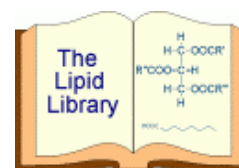


NITRO FATTY ACIDS

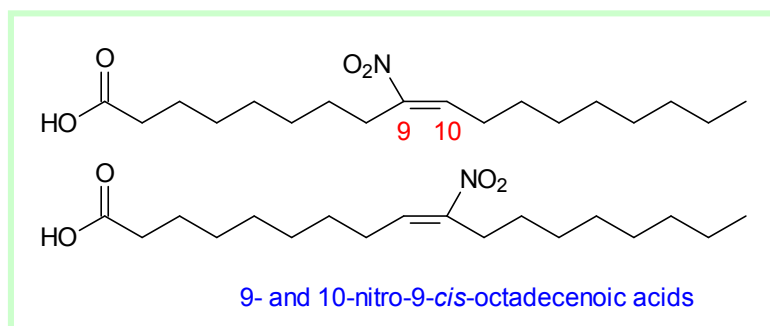
Occurrence, Chemistry and Biology



1. Occurrence

While the free radical-catalysed addition of nitric oxide (NO^{\cdot}) and nitrogen dioxide (NO_2^{\cdot}) radicals to unsaturated and hydroperoxy fatty acids *in vitro* has been known for many years, it was only in 1999 that the first paper appeared to show that nitro fatty acids were present in the membrane phospholipids of human tissues *in vitro* and *in vivo*, and at concentrations that had the potential to exert biological effects. However, their formation *in vitro* had been demonstrated earlier in studies of lipid oxidation products induced by air pollutants.

Since then, by means of sensitive analytical mass spectrometric methods, it has been demonstrated that nitrated derivatives of palmitoleic, oleic, linoleic, linolenic, arachidonic and eicosapentaenoic acids together with their nitrohydroxy derivatives are present in human plasma and urine. Of these, the two most abundant species are derived from oleic acid, i.e. 9- and 10-nitro-9-*cis*-octadecenoic acids. (Note that under the official IUPAC rules of nomenclature these should strictly speaking be designated as *trans* isomers to reflect the orientation of the nitro group relative to the alkyl substituent on the adjacent carbon atom. Those active in this area prefer the familiar lipid nomenclature).

