# Nitrated lipids: A class of cell-signaling molecules

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It is becoming increasingly evident that the inflammatory oxidants reactive oxygen species (ROS), reactive nitrogen species (RNS), and reactive halogen species (RHS) mediate diverse pathologic processes in cardiovascular, pulmonary, and neurodegenerative diseases (1, 2). RNS (peroxynitrite, nitrogen-dioxide radical, or nitronium ion) are formed from the rapid reaction between ROS (superoxide) and nitric oxide (3), oxidation of nitrite anion by peroxidases and ROS (hydrogen peroxide) (4), or oxidation of nitrite anion by RHS (hypochlorous acid) (5). RNS react with carbohydrates, DNA bases, protein tyrosine/tryptophan, and unsaturated fatty acids to form relatively stable nitrated products (6). Investigators in this area are now focusing on the role of nitrated biomolecules on cell signaling and evaluating their cellular abundance, metabolism, and bioactivity (7). In this issue of PNAS, Baker *et al.* (8) report the identification and quantitation of allylic nitro derivatives of linoleic acid (termed $LNO_2$ or nitrolinoleate) in healthy human blood samples. Data shown in this study indicate that the concentration of $LNO_2$ found in red cells and plasma represents the single largest pool of bioactive oxides of nitrogen in the vasculature. This work also represents the convergence of antiinflammatory ($^{\bullet}$NO-dependent) and proinflammatory (oxidized lipid-dependent) cell-signaling pathways. Clearly, this is an exciting finding that will likely have profound implications in advancing our understanding of inflammatory processes.

## The "Molecular Lens" Effect Induced by the Hydrophobicity of Membranes

The first obvious question is: Why should we be concerned about lipid nitration? The hydrophobic environment of biological membranes acts as a "molecular lens" and significantly enhances nitration, oxidation, and nitrosation reactions (9). Nitrogen oxides ($^{\bullet}$NO, $NO_2$, and $N_2O_3$) will freely diffuse and concentrate into the hydrophobic membrane (9–11). Both the unprotonated and protonated forms of peroxynitrite ($ONOO^-$ and ONOOH) have been reported to cross the lipid membrane through an ion channel or by passive diffusion at rates similar to that of water (12). Thus, the localization of RNS coupled with the lack of hydrolysis of RNS in the aprotic interior of biological membranes profoundly influences their reaction rates and product profiles (9, 11).

The collective works from our laboratories have demonstrated that nitric oxide is a potent inhibitor of lipid peroxidation and low-density lipoprotein oxidation (13–18). $^{\bullet}$NO reacts with lipid peroxyl radical ($LOO^{\bullet}$) at a nearly diffusion-controlled rate to form a lipid peroxynitrite (LOONO) (19). The reaction of $LOO^{\bullet}$ with $^{\bullet}$NO consumes two molecules of $^{\bullet}$NO, as the intermediate LOONO decomposes to form caged radicals [$LO^{\bullet}$ $^{\bullet}NO_2$] forming either an epoxy nitrolinoleate ($LONO_2$) after rearrangement, or dissociation and reaction with another $^{\bullet}$NO (20). Nitrogen-containing derivatives of oxidized lipids have been identified during lipoxygenase- and myeloperoxidase-catalyzed oxidation of liposome and low-density lipoprotein (LDL) in the presence of $^{\bullet}$NO and nitrite anion (15–17). The presence of cholesteryl nitrolinoleate was detected in human blood plasma and lipoproteins (21). In general, most RNS that induced protein nitration were able to nitrate lipids as well (16).

The work of Baker *et al.* (8) significantly extends our understanding of the structural characteristics of nitro-linoleic acid detected in red cells and plasma.

## Nitrated Lipid-Mediated Signal Transduction

Are these nitrated lipid products merely "footprints" of prooxidant and antioxidant action of $^{\bullet}$NO and RNS (21), do these products primarily function as a storage form of $^{\bullet}$NO, or do they have endogenous cell-signaling properties? Studies from the Freeman and O'Donnell laboratories (22, 23) have shown that nitrated membrane and lipoprotein lipids can transduce $^{\bullet}$NO-signaling reactions and mediate regulatory pathways for inflammatory signaling. These collective works reveal that nitrated linoleate exerts both cGMP-dependent and independent signaling reactions (22). Nitrolinoleate induced $^{\bullet}$NO-dependent, cGMP-mediated relaxation of smooth muscle cells (23). Nitro-linoleic acid also inhibited thrombin-mediated aggregation of human platelets by means of cAMP-dependent mechanisms, with adenylyl cyclase inhibitors partially restoring thrombin-stimulated aggregation (22). Nitrated linoleate significantly attenuated calcium mobilization and enhanced phosphorylation of vasodilator-stimulated phosphoprotein through activation of cAMP-dependent protein kinase (22). Nitrolinoleate-mediated elevation of cAMP presumably occurs through inhibition of phosphodiesterase, the enzyme responsible for hydrolysis of cAMP to AMP and by means of activation of adenylyl cyclase, leading to enhanced cAMP biosynthesis (22). Finally, nitrolinoleate, at submicromolar levels, inhibited leukocyte activation in response to phorbol 12-myristate 13-acetate (PMA) and *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), leading to decreased superoxide generation and integrin expression by means of cAMP-mediated signaling mechanism (23). These findings support the premise that nitrated unsaturated fatty acids are potent antiinflammatory lipid-signaling mediators.

