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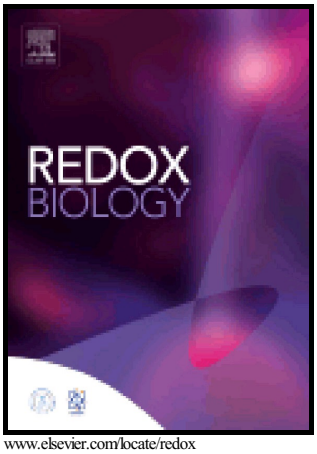

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PII: S2213-2317(18)31024-3
DOI: https://doi.org/10.1016/j.redox.2019.101106
Article Number: 101106
Reference: REDOX101106

To appear in: *Redox Biology*

Received date: 2 November 2018
Revised date: 8 January 2019
Accepted date: 10 January 2019

Cite this article as: Tânia Melo, Javier-Fernando Montero-Bullón, Pedro Domingues and M. Rosário Domingues, Discovery of bioactive nitrated lipids and nitro-lipid-protein adducts using mass spectrometry-based approaches, *Redox Biology,* https://doi.org/10.1016/j.redox.2019.101106

Discovery of bioactive nitrated lipids and nitro-lipid-protein adducts using mass spectrometry-based approaches

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Abstract

Nitro-fatty acids (NO_2-FA) undergo reversible Michael adduction reactions with cysteine and histidine residues leading to the post-translational modification (PTM) of proteins. This electrophilic character of NO_2-FA is strictly related to their biological roles. The NO_2-FA-induced PTM of signaling proteins can lead to modifications in protein structure, function, and subcellular localization. The nitro lipid-protein adducts trigger a series of downstream signaling events that culminates with anti-inflammatory, anti-hypertensive, and cytoprotective effects mediated by NO_2-FA. This lipoxidation adducts have been detected and characterized both in model systems and in biological samples by using mass spectrometry (MS)-based approaches. These MS approaches allow to unequivocally identify the adduct together with the targeted residue of modification. The identification of the modified proteins allows inferring on the possible impact of the NO_2-

FA-induced modification. This review will focus on MS-based approaches as valuable tools to identify NO_2-FA-protein adducts and to unveil the biological effect of this lipoxidation adducts.

1. Introduction

During the last decade, nitrated lipid gained the interest of the scientific community, as new endogenous signaling molecules with important regulatory role in health and disease. Research on this is aimed at understanding the reactivity of reactive nitrogen species (RNS) with lipids, to unravel their occurrence *in vi*vo and their biological roles. Among nitrated and nitroxidized lipids identified so far, the nitro-fatty acids (NO_2-FA) are best-known products of RNS. These products have been widely detected in several tissues [1–5] and biofluids [6–12], and are nowadays a hot topic in nitro lipidomics. NO_2-FA are considered important bioactive molecules and have been associated with anti-inflammatory [6,13–24], anti-hypertensive [25–32], and anti-thrombotic properties [31,33] and cytoprotective effects [2,34–37]. More recently, other nitrated and nitroxidized lipids [1,6–8,13,38] and also nitro derivatives of phospholipids (PL) [39,40] and triglycerides (TAG) [41] have been detected in biological samples and were associated with protective and beneficial effects, but they are scarcely studied. Also, esterified forms of NO_2-FA have been found as they can be generated either by direct nitrated of the esterified fatty acyl moiety or by the incorporation of NO_2-FA [41].

NO_2-FA are also known as nitroalkenes derivatives of fatty acids since it includes a nitro group linked to the double bond (alkene group) of the unsaturated fatty acyl chain, and

the nitro-alkene moiety makes these derivatives highly reactive with electrophilic properties. These endogenous electrophilic lipids are capable to covalently link to proteins, via Michael addition [42], leading to the formation of lipoxidation adducts. This type of post-translational modification (PTM) of proteins can modulate protein function, which underlies some of the biological roles attributed to the NO_2-FA (Figure 1). Some of these PTMs are shown to elicit a protective effect, which may provide clues for new therapeutic strategies and new drugs.

Detection of NO_2-FA and especially their lipoxidation adducts are still a challenge that is mostly addressed by MS approaches. MS-based approaches have been extensively applied in the study of NO_2-FA-protein adducts [4,7,34,42–44], providing detailed structural information of these adducts both *in vitro* and *in vivo*. LC-MS and MS/MS-based proteomics approaches have been performed to characterize the NO_2-FA protein adducts and the sites of adduction [34,42,45–48]. Very recently peptide adducts were also reported for NO_2-FA esterified in phospholipids using biomimetic *in vitro* studies and MS approaches [49].

In this review we will discuss the formation and type of nitrated FA found in biological systems, their structure and reactivity with proteins and characterization by a MS-based proteomic and lipidomic approach that allowed to disclose possible biological roles associate with nitrated lipids-protein adducts.

2. Endogenous nitro-fatty acids

2.1. Chemistry and analysis

NO_2-FA are endogenous chemical entities generated by the attack of nitric oxide (NO)-derived reactive species, collectively called reactive nitrogen species (RNS), with unsaturated fatty acids. Nitrogen dioxide ($^{\bullet}NO_2$), nitronium cation (NO_2^+), and peroxynitrite/peroxynitrous acid, whose decomposition yields $^{\bullet}NO_2$ and hydroxyl radical ($^{\bullet}OH$), were reported as RNS that most frequently initiate nitration or nitroxidation reactions in biomolecules, including lipids. The prevalence and the yield of one process of these processes over the others are dependent on the oxygen levels, concentration of ROS versus RNS, the presence of secondary target molecules (scavengers, thiols, and transition metals), pH, and the partition between hydrophilic and hydrophobic milieu in cellular compartments [50]. The mechanism of FA nitration and nitroxidation in biological systems is not yet wholly undisclosed, and there are some alternative routes to explain the generation of NO_2-FA (Figure 2). The free radical-induced nitration of FA mediated by $^{\bullet}NO_2$ is one of the most prominent reaction *in vivo* as a source of NO_2-FA [51]. The endogenous formation of NO_2-FA during free radical-mediated nitration reactions occurs in several biological processes such as digestion [52], metabolic stress, and inflammatory conditions [53]. Thus NO_2-FA were already identified in human red blood cells [8,9], plasma [6,8–10,12], urine [6,7], and tissues [1–5] at concentrations ranging from picomolar [12] to micromolar [6] and associated to some of the reported processes. Dietary sources of nitrite can also leads to the generation of NO_2-FA via acid-catalyzed nitration reactions [52,54–56]. Recently, NO_2-FA were also reported in plants, fresh olives, and in extra virgin olive oil [56,57], which are considered external sources of NO_2-FA and can contribute to rising the endogenous levels of NO_2-FA [58].

The most common NO_2-FA identified *in vivo* were the nitrated forms of the nitro-oleic acid (NO_2-OA), nitro-linoleic acid (NO_2-LA), and nitro-conjugated linoleic acid (NO_2-cLA) [6,8,9]. However, the reaction of RNS with fatty acids can lead to the generation of several nitroalkene derivatives of other fatty acids, such as the nitro-palmitoleic acid (NO_2-POA), nitro linolenic acid (NO_2-LNA), nitro-arachidonic acid (NO_2-AA), nitro eicosapentaenoic acid (NO_2-EPA), and nitro-docosahexaenoic acid (NO_2-Dha) [6,8,38,51]. Different stereo or positional isomers of NO_2-FA were detected *in vitro* and in biological samples [6,8,38], as represented in Figures 3 to 5 and Table 1.

Nevertheless, the recovery of NO_2-FA from biological samples, together with their detection and accurate quantification is a challenge due to their low concentration, stability issues, metabolism (β-oxidation and saturation/desaturation reactions) [59], reactivity with proteins [59] and esterification [39–41], and different distribution among tissues and biofluids [6,12]. In line with these limitations, there has been an effort for the development of specific, standardized and reproducible methodologies of sample preparation and sensitive analytical approaches. The advent of more sensitive and sophisticated instruments, allied with the possibility of high-throughput analysis prompted by MS-based approaches, combined or not with liquid chromatography (LC-MS), has been the selected tool for the identification, structural characterization and quantification of free NO_2-FA. Indeed, the detection of these lipids is an indication to disclose the bioactive properties of these nitrated derivatives. The progress in the field of MS-based approaches enabled the discovery of NO_2-FA and contributed to the knowledge of NO_2-FA biological roles giving information on the structure-function relationships [60]. The development of improved sample preparation techniques, chromatographic separations, high-resolution instruments with

great sensitivity, and innovative tools raised the possibility of detection, structural characterization and quantification of nitro lipids in human samples and animal models both under physiological and pathological conditions [1,3,4,8,59,61,62], and also in plants [56] as summarized in Table 1. The identification of NO_2-FA by MS is based on the detection of specific mass shifts compared to non-modified fatty acid (FA+45 Da). Using MS-based approaches, NO_2-FA are preferentially analyzed in negative-ion mode as $[M-H]^-$ ions [3,63]. However, positive-ion mode ionization can also occur, and NO_2-FA can also be identified as $[M+H]^+$ [26], $[M+Li]^+$ [51], $[M+NH_4]^+$ [9,41], and $[M+Na]^+$ ions [9]. Tandem mass spectra acquired both in positive- and negative-ion mode provides information that allows the structural characterization of NO_2-FA [8,38,51,63,64]. The fragmentation pattern of NO_2-FA under tandem MS (MS/MS) conditions includes the typical neutral losses of 47 Da (HNO_2) and product ions formed by cleavage of the hydrocarbon chain in the vicinity of the NO_2 group, that allow assigning this modified FA. The differentiation of isomers can be addresses by the identification of reporter fragment ion that is formed by cyclization, followed by heterolytic carbon chain fragmentation, which allows pinpointing the correct position of the NO_2 group [2,63]. These product ions have been used as diagnostic ions broadly employed for targeted analysis and quantitation of specific NO_2-FA using reversed phase LC-MS/MS approaches, in biomimetic systems and in cells, tissues and biofluids [3,6,8–10,12,17,38,59,65,66]. Structural information gathered by using MS studies can be further confirmed by infrared and nuclear magnetic resonance analysis for the confirmation of the functional groups and the final structure [6,9,10,30,31,51].