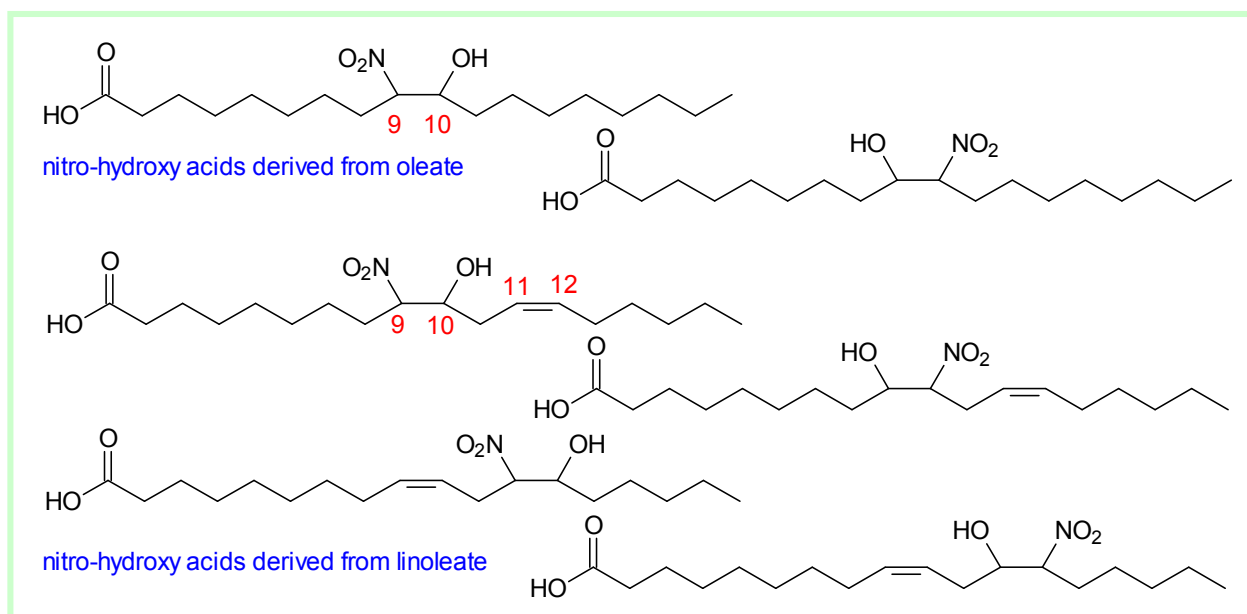


In plasma, they occur in the free form, most bound reversibly to thiol-containing proteins and glutathione, and as cholesterol esters. Free and esterified concentrations of the two regioisomers in plasma were originally estimated at 619 and 302 nM, respectively, while in red blood cells the corresponding figures were 59 and 155 nM (other stereoisomers were not detected). However, recent studies with stable isotope-dilution methodology suggests that these figures are a considerable over-estimate and that the true basal levels in plasma of healthy humans is closer to 1 nM.

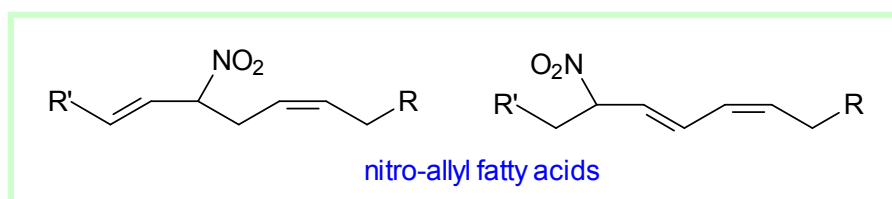
Analogous compounds derived from linoleate have been detected at significant concentrations in some studies, but not in others in healthy tissues at least. All the possible nitro-linoleate isomers have been found in tissues, but 10-nitro- and 12-nitro-9-*cis*,12-*cis*-octadecadienoic acids are the main ones found; it appears that the 9-isomer is relatively unstable and is rapidly degraded.

In addition, both nitro and nitro-hydroxy derivatives of oleate, linoleate and linolenate have been characterized. The structures of the nitrohydroxy derivatives of oleate and linoleate are illustrated. In essence, these are formed by addition of reactive nitrogen species across one of the double bonds (see below). Subsequently, nitroicosatetraenoic, α,β -nitrohydroxyeicosatrienoic and *trans*-arachidonic acids, derived from arachidonic acid via such reactions, were characterized both *in vitro* and *in vivo*. In general, there is considerable selectivity in terms of which of the various

isomers are detected in tissues. For example, the nitroicosatetraenoic acids have the NO_2 groups in positions 9, 12, 14, and 15 mainly. Such compounds are now receiving particular attention because of their potential to influence eicosanoid metabolism in addition to having biological effects in their own right.



Further related metabolites, which have been characterized and are presumed to be formed by comparable mechanisms, include nitro-allyl derivatives of various fatty acids, including oleate, in which both the position and configuration of the double bond is changed.



