydrogen abstraction, bisallylic fatty acids yield peroxides and reactive aldehydes (e.g., 4-hydroxynonenal) through cyclization and β -scission of resulting peroxy radicals [55].

When exposed to supraphysiological levels of $\bullet\text{NO}_2$, both conjugated and bisallylic fatty acids behave like isolated alkenes, highlighted in the red pathways of Scheme 1. The thermodynamically controlled $\bullet\text{NO}_2$ addition reaction becomes less reversible [46], as the resulting alkyl radical is promptly quenched by another $\bullet\text{NO}_2$ molecule, yielding either dinitro species or nitro-nitrite (quenched with O-centered $\bullet\text{NO}_2$ radical) [48]. It was hypothesized that nitro-nitrite would rapidly hydrolyze, leading to the observed nitro-hydroxy product. One minor difference between conjugated and bisallylic substrates treated with high $\bullet\text{NO}_2$ concentrations is that in a conjugated system, the resulting products assume various regional isomeric forms due to the resonance of the initial alkyl radical.

As an extension to the reactions using LA as substrate, Balazy et al. showed that AA exposure to $\bullet\text{NO}_2$ does not support lipid nitration. AA is a precursor for various enzymatically (e.g., cyclooxygenase, lipoxygenase, and cytochrome P450 enzymes) generated oxylipins and actively participates in oxidation reactions. When exposed to $\bullet\text{NO}_2$, *cis-trans* double bond isomerization is induced in arachidonic acid and can serve as a marker for $\bullet\text{NO}_2$ formation [45]. This reaction generates two major products (i.e., isomerized AA and NO_2 -OH-AA) and was shown to occur in human platelets treated with $\bullet\text{NO}_2$ gas.

Almost 10 years after the first report of CLA as the primary lipid nitration target, Salvatore et al. found that the CLnA species represent 39% of all NO_2 -FAs detected in urine [32]. CLnA encompasses conjugated 18:3 fatty acids, including those with three conjugated double bonds (i.e., conjugated trienoic fatty acids) and substrates with one pair of conjugated double bonds and an additional isolated alkene (e.g., RLA). Interestingly, the dietary sources for these conjugated triene fatty acids are not common in typical Western-type diets, as they are almost exclusively found in pomegranate and bitter melon plant seeds. The increased consumption of bitter melon by the Asian population may infer that they are exposed to higher levels of nitrated conjugated trienes, although this has not been evaluated. Regarding the role of diets, a recent study reports that gastric nitration of milk fat from ruminant species contributed to the formation of nitrated RLA (NO_2 -RLA) [56]. Nitrated RLA represents 10% of the total basal NO_2 -FA levels in human urine, a ratio consistent with the CLA and RLA levels present in milk and dairy products [32]. The presence of nitrated species with three conjugated double bonds in vivo challenges our current understanding of preferential substrates, as dietary questionnaires do not show any intake of vegetables and fruits that contribute to CLnA (e.g., punicic and eleostearic acid). Formal competitive nitration experiments have yet to be completed for CLA and CLnA to establish reaction kinetics and nitration yields.

As a continuation of this notion, the relationship between fatty acid



Scheme 1. Summary of the expected lipid nitration products for conjugated and bisallylic fatty acid substrates. Scheme 1 outlines the proposed mechanisms and outcomes of NO_2 -induced lipid oxidation and nitration. It highlights unique products and reaction pathways observed in conjugated and non-conjugated fatty acids with bisallylic C-H bonds. The blue pathways indicate lipid-radical interactions at physiological $\bullet\text{NO}_2$ concentrations [29,46], while the red pathways depict reactions and products requiring supraphysiological $\bullet\text{NO}_2$ levels [48,49,53,54]. Moreover, reaction conditions with abundant alkenes like the cell membrane, illustrated by the black pathway, can alter product distribution by generating lipid peroxy radicals, thereby influencing the final product profile [63].