The generation of NO_2-FA can be considered as the first step of nitration reactions. These species can be precursors of other nitrated and nitroxidized species because NO_2-FA

can undergo additional reaction with ROS and RNS to be further nitrated, leading to the formation of nitroso, dinitroso, nitronitroso, di and trinitro species, or oxidized generating the assorted nitroxidized species as nitrohydroxy, nitrohydroperoxy, nitro-epoxy and nitro-keto (Table 1) [3,6,8,26,54,64]. All of these derivatives were already identified by (LC)-MS and characterized by (LC)-MS/MS [1,6,9,38]. In fact, the great sensitivity of MS-based approaches allowed to identify both nitro and nitrohydroxy derivatives of palmitoleic, oleic, linoleic, linolenic, arachidonic and eicosapentaenoic acids in human plasma and urine [6]. However by far the NO_2-FA are the most studied mostly because, in opposition to other nitrated and nitroxidized FA, they are electrophilic molecules with great capability to react with protein with the formation of lipoxidation adducts.

2.2. Biological roles of nitro-fatty acids as new metabolic mediators, signaling molecules, and new therapeutic drugs candidates

NO_2-FA have raised the interest of the scientific community in last years, mainly because of their biological roles as key mediators in physiological and pathophysiological processes, as demonstrated in a variety of preclinical animal models of disease and in plants [2,5,13,15,20,28,32,34,45,56]. They were assigned as biologically relevant and putative signaling molecules in cardiovascular disease [28,33], myocardial ischemia/reperfusion and ischemia preconditioning [1,2,24], nephropathy [24], renal ischemia/reperfusion [24], diabetes and metabolic syndrome [14], pulmonary inflammation [15,67] and chronic inflammatory disease [65]. NO_2-FA reach endogenous levels that allow them to mediate pivotal signaling actions as cytoprotective and pro-survival players [2,34–37], and based on their pleiotropic actions, NO_2-FA has emerged as potential therapeutic agents with high

potential for therapeutic use (Table 2). In fact, NO_2-FA already undergo clinical trials [68]. Also, 10-NO_2-OA (CXA-10) demonstrated promising pharmacokinetic and pharmacodynamics characteristics during preclinical experiments [61,68,69]. CXA-10 is currently in phase II clinical trials for the treatment of chronic inflammatory and metabolic-related diseases, namely focal segmental glomerulosclerosis and pulmonary arterial hypertension, since it demonstrated beneficial effects when administrated via intravenous injections or through ingestion [61,68,69].

The biological actions of NO_2-FA are mediated via a) decay reactions and transduction of nitric oxide ($^{\bullet}$NO) signaling actions [29,70]. Also, NO_2FA can be considered NO donor, b) via receptor-dependent and c) via electrophilic adduction reactions to proteins [42], with formation of lipoxidation adducts. All these processes mediate important and specific signaling roles. These signaling actions are summarized in Table 2. Nitric oxide release by NO_2-FA has been associated with potential antioxidant properties through inhibition of lipid peroxidation process [71]. Additionally, the release of $^{\bullet}$NO by both NO_2-FA and nitrohydroxy FA derivatives has also been related with vasorelaxation properties of these nitrated lipid [26,29–31,51]. The nitro derivatives of arachidonic acid, NO_2-AA and nitrohydroxy-AA were also reported to be able to release $^{\bullet}$NO and thus to induce cGMP-dependent vasorelaxation in rat aortic ring in an endothelium-independent manner [26,31,51]. NO_2-LA, NO_2-cLA and nitrohydroxy-LA promoted vessel relaxation via cGMP-dependent and endothelial-independent manner in pre-constricted rat aortic rings [29,30]. Nevertheless, the $^{\bullet}$NO release by nitro lipids remains a controversial issue, and at some level, considered of minor relevance at endogenous levels [28,29,51,70,72]. Actually, $^{\bullet}$NO signaling actions mediated by NO_2-FA

mainly occurs via cGMP-independent mechanisms. NO_2-FA modulates endothelial (eNOS) and inducible nitric oxide synthase (iNOS) gene expression and activity and consequently the eNOS- and iNOS-mediated $^{\bullet}NO$ generation and reactions. Also, NO_2-FA modulates a broad array of signaling pathways that culminates with the downstream activation or inactivation of $^{\bullet}NO$ signaling [67,73,74].

The covalent adduction to key proteins propelled by the electrophilic character of NO_2-FA seems to be the most prominent mechanism by which these nitro lipids spread their modulatory and protective actions. The identification and characterization of these NO_2-FA-protein adducts in distinct biological conditions have been achieved by reversed phase LC-MS-based proteomics approaches [28,42,56,59,75,76]. This topic will be discussed in more detail in the next section.

As endogenous molecules, NO_2-FA undergo a series of metabolic, trafficking and clearance pathways that influences the regulation of activity, half-life and levels of free NO_2-FA. Protein adduction and esterification in complex lipids [70,77,78] are considered as reservoirs of NO_2-FA, allowing to regulate their endogenous levels [70,77,78]. NO_2-FA–protein adducts are reversible in biological systems [59,73,79] and NO_2-FA esterified forms can be hydrolyzed and mobilized by esterases and lipases, allowing NO_2-FA to return to free active forms [70,80]. NO_2-FA can be metabolized via β-oxidation that mediates the formation of shorter and more polar electrophilic species [59] that retains the electrophilic power, but also to inactive nitroalkane species [7,59]. In fact, in humans and rodents, the bio-elimination pathways of 10-NO_2-OA involves the generation of a series of shorter metabolites that were detected in urine using C18-HPLC-ESI-MS and MS/MS using both LTQ Velos Orbitrap and API 5000 triple quadrupole instruments [61]. However, the

electrophilic functionality of NO_2-FA is irreversibly inactivated after reduction and conversion to the correspondent nitroalkane derivative by the nitroalkene saturase prostaglandin reductase-1 [81]. Both saturation and desaturation of the double bond of NO_2-FA are related with the generation of saturated non-electrophilic NO_2-FA [59], which are nitro derivatives without signaling abilities. Adduction to peptides or proteins seems to have other proposes, such as the case of conjugation with GSH, which increases the urinary excretion rate of NO_2-FA excreted in urine [82]. Incorporation of NO_2-FA into lipoproteins is another way for NO_2-FA to enter in circulation and to be systemically distributed among tissues. The modulation of all of these diverse pathways will impact the potential reactivity, the efficacy of signaling actions and behavior of these nitration products.

The signaling actions of NO_2-FA are also mediated through the modulation of the structure and regulation of the expression and activity of anti- and pro-inflammatory proteins, heat shock proteins and phase II antioxidant response proteins. The capability of NO_2-FA to react with specific peptides and proteins determines the role of this nitrated lipids in redox regulation with consequence in cell signaling, as will be described in the following section.

3. Nitro-fatty acids and Protein lipoxidation adducts

3.1. Main target and biological significance of PTM by nitro-fatty acids

NO_2-FA are electrophilic compounds, able to react via reversible Michael addition with nucleophiles within key proteins, leading to the formation of lipid-protein adducts (lipoxidation) in a process generally denominated nitroalkylation [83,84]. The target

nucleophiles in peptides and proteins include the deprotonated thiolate group of cysteine and the nucleophilic amino group of the imidazole moiety of histidine or the amino groups of lysine and arginine [83−85]. The high electronegative olefinic NO_2 group facilitate the addition to the double bond of the unsaturated hydrocarbon chain of NO_2-FA. This addition generates an important positive density of charge in the methylenic β-carbon adjacent to the nitration site. The oxygens of the NO_2 group withdraw electrons and the double bond is rearranged over the C-N bond, generating a carbocation. This conjugation makes the β-carbon adjacent to the NO_2 group electron poor and with potential reactivity. The NO_2-FA-protein covalent adducts generated during the nitroalkylation process are reversible, which seems to be related with the possibility of redox regulation [59,73,79] and thus can be associated with the apparent lack of toxicity of these modified lipids. All of the aforementioned characteristics make NO_2-FA as promising pharmacological compounds. In fact, pre-clinical and human trials has demonstrated the NO_2-FA favorable pharmacokinetics and safety.

The formation of NO_2-FA adducts with proteins is considered a key PTM of proteins. This modification of functionally-relevant proteins can modulate the patterns of gene expression programs, transcription factors function, enzyme function and activity, metabolic and inflammatory responses, and cell signaling networks [50,59,73,84]. This lead to a series of downstream signaling events that are intrinsically related to the biological signaling roles of NO_2-FA [2,6,13–37,61,74,75,86–90] (Table 2). The activation of several of these pathways are considered essential for restoring the homeostasis and the redox balance and makes NO_2-FA promising pharmacological compounds [91].

There are several proteins reported to be targets of NO_2-FA electrophilic reactivity, for example, the p65 subunit of NF-κB [1,23,92], heme oxygenase-1 (HO-1) [17,19,22,67,89,93], mitogen-activated protein kinase (MAPK) phosphatase 1 (MPK-1) [92], Kelch-like ECH-associated protein 1 (Keap 1) [17,22,46,88], metalloproteinases (MMP-7 and MMP-9) [75], glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [42,94], protein disulfide isomerase (PDI) [95], and transient receptor potential (TRP) channels [96–99] (Table 3). NO_2-FA can also conduct their biological signaling roles by a receptor-dependent signaling action and peroxisome proliferator-activated receptor gamma (PPARγ) is one of the main targets, which is a significant route for the anti-inflammatory effect associated with NO_2-FA derivatives [6,23,47,65,93,100–102].