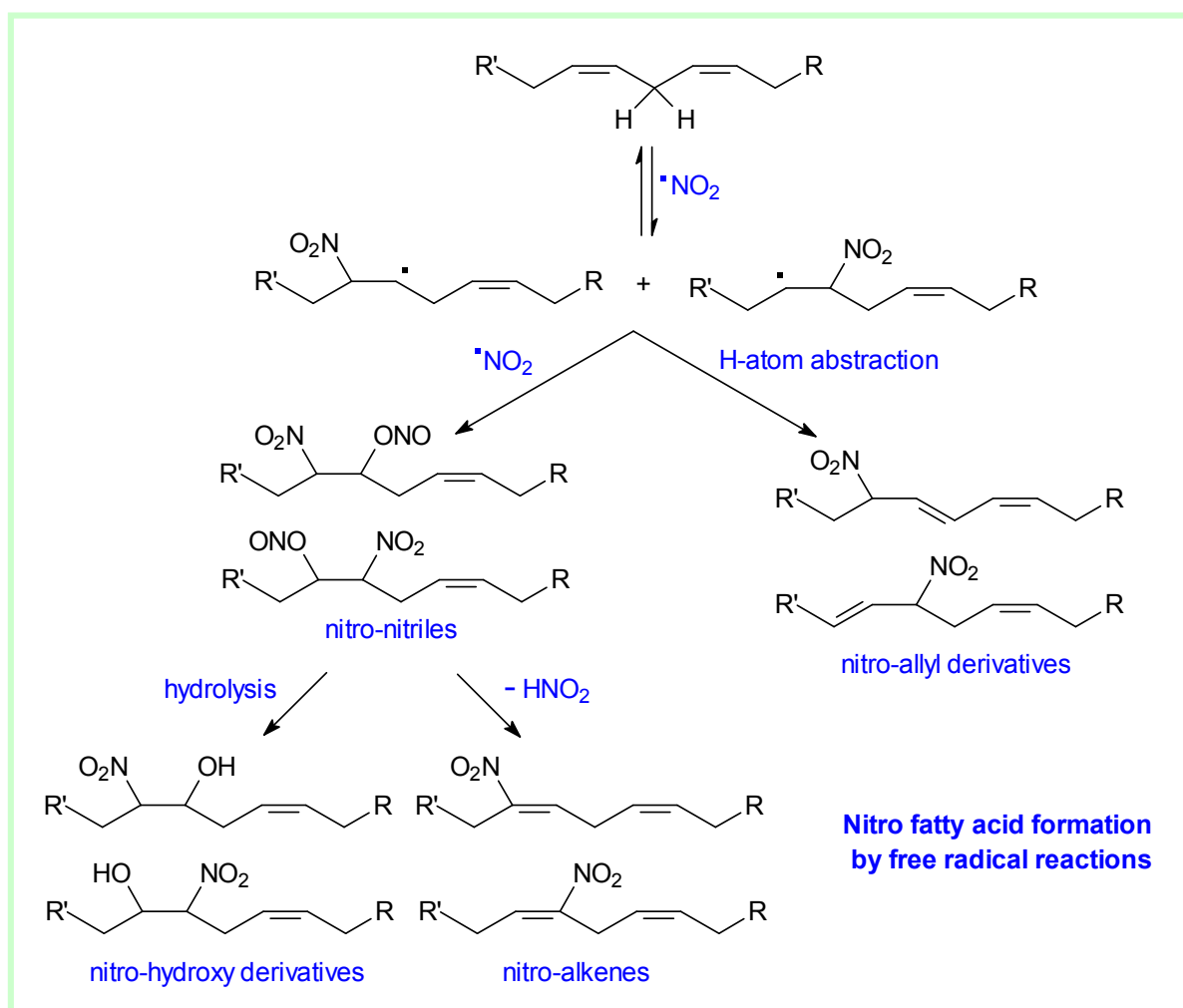
In addition, simple (non-nitrated) geometrical (*trans*) isomers of unsaturated fatty acids can be produced as a by-product of a nitration reaction. Those derived from arachidonate are of particular biological relevance.

2. Formation of Nitro Fatty Acids in Tissues

Formation of nitro fatty acids occurs in tissues through the non-enzymatic reactions of free radicals such as nitric oxide (NO^\bullet), and NO^\bullet -derived oxides of nitrogen (e.g. nitrogen dioxide (NO_2^\bullet)) and peroxynitrite (ONOO^\bullet)). These operate in conjunction with oxygen-derived inflammatory mediators such as superoxide (O_2^\bullet), hydrogen peroxide (H_2O_2) and lipid peroxyl radicals (LOO^\bullet). Many different mechanisms are involved in the production of the secondary radicals and in their subsequent reactions. These are controlled by such factors as the concentration of the NO^\bullet radicals, the site of their production, oxygen tension, and the concentrations and membrane environment of the target molecules and of any catalysts and antioxidants. The reactions have something in common with **isoprostane** formation, since they are also non-enzymatic and the reaction is with intact lipids rather than the free acids. In addition, nitro fatty acid formation can occur in foods and these could potentially reach tissues via the digestive system.

The NO_2^\cdot radical can arise from various endogenous and exogenous sources in humans. For example, immune responses to inflammatory stimuli induce nitric oxide synthase in certain cells that form NO^\cdot , which is then oxidized to NO_2^\cdot . NO_2 is a common air pollutant and can be absorbed via the lungs. Meat and other foods may contain appreciable quantities of nitrite (added as a preservative), and nitrate can be reduced to nitrite by aerobic bacteria in the mouth. In the stomach, nitrite decomposes rapidly in the acidic environment to form NO^\cdot and NO_2^\cdot and other bioactive nitrogen oxides, and these are absorbed from the intestines and thence enter into the circulation.