## Synthesis, Detection, and Quantitation of Nitrated Lipids: Technical and Experimental Challenges

A major confounding factor with regard to quantitation of nitrated lipids is their artifactual *de novo* generation in the presence of adventitious nitrite anion during sample work-up and chromatographic analysis under acidic conditions. In this study, the investigators have included extensive control experiments to ensure that the nitrated fatty acids detected in clinical specimens are not spurious byproducts of lipid extraction and analysis. For example, they included [$^{13}$C]linoleic acid as a tracer molecule before lipid extraction and analysis. In this way, they confirmed by mass spectrometry that no artifactual $^{13}$C-labeled nitro derivatives of linoleic acid were formed during *ex vivo* processing. Whether the presence of adventitious nitrite anion exaggerated lipid nitration during work-up was checked by deliberate addition of 200 $\mu$M nitrite anion to initial lipid extractions. They avoided the use of acidic pH in all critical phases of lipid extraction to avoid *ex vivo* acid-catalyzed nitration reactions and further monitored the efficiency of extraction of lipids of interest at neutral pH by using an authentic nitrolinoleate internal standard. Nitrated lipids are light-sensitive and thermally unstable. Thus, sample processing was carried out under sub-

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dued light and involved rapid freezing of nitrated lipids. Finally, they observed that hydroxyl/peroxy derivatives of fatty acids do not give rise to artifactual nitrated derivatives during sample handling. These are tedious and critical experimental precautions that must be appreciated and adhered to before one can obtain reproducible and reliable results from clinical samples. Baker *et al*. have laid the rigorous groundwork needed to move this field forward.

State-of-the-art mass spectrometric techniques [electrospray ionization (ESI)-MS/MS] were used to provide enhanced sensitivity and resolution in identifying the two structural isomers of free and esterified nitrolinoleate, 10-nitro-9-*cis*,12-*cis*-octadecadienoic acid and 12-nitro-9-*cis*,12-*cis*-octadienoic acid in human red cell and plasma lipids. Comparison between the levels of nitrolinoleate acid and the various biologically active nitrogen oxide derivatives found in human blood (see table 1 in ref. 8) reveals that the concentration of this nitrated fatty acid is nearly 100–1,000 times greater than that of nitrosothiols or nitrotyrosine. The greater abundance of membrane lipids, the molecular lens effect of membranes, and the increased reactivity of RNS with unsaturated fatty acids and their peroxidized products are deemed to be responsible for the higher quantitative yields of nitrated lipids relative to other nitrated products.

## Future Perspectives

The focus of this work has been the detection and characterization of allylic nitro derivatives of linoleic acid present in high abundance in human plasma. Literature data suggest that a significant fraction of nitrated fatty acids in clinical tissue and fluid specimens may also result from nitration of oleic, linoleic, and arachidonic acids. Thus, in addition to these other fatty acids, nitration products derived from phospholipids such as phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol, phosphatidic acid, phosphatidylserine, etc. should be considered. Nitration of lipoxygenase- and prostaglandin synthase-derived lipid oxidation products is also plausible. Future characterization of the structure and signaling of the multiple nitro derivatives and their positional isomers will pose enormous analytical and technical challenges. Thus, there is a pressing need to continuously seek more sensitive and sophisticated mass spectrometry instrumentation, as exemplified by the present investigation.

The investigators of the present study propose a biochemical convergence of $^{\bullet}$NO and oxidized lipid cell-signaling pathways (8). This view was supported by *in vitro* findings demonstrating the antagonism of the proinflammatory signaling actions of eicosanoids by allylic nitro derivatives of linoleic acid. Several *in vitro* studies have previously demonstrated the "cross-talk" between $^{\bullet}$NO and eicosanoid pathways (24, 25). Targeted deletion of inducible nitric oxide synthase was shown to regulate prostaglandin biosynthesis in rat tissues (26). Simultaneous measurements of $F_2$-isoprostane, prostaglandin $E_2$, thromboxane $B_2$, and $LNO_2$ in the presence of selective inhibitors of cyclic nucleotide pathways may shed additional light on the modulatory effect of $^{\bullet}$NO signaling on eicosanoid (thromboxane and prostacyclin)-mediated vasoconstricting (or proaggregatory) and vasorelaxing (or antiaggregatory) mechanisms.

Future research will undoubtedly focus on the mechanism of signal transduction induced by nitro lipids. Nitrolinoleic acid reportedly activates heme oxygenase-1 expression and a nuclear receptor at much lower concentrations than did linoleic acid or oxidized linoleic acid (B. A. Freeman, A. Aganwal, and E. Chen, unpublished data). Because lipid receptors are involved in the regulation of diverse inflammatory-related genes, cell growth, and cell differentiation, the differential activation by nitro lipids and oxidized eicosanoids may play an important role in the modulation of inflammatory signaling.

Finally, the finding that nitrated lipids constitute the largest fraction of bioactive nitrogen oxides in the circulation is relevant to our understanding of several fundamental issues in $^{\bullet}$NO research, such as photo-induced relaxation of smooth muscle cells. Pioneering investigations in this area suggest that photo-induced relaxation arises from $^{\bullet}$NO released from nitrite anion or nitrosothiols (27, 28). Could nitrated lipids serve as a potential reservoir for light-induced photo-relaxation of smooth muscle cells? Perhaps!


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