The nitro lipoxidation PTM of the proteins shown in the table 3 have been correlated with specific biological effects. For example, nitroalkylation of the p65 subunit of NF-κB [23], induction of HO-1 expression [93], PPARγ modulation [100], inhibition of the correct assembly of the active NADPH oxidase (NOX2) [74], and inhibition of both reductase and chaperone activities of PDI and possible prevention of NOX2 activation [95] have been associated with the anti-inflammatory properties of NO_2-FA. Another important anti-inflammatory action of NO_2-FA is attributed to their capability to induce PTM of 5-Lipoxygenase (5-LOX) limiting the inflammation induced by the 5-LOX-dependent leukotriene synthesis. This point deserves to be further explored as a potential therapeutic/pharmacological strategy due to the physiological relevance of 5-LOX, namely in inflammation [45]. Induction of HO-1 and activation of Nrf2 have been correlated with protection against oxidative stress and antioxidant actions of NO_2-FA [93]. Activation of PPARγ by NO_2-FA has also been associated with glucose uptake and anti-hyperglycemic

effects [100]. Inhibition of the catalytic activity of sHE was associated with anti-hypertensive properties of NO_2-FA [32]. Finally, neuroprotective effects associated with the decrease of protein aggregation were related with PTMs of α-synuclein by NO_2-OA [35].

3.2. Identification of protein-nitro-fatty acids adducts: tools and challenges

Identification of protein nitroalkylation by NO_2-FA has been disclosed by using different experimental approaches, as crystallographic analysis [100,101], western immunoblot-based assay [2,23,32,34,87], spectrophotometry [7,43,44,94] and MS-based approaches [4,7,34,42–44]. However, spectrophotometry and immunoassays do not give detailed structural information and crystallography requires pure proteins, being difficult to be used in the analysis of complex biological samples.

Mass spectrometry, namely using matrix assisted laser desorption/ionization (MALDI) or electrospray (ESI) MS-based proteomics approaches, often coupled to reverse phase (RP) liquid chromatography (LC–MS), are the most suitable methods for detection and characterization of adducts formed between NO_2-FA and proteins. *In vitro* generation of NO_2-FA-protein adducts, in biomimetic systems, between standards of NO_2-FA and candidate peptides or proteins has been used as strategy for the initial identification by (LC)-MS and further characterization of these adducts by MS/MS. Data obtained using these biomimetic approaches using controlled reaction conditions are more straightforward and relatively easy to analyze. This, in turn, allows to obtain knowledge on the reactivity of each individual NO_2-FA and the typical fragmentation pathways under MS/MS needed to

identify these adducts. The information gathered by tandem mass experiments concerning the typical fragmentation pathways and reporter ions can be used to identify these lipoxidation products in complex biological samples by using MS-based proteomics approaches and to develop MS target analysis, namely multiple reaction monitoring (MRM) analysis. This has contributed to achieve the ultimate goal that consists of the identification of the NO_2-FA-protein adducts in complex biological samples as cells, tissues, biological fluids, which requires specific and targeted approaches. Bottom-up proteomics approaches are usually performed. Through these analytical approaches, it is possible to unequivocally identify the modified peptides after enzymatic digestion of NO_2-FA-protein adduct, usually using trypsin, followed by the analysis of the tryptic peptides by reverse-phase (RP)-LC-MS and MS/MS. The addition of the NO_2-FA moiety increases the retention time of the modified peptides [42], which are identified on the mass spectra as singly, $[M+H]^+$ ions, or multiple charged ions, $[M+nH]^{n+}$, based on the mass shift against the unmodified peptide. This gives information on the nature of NO_2-FA covalently attached to the protein. The observed mass shift in the mass spectra for the Michael adducts will be equal to the molecular weight of the NO_2-FA. Thus, a mass shift of +327 Da and +325 Da corresponds to the addition of NO_2-OA and NO_2-LA, respectively [42]. MS/MS data allows to confirm the nature of the modification and provides information on the fragmentation pattern of NO_2-FA-peptide adducts. These data further allows to pinpoint the location of the modification site and thus the targeted residue in the peptide backbone [103,104]. Detailed information to identify the sites of adduction is revealed by a mass shift of the typical *b* and *y* product ions of the adducted peptide, when compared with the non-

modified one. The modified immonium ions are also useful to confirm the presence of a modified amino acid residue within the adducted peptide.

RP-LC-ESI-MS and MS/MS were used to detect lipoxidation adducts formed between NO_2-OA or NO_2-LA and GAPDH and GSH *in vivo* in healthy human red cells [42]. This methodology was also applied to confirm the post-translational modifications of matrix metalloproteinase by NO_2-OA [75], and the for the identification of reversible Michael adducts of NO_2-OA and thiols of proteins and GSH in liver and plasma of NO_2-OA-treated mice [59]. Significant levels of protein cysteine adducts of NO_2-OA were also observed in fresh olives, especially in the peel [56]. AT_1-R adducts with NO_2-OA were quantified by HPLC-MS/MS using MRM scan mode in the negative-ion mode as BME adducts (BME-NO_2-OA adducts) after a nucleophilic exchange of NO_2-OA from AT_1-R to BME. The presence of exchangeable NO_2-OA demonstrated the direct adduction of AT_1-R by NO_2-OA, and therefore that AT_1-R is a relevant cellular target for NO_2-OA alkylation [28]. RP-LC-MRM scan in the positive-ion mode ($[M+H]^+$ ions) was applied for the characterization of NO_2-LA-GSH adducts *in vitro* and further identification in MCF7 cells treated with NO_2-LA [76]. Nitroalkylation of albumin by NO_2-OA and NO_2-LA have been found in the plasma of mice gavage with these fatty acids [62]. Nitroalkylation of p65 subunit of nuclear factor κB (NF-κB) was observed *in vivo* in myocardial tissue of a murine model of ischaemia-reperfusion with intravenous supplementation of OA and LA [1]. One study also reported the direct analysis by MALDI-TOF-TOF MS and MS/MS, in positive-ion mode, of adenine nucleotide translocase 1 (ATN 1) adducts after NO_2-LA infusion into intact perfused heats allowing to pinpoint that the nitroalkylation of ANT1 by NO_2-LA

occurred on Cys57 [34]. Adduction of NO_2-OA to PPAR-γ [47], and to Keap1 [46] are also examples of biological detection and characterization of NO_2-OA-protein adducts by MS.

The Michael addition reactions between NO_2-FA and proteins is remarkably selective and depends on the nature and structural features of the NO_2-FA. The fatty acyl chain length and the position of the electrophilic carbon, i.e., the position of the nitroalkene group, has a pivotal effect on the reactivity of NO_2-FA [102]. Therefore both factors regulate the formation of NO_2-FA-protein adducts and the biological activity of the NO_2-FA [22,42,65,73,100,101]. In spite of its four possible isomers (at C9, C10, C12 or C13), only the NO_2-LA isomers bearing the NO_2 at C10 and C12 were reported to selectively bind to cysteine 285 (Cys285) in the ligand-binding domain and activate PPARγ [101]. The C10 isomer of NO_2-OA is more reactive toward to Cys285 in the ligand binding domain of PPARγ than the C9 isomer [47]. On the other hand, Keap1 is easily activated by the C9 isomer via nitroalkylation of Cys273 and Cys288 [22,46]. Xanthine oxidoreductase activity is preferentially inhibited by the C9 isomer of NO_2-OA or a mixture of both C9 and C10 isomers [73]. It has been reported that NO_2-FA with shorter acyl chains interact stronger with Nrf2 and NF-kB [60].

Overall, the identification of NO_2-FA-protein adducts is important, because it may give information, as shown in several examples reported earlier, on the potential protein targets whose modulation by NO_2-FA can have potential therapeutic interest.

4. Esterified nitro-fatty acids

4.1 Nitrated phospholipids and their lipoxidation adducts

In spite of their free forms, NO_2-FA can be stabilized by esterification in more complex lipids in hydrophobic compartments, as the biological membranes. Nitrated derivatives of phospholipids were identified in biomimetic model systems and also *in vivo* [39,40]. In mimetic model studies, nitrated PLs were generated after *in vitro* incubation of PL standards (phosphatidylcholines, PCs and phosphatidylethanolamines, PEs) and NO_2BF_4, and its characterization was performed using C5-LC-MS and MS/MS in a Linear ion trap [39,40]. Nitrated PCs and nitrated PEs were detected by HILIC-LC-MS and MS/MS-based lipidomic approaches in cardiac mitochondria from diabetic rats [39] and cardiomyoblasts subjected to starvation [40]. Nitrated 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine (nitrated POPC) was reported to have antioxidant properties as scavenging agent, mediated by its anti-radical potential and ability to inhibit lipid peroxidation. Anti-inflammatory properties of nitrated POPC, related with its ability to inhibit iNOS expression in LPS-activated macrophages, were also reported [105].

NO_2-FA incorporation in PLs was also reported by using C18-HPLC-ESI-MS and MS/MS in API 4000 Q-trap triple quadrupole in adipocytes supplemented with NO_2-SA, NO_2-OA, NO_2-cLA, and NO_2-LA, before and after acidic hydrolysis. The incorporation yield and profile was specific for each supplemented NO_2-FA and PL class, being PC the PL class with highest levels of incorporation of NO_2-FA [106].

Nitrated POPC was also found to have the capability to form adducts with peptides. The identification of the covalent adducts of NO_2POPC with GSH was characterized by tandem MS in different instruments and the typical fragmentation pathways were disclosed for the first time. In this study, the NO_2POPC-GSH adducts were generated under biomimetic conditions and characterized by direct infusion MS and MS/MS using different

instrumental platforms including LXQ linear ion trap, Q-TOF 2, and Q-Exactive Hybrid Orbitrap. The observed fragmentation pattern of NO_2POPC-GSH adducts included product ions that confirmed the presence of the phosphatidylcholine moiety (*m/z* 184.074 and neutral loss of 183 Da), the nitro group (neutral loss of HNO_2), and $^{*}y_2$, $^{*}b_2$ and $^{*}C_1$ fragment ions of the modified peptide. All of these product ions pinpointing that NO_2POPC was linked to a cysteine residue of GSH (Figure 6) and can be used as reporter ions applied in the search of these lipoxidation adducts in biological samples [49].