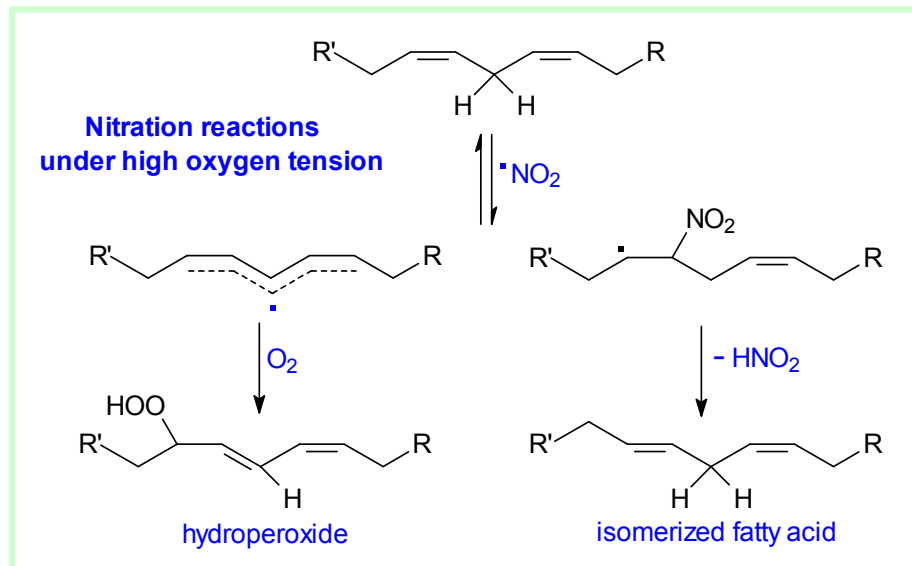
Detailed mechanistic studies of nitro fatty acid formation in human and other animal tissues are at an early stage, and the biosynthetic mechanisms proposed are largely extrapolated from chemical studies *in vitro*. The NO_2^\cdot radicals can react with unsaturated lipids and lipid radicals to form all the types of products found in tissues. Thus at low oxygen tensions, homolytic attack to the double bond yields nitroalkyl radicals, which combine with other NO_2^\cdot radicals to form nitro-nitrite intermediates. Loss of nitrous acid (HNO_2) from these intermediates results in the formation of nitroalkenes, while hydrolysis leads to the production of nitro-alcohols. In an alternative reaction, abstraction of a hydrogen atom from the nitroalkyl radicals leads to the formation of nitro-allyl derivatives.



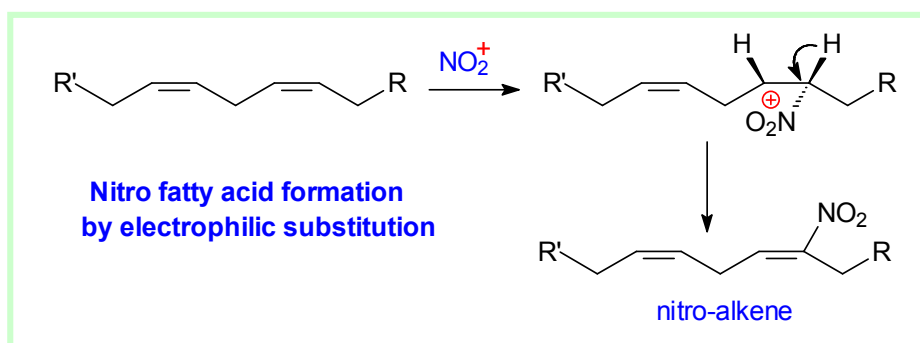
As an NO_2^\cdot radical can also initiate lipid oxidation reactions, yields of nitration versus oxidation will depend on the concentration of oxygen. For example at elevated oxygen levels, the NO_2^\cdot radical can interact with an unsaturated fatty acid to form a carbon-centred radical, which can interact with oxygen to form a lipid hydroperoxide. Unstable alkyl peroxy-nitrite intermediates can also be formed through the reactions of lipid peroxy radical (LOO^\cdot) and NO^\cdot , of peroxy-nitrite radicals, and of a lipid

hydroperoxide reaction with N_2O_4 or with HNO_2 , the last leading to the production of nitro-epoxy fatty acids.