conformation and nitration kinetics remains to be addressed. While double bond conjugation can be regarded as a structural requirement for endogenous lipid nitration, there are no reports claiming distinct reactivity of CLA isomers towards $\bullet\text{NO}_2$. The detection of 9- NO_2 -CLA and 12- NO_2 -CLA in human urine accurately reflects the endogenous availability of the *cis*-9, *trans*-11 isomer, as it accounts for up to 80% of total CLA in a standard diet [57]. Although double bond position has been reported to influence CLA oxidation, the nitration of specific CLA isomers has not been compared [57]. Not only is the conjugated system relevant for fatty acid nitration, but it also enhances the electrophilic character of nitroalkenes. Of the most studied NO_2 -FAs, NO_2 -CLA has the greatest electrophilicity, followed by NO_2 -LA and NO_2 -OA based on thermochemical assessments and stopped-flow kinetics [10,58]. Despite its favorable *in vivo* formation and reactivity towards nucleophilic targets, NO_2 -CLA degrades faster than NO_2 -OA and NO_2 -LA in aqueous environments [58]. This observation could expand the biological effects of NO_2 -CLA, for it can release $\bullet\text{NO}$. Nonetheless, the biological relevance and extent of $\bullet\text{NO}$ release have not been established, and the conjugation to thiols most likely predominates over the release of $\bullet\text{NO}$. Still, despite the experimental utility of synthetically prepared nitroalkenes (e.g., NO_2 -OA, NO_2 -LA, and NO_2 -AA), exogenous administration of these agents does not fully recapitulate the dynamic interplay of formation, target engagement, and metabolism achieved with cell-generated NO_2 -FAs.

5. Fatty acid nitration products

Like most redox reactions in physiology, fatty acid nitration is multifaceted. Its proclivity depends on several factors, including substrates and $\bullet\text{NO}_2$ local concentrations, oxygen tension, and competing radical reactions. The main product resulting from this type of endogenous nitration is NO_2 -CLA, but minor species such as NO_2 -OH-CLA and NO_2 -keto-CLA are also formed, depending on the conditions [29,33]. Higher oxygen levels promote the formation of hydroxy (NO_2 -OH) and keto (NO_2 -oxo) species through rapid reactions of oxygen with the radical intermediates [29]. These nitration products are often detected in cellular models and synthetic reaction systems (i.e., gastric fluid acidic nitration of lipids). Surprisingly, neither their presence nor any of their metabolites have been reported in preclinical animal models or humans. This is likely due to the different oxygenation levels present in biochemical and biological systems. In this regard, under standard cell culture conditions, cells are exposed to 21% O_2 , whereas tissue oxygen levels are much lower, in the 2–4% range [59,60]. This discrepancy highlights how biomimetic reactions can significantly differ from *in vivo* nitration processes. Moreover, detecting nitration intermediates in biological systems also lags, likely due to their high chemical instability. Only recently, a nitro-nitrate (NO_2 -ONO $_2$) derivative for CLA was detected and characterized in rat stomachs [30]. Upon gastric acidification of orally administered nitrite, NO_2 -ONO $_2$ -CLA (CLA delivered as a triglyceride) forms in the stomach due to the higher oxygen concentration (7.6%) [30]. At neutral and basic pH, the non-electrophilic NO_2 -ONO $_2$ -CLA decomposes into NO_2 -CLA; in plasma and urine, only NO_2 -CLA was detected [30]. The biological significance of these NO_2 -ONO $_2$ derivatives has yet to be established. Still, potential activities or roles aside from those seen with electrophilic NO_2 -FAs are possible since they produce $\bullet\text{NO}_2$ and nitrosating species during decay. Nonetheless, their detection confirms the mechanism of endogenous formation of NO_2 -CLA. Considering the physical nature of cell membranes and the abundance of $\bullet\text{NO}_2$ fostered by accelerated $\bullet\text{NO}$ autoxidation, unique nitration intermediates are expected to form in phospholipids but have yet to be characterized due to low abundance and chemical stability. Some groups have characterized nitroxidized and nitrosated derivatives of phospholipids, but the endogenous detection in such reports is limited and requires further validation [31,61].