4.2. Nitrated Triacylglycerides

Nitrated triacylglycerides (NO_2-FA-TAG) have been reported in rat plasma after oral administration of NO_2-OA, together with β-oxidation and dehydrogenation derivatives of NO_2-FA-TAG in adipocytes supplemented with NO_2-OA. These studies were performed by C18-HPLC-ESI-MS and MS/MS in API4000 Q-trap triple quadrupole and LTQ Velos Orbitrap instruments [41]. Another study reported the differential esterification profile of NO_2-FA and their metabolites in TAGs in adipose tissue of rats fed with 10-NO_2-OA. By using C18-HPLC-ESI-MS and MS/MS in API 4000 Q-trap triple quadrupole, the NO_2-FA were observed to be preferentially incorporated in monoacyl- and diacylglycerides. This was found to be in opposite to its reduced metabolites, which were favorably incorporated in TAGs. These observations were corroborated by the analysis of the lipid polar and neutral fractions from adipocytes supplemented with NO_2-SA (nitro-stearic acid), NO_2-OA, NO_2-cLA, and NO_2-LA, after acidic hydrolysis [106].

The occurrence of nitrated phospholipids and triacylglycerides can be of high relevance at biological level. The NO_2-FA-containing phospholipids and triacylglycerides

can act as a reservoir of NO_2-FA. Additionally, these esterified NO_2-FA can be further mobilized by lipases in turn to exert their adaptive and anti-inflammatory signaling actions. In the case of NO_2-FA-containing phospholipids, the NO_2-FA moiety seems to be able to retain the electrophilic character, and thus the ability to undergo reversible reactions via Michael addition with key proteins. Also, these phospholipid-esterified NO_2-FA can have an impact as anti-inflammatory and cytoprotective species. The nitration of esterified NO_2-FA or its incorporation into more complex lipids, together with the occurrence of lipoxidation products of NO_2-FA-containing phospholipids, and perhaps NO_2-FA-TAGs, can also contribute to the systemic distribution and metabolism of NO_2-FA.

5. Conclusion and future perspectives

NO_2-FA own important physiological functions that are mediated *via* formation of lipoxidation adducts and associated regulation of protein function. Several signaling proteins, with key roles in anti-inflammatory, anti-hypertensive, anti-hyperglycemic, and cytoprotective pathways, are targets of NO_2-FA adduction. This points to potential for new therapeutic strategies in important non-communicable diseases as cardiovascular, renal, pulmonary, and metabolic diseases. Mass spectrometry is a promising analytical tool in the detection of NO_2-FA-protein adducts. Nevertheless, there is a need for new methodological developments to improve the detection of these elusive lipoxidation adducts, and to obtain more insights regarding the protein targets of NO_2-FA and its roles in biological signaling pathways.

Acknowledgments

We acknowledge the European Commission's Horizon 2020 research and innovation programme for the Marie Sklodowska-Curie grant agreement number 675132 (MSCA-ITN-ETN MASSTRPLAN) to University of Aveiro and to Centro Hospitalar e Universitário de Coimbra. Thanks are due to the University of Aveiro, FCT/MEC, European Union, QREN, COMPETE for the financial support to the QOPNA (FCT UID/QUI/00062/2013) and CESAM (UID/AMB/50017 - POCI-01-0145-FEDER-007638), through national funds and where applicable co-financed by the FEDER, within the PT2020 Partnership Agreement, to the Portuguese Mass Spectrometry Network (LISBOA-01-0145-FEDER-402-022125). Tânia Melo (BPD/UI51/5388/2017) is grateful to FCT for her grant.

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Captions:

Figure 1. Schematic representation of nitrated lipids pathways: from their generation to their biological effect.

Figure 2. Representative mechanisms of nitro-fatty acid (NO_2-FA) formation. Radical-induced nitration of unsaturated fatty acids by nitrogen dioxide ($^{\bullet}NO_2$) yields a β-nitroalkyl radical that can further react with other $^{\bullet}NO_2$ generating the nitronitrite intermediates. Further loss of nitrous acid (HNO_2) leads to the generation of the nitroalkene derivatives also called NO_2-FA. Electrophilic substitution at the double bond mediated by nitronium cation (NO_2^+) also yields NO_2-FA.

Figure 3. Proposed structures for nitro-oleic (NO_2-OA) and nitro-linoleic acids (NO_2-LA), with assignment of their different positional isomers, which were previously detected in *in vitro* studies and/or biological samples.

Figure 4. Proposed structures for nitro arachidonic acid (NO_2-AA), with assignment of its different positional isomers, which were previously detected in *in vitro* studies and/or biological samples.

Figure 5. Proposed structures for nitro-docosahexaenoic acid (NO_2-DHA), with assignment of its different positional isomers, which were previously detected in *in vitro* studies and/or biological samples.

Figure 6. ESI-MS spectra of mono charged $[M+H]^+$ (A) and double charged $[M+2H]^{2+}$ ions (B) of NO_2POPC-GSH adducts, acquired in Q-Exactive Orbitrap, with identification

of major fragmentation pathways. (Reprinted with permission from Bullón et al. (2018), copyright 2018 [John Wiley & Sons]).

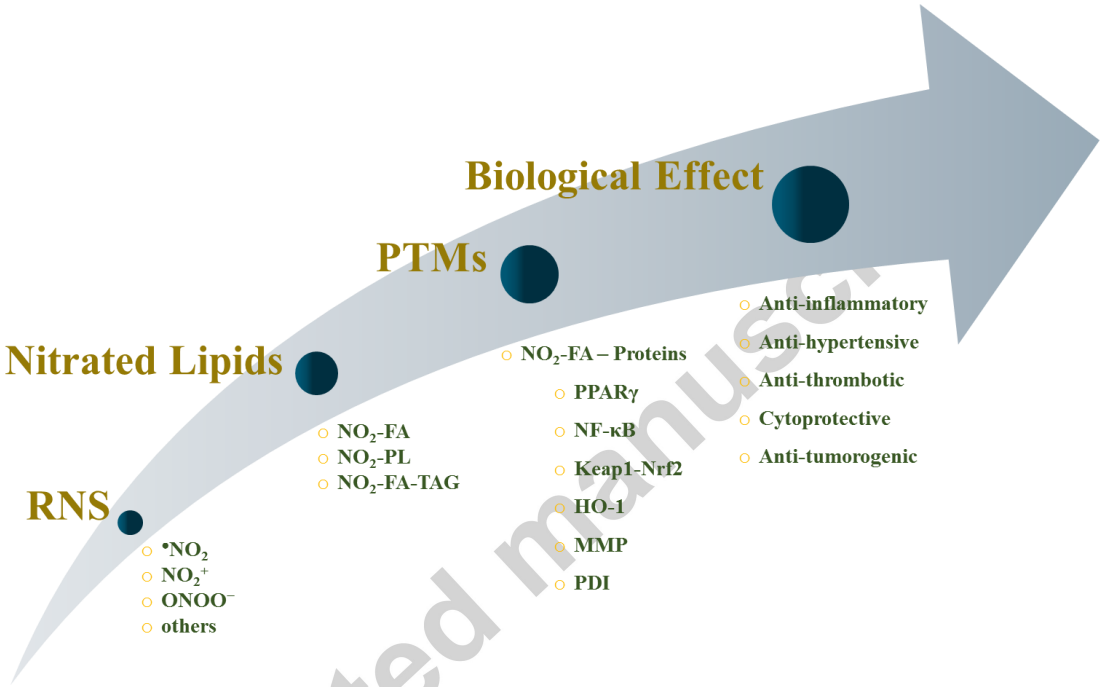


Figure 1

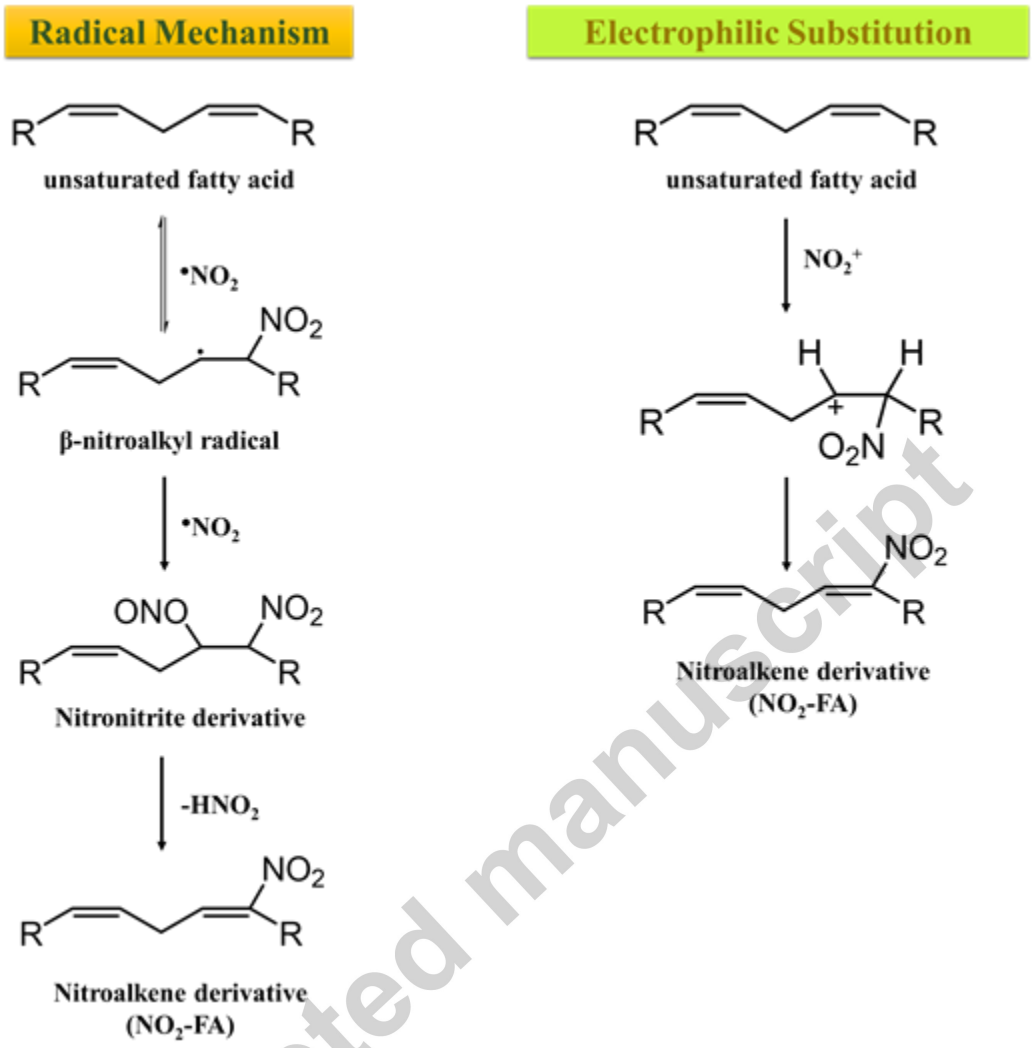
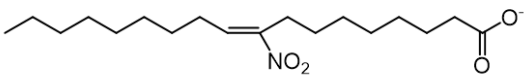