However, nitro fatty acid radicals can also be produced, which may lose HNO_2 to re-generate the unsaturated fatty acid but with one of the double bonds isomerized from the *cis* to the *trans* configuration.



A further mechanism for nitroalkene formation is addition of a nitronium ion (NO_2^+), which can be formed by reaction of a transition metal with peroxynitrite, by electrophilic substitution at the double bond.



Nitro-oleate infused into mice was hydrogenated in part to nitro-stearate and desaturated to a nitro-octadecadienoic acid. Some was subjected to β -oxidation yielding nitro-7-*cis*-hexadecenoic acid, nitro-5-*cis*-tetradecenoic acid and nitro-3-*cis*-dodecenoic acid, and their corresponding coenzyme A derivatives.

3. Biological Effects of Nitro Fatty Acids

It has long been known that nitric oxide *per se* is involved in innumerable biological processes in tissues, but the potential role of nitro fatty acids in mediating these reactions has only recently become apparent. However, in view of the new finding that basal levels of such fatty acids are a thousand times lower than had been believed, much that has been written may have to be re-evaluated, including the following.

In plasma, nitro fatty acids are stabilized by incorporation into lipoproteins, while in erythrocytes and other cells the membrane environment is similarly protective and may provide a reservoir of these compounds. However, nitroalkenoic fatty acids decay rapidly in phosphate buffers, and

presumably in the cytoplasm of cells, due to solvation reactions with release of nitric oxide radicals. A number of different mechanisms have been proposed for this reaction, which may be central to many of the biological functions of nitro acids, but detailed experimental evidence is scarce at present. Thus, it is apparent that nitrated unsaturated fatty acids are powerful electrophiles that mediate reversible nitroalkylation reactions with thiol groups of glutathione and of thio-amino acid residues of proteins, thereby regulating their structure and function of the latter. Indeed, nitro-linoleate isomers in red cells and plasma constitute the single largest pool of bioactive oxides of nitrogen in the vasculature. In particular, they bring about vasorelaxation.

In addition, intact nitro-linoleate isomers function as signalling mediators via receptor-dependent pathways as high-affinity endogenous ligands for peroxisome proliferator-activated receptors (PPAR γ), and they activate receptor-dependent gene expression at physiological concentrations. 12-Nitrolinoleate is a much more potent activator of PPAR γ than any other regioisomer. In neutrophils and platelets, nitro fatty acids activate cAMP-dependent protein kinase signalling pathways, and by such means have an anti-inflammatory role in cells. Similarly, both nitro-oleate and nitro-linoleate have been shown to be endogenous anti-inflammatory signalling mediators in a number of biological processes including the inhibition of the lipopolysaccharide-induced secretion of pro-inflammatory cytokines in macrophages, actions that are independent of nitric oxide formation or of activation of PPARs. Nitro-oleic acid is an irreversible inhibitor of the enzyme xanthine oxidoreductase, which generates proinflammatory oxidants and secondary nitrating species. In this instance, it has been established that the carboxyl group, nitration at the 9 or 10 olefinic carbons, and the double bond are all required for the inhibitory action. Therefore, nitro lipids antagonize the pro-inflammatory cell-signalling pathways, that involve oxidized lipids by a variety of mechanisms.

Nitrated derivative of arachidonic acid have also been shown to have anti-inflammatory properties via effects upon gene transcription. While studies are still at an early stage, it would not be surprising if there were appreciable influences upon the eicosanoid cascades. Similarly, the *trans*-arachidonate isomers formed as by-products of nitration reactions are emerging as biomarkers that target various biological systems. It is certain that the peroxynitrite *per se* has profound effects on the enzymes of prostanoid biosynthesis.

4. Analysis

A major difficulty in the analysis of nitrated lipids is that they are easily generated artefactually via adventitious nitrite anions during sample work-up and chromatographic analysis under acidic conditions. It is therefore necessary to include extensive control experiments to preclude the formation of spurious by-products, for example by adding unsaturated fatty acids labelled with stable isotopes as internal standards. Acidic pHs must be avoided at all critical phases of lipid extraction. It should also be noted that nitrated lipids are sensitive to light and are thermally unstable. Thereafter, modern mass spectrometric techniques, especially with electrospray ionization, provide the enhanced sensitivity and resolution required for analysis.

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