In addition to nitration reactions, the non-canonical conjugated fatty acids may yield oxidation products. The original peroxyl radical clock

described the rate by which linoleic acid autoxidation proceeds, which mainly propagates via the hydrogen atom transfer (HAT) mechanism [62]. More recently, Do et al. developed a peroxyl radical clock to calculate the contributions of HAT and peroxyl radical addition (PRA) to the overall rate of propagation for lipid peroxidation [63]. Unlike LA, the classical substrate for lipid peroxidation, CLA displayed a preference for the PRA mechanism, giving rise to products unlike those initially characterized for LA. Additionally, the overall propagation rate of lipid peroxidation for CLA was higher than that of LA [63]. In environments with high levels of alkenes, such as membranes enriched in CLA and CLnA substrates, alkene crosslinks or polymers are possible due to the additional stability of the carbon radical (Scheme 1). As depicted in the black pathway, peroxyl radicals (mainly derived from LA) initiate a radical propagation sequence, leading to lipid crosslinking and epoxidation. However, the extent of lipid crosslinking under oxidative and nitrosative stress remains incompletely understood due to challenges associated with characterizing the complex product profile. Though it is outside the scope of this review, it is important to address the complex fate of esterified CLA in membranes. Radical-mediated interactions between bisallylic and conjugated fatty acids and the abundance of oxygen versus $\text{NO}_x/\bullet\text{NO}_2$ are expected to greatly influence reactions and establish whether oxidation or nitration reactions predominate. Inflammation and cardiac ischemia-reperfusion support conditions for efficient nitration, due to high $\bullet\text{NO}$ levels established by activation of inducible nitric oxide synthase (iNOS) [64,65]. This leads to $\bullet\text{NO}_2$ levels that support fatty acid nitration. Therefore, the consequences of NO_2 -CLA formation during these pathophysiological events are of interest as these lipids are highly protective in preclinical animal models [14,41].

6. Endogenous sources of $\bullet\text{NO}_2$

With the discovery of protein nitration by peroxynitrite, the presence of biomarkers and metabolic products of endogenous nitrated proteins in the urine sparked significant interest in nitration reactions. Evidently, events characterized by elevated reactive oxygen and nitrogen species facilitate the formation of $\bullet\text{NO}_2$. However, additional “compartment-specific” reactions like gastric acidification of dietary nitrite, $\bullet\text{NO}$ autoxidation, and nitrate photolysis are also relevant in forming $\bullet\text{NO}_2$ for fatty acid nitration [52,66–68]. The ubiquitous generation of $\bullet\text{NO}_2$ is summarized in Fig. 1. Formally, fatty acid nitration proceeds via two major pathways in humans: basal nitration of dietary conjugated fatty acids in the gastrointestinal compartment and localized nitration in response to inflammation or acute tissue injury [69]. The acidic pH of the stomach facilitates the protonation of NO_2^- , which generates nitrous acid. This, in turn, yields $\bullet\text{NO}$ and $\bullet\text{NO}_2$ via N_2O_3 (the anhydride of nitrous acid), in the gastric compartment. The mimicry of this interaction was utilized in biochemical systems to generate $\bullet\text{NO}_2$ using nitrite in the presence of acid [40]. Although both products of nitrite acidification (i.e., $\bullet\text{NO}$ and $\bullet\text{NO}_2$) are radical species, $\bullet\text{NO}_2$ is a stronger one-electron oxidant ($E^{\circ\prime} = 1.04\text{V}$ vs NHE) compared to $\bullet\text{NO}$ ($E^{\circ\prime} = -0.55\text{V}$ vs NHE, pH = 7) [70,71]. To note, $\bullet\text{NO}$ and $\bullet\text{NO}_2$ are more relevant than ONOO^- in lipid oxidation given their lipophilicity and high permeation through the lipid bilayer [72]. In this regard, $\bullet\text{NO}$ and lipophilic radicals partitioning and concentrating in membranes facilitate $\bullet\text{NO}_2$ availability. Moller et al. estimated that $\bullet\text{NO}$ autoxidation is accelerated 30x in cell membranes and hydrophobic compartments, thereby efficiently generating $\bullet\text{NO}_2$ [52]. As a stronger one-electron oxidant and favored by lipid partitioning, $\bullet\text{NO}_2$ becomes an important oxidizing or nitrating reagent in complex lipids by either hydrogen abstraction or addition to unsaturated carbon-carbon bonds.