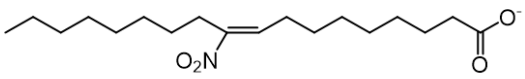
Figure 2.

Nitrated Oleic Acid (NO_2-OA) isomers



9-nitro-9-*cis*-octadecenoic acid

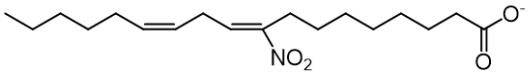
9-NO_2-OA



10-nitro-9-*cis*-octadecenoic acid

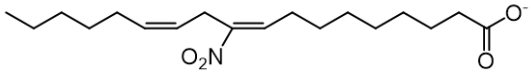
10-NO_2-OA

Nitrated Linoleic Acid (NO_2-LA) isomers



9-nitro-9,12-octadecadienoic acid

9-NO_2-LA



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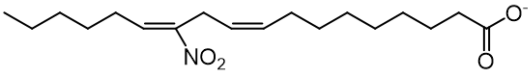
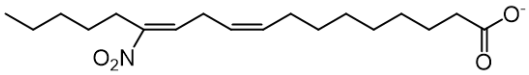


12-nitro-9,12-octadecadienoic acid

12-NO_2-LA

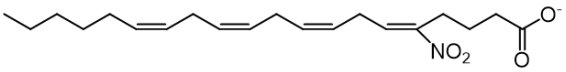


13-nitro-9,12-octadecadienoic acid

13-NO_2-LA

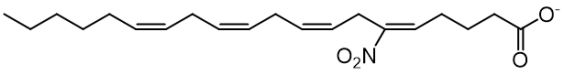
Figure 3

Nitrated Arachidonic Acid (NO_2-AA) isomers



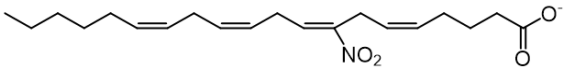
5-nitro-5,8,11,14-eicosatetraenoic acid

5-NO_2-AA



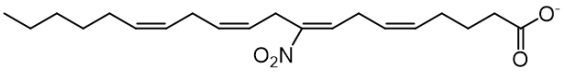
6-nitro-5,8,11,14-eicosatetraenoic acid

6-NO_2-AA



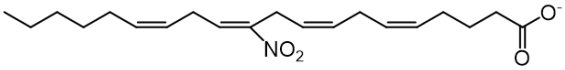
8-nitro-5,8,11,14-eicosatetraenoic acid

8-NO_2-AA



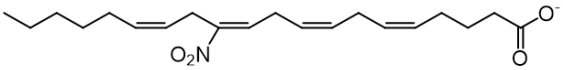
9-nitro-5,8,11,14-eicosatetraenoic acid

9-NO_2-AA



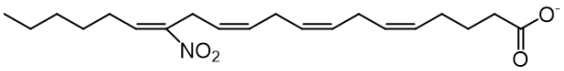
11-nitro-5,8,11,14-eicosatetraenoic acid

11-NO_2-AA



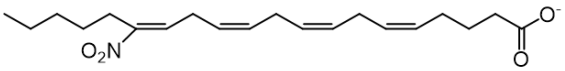
12-nitro-5,8,11,14-eicosatetraenoic acid

12-NO_2-AA



14-nitro-5,8,11,14-eicosatetraenoic acid

14-NO_2-AA

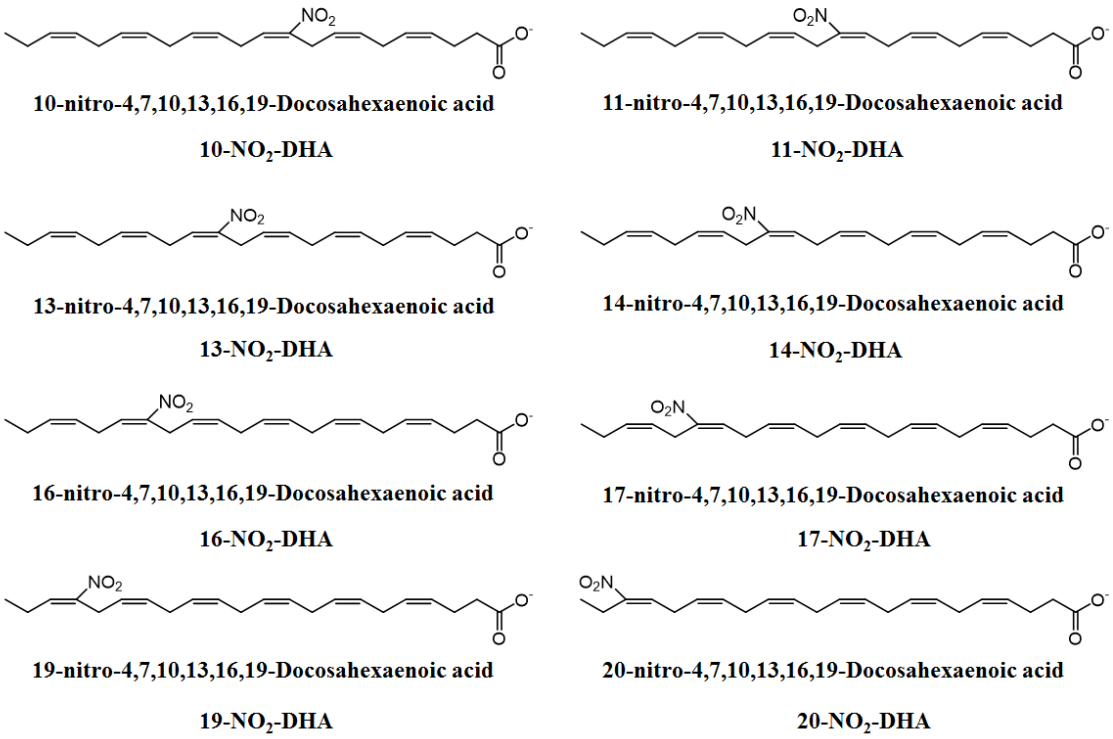


15-nitro-5,8,11,14-eicosatetraenoic acid

15-NO_2-AA

Figure 4

Nitrated Docosahexaenoic Acid (NO_2-DHA) isomers



10-nitro-4,7,10,13,16,19-Docosahexaenoic acid

10-NO_2-DHA

11-nitro-4,7,10,13,16,19-Docosahexaenoic acid

11-NO_2-DHA

13-nitro-4,7,10,13,16,19-Docosahexaenoic acid

13-NO_2-DHA

14-nitro-4,7,10,13,16,19-Docosahexaenoic acid

14-NO_2-DHA

16-nitro-4,7,10,13,16,19-Docosahexaenoic acid

16-NO_2-DHA

17-nitro-4,7,10,13,16,19-Docosahexaenoic acid

17-NO_2-DHA

19-nitro-4,7,10,13,16,19-Docosahexaenoic acid

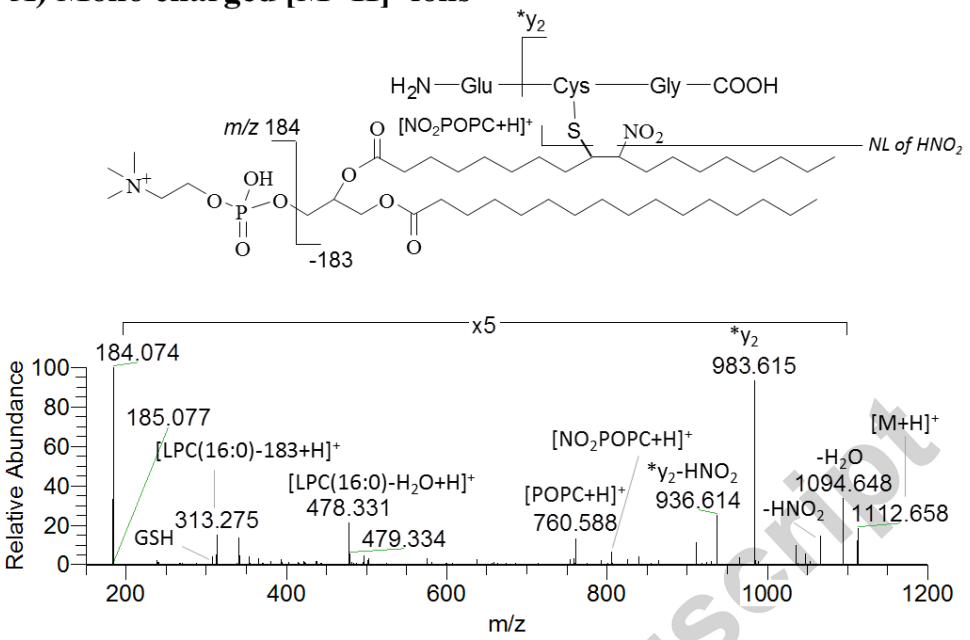
19-NO_2-DHA

20-nitro-4,7,10,13,16,19-Docosahexaenoic acid

20-NO_2-DHA

Figure 5

A) Mono charged $[M+H]^+$ ions



B) Double charged $[M+2H]^{2+}$ ions

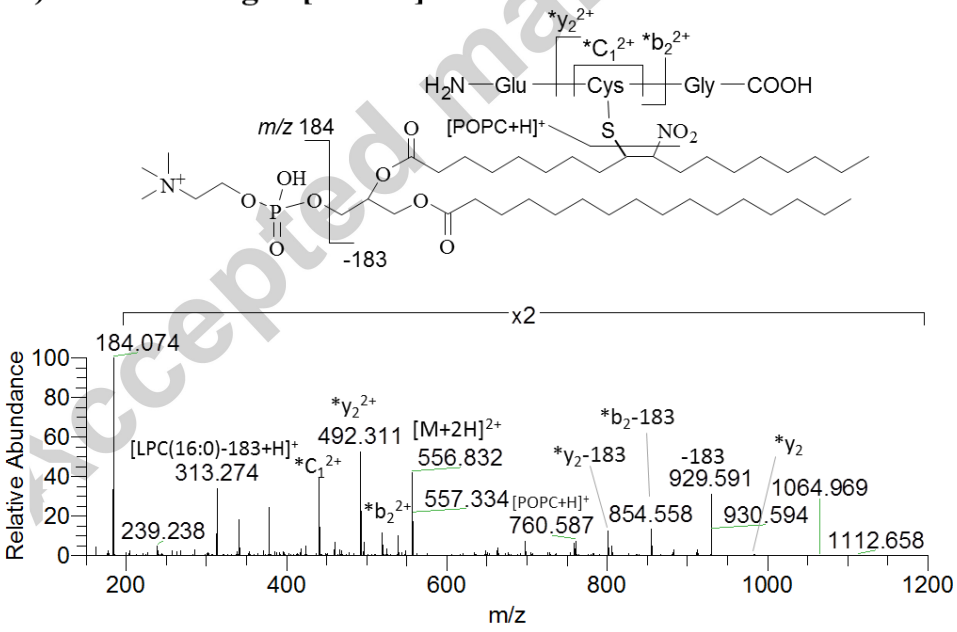


Figure 6

Table 1. Nitro-fatty acids identified in biological samples and *in vitro* mimetic model systems.

***In vitro* mimetic model systems**				
NO_2-FA	**Isomer**	**Experimental model**	**Method**	**Ref**
Nitro-oleic acid (NO_2-OA)				
NO_2-OA	9-NO_2-OA 10-NO_2-OA	Gastric juice artificial + NO_2^- Pancreatic lipase-digested EVOO	C18-HPLC-ESI-MS and MS/MS in a API 4000 triple quadrupole and LTQ Orbitrap Velos	[56]
NO_2-OA		MPO + H_2O_2 + NO_2^- $ONOO^-$ NO_2^- in acidic conditions	C18-HPLC-ESI-MS and MS/MS in a hybrid triple quadrupole-linear ion trap (4000 Q-Trap)	[6]
NO_2-OA	9-NO_2-OA 10-NO_2-OA	$^{\bullet}NO_2$	C18-HPLC-ESI-MS and MS/MS in API 2000 triple quadrupole	[80]
Nitro-linoleic acid (NO_2-LA)				
NO_2-LA	9-NO_2-LA 10-NO_2-LA 12-NO_2-LA 13-NO_2-LA	Gastric juice artificial + NO_2^- Pancreatic lipase-digested EVOO	C18-HPLC-ESI-MS and MS/MS in an API 4000 triple quadrupole and LTQ Orbitrap Velos	[56]
NO_2-LA		NO_2^- in acidic conditions	C18-HPLC-ESI-MS and MS/MS in a Quattro triple quadrupole	[10]
Nitro-conjugated linoleic acid (NO_2-cLA)				
NO_2-cLA	8-NO_2-cLA 9-NO_2-cLA 11-NO_2-cLA 12-NO_2-cLA	Gastric juice artificial + NO_2^- Pancreatic lipase-digested EVOO	C18-HPLC-ESI-MS and MS/MS in an API 4000 triple quadrupole and LTQ Orbitrap Velos	[56]
NO_2-cLA	9-NO_2-cLA 12-NO_2-cLA	MPO + H_2O_2 + NO_2^- $ONOO^-$ $^{\bullet}NO_2$	C18-HPLC-ESI-MS and MS/MS in an API 5000 triple quadrupole, API Q-Trap 4000, and Velos Orbitrap	[3]
NO_2-cLA	NO_2-cLA Multiple nitro, nitroso, and nitroxidized derivatives	Photocontrollable peroxynitrite donor 2,3,5,6-tetramethyl-4-(methylnitrosoamino)phenol (P-NAP)	ESI-MS and MS/MS in a hybrid triple quadrupole-linear ion trap (4000 Q-Trap)	[38]
Cholesteryl-nitro linoleic acid (Chol-NO_2-LA)				
Chol-NO_2-LA		NO_2^- in acidic conditions	C18-HPLLC-ESI/MS/MS in a Quattro II triple quadrupole	[9]
Chol-NO_2-LA		NO_2^- in acidic conditions	ESI–MS and MS/MS in a 2000 Q-Trap C18-HPLC-ESI–MS and MS/MS in a 2000 Q-Trap	[66]
Nitro-arachidonic acid (NO_2-AA)				
NO_2-AA		$^{\bullet}NO_2$	C18-HPLC-ESI-MS and MS/MS in an Esquire ion trap	[26]
NO_2-AA	9-NO_2-AA 12-NO_2-AA	NO_2^- in acidic conditions	C18-HPLC-ESI-MS and MS/MS in a hybrid quadrupole-	[51]

	14-NO_2-AA 15-NO_2-AA		linear ion trap	

Biological samples

Nitro-palmitoleic acid (NO_2-POA)

NO_2-POA		Human plasma and urine	C18-HPLC-ESI-MS and MS/MS in a hybrid triple Q-linear ion trap (4000 Q-Trap)	[6]

Nitrohydroxy-palmitoleic acid (NO_2OH-POA)

NO_2OH-POA		Human plasma and urine	C18-HPLC-ESI-MS and MS/MS in a hybrid triple Q-linear ion trap (4000 Q-Trap)	[6]

Nitro-oleic acid (NO_2-OA)

NO_2-OA		Human red cells, plasma and urine	C18-HPLC-ESI-MS and MS/MS in a hybrid triple Q-linear ion trap (4000 Q-Trap)	[6]
NO_2-OA	9-NO_2-OA 10-NO_2-OA	Myocardial heart tissue from a murine model of focal myocardial ischemia/reperfusion	C18-HPLC-ESI MS/MS	[1]
NO_2-OA	NO_2-OA and β-oxidation metabolites	NO_2-OA acute intravenous treatment of mice with LPS-induced inflammation	C18-HPLC-ESI-MS/MS in an API 5000 triple quadrupole	[107]
NO_2-OA	NO_2-OA and its metabolic derivatives	Human and rat urine after intravenous administration of NO_2-OA	C18-HPLC-ESI-MS and MS/MS in a LTQ Velos Orbitrap and API 5000 triple quadrupole	[61]
NO_2-OA	NO_2-OA and its metabolic derivatives	Mitochondrial extracts from rat hearts after ischemia-reperfusion	BME trans-nitroalkylation + C18-HPLC-ESI-MS and MS/MS in a 4000 Q trap hybrid triple quadrupole-linear ion trap	[62]
Dinitro-OA		Rat cardiomyocytes treated with peroxynitrite donor 3-morpholinosydnonimine (SIN-1)	C18-HPLC-ESI-MS and MS/MS in a hybrid triple quadrupole-linear ion trap (4000 Q-Trap)	[38]
NO_2-OA	NO_2-OA and its Saturation, Desaturation β-oxidation metabolic derivatives	Plasma from NO_2-OA-treated mice	C18-HPLC-ESI MS/MS coupled to an API 4000 hybrid triple quadrupole or API 5000 triple quadrupole	[59]
NO_2-OA	NO_2-OA saturation derivatives	NO_2-OA-treated BAEC cells	C18-HPLC-ESI MS/MS coupled to an API 4000 hybrid triple quadrupole or API 5000 triple quadrupole	[59]
NO_2-OA	NO_2-OA and its derivatives	Liver lipid extracts from NO_2-OA-treated mice	C18-HPLC-ESI MS/MS coupled to an API 4000 hybrid triple quadrupole or API 5000 triple quadrupole	[59]

Nitrohydroxy-oleic acid (NO_2OH-OA)

NO_2OH-OA		Human red cells, plasma and urine	C18-HPLC-ESI-MS and MS/MS in a hybrid triple Q-linear ion trap (4000 Q-Trap)	[6]

Nitro-linoleic acid (NO_2-LA)