7. Shortcomings of work detecting nitrated complex lipids

Above 99 percent of fatty acids are esterified to complex lipids *in vivo*, so triglycerides and phospholipids are envisioned to provide the largest substrate for nitration reactions. Despite the critical role of

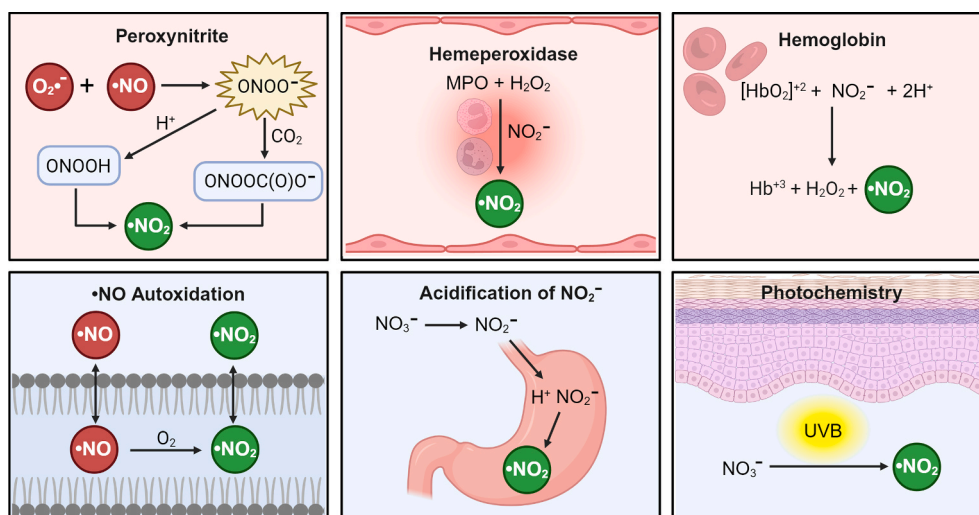


Fig. 1. The endogenous sources of nitrogen dioxide. $\bullet\text{NO}_2$ is a ubiquitous radical derived from $\bullet\text{NO}$ metabolism and radical-radical interactions. $\bullet\text{NO}_2$ formation was largely attributed to the homolytic decomposition of peroxynitrite, either after protonation or reaction with carbon dioxide. With improved understanding of $\bullet\text{NO}$ metabolism, it became clear that oxygen gradients and redox enzymes also enabled the reversible generation of $\bullet\text{NO}$ and other nitrogen oxides. These alternative mechanisms supported the formation of $\bullet\text{NO}_2$ by peroxynitrite-independent pathways, most notably by the hemeperoxidase and hydrogen peroxide-dependent oxidation of nitrite. Myeloperoxidase secreted by neutrophils (as well as eosinophil peroxidase) catalyze the oxidation of nitrite to $\bullet\text{NO}_2$ using H_2O_2 as an oxidant [81]. H_2O_2 is mainly produced by the different NADPH oxidase isoforms (depending on the biological context), xanthine oxidase, or the mitochondrial electron transfer chain. Additionally, the oxidation of nitrite by oxyhemoglobin supports $\bullet\text{NO}_2$ levels, as it is one of the initial products of this multi-step reaction pathway to produce nitrate. Basally, reactions such as $\bullet\text{NO}$ autoxidation, gastric acidification of nitrite, and photo-oxidation of nitrite in the skin support endogenous levels of the nitrating radical. In aqueous environments, $\bullet\text{NO}$ autoxidation is kinetically slow ($k = 2 \times 10^6 \text{ M}^{-2} \text{ s}^{-1}$), but hydrophobic phases accelerate the disappearance of $\bullet\text{NO}$, accelerating the formation of $\bullet\text{NO}_2$ [82]. Metabolism of dietary nitrate and nitrite generates $\bullet\text{NO}_2$ in the gastrointestinal tract. Commensal bacteria in the oral cavity possess nitrate reductase activity, readily reducing dietary nitrate obtained from leafy green vegetables into nitrite. In the stomach, protonation of nitrite into nitrous acid and subsequent homolysis of N_2O_3 generates $\bullet\text{NO}_2$. In the skin, UVB irradiation of nitrate generates $\bullet\text{NO}_2$ [67,68]. Figure created with BioRender. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