NO_2-LA		Myocardial heart tissue from a murine model of focal myocardial ischemia/reperfusion	C18-HPLC-ESI MS and MS/MS	[1]
NO_2-LA		Human plasma and urine	C18-HPLC-ESI-MS and MS/MS in a hybrid triple Q-linear ion trap (4000 Q-Trap)	[6]
NO_2-LA		Human blood plasma	C18-HPLC-ESI-MS and MS/MS in a Quattro triple quadrupole	[10]
NO_2-LA	9-NO_2-LA 12-NO_2-LA	Human red cell membranes and plasma	C18-HPLC-ESI-MS and MS/MS in a hybrid triple Q-linear ion trap	[8]
NO_2-LA	NO_2-LA and its metabolic derivatives	Mitochondrial extracts from rat hearts after ischemia-reperfusion	BME trans-nitroalkylation + C18-HPLC-ESI-MS and MS/MS in a 4000 Q trap hybrid triple quadrupole-linear ion trap	[62]
Nitrohydroxy-linoleic acid (NO_2OH-LA)				
NO_2OH-LA		Myocardial heart tissue from a murine model of focal myocardial ischemia/reperfusion	C18-HPLC-ESI MS and MS/MS	[1]
NO_2OH-LA		Human plasma and urine	C18-HPLC-ESI-MS and MS/MS in a hybrid triple Q-linear ion trap (4000 Q-Trap)	[6]
Nitrokto-linoleic acid (NO_2-oxo-LA)				
NO_2-oxo-LA		Myocardial heart tissue from a murine model of focal myocardial ischemia/reperfusion	C18-HPLC-ESI MS/MS	[1]
Nitro-conjugated linoleic acid (NO_2-cLA)				
NO_2-cLA		Plasma and vaginal lavages after cLA inoculation in the vaginal lumen from mice infected intravaginally with HSV-2	C18-HPLC-MS/MS in a 6500+ Q-trap or a API 5000	[16]
Nitro-linolenic acid (NO_2OH-LNA)				
NO_2-LNA		Human plasma and urine	C18-HPLC-ESI-MS and MS/MS in a hybrid triple Q-linear ion trap (4000 Q-Trap)	[6]
Nitrohydroxy-linolenic acid (NO_2OH-LNA)				
NO_2OH-LNA		Human plasma and urine	C18-HPLC-ESI-MS and MS/MS in a hybrid triple Q-linear ion trap (4000 Q-Trap)	[6]
Nitro-arachidonic acid (NO_2-AA)				
NO_2-AA		Human plasma and urine	C18-HPLC-ESI-MS and MS/MS into a hybrid triple Q-linear ion trap (4000 Q-Trap)	[6]
NO_2-AA		Rat cardiomyocytes treated with peroxynitrite donor 3-morpholinosydnonimine (SIN-1)	C18-HPLC-ESI-MS and MS/MS in a hybrid triple quadrupole-linear ion trap (4000 Q-Trap)	[38]
Nitrohydroxy-arachidonic acid (NO_2OH-AA)				
NO_2OH-AA		Human plasma and urine	C18-HPLC-ESI-MS and	[6]

			MS/MS in a hybrid triple Q-linear ion trap (4000 Q-Trap)	
Nitro-Eicosapentaenoic acid (NO_2-EPA)				
NO_2-EPA		Human plasma and urine	C18-HPLC-ESI-MS and MS/MS in a hybrid triple Q-linear ion trap (4000 Q-Trap)	[6]
Nitrohydroxy- Eicosapentaenoic acid (NO_2OH-EPA)				
NO_2OH-EPA		Human plasma and urine	C18-HPLC-ESI-MS and MS/MS in a hybrid triple Q-linear ion trap (4000 Q-Trap)	[6]
Nitro-Docosahexaenoic acid (NO_2-DHA)				
NO_2-DHA and dinitro-DHA		Rat cardiomyocytes treated with peroxynitrite donor 3-morpholinosydnonimine (SIN-1)	C18-HPLC-ESI-MS and MS/MS in a hybrid triple quadrupole-linear ion trap (4000 Q-Trap)	[38]
Nitrohydroxy-Docosahexaenoic acid (NO_2-DHA)				
NO_2OH-DHA		Rat cardiomyocytes treated with peroxynitrite donor 3-morpholinosydnonimine (SIN-1)	C18-HPLC-ESI-MS and MS/MS in a hybrid triple quadrupole-linear ion trap (4000 Q-Trap)	[38]
Nitrohydroxy-Docosapentaenoic acid (NO_2OH-DPA)				
NO_2OH-DPA		Rat cardiomyocytes treated with peroxynitrite donor 3-morpholinosydnonimine (SIN-1)	C18-HPLC-ESI-MS and MS/MS in a hybrid triple quadrupole-linear ion trap (4000 Q-Trap)	[38]
Nitro-conjugated linoleic acid (NO_2-cLA)				
NO_2-cLA	9-NO_2-cLA 12-NO_2-cLA	Pancreatic lipase-digested EVOO	C18-HPLC-ESI-MS and MS/MS in an API 4000 triple quadrupole and LTQ Orbitrap Velos	[56]
NO_2-cLA	9-NO_2-cLA 12-NO_2-cLA β-oxidation-metabolic derivatives of NO_2-cLA	Urine of healthy humans	C18-HPLC-ESI-MS and MS/MS in a LTQ Velos Orbitrap and AB 5000 or API4000 Q-trap triple quadrupole	[7]
		Rodents urine, plasma, and tissues (stomach, small intestine, colon, liver) after supplementation with cLA + NO_2^- and gastric acidification		
NO_2-cLA	9-NO_2-cLA 12-NO_2-cLA	Rodents liver and cardiac mitochondria incubated with NO_2^- in acidic conditions	C18-HPLC-ESI-MS and MS/MS in an API 5000 triple quadrupole, API Q-Trap 4000, and Velos Orbitrap	[3]
		Rodents cardiac tissue under ischemia-reperfusion		
NO_2-cLA	9-NO_2-cLA 12-NO_2-cLA	Raw 264.7 macrophages stimulated with LPS/IFNγ Healthy human plasma RAW264.7 macrophages stimulated with LPS/IFNγ and	C18-HPLC-ESI-MS and MS/MS in an API 5000 or a Q-	[13]

	Reduction and β-oxidation-metabolic derivatives	M1, M2 and M0 polarized bone marrow-derived macrophages (BMDM) treated with cLA	Trap 6500+ and LTQ Velos Orbitrap	
		Mice Peritoneal exudates after zymosan-A induced peritonitis and cLA supplementation		
NO_2-cLA	NO_2-cLA and β-oxidation-metabolic derivatives	Urine and plasma healthy humans after ingestion of nitrite, nitrate and cLA	C18-HPLC-ESI-MS and MS/MS in a 5000 triple quadrupole	[58]
Cholesteryl-nitro linoleic acid (Chol-NO_2-LA)				
Chol-NO_2-LA		Human blood plasma and lipoproteins from normolipidemic/healthy subjects	C18-HPLLC-ESI/MS/MS in a Quattro II triple quadrupole	[9]
Chol-NO_2-LA		J774.1 macrophages timulated with LPS/IFNγ	C18-HPLC-ESI–MS and MS/MS in a 2000 Q-Trap	[66]

Table 2. Modulation of target signaling pathways by NO_2-FA and related biological properties.

Anti-inflammatory
- NF-kB ↓ [1,13,108,15,21,23,46,60,66,86,90] - TL4R signaling ↓ [107] - PG H synthase ↓ [86,109] - 5-LOX ↓ [45] - STAT1 ↓ [87,92] - Cytokine production ↓[1,5,110–112,7,13,20,21,23,24,66,86] - MPO ↓ [24] - Leukocyte recruitment, adhesion and infiltration ↓ [13,23,24,87,108,112] - iNOS ↓ [13,24,36,51,60,66,86,112] - COX-2 ↓ [112] - PGE_2 ↓ [112] - Leukocytes number and activity ↓ [5,13,18,108] - TNF-α ↓ [23] - Xanthine oxidoreductase (XOR) ↓ [73] - MKP-1 ↑ [92] - PPARγ ↑ [6,15,21,65,100] - TRPA-1 ↑ [98] - CD36 expression ↑ [100] - Heme oxygenase 1 (HO-1) ↑ [17,19,22,23,60,66,67,89,93] - STING ↓ [16]

- PDI ↓ [95]
- NADPH oxidase (NOX 2) ↓ [24,74,113]

Vasorelaxation

- Nitric oxide ↑ [29–31,51]
- eNOS ↑ [114]
- Ang II-induced vasoconstriction ↓ [28]

Antioxidant

- Lipid peroxidation ↓ [71,86]
- Nrf2 ↑ [13,15,22,46,60,88]
- HO-1 ↑ [17,19,22,60,66,89]
- Reduction of protein nitration and oxidation [87]
- Heat shock response (HSP) proteins [22]
- XOR ↓ [73]
- NOX 2 ↓ [24,74,113]
- O_2^-, $ONOO^-$ and NO production ↓ [36,48,86,108,111,113]

Anti-hypertensive

- AT_1R ↓ [28]
- sEH ↓ [32]
- Smooth muscle cell proliferation ↓ [111]
- Prostaglandin F2↓ [111]
- Differentiation of myofibroblast through Smad2 ↓ [113]

Anti-hyperglycemic

- PPARγ ↑ [6,28,100]
- Glucose uptake ↑ [110]
- Insulin sensitivity ↑ [6,28,100]

Anti-thrombotic

- Platelets activation and clotting ↓ [18,31,33]
- Platelet production of thromboxane↓ [109]
- Thrombin-induced aggregation ↓ [33]
- PGHS ↓ [109,115,116]

Cytoprotective

- ANT1 ↑ [2,34]
- UCP-2 ↑ [2]
- Mitochondrial dysfunction ↓ [36,37]
- Mitochondrial respiratory complex ↑ [48]
- Metabolic shift ↑ [48]

Anti-tumorogenic

- Tumor cell proliferation, migration and invasion↓ [90]

Table 3. Nitro-fatty acids lipoxidation adducts identified in biological samples and *in vitro* mimetic model systems by using mass spectrometry-based approaches.

Protein/Peptide	Model system	Method	Molecular mechanism Signaling action	Biological Role	Ref.
Nitro-oleic acid (NO_2-OA)					
Cysteine	Incubation of NO_2-OA and cysteine	C18-HPLC-MS and MS/MS in a triple quadrupole API 4000			[56]
	Incubation of NO_2-OA and cysteine	C18-HPLC-MS and MS/MS in a triple quadrupole			[43]
	Whole olives, mesocarp and peel	C18-HPLC-MS and MS/MS in a triple quadrupole API 4000			[56]
GSH	NO_2-OA-Cys adduct generation after incubation between NO_2-OA and GSH	BME trans-nitroalkylation + C18-HPLC-ESI-MS and MS/MS in a Q trap 4000			[62]
	NO_2-OA-Cys adduct generation after incubation between NO_2-OA and GSH	(BME trans-nitroalkylation +) C18-HPLC-ESI-MS and MS/MS in a hybrid triple quadrupole API 4000 or API 5000 triple quadrupole			[59]
	Plasma from NO_2-OA-treated mice	(BME trans-nitroalkylation) C18-HPLC-ESI-MS and MS/MS in a hybrid triple quadrupole API 4000 or API 5000 triple quadrupole			[59]
	NO_2-OA-Cys adduct generation after incubation between NO_2-OA and GSH	ESI-MS in LCQ ion trap tryptic digestion + C18-HPLC-ESI-MS and MS/MS in an ESI-LCQ ion trap C18-HPLC-ESI-MS/MS in a Q-Trap 4000			[42]

	Red blood cells obtained from healthy humans	C18-HPLC-ESI-MS/MS in a Q-Trap 4000	Translocation to membrane	Regulation of enzyme function, cell signaling, and protein trafficking	[42]
GAPDH Cys149 His303	Cytosolic and membrane-associated protein fractions from red blood cells obtained from healthy humans	SDS-PAGE under non-reducing and denaturing conditions + Tryptic digestion + C18-nanospray LC-MS and MS/MS in a LTQ ion trap MALDI-TOF MS (Voyager DE PRO system)	Translocation to membrane	Regulation of enzyme function, cell signaling, and protein trafficking	[42]
GAPDH Cys149 Cys153 Cys244 His108 His134 His327	Incubation of NO_2-OA and GAPDH	C18-HPLC-MS in a LTQ ion trap Tryptic digestion and MALDI-TOF MS (Voyager DE PRO system) tryptic digestion + C18-HPLC-ESI-MS in an ESI-LCQ ion trap			[42]
GAPDH	Incubation of NO_2-OA and GAPDH	Tryptic digestion + C18-nanospray LC-MS and MS/MS in a LTQ ion trap electrophoresis under reducing conditions + BME trans-nitroalkylation + C18-HPLC-ESI-MS and MS/MS in a 4000 Q trap hybrid triple quadrupole-linear ion trap			[62]
5-LOX Cys416 **Cys418** His125 His360 His362 His367 His372 His432	Incubation of NO_2-OA and human recombinant 5-LOX Incubation of NO_2-OA and human polymorphonuclear leukocytes (PMNL) (intact and cell lysates)	tryptic digestion and C18-nanoHPLC-ESI-MS and MS in an Orbitrap XL	irreversible inhibition of 5-LOX activity and prevention of lung injury and systemic immune responses	Anti-inflammatory	[45]

	NO_2-OA treatment in murine model of LPS-induced inflammation (lung injury and cellular infiltration)				
Keap1 Cys38 Cys151 Cys226 **Cys273** **Cys288** Cys257 Cys489	Incubation of NO_2-OA and recombinant Keap1 Human embryonic kidney (HEK)-293T cells transfected with recombinant Keap1 and treated with NO_2-OA	tryptic digestion C18-HPLC-MS and MS/MS in a LTQ	Release of Nrf2 tracsription factor to the nucleous for induction of expression of antioxidant phase II enzymes	Antioxidant	[46]
Catephsin S (Cat S) Cys25	Incubation of NO_2-OA with a synthetic Cat S peptide (Cat S23-29)	C18-HPLC-MS and MS/MS in a Q Exactive Hybrid Quadrupole-Orbitrap tryptic digestion and C18-HPLC-MS and MS/MS in a LTQ-XL	Downregulation of Cat S expression and activity	Tissue Protection Anti-inflammatory	[5]
Fp subunit of mitochondrial complex II His2 His5 His6 Cys9 Cys14	Incubation of NO_2-OA with recombinant human complex II Fp subunit Rat heart mitochondria treated with OA-NO_2	blue native electrophoresis, BME trans-nitroalkylation, C18-HPLC-MS amd MS/MS in a hybrid triple-quadrupole linear ion trap mass spectrometer (4000 Q trap)	Inhibition of mitochondrial respiration complex II and $O_2^{\bullet-}$ production Promotion of glycolysis	Cytoprotective Antioxidant	[48]
AT_1R	HEK293 cells overexpressing AT_1R treated with NO_2-OA	immunoprecipitation of AT_1R from cell lysates, BME trans-nitroalkylation reaction of AT_1R-bound NO_2-OA, and C18-HPLC-MS and MS/MS in a 4000 Q-Trap triple quadrupole	Inhibits AT_1R-dependent vasoconstriction by reduction of heterotrimeric G-protein coupling and inhibition of IP_3 and calcium mobilization	Anti-hypertensive	[28]
MMP-7 Cys70 MMP-9 Cys100	Incubation of NO_2-OA with recombinant human proMMP-7 and proMMP-9	C18-HPLC-ESI-MS and MS/MS in a LTQ-XL C18-HPLC-ESI-MS and MS/MS in a hybrid triple quadrupole-linear ion trap 4000 Q-Trap	Modulation of proteolytic activity Decrease of enzyme expression	Anti-inflammatory	[75]

	Plasma from intraperitoneal NO_2-OA-treated mice	electrophoresis under reducing conditions + BME trans-nitroalkylation + C18-HPLC-ESI-MS and MS/MS in a 4000 Q trap hybrid triple quadrupole-linear ion trap			[62]
albumin					
	Plasma from NO_2-OA-treated mice	electrophoresis + BME trans-nitroalkylation + C18-HPLC-ESI-MS and MS/MS in a hybrid triple quadrupole API 4000 or API 5000 triple quadrupole			[59]
PPARγ **Cys285** His266 His323 His425 His449	Incubation of NO_2-OA with human recombinant PPARγ LBD HEK 293T cells were transfected with PPARγ and treated with NO_2-OA	tryptic digestion and C18-HPLC-MS and MS/MS in a LTQ immunoprecipitation, gel electrophoresis, BME- trans-nitroalkylation and ESI-MS and MS/MS in a hybrid triple quadrupole-linear ion trap mass spectrometer (4000 Q Trap,	Activation of PPARγ-related gene expression for glucose regulation and adipogenesis Decrease in adipogenesis Increase glucose uptake Restore insulin sensivity	anti-hyperglycemic anti-adipogenic effect	[47]
STING Cys88 Cys91 His16	Incubation human STING-transfected HEK293T cells with 10-NO_2-OA	Purification with magnetic beads, tryptic digestion and MALDI LTQ Orbitrap XL	Deregulation of STING palmitoylatio Inhibition of STING signaling Inhibition the release of type I IFN	Anti-inflammatory	[16]
Nitro-linoleic acid (NO_2-LA)					
cysteine	NO_2-LA-Cys adduct generation after incubation between NO_2-LA and cysteine	(C18-HPLC)-ESI-MS and MS/MS in a triple quadrupole			[107]
GSH	NO_2-LA-Cys adduct generation after incubation between NO_2-LA and GSH	ESI-MS in LCQ ion trap C18-HPLC-ESI-MS/MS in a Q-Trap 4000			[42]

	NO_2-LA-Cys adduct generation after incubation between NO_2-LA and GSH	BME trans-nitroalkylation + C18-HPLC-ESI-MS and MS/MS in a Q trap 4000			[62]
	NO_2-LA-Cys adduct generation after incubation between NO_2-LA and GSH	C18-HPLC/ESI/MS in Micromass Quattro II triple quadrupole			[76]
	Red blood cells obtained from healthy humans	C18-HPLC-ESI-MS/MS in Q-Trap 4000			[42]
	MCF7/WT and MCF7/MRP1-10 cells treated with NO2-LA	C18-HPLC/ESI/MS in Micromass Quattro II triple quadrupole			[76]
ANT1 Cys57	NO2-LA-treated intact perfused hearts	Immunoprecipitation + SDS-PAGE + in-gel digestion tryptic digestion + ABSciex 5800 MALDI-TOF-TOF MS and MS/MS	Mitochondrial uncoupling	Cytoprotective	[34]
Nitro-conjugated linoleic acid (NO_2-cLA)					
	NO_2-cLA-Cys adduct generation after incubation between NO_2-cLA and cysteine	C18-HPLC-MS and MS/MS in LTQ Velos Orbitrap and AB 5000 or API4000 Q-trap triple quadrupole			[7]
cysteine	Urine from healthy humans	C18-HPLC-MS and MS/MS in LTQ Velos Orbitrap and AB 5000 or API 4000 Q-trap triple quadrupole			[7]
	NO_2-cLA-Cys adduct generation after incubation between NO_2-cLA and cysteine	C18-HPLC-MS and MS/MS in API 5000 triple quadrupole			[44]
	Urine from healthy humans	C18-HPLC-MS and MS/MS in API 5000 triple quadrupole			[44]
Nitro-arachidonic acid (NO_2-AA)					
PDI Cys397 Cys400	Incubation of human recombinat PDI with NO_2-AA	C4-HPLC-MS of intact protein in a hybrid triple quadrupole/linear ion trap mass spectrometer (Q-trap 4500)	Inhibition of reductase and chaperone activity of PDI	Anti-inflammatory	[95]
		digestion tryptic			

	digestion and C18- HPLC-MS and MS/MS

Highlights

- Nitro-fatty acids (NO_2-FA) are endogenous bioactive lipids
- NO_2-FA form reversible Michael adducts with proteins leading to PTMs
- Adduction of NO_2-FA with proteins culminates to anti-inflammatory, anti-hypertensive, and cytoprotective effects
- Mass spectrometry (MS)-based approaches allows to identify NO_2-FA-protein adducts and to unveil their biological effects.