phospholipids in generating signaling-capable NO_2 -FAs and other nitrated products, characterization of the nitration products in complex lipid presents significant analytical challenges. The chemical nature of complex lipids (i.e., diversity in head group polarity and charge state, the combinatorial complexity of phospholipids) and the sensitivity of nitroalkenes to the conditions of chemical hydrolysis reactions amplify the analytical challenges associated with detecting the $\bullet\text{NO}_2$ -modified species. Additionally, when measuring the formation of these nitrated products in cells or tissue, the modified constituents are diluted amongst the various lipid species, limiting their abundance in the context of much greater contributions of native phospholipids or triglycerides. With these considerations in mind, efforts have centered around the detection of nitrated phospholipids in synthetic reaction systems. To date, 11 studies have identified nitrated and nitroxidized derivatives of phospholipids and triglycerides [73]. Despite the important advances in detecting these $\bullet\text{NO}_2$ -mediated derivatives of lipids and encouraging findings showing the presence of nitrated species, significant effort is still needed to validate these observations. In this regard, many of the reported products correspond to nitration of OA and LA, findings that contradict cell and biochemical nitration studies that failed to show the nitration of these fatty acids. The use of the potent nitrating agent nitronium ion (NO_2^+) to induce phospholipid nitration is concerning, as the mechanism of this reaction considerably deviates from biological nitration condition in which $\bullet\text{NO}_2$ and not NO_2^+ is the nitrating agent [31,74–78]. NO_2^+ readily reacts with any fatty acid double bond, expanding the scope of nitration targets and eliminating the requirement for conjugated dienes. In biological settings, NO_2^+ , if formed, is expected to rapidly decompose due to its considerable hygroscopic behavior [79]. Ultimately, efforts toward characterizing nitrated complex lipids in biomimetic reaction systems will strengthen the ability to detect endogenous nitrated lipids. In this regard, caution should be used when measuring species with one degree of unsaturation, more so considering that oleic acid is not a substrate for nitration and that the

main metabolic product of NO_2 -CLA in vivo is dihydro- NO_2 -CLA (a mixture of 9-nitrooctadec-11-enoic acid and 12-nitrooctadec-9-enoic acid), a constitutional isomer of NO_2 -OA that is not electrophilic, does not contain a nitroalkene and can be easily assigned as NO_2 -OA [15,80].

8. Conclusions

Starting from early reports in the field, it was evident that not all fatty acid substrates efficiently generate nitrated products, and only supraphysiological levels of $\bullet\text{NO}_2$ support nitration of bisallylic alkenes via addition reactions. However, the concentration of $\bullet\text{NO}_2$ in physiological and pathological conditions does not reach levels that support the nitration of linoleic acid or arachidonic acid, promoting radical-catalyzed oxidation reactions via hydrogen abstraction instead. Due to differences in chemical reactivity and resonance stabilization of intermediates, conjugated fatty acids are the preferential nitration targets in biology, forming NO_2 -FAs that can modulate cytoprotective signaling cascades. Evidently, there is a striking difference between conjugated vs. non-conjugated fatty acids regarding the reactivity towards physiological levels of $\bullet\text{NO}_2$. This discrepancy presents an opportunity to emphasize the importance of selecting the appropriate substrate and nitrating agent when developing analytical methods to detect lipids with a nitro group or other nitroxidized modifications. Over the last few years, efforts have been focused on detecting nitrated complex lipids, as they are the precursors to electrophilic NO_2 -FAs. Strong nitrating agents like NO_2BF_4 expand the scope of potential nitration targets, but the reaction mechanism deviates from that of $\bullet\text{NO}_2$ radical-mediated nitration. These conditions are not biomimetic and allow for a product profile that may not accurately represent species that form endogenously. Ultimately, when measuring the formation of nitrated lipids in any biological sample, the specificity of nitration for conjugated fatty acids in physiological conditions is a critical factor to consider.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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